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HOST-PARASITE INTERACTIONS MEDIATED BY TOLL-LIKE RECEPTORS IN MALARIA LIVER INFECTION

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DOUTORAMENTO EM CIÊNCIAS BIOMÉDICAS
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As opiniões expressas são da exclusiva responsabilidade do seu autor.

Resumo

A malária constitui uma das doenças infecciosas mais devastadoras, afectando actualmente 5 a 10% da população mundial. O audacioso plano de erradicação da malária é um objectivo em curso que requer o desenvolvimento de novas estratégias de combate à infecção e que é limitado pelo conhecimento insuficiente da biologia do *Plasmodium*, o parasita que provoca a malária, e das complexas interacções que mantém com o hospedeiro.

O *Plasmodium* é um organismo protozoário com um ciclo de vida complexo que envolve um mosquito vector do género *Anopheles* e um hospedeiro vertebrado. A fase hepática da malária é a primeira etapa do ciclo de vida do parasita no hospedeiro mamífero, durante a qual não é revelado nenhum sintoma da doença. É, no entanto, nos hepatócitos que o *Plasmodium* se desenvolve e replica originando milhares de novos parasitas que são depois libertados na corrente sanguínea. A passagem do parasita para o sangue dará lugar à fase sintomática da doença. Assim, apesar de ser clinicamente silenciosa, a infecção do fígado pelo *Plasmodium* é determinante no estabelecimento de qualquer infecção de malária. A fase hepática da malária constitui um excelente alvo para a criação de uma vaccina ou de novas abordagens terapeúticas uma vez que conferirá protecção completa ao hospedeiro humano, impedindo a manifestação clínica de sintomas e, mais importante, a transmissão da doença para outros indivíduos. A compreensão dos mecanismos fundamentais de controlo da infecção no fígado irá certamente contribuir para o desenvolvimento de novas estratégias de combate à infecção. Considerando que as terapias existentes actualmente tenderão a tornar-se insuficientes ou obsoletas, devido em grande parte ao desenvolvimento de resistências pelo parasita, será crucial produzir vacinas eficazes contra a infecção por *Plasmodium*.

O principal desafio para qualquer hospedeiro é o de detectar o parasita e de induzir uma resposta defensiva rápida. Os *Toll* e os *Toll-like receptors* (TLRs) constituem uma família de receptores do sistema imune inato, que desempenham esse papel essencial de reconhecimento e defesa contra agentes infecciosos. Estão presentes tanto em organismos invertebrados como vertebrados, reflectindo uma notável conservação de

função em termos evolutivos. A análise funcional dos TLRs dos mamíferos revelou que estes reconhecem moléculas ou famílias moleculares específicas conservadas evolutivamente que se encontram presentes em microrganismos. Estas moléculas não são encontradas em mamíferos, excepto em condições de stress celular ou inflamação, tornando-as alvos importantes no desenvolvimento de novas estratégias de intervenção imunológica. Os diferentes membros desta família de receptores reconhecem lípidos, proteínas, lipoproteínas, carbohidratos, péptidos, lipopeptídeos e estruturas de ácidos nucleicos que são na generalidade expressas por grupos específicos de organismos patogénicos. Quando reconhecidas pelos TLRs, induzem a activação da imunidade inata, assegurando a geração de uma resposta imune adaptativa contra os parasitas.

Actualmente, embora seja consensual a noção de que os TLRs estão envolvidos na detecção de parasitas, e tendo sido previamente descrito que os hepatócitos expressam os TLRs 1 a 9, a nossa compreensão acerca da sua função na resposta imune inata contra a infecção do fígado pelo *Plasmodium* ainda é limitada.

O trabalho apresentado nesta tese focou-se essencialmente na determinação do papel de alguns destes receptores e de uma molécula adaptadora envolvida na via de sinalização dos mesmos, MyD88, durante a fase hepática da malária.

Enquanto que a infecção de ratinhos mutantes para TLR2 por esporozoítos de *P. berghei* – a forma invasiva transmitida por mosquitos *Anopheles* e que infecta hepatócitos de roedores – é semelhante à dos ratinhos controlo *wild-type*, a infecção de ratinhos que não expressam os receptores TLR4 e TLR9 apresenta diferenças significativas importantes relativamente aos controlos. Ratinhos com deficiências na expressão de TLR4 e TLR9 revelam uma maior susceptibilidade e resistência, respectivamente, à infecção do fígado pelo parasita. A expressão reduzida ou ausência de TLR4 reduz a intensidade da resposta imune, permitindo uma maior proliferação do parasita no fígado. Pelo contrário, a ausência de expressão de TLR9 parece ser prejudicial para o parasita, uma vez que os níveis de infecção pelo parasita são reduzidos em mutantes para TLR9. Estas observações sugerem que estes dois receptores desempenham papéis importantes na infecção pela forma hepática de *P. berghei*. De facto, o tratamento com LPS e CpG, ligandos de TLR4 e TLR9 respectivamente, em simultâneo com a infecção, provoca uma diminuição nos níveis de infecção no fígado. O receptor TLR9 poderá ser importante na infecção e desenvolvimento do parasita durante a primeira fase do seu ciclo de vida no

hospedeiro. Adicionalmente, a molécula recrutada por todos os TLRs na sua via de sinalização, MyD88, é aqui descrita como mediadora da imunidade protectora induzida por esporozoítos de *P. berghei* atenuados por irradiação, a única forma até agora descrita de imunização efectiva contra a forma hepática do parasita. Embora a molécula adaptadora MyD88 seja relevante no processo de imunização com esporozoítos irradiados, os ratinhos que não expressam MyD88, quando injectados com parasitas viáveis, revelam níveis de infecção muito semelhantes aos ratinhos controlo *wild-type*.

Os resultados apresentados nesta tese sugerem que os TLRs são mediadores de interacções entre o parasita e o hospedeiro durante a fase hepática da malária e poderão ainda estar envolvidos no desenvolvimento de imunidade conferida pela imunização com esporozoítos irradiados. A activação destes receptores na fase inicial da infecção poderá ainda ditar o grau de desenvolvimento da patologia em etapas mais tardias da doença, e consequentemente, determinar o aparecimento ou a ausência de malária cerebral, a manifestação mais letal desta doença.

Abstract

Malaria is one of the most severe human infectious diseases, affecting 5 to 10% of the world's population every year. Although malaria eradication has emerged as a desirable if audacious goal, it is consensual that the development of novel intervention strategies is limited by our current understanding of the biology of *Plasmodium*, the causative agent of malaria, and of the complex relationships that the parasite maintains with its hosts.

Plasmodium has a complex life cycle that oscillates between a mosquito vector and a vertebrate host. Upon infection of its mammalian host, each *Plasmodium* sporozoite establishes itself in liver hepatocytes where it replicates into thousands of new parasites (merozoites) that are subsequently released into the bloodstream, infecting red blood cells and causing malaria. Liver infection by *Plasmodium* is an ideal target for the development of anti-malaria strategies, as it is the first step of infection and it is clinically silent. Indeed, *Plasmodium* liver stage is the epitome of a perfect malaria vaccine or drug target since complete protection from infection of the treated human host abrogates clinical manifestation and, importantly, transmission of the disease. Thus, understanding the key events during liver infection will certainly facilitate our progress towards novel intervention strategies against malaria.

The prime challenge for any invaded host is to detect the pathogen and orchestrate a rapid defensive response. A set of essential surface and endosomal molecules that comprise the Toll or Toll-like family of receptors perform this role in invertebrate and vertebrate organisms, reflecting a remarkable conservation of function. Functional analysis of mammalian Toll-like receptors (TLRs) has revealed that they recognize specific evolutionarily conserved microbial molecules or molecular families that are present among pathogens and are usually critical to the pathogen's function. These molecules are not found in mammals except in cell stress and inflammation, making them crucial targets for immune intervention. The members of the TLR family recognize lipids, proteins, lipoproteins, carbohydrates, peptides, lipopeptides and nucleic acid structures that are broadly expressed by specific groups of pathogens, providing the

basis for innate immunity and ensuring multiple mechanisms of the adaptive immune response against parasites.

Although it is clear that pathogen detection involves members of the TLR family and that hepatocytes express the majority of all known TLRs, our current understanding about their function in the innate response to *Plasmodium* liver infection remains elusive.

The work presented in this thesis aimed to determine the role of some of these innate receptors and their adaptor molecule, MyD88, not only in the establishment of *Plasmodium* in the liver but also during an immunization process with attenuated forms of *Plasmodium* sporozoites.

Liver stage infection by *Plasmodium* is strongly affected by manipulation of TLR4 and TLR9. While TLR2-deficient mice are infected as wild-type control mice, TLR4 and TLR9-deficient mice show increased susceptibility and resistance to liver stage infection, respectively. While TLR4 absence dampens the inflammatory response, increasing the parasite load in the liver, the lack of TLR9 seems to be detrimental for the parasite. On the other hand, administration of LPS and CpG (TLR4 and TLR9 ligands, respectively), at the time of sporozoite injection, strongly reduces the levels of *Plasmodium* liver load.

In humans, immunization with large numbers of radiation-attenuated sporozoites (RAS) remains the only protocol that leads to the induction of sterile immunity. MyD88 is a mediator of protective immunity induced by *P. berghei* RAS, despite the fact that when injected with viable sporozoites, MyD88-deficient mice are infected as normal wild-type control mice.

Altogether, the findings presented herewith suggest that TLRs not only mediate major events in the establishment of *Plasmodium* liver stage infection but are also key players during the establishment of sterile immunity.

Palavras-chave

Malária
Plasmodium
Fígado
Hepatócito
Imunidade inata
Toll-like receptors
Interacções hospedeiro-parasita

Keywords

Malaria
Plasmodium
Liver
Hepatocyte
Innate immunity
Toll-like receptors
Host-pathogen interactions

Abbreviations

cDNA	complementary deoxyribonucleic acid
CM	cerebral malaria
CSP	circumsporozoite protein
CTL	cytotoxic T-cell
DAPI	4'6-diamidino-2-phenylindole
DCs	dendritic cells
DDT	dichloro-diphenyl-trichloroethane
DMEM	Dulbecco's modified eagle medium
EEF	exoerythrocytic form
ERK 1/2	extracellular signal-related kinase ½
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
GAS	genetically attenuated sporozoites
GFP	green fluorescence protein
HPRT	hypoxanthine guanine phosphoribosyltransferase
Hsp60	heat shock protein 60
Hsp70	heat shock protein 70
HSPGs	heparan sulphate proteoglycans
i.p.	intraperitoneal
i.v.	intravenous
IL-6	interleukin-6
IRAK	IL-1 receptor associated kinase
iRBC	infected RBC
JNK	jun NH2-terminal kinase
KO	knockout
LRR	leucin-rich repeat
MALP2	dipalmitoylated mycoplasm lipoprotein-2
MMTV	mouse mammary tumor virus
mRNA	messenger RNA

MyD88	myeloid differentiation factor 88
NF-κB	nuclear factor kappa-B
NLRs	Nod-like receptors
P.	*Plasmodium*
PAMP	pathogen-associated molecular pattern
PbA	*Plasmodium berghei* ANKA
PBS	phosphate buffered saline solution
PCR	polymerase chain reaction
pen/strep	penicillin/streptomycin
PFA	paraformaldehyde
PfEMP1	*P. falciparum*-erythrocyte membrane protein 1
PRR	pattern recognition receptor
PV	parasitophorous vacuole
qRT-PCR	quantitative real-time polimerase chain reaction
RAS	radiation attenuated sporozoites
RLRs	RIG-I-like receptors
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute (medium)
rRNA	ribosomal RNA
RSV	respiratory syncytical virus
RT-PCR	reverse transcriptase PCR
s.d.	standard deviation
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF-α	tumour necrosis factor α
TRAF6	tumor necrosis factor receptor-associated factor 6
TRAP	thrombospondin related anonymous protein

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Chapter 1 General Introduction

Plasmodium, the causative agent of malaria, is a protozoan parasite that has been discovered more than 125 years ago. Still, malaria remains a major global-health problem today. According to the World Health Organization (WHO) World Malaria Report 2008 the most recent numbers shows that malaria still kills approximately one million people each year, essentially young children and pregnant women. This public health burden creates a significant barrier to economic and social development of the most affected countries (Snow, Trape et al. 2001). So far, due to the complexity of the *Plasmodium* parasite and its life cycle, the available options for preventing malaria remain limited to vector control and chemoprophylaxis. While drug-resistant strains of the parasite are emerging and insecticide-resistant strains of the mosquito vector are spreading around, the efforts to design an effective vaccine or to develop new drugs capable of reducing malaria morbidity, mortality or transmission remain unsuccessful.

Malaria – Past, present and future

The term malaria is derived from the Italian *mala aria*, which means “bad air”, from the early association of the disease with marshy areas. Towards the end of the 19th century, Charles Laveran, a French army surgeon, observed parasites in the blood of a patient suffering from malaria, and Dr Ronald Ross, a British medical officer in Hyderabad, India, discovered that mosquitoes were responsible for transmitting malaria. The Italian professor Giovanni Grassi subsequently showed that human malaria could only be transmitted by *Anopheles* mosquitoes (reviewed inTuteja 2007).
Currently, over two billion people, representing more than 40% of the world’s population, are at risk of malaria (Snow, Guerra et al. 2005). The most affected populations are from developing countries in both the subtropical and tropical regions, many of which are endemic for the disease. Despite its broad distribution, most of the malaria cases and deaths occur in sub-Saharan Africa. Nevertheless, Asia, Latin America, the Middle East and parts of Europe are also affected (World Health Organization, 2007) (see Figure 1). Malaria is widespread where the temperature and rainfall are most suitable for the development of *Anopheles* mosquitoes, the transmission vector of the malaria parasite, *Plasmodium*. In the past, malaria also occurred widely in other regions with temperate

climate conditions such as Western Europe and the United States. However, public health measures and economic development have been successful in achieving complete eradication of the disease, except for cases imported via international travel.

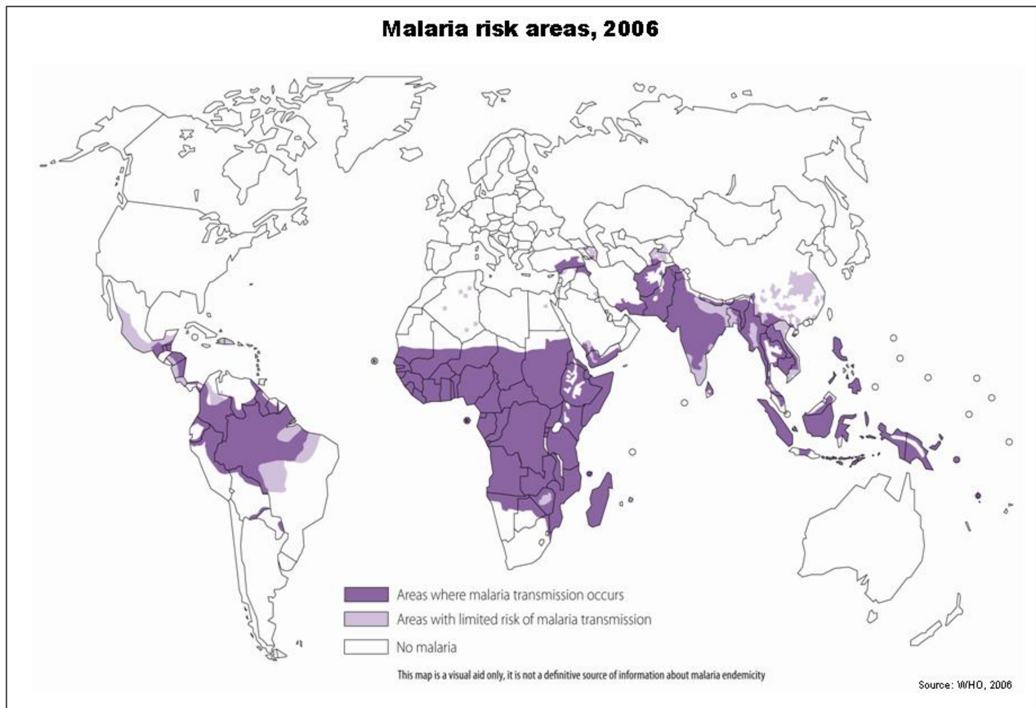


Figure 1 Malaria risk areas, 2006 (World Health Organization, 2007).

To counteract this major public health problem *The Global Malaria Eradication Programme* was created by the WHO in 1955 and was based on two different kinds of approaches. First, chloroquine was used for treatment and prevention of malaria infection, and second, the insecticide dichloro-diphenyl-trichloroethane (DDT) was applied as a vector control strategy. The implementation of these two types of measures led to some beneficial effects, especially in areas with lower transmission rates, like Sri Lanka and India (WHO, The World Health Report 1999). However, global eradication was officially abandoned in 1972, mainly due to the emergence of chloroquine-resistant

Plasmodium parasites and DDT-resistant *Anopheles* mosquitoes (Brito 2001), but also due to lack of political motivation. Still, in certain countries like Thailand, where better health infrastructure and sustained anti-vector measures, associated to economic development, were implemented, parasite transmission has actually declined. In other countries, like Sri Lanka and Madagascar, the resurgence of malaria caused devastating epidemics, which have affected entire populations (Roberts, Manguin et al. 2000). Then, the emergence of chloroquine and sulfadoxine-pyrimethamine-resistant *Plasmodium* parasites in Africa (Snow, Trape et al. 2001) became a major issue that required a serious intervention from the international community, which led to new vector controlling measures and new drug combinations with artemisinin derivatives. These measures were very successful in some areas, reinvigorating the program for global eradication (Greenwood, Fidock et al. 2008).

Currently, the WHO's *Global Malaria Programme* (GMP), as well as other international initiatives that have been launched to challenge malaria, postulates that malaria control requires an integrated approach that comprises prevention and treatment with effective antimalarials. Several other organizations support the implementation of prevention and treatment programs. In fact, there are several ways of decreasing malaria prevalence but none currently offers an absolute blockade (Greenwood and Mutabingwa 2002). Thus, it is urgent to develop new tools to prevent and treat malaria and to overcome the increasing parasite drug resistance observed over the past few decades. Many believe that the three combined strategies of drug treatment, vaccination and vector control will ultimately be a requisite to significantly decrease malaria infection and transmission (Miller and Greenwood 2002; Ballou, Arevalo-Herrera et al. 2004).

Vaccines, drugs and anti-vector measures are being developed to prevent infection, disease, and transmission. Interestingly, the most widely used old compounds are both derived from ancient herbal therapies. Quinine, isolated from cinchona bark in 1820, and artemisinin, which was obtained from the plant *Artemisia annua* in 1972 and currently constitutes the most used drug for treatment, being an extremely effective antimalarial. To overcome parasite drug resistance and to extend the useful life of current drugs, combination therapy is being progressively more employed. Further, progress towards developing a vaccine is incomplete. There is no clinically approved malaria vaccine available thus far, even though some are currently in the clinical development and

testing phases (Girard, Reed et al. 2007; Todryk and Hill 2007). Another prospective option for reducing malaria transmission is by using genetically modified mosquitoes that are refractory to parasite transmission (Christophides 2005). Nevertheless, to be effective, these mosquitoes need to be successful in the wild, competing with the already evolutionary and ecologically established mosquitoes.

The availability of genome sequences for humans, *Anopheles* mosquitoes and *Plasmodium* parasites has created new expectations for future research achievements. As part of a major and global effort to eradicate malaria, research must be directed to a deeper knowledge of *Plasmodium* biology but also to the mammalian host and vector response to the parasite. In that respect, a better understanding of the host immune mechanisms operating against *Plasmodium* infection can also give rise to more powerful interventions. These would be critical to counteract antimalarial drug resistance and the requirement of an effective vaccine against *Plasmodium*, which hinders malaria control.

Plasmodium

Malaria parasites are eukaryotic unicellular microorganisms that belong to the genus *Plasmodium*. More than 100 species of *Plasmodium* can infect several animal species such as birds, reptiles, and a variety of mammals, although only five different species of *Plasmodium* infect humans under natural conditions: *P. falciparum, P. vivax, P. malariae, P. ovale* and more recently *P. knowlesi*. These five species differ in their morphology, geographical distribution, relapse patterns and drug responses. Host immunity against them is also distinct. Among these five species, *P. falciparum* is the most virulent and the major cause of mortality. It is also distinguished by its ability to adhere to the endothelium during the blood stage of the infection and to sequester in several organs, including the brain. The sequestration of *P. falciparum*-infected erythrocytes in the brain has been thought to be a major cause of severe malaria-associated pathology including cerebral malaria, a complication that is often fatal. However, this view is today contested by proposals of alternative pathogenic mechanisms. *Plasmodium vivax* is usually found in tropical areas outside Africa because most Africans lack the Duffy blood group antigen, essential for *P. vivax* invasion, which

is expressed on the surface of erythrocytes. Both *P. vivax* (the most widespread species) and *P. ovale* (the least common malaria parasite, restricted to West Africa) are capable of remaining latent in the liver for weeks to many years as quiescent liver stage forms – hypnozoites, which turns infection with these parasites difficult to eliminate. The beginning of a new round of pre-erythrocytic schizogony results in relapses of malaria infection. Despite not being able to form hypnozoites, *P. malariae* can persist for decades as a long-lasting asymptomatic blood stage infection. It is found worldwide, but with relatively low frequency (reviewed in Collins and Jeffery 2007). A fifth species, *P. knowlesi*, which was originally described as a malaria parasite of non-human primates, has been recently described in naturally infected humans in specific regions, such as Malaysia, where the monkey population is highly prevalent (Singh, Kim Sung et al. 2004).

***Plasmodium* life cycle**

Plasmodium parasites have a complex life cycle in their mosquito vector and vertebrate hosts (see Figure 2). Vertebrate infection begins with the bite of an infected female *Anopheles* mosquito. Sexual stage differentiation and development of sporozoites, the liver infective form of the parasite, take place in the mosquito vector and its transfer occurs during a blood meal. It was thought that sporozoites moved rapidly away from the site of injection. However it was recently described that most of the infective sporozoites remain at the injection site for hours, with only slow release into the bloodstream (Yamauchi, Coppi et al. 2007). Once in circulation, sporozoites travel to the liver and enter parenchymal cells to initiate the hepatic phase of the disease. Prior to invasion of a final hepatocyte where the parasite will develop and replicate, sporozoites traverse several hepatocytes before invading a final one (Mota, Pradel et al. 2001). The structures on sporozoites surface responsible for hepatocyte invasion are mainly the thrombospondin domains on the circumsporozoite protein (CSP) and on thrombospondin-related adhesive protein (TRAP). These domains specifically bind to heparan sulfate proteoglycans (HSPGs) on the hepatocytes (Frevert, Sinnis et al. 1993). Once inside the hepatocyte, each sporozoite gives rise to tens of thousands of

merozoites through a process termed schizogony. The time for asexual replication in the liver differs, depending on the infecting parasite and infected host. The prepatent period – the time between sporozoite inoculation and the appearance of parasites in the blood – takes 6 to 18 days for human species, and 42 to 48 hours for murine malaria parasites. Using a rodent malaria parasite, *P. berghei*, it has been shown that liver stage parasites "manipulate" their host cells to ensure the safe release of merozoites into the blood. Hepatocyte-derived merosomes appear to act as shuttles that ensure the protection of parasites from the host immune system and the release of viable merozoites directly into the bloodstream (Sturm, Amino et al. 2006). The release of many thousands of merozoites into the blood, initiates the erythrocytic stage of malaria, a pathogenic cyclic phase in which merozoites develop into mature schizonts within erythrocytes, which rupture and release a new wave of merozoites that invade a new batch of red blood cells (reviewed in Prudencio, Rodriguez et al. 2006).

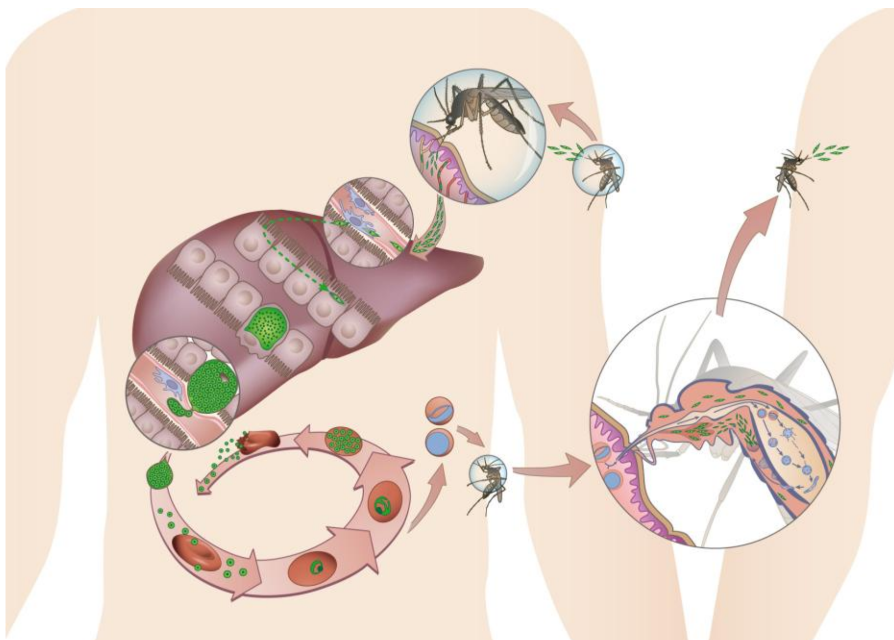


Figure 2 *Plasmodium* life cycle.

Each merozoite invades an erythrocyte and divides by schizogony to form an erythrocytic schizont, containing up to 20 daughter merozoites. These merozoites can reinfect other erythrocytes, giving rise to a cyclical blood-stage infection with a periodicity of 48–72 hours, depending on the *Plasmodium* species.

During the blood stage of infection, unknown factors trigger a subset of developing merozoites to differentiate into male and female gametocytes, which, when taken up by a feeding mosquito, give rise to extracellular gametes, carrying on the *Plasmodium*'s life cycle. In the mosquito mid-gut, the gametes fuse to form a motile zygote (ookinete), which penetrates the mid-gut wall and forms an oocyst, within which meiosis takes place and genetically distinct haploid sporozoites develop (Aravind, Iyer et al. 2003). The mosquito becomes infectious to its next blood meal donor around two weeks after ingesting gametocytes.

To complete its life cycle, *Plasmodium* must pass through the mosquito midgut and salivary glands, penetrate skin vessels, traverse macrophages and several hepatocytes prior to enveloping itself in a vacuole and finally, to attach to the surface of erythrocytes before invasion. The unicellular malaria parasite exploits a pool of around 5000 genes (Gardner, Hall et al. 2002) to undergo remarkable changes and face distinct environments and barriers to parasite infection and development. Since all these processes require a series of specific molecular interactions, they are considered as potential targets for the development of new tools against malaria.

The pre-liver infection

The liver stage of *Plasmodium* infection appears as a good target for developing anti-malaria vaccines or prophylactic drugs. Given that it is the first obligatory step of infection and that *Plasmodium* remains in the liver for about a week in human malaria infections, the immune system has enough time to mount a response. However, the immune response is usually unsuccessful as the blood stage of infection will follow. Even so, it can be improved to make it more effective. Furthermore, the liver stage is a relevant target to prevent the infection at an early stage, once it is clinically silent and either an effective vaccine or prophylaxis would avoid the development of disease. In

addition, the infection is achieved with only a small number of infected hepatocytes and so, there are only small numbers to eradicate (Rosenberg, Wirtz et al. 1990). In recent years, despite some experimental constraints, particularly in humans, some advances have been made to understand both parasite and host mechanisms of infection and response, respectively. The use of malaria rodent models to study the liver stage of infection has been crucial to allow manipulation of liver infection, intravital imaging, gene knockouts and a sort of scientific approaches that would never be possible in humans. These progresses are having important implications in the development of potential tools for novel clinical approaches and for vaccine design.

During a blood meal, infected *Anopheles* mosquitoes inoculate *Plasmodium* sporozoites into the dermal and subdermal tissue of the host. Over time, the sporozoites migrate through the skin until they reach a blood vessel, then traversing the endothelium, sporozoites move into the blood circulation, from where they reach the liver to initiate a malaria infection. Using intravital microscopy of the skin during a mosquito bite, Vanderberg and colleagues, directly evidenced that mosquito probing introduces sporozoites into dermal tissue and these migrate through the dermis and into blood vessels. Furthermore, they also pointed out the role of anti-sporozoite antibodies in blocking sporozoite invasion of these dermal blood vessels (Vanderberg and Frevert 2004).

Interestingly, a quantitative real-time analysis of the fate of sporozoites was used after a mosquito bite in rodents, to demonstrate that only a portion of the parasites entered circulation, whereas some are drained by lymphatics and stop at the proximal lymph node (Amino, Thiberge et al. 2006). Once there, the authors postulate that most are degraded by dendritic cells, but a few sporozoites are capable of partially differentiating into exoerythrocytic stages. This event occurs during sporozoite migration after mosquito probing, and the fact that parasites can partially develop in lymph nodes might have a major influence in host immunity against *Plasmodium* infection and therefore must be considered in vaccine development against the preerythrocytic stages of the parasite. It was also proposed that host cell traversal is important for progression of the malaria parasite through the dermis to the liver. Using a murine parasite, *P. berghei*, it has been recently shown that cell traversal is important in the host dermis for preventing sporozoite destruction by phagocytes and arrest by nonphagocytic cells (Amino,

Giovannini et al. 2008). In addition, during the migration in the skin before entering the liver, sporozoites are very susceptible to neutralization by antibodies. These bind sporozoite surface proteins, mainly the CSP (Yoshida, Nussenzweig et al. 1980) that uniformly covers the external surface of sporozoites, and effectively opsonize the parasite (Vanderberg and Frevert 2004). Still, when sporozoites are able to rapidly reach a blood vessel, they are then carried to the liver in a very short period of time, entering the sinusoids through the portal fields. Sinusoids display a very specialized endothelium, essentially consisted by fenestrated endothelial cells and resident liver macrophages, known as Kupffer cells (Bouwens, De Bleser et al. 1992; Prudencio, Rodriguez et al. 2006).

The liver infection

Sporozoites attach to the endothelial inner layer of liver sinusoids by interactions between parasite surface proteins, both CSP and TRAP (Robson, Hall et al. 1988), with host extracellular matrix proteoglycans (reviewed in Kappe, Buscaglia et al. 2004). These parasite surface proteins, CSP and TRAP, are also thought to play essential roles in sporozoite gliding motility and host cell infection (Sultan, Thathy et al. 1997). Since sporozoites do not adhere to sinusoidal endothelia *in vitro* (Pradel and Frevert 2001), the gliding along the sinusoid lining is thought to be mediated by the interaction of CSP and TRAP with the unique highly sulphated liver HSPGs protruding from the extracellular matrix in the space of Disse and through fenestrae of the endothelial cells (Robson, Frevert et al. 1995; Pradel, Garapaty et al. 2002).
After attaching to sinusoids endothelium, sporozoites must cross the sinusoidal barrier to reach hepatocytes. Surprisingly, Frevert and colleagues suggested that instead of taking what would seem a safer route through endothelia, the parasites traverse Kupffer cells without suffering any harm. The authors proposed a model in which these liver macrophages function as the sporozoite gate to the liver. Thus, according to this model, sporozoites glide along the endothelium, then penetrate and traverse Kupffer cells to reach hepatocytes. Notably, the CSP from sporozoites, by binding to ribosomes and inhibiting protein synthesis (Frevert, Galinski et al. 1998), seems to suppress the

respiratory burst in Kupffer cells (Usynin, Klotz et al. 2007) and to inhibit antigen presentation and cytokine release, thereby disabling the Kupffer cells' potential anti-parasitic function (Steers, Schwenk et al. 2005). However, it has been shown that Kupffer cells can phagocytize sporozoites (Meis, Verhave et al. 1983) and interfere with the development of the exo-erythrocytic forms (Vreden, Sauerwein et al. 1993) presumably by the production of IL-6 (Vreden, van den Broek et al. 1992). In addition, *in vivo* depletion of Kupffer cells prior to infection with *P. berghei* sporozoites results in increased parasitemia (Vreden, Sauerwein et al. 1993).

Once inside the liver parenchyma, sporozoites traverse several hepatocytes before infecting a final one (Mota, Pradel et al. 2001). Mota and colleagues described that sporozoite host cell traversal caused breaching of the plasma membrane of the hepatocyte, followed by rapid repair. These observations emphasized that sporozoite migration through several cells in the mammalian host seems to be required for liver stage development and maintenance of the life cycle. The same authors proposed that sporozoites must cross the cytosol of several hepatocytes before invading a final one by the formation of a parasitophorous vacuole (PV), a specialized membrane compartment. Cell traversal seems to depend on at least two sporozoite secretory proteins – SPECT (sporozoite microneme protein essential for cell traversal) and SPECT2 (Ishino, Yano et al. 2004; Ishino, Chinzei et al. 2005). Inside the PV, in which the parasite is confined from the host cell cytoplasm (Mikolajczak and Kappe 2006), the parasite proliferates and differentiates into thousands of erythrocyte-invasive forms, the merozoites (Hollingdale 1985). The initial formation of the vacuole is dependent on secretory proteins that are characterized by 6-cysteine domains. Indeed, sporozoites lacking 6-cysteine proteins enter hepatocytes but cannot form a PV (Labaied, Harupa et al. 2007) and do not undergo the subsequent liver stage development.

Several other parasite proteins have been described as being important in nutrient uptake from the host infected hepatocyte. UIS3 protein (Mikolajczak, Jacobs-Lorena et al. 2007) is one these proteins that seem to be inserted in the PV membrane. Genetically modified parasites that lack the expression of these proteins, display arrested development early in infection (Mueller, Camargo et al. 2005; Tarun, Dumpit et al. 2007). In fact, these proteins are critical for parasite development, since both 6-Cysteine protein-deficient (Labaied, Harupa et al. 2007) and PV protein-knockout

parasites are consequently attenuated and cannot give rise to a blood stage infection (Mueller, Camargo et al. 2005; Tarun, Dumpit et al. 2007).

Liver innate immunity

Vertebrate immunity can be divided into innate and adaptive, both of which are responsible for the recognition and elimination of pathogens. The innate immune system, also present in invertebrates, constitutes the first line of defense against parasites. The innate immune response is mediated largely by polymorphonuclear leukocytes (PMNs) such as neutrophils, macrophages, dendritic cells (DCs), mast cells and NK cells. The functions of these innate cellular components comprise opsonization, activation of complement and phagocytosis that kills the pathogens and concurrently coordinate additional host responses by synthesizing a wide range of inflammatory mediators and cytokines (Medzhitov 2001).

In general, the infectious agent is killed and degraded in antigen-presenting cells within the maturing phagossome, and components of the pathogen are presented to T cells, resulting in the activation of the adaptive immune response and in the establishment of protective immunity (Aderem and Ulevitch 2000). The recognition by the adaptive immune system and the efficiency of the response relies on the generation of random and highly diverse repertoires of B and T cell antigen receptors, clonal selection and expansion of lymphocytes with receptors of relevant specificity, followed by subsequent memory acquisition in most cases.

The liver is an organ that stands between the gastrointestinal tract and the systemic circulation. Blood from the intestines, rich in bacterial products and in food-derived antigens, encounters the resident population of immune cells. The vast majority of the non-parenchymal cells found in the liver are specialized macrophages called Kupffer cells (KCs). Additionally, CD4 and CD8 T cells, DCs, natural killer (NK) cells and NKT cells are also found in the liver (reviewed in Crispe 2003).

The primary antigen-presenting cells (APCs) in the liver are DCs, which migrate to lymph nodes in order to present antigens to antigen-specific lymphocytes, which in turn migrate to the site of infection. Not only DCs but also KCs as well as liver sinusoidal

endothelial cells (LSECs), hepatic stellate cells (HSCs) and hepatocytes are able to present antigens in the liver. These are resident cells and do not enter the draining lymph nodes.

KCs constitute the liver resident macrophages and their interaction with leucocytes has been found to be relevant for host defense, liver regeneration and establishment of liver immune tolerance (reviewed in Bilzer, Roggel et al. 2006). By residing in liver sinusoids, KCs are among the first immune cells to come in contact with bacteria or bacterial components derived from the gastrointestinal tract. Additionally, KCs are thought to be responsible for the rapid clearance of bacteria from the blood stream (Klein, Zhadkewich et al. 1994).

The most relevant factors in KC activation are endogenous complement factors C3a and C5a, β-glucans from bacteria and fungi, and LPS. In particular, KC activation by LPS, involving the LPS-binding protein CD14 and TLR4, activates the NF-κB pathway that ultimately will lead to production of pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) and IL-1, as well as large amounts of nitric oxide (NO). The release of pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α, promotes the infiltration of neutrophils to eliminate bacteria. TNF-α also induces apoptosis in hepatocytes under pathological conditions (Schumann and Tiegs 1999). Additionally, KCs produce IL-12 and IL-18 which activate NK cells to produce anti-viral interferon-γ (IFN-γ). However, following initial activation to produce pro-inflammatory cytokines, KCs release IL-10 which down-regulates the production of TNF-α, IL-6 and other cytokines (Knolle, Schlaak et al. 1995) and thereby probably contributes to the intrahepatic cell populations capability to induce tolerance.

LSECs can also be efficient APCs in the liver (Crispe 2009). They constitutively express MHC class I and II, as well as the co-stimulatory molecules CD40, CD80, and CD86. LSECs take up the antigen and present it to CD4 and CD8 T cells.

Physiological concentrations of endotoxin, which are continuously present in portal venous blood (Crispe 2009), seem to induce the release of IL-10 from LSECs and KCs (Knolle, Schlaak et al. 1995) and to downregulate CD4 T cell activation by LSECs through down-modulation of the expression of MHC class II, CD80 and CD86 (Knolle, Germann et al. 1999). In contrast, TLR4 activation of APCs by endotoxin induces T cell activation (Pasare and Medzhitov 2005). These observations indicate that the liver

tolerogenic effect might be related to the continuous exposure of sinusoidal cells to bacterial products from the gut.
Nevertheless, in contrast to TLR4 activation, activation of TLR3 by polyI:C or activation of TLR9 by CpG oligonucleotides, has been shown to induce CD8 T cell-mediated hepatitis in transgenic mouse models and has been suggested to induce autoimmunity by breaking immune tolerance in the liver (Sacher, Knolle et al. 2002; Lang, Georgiev et al. 2006). As well, TLR9 activation has been described to exacerbate CD4 T cell induced hepatitis (Jiang, Sun et al. 2009).
HSCs are well known for their involvement in hepatic fibrosis and storage of vitamin A, and were recently shown to function as APCs (Winau, Hegasy et al. 2007). They are able to present protein or lipid antigens to MHC class I and II or to CD8 and CD4 T cells as well as to NKT cells. Upon bacterial infection, HSC elicited a protective antigen-specific T cell response that eliminated the pathogens (Winau, Hegasy et al. 2007).
Hepatocytes constitute the parenchymal cell population in the liver, which primarily perform metabolic functions. Still, they are also involved in immunoregulation by their ability to function as APCs. It has been shown recently that direct interactions occur between lymphocytes and hepatocytes through cytoplasmic extensions penetrating the liver endothelial fenestrations (Warren, Le Couteur et al. 2006). Apart from their constitutive MHC class I expression, hepatocytes also express MHC class II under inflammatory conditions, present the antigen and activate CD4 T cells (Herkel, Jagemann et al. 2003).
T lymphocytes in the liver display an activated phenotype, constituting a source of both IFN-γ and IL-4 (Klugewitz, Blumenthal-Barby et al. 2002). However, the primary consequence of T cell priming in the liver seems to be tolerance (Knolle, Schmitt et al. 1999; Limmer, Ohl et al. 2000). Indeed, as mentioned above, antigen presentation by LSECs induces apoptosis in CD8 T cells (Bertolino, Trescol-Biemont et al. 1998), a mechanism which might frustrate efforts to prime an effective local immune response.
NK cells are present at an unusual high frequency among liver lymphocytes. By synthesizing IFN-γ, which promotes secretion of chemokine CXCL9 by hepatocytes, NK cells are thought to have a crucial role in T cell recruitment, and thus in promoting T cell-mediated immunity (Itoh, Morita et al. 2001). As for NKT cells, even though having a limited T cell receptor (TCR) diversity, they were shown to play a role in the immune

response to the liver stage of malaria infection (Gonzalez-Aseguinolaza, de Oliveira et al. 2000) and to be crucial for the establishment of anti-tumour immunity (Cui, Shin et al. 1997).

Immune responses to malaria

The life cycle of *Plasmodium* includes several stages, which can be targeted by host immune responses, either by blocking the intermediary critical steps between the different infection stages, or by directly killing the parasite.

Following the injection of *Plasmodium* parasites by a female *Anopheles* mosquito into the human dermis, sporozoites have to evade antibodies and other opsonizing molecules such as complement, when accessing blood vessels in the skin and then to pass through liver macrophages and hepatocytes to initiate liver stage infection.

Liver developing parasites are a target of cytotoxic T lymphocytes (CTLs) and at the time of merosome release, merozoites have to evade circulating antibodies to invade erythrocytes. Blood stage parasites are susceptible to opsonizing antibodies and macrophages, and cytokine responses have been related to both protection and disease during this stage of infection. In fact, antibodies that block binding of *P. falciparum*–infected erythrocytes to endothelium might prevent disease and control parasitemia. Human antibodies specific for gametocytes can block transmission to mosquitoes, although these might require complement for parasite killing. Parasites can also be killed by *Anopheles* mosquito innate immune responses during early or late sporogonic stages and lead to refractoriness to infection (reviewed in Greenwood, Fidock et al. 2008).

It has been demonstrated that the main anti-parasite effector mechanism in the liver is IFN-γ produced mainly by CS-specific CD8 and CD4 T cells that inhibit parasite development within hepatocytes (Schofield, Ferreira et al. 1987; Weiss, Sedegah et al. 1988; Hoffman, Isenbarger et al. 1989; Romero, Maryanski et al. 1989; Rodrigues, Cordey et al. 1991). CD8 CTLs that are capable of recognizing *Plasmodium* antigens presented by MHC class I molecules on the surface of infected hepatocytes seem also to play a role. CTLs kill the hepatocytes through pore-forming perforin proteins, which allow apoptosis-inducing granzymes to enter and kill the target cells. However, immunity

can also be achieved in perforin knockout mice immunized with irradiated sporozoites (Renggli, Hahne et al. 1997). Moreover, IFN-γ production may enhance cytolytic mechanisms by increasing class I molecules on the surface of hepatocytes, making those hepatocytes better targets for CTL lysis, and activating supplementary effector cells such as natural killer cells and macrophages, which are also able to kill the target cells through CTL-like mechanisms, TNF-α induction and NO production.

In the erythrocytic stage, the potential targets for an immune response are merozoites in the blood stream or intraerythrocytic parasites. Since HLA class I or II molecules are absent from the surface of the parasite or the infected red blood cell (RBC), it is generally agreed that humoral responses are critical in blood-stage immunity. It was shown in mouse models that B cells and antibodies are important in parasite elimination (Langhorne, Cross et al. 1998). The mechanisms by which antibodies are effective consist of blockade of the invasion of RBCs by merozoites (Blackman, Heidrich et al. 1990), antibody-dependent cellular killing mediated by cytophilic antibodies (Bouharoun-Tayoun, Oeuvray et al. 1995) and binding of antibody to parasite-induced molecules on the RBC surface, leading to greater clearance of infected RBCs (Bull, Lowe et al. 1998). Several studies suggest that responses to many antigens are involved in protection (Gray, Corran et al. 2007). Many presumed target antigens on the infected RBC surface and antigens either on the merozoite surface or released during merozoite invasion have been identified as being potentially protective.

The immune response to malaria is extremely complex, and is both species- and stage-specific. The generation and maintenance of clinically protective immune responses requires repeated infections over the lifetime of the individual. There are several mechanisms playing a role in adaptive immunity against malaria: antibodies that prevent invasion of sporozoites into liver cells, CD8 T cells and IFN-γ that inhibit parasite development in infected hepatocytes, antibodies that block invasion of merozoites into erythrocytes, antibodies that prevent sequestration of infected erythrocytes by preventing binding to adhesion molecules on the vascular endothelium, IFN-γ and CD4 T cells that activate macrophages to phagocytose intra-erythrocytic parasites and free merozoites, antibodies that neutralize parasite glycosylphosphatidylinositol and inhibit induction of the inflammatory cytokine cascade and, finally, antibodies that mediate

complement-dependent lysis of extracellular gametes, prevent fertilization of gametes and the development of zygotes (reviewed in Stevenson and Riley 2004).
As described above, the adaptive immune responses known to be protective at each stage of the parasite's life cycle have been the subject of many studies in mice models of malaria infection and are quite well known. However, unlike other infections with intracellular pathogens, including viruses, bacteria and some protozoan parasites (Janeway and Medzhitov 2002; Akira, Uematsu et al. 2006; Beutler, Jiang et al. 2006), the role of the innate immune mechanisms against *Plasmodium* infection has not been often addressed.

Vaccine development against malaria

It is known that older children and adults with exposure to malaria infection can develop complete protection from severe illness and death, even though sterile immunity is probably never achieved. However, the viability of malaria vaccination is supported by a number of observations. Both epidemiological data and mathematical models demonstrate that immunity to malaria might be acquired with age, since only a few adults die of malaria in endemic regions (Gupta and Day 1994). This might be due either to the acquisition of tolerance – the infection proceeds at the same rates, but there are no clinical symptoms (clinical immunity) – or to the development of immunity to the parasite infection, in individuals that are continuously exposed to the disease throughout their life time. Some epidemiological data correlate specific immune responses with reduced malaria incidence, which indicates that it might be possible to develop immunity against the parasite. For instance, a considerable number of associations were done with immune responses to the main highly variable protein on the surface of infected erythrocytes, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which constitutes a difficult target for immunization due to its high rates of antigenic switching and considerable diversity (Bull, Lowe et al. 1998; Dodoo, Staalsoe et al. 2001). Additionally, in the 1970s, it was shown that both humans and mice could be immunized with RAS, meaning that immunity against the pre-erythrocytic malarial stages by itself could induce

sterile protection for several months (Nussenzweig, Vanderberg et al. 1967; Clyde, Most et al. 1973).

Despite it being possible to culture sporozoites *in vitro* (Al-Olayan, Beetsma et al. 2002), an extensive production is not a viable process, while immunization by thousands of infected mosquitoes is unfeasible as well. Thus, a huge effort has been made in the past 25 years seeking to understand the immune mechanisms responsible for this protection and to identify the parasite proteins that are the targets of these protective immune responses.

Thus far, sterile protective immunity against challenge with *Plasmodium* spp. sporozoites has been induced in several model systems and in humans by immunization with RAS. The primary target of this RAS-induced protection is the infected hepatocyte and several immune mechanisms have been identified as critical mediators of protection against sporozoite infection. Studies in mice indicate that CD8 effector T cells producing IFN-γ are the main mechanism by which these vaccines may act (Schofield, Villaquiran et al. 1987).

T cells specific for parasite-derived peptide/class I MHC molecule complexes on the surface of infected hepatocytes are the primary immune effectors, which kill parasites in infected hepatocytes, while antibodies against sporozoite surface proteins, such as CSP, are thought to have a minor function. CD4 T cells that recognize parasite-derived peptides presented by class II MHC molecules on the surface of infected hepatocytes as well as other factors, including NO, are also important effectors in protection against sporozoite infection (reviewed in Doolan and Martinez-Alier 2006).

It was recently shown in mice that parasites deficient in certain proteins, which induce arrested development in the liver, were also able to induce sterile protection. Indeed, a single-dose immunization with specific genetically attenuated sporozoites (GAS) can induce full protection against subsequent sporozoite infection. Moreover, prime-boost immunizations are capable of inducing protection for at least 6 months. Several GAS that infect hepatocytes but are unable to establish blood-stage infections *in vivo* have been described: UIS3-deficient sporozoites (Mueller, Labaied et al. 2005), UIS4-deficient sporozoites (Mueller, Camargo et al. 2005), P36p-deficient sporozoites (van Dijk, Douradinha et al. 2005; Douradinha, van Dijk et al. 2007) and P52-deficient sporozoites, in *P. falciparum*, an ortholog of the rodent parasite gene P36p (van Schaijk,

Janse et al. 2008). The protection conferred by GAS is, to some extent, achieved by antibodies that prevent sporozoite invasion of hepatocytes. However, CD8 T cells are essential to eliminate the remaining infected hepatocytes both by direct cytotoxicity and by IFN-mediated mechanisms (Jobe, Lumsden et al. 2007; Mueller, Deckert et al. 2007), similar to those conferring protection induced by RAS (Doolan and Hoffman 2000).

Because many do not believe a live-attenuated vaccine will ever be feasible, research has also been directed towards identifying parasite antigens that should be included in a vaccine. Despite extensive work on this subject, the number or the identity of the antigenic determinants involved in the protection against liver infection conferred by immunization with RAS remain unclear. The genes that need to be targeted in a designed subunit vaccine in order to elicit similar levels of immunity are still undefined. There may be numerous antigens that contribute to the sterile protective immunity elicited by the immunization with RAS. This means that subunit vaccines that comprise only one or a few antigens might need to induce immune responses that are significantly stronger than those that are induced by years of natural exposure to the parasite, in order to be able to confer worthwhile protection (Hill 2006).

Although hundreds of other *Plasmodium* genes are expressed in sporozoites and exoerythrocytic forms (EEFs), the CSP seems to be an immunodominant protective antigen in RAS (Kumar, Sano et al. 2006). The CSP is a surface protein that covers *Plasmodium* sporozoites and the plasma membrane of EEFs and has been detected in the cytoplasm of the infected hepatocytes (Hollingdale, Leland et al. 1983). Recently, CSP was shown to influence the expression of over one thousand host genes involved in several metabolic processes in order to create an appropriate niche for the parasite growth and development in liver hepatocytes (Singh, Buscaglia et al. 2007). The presence of CSP in the hepatocyte seems to enhance parasite growth of the liver stages both *in vitro* and *in vivo* (Singh, Buscaglia et al. 2007). Nevertheless, regardless of being considered as a major candidate antigen for vaccines targeting the pre-erythrocytic stages of *Plasmodium* infection, sterile immunity can be induced despite the absence of specific immune responses to the CSP expressed by the parasite used for challenge (Gruner, Mauduit et al. 2007).

The sequencing of the genome of *P. falciparum* (Gardner, Hall et al. 2002) provided a most useful tool for vaccine research. However, this might become a double-edged

sword: the approximately 5300 open reading frames that were identified, many of completely unknown function, may scatter the enormous efforts that are being developed thus far. It is not obvious whether the identification of thousands of putative candidate antigens to generate a vaccine would be helpful or not in a field that is already struggling with a small number of putative vaccine candidates. Still, malaria research is already gaining from the genome sequencing and related proteome project (Florens, Washburn et al. 2002; Hall, Karras et al. 2005). For instance, the stage-specificity of expression of many antigens in the *Plasmodium* life cycle is being better defined, revealing the unique expression of liver and blood stage antigens.

Four antigens expressed by *P. falciparum* RAS in hepatocytes have been extensively investigated as putative vaccine targets: PfCSP, Pf Sporozoite Surface Protein 2/Thrombospondin related anonymous protein (PfSSP2/TRAP), Pf Exported Protein-1 (PfEXP-1), and Pf Liver Stage Antigen-1 (PfLSA1). Immunizing mice with the rodent malaria orthologues of PfCSP, PfSSP2/TRAP, and PfEXP-1 protects them from sporozoites infection. Moreover, immunization of people with PfCSP partially protects them against experimental and natural challenge (reviewed in Hoffman and Doolan 2000). So, currently, despite a long history of disappointing results with other candidates, the most success has been achieved using a PfCSP-based recombinant protein, called RTS,S, one subunit vaccine which has shown promise in phase IIb clinical trials (Bojang, Milligan et al. 2001; Alonso, Sacarlal et al. 2004; Alonso, Sacarlal et al. 2005; Aponte, Aide et al. 2007). This leading malaria vaccine candidate incorporates a fusion protein, which comprises CSP and the HBV surface antigen and aggregates as virus-like particles, together with the adjuvant AS02, which is based on monophosphoryl lipid A and QS-21 (Stoute, Slaoui et al. 1997). However, the RTS,S immunized volunteers were not protected after six months post−immunization and the vaccine only induced antibody and CD4 T cell responses, while CD8 T cell responses were not detectable (Lalvani, Moris et al. 1999).

A second approach has been to elicit CD8 T cell responses, against the known Pf proteins expressed by irradiated sporozoites in hepatocytes. A PfCSP DNA vaccine has been reported to be safe (Le, Coonan et al. 2000) to induce CD8 CTL in malaria-naïve volunteers (Wang, Doolan et al. 1998) and to elicit CD8 T cell-dependent IFN-γ production (Wang, Epstein et al. 2001).

These promising results are also stimulating the search for additional vaccines. However, the field must be aware that natural immunity to malaria might consist of a complex mixture of diverse immune responses, some of probably no protective value and some potentially counter-protective, such as pro-inflammatory responses that have been implicated in the pathology of cerebral malaria (CM).

Likewise, ongoing large efforts are testing *P. falciparum* attenuated parasites, by deletion of essential genes, as vaccine candidates in humans and developing a mean to deliver attenuated vaccines.

Toll-like receptors

For a long time a dogma persisted according to which the innate branch of immunity was considered to be entirely unspecific and somewhat less evolved then the adaptive immunity branch. With the discovery of a class of recognition receptors, which bind highly conserved specific molecules among pathogens, the unforeseen specificity of innate immunity was recognized, despite at a much lower level then the specificity observed in adaptive immunity.

TLRs are one subset of a group known as pattern recognition receptors (PRRs). This set of recognition receptors of the innate immune system recognizes conserved molecular structures known as pathogen-associated molecular patterns (PAMPs), which are present in large groups of pathogens (Medzhitov and Janeway 1997). Mannose receptors, such as C-type lectins, scavenger receptors, opsonins (acute phase proteins, complement proteins), NOD (nucleotide-binding oligomerization domain) receptors and CARD (caspase activating and recruitment domain)-containing proteins, such as RIG-1 (retinoic acid-inducible gene-1) and MDA-5 (melanoma differentiation-associated gene-5) are also other members of this functionally-defined family.

The Toll receptor was originally identified in *Drosophila* as an essential receptor for the establishment of the dorso-ventral pattern in developing embryos (Hashimoto, Hudson et al. 1988). In 1995, it was demonstrated that the Toll pathway in *Drosophila* embryos is crucial for immune responses (Lemaitre, Meister et al. 1995), and that Toll-mutant flies were highly susceptible to fungal infection (Lemaitre, Nicolas et al. 1996). The

involvement of Toll in flies' immune defense drove the research to other animal models. In 1997, the first mammalian homologue of the *Drosophila* Toll receptor was identified. A mammalian homologue of *Drosophila* Toll receptor, currently designated as TLR4, was shown to induce the expression of genes involved in inflammatory responses (Medzhitov, Preston-Hurlburt et al. 1997). Afterwards, several other proteins that are structurally similar to TLR4 were also identified and designated as TLRs (Rock, Hardiman et al. 1998). Since then, mammalian TLRs have been a major focus in the immunology field. They participate in the first line of defense against invading pathogens and play a significant role in inflammation, immune cell regulation, survival and proliferation. TLRs are Type I transmembrane proteins characterized structurally by the presence of a Leucin-Rich Repeat (LRR) domain in the extracellular part and a TIR (Toll IL-1 receptor) domain in the intracellular portion.

Thus far, 11 human TLRs (TLR1–TLR11) and 13 (TLR1–TLR13) mouse TLRs have been identified. Toll homologues can also be found in plants (O'Neill and Greene 1998) and in worms (Rich, Allen et al. 2000). In *Drosophila*, nine Toll receptors were identified to date, but only Toll 1, was shown to play a role in host defense (Hoffmann and Reichhart 2002).

Mammalian TLRs recognize a wide spectrum of ligands that comprise modified lipids, proteins and nucleic acids. TLR2 recognizes an extensive array of microbial compounds, such as bacterial lipopeptides (Aliprantis, Yang et al. 1999), peptidoglycan and lipoteicoic acid from Gram-positive bacteria (Schwandner, Dziarski et al. 1999). TLR2 can form heterodimers with both TLR1 and TLR6 (Ozinsky, Underhill et al. 2000) recognizing several other PAMPs including triacylated lipoproteins (Alexopoulou, Thomas et al. 2002), the dipalmitoylated mycoplasm lipoprotein 2 (MALP2) (Takeuchi, Kawai et al. 2001) and fungal zymosan (Ozinsky, Underhill et al. 2000).

TLR4 recognizes the Gram-negative bacterial lipopolysaccharide (LPS). Already in the early 1970's it was reported that antibody production by B cells could be induced by the mitogen LPS (Coutinho, Moller et al. 1973). Despite there being no specificity associated to LPS stimulation alone, B cells stimulated with this microbial component together with antigen give rise to an antigen-specific response (Coutinho, Gronowicz et al. 1974). Following these findings, the mouse strain C3H/HeJ was reported to be hyporresponsive to LPS, and the authors speculated that the observed defect in B cell

activation could be due to the absence of the receptor for LPS (Coutinho 1976). This was later confirmed by showing that using an antibody which bound the surface of B cells inhibited their LPS-mediated activation and immunoglobulin production, competing with LPS for the receptor (Coutinho, Forni et al. 1978).

Later, two independent studies identified a mutation in the gene responsible for the LPS hyporesponsiveness in two mouse strains that were hyporesponsive to LPS, the C57Bl10/ScCr and the C3H/HeJ (Poltorak, He et al. 1998; Qureshi, Lariviere et al. 1999).

Afterwards, other TLR4 agonists with similar immunostimulatory properties to LPS in mice were identified: Taxol (Kawasaki, Akashi et al. 2000), the envelope proteins of mouse mammary tumour virus (MMTV) (Rassa, Meyers et al. 2002), the fusion protein of respiratory syncytical virus (RSV) (Kurt-Jones, Popova et al. 2000) and a few fungal structural components (Shoham, Huang et al. 2001; Wang, Warris et al. 2001). More recently, the heat shock protein 60 (Hsp60) (Ohashi, Burkart et al. 2000), Hsp70 (Asea, Rehli et al. 2002; Correia, V., personal communication; Vabulas, Ahmad-Nejad et al. 2002), gp96 (Vabulas, Braedel et al. 2002) and β-defensin (Biragyn, Ruffini et al. 2002) have been described as endogenous ligands for TLR4. Fibronectin, heparan sulfate and hyaluronic acid are extracellular matrix components that are produced in response to tissue injury during inflammation. The extra domain A of fibronectin (Okamura, Watari et al. 2001), polysaccharide fragments of heparan sulphate (Johnson, Brunn et al. 2002) and oligosaccharides of hyaluronic acid (Termeer, Benedix et al. 2002) have also been reported as powerful agonists of TLR4. In addition, host fibrinogen is able of triggering chemokine secretion by macrophages, through recognition by TLR4 (Smiley, King et al. 2001).

TLR9 recognizes bacterial DNA and synthetic oligodeoxynucleotides containing CpG dinucleotides (Hemmi, Takeuchi et al. 2000). TLR9 has also been reported to be required in the recognition of host chromatin-IgG complexes (Leadbetter, Rifkin et al. 2002). Recently, hemozoin purified from *P. falciparum* was described as a new non-DNA ligand for TLR9 (Coban, Ishii et al. 2005).

TLRs can be expressed in two different cellular localizations. TLRs 3, 7, 8, and 9, are expressed in intracellular compartments, the endosomes. Intracellular TLRs sense viral

and bacterial nucleic acids in particular. TLRs 1, 2, 4, 5, and 6 are located on the cell surface.

TLRs signal via common pathways that converge on NF-κB activation and lead to the expression of several inflammatory genes. Each TLR elicits selective cellular responses to pathogens due to a differential usage of the intracellular adaptor proteins myeloid differentiation factor 88 (MyD88), MyD88-adaptor-like (MAL, also known as TIRAP), Toll/IL-1 receptor (TIR) domain-containing adaptor inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM) (Kawai and Akira 2007). The recruitment of distinct accessory molecules is thought to confer ligand specificity and response selectivity. TLR stimulation leads to cytokine production and antimicrobial responses through NF-κB activation (Medzhitov, Preston-Hurlburt et al. 1997).

Upon binding of a TLR agonist, all the receptors sequentially recruit the adaptor molecule MyD88, IL-1 receptor associated kinase (IRAK), and tumour necrosis factor receptor-associated factor 6 (TRAF6). These adaptors mediate the activation of the jun NH2-terminal kinase (JNK), nuclear factor NF-κB, p38, extracellular signal-related kinase 1/2 (ERK 1/2) and phosphoinositide 3-kinase signaling pathways, inducing the activation of target genes (Takeuchi and Akira 2001).

Despite the fact that all mammalian TLRs seem to require MyD88 to signal, there are some reports that suggest that TLR signaling can trigger at least two pathways (Adachi, Kawai et al. 1998). The first of these two pathways to be described, dependent on MyD88, induces the production of pro-inflammatory cytokines; the second, MyD88 independent, leads to the expression of IFN-β inducible genes. More recently, the TLR adaptor molecules Toll-interacting protein (TOLLIP), TIR-containing adaptor protein (TIRAP), TRIF and TRAM were identified (Burns, Clatworthy et al. 2000). TRAM and TIRAP are believed to confer the specificity for the MyD88 independent or dependent signaling cascade, respectively. Contrary to intracellular TLRs, which recruit MyD88 or TRIF, cell surface TLRs use TIRAP and/or TRAM as additional adaptors suggesting a link between adaptor usage and TLR localization.

The understanding of how TLR activation induces inflammatory and antimicrobial responses has considerably progressed. Upon TLR activation, pro-inflammatory cytokines are released, inflammatory cells are recruited and direct antimicrobial activity is elicited in macrophages, for instance. Moreover, TLR signaling increases the

expression of co-stimulatory molecules and MHC class II on the surface of APCs, thus allowing efficient presentation of microbial products to T cells. Accordingly, TLRs provide a major contribution for the initiation of the adaptive immune responses (Medzhitov 2001), particularly when in association with B cell receptor stimulation in the activation of antigen-specific B cells, to allow for specificity of the immune response (Coutinho, Gronowicz et al. 1974).

Since uncontrolled inflammation is deleterious, TLR-mediated responses are tightly controlled by many mechanisms to induce appropriate responses against diverse microbial pathogens and to prevent excessive inflammation. For instance, TLR expression is modulated by IL-10 and TGF-β (McCartney-Francis, Jin et al. 2004). Additionally, negative regulators (such as an alternative spliced form of MyD88, IRAKM, suppressors of cytokine signaling protein) are induced by TLR ligands themselves to avoid exacerbated detrimental immune responses (Liew, Xu et al. 2005).

Finally, TLR4 expression was found specifically in a subset of CD4 T cells, the regulatory T cells (Caramalho, Lopes-Carvalho et al. 2003). LPS stimulation of regulatory T cells led to an improvement in their suppressive function, thus uncovering a new anti-inflammatory role of TLR. The discovery of an anti-inflammatory effect of TLR activation might be extremely relevant in the evolution of the immune system, as it constitutes a rare negative feedback mechanism during potentially-deleterious inflammatory immune responses.

Currently, it is unanimously accepted that innate immunity has evolved to recognize immunologically active molecules from either pathogens or the host by a variety of recognition receptors expressed on the plasma membrane and in endosomes, such as the TLRs.

As well, other receptors have been identified: the Nod-like receptors (NLRs) and the recently described RIG-I-like receptors (RLRs), both of which reside in the cytoplasm (Creagh and O'Neill 2006). These recognition receptors are widely distributed in a variety of tissues and cell types to detect and respond to infection by triggering strong but defined innate immune activation through specific intracellular adaptor(s) and signaling molecules (Meylan, Tschopp et al. 2006).

Toll-like receptors in malaria

TLRs, which are highly expressed in specialized immune cells such as macrophages, dendritic cells and B cells, play important roles not only in clearing the initial burden of infectious organisms, but also in the induction of adaptive (antigen-specific) immune responses to achieve long-lasting protection against infection. However, this scenario is much less simple in the case of pathogens that can chronically infect the host, such as *Plasmodium*. *Plasmodium* is still one of the most successful pathogens in the world. For a long time, *Plasmodium* coevolved with humans and other vertebrates, resulting in an extremely well-adapted parasite. The different *Plasmodium* spp undergo morphological changes during their complex life cycle, allowing them to evade or even suppress the host immune response. Thus, it is necessary to understand the nature of both innate and adaptive immune responses elicited during malaria infection and the mechanisms that allow parasite evasion and survival throughout its life cycle.

Gene expression for all the nine TLRs that were first identified was observed in both human primary hepatocytes and in the human hepatoma cell line HepG2, as well as their associated accessory molecules, like MyD88 and MD-2 (in mouse hepatocytes) (Liu, Gallo et al. 2002). Considering that expression of Toll-like receptors and their associated accessory molecules impart responsiveness to microbial products, it is clear that the role of Toll-like receptors in hepatocytes is a key issue that remains to be elucidated in the liver stage of malaria infection.

It is widely accepted that activation of the cellular components of the innate immune response plays an essential role in the outcome of infection with protozoan parasites. Proinflammatory cytokines produced by immune cells induce the activation of effector mechanisms responsible for restraining parasite development at early stages of infection. Several reports support the idea that TLRs are involved in the initial recognition of protozoan parasites by the immune system of the vertebrate host in early resistance to infection, for development of acquired immunity and also in pathology (reviewed in Gazzinelli and Denkers 2006).

Immunological and biochemical studies have shown a significant role of parasite surface molecules. Among them, the glycosylphosphatidylinositols (GPIs) are glycolipids that cover the surface of most protozoan parasites and have been described to be involved

in the modulation of host immune responses (Ropert and Gazzinelli 2000). GPI-anchored glycoproteins purified from *P. falciparum* are capable of inducing the synthesis of proinflammatory cytokines, like TNF-α and IL-1, by macrophages. Evidence shows that GPI anchors from *P. falciparum* contribute to the pathology of infection having led to the classification of GPI as the "malaria toxin". Indeed, GPI alone is enough to originate symptoms comparable to acute malaria infection in mice (Schofield and Hackett 1993; Tachado, Gerold et al. 1996). More recently, *P. falciparum* GPI has been shown to induce proinflammatory cytokine production in macrophages through the interaction of the three fatty acyl chains of the GPI anchor with the TLR2/TLR1 complex, also involving a small contribution of TLR4 (Naik, Branch et al. 2000; Krishnegowda, Hajjar et al. 2005).

In addition, it is also believed that TLR9, a well known intracellular receptor for unmethylated bacterial CpG DNA motifs (Hemmi, Takeuchi et al. 2000), plays a crucial role in induction of proinflammatory cytokines during infection with protozoan parasites. Although this is becoming obvious for several protozoan parasites, the role of TLR9 in malaria remains unclear.

According to some findings, hemozoin, a product of hemoglobin digestion by *Plasmodium*, seems to play an immunomodulatory role during infection. Hemozoin has been reported to induce (Coban, Ishii et al. 2002; Coban, Ishii et al. 2005) or inhibit (Taramelli, Basilico et al. 1995; Skorokhod, Alessio et al. 2004; Millington, Di Lorenzo et al. 2006; Urban and Todryk 2006) DC maturation and to trigger the production of proinflammatory cytokines such as TNF-α and IL-12, chemokines (Jaramillo, Gowda et al. 2003; Huy, Trang et al. 2006), and IL-10 (Deshpande and Shastry 2004; Keller, Yamo et al. 2006). Hemozoin might also be involved in the impairment of macrophage functions and in the reduction in expression of major histocompatibility complex class II antigen, CD54, and CD11c in human monocytes (Schwarzer, Turrini et al. 1992; Schwarzer, Alessio et al. 1998). These contradictory results might be attributed to different hemozoin isolates that may be contaminated with other compounds such as glycolipids, or nucleic acid derived from *P. falciparum* (Parroche, Lauw et al. 2007). Moreover, synthetic hemozoin displays diverse activities, depending on the source of the heme employed to prepare the β-hematin (Jaramillo, Plante et al. 2004; Coban, Ishii et al. 2005).

Hemozoin was described as a TLR agonist and a TLR9/MyD88-dependent pathway was shown to mediate murine DC activation by hemozoin during malaria, thus identifying the first non-DNA ligand for TLR9 (Coban, Ishii et al. 2005). TLR9 was known as a receptor for DNA, mostly of unmethylated CpG-containing DNA, and the description of hemozoin as TLR9 ligand was a surprising and controversial finding. Indeed, it was recently claimed that hemozoin plays a specific role in presenting the DNA to the intracellular TLR9, although it is not capable of stimulating the innate immune system by itself (Parroche, Lauw et al. 2007).

As illustrated above for the malaria parasite, and reported in the literature for other protozoan parasites, different pathogens contain several agonists that trigger TLRs. Nevertheless, the specific role of MyD88 – the adaptor molecule common to all TLRs – and TLRs during malaria infection is under intense discussion, and it is not yet clear whether TLRs and their signalling pathways are critical in *Plasmodium* infection. Genetic studies in human patients have uncovered correlations of TLR polymorphisms to disease susceptibility: a frequent TLR4 allele (Asp299Gly) has been associated with severe malaria (Mockenhaupt, Cramer et al. 2006) and risk of maternal malaria, whereas the TLR9 allele T-1486C increases the risk of maternal malaria but was not associated with severe malaria (Mockenhaupt, Hamann et al. 2006). Recently, it was also reported that heterozygosity for a variant of MAL/TIRAP – an adaptor molecule for TLR2 and TLR4, is associated with protection to *P. falciparum* infection (Khor, Chapman et al. 2007). In addition, data concerning the regulation of TLR expression shows that there is an upregulation of TLR expression in patients infected with *P. falciparum* (Loharungsikul, Troye-Blomberg et al. 2008). Consistently, it has been shown that *P. falciparum* infection causes proinflammatory priming of human TLR responses during the acute phase of the disease (McCall, Netea et al. 2007). Supporting these observations, an upregulation of messenger ribonucleic acid transcripts coding for various components of inflammatory pathways (such as several TLRs, MyD88, NF-κB, and IFN-γ) was described in PMBC from patients during presymptomatic infection (Ockenhouse, Hu et al. 2006). The rupture of blood-stage schizonts releasing *Plasmodium*-derived TLR ligands, like GPI or hemozoin-bound nucleic acids, can induce differences in TLR expression, particularly when a correlation between TLR expression and severity of malaria has been evoked.

Several studies have been carried out in MyD88-knockout mice in the context of infectious disease models caused by protozoans, showing that these mice are generally immuno-compromised in terms of their capacity to fight pathogens. MyD88-knockout mice infected with *T. gondii* (Scanga, Aliberti et al. 2002), *T. cruzi* (Campos, Closel et al. 2004), *T. brucei* (Drennan, Stijlemans et al. 2005), and *Leishmania* spp. (de Veer, Curtis et al. 2003; Muraille, De Trez et al. 2003) are described as being highly susceptible to infection. However, rodent malaria models behave differently in what concerns the role of MyD88. Despite being described as responsible for increased IL-12 production during infection with the *P. berghei* NK65, and thus for the pathology during infection, MyD88 is not critical in the control of parasite replication (Adachi, Tsutsui et al. 2001). As expected, MyD88 and TLR signalling-dependent activation of target cells is also at the origin of the deleterious consequences of unregulated innate immunity responses. Thus, in an experimental model of CM using *P. berghei* ANKA, MyD88 signaling is involved in CM development (Coban, Ishii et al. 2007), such that a reduction in mortality is observed in mice lacking MyD88. However, conflicting reports described CM development as not dependent of MyD88 and TLR signaling (Togbe, Schofield et al. 2007). In addition, another report stated that there are no differences between infected TLR2/4/9-deficient triple-knockout mice, which are MyD88-dependent TLRs, and wild-type mice regarding survival and pathogenesis (Lepenies, Cramer et al. 2008). TLR2 and TLR9, previously shown to be involved in the recognition of GPI anchors and DNA from *Plasmodium*, were proposed as being responsible in part for the fatal result of the infection. In summary, the role of TLRs and MyD88 in CM pathogenesis is not clearly understood.

Aims

Over the past few years, there were some substantial advances in the understanding of host immune responses to *Plasmodium*. Still, one of the underlying difficulties hindering successful vaccine design is the incomplete knowledge of the specific types of immune response to aim for, and then how to induce them. A deeper insight into the mechanisms of innate immunity during *Plasmodium* infection could provide critical clues

on how manipulation of the immune system may best be achieved. Thus, we proposed to elucidate some key features of the innate immune response to malaria liver stage.
We sought to determine whether TLRs mediate host-pathogen interactions during liver stage infection, namely if TLRs present in hepatocytes play a role in parasite recognition, notwithstanding the role of TLRs in immune cells.
To accomplish our goal, TLR2, TLR4, TLR9 and MyD88-deficient mice were injected with *P. berghei* sporozoites and the subsequent infection was characterized both in terms of parasite liver load, including the level of *Plasmodium* development inside host cells, and the host immune response. This approach aimed to unveil the nature of the effects induced by the innate response to *Plasmodium* and whether these are detrimental or beneficial for the parasite. Additionally, we also intended to investigate if TLR MyD88-mediated signalling is important in the protection against *Plasmodium* sporozoite infection that is conferred by immunization with RAS.

Chapter 2 Materials and Methods

Hepatoma cell lines

Hepa1-6 (murine hepatoma cell line) and HepG2 (human hepatoma cell line) cells were cultured in Dulbecco's MEM medium (DMEM) with 1mM glutamine supplemented with 10% Fetal Calf Serum (FCS), 1% penicillin/streptomycin (100U/mL penicillin, 0.1mg/mL streptomycin) and maintained at 37ºC in a 5% CO_2 atmosphere. HuH7 cells (human hepatoma cell line) were cultured in RPMI supplemented with 10% FCS, 1% penicillin/streptomycin, 10mM HEPES, 2mM glutamine and 0.1mM non-essential aminoacids at 37ºC in 5% CO_2. All cell culture media and supplements were purchased from Gibco, Invitrogen. Cells were routinely screened and found to be *Mycoplasma* free.

Hepatocytes cultures

Primary hepatocytes were prepared from 8 to 12 week old C57BL/6 wild-type, TLR2, TLR4 and TLR9-knockout mice as previously described, with minor modifications (Renia, Mattei et al. 1990). Cells were isolated by perfusion of liver lobes with Liver Perfusion Medium and Liver Digest Medium (Gibco, Invitrogen) and were further purified over a 60% Percoll gradient (GE Healthcare). Hepatocyte purity and viability were >95%, as assessed by trypan blue dye exclusion. Cells were cultured in eight-chamber plastic Lab-Tek slides (Nunc) in William's E Medium (Gibco, Invitrogen) supplemented with 4% FCS (Gibco, Invitrogen) and 1% penicillin/streptomycin (Gibco, Invitrogen) and incubated at 37ºC in 5% CO_2.

Parasites

Green fluorescent protein (GFP)-expressing *Plasmodium berghei* sporozoites, parasite line 259cl2 (Franke-Fayard, Trueman et al. 2004) were obtained from dissection of infected female *Anopheles stephensi* mosquito salivary glands. Dissections of mosquito salivary glands were performed in RPMI 1640 medium (Gibco, Invitogen). The glands were collected and mechanically disrupted to release the parasites. The debris was pelleted and the sporozoites in the supernatant were collected after three consecutive centrifugations at 20 g for 5 min, at 4ºC. Sporozoites were then counted and maintained on ice until use (adapted from Ozaki *et al.*, 1984). Sporozoite quantification was determined using a haemocytometer.

Mosquitoes were provided from the Department of Medical Microbiology, University Medical Centre St. Radboud, HB Nijmegen, Netherlands and from Unidade de Malária, Instituto de Medicina Molecular, Lisbon, Portugal.

Mice

BALB/c, C3H/HeN, C3H/HeJ, C57BL/10 ScSn, C57BL/10 ScN, C57BL/6 (Thy1.2), C57BL/6 Thy1.1 and C57BL/6 TLR2, TLR4, TLR9 and MyD88-knockout mice were bred and maintained under specific pathogen-free conditions at the Instituto Gulbenkian de Ciência mouse housing facilities. MyD88, TLR2, TLR4, and TLR9-knockout mice were provided by S. Akira (Osaka University, Osaka, Japan).

All animals were used between 7 and 12 weeks old. Mice experimental protocols were approved by the institutional ethical committee as well as the Portuguese Veterinary General Division.

***In vitro* infections**

One day prior to infection, $2x10^5$ Hepa 1-6, HepG2 or HuH7 cells were seeded in 24-well plates and grown at 37ºC in 5% CO_2. Cells with approximately 80% confluence were infected with $2x10^4$ *P. berghei* sporozoites, per well. The plates were then centrifuged for 5 min at 2000 g and incubated at 37ºC in 5% CO_2. Primary hepatocytes were seeded 24 hours before infection in 8-chamber plastic Lab-Tek slides and incubated at 37ºC in 5% CO_2. After medium removal, $4x10^4$ sporozoites were added in 100µl of fresh supplemented medium. The culture medium was removed 42 hours post-infection and the cells lysed with a denaturing solution. After homogenization, lysates were used for RNA extraction. Total RNA from a pool of cells from 3 to 4 wells was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and quality were determined using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies).

Quantification of infection by immunofluorescence

Cells were washed with Phosphate Buffered Saline (PBS) 24 hours after infection, fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature (RT) and permeabilized for 1 hour with 0.1% saponin in a "protein blocking" solution (3% Bovine Serum Albumin,

100mM glycine and 10% FCS; Sigma) to avoid unspecific reactions. Cells were then incubated for 45 min at RT with a mouse monoclonal antibody against the parasite heat shock protein 70 (Hsp70) (Tsuji, Mattei et al. 1994). After washing twice with PBS, cells were incubated for 45 min at RT with a secondary goat anti-mouse antibody AlexaFluor 555 (Molecular Probes, Invitrogen) diluted in blocking solution, for detection. Hepatoma cells were then washed twice with PBS and incubated with 4'6-diamidino-2-phenylindole (DAPI, Sigma) diluted in PBS, to stain the nuclei. Finally, cells were washed with PBS and mounted on a slide with mounting medium Mowiol (Calbiochem) and observed on a fluorescence microscope. Infection was assessed by quantifying the number of EEFs per coverslip, a slide placed in the bottom of the well prior to cell seeding.

Gene-specific expression and infection quantification by qRT-PCR

Extraction of total RNA from liver was performed using RNeasy Mini Kit (Qiagen), according to the instructions provided by the manufacturer. After extraction, RNA concentration and quality were determined using a NanoDrop spectrophotometer (NanoDrop Technologies). One microgram of total RNA was reverse-transcribed to single strand cDNA using the AMV Reverse Transcriptase protocol (Roche Applied Science). The transcripts in the cDNA pool obtained from the reverse transcriptase reaction were quantified by quantitative real time PCR. The reaction was performed in the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems).

Infection load in the liver was determined by qRT-PCR using *P. berghei* 18S rRNA-specific primers as previously described (Bruna-Romero, Hafalla et al. 2001). Gene-specific expression in liver was determined in a similar way using gene-specific primers. The levels of gene expression were quantified and normalized to the mRNA expression levels of the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT) using the following primer sequences 5'-TGCTCGAGATGTGATGAAGG-3' and 5'-TCCCCTGTTGACTGGTCATT-3'. The following primers were used to quantify the number of parasite copies: *P. berghei* 18S rRNA, 5'-GGAGATTGGTTTTGACGTTT ATGTG-3' and 5'-AGCATTAAATAAAGCGAATACATCCTTAC-3'.

The following primer sequences were used to quantify the expression of leukocyte markers: Cd3e, 5'-TCTCGGAAGTCGAGGACAGT-3' and 5'-ATCAGCAAGCCCAGAGT

GAT-3'; Klrd1, 5'-TCACTCGGTGGAGACTGATG-3' and 5'-AGGCAAACACAGCATTCA GA-3'; Mgl2, 5'-GGATCCCAAAATTCCCAGTT-3' and 5'-TCCCTCTTCTCCAGTGTGCT-3'; Ncf2, 5'-GCAGTGGCCTACTTCCAG AG-3' and 5'-CTTCATGTTGGTTGCCAATG-3'.

TLR agonists (LPS and CpG) treatment *in vivo*

C57BL/6 mice were injected intraperitoneally (i.p.) with 10µg of LPS in PBS per mouse. PBS was prepared from double processed tissue culture water, endotoxin free (Sigma) and tissue-culture 10x PBS solution (Gibco). Ultra-pure LPS (*Escherichia coli* 0111:B4) and CpG were purchased from Sigma and InvivoGen, respectively.

Sporozoite immunization and challenge

GFP-expressing *P. berghei* RAS were submitted to a radiation dose of 16 krad, using a gamma-irradiator (Co source).

Wild-type, TLR2, TLR4, TLR9 and MyD88-knockout mice were primed with the injection of $5x10^4$ *P. berghei* RAS and given booster immunizations with two intravenous (i.v.) injections of $2x10^4$ RAS in 7 to 10 days intervals. After challenge with $1x10^4$ viable *P. berghei* sporozoites, all animal groups were monitored for blood-stage infections. Sterile immunity was assayed by Giemsa-stained blood smears and FACS analysis of blood drops, based on parasite GFP detection, obtained on days 1 to 30 post-sporozoite challenge. Sterile immunity was defined as the complete absence of blood-stage parasitemia at all time points. Naïve control mice groups were used for all experiments performed.

Isolation of liver non-parenchymal cells and flow cytometry analysis

Mice were infected with $1x10^5$ *P. berghei* sporozoites and sacrificed 42 hours after infection to collect livers for isolation of liver non-parenchymal cells. Liver fragments were perfused using Liver Perfusion Medium and cells were dissociated using Liver Digestion Medium (Gibco, Invitrogen). Cells were centrifuged at 500 g for 10 min at 4ºC in RPMI with 10% FCS, 1% pen/strep, 1% HEPES, 1% sodium pyruvate and 0.1% β-mercaptoethanol. The pellet was ressupended in supplemented RPMI and, to eliminate hepatocytes, centrifuged twice over Percoll (60% and 30%, respectively) at 900 g for 10 minutes at 4ºC without brake. Pelleted lymphocytes were washed once in complete

RPMI and treated with ACK (ammonium, chloride and potassium; 0.15M NH_4Cl, 1.0 mM $KHCO_3$, 0.5 mM EDTA, pH 7.2), to remove erythrocytes.
Cells were then resuspended in PBS containing 2% FCS and 0.01% sodium azide, and were incubated with Fc-block (anti-CD16/CD32, produced in house) before antibody staining. Data were acquired on a FACSCaliburTM (BD Biosciences) and analyzed with Flowjo software (TriStar Inc.). Live cell counts were calculated from the acquisition of a fixed number of 10μm latex beads (BeckmanCoulter) mixed with a known volume of unstained cell suspension in propidium iodide. The antibodies used were αCD4, αCD8, αNK1.1, αCD3, αF4/80, αCD11b, αCD11c, αCD19, αIgM, αIgD, αCD69, αGr-1, αLy6g, αMHC-II, αTNF-α and αIFN-γ with one of the following conjugations: Allophycocyanin (APC), Fluorescein isothiocyanate (FITC), Cy5, PerCP, Phycoerythrin (PE) or Alexa 488.

Bone marrow mouse chimeras

C57BL/6 mice (Thy1.2+) were gamma-irradiated with 900 rad and, on the following day, received i.v. $2x10^6$ bone marrow cells in PBS, from either WT or TLR-knockout mice donors (Thy1.1+). Reconstitution was assessed by recovering peripheral blood lymphocytes and staining for CD4, Thy1.1 and Thy1.2.

Histopathology and morphometric analysis

Liver tissues were harvested from infected mice 40 hours after infection. Tissues were fixed in 10% formalin for paraffin embedding and hematoxylin-eosin (HE) staining. Inflammatory foci were assessed in pictures of HE-stained sections by morphometric analysis using the ImageJ 1.34s software. The area of inflammatory foci was normalized to the total area observed.

Statistical analysis

All data are presented as mean ± SD. Statistical significance between experimental groups was determined using the two-tailed Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

Chapter 3 Results

TLR4 deficiency is associated with impaired immune response to malaria liver stage infection

Abstract

Toll-like receptors (TLRs) are known to play an essential role in the innate recognition of pathogens and subsequent induction of host immune responses. However, the role of TLRs during liver infection of *Plasmodium*, the malaria protozoan parasite, has never been addressed. We found that TLR4-deficient mice display enhanced susceptibility to *P. berghei* sporozoite infection, whereas mice lacking TLR2 show no significant differences from wild-type control mice. Using both infected bone marrow chimeras and primary hepatocytes, we demonstrated that the resistance associated to the presence of TLR4 is mediated not only by bone marrow-derived cells but also by hepatocytes. Increased susceptibility in TLR4-mutant mice is first associated with a reduction in macrophage recruitment in response to sporozoite infection and, secondly, with impaired TNF production by macrophages in the liver. Moreover, treatments with LPS, a TLR4 ligand, at the time of sporozoite injection, decreased the levels of liver stage infection. Altogether, these findings suggest that TLR4 is implicated in the control of *Plasmodium berghei* liver infection, playing an important role in parasite recognition by the host.

Introduction

Malaria, one of the most prevalent infectious diseases, is caused by the protozoan parasite *Plasmodium* and represents a major threat to public health, creating a significant barrier to economic and social development of the most affected countries. The characterization of fundamental aspects of parasite-host interactions is critical to identify new therapeutic targets for clinical intervention.

Plasmodium sporozoites infect the liver of mammalian hosts after an *Anopheles* mosquito bite. This gives rise to a clinically silent but decisive process, in which the invading parasites replicate and give rise to thousands of merozoites. The blood stage of the infection, which constitutes the symptomatic phase of the disease, is initiated by the release of merozoites in the blood stream and subsequent erythrocyte invasion. Parasite recognition in the liver is likely to be determinant to the outcome of the disease. Therefore, the generation of an early effective immune response against malaria may well be fundamental for the control of parasite replication.

TLRs are a major, structurally related, receptor family known to be required for innate defense against most parasites. These receptors recognize distinct evolutionary conserved molecular structures derived from pathogens, and also inflammatory mediators that represent danger signals from the host, playing a key role in innate immune recognition. Acting as sensors of parasite infection, TLRs induce inflammatory and antiparasitic effector responses. Upon activation, TLRs transduce signals via a common adaptor molecule, MyD88, leading to NF-kB activation that consequently leads to the induction of a wide range of inflammatory genes (Medzhitov, Preston-Hurlburt et al. 1998; Kawai, Adachi et al. 1999; Akira and Takeda 2004). So far, 11 human TLRs and 13 mouse TLRs have been identified. TLR2 recognizes molecules from Gram-positive bacteria (peptidoglycan, lipoteichoic acid, and lipoproteins) while TLR4 has been identified as a receptor for LPS being, therefore, a key player in the immune response to Gram-negative bacteria (Poltorak, He et al. 1998; Hoshino, Takeuchi et al. 1999; Lien, Sellati et al. 1999; Schwandner, Dziarski et al. 1999; Yoshimura, Lien et al. 1999).

TLRs have been implicated in the initial recognition of protozoan parasites and early resistance to protozoan infection by the host immune system (Campos and Gazzinelli 2004; Kropf, Freudenberg et al. 2004; Nebl, De Veer et al. 2005; Minns, Menard et al. 2006). Activation of TLR and MyD88 signaling pathway has been associated with a protective effect during infection with *T. cruzi*, *T. gondii* and *Leishmania* spp (Campos and Gazzinelli 2004; Kropf, Freudenberg et al. 2004; Minns, Menard et al. 2006). Nevertheless, TLRs constitute a double-edge sword, as their activation throughout infection was also shown to be involved in the establishment of immune pathology (Gazzinelli, Ropert et al. 2004; Gazzinelli and Denkers 2006).

Although a controversial role of TLRs in mediating innate immune responses to parasite-derived molecules during malaria blood stage has been described before (Naik, Branch et al. 2000; Krishnegowda, Hajjar et al. 2005; Parroche, Lauw et al. 2007), their involvement in the recognition of *Plasmodium* liver infection has not yet been demonstrated. MyD88 signaling was reported to be implicated in the development of CM, and TLR2 and TLR9 were described as partially responsible for a fatal outcome of the infection (Coban, Ishii et al. 2007). The same authors have previously demonstrated that TLR2 and TLR9 were involved in the recognition of GPI anchors and DNA from *Plasmodium* (Coban, Ishii et al. 2005; Krishnegowda, Hajjar et al. 2005). Human patients infected with *P. falciparum* show an upregulation of TLR expression (Loharungsikul, Troye-Blomberg et al. 2008), and human genome studies link them to disease susceptibility (Mockenhaupt, Cramer et al. 2006; Mockenhaupt, Hamann et al. 2006; Khor, Chapman et al. 2007). Further evidence of TLR involvement in malaria infection was provided by the finding that *P. falciparum* infection in humans causes proinflammatory priming of human TLR responses (Ockenhouse, Hu et al. 2006; McCall, Netea et al. 2007). Accordingly, mRNA coding for several receptors and inflammatory molecules such as TLRs, MyD88, NF-κB and IFN-γ was shown to be upregulated in PBMC from patients during presymptomatic infection (Ockenhouse, Hu et al. 2006). Eventhough the above findings pointing to an important role of TLRs during malaria infection, several contradictory reports were described in the literature, most of them showing that CM development seems to be independent of MyD88 and TLR signaling (Togbe, Schofield et al. 2007). It was recently described that infected TLR2/4/9-deficient mice (MyD88-dependent TLRs) showed no differences in survival and pathogenesis after a blood-stage infection when compared to wild-type mice (Lepenies, Cramer et al. 2008). The reasons for these discrepancies are not fully understood, especially in what concerns inconsistent data obtained with similar mouse models in different laboratories.

Despite extensive studies on the role of TLRs in the blood stage of *Plasmodium* infection, the knowledge about their function in the liver stage is still very limited. Recently, Torgler *et al* proposed that cell traversal by sporozoites has a detrimental effect on parasite survival (Torgler, Bongfen et al. 2008). Non-traversed hepatocytes have the ability to respond to released host factors, resulting in a NF-κB-dependent innate immune response and, consequently, in a reduction of the infection load. Despite

hypothesizing that *Plasmodium* sporozoite-mediated hepatocyte wounding induces an innate immune response, which limits the extent of parasite infection, the authors do not demonstrate whether members of the TLR family are implicated in NF-κB activation during sporozoites infection.

Gene expression for all nine TLRs, as well as for their associated accessory molecules MyD88 and MD-2, was identified both in human primary hepatocytes and in the human hepatoma cell line HepG2 (Liu, Gallo et al. 2002). Hence, we sought to investigate the role of TLR2 and TLR4 – two TLRs known to participate in the immune response to other protozoan infections – in the innate immune response to *P. berghei* sporozoites during liver infection.

Using a murine model, we demonstrated that TLR4, but not TLR2, plays a critical role in host control of *P. berghei* sporozoite infection. We showed that two independent TLR4-natural mutant mouse strains and genetically engineered TLR4-knockout mice are more susceptible to *P. berghei* sporozoite infection. The enhanced resistance of TLR4-competent mice is mainly mediated by bone marrow-derived cells but an additional protective effect, that seems to be hepatocyte-dependent, is observed in primary hepatocyte cultures. The increase in infection levels in TLR4-knockout mice correlates with impaired TNF production by macrophages in the liver and also with decreased macrophage and DC recruitment in TLR4-knockout infected livers. Moreover, LPS treatment significantly reduces the levels of *P. berghei* sporozoite infection, providing additional evidence for a role of TLR4 activation in the modulation of host response early during infection.

Results

Mice lacking a functional TLR4 show increased susceptibility to *P. berghei* sporozoites infection

Two independent natural mutant mouse strains, B10ScN and C3H/HeJ, which have an inactivating mutation in TLR4, as well as C57BL/6 TLR4-knockout mice were infected with *P. berghei* sporozoites. An increase in the number of parasite copies in all three mutant strains relative to wild-type controls, which have a functional TLR4 molecule,

was observed by qRT-PCR of mRNA from livers of infected mice, 40 hours post-infection. Analysis of mRNA from livers of infected mice at 20 hours post-infection reveals that this increase is already observed by then (Figure 1).

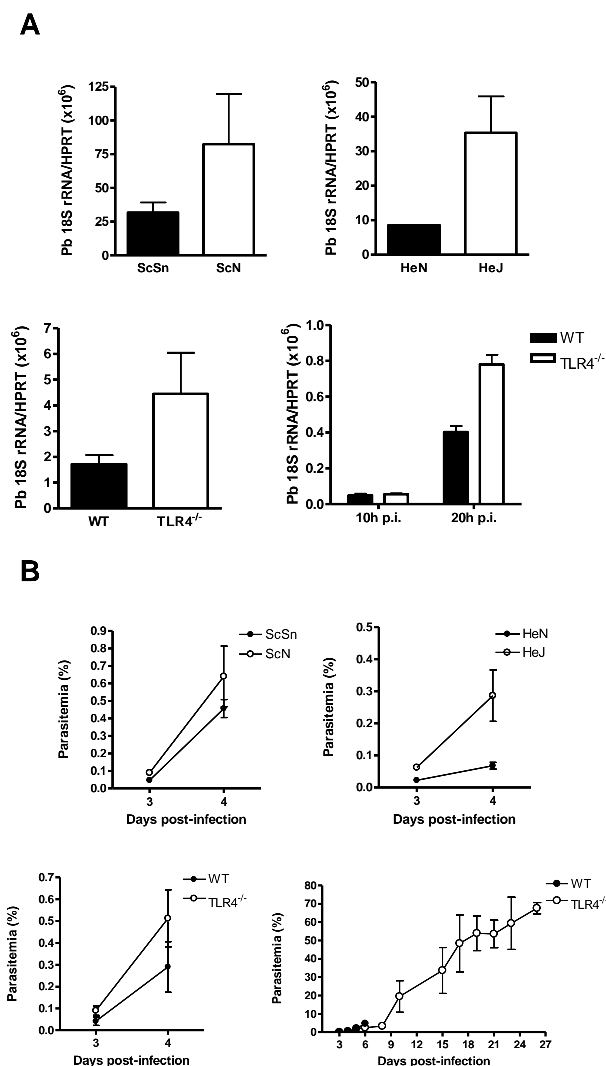


Figure 1 Mice lacking functional TLR4 are more susceptible to *P. berghei* sporozoite infection. Groups of wild-type (C3H/HeN, C57BL/10 ScSn and C57BL/6) mice, and TLR4-deficient mice (C3H/HeJ, C57BL/10 ScN and C57BL/6 TLR4-knockout), were infected with $2x10^4$ *P. berghei* sporozoites. After liver removal, 10, 20 and 40 hours after infection, the number of parasite copies was quantified by qRT-PCR of mRNA extracted from the infected livers (A).

Blood parasitemias were measured in all experimental groups by FACS analysis of blood drops, starting at day 3 post-infection. Parasitemias from mice that did not develop CM were measured until day 26 post-infection (B). Results are representative of two to ten independent experiments (mean ± SD, n=4-6, $p < 0.05$).

TLR4-mutant mice showed higher blood parasitemias at day 3 post-infection, which confirms that sporozoites have increased rates of infection in the liver and that consequently more erythrocyte-infective parasites are released in the blood stream 42 hours after liver infection (Figure 1). In contrast, mice lacking TLR2 do not show significant differences in susceptibility to infection, as confirmed by RT-PCR and by measuring blood parasitemias at day 3 post-infection (Figure 2).

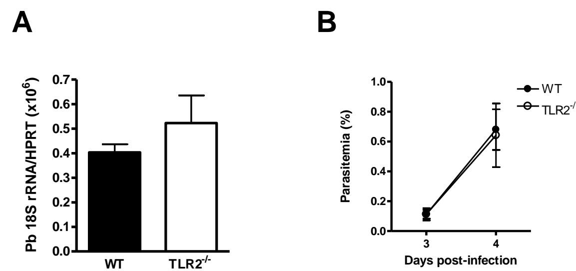


Figure 2 TLR2-knockout mice do not show significant differences from wild-type controls after infection with *P. berghei* sporozoites. Wild-type and TLR2-mutant C57BL/6 mice were infected with $2x10^4$ *P. berghei* sporozoites. After liver extraction, 40 hours post-infection, the number of parasite copies was quantified by qRT-PCR of mRNA extracted from the infected livers (A). Blood parasitemias were measured after day two post-infection in all experimental groups by FACS analysis of blood drops (B) (mean ± SD, n=5).

Altogether, the results we obtained after infection of TLR4-deficient mice with three different genetic backgrounds indicates that TLR4 plays a critical role in the immune response to *P. berghei* sporozoites infection. Host response to the parasite infection in the liver might determine the outcome of the disease, once a higher percentage of

parasites will develop in the blood stage of the infection due to inefficient parasite recognition. In fact, none of the TLR4-deficient mice infected with sporozoites died with CM, dying of hiperparasitemia after day 20 post-infection instead. C57BL/6 wild-type controls all died from CM at day 6 or 7 post-infection (Figure 3). Thus, these observations suggest that the immune mechanisms elicited throughout infection and development of *Plasmodium* parasites in the liver will be important in the outcome of the blood stage of the disease.

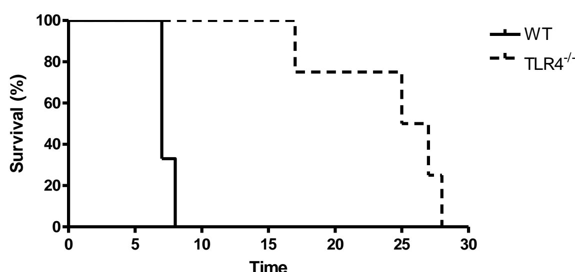


Figure 3 Increased survival of infected TLR4-knockout mice. Wild-type and TLR4-knockout C57BL/6 mice were infected with $2x10^4$ *P. berghei* sporozoites. Infected mice were monitored daily for clinical symptoms (n=6).

LPS treatment decreases the levels of infection

Our findings suggest that TLR4 might be activated during *P. berghei* sporozoite liver infection, which then interferes with the normal course of infection. Thus, we next sought to test whether infection-independent TLR4 activation interferes with the levels of infection. To this end, we used the TLR4 ligand LPS, a component of the cell wall of Gram-negative bacteria like *E. coli*, in *in vivo* and *in vitro* infections. Binding of LPS to TLR4 leads to the activation of an intracellular signaling cascade resulting in a proinflammatory response characterized by the expression of TLR-response genes such as IL-1, IL-6, and TNF. Both C57BL/6 and BALB/c mice were injected i.p. with 10 micrograms of LPS per mouse, immediately prior to *P. berghei* sporozoites infection. The C57BL/6 mice treated with LPS revealed a 94% reduction in the number of parasite copies in the liver, collected 40 hours after infection, when compared to the control group (Figure 4A). Similar reductions were observed when mice were allowed to go through blood stage infection. By day 4, while the average parasitemia of the control group was 0.15%, blood-stage parasites were only detectable in Giemsa-stained blood

smears by day 6 post-infection in the mice treated with LPS (Figure 4B). Moreover, eight out of nine of the infected BALB/c mice have never developed detectable parasitemia after injection of $2x10^4$ *P. berghei* sporozoites.

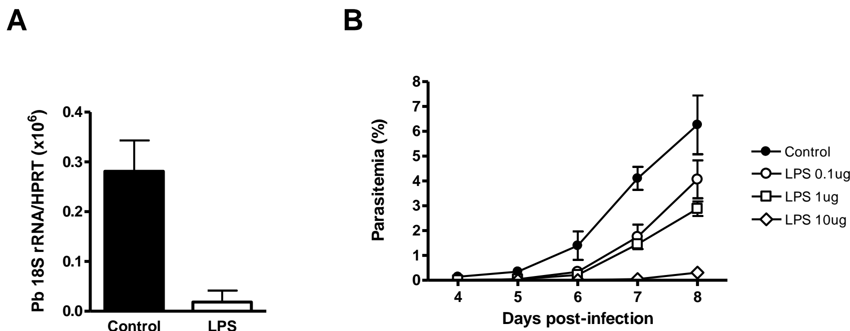


Figure 4 *In vivo* treatment with LPS reduces the number of developing parasites. Wild-type C57BL/6 mice were injected i.p. with 0.1, 1 and 10µg of LPS per mouse and right after, infected i.v. with $2x10^4$ *P. berghei* sporozoites. The number of parasite copies after 10µg of LPS injection was quantified by qRT-PCR of mRNA extracted from the infected livers collected 40 hours after infection (A). Blood parasitemias were measured after day three post-infection in all experimental groups, by FACS analysis of blood drops (B). Results are representative of five independent experiments (mean ± SD, n=3-6, $p < 0.01$).

Next, we tested whether the protection conferred by LPS was hepatocyte-mediated. Thus, we treated LPS responsive Hepa1-6, HepG2 cells (mouse and human hepatoma cell lines, respectively) and C57BL/6 mouse-derived hepatocytes with 10 micrograms of LPS per ml, at the time of sporozoite infection. We found that TLR4 activation by LPS decreases the infection levels in hepatoma cells (Figure 5) and mouse primary hepatocytes. To assess the effect of LPS treatment in the development of the parasite *in vitro*, we quantified the number and the area of parasite EEFs at 24 hours after infection. LPS treatment not only decreases the number of EEFs (Figure 5A) but also leads to a reduction in the area of the developing EEFs (Figure 5B), which constitutes a sign of arrested or abnormal development.

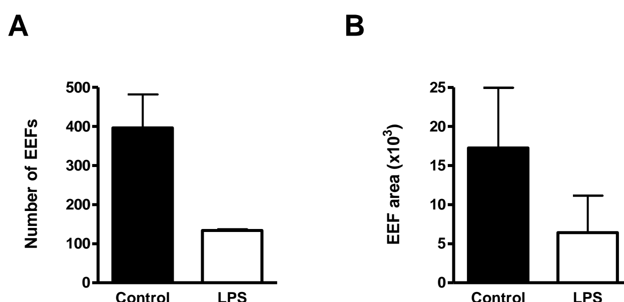


Figure 5 LPS is detrimental to parasite development *in vitro.* Hepa1-6 cells were incubated with 10µg of LPS troughout infection with $2x10^4$ *P. berghei* GFP-sporozoites. After fixation, 24 hours post-infection, GFP-positive EEFs were counted (A). The area of the EEFs was quantified by ImageJ (B) (mean ± SD, n=3, $p < 0.05$).

The *in vitro* effect of LPS does not represent as dramatic a decrease in infection as that observed *in vivo* suggesting that LPS-mediated protection *in vivo* might be the result of cumulative protective effects from immune cells and hepatocytes. In support of this hypothesis, primary hepatocytes lacking TLR4 expression reveal only a small but significant increase in susceptibility to *P. berghei* sporozoite infection, when compared to primary hepatocytes from wild-type control mice (Figure 6). Altogether, these data suggest that hepatocytes might respond to *Plasmodium* liver stage infection through TLR4 activation by initiating a cascade of immune events that will be detrimental for parasite development and disease outcome. Moreover, other effects besides those occurring on hepatocytes seem to be important for the phenotype observed *in vivo*.

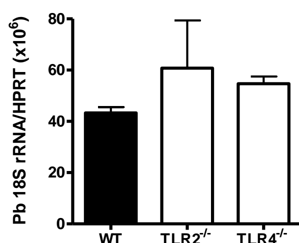


Figure 6 Primary hepatocytes from mice that lack TLR4 expression are more susceptible to sporozoite infection. Wild-type, TLR2- and TLR4-knockout C57BL/6 mouse-derived hepatocytes were cultured *in vitro* and infected with *P. berghei* sporozoites. Quantitative PCR analysis was performed to measure the number of parasite copies from cell lysates collected 40 hours after infection (mean ± SD, n=3, $p < 0.05$).

TLR4 bone marrow chimeras (WT BM > WT or TLR4$^{-/-}$ mice) lose the increased susceptibility to infection

In the absence of TLR4, mice reveal an impaired immune response and primary hepatocytes show increased susceptibility to infection. However, the role of TLR4-expressing immune cells in this process remains unclear. To clarify the relative importance of TLR4-associated resistance in hepatocytes and/or hematopoietic (immune) cells, we performed mouse bone-marrow chimeras. First, wild-type and TLR4-knockout C57BL/6 mice (Thy1.2) were lethally irradiated and reconstituted with bone marrow-derived cells from TLR4-suficient mice (Thy1.1). We performed a flow-cytometry analysis screening after staining cells for Thy1.1 and Thy1.2 to confirm that both groups of mice were fully reconstituted with wild-type bone marrow-derived cells from the C57BL/6 Thy1.1 donors. Two to three months later, to allow the regular turnover of the macrophage population, which is to some extent resistant to irradiation and consequently not fully depleted, the bone marrow chimeras were infected with *P. berghei* sporozoites. TLR4-knockout mice that had been reconstituted with TLR4-competent bone marrow lose the increased susceptibility to infection, as shown by qRT-PCR of mRNA from livers of mice sacrificed 40 hours post-infection (Figure 7A). These results were corroborated by following the parasitemias of chimeric C57BL/10 ScSn and C57BL/10 ScN (TLR4-deficient) fully reconstituted with wild-type bone marrow. By day 3 post-infection, these mouse chimeras did not display significant differences in blood parasitemias (Figure 7B). These data demonstrate a role for hematopoietic cells in mediating the TLR4-associated resistance to infection.

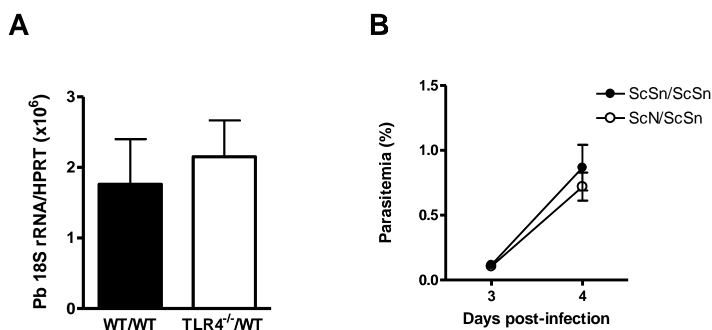


Figure 7 Bone marrow chimeric mice (WT BM > WT mice and WT BM > TLR4$^{-/-}$ mice) show similar levels of infection. Wild-type C57BL/6 and C57BL/10 ScSn and TLR4-deficient C57BL/6 and C57BL/10 ScN recipient mice (Thy1.2) were lethally irradiated and reconstituted with wild-type bone marrow from C57BL/6 Thy1.1 and C57BL/10 ScSn donor mice. The degree of reconstitution was 96-100%. Mouse chimeras were infected with $2x10^4$ *P. berghei* sporozoites. The number of parasite copies was quantified by qRT-PCR of mRNA extracted from infected livers from C57BL/6 chimeric mice, collected 40 hours post-infection (A) Blood parasitemias were measured after day two post-infection in C57BL/10 mouse chimeras, by FACS analysis of blood drops (B) (mean ± SD, n=6-8).

Mice with TLR4-deficient hematopoietic cells (TLR4$^{-/-}$ BM > WT or TLR4$^{-/-}$ mice), show a small but significant difference in susceptibility to liver infection

After analyzing the outcome of introducing wild-type bone marrow-derived cells in mice lacking TLR4 in the response to infection, we sought to determine the effect of TLR4 absence in tissues other than bone marrow-derived cells. Mouse bone marrow chimeras were obtained as described before. Here, wild-type and TLR4-knockout C57BL/6 mice were lethally irradiated and reconstituted with bone marrow-derived cells from TLR4-deficient mice. Such chimeras, therefore, do not differ in TLR4 expression by bone marrow-derived (immune) cells, but show differential TLR4 expression in all other body tissues, including hepatocytes. We found that when bone marrow-derived (immune) cells lack TLR4 expression, TLR4 absence in other tissues imparts a moderate but significant increase in susceptibility to infection (Figure 8). Again, these observations suggest that the increase in the number of parasite copies described in infected TLR4-deficient mice also appears to be mediated by hepatocytes, as previously indicated by the infection of wild-type and TLR4-knockout C57BL/6 mouse-derived primary hepatocytes.

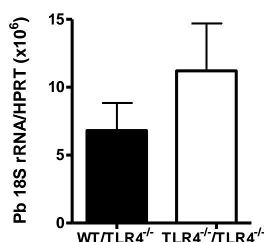
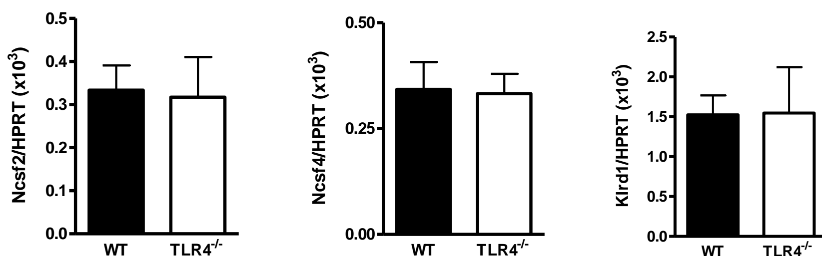


Figure 8 TLR4-/- BM > TLR4-/- mouse chimeras are more susceptible to *P. berghei* sporozoite infection than TLR4-/- BM > WT mice. TLR4-suficient and TLR4-deficient C57BL/6 recipient mice were lethally irradiated and reconstituted with TLR4-deficient bone marrow from C57BL/6 TLR4-knockout donor mice. Chimeric mice

were infected with $2x10^4$ *P. berghei* sporozoites. The number of parasite copies was quantified by qRT-PCR of mRNA extracted from the infected livers collected 40 hours post-infection (mean ± SD, n=8, $p < 0.05$).

Macrophage recruitment is reduced in TLR4-knockout mice during *P. berghei* sporozoite infection

In order to clarify which immune cells are responsible for the increased susceptibility to sporozoite infection in TLR4-deficient mice, we analysed the expression of leukocyte markers throughout infection in the liver. We compared wild-type and TLR4-knockout C57BL/6 mice for expression of the following genes: Ncf2 and Ncf4 (neutrophil cytosolic factor 2 and 4, a neutrophil marker), Klrd1 (killer cell lectin-like receptor subfamily D member 1, a NK cell marker), CD68 antigen (expressed by tissue macrophages and cells of myeloid/mononuclear lineage), Mgl2 (macrophage galactose N-acetyl-galactosamine specific lectin 2, a macrophage cell surface carbohydrate-binding molecule) and CD3 (a TCR adaptor molecule). Among these genes, we found that the expression of CD68 and Mgl2 in the livers of TLR4-knockout infected mice was decreased at 40 hours post-infection, suggesting that there is less macrophage recruitment in TLR4-deficient infected livers than in the livers of wild-type mice (Figure 9). The other cell markers did not show significant differences in expression between the two groups of mice, indicating that there are no differences in numbers of neutrophils, NK cells and T cells in infected mice that either lack or express TLR4 (Figure 9).



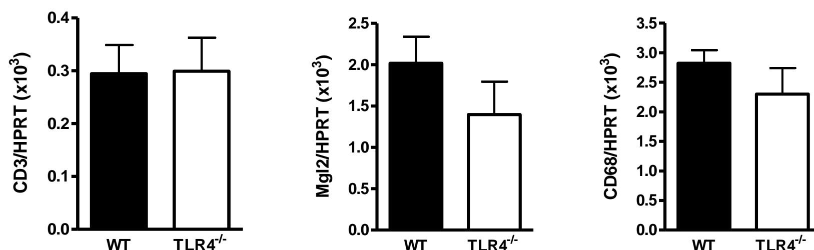
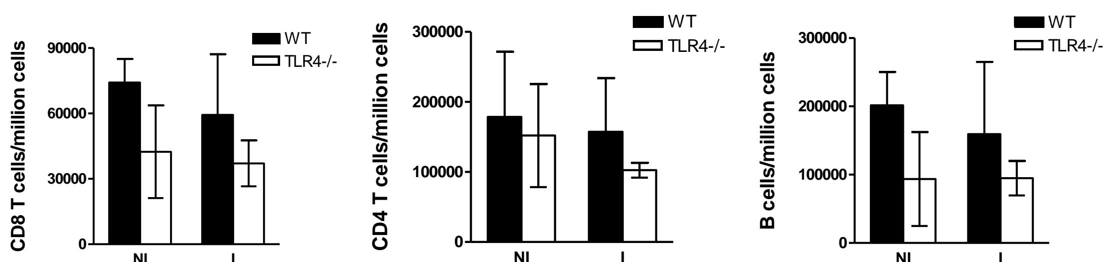


Figure 9 CD68 and Mgl2 are less expressed in livers of TLR4-knockout infected mice. Wild-type and TLR4-knockout C57BL/6 mice were infected with $2x10^4$ *P. berghei* sporozoites. Livers were collected 40 hours after infection and gene expression levels of several leukocyte markers, Ncf2 and Ncf4, Klrd1, CD3, Mgl2 ($p < 0.05$) and CD68 ($p < 0.05$), were determined by quantitative RT-PCR (mean ± SD, n=6).

Macrophage and DC recruitment in livers of infected mice is TLR4-mediated

To characterize the immune response in the liver and to determine whether TLR4 deficiency would lead to a decrease in activation and recruitment of immune cells, we isolated liver non-parenchymal cells from wild-type and TLR4-knockout C57BL/6 infected mice, 40 hours after infection.

We show that there are no significant differences in lymphocyte numbers between naïve and infected wild-type C57BL/6 mice at 40 hours post-infection (Figure 10). There is a small but non-significant increase in the frequency of both CD4 and CD8 activated T cells and, consequently, IFN-γ production, and a clear activation of NK T cells, which indicates that T cells are activated at this stage of the infection (Figure 11).



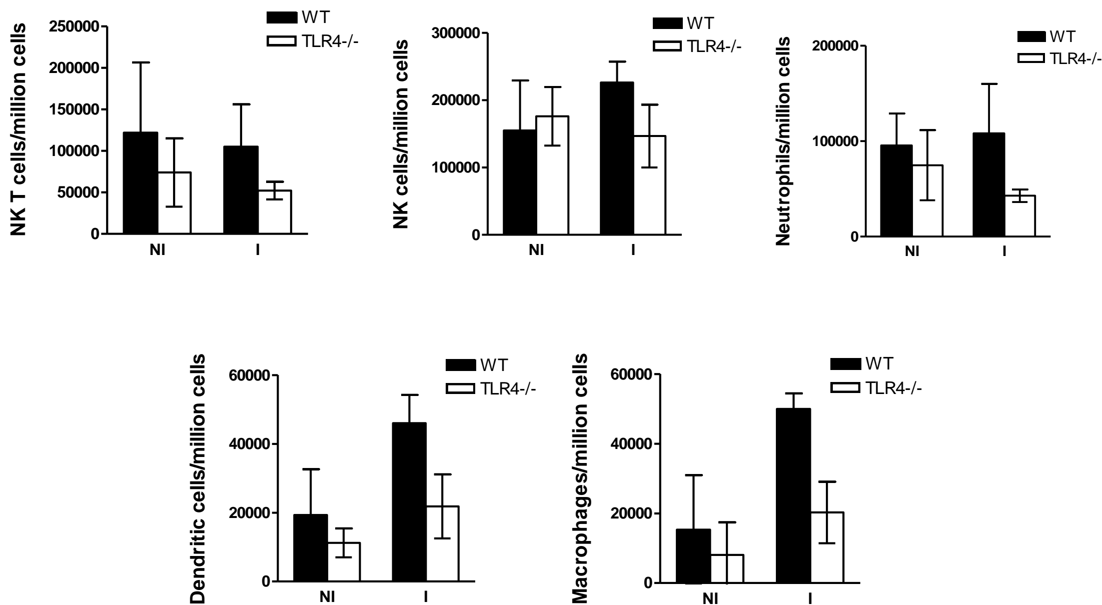


Figure 10 Cell numbers of non-parenchymal populations in the liver. Liver non-parenchymal cells from naïve and infected wild-type and TLR4-knockout C57BL/6 mice were purified and stained for different cell markers: CD8 T cells, CD4 T cells, B cells, NK T cells, NK cells, neutrophils, DCs ($p < 0.05$) and macrophages ($p < 0.05$). Mice were infected with $1x10^5$ *P. berghei* sporozoites (NI-non-infected mice, I-infected mice; mean ± SD, n=4).

Infected wild-type mice also display a well-defined increase in both DC and macrophage populations, and a non significant increase in NK cells and neutrophils (Figure 10). This increment in cell populations does not occur in TLR4-knockout mice, which indicates that both macrophage and DC recruitment to the tissues is TLR4-mediated. Additionally, CD4 and CD8 activation also seems to be at least partially mediated by TLR4 since both CD4 and CD8 do not increase their expression of CD69, an early activation marker, in infected mice lacking TLR4. In contrast, NK T cells are activated as infected wild-type mice, suggesting that NKT cell activation is TLR4-independent. It is interesting to observe that macrophages from TLR4-knockout infected mice produce less TNF than wild-type infected controls, which shows that macrophage activation is also mediated by TLR4 during liver stage of infection.

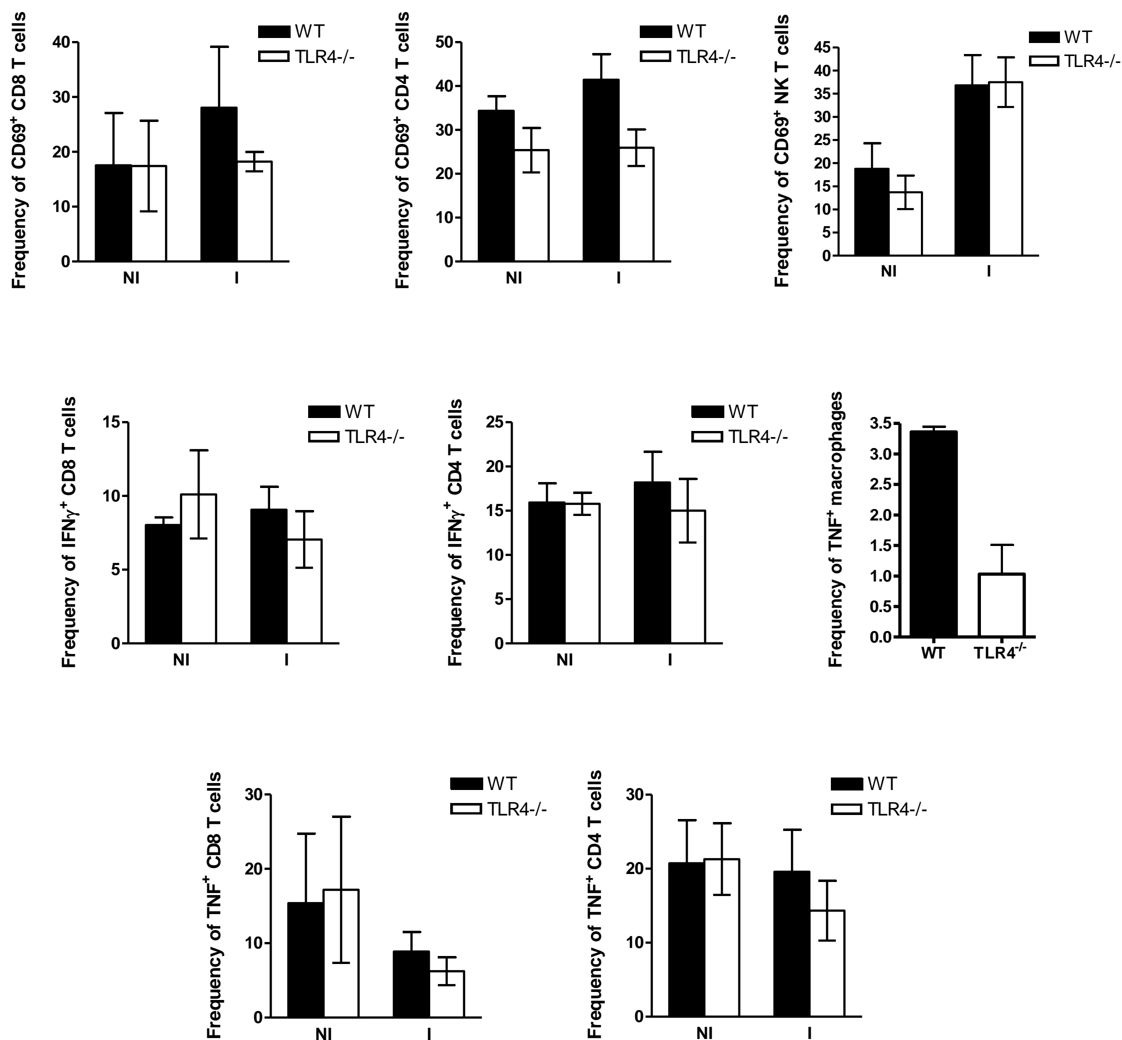


Figure 11 Frequencies of activated non-parenchymal populations in the liver. Liver non-parenchymal cells from naïve and infected wild-type and TLR4-knockout C57BL/6 mice were purified and stained for different cell markers: CD69+ CD8 T cells, CD69+ CD4 T cells ($p < 0.05$), CD69+ NK T cells ($p < 0.05$); CD8 T cells and CD4 T cells stained for intracellular expression of IFN-γ and TNF, respectively and macrophages from infected mice stained for intracellular expression of TNF ($p < 0.05$). Mice were infected with 1×10^5 *P. berghei* sporozoites (NI-non-infected mice, I-infected mice; mean ± SD, n=4).

TLR4 activation during liver infection partially mediates the infiltration of immune cells in the tissues

Activation of TLR4 by sporozoites in hepatocytes as well in circulating immune cells is necessary for the activation of the immune response. In the absence of TLR4, macrophage recruitment is reduced, which results in an increased parasite proliferation in the liver. To observe the extent of tissue inflammatory response, we analyzed histological sections of wild-type and TLR4-knockout C57BL/6 mouse livers, infected with *P. berghei* sporozoites. As expected, we observed the existence of small inflammatory foci surrounding infected hepatocytes in wild-type mice (van de Sand, Horstmann et al. 2005). However, the total area of these inflammatory foci was significantly lower in TLR4-knockout mice (Figure 12A). Morphometric analysis was used to quantify the size occupied by infiltrated inflammatory cells and revealed that the area occupied by the inflammatory foci on C57BL/6 wild-type mouse liver sections is 2.5-fold increased when compared to TLR4-knockout mice (Figure 12B). TLR4 activation during *P. berghei* liver infection induces an inflammatory response which leads to the recruitment of immune cells to the infection site.

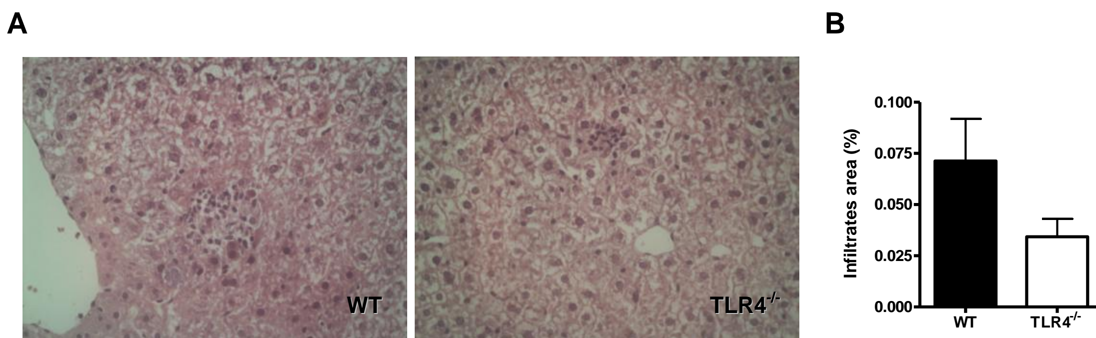


Figure 12 Recruitment of immune cells to *P. berghei* infected livers is partially mediated by TLR4. Liver sections showing representative inflammatory foci in wild-type and TLR4-knockout C57BL/6 mice after infection with $1x10^5$ *P. berghei* sporozoites (A). The area of cell infiltrates was quantified by ImageJ (B) (mean ± SD, n = 3, $p < 0.05$).

Discussion

In recent years, substantial advances have been made in our knowledge about host immune responses to parasite infections. Despite considerable progress, our understanding of how *Plasmodium* interacts with the host immune system is far from being fully elucidated. *Plasmodium* infection is still increasing the burden of malaria worldwide and the associated drug resistance is a rising phenomena that requires new clinical approaches. Thus, it is essential to improve our knowledge about the biology of malaria infection. We focused on the interactions between the host immune system and *P. berghei* liver parasites mediated by some members of the TLR family. Their role in the blood stage of malaria infection, and namely in the development of cerebral malaria, is controversial. In addition, very little is known concerning the role of TLRs during the liver stage of infection. Recently, it was reported that the wounding of hepatocytes by sporozoites activates NF-κB, which might implicate triggering of TLR signaling. In the present chapter, we show that while we could not determine a role for TLR2 in the immune response to sporozoite infection, mice lacking TLR4 display enhanced susceptibility to *P. berghei* liver infection. Increased levels of parasite copies were observed in the liver of TLR4-deficient mice, as well as the expected higher parasitemias at the beginning of blood stage infection. To our knowledge this is the first report that Plasmodium infection triggers a TLR, and stresses the importance of the liver phase of malaria as an immunological significant stage.

Mouse chimeras reconstituted with wild-type bone marrow reveal that the increased susceptibility associated with the absence of TLR4 is mainly mediated by hematopoietically-derived immune cells. However, by infecting primary hepatocytes and bone marrow chimeric mice reconstituted with a TLR4-deficient hematopoietic cell population, we showed that this phenotype is also partially mediated by hepatocytes. This finding is not entirely unexpected: hepatocytes constitute obligatory host cells in the *Plasmodium* life cycle, and the fact that they express a plethora of innate receptors like TLRs increases the chance for the appearance of recognition mechanisms during the co-evolution of host and parasite. The finding that TLR4 is activated in hepatocytes during Plasmodium infection calls for the question of what are the consequences of the recognition of the parasite. By showing that TLR4 activation by LPS, remarkably

reduces the infection levels and hinders parasite development, it appears that TLR4 activation in hepatocytes has significant consequences during liver invasion by *Plasmodium*. Remarkably, we show that injection of 10 micrograms of LPS per mouse, at the time of sporozoite infection, can prevent BALB/c mice infection and decrease by 94% the infection of C57BL/6 mice, which are more susceptible to *P. berghei* sporozoite infection.

At twenty hours post-infection we already observe an increased susceptibility of TLR4-deficient mice to infection. This phenotype correlates with defective macrophage responses *in vivo*, not only because there is a decrease in macrophage recruitment in TLR4-deficient infected livers, but also because they are less activated and therefore produce less TNF than wild-type controls. TNF release induces a pro-inflammatory response, which appears to be detrimental for the parasite and thus, limits the extent of parasite infection. Moreover, there is a clear increase in the number of DCs in the liver of infected wild-type mice whereas in the absence of TLR4, this influx does not occur. It was previously described that when DCs are sensitized directly with viable sporozoites, they stimulate CD8 T cells *in vivo* (Plebanski, Hannan et al. 2005), therefore, TLR4-mediated recruitment and activation of DCs might play an important role in the immune response against *P. berghei* infection. The increase in the number of DCs present in the liver after infection allows for the mounting of an adaptive, specific immune response by inducing differentiation of helper T cells, a process that can is augmented by TLR activation. The fact that we do not observe significant differences in T cell numbers is expected at this earlier stage of the infection. However, we could already observe an increase in the activation of both CD8 and CD4 T cells (expressing CD69) and of IFN-γ-producing Th1 and CD8 T cells, which while not being significant, can still be relevant if we consider the reduced fraction of the total number of resident T cells in the liver present at infection foci. These cells were probably mainly activated by the considerable amount of DCs in the liver at this stage. Furthermore, there might be also some direct activation of T cells TLRs by the parasite or by any endogenous ligands released during infection (Caramalho, Lopes-Carvalho et al. 2003). To get a cleared view at the immune cell activation process at this stage, injection of millions of sporozoites might induce a more vigorous and clearcut response, but this would be further away from how natural infections occur, where a reduced number of sporozoites infect the liver. The small

proportion of infiltrates in wild-type mice infected with $1x10^5$ sporozoites – less than 0.1% – also demonstrates how small is the percentage of infected hepatocytes. Nevertheless there is a significant difference in the number of infiltrates in TLR4-knockout mice, which is clearly diminished.

In summary, first, both macrophage and DC numbers are increased during infection as well as activated T cells, NK cells and neutrophils, to a smaller extent, which indicates that they are all involved in host immune response to *Plasmodium* infection. Secondly, their recruitment to the infection site seems to be clearly mediated by TLR4 both in macrophages and DCs and at least partially mediated in the remaining populations of immune cells.

Finally, we showed that C57BL/6 mice lacking TLR4 display increased susceptibility to liver stage infection with *P. berghei* sporozoites, suggesting that TLR4 is required for a stronger immune response to *P. berghei* sporozoite infection in the liver. Nevertheless, unlike C57BL/6 controls, these mice do not develop CM. All wild-type mice die of CM between day 6 and 8 post-infection, while TLR4-knockouts only die later, between days 17 and 27, reaching very high atypical levels of blood parasitemias. These data indicate that TLR4 might be required for the development of CM when mice have been infected with the liver form of the parasite. It has been reported in the literature that mice with a C57BL/6 background, without a functional TLR4, develop CM as wild-type controls after a blood stage infection. Altogether, these findings emphasize the relevance of the immune events occurring during liver stage following sporozoite infection, and suggest that they might be important in the outcome of the disease.

We propose that TLR4 activation both in immune cells, namely in macrophages and DCs, and in hepatocytes is required for a more efficient control of parasite infection. These data suggest an important role for TLR4 in parasite recognition by the host innate immune system, whether by direct recognition of a parasite TLR ligand or by indirect recognition of exposed or released endogenous ligands induced by parasite infection. An extensively described process like cell traversal is one possible event that can trigger TLR4-mediated signaling, activating host defense mechanisms.

The findings presented herein thus confirm that TLR4 plays a role in host resistance to *P. berghei* and provide the first clear evidence for TLR involvement in the control of malaria infection *in vivo*.

Chapter 4 Results

TLR9 is required for *Plasmodium berghei* liver infection

Abstract

Through recognition of pathogen-associated molecules, TLRs are known to participate in the control of infectious diseases. TLR9 is an intracellular receptor which recognizes foreign DNA molecules and is expressed by hepatocytes. Given that *Plasmodium* is an obligatory intracellular parasite, we hypothesized that TLR9 could be involved in the immune response in the liver stage of malaria. Our results show that unlike TLR4, which is implicated in the control of *P. berghei* liver infection and in other parasitic infections, TLR9 does not seem to play a role in host resistance to *Plasmodium* infection. In fact, TLR9 is shown to be beneficial for the parasite at early stages of *P. berghei* malaria infection. By infecting mice lacking TLR9 expression, we observed increased resistance to liver infection with *P. berghei* sporozoites. In addition, by infecting TLR9-deficient primary hepatocytes and mouse chimeras, it appears that this resistance is hepatocyte-mediated, and not caused by lack of TLR9 expression in hematopoietic immune cells. However, treatment of mice and cells with CpG, a TLR9 ligand, also led to an increase in resistance to sporozoite infection, indicating that TLR9 is differentially activated or having distinct effects in different cell types.
The role of this innate receptor during *P. berghei* sporozoites infection still requires more attention. One can predict that parasite-host interactions mediated by TLRs are complex and differ among protozoan parasites, and that TLR activation may differentially affect the outcome of the disease.

Introduction

We showed previously that TLR4-deficient mice display enhanced susceptibility to liver infection by *P. berghei* sporozoites. This suggests that TLR4-dependent signaling is

activated during infection and is involved in host control of *P. berghei* sporozoite infection.

We then sought to determine whether TLR9 would influence the course of malaria liver infection. TLR9 is known as a receptor for DNA, mostly of unmethylated CpG-containing DNA. In malaria blood-stage infection, it has been shown that DCs are activated via a TLR9/MyD88-dependent pathway and that hemozoin mediates this activation (Coban, Ishii et al. 2005). More recently, others have shown that hemozoin plays a specific role in presenting parasite DNA to the intracellular TLR9, although it is not capable of stimulating the innate immune system by itself (Parroche, Lauw et al. 2007). Still, the importance of TLR9 signaling for the course of blood stage infection has been questioned, as infection of TLR2/4/9-deficient triple-knockout mice with blood stage parasites showed no differences regarding survival and pathogenesis, when compared to wild-type control mice (Lepenies, Cramer et al. 2008).

Despite the above mentioned data concerning the effect of TLR9 in malaria infection with blood-stage parasites, it remains an open question whether the same is observed during liver stage infection.

Concerning the role of TLR9 in *P. berghei* liver infection, we observed a drastic reduction in parasite copies detected in the liver of TLR9-knockout mice following sporozoite infection. By infecting primary hepatocytes and bone-marrow mouse chimeras we concluded that this effect is only mediated by hepatocytes, differently from what was found for infected TLR4-knockout mice, where immune cells also played an important role. Surprisingly, similarly to what happens after LPS treatment, generalized TLR9 activation by administration of CpG to infected mice or cultured hepatocytes *in vitro* decreases the levels of infection.

It is commonly accepted that TLRs play a role in recognition and activation of the innate immune response against parasites. In this chapter, we show that TLR9 expression in hepatocytes seems to be beneficial for *P. berghei* liver infection. So far, it has never been shown that a TLR accounts for an increased resistance to infection, and these results open important questions about the interaction between host and parasite during the initial steps of malaria infection.

Results

TLR9-knockout mice display increased resistance to *P. berghei* sporozoite infection

We demonstrated previously that mice lacking TLR2 expression do not display significant differences from wild-type controls in liver infection with *P. berghei* sporozoites, whereas in the absence of TLR4, mice showed to be more susceptible to infection. This observation suggests an important role of TLR4 in *Plasmodium* recognition and, therefore, in the control of infection. To date, lack of TLR expression has been associated with susceptibility to infection, not only in malaria, as we described, but also in other infectious diseases. We then sought to characterize the role of TLR9 during liver stage and a distinct response to infection was observed in TLR9-deficient mice infected with *P. berghei* sporozoites. Strikingly, in the absence of TLR9, C57BL/6 mice are more resistant to *P. berghei* sporozoites infection than wild-type controls (Figure 1).

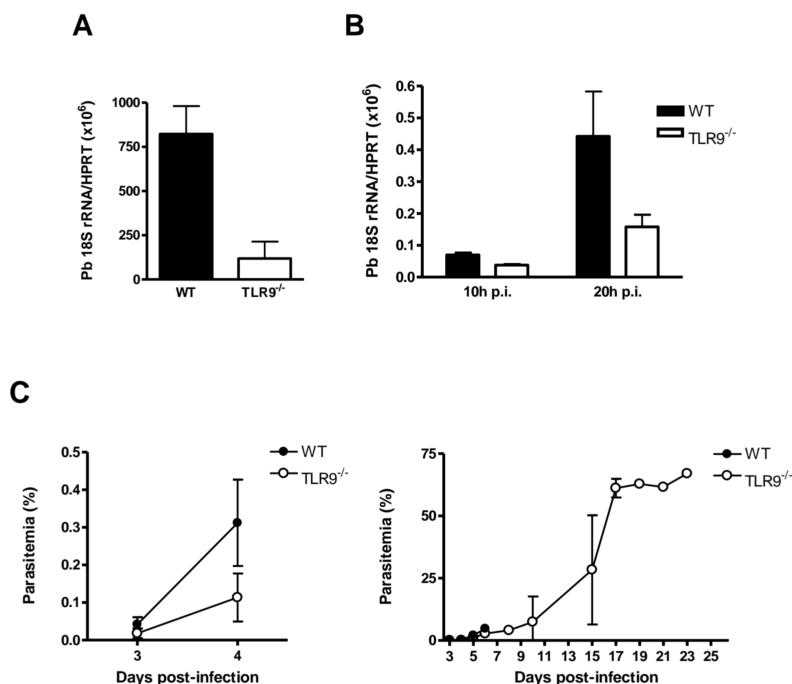


Figure 1 Mice lacking TLR9 expression are more resistant to *P. berghei* sporozoite infection. Wild-type and TLR9-knockout C57BL/6 mice were infected with $2x10^4$ *P. berghei* sporozoites. After liver removal, 10, 20 (B) and 40 (A) hours after infection, the number of parasite copies was quantified by qRT-PCR of mRNA extracted from the livers. Blood parasitemias were measured in both experimental groups by FACS analysis of blood drops collected from day 2 post-infection. Parasitemias from mice that did not develop CM were measured until day 25 post-infection (C). Results are representative of two to six independent experiments (mean ± SD, n=4-6, $p < 0.05$).

A reduction in the number of parasite copies was observed by quantitative RT-PCR of mRNA from livers of TLR9-knockout infected mice when compared to wild-type infected mice, collected 40 hours post-infection. Mice lacking TLR9 expression displayed an 80% reduction in the number of parasite copies when compared to wild-type controls (Figure 1A). Additionally, parasites were only detected in the blood of these mice at day 4 post-infection, while in wild-type controls blood stage parasitemias were already positive at day 3 post-infection (Figure 1C). Moreover, the resistance to infection associated with TLR9 deficiency was observed at early stages of parasite development in the liver. At 10 hours post-infection the parasite load in livers of mice infected with $2x10^4$ *P. berghei* sporozoites were clearly reduced when compared to wild-type controls (Figure 1B). These lower numbers of parasite copies were maintained throughout infection, suggesting that TLR9 plays a role at very early stages of liver infection, but might influence the blood stage of infection as well. To access survival after infection with *P. berghei* sporozoites, both wild-type and TLR9-knockout C57BL/6 mice were monitored daily for clinical symptoms. It was observed that most of the TLR9-knockout mice (70%) died with CM, as did the wild-type controls (Figure 2).

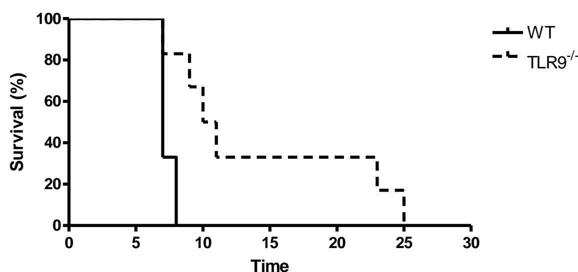


Figure 2 Survival of infected mice. Wild-type and TLR9-knockout C57BL/6 mice were infected with $2x10^4$ *P. berghei* sporozoites. Infected mice were monitored daily for clinical symptoms (n=6).

TLR9-deficient hepatocytes display an increased resistance to *P. berghei* sporozoite infection

To better understand which cells are responsible for the resistance to infection observed *in vivo*, hepatocytes from wild-type and TLR9-knockout C57BL/6 mice were purified, cultured and infected with *P. berghei* sporozoites. In the absence of TLR9, primary hepatocytes also reveal an increase in resistance to infection when compared to wild-type controls (Figure 3). These observations suggest that the resistance described *in vivo*, which is associated to lack of TLR9 expression, is at least partially mediated by hepatocytes. Importantly, we can conclude that TLR9 plays a role in hepatocyte susceptibly to infection.

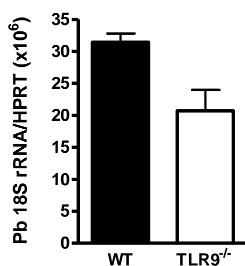


Figure 3 Primary hepatocytes from TLR9-knockout mice are more resistant to *P. berghei* sporozoite infection than wild-type controls. Pools of wild-type and TLR9-knockout C57BL/6 mouse-derived hepatocytes were cultured *in vitro* and infected with *P. berghei* sporozoites. Quantitative RT-PCR analysis was performed to measure the number of parasite copies from cell lysates collected 40 hours after infection (mean ± SD, n=3, $p < 0.05$).

TLR9 chimeras (WT BM > TLR9$^{-/-}$ mice) maintain an increased resistance to infection

In the absence of TLR9, both mice and primary hepatocytes show an enhanced resistance to infection. To determine the role of hematopoietically-derived immune cells, during liver infection with *P. berghei*, we produced mouse bone-marrow chimeras. First, wild-type and TLR9-knockout C57BL/6 mice (Thy1.2) were lethally irradiated and reconstituted with bone marrow-derived cells from TLR9-suficient mice (Thy1.1). In order to confirm that both groups of mice were fully reconstituted with wild-type bone marrow-derived cells from the C57BL/6 Thy1.1 donors, we performed a flow-cytometry analysis after staining peripheral blood lymphocytes for Thy1.1 and Thy1.2. Two to three months later, to allow the regular turnover of the radiation-resistant macrophage population, the chimeras were infected with $2x10^4$ *P. berghei* sporozoites. qRT-PCR

analysis of mRNA from livers of mice sacrificed 40 hours post-infection revealed that TLR9 chimeric mice maintain the increased resistance to infection observed in the TLR9-knockout mice (Figure 4A). These data were supported by following parasitemias from the infected TLR9 chimeras, fully reconstituted with wild-type bone marrow, which showed a significant decrease in blood parasitemias at day 3 post-infection when compared to wild-type control mice, also irradiated and reconstituted with wild-type bone marrow (Figure 4B). These data exclude a potential role of hematopoietically-derived cells in the TLR9-mediated resistance to infection.

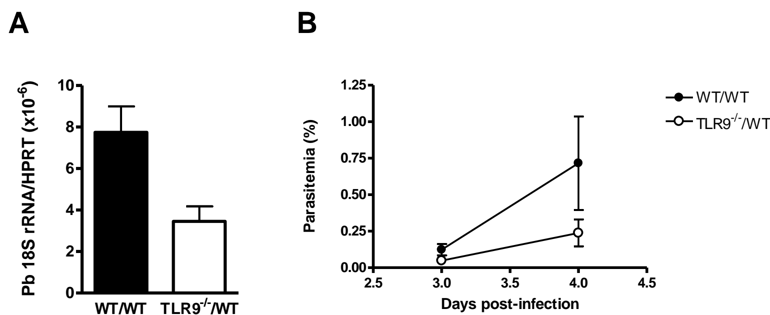


Figure 4 TLR9-knockout increased resistance to *P. berghei* infection is not mediated by bone marrow-derived cells. Wild-type and TLR9-knockout C57BL/6 recipient mice (Thy1.2) were lethally irradiated and reconstituted with wild-type bone marrow from C57BL/6 Thy1.1 donor mice. The degree of reconstitution was >96%. Bone marrow chimeric mice were infected with $2x10^4$ *P. berghei* sporozoites. The number of parasite copies was quantified by qRT-PCR of mRNA isolated from the infected livers collected 40 hours post-infection (A). Blood parasitemias were measured from day two post-infection in all animal groups, by FACS analysis of blood drops (B) (mean ± SD, n=6, $p < 0.05$).

TLR9-deficient bone marrow chimeras ($TLR9^{-/-}$ BM >$TLR9^{-/-}$ mice) show a significant increase in resistance to infection with *P. berghei* sporozoites

The introduction of wild-type bone marrow-derived cells in mice lacking TLR9 led to the conclusion that these TLR9-suficient hematopoietic cells are not mediating the increased resistance to infection observed in these mice. The converse experiment was

then performed, where we sought to confirm whether TLR9-deficient hematopoietically-derived cells would have any impact on the disease progression. We produced mouse bone-marrow chimeras as described above, where wild-type and TLR9-knockout C57BL/6 mice were lethally irradiated and reconstituted with bone marrow derived cells from TLR9-mutant mice. We show that when immune cells lack TLR9 expression, there is still a significant difference in parasite loads between both wild-type and TLR9-knockout chimeras, as we observed in naive mice, despite to lower extent (Figure 5). These observations indicate that TLR9 deficiency in non-hematopoietic cells leads to increased resistance to *P. berghei* infection.

As mentioned above, by infecting primary hepatocytes we confirmed that absence of TLR9 only in hepatocytes correlates with the *in vivo* reduction in the number of developing parasites in the liver of infected TLR9-knockout mice.

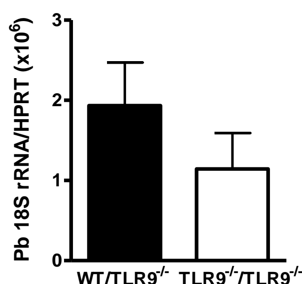


Figure 5 TLR9-deficiency in non-hematopoietic cells leads to increased resistance to malaria liver stage infection. TLR9-sufficient and TLR9-deficient C57BL/6 recipient mice were lethally irradiated and reconstituted with TLR9-deficient bone marrow from C57BL/6 TLR9-knockout donor mice. Chimeric mice were infected with $2x10^4$ *P. berghei* sporozoites. The number of parasite copies was quantified by qRT-PCR of mRNA isolated from the infected livers collected 40 hours post-infection (mean ± SD, n=5, $p < 0.05$).

To determine whether the irradiation of mice followed by the injection of hematopoietic cells to reconstitute the depleted bone-marrow could have interfered with the levels of infection, we produced control chimeric mice: wild-type and TLR9-knockout C57BL/6 mice were lethaly irradiated and reconstituted with bone marrow-derived cells from TLR9-suficient and TLR9-deficient mice, respectively. The infection of these mouse chimeras clearly showed that the irradiation and reconstitution procedures do not affect the phenotype observed in naïve mice infected with *P. berghei* sporozoites (Figure 6).

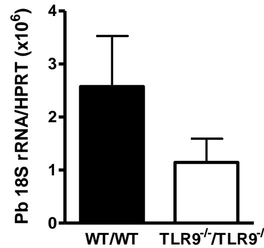


Figure 6 The irradiation and reconstitution procedures do not affect the susceptibility to infection associated to TLR9. TLR9-sufficient and TLR9-deficient C57BL/6 recipient mice were lethally irradiated and reconstituted with wild-type and TLR9-knockout bone marrow, respectively, from wild-type and TLR9-knockout C57BL/6 donor mice. Bone marrow chimeras were infected with $2x10^4$ *P. berghei* sporozoites. The number of parasite copies was quantified by qRT-PCR of mRNA isolated from the infected livers collected 40 hours post-infection (mean ± SD, n=4, $p < 0.05$).

CpG treatment inhibits liver infection *in vivo* and decreases the levels of infection *in vitro*

We showed that TLR9 expression in hepatocytes is responsible for an increased susceptibility to infection. To test whether TLR9 triggering would interfere with the infection levels we used CpG, a TLR9 agonist, to activate TLR9 both *in vivo* and *in vitro*. Both BALB/c and C57BL/6 mice were injected i.p. with 10 micrograms of CpG per mouse at the same time as sporozoite infection. Surprisingly, BALB/c mice did not become infected after injection of $2x10^4$ *P. berghei* sporozoites while 50% of the C57BL/6 mice did. Additionally, we observed by qRT-PCR that the C57BL/6 mice that became infected after treatment with CpG displayed a dramatic reduction in the number of developing parasites in the liver (Figure 7A). Periodically, blood parasitemias were measured in mice in which the infection was prevented by CpG injection. These mice never developed positive parasitemia and remained alive for several months, dying later of natural causes (Figure 7B).

To assess if CpG-mediated protection also occurs in hepatocytes infected *in vitro*, we treated Hepa1-6, HepG2 and Huh7 cells (mouse and two human hepatoma cell lines, respectively) and C57BL/6 primary hepatocytes with 10 micrograms of CpG per well, at

the time of sporozoite infection. We observed that CpG treatment *in vitro* also decreased the infection levels in hepatoma cells (Figure 7C) and in mouse primary hepatocytes (Figure 7D). We then did a titration in Huh7 cells with different concentrations of CpG, which resulted in an inversed correlation between CpG doses and infection levels (Figure 7C).

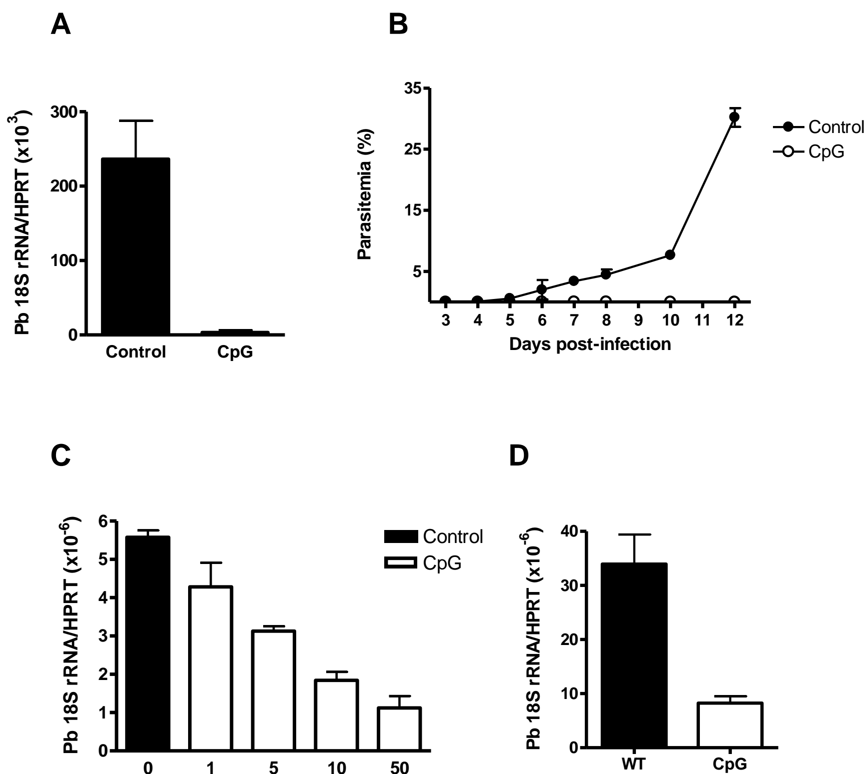


Figure 7 CpG treatment both *in vivo* and *in vitro* results in a major reduction in parasite load. Wild-type C57BL/6 mice were injected i.p. with 10μg of CpG per mouse and, at the same time, infected with $2x10^4$ *P. berghei* sporozoites. The number of parasite copies was quantified by qRT-PCR of mRNA isolated from the infected livers collected 40 hours post-infection (A). Likewise, BALB/c mice were infected and blood parasitemias were measured after day 2 post-infection, by FACS analysis of blood drops (B). Huh7 hepatoma cells were treated with 0, 1, 5, 10 and 50μg of CpG (C) and C57BL/6 primary hepatocytes were treated with 10μg of CpG (D). Then, both cells were infected with $2x10^4$ *P. berghei* sporozoites per well. The number of parasite

copies was quantified by qRT-PCR of mRNA extracted from cell lysates collected 24 (C) and 42 hours after infection (D) (mean ± SD, n=3-5, $p < 0.05$).

Discussion

Plasmodium is a protozoan parasite which has coevolved with its mammalian hosts for a long period of time and might have developed, as well as other parasites, strategies to evade and manipulate the host immune response.

Our understanding of how *Plasmodium* sporozoites might evade the immune response in the liver is scarce. Studying the function of TLRs, major parasite immune sensors, in *Plasmodium* liver infection, may contribute to shed some light on the nature of host-parasite interactions at this stage.

We demonstrated that TLR signaling is activated during *Plasmodium* infection, as TLR4-deficient mice show enhanced susceptibility to infection. Likewise, in recent years it has been reported that several TLRs are important for the recognition of pathogens and activation of the innate immune response against protozoan parasites and against pathogens in general. So far, it has never been shown that lack of TLR accounts for an increased resistance displayed to an infection. However, our data indicates that mice lacking TLR9 are more resistant to *P. berghei* liver infection, suggesting that TLR9 increases susceptibility to this parasite. Our findings can be interpreted as a mechanism used by *Plasmodium* to evade the immune response or to somehow facilitate the early stages of liver infection. Given that the phenotype displayed by TLR9-deficient mice appears to be hepatocyte-mediated, one can speculate that while traversing and infecting hepatocytes the parasite activates TLR9, and that this activation is beneficial for the infection. TLR9 triggering in this case could be attained by endogenous ligands or by the parasite itself. The use of an immune receptor for facilitating an infection might seem a surprising and unexpected ability from the *Plasmodium* protozoan, but the long history of evolution between this parasite and mammalian hosts could well give rise to such interactions. The role of TLR9 in facilitating the infection of *Plasmodium* can be thought to happen in one of two processes: the infection itself and subsequent parasite development inside the hepatocyte; and a dampening effect on the ensuing immune

response. In the first case it is possible that TLR9 triggering during parasite infection of hepatocytes somehow provokes the liver cell to undergo alterations which are either beneficial or even necessary for optimal infection, and can include cellular compartment rearrangement or de novo synthesis of proteins, for example. The second case could be an indirect effect in the immune response to liver infection, as TLR9 activation in hepatocytes might inhibit or otherwise redirect the danger signaling molecules released in response to cellular stress.

Contrary to TLR4, TLR9 expression in hematopoietic cells appears to have no effect on liver infection. This result rules out Kupffer cells as being responsible for the phenotype observed in TLR9-deficient mice, despite these cells being also traversed by the parasite. It is however possible that in the bone marrow chimeras we produced, the reconstitution of Kupffer cells was not complete, even after the long period we allowed after bone marrow transfer before infecting the mice.

While it is clear from our results that TLR9 expression in hepatocytes has a supporting effect in *Plasmodium* infection, our experiments do not exclude that triggering of TLR9 in hematopoietic cells impacts on the liver stage of malaria infection. In particular, the inhibition in infection levels observed after CpG administration is likely to activate the immune system and consequently negatively affect the infection of hepatocytes. To address this issue, different bone marrow chimeras must be studied where bone marrow deficient or sufficient for TLR9 is injected into irradiated wild-type mice.

TLR9 activation with CpG, similarly to what was described for TLR4 stimulation using LPS, led to an increase in resistance to sporozoite infection. The mechanism or the target cells where TLR9 activation is triggered, either by the parasite infection or by CpG, must be distinct, giving rise to dissimilar effects in parasite load. Further work would be necessary to unravel the mechanisms underlying TLR9-associated host susceptibility to *P. berghei* liver infection.

Chapter 5 Results

MyD88 is a mediator of protective immunity induced by *Plasmodium* radiation-attenuated sporozoites

Abstract

Sterile protective immunity against malaria can be elicited through immunization with radiation-attenuated sporozoites (RAS). This experimental model has been unraveling essential defense mechanisms involved in the establishment and maintenance of protective immunity against malaria liver infection. However, these have not been fully elucidated.

TLRs play a critical role in the activation of innate immunity by the recognition of parasite specific molecules. In signaling pathways via TLRs, MyD88 is a common adaptor which is essential for the production and release of inflammatory cytokines. In order to determine whether these host immune players are involved in the protection against *Plasmodium* liver infection conferred by immunization with RAS, we tested if sterile immunity could be induced in TLR2, TLR4, TLR9 and MyD88-knockout mice. Upon immunization with one or two doses of irradiated sporozoites, MyD88-knockout mice are able to develop some degree of protection, although to a smaller extent when comparing to wild type mice, and not enough to confer full protection after challenge with viable sporozoites. Accordingly, mice that lack MyD88 failed to develop protective immunity against *P. berghei* sporozoites upon three doses of irradiated sporozoites, whereas in immunized wild-type, TLR2, TLR4 and TLR9-knockout mice, parasite development was arrested.

These findings point out the essential but not exclusive role of MyD88 (TLR2, TLR4 and TLR9-independent) in the protection elicited by irradiated sporozoites, emphasizing the relevance of innate immune mechanisms in the development of protective immunity to *Plasmodium*.

Introduction

The experimental induction of sterile and lasting immunity against sporozoite infection, whereby the development of an erythrocytic stage infection is prevented, has been shown in the late 60s using gamma-irradiated rodent malaria sporozoites (Nussenzweig, Vanderberg et al. 1967). Later, immunization with RAS was also achieved in humans (Clyde, Most et al. 1973), leading to a global effort to develop a malaria vaccine targeting the pre-erythrocytic stages of *Plasmodium* parasites. This became a unique model to elucidate the immune mechanisms in the liver that are responsible for the establishment and maintenance of protective immunity.

Recent accumulating evidence has demonstrated that irradiated sporozoites are parasite attenuated forms, which are viable and able to invade hepatocytes, but that undergo arrested development and fail to establish a blood-stage infection. Therefore, the immunization with RAS is able to induce protective immune responses against subsequent challenges with viable sporozoites. It has been shown that the protection conferred by RAS is mediated by both CD8 and CD4 T cells as well as by antibodies that recognize surface proteins in sporozoites. Although these mechanisms of protection play a major role in RAS-mediated protective immunity, a wide array of other immune effectors is being described in mice infected with the rodent species of *Plasmodium*, which represent a relevant model for studding the immune mechanisms in malaria infection. Thus, RAS-mediated protection also relies on cytokines such as IFN-γ and IL-12, on molecules like NO and on NK cells for parasite clearance (reviewed in Doolan and Martinez-Alier 2006). Regardless of the extensive acquired knowledge about the defense mechanisms implicated in the establishment of a protective immune response, they have not been entirely elucidated.

TLRs play an essential role in the direct recognition of infectious agents, leading to the establishment of innate and adaptive immune responses (Pasare and Medzhitov 2005; Akira, Uematsu et al. 2006). In mammals, signalling events downstream of TLRs are mediated by different TIR-containing adaptor proteins, namely MyD88, which is common to all TLRs. Therefore, the fact that TLRs are major players in host defense lead us to attempt to define the relevance of MyD88, TLR2, TLR4 and TLR9 in the protection

conferred by irradiated sporozoites. Our results illustrate that unlike TLR2, TLR4 and TLR9, MyD88 is a critical effector in the process of immunization with RAS.

Results

MyD88 is a major mediator in the establishment of protection elicited by immunization with RAS

To evaluate the contribution of MyD88, TLR2, TLR4 and TLR9 in the mechanisms of protection against malaria liver infection, wild-type, MyD88, TLR2, TLR4 and TLR9-knockout C57BL/6 mice received one immunization and two extra boosts of *P. berghei* RAS in 10-day intervals, before challenge with $1x10^4$ sporozoites. All non-immunized mice, used as controls in the challenge infection, developed a patent blood-stage infection. In contrast, none of the wild-type, TLR2, TLR4 and TLR9-knockout C57BL/6 mice immunized with *P. berghei* RAS developed blood parasitemia, indicating that it is possible to induce sterile protection to challenge with viable sporozoites in the absence of TLR2, TLR4 and TLR9. However, 86% of MyD88-knockout mice were not protected against the challenge. Blood-stage parasites were detectable in challenged MyD88-knockout mice, both in Giemsa-stained blood smears and in blood drops analyzed by flow cytometry (Table 1 and Figure 1), suggesting that immunization with RAS is at least partially mediated by MyD88 and TLR2, TLR4 and TLR9-independent.

Table 1 Immunization with RAS fails to elicit sterile immunity in the absence of MyD88 but not in the absence of TLR2, TLR4 and TLR9

Mouse strain	No. Protected/total no. challenged mice	Sterile protection (%)
WT	21/21	100
TLR2-/-	11/11	100
TLR4-/-	5/5	100
TLR9-/-	10/10	100
MyD88-/-	1/7	14
Naive	30/30	0

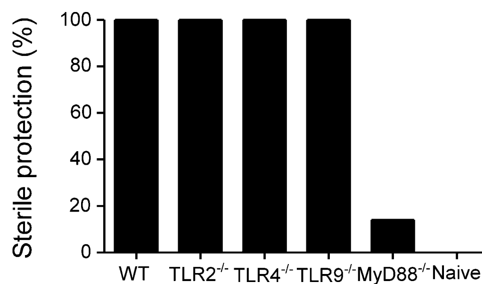


Figure 1 The vast majority of MyD88-knockout mice do not develop sterile immunity after immunization with RAS. Wild-type, TLR2, TLR4, TLR9 and MyD88-knockout C57BL/6 mice were primed with $5x10^5$ *P. berghei* RAS and given booster immunizations with two injections of $2x10^4$ RAS. After challenge with $1x10^4$ viable *P. berghei* sporozoites, all animal groups were monitored for blood-stage infections. Protection was determined by measuring parasitemia in Giemsa-stained blood smears and FACS analysis of blood drops, obtained on a regular basis from day 1 to day 42 post-challenge.

Naïve wild-type and MyD88-knockout C57BL/6 mice infected with $1x10^4$ viable *P. berghei* sporozoites develop a patent blood-stage infection at day 3 to 4 post-infection. Immunized wild-type, TLR2, TLR4 and TLR9-knockout mice do not become infected after chalenge with viable sporozoites while immunized MyD88-knockout mice show positive parasitemias after day 5 post-infection. Parasites were not detected in the blood in the first days of infection as in naïve infected mice, both by analysing Giemsa-stained blood smears and blood drops by flow cytometry. Interestingly, while all naïve wild-type mice died at day 7 post-infection, immunized MyD88-knockout mice died later on, at days 16 and 27 post-infection (Figure 2). MyD88 seems to be necessary to the the establishment of protection during the immunization with RAS since the majority of RAS immunized mice did not develop immunity. However, other factors appear to be of importance in the process of immunization, as one out of seven MyD88-knockout mice developed protection and that there is a delay in the appearance of parasites in the blood of the remaining mice on the group, which indicates that immunized MyD88-knockout mice developed some degree of protection.

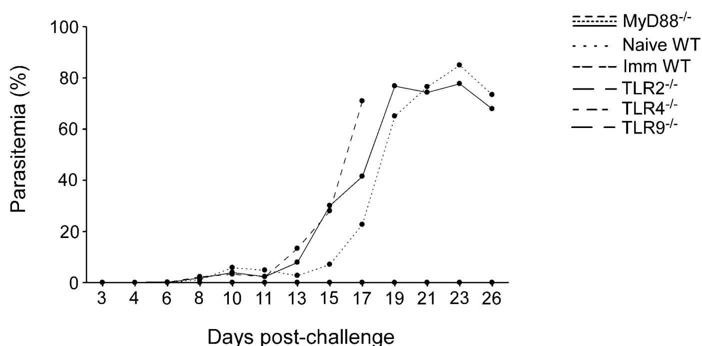


Figure 2 Immunized MyD88-knockout mice developed a patent blood-stage infection after day 5 post-challenge. Wild-type, TLR2, TLR4, TLR9 and MyD88-knockout C57BL/6 mice were immunized with three injections of *P. berghei* RAS and challenged with $1x10^4$ viable *P. berghei* sporozoites. Blood parasitemias were monitored in all animal groups, by examination of Giemsa-stained blood smears and FACS analysis of blood drops on a regular basis after day one post-challenge. Naïve wild-type and MyD88-knockout mice were the only mice that developed parasitemia at day 3 to 4 and at day 5 post-challenge, respectively. Parasitemias are shown for individual mice in the MyD88-knockout group (n=5-10).

We show that MyD88 seems to be necessary for the establishment of protection against sporozoite infection, which is obtained in C57BL/6 mice by one immunization followed by two boosts of RAS. We investigated the immunization process further and asked whether MyD88 is particularly important in the establishment of the initial partial protection conferred by the first injection of RAS. Mice that were injected with one or multiple immunizing doses of RAS were challenged with live sporozoites and the insuing infection was monitored. The degree of protection was assessed by quantifying the number of parasite copies by qRT-PCR of mRNA isolated from the livers at 40 hours after the challenge with $1x10^4$ viable *P. berghei* sporozoites (Figure 3). A single injection with RAS is sufficient to significantly decrease the infection levels after challenge with viable sporozoites, both in wild-type and in MyD88-knockout mice. We observed that the level of protection is reduced but not significantly in MyD88-knockout mice when comparing to wild-type mice. This reduction in protection against the challenge with viable sporozoites was observed in MyD88-knockout mice upon the first immunization

and was enhanced when followed by a single immunization boost, suggesting that MyD88 is important during the course of immunization and not only in the initial immunization (Figure 3). In addition, there are no significant differences in parasite load at 40 hours post-infection between naïve wild-type and MyD88-knockout C57BL/6 mice infected with viable *P. berghei* sporozoites (Figure 3). This indicates that MyD88 itself does not play a role during liver infection with *P. berghei* or that different TLRs, MyD88-dependent, play opposite roles (resistance and susceptibility) during infection, compensating each other effects.

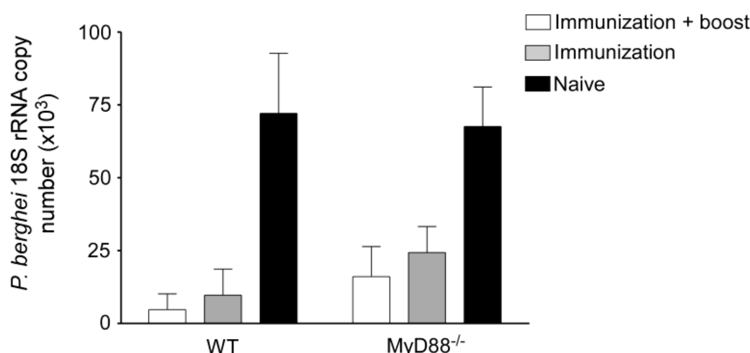


Figure 3 MyD88 is implicated in the protective mechanisms elicited during immunization with RAS. Wild-type and in MyD88-knockout C57BL/6 mice were immunized with one and two injections of *P. berghei* RAS. The degree of protection was assessed by quantifying the number of parasite copies by qRT-PCR of mRNA isolated from the livers at 40 hours after the challenge with $1x10^4$ viable *P. berghei* sporozoites (mean ± SD, n=4-6).

We could not observe significant differences in initial parasitemias from wild-type and MyD88-knockout C57BL/6 naïve mice, which is expected after seing no differences in parasite load in the liver at 40 hours post-infection. However, we observed that while wild-type mice died with CM, all of the MyD88-knockout mice died later on, even though they presented CM symptoms, and reached parasitemias higher than 80%, which is not usually observed in mice infected with *P. berghei* parasites (Figure 4). In two out of five mice, the parasites were almost undetectable around two months post-infection, and

then, reached again very high parasitemias titres, suggesting that absence of MyD88 might be relevant for parasite clearance.

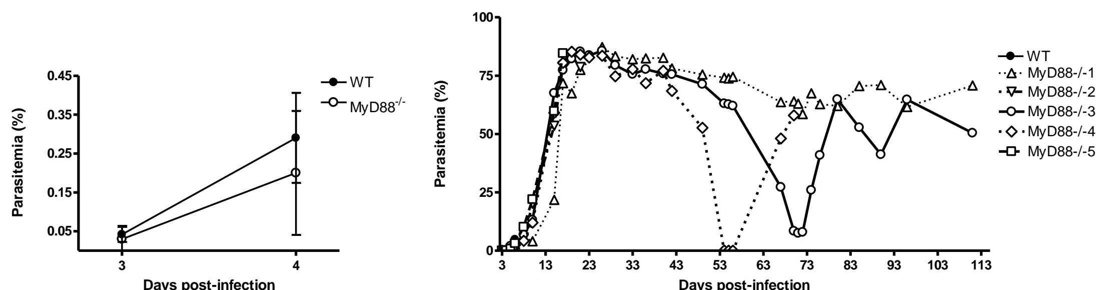


Figure 4 Mice lacking a functional MyD88 do not show any differences from wild-type controls at day 3 post-infection but display very high parasitemias during blood stage infection. Wild-type and MyD88-knockout mice were infected with $2x10^4$ *P. berghei* sporozoites. Blood parasitemias were measured by FACS analysis of blood drops. Parasitemias are shown for individual mice in the MyD88-knockout group on the right panel (mean ± SD, n=5).

TLR and MyD88-knockout mice can develop a patent blood-stage infection throughout the immunization course with RAS

During the course of immunization with RAS, the parasitemia of all mice was monitored weekly, by examination of Giemsa-stained blood smears and FACS analysis of blood drops. Interestingly, while during the immunization protocol none of the wild-type mice showed positive parasitemias, several TLR2, TLR9 and MyD88-knockout mice, immunized at the same time, developed a persistent blood-stage infection following the injection of RAS. Only the mice that did not become infected during the immunization course were challenged (Figure1, Table1). The incidence of infected mice after RAS injection was significantly higher in MyD88-knockout mice since 50% of mice injected with RAS became infected before the challenge with viable sporozoites (Table 2). The fact that none of the wild-type mice become infected while some TLR-MyD88-deficient did suggests that these mice were not able to counteract the infection by sporozoites which have resisted the radiation and were in fact not attenuated, and consequently,

were able to successfully infect the liver and continue their life cycle, progressing to the blood. The blood-stage infection developed by some of the mice in the course of immunization was found to be persistent and without significant variations in parasitemia (Figure 5).

Table 2 A proportion of TLR2, TLR9 and MyD88-knockout mice developed a patent blood-stage infection throughout the immunization course with RAS

Mouse strain	No. Infected/RAS injected mice
WT	0/21
TLR2-/-	1/11
TLR4-/-	0/5
TLR9-/-	2/10
MyD88-/-	7/14

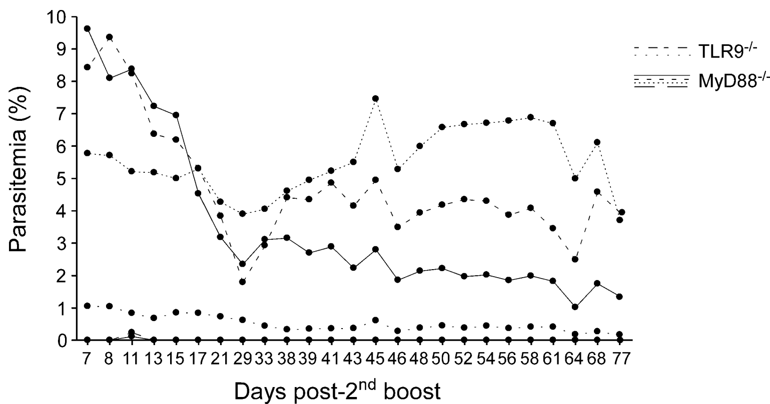


Figure 5 Mice infected during the immunization process developed a chronic blood-stage infection throughout time. TLR9 and MyD88-knockout mice were injected with RAS and never challenged with viable sporozoites. Parasitemias were assessed by FACS analysis of blood drops obtained regularly after the first immunization and the two following boosts. Parasitemias are shown for individual mice (n=2-4).

Discussion

To further determine whether MyD88 was particularly important in the establishment of the initial partial protection following RAS immunization, we quantified the degree of protection after immunization either without boosting or followed by a single boost. The results indicate that MyD88 mediates protection at the time of the first immunization and also seems to play a role in the subsequent boosts.

In the present chapter, we explored the possibility that TLRs and MyD88 might be required for the development of protective immune responses during immunization with RAS. This hypothesis was driven by the fact that TLRs are known to play a critical role in the direct recognition of pathogens, leading to the induction of innate and adaptive immune responses. Therefore, by adressing their importance in the protection conferred by RAS, we demonstrate that while TLR2, TLR4 and TLR9 are not implicated in RAS-mediated protection, MyD88 is a major effector in the immunization against malaria liver infection.

We did not observe a role for TLR2, TLR4 and TLR9 in the establishment of protection against the infection with *P. berghei* sporozoites. However, RAS fail to elicit sterile immunity in the absence of MyD88 which indicates that this TLR-adaptor molecule is involved in the mechanisms of protection conferred by RAS immunization. It is important to notice that the fact that TLR2, TLR4 and TLR9 are not mediating protection does not exclude the hypothesis that other TLRs or IL-1R signaling pathways might be involved in this process.

Interestingly, despite not being implicated in the establishment of RAS-mediated protection, some of these TLRs are important in the response to putative radiation-resistant sporozoites. As shown in Table 2 and Figure 5, a proportion of TLR2, TLR9 and MyD88-knockout mice developed a patent blood-stage infection throughout the immunization course with RAS, while wild-type mice never displayed positive parasitemias. The blood-stage infection developed by some of the mice during the course of immunization was found to be persistent and without significant parasitemia variations. These mice, which were never challenged with viable sporozoites, developed a chronic blood-stage infection throughout time, which may be due to the development of a certain degree of immunization that does not allow parasite replication as efficiently

as in nonimmunized mice. These observations suggest that an immunodeficient mouse can have compromised adaptive immune responses, as wild-type mice never became infected during course of immunization with RAS against the few sporozoites that might have resisted the attenuation by irradiation. One may possibly conclude that vaccination with RAS is only effective in mice that are not immunocompromised, since the range of irradiation doses that can be employed is very limited, ranging between a value of radiation that can kill rather than attenuate the sporozoites and an insufficient amount of radiation that allows sporozoites to remain viable.

Surprisingly, there are no significant differences between parasite load in the liver of naive wild-type and MyD88-knockout C57BL/6 mice infected with viable sporozoites at 40 hours post-infection. Despite being involved in the development of protection confered by immunization with RAS and contrary to what we were expecting after describing TLR4-associated resistance and TLR9-mediated susceptibility to liver infection, MyD88 does not play a role during liver infection with *P. berghei* sporozoites. However, we can hypothesize that the parasite load observed in infected MyD88-knockout mice is the result of the sum of several TLR-MyD88-mediated effects. For instance, considering that the opposite effects of TLR4 and TLR9 activation during *P. berghei* sporozoites infection are MyD88-mediated, they can cancel out each other, as they represent opposite effects.

We also observed that MyD88-knockout mice infected with sporozoites did not develop CM as wild-type mice and remained alive for months after infection, reaching parasitemias of around 90%. These results obtained with liver stage infection are in contradiction to what we observed and was described previously for blood-stage infection of these mice given that MyD88-knockout mice were found to be as sensitive to fatal CM development as wild-type control mice after blood-stage infection (Correia et al. unpublished results; Togbe, Schofield et al. 2007). This observation suggests that MyD88 is involved in CM development after sporozoite infection. Also, the immune events in the liver may influence disease progression, since MyD88-knockout mice develop CM after a blood-stage infection. Furthermore, it seems likely that what probably kills mice at later stages of infection is not hyperparasitemia, since those mice reached extremely high levels of parasitemia, but MyD88-mediated deleterious inflammation. It is worth noting that two of the mice were even able to nearly clear

parasitemia after having reached around 90% of parasite-infected erythrocytes. This indicates that while erythropoiesis is normally taking place at the bone marrow, the mice are still able to recover from massive destruction of red-blood cells. However, as disease persists for months, bone-marrow depletion can also occur, rendering mice unable to recover.

The immunization with RAS and, more recently, with GAS is, thus far, the only effective known vaccine against malaria. Despite being extensively studied, the mechanisms by which the response to these parasites is triggered are still undefined. Our findings indicate that MyD88 is a new player in this process. Still, the mechanisms by which MyD88 is initiating a response against RAS and their dependency of TLRs must be object of further work. It also remains to be elucidated if these mechanisms are similar to the ones developed during GAS immunization.

Moreover, the role of MyD88 during CM and at later stages of blood infection needs to be further investigated. The immune events that take place during liver infection and their effect in the outcome of the disease must be considered in the future and data from blood-stage infections must be analysed regarding the absence of a primary liver infection. That these early immune responses participate in the outcome of blood-stage disease is a possibility that cannot be excluded.

Chapter 6 General Discussion

Malaria is one of the most devastating infectious diseases and one of the major causes of death in developing countries, representing a significant barrier to economical and social development. An expanded global effort in control programs using drug treatment of infected individuals and populations at high risk of infection, and mosquito control with insecticide-treated bed nets and indoor-insecticide spraying, are being responsible for the retreating of malaria incidence in some areas of the developing world. Still, more than 40% of the world's population is at risk of contracting malaria, mainly owing to the widespread emergence of drug-resistant *Plasmodium* variants. Research into the basic biology of malaria infection is, therefore, essential to provide new intervention targets for antimalarial drugs and vaccines.

In recent years, remarkable findings have been made, widening our understanding of the innate immune system – the primary host defense barrier against a variety of microbial pathogens such as bacteria, fungi, viruses and parasites. The characterization of TLRs, a family of pathogen recognition receptors, was one of the most relevant findings in immunology in the last decade. This major breakthrough made immunologists aware of the specificity of the nature of the primary response, responsible for pathogen recognition and necessary for the induction of immune responses. To date, TLRs have been implicated in the recognition of different groups of pathogens and their activation has been associated with a protective effect during infection with *T. gondii*, *T. cruzi*, and *Leishmania* spp, other protozoan parasites which are *Plasmodium* related. Thus far, malaria seems to represent an exception in this field. One study has shown that CM pathogenesis and lethality is TLR-MyD88-dependent, and the activation of MyD88 signalling during blood-stage infection seems to be involved in the excessive proinflammatory cytokine production responsible for the observed symptoms (Coban, Ishii et al. 2007). However, malaria research has been mainly focused on studying the immune responses to blood stage infection, while in liver stage infection the importance of TLRs, or other innate receptors, in parasite recognition was not addressed. It is known that the cellular inflammatory response against *P. berghei* is triggered shortly after the injection of viable sporozoites (Khan and Vanderberg 1991; Khan, Ng et al. 1992) indicating that parasite recognition might occur at very early stages of infection. Furthermore, the primary site of replication for *Plasmodium* parasites in the mammalian host is the liver, within hepatocytes. The liver is considered to offer unique advantages

for the extensive parasite replication that occurs before the erythrocytic phase of the life cycle. As happens for other parasites, *Plasmodium* is believed to exploit the liver's environment and subvert immunity to establish a successful infection. *Plasmodium* is thought to manipulate the phagocytic function of Kupffer cells to cross liver endothelium and gain access to hepatocytes (Frevert, Engelmann et al. 2005; Baer, Roosevelt et al. 2007; Usynin, Klotz et al. 2007). In fact, by constituting a mandatory step for the progression of the infection and the establishment of the disease, the liver is likely to be an important target both for the generation of more powerful innate immune responses and for vaccine-induced adaptive immunity.

The hepatocytes are known to express nine TLRs (Liu, Gallo et al. 2002). The work presented herein was focused on the role of some TLRs, expressed by both hepatocytes and immune cells, during the liver stage of infection and in the outcome of the disease. A murine parasite, *P. berghei*, was used to investigate the role of TLR2, TLR4, TLR9 and MyD88 in the innate immune response to *P. berghei* sporozoite infection. These TLRs and adaptor molecule have been previously described to be involved in host protection against other protozoan parasites, playing a role in pro-inflammatory responses against the parasite during malaria blood-stage infection (Franklin, Rodrigues et al. 2007), and as being responsible by the pathology observed during blood-stage infection by being implicated in CM (Coban, Ishii et al. 2007).

TLR4 and TLR9 seem to have distinct and independent roles during malaria infection, as our results show that mice lacking TLR4 and TLR9 expression display enhanced susceptibility and resistance, respectively. Consequently, TLR4 appears to be involved in the host response to the parasite infection and *Plasmodium* seems to take advantage of TLR9 during liver infection.

TLR4-mediated resistance mechanisms pertain mostly to bone marrow-derived cells, with a minor involvement of hepatocytes. This seems to indicate that the activation of immune cells via TLR4 during *Plasmodium* infection is crucial to mount an effective response to the parasite. Among the immune cells present in the liver, Kupffer cells, the liver macrophages, seem the most likely to be involved in such an early response, particularly by taking into account our finding that TLR4-deficient mice show impaired TNF production by macrophages in the liver. Kupffer cells were described previously as being traversed by sporozoites in liver sinusoids as a mean of reaching the liver

parenchyma. During this interaction, the parasite or an endogenous ligand might activate TLR4 leading to the activation and recruitment of other immune cells, which will infiltrate the tissues, initiating an inflammatory response. We show that in a natural infection TLR4 activation upon infection is partially responsible for the recruitment of macrophages and DCs to the tissues and which consequently decreases the number of successful developing parasites in the liver. It is important to state that TLR4 activation can happen either by interaction with a TLR ligand from the parasite, from bacteria present in mosquito salivary glands, or by recognition of exposed or released endogenous ligands induced by parasite infection. TLR4 activation in hepatocytes during sporozoite infection also seems to play an important role in parasite elimination. The hepatocyte might be responsible for TLR4-mediated direct parasite killing by the production of reactive oxygen species. Moreover, it might potentially contribute to the immune response by presenting parasite antigens to immune cells.

TLR4 activation by LPS is capable of remarkably reduce the infection levels, both *in vivo* and *in vitro*, by interfering with parasite infection and development, emphasizing the relevance of TLR4 in the modulation of host response early during infection and how its activation can be used to achieve stronger responses against *Plasmodium*. This clearly opens new research lines in what concerns vaccine development, namely by using TLR4 agonists as adjuvants in the induction of more effective immune responses.

In opposition to the importance of TLR4 for the immune response against *Plasmodium*, TLR9 expression on hepatocytes is responsible for increased susceptibility to sporozoite infection. TLR9-associated susceptibility is only mediated by hepatocytes, differently from the phenotype described for TLR4. The triggering of TLR9 might activate the immune system in a way that, while being effective against intracellular viral or bacterial infections, will deviate the immune response towards effector mechanisms which are less harmful to a protozoan parasite like *Plasmodium*. Of note is the fact that TLR9 can have anti-inflammatory effects, as observed in the gut where TLR9-triggered type I IFN has anti-inflammatory functions in colitis (Rachmilewitz, Katakura et al. 2004; Katakura, Lee et al. 2005). As such it is still a possibility that TLR9 activation in hepatocytes might lead to an anti-inflammatory response that benefits the parasite.

Treatment with the TLR9 ligand CpG seems to activate innate immunity in bone marrow-derived cells and, similarly to LPS, increases resistance to infection. It seems that

activation of TLR signalling might be useful in designing more powerful vaccines. However, TLR-based immunotherapeutic strategies must take into account the cells that are being targeted. The reason why TLR9 activation by CpG induces a decrease in infection, while TLR9 triggering in sporozoite infection leads to a better parasite development, remains to be elucidated.

Still, we report for the first time, the unique role of TLR9 during a protozoan infection. Never a Toll-like receptor has been implicated in a pathogen infection as a mean to more successfully infect and/or develop in the host and evade the immune system.

Considering the role of TLR4 and TLR9 in malaria, it was somehow surprising to notice that MyD88-knockout mice do not display a substantial impairment of host immune responses during *P. berghei* liver infection. Indeed, they do not show significant differences in the levels of infection, when compared to wild-type controls. This observation might be explained by the fact that, as described above, at least two different TLRs, TLR4 and TLR9, play a role in *P. berghei* infection. Assuming that both effects are MyD88-mediated and if there is no contribution from other TLRs, TLR4 and TLR9-mediated resistance and susceptibility, respectively, might compensate one another cancelling out each other's effects. However, this might also not be the case: on the one hand, there is the possibility that the receptors signal through MyD88-independent pathways; on the other hand, the use of one of the receptors by the parasite might not require its signalling, as it would be the case if the receptor constitutes a gateway into the cell. It is interesting to note that MyD88-knockout mice do not die of *P. berghei* acute infection and become chronically infected. This finding underlines the importance of MyD88 and putatively of the Toll pathway in the immunopathology of malaria infection, namely for CM, which is the main cause of death of *Plasmodium*-infected individuals.

These findings reinforce the idea that host defence against protozoan pathogens might depend on the engagement of multiple TLRs, but also that this engagement can be manipulated for the parasite's own benefit.

A second aim of this work was to determine whether TLRs are involved in the protection conferred by RAS against *Plasmodium* liver infection. Thus far, the RAS vaccine is the only effective vaccine against malaria that was shown to elicit sterile immunity against *Plasmodium* parasites, both in experimental rodent hosts and in human volunteers.

More recently, several GAS vaccines have been proposed as alternative whole-parasite attenuated vaccines, which are a more feasible approach than RAS vaccines. By knocking out specific genes, GAS immunization is safer and more reproducible, and the GAS are easier to produce and also induce protective immunity in mice. Several studies have described other players implicated in the process of immunization, although it is still unknown how the immune response to attenuated parasites is initiated. In this scenario, the role of the innate immune receptors and associated signaling molecules, such as TLRs and MyD88, remains to be elucidated. MyD88-knockout mice failed to develop protective immunity against *P. berghei* sporozoites upon three doses of irradiated sporozoites, whereas in immunized wild-type, TLR2, TLR4 and TLR9-knockout mice, parasite development was arrested. These findings highlight the essential and exclusive role of MyD88 (TLR2, TLR4 and TLR9 independent) in the protection elicited by irradiated sporozoites, emphasizing the importance of MyD88-dependent innate immune mechanisms in the development of protective immunity to *Plasmodium*.

It would be interesting to determine whether any of the TLRs is involved in MyD88-mediated protection in the process of RAS immunization. Knowing that MyD88 is involved not only in the signaling of TLRs, but also in both IL-1 and IL-18R/IL-1R-associated kinase signaling, one must not exclude the contribution of these other receptors in the absence of protection observed in RAS-immunized MyD88-knockout mice. It would also be interesting to address the relative contributions of IL-1 and IL-18 *versus* TLR signaling in MyD88-mediated protection against sporozoites infection, induced by RAS immunization.

The mechanism by which MyD88 mediates the development of protection remains unclear and would be relevant to be addressed in the future. Nevertheless, the data presented herein already reveal a new player in the protection against *Plasmodium* infection by immunization with RAS, which must be considered in vaccine development. These results might also change our view about the significance of innate immunity in a process where adaptive immunity was considered to date as the major player and focus. From these findings, two main conclusions can be drawn. First, TLRs are mediators of host-parasite interactions in malaria and, contrary to what would have been expected and described in the literature in recent years, they play distinct roles during infection.

TLRs seem to be involved in *Plasmodium* recognition, as is the case with other protozoan parasites, and their activation might be crucial for the development of protective immune responses. Secondly, the work described highlights the importance of liver infection in disease outcome. The immune events that take place during liver infection and their effect in the outcome of the disease must be taken into account, and the study of blood-stage infection must be analysed considering the absence of a primary liver infection. Whether these early immune responses participate in the blood-stage disease outcome cannot be formally excluded.

Once we acquire a deeper knowledge of the interactions between *Plasmodium* parasites and the host in the liver, especially in what concerns host immune responses, we can define better approaches to counteract this very well adapted parasite. Our findings also corroborate the established view about the complexity of the intrinsic mechanisms of infection developed by *Plasmodium*, and remind us that, despite decades of research, understanding malaria still remains a major challenge.

Perspectives

This work contributed to achieve a better understanding of host innate immune responses against malaria but also raised several questions that have not been yet clarified. Furthermore, recent findings have described important issues where TLRs might also be involved that should be addressed in the future. Some intriguing descriptions on the fate of sporozoites that are inoculated into the host dermis were reported. If sporozoites are drained to and partially develop in the lymph nodes (Amino, Thiberge et al. 2006), delivering exoerythrocytic antigens to a place other than hepatocytes, one might expect that it will have relevant implications in the immune response against *Plasmodium*. In the work presented here, given the model used to study the role of TLRs during *Plasmodium* liver infection in mice, we could not address questions related with sporozoite development in the lymph nodes. It would be important to determine the influence of TLRs in this additional step, where dendritic cells might become activated through the same TLRs playing a role in liver infection. The fact that sporozoites can escape immune cells and destructive mechanisms to go on to develop

in lymph nodes, where antigen presentation occurs and where there is priming of immune responses, is of immunological significance and might be influenced by TLRs. This issue will require further studies using the same kind of approach but instead of infecting mice by injecting sporozoites directly into the blood stream, the infection should be performed by mosquito bite. Malaria, as well as other infectious diseases, constitutes a serious threat to several populations and human health worldwide, urging for new strategies to treat and prevent disease. Based on a deeper understanding of parasite-host interactions mediated by TLRs we can attempt to develop new TLR-based strategies against protozoan infection. Using pathogen-derived TLR ligands as therapeutic and prophylactic tools may constitute a future promising approach. In fact, most of the chemically defined microbial products being used as adjuvants or immunostimulants are TLR agonists, such as polyinosinic–polycytidylic acid (poly I:C), (TLR3 agonist), lipid A (TLR4 agonist), flagellin (TLR5 agonist), imiquimod (TLR7 and TLR8 agonist) and CpG DNA (TLR9 agonist) (Akira, Uematsu et al. 2006), which act as potentiators of the immune response. Currently, some approaches that use TLR agonists as adjuvants are already in progress. Monophosphoryl lipid A (MPL), a TLR4 agonist, is in advanced stages of development for use as an adjuvant in vaccine formulations using recombinant antigens of *P. falciparum* (Richards, 1998) and *Leishmania* spp. (Skeiky, Coler et al. 2002), both of which are protozoans. We also showed that by using a TLR4 or a TLR9 ligand, we are able to prevent or interfere with liver parasite development. Synthetic compounds designed to mymic the structure of the ligand without having the strong and deleterious inflammatory effect like LPS has may be considered as alternative strategies to be used. Furthermore, CpG-containing oligodeoxynucleotides have been successfully used as adjuvants in vaccines that induce effective protective immunity in experimental models against challenge from different protozoan parasites, including *Plasmodium* spp. (Coban, Ishii et al. 2004; Kumar, Jones et al. 2004).

In a different approach, when the activation of TLR pathway seems to be involved in pathology, like in the case of MyD88 (Adachi, Tsutsui et al. 2001), the use of TLR antagonists during acute malaria episodes might be beneficial, but only if it is confirmed that MyD88 and TLRs are not crucial for protective immunity and parasite clearance at later stages of natural infection. Similarly, if we demonstrate that the activation of TLR9

signaling during sporozoite infection is detrimental for the host, we can propose the use of a TLR9 antagonist as a prophylactic agent. As mentioned previously, vaccination with the synthetic carbohydrate moiety of *P. falciparum* GPI anchors protects mice from cytokine-mediate pathology observed during acute malaria (Schofield, Hewitt et al. 2002). Being recognized by TLRs (Krishnegowda, Hajjar et al. 2005), parasite GPI anchors can be mimicked by synthetic ones which can be used to block the pathological effects of malaria, working as an anti-toxic vaccine (Gazzinelli and Denkers 2006).

The potential of TLR-based therapeutical interventions must not be neglected. The available knowledge about the biology of TLRs and their signalling pathways and about the nature of TLR-mediated host-parasite interactions will likely contribute to the development of new immunotherapeutic strategies to control malaria.

References

Adachi, K., H. Tsutsui, et al. (2001). "Plasmodium berghei infection in mice induces liver injury by an IL-12- and toll-like receptor/myeloid differentiation factor 88-dependent mechanism." J Immunol 167(10): 5928-34.

Adachi, O., T. Kawai, et al. (1998). "Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function." Immunity 9(1): 143-50.

Aderem, A. and R. J. Ulevitch (2000). "Toll-like receptors in the induction of the innate immune response." Nature 406(6797): 782-7.

Akira, S. and K. Takeda (2004). "Toll-like receptor signalling." Nat Rev Immunol 4(7): 499-511.

Akira, S., S. Uematsu, et al. (2006). "Pathogen recognition and innate immunity." Cell 124(4): 783-801.

Al-Olayan, E. M., A. L. Beetsma, et al. (2002). "Complete development of mosquito phases of the malaria parasite in vitro." Science 295(5555): 677-9.

Alexopoulou, L., V. Thomas, et al. (2002). "Hyporesponsiveness to vaccination with Borrelia burgdorferi OspA in humans and in TLR1- and TLR2-deficient mice." Nat Med 8(8): 878-84.

Aliprantis, A. O., R. B. Yang, et al. (1999). "Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2." Science 285(5428): 736-9.

Alonso, P. L., J. Sacarlal, et al. (2005). "Duration of protection with RTS,S/AS02A malaria vaccine in prevention of Plasmodium falciparum disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial." Lancet 366(9502): 2012-8.

Alonso, P. L., J. Sacarlal, et al. (2004). "Efficacy of the RTS,S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial." Lancet 364(9443): 1411-20.

Amino, R., D. Giovannini, et al. (2008). "Host cell traversal is important for progression of the malaria parasite through the dermis to the liver." Cell Host Microbe 3(2): 88-96.

Amino, R., S. Thiberge, et al. (2006). "Quantitative imaging of Plasmodium transmission from mosquito to mammal." Nat Med 12(2): 220-4.

Aponte, J. J., P. Aide, et al. (2007). "Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial." Lancet 370(9598): 1543-51.

Aravind, L., L. M. Iyer, et al. (2003). "Plasmodium biology: genomic gleanings." Cell 115(7): 771-85.

Asea, A., M. Rehli, et al. (2002). "Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4." J Biol Chem 277(17): 15028-34.

Baer, K., M. Roosevelt, et al. (2007). "Kupffer cells are obligatory for Plasmodium yoelii sporozoite infection of the liver." Cell Microbiol 9(2): 397-412.

Ballou, W. R., M. Arevalo-Herrera, et al. (2004). "Update on the clinical development of candidate malaria vaccines." Am J Trop Med Hyg 71(2 Suppl): 239-47.

Bertolino, P., M. C. Trescol-Biemont, et al. (1998). "Hepatocytes induce functional activation of naive CD8+ T lymphocytes but fail to promote survival." Eur J Immunol 28(1): 221-36.

Beutler, B., Z. Jiang, et al. (2006). "Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large." Annu Rev Immunol 24: 353-89.

Bilzer, M., F. Roggel, et al. (2006). "Role of Kupffer cells in host defense and liver disease." Liver Int 26(10): 1175-86.

Biragyn, A., P. A. Ruffini, et al. (2002). "Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2." Science 298(5595): 1025-9.

Blackman, M. J., H. G. Heidrich, et al. (1990). "A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies." J Exp Med 172(1): 379-82.

Bojang, K. A., P. J. Milligan, et al. (2001). "Efficacy of RTS,S/AS02 malaria vaccine against Plasmodium falciparum infection in semi-immune adult men in The Gambia: a randomised trial." Lancet 358(9297): 1927-34.

Bouharoun-Tayoun, H., C. Oeuvray, et al. (1995). "Mechanisms underlying the monocyte-mediated antibody-dependent killing of Plasmodium falciparum asexual blood stages." J Exp Med 182(2): 409-18.

Bouwens, L., P. De Bleser, et al. (1992). "Liver cell heterogeneity: functions of non-parenchymal cells." Enzyme 46(1-3): 155-68.

Brito, I. (2001). "Eradicating malaria: high hopes or a tangible goal? ." Health Policy at Harvard 2: 61–66.

Bruna-Romero, O., J. C. Hafalla, et al. (2001). "Detection of malaria liver-stages in mice infected through the bite of a single Anopheles mosquito using a highly sensitive real-time PCR." Int J Parasitol 31(13): 1499-502.

Bull, P. C., B. S. Lowe, et al. (1998). "Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria." Nat Med 4(3): 358-60.

Burns, K., J. Clatworthy, et al. (2000). "Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor." Nat Cell Biol 2(6): 346-51.

Campos, M. A., M. Closel, et al. (2004). "Impaired production of proinflammatory cytokines and host resistance to acute infection with Trypanosoma cruzi in mice lacking functional myeloid differentiation factor 88." J Immunol 172(3): 1711-8.

Campos, M. A. and R. T. Gazzinelli (2004). "Trypanosoma cruzi and its components as exogenous mediators of inflammation recognized through Toll-like receptors." Mediators Inflamm 13(3): 139-43.

Caramalho, I., T. Lopes-Carvalho, et al. (2003). "Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide." J Exp Med 197(4): 403-11.

Christophides, G. K. (2005). "Transgenic mosquitoes and malaria transmission." Cell Microbiol 7(3): 325-33.

Clyde, D. F., H. Most, et al. (1973). "Immunization of man against sporozite-induced falciparum malaria." Am J Med Sci 266(3): 169-77.

Coban, C., K. J. Ishii, et al. (2005). "Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin." J Exp Med 201(1): 19-25.

Coban, C., K. J. Ishii, et al. (2004). "Effect of CpG oligodeoxynucleotides on the immunogenicity of Pfs25, a Plasmodium falciparum transmission-blocking vaccine antigen." Infect Immun 72(1): 584-8.

Coban, C., K. J. Ishii, et al. (2002). "Purified malaria pigment (hemozoin) enhances dendritic cell maturation and modulates the isotype of antibodies induced by a DNA vaccine." Infect Immun 70(7): 3939-43.

Coban, C., K. J. Ishii, et al. (2007). "Pathological role of Toll-like receptor signaling in cerebral malaria." Int Immunol 19(1): 67-79.

Collins, W. E. and G. M. Jeffery (2007). "Plasmodium malariae: parasite and disease." Clin Microbiol Rev 20(4): 579-92.

Coutinho, A. (1976). "Genetic control of B-cell responses. II. Identification of the spleen B-cell defect in C3H/HeJ mice." Scand J Immunol 5(1-2): 129-40.

Coutinho, A., L. Forni, et al. (1978). "Genetic and functional characterization of an antiserum to the lipid A-specific triggering receptor on murine B lymphocytes." Eur J Immunol 8(1): 63-7.

Coutinho, A., E. Gronowicz, et al. (1974). "Mechanism of thymus-independent immunocyte triggering. Mitogenic activation of B cells results in specific immune responses." J Exp Med 139(1): 74-92.

Coutinho, A., G. Moller, et al. (1973). "In vitro activation of mouse lymphocytes in serum-free medium: effect of T and B cell mitogens on proliferation and antibody synthesis." Eur J Immunol 3(5): 299-306.

Creagh, E. M. and L. A. O'Neill (2006). "TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity." Trends Immunol 27(8): 352-7.

Crispe, I. N. (2003). "Hepatic T cells and liver tolerance." Nat Rev Immunol 3(1): 51-62.

Crispe, I. N. (2009). "The liver as a lymphoid organ." Annu Rev Immunol 27: 147-63.

Cui, J., T. Shin, et al. (1997). "Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors." Science 278(5343): 1623-6.

de Veer, M. J., J. M. Curtis, et al. (2003). "MyD88 is essential for clearance of Leishmania major: possible role for lipophosphoglycan and Toll-like receptor 2 signaling." Eur J Immunol 33(10): 2822-31.

Deshpande, P. and P. Shastry (2004). "Modulation of cytokine profiles by malaria pigment--hemozoin: role of IL-10 in suppression of proliferative responses of mitogen stimulated human PBMC." Cytokine 28(6): 205-13.

Dodoo, D., T. Staalsoe, et al. (2001). "Antibodies to variant antigens on the surfaces of infected erythrocytes are associated with protection from malaria in Ghanaian children." Infect Immun 69(6): 3713-8.

Doolan, D. L. and S. L. Hoffman (2000). "The complexity of protective immunity against liver-stage malaria." J Immunol 165(3): 1453-62.

Doolan, D. L. and N. Martinez-Alier (2006). "Immune response to pre-erythrocytic stages of malaria parasites." Curr Mol Med 6(2): 169-85.

Douradinha, B., M. R. van Dijk, et al. (2007). "Genetically attenuated P36p-deficient Plasmodium berghei sporozoites confer long-lasting and partial cross-species protection." Int J Parasitol 37(13): 1511-9.

Drennan, M. B., B. Stijlemans, et al. (2005). "The induction of a type 1 immune response following a Trypanosoma brucei infection is MyD88 dependent." J Immunol 175(4): 2501-9.

Florens, L., M. P. Washburn, et al. (2002). "A proteomic view of the Plasmodium falciparum life cycle." Nature 419(6906): 520-6.

Franke-Fayard, B., H. Trueman, et al. (2004). "A Plasmodium berghei reference line that constitutively expresses GFP at a high level throughout the complete life cycle." Mol Biochem Parasitol 137(1): 23-33.

Franklin, B. S., S. O. Rodrigues, et al. (2007). "MyD88-dependent activation of dendritic cells and CD4(+) T lymphocytes mediates symptoms, but is not required for the immunological control of parasites during rodent malaria." Microbes Infect 9(7): 881-90.

Frevert, U., S. Engelmann, et al. (2005). "Intravital observation of Plasmodium berghei sporozoite infection of the liver." PLoS Biol 3(6): e192.

Frevert, U., M. R. Galinski, et al. (1998). "Malaria circumsporozoite protein inhibits protein synthesis in mammalian cells." Embo J 17(14): 3816-26.

Frevert, U., P. Sinnis, et al. (1993). "Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes." J Exp Med 177(5): 1287-98.

Gardner, M. J., N. Hall, et al. (2002). "Genome sequence of the human malaria parasite Plasmodium falciparum." Nature 419(6906): 498-511.

Gazzinelli, R. T. and E. Y. Denkers (2006). "Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism." Nat Rev Immunol 6(12): 895-906.

Gazzinelli, R. T., C. Ropert, et al. (2004). "Role of the Toll/interleukin-1 receptor signaling pathway in host resistance and pathogenesis during infection with protozoan parasites." Immunol Rev 201: 9-25.

Girard, M. P., Z. H. Reed, et al. (2007). "A review of human vaccine research and development: malaria." Vaccine 25(9): 1567-80.

Gonzalez-Aseguinolaza, G., C. de Oliveira, et al. (2000). "alpha -galactosylceramide-activated Valpha 14 natural killer T cells mediate protection against murine malaria." Proc Natl Acad Sci U S A 97(15): 8461-6.

Good, M. F. (2001). "Towards a blood-stage vaccine for malaria: are we following all the leads?" Nat Rev Immunol 1(2): 117-25.

Gray, J. C., P. H. Corran, et al. (2007). "Profiling the antibody immune response against blood stage malaria vaccine candidates." Clin Chem 53(7): 1244-53.

Greenwood, B. and T. Mutabingwa (2002). "Malaria in 2002." Nature 415(6872): 670-2.

Greenwood, B. M., D. A. Fidock, et al. (2008). "Malaria: progress, perils, and prospects for eradication." J Clin Invest 118(4): 1266-76.

Gruner, A. C., M. Mauduit, et al. (2007). "Sterile protection against malaria is independent of immune responses to the circumsporozoite protein." PLoS ONE 2(12): e1371.

Gupta, S. and K. P. Day (1994). "A theoretical framework for the immunoepidemiology of Plasmodium falciparum malaria." Parasite Immunol 16(7): 361-70.

Hall, N., M. Karras, et al. (2005). "A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses." Science 307(5706): 82-6.

Hashimoto, C., K. L. Hudson, et al. (1988). "The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein." Cell 52(2): 269-79.

Hemmi, H., O. Takeuchi, et al. (2000). "A Toll-like receptor recognizes bacterial DNA." Nature 408(6813): 740-5.

Herkel, J., B. Jagemann, et al. (2003). "MHC class II-expressing hepatocytes function as antigen-presenting cells and activate specific CD4 T lymphocyutes." Hepatology 37(5): 1079-85.

Hill, A. V. (2006). "Pre-erythrocytic malaria vaccines: towards greater efficacy." Nat Rev Immunol 6(1): 21-32.

Hoffman, S. L. and D. L. Doolan (2000). "Malaria vaccines-targeting infected hepatocytes." Nat Med 6(11): 1218-9.

Hoffman, S. L., D. Isenbarger, et al. (1989). "Sporozoite vaccine induces genetically restricted T cell elimination of malaria from hepatocytes." Science 244(4908): 1078-81.

Hoffmann, J. A. and J. M. Reichhart (2002). "Drosophila innate immunity: an evolutionary perspective." Nat Immunol 3(2): 121-6.

Hollingdale, M. R. (1985). "Malaria and the liver." Hepatology 5(2): 327-35.

Hollingdale, M. R., P. Leland, et al. (1983). "Serological reactivity of in vitro cultured exoerythrocytic stages of Plasmodium berghei in indirect immunofluorescent or immunoperoxidase antibody tests." Am J Trop Med Hyg 32(1): 24-30.

Hoshino, K., O. Takeuchi, et al. (1999). "Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product." J Immunol 162(7): 3749-52.

Huy, N. T., D. T. Trang, et al. (2006). "Leukocyte activation by malarial pigment." Parasitol Int 55(1): 75-81.

Ishino, T., Y. Chinzei, et al. (2005). "A Plasmodium sporozoite protein with a membrane attack complex domain is required for breaching the liver sinusoidal cell layer prior to hepatocyte infection." Cell Microbiol 7(2): 199-208.

Ishino, T., K. Yano, et al. (2004). "Cell-passage activity is required for the malarial parasite to cross the liver sinusoidal cell layer." PLoS Biol 2(1): E4.

Itoh, Y., A. Morita, et al. (2001). "Time course profile and cell-type-specific production of monokine induced by interferon-gamma in Concanavalin A-induced hepatic injury in mice: comparative study with interferon-inducible protein-10." Scand J Gastroenterol 36(12): 1344-51.

Janeway, C. A., Jr. and R. Medzhitov (2002). "Innate immune recognition." Annu Rev Immunol 20: 197-216.

Jaramillo, M., D. C. Gowda, et al. (2003). "Hemozoin increases IFN-gamma-inducible macrophage nitric oxide generation through extracellular signal-regulated kinase- and NF-kappa B-dependent pathways." J Immunol 171(8): 4243-53.

Jaramillo, M., I. Plante, et al. (2004). "Hemozoin-inducible proinflammatory events in vivo: potential role in malaria infection." J Immunol 172(5): 3101-10.

Jiang, W., R. Sun, et al. (2009). "TLR-9 activation aggravates concanavalin A-induced hepatitis via promoting accumulation and activation of liver CD4+ NKT cells." J Immunol 182(6): 3768-74.

Jobe, O., J. Lumsden, et al. (2007). "Genetically attenuated Plasmodium berghei liver stages induce sterile protracted protection that is mediated by major histocompatibility complex Class I-dependent interferon-gamma-producing CD8+ T cells." J Infect Dis 196(4): 599-607.

Johnson, G. B., G. J. Brunn, et al. (2002). "Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4." J Immunol 168(10): 5233-9.

Kappe, S. H., C. A. Buscaglia, et al. (2004). "Plasmodium sporozoite molecular cell biology." Annu Rev Cell Dev Biol 20: 29-59.

Kawai, T., O. Adachi, et al. (1999). "Unresponsiveness of MyD88-deficient mice to endotoxin." Immunity 11(1): 115-22.

Kawai, T. and S. Akira (2007). "TLR signaling." Semin Immunol 19(1): 24-32.

Kawasaki, K., S. Akashi, et al. (2000). "Mouse toll-like receptor 4.MD-2 complex mediates lipopolysaccharide-mimetic signal transduction by Taxol." J Biol Chem 275(4): 2251-4.

Keller, C. C., O. Yamo, et al. (2006). "Acquisition of hemozoin by monocytes down-regulates interleukin-12 p40 (IL-12p40) transcripts and circulating IL-12p70 through an IL-10-dependent mechanism: in vivo and in vitro findings in severe malarial anemia." Infect Immun 74(9): 5249-60.

Khan, Z. M., C. Ng, et al. (1992). "Early hepatic stages of Plasmodium berghei: release of circumsporozoite protein and host cellular inflammatory response." Infect Immun 60(1): 264-70.

Khan, Z. M. and J. P. Vanderberg (1991). "Role of host cellular response in differential susceptibility of nonimmunized BALB/c mice to Plasmodium berghei and Plasmodium yoelii sporozoites." Infect Immun 59(8): 2529-34.

Khor, C. C., S. J. Chapman, et al. (2007). "A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis." Nat Genet 39(4): 523-8.

Klein, A., M. Zhadkewich, et al. (1994). "Quantitative discrimination of hepatic reticuloendothelial clearance and phagocytic killing." J Leukoc Biol 55(2): 248-52.

Klugewitz, K., F. Blumenthal-Barby, et al. (2002). "Immunomodulatory effects of the liver: deletion of activated CD4+ effector cells and suppression of IFN-gamma-producing cells after intravenous protein immunization." J Immunol 169(5): 2407-13.

Knolle, P., J. Schlaak, et al. (1995). "Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge." J Hepatol 22(2): 226-9.

Knolle, P. A., T. Germann, et al. (1999). "Endotoxin down-regulates T cell activation by antigen-presenting liver sinusoidal endothelial cells." J Immunol 162(3): 1401-7.

Knolle, P. A., E. Schmitt, et al. (1999). "Induction of cytokine production in naive CD4(+) T cells by antigen-presenting murine liver sinusoidal endothelial cells but failure to induce differentiation toward Th1 cells." Gastroenterology 116(6): 1428-40.

Krishnegowda, G., A. M. Hajjar, et al. (2005). "Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of Plasmodium falciparum: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity." J Biol Chem 280(9): 8606-16.

Kropf, P., M. A. Freudenberg, et al. (2004). "Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite Leishmania major." Infect Immun 72(4): 1920-8.

Kumar, K. A., G. Sano, et al. (2006). "The circumsporozoite protein is an immunodominant protective antigen in irradiated sporozoites." Nature 444(7121): 937-40.

Kumar, S., T. R. Jones, et al. (2004). "CpG oligodeoxynucleotide and Montanide ISA 51 adjuvant combination enhanced the protective efficacy of a subunit malaria vaccine." Infect Immun 72(2): 949-57.

Kurt-Jones, E. A., L. Popova, et al. (2000). "Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus." Nat Immunol 1(5): 398-401.

Labaied, M., A. Harupa, et al. (2007). "Plasmodium yoelii sporozoites with simultaneous deletion of P52 and P36 are completely attenuated and confer sterile immunity against infection." Infect Immun 75(8): 3758-68.

Lalvani, A., P. Moris, et al. (1999). "Potent induction of focused Th1-type cellular and humoral immune responses by RTS,S/SBAS2, a recombinant Plasmodium falciparum malaria vaccine." J Infect Dis 180(5): 1656-64.

Lang, K. S., P. Georgiev, et al. (2006). "Immunoprivileged status of the liver is controlled by Toll-like receptor 3 signaling." J Clin Invest 116(9): 2456-63.

Langhorne, J., C. Cross, et al. (1998). "A role for B cells in the development of T cell helper function in a malaria infection in mice." Proc Natl Acad Sci U S A 95(4): 1730-4.

Laveran, A. (1882). "De la nature parasitaire de l'impaludisme." Bull. Mem. Soc. Med. Hop. Paris 18: 168–176.

Le, T. P., K. M. Coonan, et al. (2000). "Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers." Vaccine 18(18): 1893-901.

Leadbetter, E. A., I. R. Rifkin, et al. (2002). "Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors." Nature 416(6881): 603-7.

Lemaitre, B., M. Meister, et al. (1995). "Functional analysis and regulation of nuclear import of dorsal during the immune response in Drosophila." Embo J 14(3): 536-45.

Lemaitre, B., E. Nicolas, et al. (1996). "The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults." Cell 86(6): 973-83.

Lepenies, B., J. P. Cramer, et al. (2008). "Induction of experimental cerebral malaria is independent of TLR2/4/9." Med Microbiol Immunol 197(1): 39-44.

Lien, E., T. J. Sellati, et al. (1999). "Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products." J Biol Chem 274(47): 33419-25.

Liew, F. Y., D. Xu, et al. (2005). "Negative regulation of toll-like receptor-mediated immune responses." Nat Rev Immunol 5(6): 446-58.

Limmer, A., J. Ohl, et al. (2000). "Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance." Nat Med 6(12): 1348-54.

Liu, S., D. J. Gallo, et al. (2002). "Role of toll-like receptors in changes in gene expression and NF-kappa B activation in mouse hepatocytes stimulated with lipopolysaccharide." Infect Immun 70(7): 3433-42.

Loharungsikul, S., M. Troye-Blomberg, et al. (2008). "Expression of toll-like receptors on antigen-presenting cells in patients with falciparum malaria." Acta Trop 105(1): 10-5.

McCall, M. B., M. G. Netea, et al. (2007). "Plasmodium falciparum infection causes proinflammatory priming of human TLR responses." J Immunol 179(1): 162-71.

McCartney-Francis, N., W. Jin, et al. (2004). "Aberrant Toll receptor expression and endotoxin hypersensitivity in mice lacking a functional TGF-beta 1 signaling pathway." J Immunol 172(6): 3814-21.

Medzhitov, R. (2001). "Toll-like receptors and innate immunity." Nat Rev Immunol 1(2): 135-45.

Medzhitov, R. and C. A. Janeway, Jr. (1997). "Innate immunity: the virtues of a nonclonal system of recognition." Cell 91(3): 295-8.

Medzhitov, R., P. Preston-Hurlburt, et al. (1997). "A human homologue of the Drosophila Toll protein signals activation of adaptive immunity." Nature 388(6640): 394-7.

Medzhitov, R., P. Preston-Hurlburt, et al. (1998). "MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways." Mol Cell 2(2): 253-8.

Meis, J. F., J. P. Verhave, et al. (1983). "An ultrastructural study on the role of Kupffer cells in the process of infection by Plasmodium berghei sporozoites in rats." Parasitology 86 (Pt 2): 231-42.

Meylan, E., J. Tschopp, et al. (2006). "Intracellular pattern recognition receptors in the host response." Nature 442(7098): 39-44.

Mikolajczak, S. A., V. Jacobs-Lorena, et al. (2007). "L-FABP is a critical host factor for successful malaria liver stage development." Int J Parasitol 37(5): 483-9.

Mikolajczak, S. A. and S. H. Kappe (2006). "A clash to conquer: the malaria parasite liver infection." Mol Microbiol 62(6): 1499-506.

Miller, L. H. and B. Greenwood (2002). "Malaria--a shadow over Africa." Science 298(5591): 121-2.

Millington, O. R., C. Di Lorenzo, et al. (2006). "Suppression of adaptive immunity to heterologous antigens during Plasmodium infection through hemozoin-induced failure of dendritic cell function." J Biol 5(2): 5.

Minns, L. A., L. C. Menard, et al. (2006). "TLR9 is required for the gut-associated lymphoid tissue response following oral infection of Toxoplasma gondii." J Immunol 176(12): 7589-97.

Mockenhaupt, F. P., J. P. Cramer, et al. (2006). "Toll-like receptor (TLR) polymorphisms in African children: Common TLR-4 variants predispose to severe malaria." Proc Natl Acad Sci U S A 103(1): 177-82.

Mockenhaupt, F. P., L. Hamann, et al. (2006). "Common polymorphisms of toll-like receptors 4 and 9 are associated with the clinical manifestation of malaria during pregnancy." J Infect Dis 194(2): 184-8.

Mota, M. M., G. Pradel, et al. (2001). "Migration of Plasmodium sporozoites through cells before infection." Science 291(5501): 141-4.

Mueller, A. K., N. Camargo, et al. (2005). "Plasmodium liver stage developmental arrest by depletion of a protein at the parasite-host interface." Proc Natl Acad Sci U S A 102(8): 3022-7.

Mueller, A. K., M. Deckert, et al. (2007). "Genetically attenuated Plasmodium berghei liver stages persist and elicit sterile protection primarily via CD8 T cells." Am J Pathol 171(1): 107-15.

Mueller, A. K., M. Labaied, et al. (2005). "Genetically modified Plasmodium parasites as a protective experimental malaria vaccine." Nature 433(7022): 164-7.

Muraille, E., C. De Trez, et al. (2003). "Genetically resistant mice lacking MyD88-adapter protein display a high susceptibility to Leishmania major infection associated with a polarized Th2 response." J Immunol 170(8): 4237-41.

Naik, R. S., O. H. Branch, et al. (2000). "Glycosylphosphatidylinositol anchors of Plasmodium falciparum: molecular characterization and naturally elicited antibody response that may provide immunity to malaria pathogenesis." J Exp Med 192(11): 1563-76.

Nebl, T., M. J. De Veer, et al. (2005). "Stimulation of innate immune responses by malarial glycosylphosphatidylinositol via pattern recognition receptors." Parasitology 130 Suppl: S45-62.

Nussenzweig, R. S., J. Vanderberg, et al. (1967). "Protective immunity produced by the injection of x-irradiated sporozoites of plasmodium berghei." Nature 216(5111): 160-2.

O'Neill, L. A. and C. Greene (1998). "Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects, and plants." J Leukoc Biol 63(6): 650-7.

Ockenhouse, C. F., W. C. Hu, et al. (2006). "Common and divergent immune response signaling pathways discovered in peripheral blood mononuclear cell gene expression patterns in presymptomatic and clinically apparent malaria." Infect Immun 74(10): 5561-73.

Ohashi, K., V. Burkart, et al. (2000). "Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex." J Immunol 164(2): 558-61.

Okamura, Y., M. Watari, et al. (2001). "The extra domain A of fibronectin activates Toll-like receptor 4." J Biol Chem 276(13): 10229-33.

Ozinsky, A., D. M. Underhill, et al. (2000). "The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors." Proc Natl Acad Sci U S A 97(25): 13766-71.

Parroche, P., F. N. Lauw, et al. (2007). "Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9." Proc Natl Acad Sci U S A 104(6): 1919-24.

Pasare, C. and R. Medzhitov (2005). "Toll-like receptors: linking innate and adaptive immunity." Adv Exp Med Biol 560: 11-8.

Plebanski, M., C. M. Hannan, et al. (2005). "Direct processing and presentation of antigen from malaria sporozoites by professional antigen-presenting cells in the induction of CD8 T-cell responses." Immunol Cell Biol 83(3): 307-12.

Poltorak, A., X. He, et al. (1998). "Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene." Science 282(5396): 2085-8.

Pradel, G. and U. Frevert (2001). "Malaria sporozoites actively enter and pass through rat Kupffer cells prior to hepatocyte invasion." Hepatology 33(5): 1154-65.

Pradel, G., S. Garapaty, et al. (2002). "Proteoglycans mediate malaria sporozoite targeting to the liver." Mol Microbiol 45(3): 637-51.

Prudencio, M., A. Rodriguez, et al. (2006). "The silent path to thousands of merozoites: the Plasmodium liver stage." Nat Rev Microbiol 4(11): 849-56.

Qureshi, S. T., L. Lariviere, et al. (1999). "Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4)." J Exp Med 189(4): 615-25.

Rassa, J. C., J. L. Meyers, et al. (2002). "Murine retroviruses activate B cells via interaction with toll-like receptor 4." Proc Natl Acad Sci U S A 99(4): 2281-6.

Renggli, J., M. Hahne, et al. (1997). "Elimination of P. berghei liver stages is independent of Fas (CD95/Apo-I) or perforin-mediated cytotoxicity." Parasite Immunol 19(3): 145-8.

Renia, L., D. Mattei, et al. (1990). "A malaria heat-shock-like determinant expressed on the infected hepatocyte surface is the target of antibody-dependent cell-mediated cytotoxic mechanisms by nonparenchymal liver cells." Eur J Immunol 20(7): 1445-9.

Rich, T., R. Allen, et al. (2000). "How low can toll go?" Trends Genet 16(7): 292-4.

Roberts, D. R., S. Manguin, et al. (2000). "DDT house spraying and re-emerging malaria." Lancet 356(9226): 330-2.

Robson, K. J., U. Frevert, et al. (1995). "Thrombospondin-related adhesive protein (TRAP) of Plasmodium falciparum: expression during sporozoite ontogeny and binding to human hepatocytes." Embo J 14(16): 3883-94.

Robson, K. J., J. R. Hall, et al. (1988). "A highly conserved amino-acid sequence in thrombospondin, properdin and in proteins from sporozoites and blood stages of a human malaria parasite." Nature 335(6185): 79-82.

Rock, F. L., G. Hardiman, et al. (1998). "A family of human receptors structurally related to Drosophila Toll." Proc Natl Acad Sci U S A 95(2): 588-93.

Rodrigues, M. M., A. S. Cordey, et al. (1991). "CD8+ cytolytic T cell clones derived against the Plasmodium yoelii circumsporozoite protein protect against malaria." Int Immunol 3(6): 579-85.

Romero, P., J. L. Maryanski, et al. (1989). "Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria." Nature 341(6240): 323-6.

Ropert, C. and R. T. Gazzinelli (2000). "Signaling of immune system cells by glycosylphosphatidylinositol (GPI) anchor and related structures derived from parasitic protozoa." Curr Opin Microbiol 3(4): 395-403.

Rosenberg, R., R. A. Wirtz, et al. (1990). "An estimation of the number of malaria sporozoites ejected by a feeding mosquito." Trans R Soc Trop Med Hyg 84(2): 209-12.

Sacher, T., P. Knolle, et al. (2002). "CpG-ODN-induced inflammation is sufficient to cause T-cell-mediated autoaggression against hepatocytes." Eur J Immunol 32(12): 3628-37.

Scanga, C. A., J. Aliberti, et al. (2002). "Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and regulates parasite-induced IL-12 production by dendritic cells." J Immunol 168(12): 5997-6001.

Schofield, L., A. Ferreira, et al. (1987). "Interferon-gamma inhibits the intrahepatocytic development of malaria parasites in vitro." J Immunol 139(6): 2020-5.

Schofield, L. and F. Hackett (1993). "Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites." J Exp Med 177(1): 145-53.

Schofield, L., M. C. Hewitt, et al. (2002). "Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria." Nature 418(6899): 785-9.

Schofield, L., J. Villaquiran, et al. (1987). "Gamma interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites." Nature 330(6149): 664-6.

Schumann, J. and G. Tiegs (1999). "Pathophysiological mechanisms of TNF during intoxication with natural or man-made toxins." Toxicology 138(2): 103-26.

Schwandner, R., R. Dziarski, et al. (1999). "Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2." J Biol Chem 274(25): 17406-9.

Schwarzer, E., M. Alessio, et al. (1998). "Phagocytosis of the malarial pigment, hemozoin, impairs expression of major histocompatibility complex class II antigen, CD54, and CD11c in human monocytes." Infect Immun 66(4): 1601-6.

Schwarzer, E., F. Turrini, et al. (1992). "Impairment of macrophage functions after ingestion of Plasmodium falciparum-infected erythrocytes or isolated malarial pigment." J Exp Med 176(4): 1033-41.

Shoham, S., C. Huang, et al. (2001). "Toll-like receptor 4 mediates intracellular signaling without TNF-alpha release in response to Cryptococcus neoformans polysaccharide capsule." J Immunol 166(7): 4620-6.

Singh, A. P., C. A. Buscaglia, et al. (2007). "Plasmodium circumsporozoite protein promotes the development of the liver stages of the parasite." Cell 131(3): 492-504.

Singh, B., L. Kim Sung, et al. (2004). "A large focus of naturally acquired Plasmodium knowlesi infections in human beings." Lancet 363(9414): 1017-24.

Skeiky, Y. A., R. N. Coler, et al. (2002). "Protective efficacy of a tandemly linked, multi-subunit recombinant leishmanial vaccine (Leish-111f) formulated in MPL adjuvant." Vaccine 20(27-28): 3292-303.

Skorokhod, O. A., M. Alessio, et al. (2004). "Hemozoin (malarial pigment) inhibits differentiation and maturation of human monocyte-derived dendritic cells: a peroxisome proliferator-activated receptor-gamma-mediated effect." J Immunol 173(6): 4066-74.

Smiley, S. T., J. A. King, et al. (2001). "Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4." J Immunol 167(5): 2887-94.

Snow, R. W., C. A. Guerra, et al. (2005). "The global distribution of clinical episodes of Plasmodium falciparum malaria." Nature 434(7030): 214-7.

Snow, R. W., J. F. Trape, et al. (2001). "The past, present and future of childhood malaria mortality in Africa." Trends Parasitol 17(12): 593-7.

Steers, N., R. Schwenk, et al. (2005). "The immune status of Kupffer cells profoundly influences their responses to infectious Plasmodium berghei sporozoites." Eur J Immunol 35(8): 2335-46.

Stevenson, M. M. and E. M. Riley (2004). "Innate immunity to malaria." Nat Rev Immunol 4(3): 169-80.

Stoute, J. A., M. Slaoui, et al. (1997). "A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation Group." N Engl J Med 336(2): 86-91.

Sturm, A., R. Amino, et al. (2006). "Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids." Science 313(5791): 1287-90.

Sultan, A. A., V. Thathy, et al. (1997). "TRAP is necessary for gliding motility and infectivity of plasmodium sporozoites." Cell 90(3): 511-22.

Tachado, S. D., P. Gerold, et al. (1996). "Glycosylphosphatidylinositol toxin of Plasmodium induces nitric oxide synthase expression in macrophages and vascular endothelial cells by a protein tyrosine kinase-dependent and protein kinase C-dependent signaling pathway." J Immunol 156(5): 1897-1907.

Takeuchi, O. and S. Akira (2001). "Toll-like receptors; their physiological role and signal transduction system." Int Immunopharmacol 1(4): 625-35.

Takeuchi, O., T. Kawai, et al. (2001). "Discrimination of bacterial lipoproteins by Toll-like receptor 6." Int Immunol 13(7): 933-40.

Taramelli, D., N. Basilico, et al. (1995). "The heme moiety of malaria pigment (beta-hematin) mediates the inhibition of nitric oxide and tumor necrosis factor-alpha production by lipopolysaccharide-stimulated macrophages." Exp Parasitol 81(4): 501-11.

Tarun, A. S., R. F. Dumpit, et al. (2007). "Protracted sterile protection with Plasmodium yoelii pre-erythrocytic genetically attenuated parasite malaria vaccines is independent of significant liver-stage persistence and is mediated by CD8+ T cells." J Infect Dis 196(4): 608-16.

Termeer, C., F. Benedix, et al. (2002). "Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4." J Exp Med 195(1): 99-111.

Todryk, S. M. and A. V. Hill (2007). "Malaria vaccines: the stage we are at." Nat Rev Microbiol 5(7): 487-9.

Togbe, D., L. Schofield, et al. (2007). "Murine cerebral malaria development is independent of toll-like receptor signaling." Am J Pathol 170(5): 1640-8.

Torgler, R., S. E. Bongfen, et al. (2008). "Sporozoite-mediated hepatocyte wounding limits Plasmodium parasite development via MyD88-mediated NF-kappa B activation and inducible NO synthase expression." J Immunol 180(6): 3990-9.

Tsuji, M., D. Mattei, et al. (1994). "Demonstration of heat-shock protein 70 in the sporozoite stage of malaria parasites." Parasitol Res 80(1): 16-21.

Tuteja, R. (2007). "Malaria - an overview." Febs J 274(18): 4670-9.

Urban, B. C. and S. Todryk (2006). "Malaria pigment paralyzes dendritic cells." J Biol 5(2): 4.

Usynin, I., C. Klotz, et al. (2007). "Malaria circumsporozoite protein inhibits the respiratory burst in Kupffer cells." Cell Microbiol 9(11): 2610-28.

Vabulas, R. M., P. Ahmad-Nejad, et al. (2002). "HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway." J Biol Chem 277(17): 15107-12.

Vabulas, R. M., S. Braedel, et al. (2002). "The endoplasmic reticulum-resident heat shock protein Gp96 activates dendritic cells via the Toll-like receptor 2/4 pathway." J Biol Chem 277(23): 20847-53.

van de Sand, C., S. Horstmann, et al. (2005). "The liver stage of Plasmodium berghei inhibits host cell apoptosis." Mol Microbiol 58(3): 731-42.

van Dijk, M. R., B. Douradinha, et al. (2005). "Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells." Proc Natl Acad Sci U S A 102(34): 12194-9.

van Schaijk, B. C., C. J. Janse, et al. (2008). "Gene disruption of Plasmodium falciparum p52 results in attenuation of malaria liver stage development in cultured primary human hepatocytes." PLoS ONE 3(10): e3549.

Vanderberg, J. P. and U. Frevert (2004). "Intravital microscopy demonstrating antibody-mediated immobilisation of Plasmodium berghei sporozoites injected into skin by mosquitoes." Int J Parasitol 34(9): 991-6.

Vreden, S. G., R. W. Sauerwein, et al. (1993). "Kupffer cell elimination enhances development of liver schizonts of Plasmodium berghei in rats." Infect Immun 61(5): 1936-9.

Vreden, S. G., M. F. van den Broek, et al. (1992). "Cytokines inhibit the development of liver schizonts of the malaria parasite Plasmodium berghei in vivo." Eur J Immunol 22(9): 2271-5.

Wang, J. E., A. Warris, et al. (2001). "Involvement of CD14 and toll-like receptors in activation of human monocytes by Aspergillus fumigatus hyphae." Infect Immun 69(4): 2402-6.

Wang, R., D. L. Doolan, et al. (1998). "Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine." Science 282(5388): 476-80.

Wang, R., J. Epstein, et al. (2001). "Induction of CD4(+) T cell-dependent CD8(+) type 1 responses in humans by a malaria DNA vaccine." Proc Natl Acad Sci U S A 98(19): 10817-22.

Warren, A., D. G. Le Couteur, et al. (2006). "T lymphocytes interact with hepatocytes through fenestrations in murine liver sinusoidal endothelial cells." Hepatology 44(5): 1182-90.

Weiss, W. R., M. Sedegah, et al. (1988). "CD8+ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites." Proc Natl Acad Sci U S A 85(2): 573-6.

Winau, F., G. Hegasy, et al. (2007). "Ito cells are liver-resident antigen-presenting cells for activating T cell responses." Immunity 26(1): 117-29.

Yamauchi, L. M., A. Coppi, et al. (2007). "Plasmodium sporozoites trickle out of the injection site." Cell Microbiol 9(5): 1215-22.

Yoshida, N., R. S. Nussenzweig, et al. (1980). "Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite." Science 207(4426): 71-3.

Yoshimura, A., E. Lien, et al. (1999). "Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2." J Immunol 163(1): 1-5.

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