UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

EXPERIMENTAL PREGNANCY-ASSOCIATED MALARIA: IMMUNOPATHOLOGICAL COMPONENTS

Rita Maria de Almeida Neres

Doutoramento em Farmácia

(Microbiologia)

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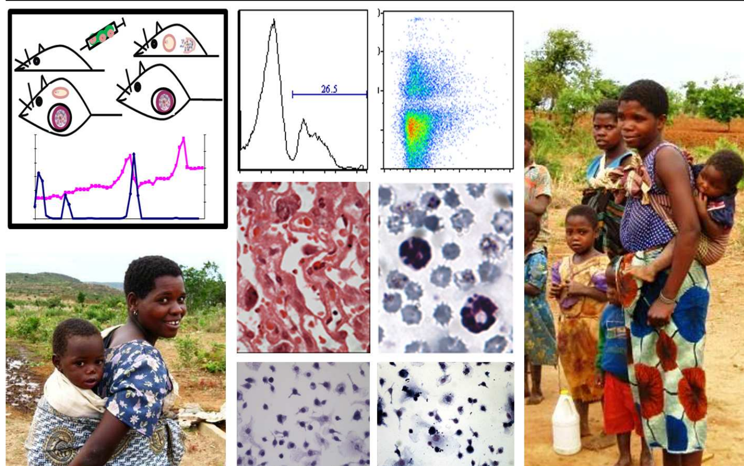

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Science and Human needs

Investigating for happiness

The panel presented in the previous page is composed by:
Outdoor (upper image), São Tomé e Príncipe (2005).
Mozambican pregnant women with children, by Ana Neres.
The remaining images are experimental pregnancy-associated malaria pictures:
Flow Cytometry charts; HE staining of an infected placenta; Blood smear; Mouse placental cell cultures.
Composition by Sergio Saraiva.

Ao Sebastião

Para o Francisco e para o Henrique

“We ourselves feel that what we are doing is just a drop in the ocean.

But the ocean would be less because of that missing drop.”

Mother Teresa of Calcuta

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PUBLICATIONS

The work presented in this thesis includes data reported in the following publications:

Neres R, Marinho CR, Goncalves LA, Catarino MB & Penha-Goncalves C. (2008). Pregnancy outcome and placenta pathology in Plasmodium berghei ANKA infected mice reproduce the pathogenesis of severe malaria in pregnant women. *PLoS One* **3,** e1608.

Marinho CR, **Neres R**, Epiphanio S, Goncalves LA, Catarino MB & Penha-Goncalves C. (2009). Recrudescent Plasmodium berghei from pregnant mice displays enhanced binding to the placenta and induces protection in multigravida. *PLoS One* **4,** e5630.

SUMMARY

Pregnancy-associated malaria (PAM) represents a major health concern worldwide. Current knowledge of this form of human malaria is concurred by epidemiological, pathological, immunological and parasite biology evidence. Nevertheless, the lack of animal models to study PAM constitute a limitation to in-depth identification of cellular and molecular components of PAM pathogenesis and to further understand the susceptibility and protection mechanisms underlying PAM.

The aim of this thesis was firstly to establish an experimental system enabling pregnancy-associated malaria (PAM) studies in mouse models and secondly to characterize the immunological and pathological features in murine pregnancy-associated malaria.

Using *P. berghei*-ANKA-GFP parasites and the BALB/c mouse strain the experimental systems were developed taking in consideration that human PAM has distinct presentations and consequences depending on the previous maternal immunity to malaria (premunition). In one scenario, women have not developed an immune protection against malaria before pregnancy (low premunition), which is typical of regions with low malaria transmission or unstable malaria. To model the low premunition scenario the mouse females contacted with malaria parasites for the first time when infected with iRBC during pregnancy (Model of infection during pregnancy). On the other hand, in the high-transmission areas women experience prolonged exposure to malaria and are relatively well protected against malaria before pregnancy (high premunition). To model high premunition before pregnancy, the females were immunized by infection with iRBC before pregnancy (Model of pre-exposure).

These models were scrutinized for pregnancy outcome, placenta pathology and PAM protective responses. The experimental model established in non-immune mice showed enhanced disease severity, poor pregnancy outcomes and the prominent histological alterations. The pathology of mouse placenta infected with *P. berghei* resembles the acute *P. falciparum* placental malaria in humans allowing us to identify trophoblast thickening and vascular space reduction as hallmarks of placenta malaria induced by the *P.berghei*. In the pre-exposure (pregnancy-induced malaria recrudescence) model the intensity of parasite recrudescence showed to be quantitatively correlated with the placenta pathology while the recrudescence incidence and the adverse pregnancy outcomes decreased with parity.

P. berghei-GFP adhesion experiments indicate that iRBC express ligands for different receptors in the mouse placenta. iRBC from recrudescent females displayed enhanced adhesion to the placenta suggesting that *P. berghei* parasites mediating PAM have increased specificity for placenta receptors. Pre-exposed females showed a long-term malaria protection state that is abrogated by pregnancy, strongly suggesting that the host mechanisms that confer protection against pregnancy–associated *P. berghei* appear not to protect from non-placental *P. berghei*.

The data provided in this thesis demonstrate that the experimental systems based on *P.berghei*-BALB/c mouse are valid models to study the pathogenesis of placenta malaria, the adhesion of placental parasites, the parasite-placenta interaction and the mechanisms of PAM protection elicited during pregnancy. The experimental systems presented in this thesis could prove useful in drawing new hypothesis and testing analogies on the factors and mechanisms that are considered relevant for human PAM.

Keywords: malaria, pregnancy, placenta, *Plasmodium berghei*, pathology, parity, mouse models, BALB/c, recrudescence, premunition, immunopathology.

RESUMO

Em áreas endémicas de malária, estima-se a ocorrência de mais de 50 milhões de gravidezes por ano, aproximadamente metade das quais se verificam na África Sub-Sahariana, onde a transmissão de *P. falciparum* é mais intensa. A Malária Associada à Gravidez é um dos mais importantes problemas de saúde pública em África, contribuindo para uma alta carga da morbilidade materna e fetal, que pode ser responsável por 100,000 mortes de bebés por ano. As mulheres grávidas são mais susceptíveis à malária e podem exibir manifestações clínicas mais severas. Para além disso, a malária durante a gravidez está associada a maiores taxas de aborto e os recém-nascidos têm maior probabilidade de manifestar restrição de crescimento intra-uterino em combinação com um baixo peso à nascença, que constituem factores de risco fortemente associados à mortalidade neonatal.

Nem todos os aspectos da malária na gravidez estão totalmente entendidos, tanto do lado do parasita, como do lado do hospedeiro e constituem prioridades para a investigação. A maior parte do conhecimento que suporta a base biológica e imunopatológica da Malária Associada à Gravidez apenas deriva de estudos realizados em mulheres grávidas que vivem em áreas endémicas de malária. Uma vez que, por razões éticas ou logísticas, algumas questões importantes não podem ser abordadas em seres humanos, o conjunto de resultados apresentado na presente tese demonstra as potencialidades em usar modelos de ratinho para estudar a Malária Associada à Gravidez.

Na primeira parte da tese, apresentam-se os aspectos principais da Malária Associada à Gravidez em humanos, sendo abordados os pontos seguintes: (i) a importância da doença, realçando-se a epidemiologia e o seu impacto, tanto na mãe, como no feto/recém-nascido; (ii) caracterização da placenta e de alguns aspectos da imunologia associada à gravidez, realizando-se uma descrição subsequente das modificações conhecidas que são causadas pela malária; (iii) descrição da interacção entre os parasitas *P. falciparum* e o hospedeiro (grávida), realçando-se as características do parasita essenciais para a doença, bem como os mediadores placentários reconhecidos como intervenientes nesta interacção; e finalmente (iv) fundamenta-se a necessidade e as vantagens de usar modelos de ratinho para estudar a Malária Associada à Gravidez, salientando-se as semelhanças/diferenças na estrutura e no desenvolvimento da placenta entre o ratinho e o ser humano e, por último, refere-se os estudos anteriores que se debruçaram sobre a malária na gravidez em ratinho.

Os sistemas experimentais desenvolvidos tiveram em consideração que a malária na gravidez humana tem apresentações e consequências distintas conforme o grau de protecção da mãe. Este nível de imunidade materna à malária prévia à gravidez (premunição) está correlacionado com a exposição à malária e, portanto, com o nível de endemicidade, ou intensidade da

transmissão da malária, podendo considerar-se separadamente uma de duas situações. Na primeira, as mulheres não desenvolveram uma protecção contra a malária antes da gravidez (premunição baixa), o que é típico de regiões com baixa transmissão de malária ou com malária instável. No outro cenário, no qual as mulheres vivem em regiões que facilitam a exposição permanente à malária, as futuras mães estão relativamente bem protegidas contra a malária antes da gravidez (alta premunição). Consequentemente, decidiu-se pelo estabelecimento de dois modelos de ratinho que mimetizassem cada um destes cenários.

No modelo associado a uma premunição baixa, as fêmeas contactam com o parasita pela primeira vez apenas quando infectadas durante a gravidez (Modelo de Infecção Durante a Gravidez). No outro modelo, é necessário garantir que as fêmeas tenham uma alta premunição antes da gravidez, pelo que as fêmeas são infectadas e imunizadas antes da gravidez (Modelo de Pré-Exposição). Ambos os modelos foram estabelecidos usando a estirpe de ratinho BALB/c e os parasitas *P. berghei*-ANKA. Além disso, os modelos foram analisados considerando a reprodutibilidade das características principais da doença humana, tais como as modificações na placenta, os efeitos na prole e o tipo de interacção entre o parasita e a placenta.

Estes modelos foram avaliados para o resultado da gravidez, patologia da placenta e respostas protectoras à malária associadas à gravidez. O modelo experimental estabelecido em ratinhos não imunes mostrou uma maior severidade da doença, efeitos adversos na prole e alterações histológicas proeminentes. A patologia da placenta de ratinho infectada com *P. berghei* assemelha-se às manifestações agudas de infecções de *P. falciparum* em seres humanos. Particularmente, é possível observar o espessamento do trofoblasto e a redução dos espaços intervilosos, características da malária placentária induzida por *P. berghei*. No modelo de pré-exposição (recrudescência induzida pela gravidez) a intensidade da recrudescência, em termos de percentagem de parasitas no sangue periférico, mostrou estar quantitativamente correlacionada com a patologia da placenta. Por outro lado, a incidência de recrudescência e os resultados adversos da gravidez diminuíram com a paridade.

As experiências de adesão com *P. berghei*-GFP indicam que os eritrócitos infectados expressam ligandos diferentes para os receptores na placenta de ratinho. Os eritrócitos infectados de fêmeas recrudescentes manifestam uma maior intensidade de adesão na placenta, o que sugere que os parasitas de *P. berghei* que medeiam a malária na gravidez tenham aumentado a sua especificidade para se ligarem a receptores das placentas. As fêmeas pré-expostas à malária mostraram um estado de protecção por longos períodos contra a doença, mas este estado é alterado pela gravidez, sugerindo que os mecanismos que conferem protecção contra

os parasitas *P. berghei* associados à gravidez não protegem de parasitas *P. berghei* não placentários.

A descrição das características de ambos os modelos permitiu a sua validação como representações congruentes da Malária Associada à Gravidez humana. Consequentemente, estes modelos podem ser explorados no sentido de contribuir para o avanço do conhecimento da doença, nomeadamente dos aspectos da imunopatologia.

A investigação que serviu de base a esta tese permitiu a observação e a descrição da doença murina e das suas consequências. Para além disso, com este trabalho foi possível perspectivar algumas linhas de pesquisa, umas relacionados com a biologia de parasita, como a exploração da variação antigénica em *P. berghei* através do uso de parasitas recrudescentes, e outras referentes aos mecanismos da doença, como a imunopatologia. Nesse sentido, estes modelos podem contribuir para elucidar várias questões que estão ainda por esclarecer, como os factores que provocam a recrudescência de parasitas na ausência de reinfecção, o papel de anticorpos assimétricos na Malária Associada à Gravidez, a relação entre pre-eclampsia e malária, a activação policlonal e os processos associados à memória imunológica. Finalmente, a exploração das futuras potenciais direcções da pesquisa da doença pode fornecer novos avanços na compreensão dos mecanismos imunopatológicos, bem como servir de base para testes de segurança e eficácia de medicamentos ou mesmo contribuir para encontrar uma vacina que confira protecção durante a gravidez. Em suma, os modelos de ratinho parecem ser um instrumento promissor para compreender melhor os mecanismos desconhecidos da Malária Associada à Gravidez humana, constituindo uma esperança para melhorar a prevenção e o controlo da doença.

Palavras-Chave: malária, gravidez, placenta, *Plasmodium berghei*, patologia, paridade, modelos murinos, recrudescência, premunição, BALB/c.

ABBREVIATIONS

ADCC	Antibody-Dependent Celular Cytotoxicity
ADCI	Antibody-Dependent Cell-mediated Inhibition
APC	Antigen Presenting Cell
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CHO	Chinese Hamster Ovary cells
CSA	Chondroitin Sulfate A
CTL	Cytotoxic T Lymphocytes
DC	Dendritic Cells
EIR	Entomological Inoculation Rate
ELISA	Enzyme-Linked Immunoabsorbent Assay
G	Gestational day
GAG	Glycosaminoglycan
GFP	Green Fluorescent Protein
GPI	Glycosylphosphatidylinositol
HÁ	Hyaluronic Acid
HE	Hematoxilyn-Eosin staining
HLA	Human Leucocyte Antigen
HO	HemeOxygenase
i.p.	Intraperitoneal
i.v.	Intravenous
ICAM-1	Intercellular-Adhesion Molecule 1
IFN	Interferon
Ig	Immunoglobin
IL	Interleukin
IPTp	Intermittent Preventing Treatment in pregnancy
iRBC	Infected Red Blood Cell
ITN	Insecticide-Treated bed Nets
IUGR	IntraUterine Growth Retardation
IVS	InterVillous Space
LBW	Low Birth Weight

LIF	Leukemia Inhibitory Factor
MCP	Monocyte Chemotactic Protein
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
NK	Natural Killer cell
NO	Nitric Oxide
PAM	Pregnancy-Associated Malaria
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PfEMP1	*Plasmodium falciparum* Erythrocyte Protein 1
PR	Parasite Rate
PTD	PreTerm Delivery
RBC	Red Blood Cell
RT-PCR	Reverse Transcriptase PCR
s.e.m.	Standard Error of the Mean
TGF	Transforming Growth Factor
Th	T helper
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
Treg	Regulatory T cell
uNK	Uterine Natural Killer cell
VSA	Variant Surface Antigen
WHO	World Health Organization

INTRODUCTION

Pregnancy-associated malaria (PAM) represents a major health concern worldwide. Current knowledge of this form of human malaria is concurred by epidemiological, pathological, immunological and parasite biology evidence. In this section we summarize the background evidence that supports current hypotheses on PAM pathogenesis and elaborate considerations on the potential contribution of animal models to further understand the susceptibility and protection mechanisms underlying PAM.

MALARIA IN HUMAN PREGNANCY

Global distribution and disease burden

Pregnant women appear to be more susceptible to malaria than other adults and malaria is widely recognized as an infection that can seriously jeopardize the outcome of pregnancy, especially in women pregnant for the first time (Bray & Anderson, 1979; Brabin, 1983). Each year more than 50 million pregnancies occur in malaria-endemic areas, half of which in Africa mostly in areas of relatively stable malaria transmission and less than 5% of pregnant women have access to effective proposed interventions (Steketee *et al.*, 2001; WHO, 2004). Malaria infection of the placenta and malaria-caused maternal anemia contribute to low birth weight (LBW) which is a risk factor for infant mortality and impaired child development.

The burden of malaria infection during pregnancy is caused chiefly by *Plasmodium falciparum* (*P. falciparum*) (Figure 1), the most virulent/lethal, and also the most common Plasmodium species in Africa (Newbold *et al.*, 1997). The global population at risk of *P. falciparum* transmission in 2007 was estimated in 2.37 billion across 87 countries and included 0.98 billion people living in areas with low, unstable transmission risk (Snow *et al.*, 2008). Although *P. vivax* is responsible for infecting an estimated 2.59 billion people annually (Guerra *et al.*, 2006), it is commonly considered more benign than *P. falciparum* and its impact in pregnancy has recently started to be assessed (ter Kuile & Rogerson, 2008).

It is generally acknowledged that during pregnancy there is an increase in prevalence and density of *P. falciparum* malaria (Bray & Anderson, 1979; Brabin, 1983) and the impact of the other three human malaria parasites (*P. vivax, P. malariae* and *P. ovale*) is less clear in pregnancy, but they are not associated with severe disease. *P. falciparum* is normally present in tropical, subtropical and warm temperate regions. *P. vivax* is instead the most frequent parasite found in Asia, Central- and South-America and less common in West Africa as the majority of the population is negative for the Duffy blood group antigen that serves as a receptor for *P. vivax* parasites to enter the host red blood cells (RBC). *P. malariae* is much less common, appearing only in few African regions and in Western Pacific. Finally, *P. ovale* which

has the most limited distribution of all the malaria parasites of humans, can occur throughout most of sub-Saharan Africa and is known to be endemic only in New Guinea and in Philippines (Carter & Mendis, 2002). Acquired infections in humans have been recently reported in East Malaysia and Singapore for *P. knowlesi*, a simian malaria parasite that is generally misidentified as *P. malariae* since the blood stages are morphologically similar on microscopy, needing molecular methods of detection for correct diagnosis (Singh *et al.*, 2004; Ng *et al.*, 2008).

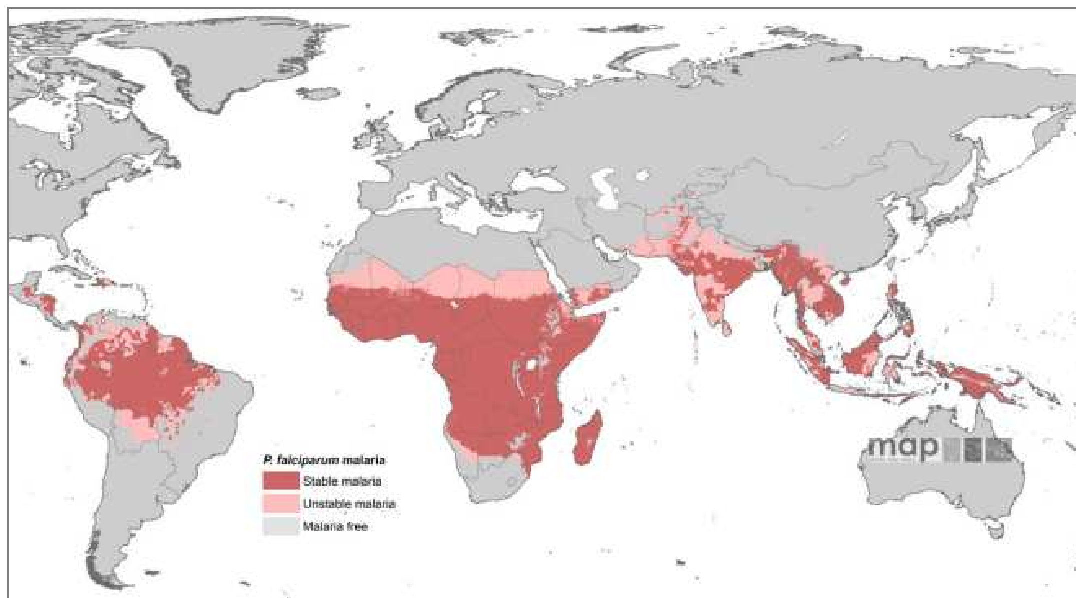


Figure 1. Geographical distribution of *P. falciparum* malaria risk defined by annual parasite incidence, temperature and aridity. Populations at risk in areas defined as having stable (dark pink) and unstable transmission (light pink). No transmission was assumed where assembled data stated no malaria risk, because not a single *P. falciparum* clinical case had been reported over several years, or where temperature was too low for sporogony to complete within the average life span of the local dominant vector species, or conditions were too arid for anopheline mosquito survival. In this map unstable malaria was used to define areas where transmission was biologically plausible and/or had been documented but where incidence was likely to be less than one case per 10,000 population *per annum*. Stable malaria areas represent populations at risk of significant disease burdens, including populations exposed to infrequent malaria infection risks and those subject to repeated infections and thus high disease burden risks with great public health needs. From Snow *et al*.(2008).

Effective interventions recommended by WHO have proved capable of reducing substantially the adverse disease outcomes in pregnancy (Steketee *et al.*, 2001). These interventions include intermittent preventive malaria treatment in pregnancy (IPTp), insecticide-treated bed nets (ITN) and effective case management of malaria illness and anemia, which in turn includes nutritional supplementation with iron/vitamin/mineral preparations, screening for anemia and monitoring of other diseases such as helminthes or bacterial infections (WHO, 2004). Nevertheless the majority of pregnant women still have no access to those measures, some of which are expensive and logistically difficult to apply, and efforts are required to accelerate implementation of strategies to prevent and control malaria in pregnancy. PAM focused research is needed to fulfill gaps of knowledge and thus contribute to disease impact reduction. The work in progress towards a better understanding of the disease mechanisms, parasite biology and maternal immunopathology, is a critical contribution for a stronger

interaction between science and strategic programs developing new control/clinical measures including a vaccine specific for PAM.

Epidemiology

Malaria exists in many parts of the world but the incidence varies from place to place and the same is observed for pregnancy malaria. The term "malaria" is derived from the belief of the ancient Romans that the disease was caused by the "bad air" of the marshes surrounding Rome, which denotes the past occurrence of the disease in places where any cases are currently observed.

The disease distribution is determined by several factors, including parasite life cycle attributes. The completion of *Plasmodium* spp. life cycle usually needs two obligatory hosts: a vertebrate intermediate host (mammals, birds and reptiles) and an invertebrate definitive host (hematophagous insect of the genus *Anopheles*). The life cycle of *P. falciparum* is outlined in Figure 2.

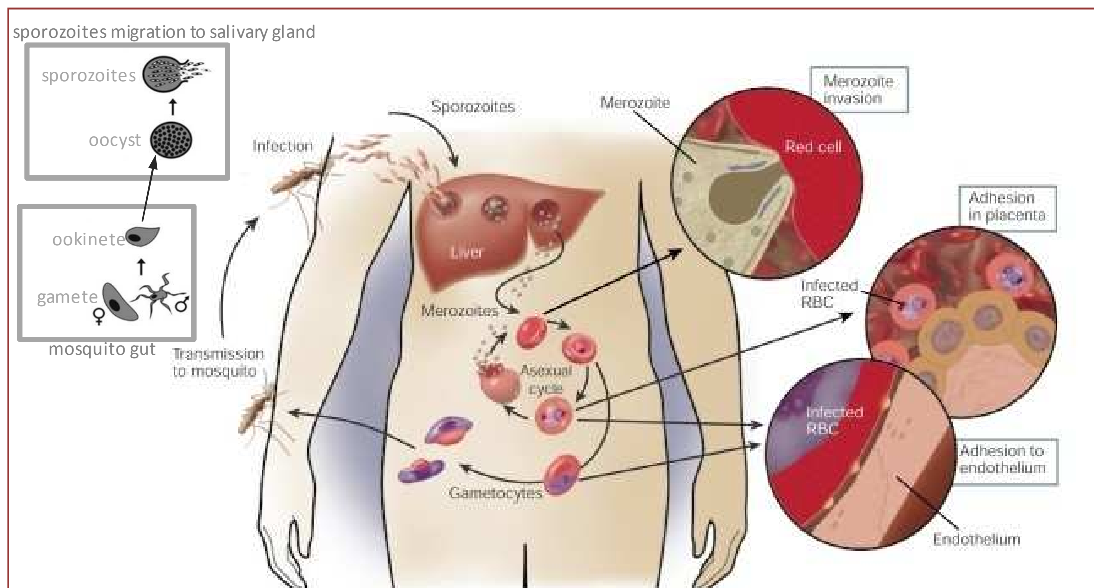


Figure 2. *Plasmodium falciparum* life cycle. Female mosquitoes of the genus *Anopheles* carrying malaria parasites inject sporozoites into the blood stream with the saliva while feeding blood. Within few minutes the sporozoites travel to the liver where they transverse several hepatocytes before invade and replicate in one (Mota *et al.*, 2001). Liver stage infection is asymptomatic. About 10-12 days later each sporozoite develops into thousands of merozoites that are released back into the bloodstream and will invade RBC. Inside a RBC, the parasite undergoes a new phase of asexual division to form a multinucleated schizont while expressing proteins in the outer RBC membrane, some of which have adhesive properties, enabling the mature parasite to bind receptors expressed by endothelial cells and in the deep vascular beds of organs such as the brain, lungs and placenta. Every 48 hours the parasite multiplies, each iRBC bursts and releases around 20 daughter merozoites, thereby continuing the blood stage cycle. Some merozoites follow a different developmental path and, rather than form another schizont, they differentiate into the sexual stage, forming either a male or female gametocyte, which, when taken by another feeding mosquito, perpetuate the sexual cycle in the insect. In the mosquito stomach the gametocytes develop into female and male gametes and after fertilization the diploid ookynetes migrate into the gut wall where they mature to form oocysts. Meiosis occurs within the oocysts leading to development of haploid infective sporozoites. When oocysts rupture the sporozoites migrate to the salivary glands, ready to be injected during the next mosquito blood meal. Adapted from Miller *et al*. (2002) and Carvalho *et al*. (2002). Malaria is considered

endemic in a region when it occurs at a relatively constant incidence by natural transmission over successive years. Endemicity levels can be separated in classes according to the parasite prevalence in the population. Following Lysenko's classification cited by Hay *et al*. (Hay *et al.*, 2004), endemicity classes are defined by the parasite rate (PR) in the 2–10-year age cohort as follows: hypoendemic < 10% infection prevalence; mesoendemic 11–50% infection prevalence; hyperendemic 51–75% infection prevalence. The exception is for the holoendemic class (> 75% infection prevalence) where the PR refers to the 1-year age group (Snow *et al.*, 2005).

Stable malaria transmission is associated with little seasonal or annual fluctuation in the disease incidence and leads to a characteristic pattern of immunity whereby older children and adults become progressively immune to the worst effects of the disease. In areas of stable malaria, the parasite is transmitted by a strongly anthropophilic rather than zoophilic *Anopheles* vector species that is found in the warmer regions of the world where the climatic conditions facilitate a rapid parasite development inside the mosquito (Carter & Mendis, 2002). *P. falciparum* is commonly the most prevalent parasite in areas with stable malaria transmission. Malaria is considered unstable in places where there is no reliable periodic transmission and sporadic epidemics may occur after long periods of almost no transmission. Unstable malaria is often associated with a short-lived vector or a more zoophilic mosquito, with low probability of taking two consecutive meals from a human host. In these areas typically temperatures are lower than in areas with stable malaria, sporogony (the nuclear divisions that give rise to sporozoites inside the mosquito) is slower and the most prevalent pathogen is *P. vivax*.

Malaria transmission intensity can be quantified by the Entomological Inoculation Rate (EIR) that expresses the average number of infective bites per person per year. Climatic factors, including temperature, humidity and precipitation, influence the density and development of the parasite inside the vector. For instance, the sporogony does not occur bellow 16°C or above 33°C, and open-air water is needed for the oviposition by fertilized females, where the eggs develop to adults in 7 to 20 days depending on the temperature. In addition, other factors such as mosquito longevity, predilection of the vector to feed on humans (anthropophily), mosquito endophily (tendency to preferentially rest inside the houses, in opposition to exophily when the mosquitoes likely abandon the house after a blood meal) or human factors (density and behavior of the human population), are also critical to shape human malaria epidemiology (Forattini *et al.*, 1987; Guerra *et al.*, 2008). Furthermore, the knowledge of disease epidemiology and the understanding of mosquitoes preferences and habits are very important to the design of preventive measures. For instance, if a parasite shows exophilic behavior and uses outside refuges, convincing people to use personal protection such as a bed net and eliminating open-air water, instead of indoor residual spraying, may be considered as a more effective malaria control measure in those conditions.

The outcome of malaria disease is determined by factors besides the transmission intensity, which include parasite features (virulence, drug resistance), host factors (age, immunity, genetics) and even socio-economic factors (access to treatment, politics, gender, economic condition, etc.). Relatively to the last point, it is worth to strengthen that malaria and poverty are closely related. Endemic countries are among the ones with lower rates of economic growth, making more difficult the access to preventive and curative measures. In addition, low socioeconomic status, normally associated with low maternal educational levels, can even complicate the consequences of malaria in pregnancy. For instance, low socioeconomic conditions are connected with poor nutritional status of the mother and hence poor fetal nutrition, limited access to complementary foods/supplements due to their cost and standard living conditions that facilitate diseases transmission, including malaria (De Pee *et al.*, 2002). On the other way round, malaria can be seen as an obstacle to development and a cause of poverty, because it can affect population growth, worker productivity, absenteeism and premature mortality (Sachs & Malaney, 2002). Specifically, malaria in pregnancy, as a major public health concern in low-income countries, also has a negative impact in the development and its resolution is constrained by the lack of resources to implement specific and effective interventions. The costs associated to malaria in pregnancy include the expenditures coming up from measures directed to pregnant women (for example, ITN and IPTp) but also the additional costs arising as a consequence of PAM, namely the long-term costs of treating the effects of maternal infection in the infant (Worrall *et al.*, 2007).

Together, all these epidemiological features of malaria determine the worldwide distribution of the disease in pregnancy. Africa bears 90% of the world burden of *P. falciparum* malaria and consequently the greatest problems of malaria in pregnancy are in sub-Saharan Africa, where it is one of the leading causes of maternal and perinatal mortality and morbidity (WHO, 2004). In Asia and South America there is a different scenario because transmission intensities are generally lower, despite the existence of discrete areas of very high transmission (Singh *et al.*, 2001). In those regions *P. vivax* is the most prevalent species and thus is the major parasite to be aware of.

It is noteworthy the efforts put in the construction of a map of estimates of *P. falciparum* infection prevalence worldwide (http://www.map.ox.ac.uk). This project appears as a tool to the commitment of reducing/eliminating malaria, as part of a global effort to tackle diseases of poverty through the Millennium Development Goals (Hay & Snow, 2006). These maps will constitute a support to identify intervention needs and may provide tools to evaluate the implementation of future strategies on malaria control, including malaria in pregnancy.

Factors affecting Pregnancy-Associated Malaria (PAM)

The consequences of *P. falciparum*-PAM are determined by a range of factors, including the number of previous pregnancies, the existence of co-infections and the level of immunity acquired by the woman throughout her life. This level of pre-pregnancy immunity, or premunition, depends largely on the epidemiological setting where the woman is living. Therefore, the prevalence of malaria infection on pregnant women can range from 10% to 65% across the different settings (Steketee *et al.*, 2001). A description of PAM in high and low transmission regions is reviewed in detail by Nosten *et al*. (Nosten *et al.*, 2004).

- **PAM in regions with high malaria transmission**

In areas with high endemicity or with stable malaria transmission, adults usually manifest a protective semi-immunity against *P. falciparum*, which is maintained only by continued exposure to malaria and most of the malaria infections are asymptomatic (do not produce fever or clinical illness) (Desai *et al.*, 2007). Women with previously reasonable immunity appear to lose part of that protection in pregnancy and are more likely to be parasitemic than non-pregnant women (WHO, 2000). Parasite prevalence and parasitemia density are maximal in the second pregnancy trimester, and this is most evident in first and second pregnancies with decreased risk in each successive pregnancy (Singh *et al.*, 2001; Rogerson *et al.*, 2007). In fact, in contrast with other infectious diseases that induce complications in pregnant women, malaria burden in pregnancy decreases in incidence with successive pregnancies (Duffy & Fried, 1999). Despite the higher parasite incidence in pregnancy, severe disease is uncommon, infection is frequently asymptomatic in these settings and, consequently, malaria may go unsuspected and undetected (Diagne *et al.*, 1997). Nevertheless, immune women can display heavy placental infection, despite negative peripheral blood smears, which can result in the development of maternal anemia and in babies with LBW (Dorman & Shulman, 2000).

- **PAM in regions with low malaria transmission**

In regions where malaria transmission is unstable, less intense, sporadic or periodic, the state of premunition is not attained and people are at greater risk of developing severe and symptomatic disease, and even of death, at all ages (Luxemburger *et al.*, 1997; WHO, 2004). In this epidemiological context, malaria infection in pregnant women may result in a variety of adverse outcomes, including a high risk of developing complicated malaria, with central nervous system problems, maternal death (as a direct result of severe malaria or as an indirect result of malaria-related severe anemia), spontaneous abortion, neonatal death and LBW (Nosten *et al.*, 1991; WHO, 2004). Since in these settings malaria is usually symptomatic, it is likely to be detected at early stages and, if treated, might result in the reduction of the harmful consequences of malaria in pregnancy, often allowing parasite clearance before placental colonization (McGready *et al.*, 2004).

Malaria pathogenesis and PAM consequences

Symptomatic malaria disease begins only after multiplication of blood stage asexual parasites, leading to massive destruction of RBC, release of bioactive parasite molecules and toxins, eventual iRBC sequestration and stimulation of host innate immune system to produce proinflammatory mediators that are the main components of malaria pathogenesis.

The common symptoms of non-pregnant individuals to all four human malaria parasite species are headache and muscle aches, periodic fever and chills. However, some *P. falciparum* infections can progress to severe anemia, metabolic acidosis, hypoglycemia and some organ-related pathological conditions (Miller *et al.*, 2002). The destruction of iRBC is also part of malaria pathogenesis that, collectively with the acute hemolysis of uninfected RBC and dyserythropoiesis, leads to anemia, compromising the oxygen delivery in the tissues. Only *P. falciparum*, and not the other three species, causes fatal forms of disease due to its two main characteristics, that are its higher levels of parasitemia when compared with the other species and its property of being sequestered in the microvascular endothelial surface (Kyes *et al.*, 2001). This distinct ability of *P. falciparum* to sequester favors an extensive accumulation of the iRBC in vital organs. In addition, the local and/or systemic action of parasite released products, as well as local and/or systemic production of inflammatory cytokines and chemokines in response to the infection, and the activation, recruitment and infiltration of inflammatory cells, can all together influence the progress to pathology, the clinical manifestations and the outcome of the disease (Schofield & Grau, 2005).

The effect of *P. vivax* in pregnancy has received little attention but is considered less severe than *P. falciparum*. *P. vivax* infections in pregnancy are likely to result in febrile illness; infected women are more likely to be anemic and to deliver neonates with lower birth weight as compared to uninfected women, but less pronouncedly than with *P. falciparum* cases (Nosten *et al.*, 1999; ter Kuile & Rogerson, 2008).

- **Effects of malaria on maternal health**

The effect of the infection on the mother may range from insignificant to severe, depending on the previous acquired immunity, parity and on other health conditions such as co-infections, being the human immunodeficiency virus (HIV) the most worrying at present (Desai *et al.*, 2007; Van geertruyden & D'Alessandro, 2007). Pregnant women are also more prone to develop hypoglycemia, which may be worsen during *P. falciparum* infection, and possibly contribute for LBW associated with peripheral parasitemia (Menendez, 1995). Anemia is the most common consequence of maternal malaria regardless the level of endemicity. WHO defines anemia when hemoglobin is lower than 11 g/dl and severe anemia when hemoglobin is lower than 5 g/dl (WHO, 2000). Anemia is more common in pregnant women than in non-pregnant and even asymptomatic infections frequently worsen maternal anemia. In areas with stable *P. falciparum* transmission, PAM can contribute to approximately 2% to 15% of

maternal anemia (Steketee *et al.*, 2001). Severe maternal anemia increases the risk of maternal death and is estimated to cause 10.000 deaths in Africa per year (Guyatt & Snow, 2001). Generally, anemia causes are multifactorial and include inadequate nutrition, iron and folate deficiency, helminthes infections, hemoglobinopaties and HIV infections. In malaria-endemic regions, malaria-induced anemia in pregnancy is caused by destruction of parasitized and non-parasitized erythrocytes, suppression of hematopoiesis and intense sequestration of infected erythrocytes in the placenta (Fleming, 1989; Menendez *et al.*, 2000).

- **Effects of malaria on fetus and infant health**

PAM may affect the fetus/infant by a variety of mechanisms, including severe maternal disease, placental damage or even by direct infection. Firstly, maternal infection may lead to a systemic illness and, because of the high maternal fever, anemia, respiratory distress or systemic reactions to malaria, the fetus can suffer, display Intrauterine Growth Retardation (IUGR) and even die in absence of parasite sequestration in the placenta. Secondly, the placenta may be directly infected, resulting in placental insufficiency by impaired blood flow and reduced placental exchanges. Thirdly, the fetus may be directly infected through the placenta (Menendez & Mayor, 2007). Moreover, maternal infection may precipitate preterm delivery, when the fetus is still unable to tolerate life outside uterus or with LBW, which in turn is a cause of poor infant survival and development (van Geertruyden *et al.*, 2004; Menendez & Mayor, 2007).

Epidemiological data reveal that neonates appear to be relatively well-protected from clinical malaria and from severe consequences of malaria infection for the first 3-6 months of life (Snow *et al.*, 1998). Several attempts have been described to explain the reduced malaria incidence in infants. The relative protection of the infants from infection could be due to behavioral practices, such as the constant supervision by the mother who may repel biting mosquitoes away from them and because they tend to be kept well covered up (Riley *et al.*, 2001). Furthermore, there are some physiological mechanisms that may inhibit the replication of blood stage parasites in neonates conferring them clinical protection during early infancy. For instance, malaria parasites grow much more slowly in RBC containing fetal hemoglobin (HbF) than in those with normal adult hemoglobin (Pasvol *et al.*, 1977) and infants diet may lack some of the essential nutrients for parasite replication, such as the p-amino-benzoic acid (pABA) that is vital for parasites and it is present in low levels in breast milk (Riley *et al.*, 2001)
Depending on the level of malaria transmission intensity, malaria contributes to an estimated 8% to 36% of cases of prematurity and 13% to 70% of IUGR. Maternal malaria, especially in areas of low or unstable transmission, can result in abortion (delivery of a dead fetus before 28 weeks of gestational age) or stillbirth (delivery of a dead fetus after 28 weeks), and is estimated to account for 3% to 8% of all infant deaths (Steketee *et al.*, 2001).

PLACENTAL IMMUNOPATHOLOGY CAUSED BY MALARIA

The aims of this section are firstly to provide an overview of the main characteristics of the placenta and pregnancy immunology, and secondly to depict the major malaria-induced modifications in both domains.

Placenta structure

The word placenta has Latin origin and means 'flat cake' (Cross, 2005). The placenta is an organ that develops during pregnancy, deriving at a large extent from embryonic tissue and indispensable for pregnancy success (Benirschke, 1998) and performs a multitude of nutritional, respiratory, hormonal, excretory and immunological functions. By being attached to the uterus wall the by blood vessels, it conveys supplies to the fetus and removes waste from the fetus transferring it to the mother.

The placenta is essential for sustaining the fetus growth during gestation and defects in its function result in fetal growth restriction or, if more severe, fetal death. The pathological changes are best understood in the context of the placenta structure and anatomy (Figure 3).

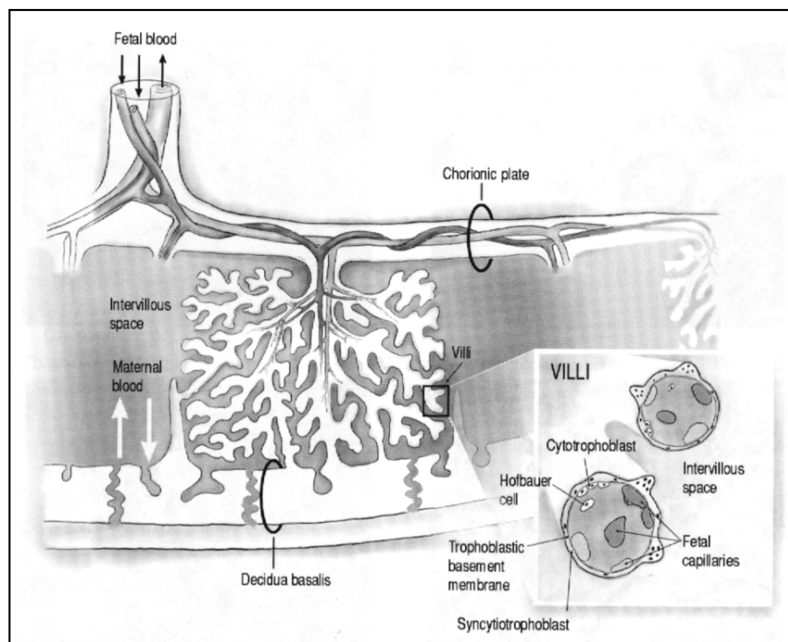


Figure 3. Schematic representation of the human placenta. The human placenta can be described as a disk full of blood where the villous trees (fetal part) are bathing. Inset showing a cross-section of terminal villi, the "placenta barrier". From Duffy (2001).

The fetus side (chorion) of the human placenta has a flat form, where the umbilical cord is inserted, and the opposite face, bordering the uterus, has a u-shaped surface. The umbilical cord is a channel that carryies fetal blood between the fetus and the placenta and normally contains two arteries and one vein, surrounded by extensive mesenchymal tissue. The villi, consisting of connective tissues (mostly fibroblasts) in which fetal blood vessels are found,

extend from the chorion plate into the vascular interior of the placenta and continue to branch throughout the pregnancy. The spaces between villi, the intervillous spaces (IVS), are filled with maternal blood supplied by spiral arteries in the decidua. Some villi end freely in the vascular space while others extend to the opposing decidua anchoring to the pregnant uterus in the decidua basalis, contributing to stabilize the mechanical integrity of the placental-maternal interface. The decidual cells are lining the uterus and result from the differentiation of maternal endometrial stromal tissue adjacent to the placenta. Fetal circulation is entirely closed, confined to vessels within the chorionic villi, whilst maternal blood flow through the placenta is open. The inset of Figure 3 outline a cross-section of terminal villi, known as "placenta barrier" that separates maternal blood and fetal blood, across which occur all exchanges of gases, nutrients, hormones and wastes. The "placenta barrier" is constituted by three cell layers: the outer layer, covering the villi named syncytiotrophoblast (a syncytium with many dark-stained nuclei), the trophoblastic basement membrane and the fetal capillary endothelium. The villous cytotrophoblastic cells (Langhan's layer) at early pregnancy form a continuous single layer of stem cells over the connective tissue of the chorionic villi that, during differentiation, fuse together into the multinucleated syncytiotrophoblast and add the cellular components to the syncytium. Later in pregnancy the cytotrophoblast is reduced to a few scattered large pale cells. In addition to the regulation of the exchange between the mother and the fetus, the syncytiotrophoblast is also responsible for placental hormone and enzyme production (Benirschke, 1998). During the latter half of the pregnancy, or under stress conditions, the villi might shrink allowing the syncytial epithelium to buckle and leading to the production of an excessive number of syncytial knots. Hofbauer cells are the placental macrophages, within the villous stroma and chorio-amniotic membranes, which naturally have phagocytic activity (Benirschke, 1998).

Placenta pathology associated to malaria

Placental malaria is an important component of the deleterious effects of malaria in pregnancy and the connection between placental histological changes and pregnancy outcome was reviewed in detail by Brabin *et al*. (Brabin *et al.*, 2004b). The accumulation of *P. falciparum* parasites in the intervillous spaces (IVS) of the placenta is a pathological feature of malaria in pregnancy. Other characteristics of placental malaria include malaria pigment (hemozoin) accumulation, fibrinoid deposits, thickening of the trophoblastic basement membrane, focal necrosis, damage of the syncytiotrophoblastic membrane and mononuclear inflammatory infiltration of the IVS (Walter *et al.*, 1982; Crocker *et al.*, 2004).

The striking alterations caused by placental malaria occur within the IVS, but typical pathological features are found both in maternal and fetal parts of the placenta. It is possible to evaluate the presence of parasites/pigment in IVS, in erythrocytes and monocytes and in polymorphonuclear leucocytes. It is also in the IVS that parasites can be sequestered and inflammatory infiltrates accumulated. Fibrin-type fibrinoid deposits (blood-clotting product)

can be evident in intervillous/perivillous and in the basal plate. Parasites, hemozoin and fibrinoid necrosis can be present in the syncytiotrophoblast. In the trophoblastic basement membrane a matrix-type fibrinoid can occur resulting in its thickening. Excessive syncytial knots are also observed in infected placentas (Walter *et al.*, 1982; Bulmer *et al.*, 1993; Crocker *et al.*, 2004).

The first classification of human placental infection was introduced by Bulmer *et al*. (Bulmer *et al.*, 1993). The rationale of this histological classification was the assumption of the progression of infection and allows distinguishing current and past placental malaria infection. Thus, active infections are associated with the presence of parasites and can be either acute, if the parasites are present in maternal RBC as well as hemozoin (which can be found in iRBC or within macrophages) or chronic, when iRBC are also present but hemozoin is covered by fibrin. Past infections involve the presence of hemozoin, usually mixed with fibrin but with the absence of parasites, since after clearance of the infection the pigment can persist in fibrin (Bulmer *et al.*, 1993).

The cause of placental pathology associated to malaria is still not clear and could include direct effects of malaria infection by iRBC and hemozoin deposition as well as accumulation of inflammatory infiltrates in the IVS. For instance, intervillous accumulation of mononuclear cells induces an alteration in the cytokine balance and may contribute to pathological changes resulting in the damage of the syncytiotrophoblastic membrane (Walter *et al.*, 1982; Crocker *et al.*, 2004). When the degree of infiltration is intense it results in a massive chronic intervillousitis with a prominent inflammatory infiltrate in the IVS mainly composed by monocytes and macrophages, frequently associated with fibrin deposition, syncytial knots and malarial pigment (Ordi *et al.*, 1998).

The consequences of these pathologic changes on maternal disease and poor fetal outcomes have been discussed (Duffy, 2001; Brabin *et al.*, 2004b). In sum, several observations invoke that the dense accumulation of parasites and inflammatory cells, together with the increased fibrinoid deposition and the trophoblast basement membrane thickening, could collectively cause an obstacle to gas and nutrients placental exchange, between the mother and the fetus.

Components of immunological response to pregnancy

Locally in the placenta a correct balance of cytokines produced by various immune cells (T lymphocytes, uterine NK (uNK) cells, macrophages) is essential for the pregnancy success (Veenstra van Nieuwenhoven *et al.*, 2003). Many cells within the placenta, including Hofbauer cells, decidual cells, cells of the syncytiotrophoblast, uterine epithelial cells, uNK cells, amniotic membranes, the fetal tissues and intervillous maternal leucocytes, can produce a wide variety of cytokines and chemokines having a determinant role in placental development and immunology (Robertson *et al.*, 1994).

The uNK cells have a NK cell-like function but they are specific for the uterus as they show a different phenotype when compared with peripheral NK cells. It has been suggested that uNK cells affect implantation and placentation and, at the same time, they play an important role in the protection against infections and in the regulation of immune response. For instance, decreased numbers of uNK cells were associated with significantly higher rates of miscarriage and low numbers of uNK cells were also found in the decidua of women with a genetically abnormal fetus as compared with women pregnant with a normal fetus. One recognized function of the uNK cells is the production of cytokines, usually influencing placentation, such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF) and leukemia inhibitory factor (LIF) that stimulate growth of the trophoblast and promote trophoblast cell proliferation and differentiation (Veenstra van Nieuwenhoven *et al.*, 2003).

T lymphocytes are the best studied peripheral immune cells in human pregnancy (Veenstra van Nieuwenhoven *et al.*, 2003). There are various types of T-lymphocytes namely the helper T lymphocytes (Th) and the cytotoxic T lymphocytes (CTL). The former provide help to other immune cells by producing cytokines, whereas CTL lymphocytes can directly kill foreign or infected cells. T lymphocytes can also be classified into different functional subsets based on their profile of cytokine production. Type 1 T (Th1) cells produce cytokines that promote cellular immune responses, whereas the cytokines produced by type 2 T cells (Th2) provide optimal help for humoral responses (Raghupathy, 2001).

Each pattern of cytokines has different roles in immune responses. Th1 cells produce, for example, IFN-γ, TNF-α and IL-2, which are soluble mediators of the cellular response by activating macrophages and cell-mediated reactions, important in resistance to infection with intracellular pathogens. Th2 cells produce IL-4, IL-5, IL-9, IL-10 and IL-13 that encourage humoral responses, promoting antibody production which is important in combating infections with extracellular agents (Veenstra van Nieuwenhoven *et al.*, 2003). However, Th1 and Th2 are not the only types of Th-mediated responses and other cytokine patterns also exist. For instance, Th3 cells secrete TGF-β but do not secrete IFN-γ, IL-2, IL-4 or IL-10, and are capable of down-regulating Th1 cells (Raghupathy, 2001). Th1 and Th2 cells are reciprocally inhibitory to each other. For instance, IL-10, a product of Th2 cells, inhibits the development of Th1 cells by acting on antigen-presenting cells, whereas IFN-γ, a product of Th1 cells, prevents the activation of Th2 cells. The overall effect is that a given immune response can be dominated by either a cell-mediated profile (Th1) or humoral immunity (Th2) (Raghupathy, 2001).

Typically, pregnancy is considered a Th2 dominant state because during pregnancy it is observed a decrease in the ratio between type 1 and type 2 cytokine production by peripheral lymphocytes, as compared with non-pregnant women, which is beneficial for pregnancy. However, Th1 or Th2 balance might be a dynamic process throughout the course of pregnancy. For instance, at pregnancy start a Th1 environment is necessary to promote endometrium

invasion and implantation. Later, Th2 dominance is adequate for the maintenance of the pregnancy and to tolerate the fetus, since Th1 cytokines are harmful as they inhibit embryonic and fetal development. Finally at pregnancy termination, the shift towards Th1 is essential for delivery.

The Th1/Th2 paradigm provides an explanation to understand immune responses in pregnancy and how the immune system directs responses to different types of pathogens and stimuli. However, there is no consensus to whether the decreased type 1/type 2 cytokine production ratio is due to a decreased production of Th1 cytokines or to an increased production of Th2 cytokines (Veenstra van Nieuwenhoven *et al.*, 2003). This decreased ratio can be explained by different mechanisms. Firstly, the increase in pregnancy hormones (e.g. progesterone and estrogen) may directly affect lymphocytes by shifting their cytokine production towards type 2. Secondly, the placenta may interfere with lymphocyte cytokine production, since trophoblast cells also produce cytokines (mainly type 2), which may direct the maternal immune response towards a Th2 immune response, and could produce factors that inhibit cytotoxic T-lymphocyte activity (Veenstra van Nieuwenhoven *et al.*, 2003).

In sum, successful pregnancy and fetal growth are associated with a predominant Th2 response in the placenta, accompanied by an adequate hormonal regulation. Thus, production of Th2 cytokines locally in the placenta favor the maintenance of pregnancy, whereas any stimuli that increase Th1 cytokine production locally or systemically may lead to unsuccessful pregnancies, since a Th1 dominance is associated with fetal rejection and miscarriage because they cause an inflammatory environment (Veenstra van Nieuwenhoven *et al.*, 2003).

Immunological tolerance of the fetus

The description of the known pregnancy immunological mechanisms is essential for pregnancy-associated malaria studies, because they establish a pattern of comparison with the malaria-induced modifications. For instance, abnormalities of maternal immune tolerance to the fetal semi-allograft have been implicated in several common disease processes occurring during pregnancy, leading to recurrent early miscarriage, pre-eclampsia and eclampsia (Zenclussen *et al.*, 2007). These conditions are characterized by inflammation in the fetal-maternal interface and/or systemic manifestations, which are common to the malaria disease.

The fetus is often compared to an allograft because it is genetically different from the host (mother) and thus must find strategies to evade immune defenses and avoid “rejection”. The embryo in early development divides into two groups of cells that originate the fetus and the trophoblast, and the last are the only cells to directly interact with the cells of the maternal immune system (Veenstra van Nieuwenhoven *et al.*, 2003). The trophoblast cells encounter a systemic immune response in the maternal circulation as well as when they are invading into the uterine wall, and several specialized mechanisms have evolved to help the fetus, which expresses paternal antigens, to evade maternal immune attack. Indeed, the maternal immune

system not only becomes aware of the fetus presence but dynamically tolerates it. Those mechanisms of tolerance were recently reviewed and summarized (Zenclussen *et al.*, 2007).

Trophoblast cells, which include the syncytiotrophoblast, villous cytotrophoblast and non-villous cytotrophoblast, lack expression of major histocompatibility complex (MHC) class Ia molecules and so they cannot be recognized as non-self by maternal T cells. However, invading non-villous cytotrophoblasts (the tissue of implantation) in endometrium are at risk of lysis by uNK cells, which are present in endometrium and in the decidua in large amounts. Nevertheless, they do not attack the semi-allogeneic non-villous cytotrophoblast, because uNK express inhibitory receptors that bind to the MHC Ib, the non-classical HLA (HLA-C, HLA-E and HLA-G), on cytotrophoblasts (Veenstra van Nieuwenhoven *et al.*, 2003).

Furthermore, trophoblast cells express apoptosis-inducing ligand (FasL) that may cause apoptosis of activated maternal lymphocytes expressing the cognate receptor (Fas). In fact, Fas and its ligand (FasL) play an important role in the regulation of immune tolerance. Fas is highly expressed in several immune cells including activated T and B lymphocytes, NK cells, monocytes and macrophages. FasL is expressed on the surface of fetal cytotrophoblasts as well as on maternal decidual cells of the placenta, that is, in cells located at the interface between the fetal placenta and maternal endometrium (Veenstra van Nieuwenhoven *et al.*, 2003).

There are other mechanisms acting locally at the site of fetal antigen exposure which may operate in parallel to sustain gestation. Complement activation promotes inflammatory and immune responses, by inducing chemotaxis of inflammatory cells, enhancing phagocytosis by neutrophils and monocytes, facilitating immune complex clearance and mediating cell lysis by the membrane attack complex. Complement can also bind and attack self tissues, especially in areas of active inflammation but its deleterious effects can be avoided by complement control proteins. In pregnancy, complement regulation is essential for the maintenance of a normal pregnancy and complement activation is inhibited by expression of some proteins, such as Crry which has been implicated as a negative regulator of complement activation in mice, promoting maternal-fetal tolerance and survival (Xu *et al.*, 2000).

Moreover, in a normal pregnancy there is an increase on the levels of IgG asymmetric antibodies that have an effective participation in fetal protection. These antibodies behave as univalent for having one of the paratopes blocked with a carbohydrate chain and, consequently, they do not form antibody-antigen complexes. Thus, due to their molecular asymmetry and functional univalency, these immunoglobulins are unable to generate a classical immune response and, therefore, to trigger effector immune responses (Margni & Zenclussen, 2001). Asymmetric antibodies are present in humans as well as in mice and are synthesized systemically, representing 15% of the total serum antibodies (Zenclussen *et al.*, 2001). When specific for self-antigens, asymmetric antibodies are beneficial for the host due to their blocking functions, namely in allergic manifestations, auto-immune diseases and especially in pregnancy. During pregnancy asymmetric antibodies were found in serum and in

the placenta with specific activity to paternal antigens. Acting locally in the placenta, they block paternal antigens without generating the classical immune response (Malan Borel *et al.*, 1991). In a normal pregnant woman, IgG asymmetric antibodies are elevated in sera and in the placenta, constituting around 50% of the IgG population in that organ and 80% of them have anti-paternal activity (Malan Borel *et al.*, 1991), being their synthesis modulated by placental secreted factors, such as IL-6. High levels of this cytokine are associated with low levels of asymmetric antibodies which can endanger pregnancy success (Margni & Zenclussen, 2001).

The placenta also grants an effective physical barrier to the immune attack, because it provides a separation between the maternal and fetal blood, and the villous syncytiotrophoblast (the placental membrane directly exposed to maternal blood) is semi-permeable acting as a filter for two way transport including diffusion of certain molecules and active transport (Myren *et al.*, 2007).

In addition to locally acting mechanisms, systemic changes are also needed to facilitate fetal tolerance. For instance, it was shown a peripheral expansion of regulatory T cells (Treg) during pregnancy as a result of continuous alloantigen release from the placenta (Zenclussen *et al.*, 2007). Treg are known to play a major role in preventing autoimmunity but also in tolerating allogeneic organ grafts, acting either by cell-cell contact or by secreting immunomodulatory factors such as IL-10 and TGF-β. In pregnancy, Treg play a role in promoting additional acceptance of the fetus because in certain pathologic scenarios the recruitment and function of Treg appear to be impaired and the proportion of Treg is lower in cases of spontaneous abortion, when compared with induced abortions in mice (Aluvihare *et al.*, 2004). Particularly, at the maternal-fetal interface, Treg are thought to inhibit maternal effector cells and up-regulate tolerant molecules such as heme oxygenase 1 (HO-1), LIF, TGF-β and IL-10. LIF is essential for implantation success but is involved in tolerance of allografts and also has an important role on inducing tolerance at the fetal-maternal interface. HO-1 beneficial effects are related to the avoidance of the toxic accumulation of free heme. In fact, free heme, originated from senescent RBC for example, can cause cell damage and tissue injury as heme catalyses the formation of reactive oxygen intermediates (ROI), resulting in oxidative stress (Zenclussen *et al.*, 2007).

General aspects of the immunity to malaria

It is generally accepted that repeated malaria exposure will lead to increasing immunity to malaria. Since protection increases with exposure, the acquisition of immunity is faster in high transmission regions, where the age group more affected by the disease is infants under one year, who are at higher risk of death. As both age and exposure increase, the individuals also acquire higher ability to limit the consequences of infection, namely they are more protected from severe illness and death. However sterile immunity is seldom attained, since many adults continue to have circulating parasites in the blood (Langhorne *et al.*, 2008). These

observations, together with the fact that adult travelers from non-endemic areas are likely to have severe clinical manifestations of the disease, suggest that malaria protection can be immune-mediated. Clinical immunity to malaria could therefore be attained when the immune responses are regulated to perform parasite clearance while avoiding detrimental effects and pathology (Artavanis-Tsakonas *et al.*, 2003). There are immune mechanisms that effectively act against each parasite stage, some of which are represented in Figure 4.

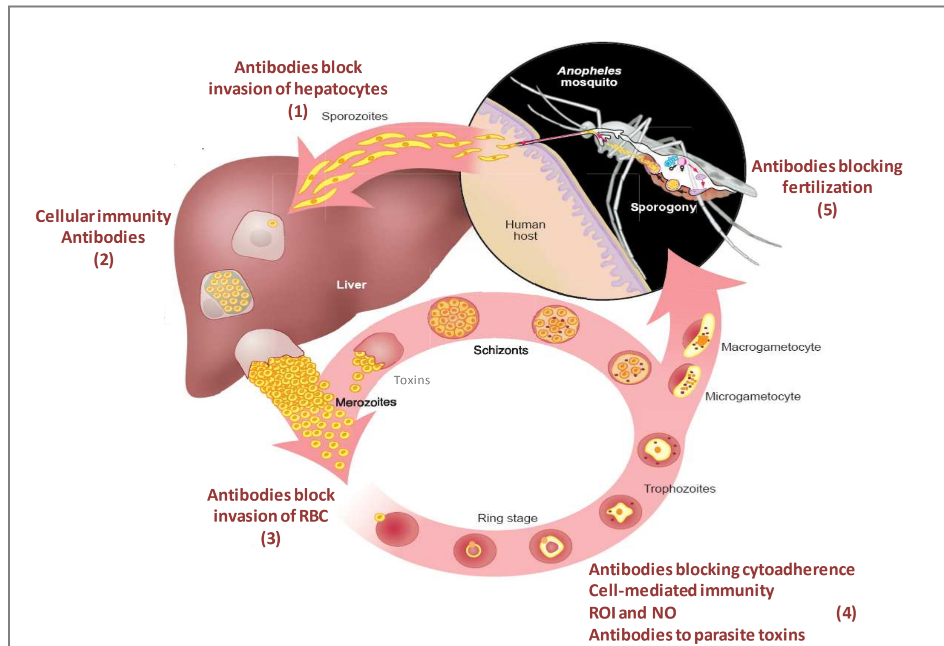


Figure 4. Possible immune protection mechanisms at various stages of the plasmodium life cycle in the mammalian host. (1) Antibodies to sporozoite antigens neutralize sporozoites and/or block invasion of hepatocytes. (2) At liver stage cellular immunity is essential: IFN-γ released by $CD8^+$ and $CD4^+$ T cells induces NO production by hepatocytes; $CD8^+$ CTL and NK cells exert cytotoxicity and induce Fas/FasL-mediated apoptosis; NK cells plus antibodies exert ADCC (antibody dependent cellular cytotoxicity); γδ T cells and NKT cells also kill intrahepatic parasites. (3) Antibodies to merozoites block invasion of RBC, mediate merozoites opsonization and ADCI (antibody dependent cell-mediated inhibition), facilitating merozoite phagocytosis by macrophages. (4) Antibodies to iRBC surface proteins opsonize iRBC for phagocytosis and/or block the adhesion of iRBC to endothelium; $CD4^+$ T cells and monocytes secrete cytokines with parasiticide / parasitostatic effects: TNF-α and IFN-γ activate macrophages to phagocytose and/or kill iRBC and merozoites; antibodies can neutralize parasite toxins (GPI) and prevent severe clinical disease, though with no effect on parasite itself; NO (nitric oxide) and TNF-α, released from activated macrophages and ROI (reactive oxygen intermediate), have a parasiticidal effect on merozoites or intra-erythrocytic parasites. (5) Antibody and complement taken up in the blood meal mediate the lysis of gametocytes and prevent fertilization and further development of the parasite in the mosquito. Figure from Long & Hofman (2002) and adapted with Bolad & Berzins (2000) and Langhorne *et al*. (2008).

The relative importance of each of these mechanisms is still debatable (Langhorne *et al.*, 2008). At the pre-erythrocytic stage, following the sporozoites inoculation into the host, antibodies to sporozoites antigens could protect both through opsonization of the sporozoite and its clearance before reaching the hepatocyte or by blocking hepatocytes invasion. However antibodies to sporozoites are thought of reduced importance (Langhorne *et al.*, 2008)

as the intracellular parasite, within a cell expressing MHC classes I and II, is the major target of the immune system in the pre - erythrocytic stage. $CD8^+$ T cells, recognizing parasite-derived peptides presented by MHC class I have been shown to be important in eliminating intracellular parasites that successfully invade and replicate within hepatocytes. This activity is mediated essentially by perforin, Fas ligand (FasL) and IFN-γ (Overstreet *et al.*, 2008). The process of $CD8^+$ T cells priming by DCs was thought to occur after hepatocytes infection, when DCs acquire antigens from apoptotic hepatocytes, migrate to lymph nodes draining the liver where they prime T cells, a process called “cross-presentation” (Leiriao *et al.*, 2005). However, it was further demonstrated that protective $CD8^+$ T cells are primed primarily in the lymph node that drains sporozoite from the skin inoculation site (Chakravarty *et al.*, 2007). The parasite antigens are internalized by immature DCs that prime the T cell response specific for the parasite through antigen presentation. Thus, $CD8^+$ T cells against malaria are found early in the lymphoid tissues linked to the cutaneous infection site.

As infection progresses to blood stages, the potential targets for an immune response are free merozoites or iRBC. Given that RBC do not have MHC I or II, and so, contrary to the infected hepatocyte, they cannot be targeted by $CD8^+$ T cells, the humoral responses are usually assumed as having a key role in the blood-stage immunity. Antibodies can be effective in protection by several mechanisms. Anti-merozoite antibodies may mediate blockade of RBC invasion, lead to opsonization of merozoites for uptake through Fc receptors and/or complement receptors on phagocytes. Antibodies against parasite molecules on the RBC surface membrane could act by either allowing antibody-dependent cell-mediated inhibition (ADCI) mediated by cytophilic antibodies (IgG1 and IgG3 in humans, which are functionally similar to IgG2a in mice) or complement-mediated lysis of iRBC or by mediating opsonisation of iRBC for phagocytosis. In the case of *P. falciparum* some of the surface proteins mediate the iRBC sequestration to endothelium, and thus, antibodies blocking adhesion to host receptors could allow phagocytes to act and avoid severe malaria syndromes, such as cerebral malaria.

Malaria infection symptoms in non-immune individuals arise as the rupture of schizont-infected erythrocytes triggers a cascade of inflammatory responses, owing to molecules released at that moment by the synchronic parasites, which, if not controlled, can lead to death. This wave of toxaemia and cytokines release from cells of both the innate and adaptive immune systems trigger the classical symptoms of fever and chills, but also contribute to control parasite replication, maintaining densities at levels compatible with host survival. Glycolipids have been identified as the major candidates to trigger toxins release, having a potent capacity of stimulate TNF-α production by macrophages (Schofield & Hackett, 1993). Thus, the antibodies that neutralize malaria toxins can contribute to reduce disease severity. For instance, antibodies to malarial glycosylphosphatidylinositol (GPI) and other glycolipids block induction of TNF-α from macrophages thereby down-regulating the inflammatory cascade and preventing immunopathology (Artavanis-Tsakonas *et al.*, 2003).

Antibody-independent cellular-mediated immunity might also play a role in natural immunity against the erythrocytic stage. $CD4^+$ cells of the Th2 type have a role in humoral immunity as helper cells for B cells (Perlmann & Troye-Blomberg, 2000). $CD4^+$ T cells of the Th1 type activate macrophages and other cells to produce TNF, nitric oxide (NO), reactive oxygen intermediates (ROI) and other mediators, through the release of cytokines, such as IFN-γ (Good *et al.*, 2004). These inflammatory molecules and released products have been shown capable of killing parasites, which probably occurs in the spleen, where blood flow slows in marginal sinuses, allowing iRBC to be removed by marginal zone macrophages and DCs.

The spleen has several crucial functions to control malaria infection including the removal of damaged iRBC and the restitution of blood cells, since it is a major site for erythropoiesis, and it is a place where pathogen-specific T and B cells are generated (Engwerda *et al.*, 2005). In Figure 5, it is represented an accepted model by which cell-mediated immunity clears the parasite in the spleen. Parasite-specific $CD4^+$ T cells can be activated specifically by parasite epitopes expressed on APCs, but parasites are killed by non-specific mechanisms.

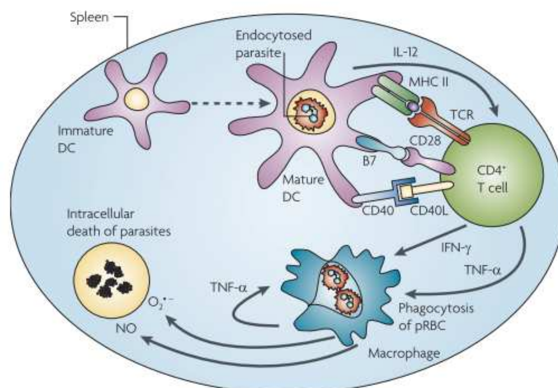


Figure 5. Schematic representation of the possible mechanism by which cell-mediated immunity clears malaria parasites. Immature dendritic cells (DC) are "sentinels" that endocytose and process parasites in their immediate environment, which leads to its activation and presentation to $CD4^+$ T cells (and also to $CD8^+$ T cells), together with activation signals (IL-12) to initiate immune response. Activation of $CD4^+$ T cells lead to macrophage activation, phagocytosis of parasitized RBC and production of cytokines and inflammatory molecules, such as NO and ROI. From Wykes & Good (2008).

Innate immune cells have an important role in controlling the primary wave of blood-stage parasitemia. Monocyte-derived macrophages, polymorphonuclear leukocytes, NK cells and γδ T cells are able to kill mature stages of parasitized iRBC in the absence of antibodies, which may be due to the expression of proteins (such as PfEMP1) on the iRBC surface containing binding sites for CD36 or ICAM-1, promoting binding of leukocytes and phagocytosis (Bolad & Berzins, 2000). Moreover NK cells and γδ T cells can have an important role in controlling the disease by producing IFN-γ. Additionally, iRBC or parasite products can interact with various Toll-like receptors (TLR) present in immune cells. GPI anchors from certain protozoans have been shown to bind TLR2-TLR1 complex on human DC, macrophages and B cells, and hemozoin (or more likely contaminating DNA) activates DC through TLR9, inducing potent immunostimulatory properties and pro-inflammatory cytokines (Langhorne *et al.*, 2008).

In the murine malaria model using *P. chabaudi chabaudi* AS both cell-mediated immunity and humoral immunity act sequentially to clear the infection. The predominant response during the first/acute phase of the infection is of a Th1-type cell, producing IL-2 and IFN-γ, while the control of the parasites at later phases, after the clearance of the first phase, also involves a specific response where Th2 cells provide B cell help by producing IL-4, which generate specific IgG antibodies important effectors in controlling the disease in these phases (Langhorne *et al.*, 1989).

It is noteworthy to remark that in malaria endemic regions the majority of malaria-infected individuals are also concomitantly infected with a range of other pathogens, such as other protozoan, helminthes, bacteria and viruses. The immune response to intracellular microorganisms, such as protozoan, bacteria, viruses, is often characterized by cellular cytolytic activity and the production of inflammatory cytokines, such as IFN-γ and TNF-α. Conversely, the immune protection to extracellular pathogens, helminthes for example, depends on humoral responses and on specific Igs generated to neutralize the foreign agent (Constant & Bottomly, 1997). Thus, individuals with co-infections can display different abilities in mounting an effective immune response to malaria. For instance, an individual with helminthes may bias the immune response towards Th2-type, which may reduce the levels of IFN-γ necessary in early response to malaria infection.

Immune memory in malaria

It is often observed that immunity to malaria wane quickly when immune adults leave malaria-endemic regions, proposing that continued exposure to malaria antigens is necessary not only for the generation of effector and memory cells but also for their persistence (Langhorne *et al.*, 2008). It has been difficult to identify the several immune players involved in immunological memory, as these cells are ill-defined by available methods, both in humans and in mouse models (Langhorne *et al.*, 2008). It is established that the formation of both central and effector $CD8^+$ memory T cells in the liver stage requires priming by DC in the draining lymph nodes (Chakravarty *et al.*, 2007) and are long-lived (up to 6 months). Immune responses to blood-stages malaria antigens have also memory cells associated. However, the normal immune response and memory establishment can be hampered by a chronic plasmodium infection (Figure 6) (Urban & Roberts, 2002; Langhorne *et al.*, 2008). In fact the parasite seems to be able to manipulate the host immune system during infection and to interfere with B and T cell activation, impairing the generation of immunological memory, which might result in a short-lived memory.

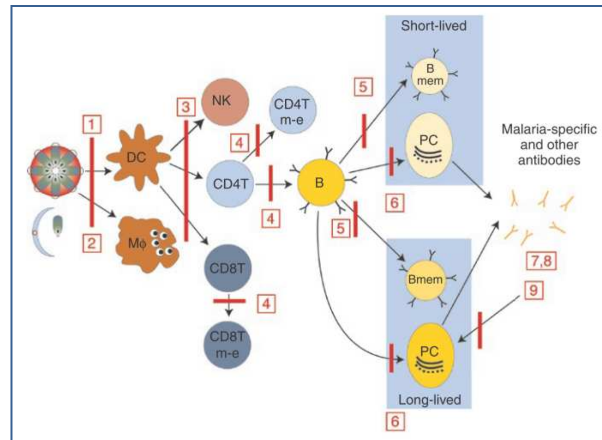


Figure 6. Possible mechanisms interfering with B and T cells activation and with the generation of immunological memory induced by malaria parasites. Red vertical bars indicate points in the B and T cell response at which parasites could interfere (inhibit, suppress or change the immune response). Numbers indicate possible mechanisms of interference: 1, interaction between iRBC–parasite and DC can inhibit DC maturation and reduce its capacity to stimulate T cells; 2, hemozoin can inhibit macrophage (Mϕ)–monocyte function; 3, shift of IL-12 to IL-10 secretion by parasite-modulated DC and macrophages may inhibit $CD4^+$ T cell (CD4T) activation; 4, $CD4^+$ T cells produce IL-10 and TGF-β, which inhibits the generation of central and memory-effector (m-e) cells; 5, iRBC induce apoptosis and/or depletion of memory B cells (Bmem); 6, limiting specificities repertoire of plasma cells; 7, the *P. falciparum* genome encodes a large number of predicted proteins and most induced responses to many polymorphic targets may not be protective and act as a 'smoke-screen'. Hypergamma-globulinemia, a common feature of *P. falciparum* infection, may accelerate the catabolism of immunoglobulin molecules. 8, antigenic variation of proteins on the iRBCs may be an effective mechanism for immune escape; 9, circulating immune complexes and low-affinity immunoglobulin molecules can trigger apoptosis of long-lived plasma cells (PC) through FcγRIIB. From Langhorne *et al*. (2008)

Polyclonal B cell activation and hypergammaglobulinemia are prominent features of human malaria (Achtman *et al.*, 2005), which are caused by parasite molecules that can directly induce the proliferation and differentiation of antibody-secreting cells from different B cell, regardless of their antigen specificity. Particularly, malarial chronic infections lead to severe deregulation of the immune system and B cells are overactivated resulting in hypergammaglobulinemia, the secretion of an array of autoantibodies and the frequent occurrence of B-cell tumors (Burkitt's lymphoma) (Donati *et al.*, 2004). The antibodies secreted by B cells stimulated with polyclonal activators are nonspecific and normally recognize antigens such as actin, myoglobin, myosin and DNA (Montes *et al.*, 2007). The antigens and mechanisms that lead to polyclonal activation are poorly understood. Polyclonal activators can be components of cell membranes, the cytosol or excretion/secreted products. For instance, it was identified the exact region of the PfEMP1 (the CIRD1α domain) that induces the polyclonal activation in malaria (Donati *et al.*, 2004). Polyclonal activators have the capacity of inducing the proliferation of multiple B cell clones and the up-regulation of MHC class II, CD69, CD25 and costimulatory receptor molecules such as CD80 and CD86 (Montes *et al.*, 2007). It has been debated whether polyclonal activation produces detrimental or beneficial effects in the host (Achtman *et al.*, 2005; Montes *et al.*, 2007). Polyclonal activation can be seen as a strategy of the infectious agents to avoid the host-specific immune response. Conversely, two potential beneficial effects of polyclonal activation were described. First, by enhancing natural antibodies production, recognizing a conserved range of antigens in many pathogens that can activate the innate immune system via the classical pathways of complement activation. Thus natural

antibodies represent a first line of defense while the adaptive response is not mounted (Montes *et al.*, 2007). Second, the polyclonal stimuli can be responsible for memory B cells maintenance. In this sense, it was demonstrated that in contrast to naïve B cells, memory B cells proliferate and differentiate into antibody-secreting cells after *in vitro* stimulation with polyclonal stimuli. This continuous stimulation and differentiation of memory B cells has been proposed as a plausible mechanism for the sustainment of “a long term serological memory” in the absence of a specific antigen. Contrarily, a “short term serological memory” is antigen-dependent and lasts for few months (Bernasconi *et al.*, 2002). In respect to malaria, it has been debated how malaria antibodies can persist for long periods after termination of the infection and the role of polyclonal activation in malaria, namely whether it gives rise to long-lived plasma cells (Achtman *et al.*, 2005).

Immunological response to malaria in pregnancy

Malaria in pregnancy might be the overall result of combining humoral immunity and cellular immunity mechanisms to the modifications associated to pregnancy, which include alterations in hormones balance and the development of the placenta.

Human PAM antibodies are directed to antigens specific of *P. falciparum* selected for their affinity to placental receptors, namely chondroitin sulfate A (CSA) (Staalsoe *et al.*, 2004). Through exposure to CSA-binding parasite variants over successive pregnancies women might acquire specific immunity to this parasite subpopulation. This notion is supported by several studies showing that primigravida women do not have antibodies that block binding of parasites to CSA. By contrast, multigravida possess serum IgG that inhibit iRBC adhesion to CSA (Ricke *et al.*, 2000). Moreover, women with high levels of anti-CSA-binding antibodies have reduced anemia and deliver babies with increased birth weight (Staalsoe *et al.*, 2004).

Decreased cellular immunity can also be a reason for increased malaria susceptibility, since several reports showed reduced responses to malaria antigens in pregnancy (Rasheed *et al.*, 1993; Fievet *et al.*, 2002). Placental infections are frequently characterized by the presence of inflammatory cells that are not usually present in sites of peripheral circulation. These recruited cells are predominantly monocytes that can be activated by parasites or parasite-derived products, such as hemozoin (Pichyangkul *et al.*, 1994) and GPI (Nebl *et al.*, 2005). Activated macrophages release potent anti-microbial molecules to aid parasite elimination, such as ROI, NO and TNF-α, contributing to an altered Th1 cytokine milieu in the placenta.

Changes in cytokines levels during malaria in pregnancy and associated to parity have also been documented. PAM typically induces a Th1/Th2 disequilibrium favoring the Th1-type pathway, leading to increased levels of inflammatory cytokines in the placenta and maternal peripheral blood. This event is associated with poor outcomes principally in primigravida and the role of inflammatory cytokines, such as IFN-γ and TNF-α, on PAM pathogenesis has been intensively investigated. Conversely, it has been observed a counter-regulatory effect in response to the inflammation, possibly to compensate immune-mediated damage in the

placenta. Th2 cytokines concentrations have also modified concentrations among pregnant infected women in comparison with non-infected. For example, in a study from Kenya (Fried *et al.*, 1998a) malaria-infected women had abundant levels of pro-inflammatory cytokines in concert with increased levels of the regulatory cytokine TGF-β. In fact, this cytokine is recognized by its immunosuppressive role, for example by reducing macrophages activation. In the placenta the main sources of TGF-β are the decidual and trophoblast cells (Robertson *et al.*, 1994) and it is known that it plays an important role in pregnancy by inhibiting strong proliferative cellular responses and that low levels of this cytokine are associated with spontaneous abortions (Raghupathy, 2001).

Increased levels of TNF-α has been associated with adverse birth outcomes (Fried *et al.*, 1998a; Fievet *et al.*, 2001; Rogerson *et al.*, 2003a). Besides having a role in protection, this cytokine has also been implicated in the pathogenesis of malarial disease, in particular in the malarial fever (Schofield & Hackett, 1993). Despite its potent cytotoxic effects, TNF-α also plays a role in normal fetal development and parturition and is produced in normal placentas by trophoblast cells and by resident macrophages (Hofbauer cells) (Robertson *et al.*, 1994). Overproduction of TNF-α and other pro-inflammatory cytokines in the placenta were observed in women that experienced spontaneous abortion (Raghupathy, 2001) and pre-eclampsia (Azizieh *et al.*, 2005). Higher levels of TNF-α were observed in malaria-exposed pregnant women and associated with LBW in a study realized in Kenya (Fried *et al.*, 1998a), and also in another report on placental malaria from Malawi (Rogerson *et al.*, 2003a), suggesting that this cytokine has also an immunopathologic role. TNF also primes neutrophils, regulates macrophage IL-12 production and is a co-factor for IL-12-induced IFN-γ production by T cells, and can even up-regulate the expression of ICAM-1 on endothelial cells (Robertson *et al.*, 1994).

IFN-γ is extremely potent in mediating host defense and is released from NK cells, T-lymphocytes and γδ T cells, upon stimulation by other T-cell or macrophage immune-mediators such as IL-12, TNF-α and hydrogen peroxide. In turn, high levels of IFN-γ may activate mononuclear cell production of TNF-α. In the placenta IFN-γ can be produced by cytotrophoblasts and by villous syncytiotrophoblast (Robertson *et al.*, 1994). Although elevated levels of TNF-α associated to LBW in PAM have been a consistent finding, the effect of IFN-γ has been controversial. In the study from Malawi mentioned above, IFN-γ levels are higher in plasma of women with placental malaria and in malaria-infected placentas than in uninfected placentas but were not associated with LBW (Rogerson *et al.*, 2003a). In the Kenyan study, placental plasma showed higher levels of IFN-γ associated with LBW especially in aparasitemic exposed primigravida, meaning that even after resolving infection IFN-γ might have harmful effects (Fried *et al.*, 1998a). In a different report from Kenya, IFN-γ response, elicited by placental leucocytes in response to malaria antigen stimulation, correlates with protection against placental malaria and in fact multigravida produce higher levels of this

cytokine. In particular, parasitemic multigravida cells were low IFN-γ responders, producing 60-fold less IFN-γ than cells from uninfected multigravida (Moore *et al.*, 1999).

IL-10, as a Th2 cytokine induces B cell proliferation, plasma cell differentiation and immunoglobin production. However, IL-10 has other tasks than the stimulation of humoral activity and the development and maturation of antimalarial antibodies. It also has a role on the decrease of anti-inflammatory responses, by reducing the production of IL-6, TNF-α, IFN-γ and IL-12, which make it also a regulatory cytokine. Trophoblast cells and maternal leucocytes are the main source of placental IL-10 (Robertson *et al.*, 1994; Fievet *et al.*, 2001). The effective role of IL-10 in protecting the placenta and the fetus by preventing the termination of the pregnancy has been demonstrated in several studies (Moore *et al.*, 1999; Fievet *et al.*, 2001; Suguitan *et al.*, 2003b), even though the elevated levels of IL-10 may suppress anti-parasite inflammatory responses resulting in high placental parasitemias and anemia (Suguitan *et al.*, 2003a).

Nevertheless, the relationship between peripheral and placental cytokine levels in the same individual has not been established. In human studies only peripheral parameters are available and it is not possible to access the concentrations in the placenta in order to infer their role in pathogenesis. A correlation between peripheral and placental concentrations of TNF-α, IFN-γ and IL-4 has been reported at delivery, suggesting that placental responses might determine the systemic profile of some cytokines (Fried *et al.*, 1998a). Since placentas are only available at delivery, these evaluations are limited and it is not possible to perform kinetics of this correlation.

The balance between pro- and anti-inflammatory cytokines is required for adequate protection and influences the pathology, namely the degree of anemia, clinical severity and disease outcome (Stevenson & Riley, 2004). Th1 cytokines are important in controlling early parasitemia, but they need to be counterbalanced later in the infection by a Th2 response leading to antibody production. In fact, cytokines such as IFN-γ appear to play a role in protecting against placental parasitemia whilst IL-10, for example, appears to avert inflammation, having a regulatory role in avoiding the detrimental effects of IFN-γ and TNF-α. Cytokines may be determinants of malaria severity and disease outcome and, once their effects are better understood, are potential targets for therapeutic interventions or even for placental malaria diagnosis. In conclusion, unraveling the cytokines interplay in the context of placental malaria should be helpful in scrutinizing the PAM pathogenesis mechanisms and define possible correlations of altered levels of cytokines with poor pregnancy outcomes.

Vertical transmission of disease and protection

There is no clear agreement on the definition of congenital malaria. Vertical transmission of parasites, from mother to child, may occur during pregnancy or perinatally during labour. Some authors stated that only the former should be considered true congenital malaria (Menendez, 1995), although more recently they do not discriminate both courses of

transmission (Menendez & Mayor, 2007). The overall incidence of congenital malaria is unknown and difficult to estimate owing also to its unclear definition. It is thought that congenital transmission is an uncommon event due mainly to the effectiveness of the placenta as a barrier to parasites. However, some reports have described cord blood infections in 35% of babies born to women with placental malaria infection (Redd *et al.*, 1996), or up to 10-32% of all newborns independently of the placental infection using sensitive diagnostic molecular methods, suggesting that cord malaria parasites were acquired antenatally and that they could cross the placenta with higher frequency than what was previously expected (Brabin, 2007; Menendez & Mayor, 2007). Indeed, placental malaria may be accompanied by damage of the syncytiotrophoblastic membrane, which may compromise the integrity of the placenta, allowing the parasites to enter the fetal circulation, establishing a route for malaria transmission (Crocker *et al.*, 2004). Moreover, some immunological evidence also points to fetal lymphocyte exposure to malaria antigens in uterus (Menendez & Mayor, 2007).

Whatever the course of parasite transmission to fetus/newborn is, the exposure to malaria parasites or to malaria antigens is likely to have important implications in their development. Certainly this exposure has immunological effects by priming the immune response or by inducing immune tolerance in the fetus, conferring reduced or increased malaria susceptibility, respectively (Menendez & Mayor, 2007). Thus, infection during pregnancy may be either harmful or beneficial for the infant, and the outcome will depend mainly on the time of pregnancy at which the first infection has occurred (Menendez, 1995).

Transfer of maternal antibodies occurs *in utero* across the placenta and vertical transmission of malaria maternal antibodies has been observed (Hviid & Staalsoe, 2004). The Ig transplacental transfer is restricted to the IgG isotype and the mechanism of transplacental transfer of maternal IgG was reviewed few years ago (Kristoffersen, 2000). Briefly, the transfer of IgG across the placental barrier (Figure 7) is an active process involving Fc receptor (FcR) molecules. The maternal IgG must pass two cellular barriers: the syncytiotrophoblast and the fetal endothelial cells.

Passive transfer of antibodies continues after birth via breast feeding, since anti-malaria antibodies have been detected in human breast milk (Leke *et al.*, 1992). However these antibodies are believed to act only within the gut to protect the infant from enteric pathogens, as the majority of the immunoglobulins are degraded in the intestine and very little if any Ig isotype is absorbed in an active form into circulation (Riley *et al.*, 2001).

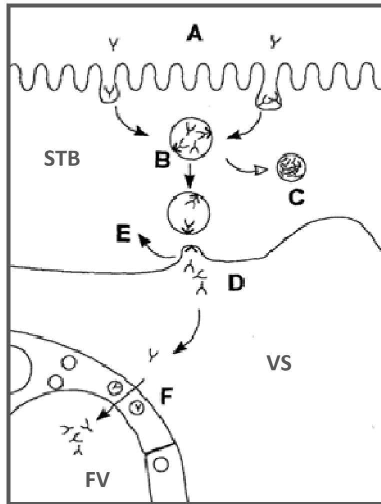


Figure 7. **Steps in the IgG transport through the placental barrier**. Maternal IgG (A) enters the syncytiotrophoblast (STB) by an active receptor-mediated process, or by liquid fluid phase endocytosis (not established). Once internalized (B), by either mode, vesicles fuse and molecules are sorted. Transport within the endocytotic compartment involves IgG binding to the high-affinity FcRn of the early endosome and transcytosis (the complex is internalized and carried through the cytoplasm of the syncytiotrophoblast in a transport vesicle to its luminal surface), whereas unbound molecules will enter the lysosomal pathway (C) and are degraded. Once brought to the basal side (D), the FcRn/IgG complex is exocytosed and IgG dissociated from FcRn. The FcRn is then probably recycled (E). IgG then diffuse through the villous stroma (VS) and is transferred to the fetal vessels (FV) through the endothelial cells in caveolae by an unknown mechanism (F). From Kristoffersen (2000).

Placental pathologic changes caused by malaria may inhibit the transfer of maternal proteins generically and reduce the transfer of antibodies, even those associated to other diseases (Riley *et al.*, 2001). Systemic antibody transfer to the infant decays abruptly at birth and IgG concentrations decline as it is catabolized and thus the persistence of antibody titers in the infant depends on the starting concentration of antibody at birth (Riley *et al.*, 2001). Furthermore, it has been observed that maternal antibodies do not protect infants against malaria as it could be expected. In a longitudinal study with 143 children in Ghana there was no association between levels of maternal antibodies and protection against malaria over the first 20 weeks of age, but in the contrary infection risk was higher in children with higher maternally derived antibody levels (Riley *et al.*, 2000). This increased susceptibility to infection in presence of maternal infection, rather than an expected protection, might be due to immunologic tolerance induced by *in utero* exposure to parasite antigens (Lammie *et al.*, 1991). Later, a study conducted in Cameroon evaluated the levels of antibodies specific for PAM and for non PAM parasites in cord blood of 79 neonates, who were followed up to 2 years until the first appearance of *P. falciparum* parasites (Cot *et al.*, 2003). They concluded that maternally transmitted anti-PAM antibodies, but not antibodies to any other specificity, were negatively related to the length of time until the first appearance of parasites in peripheral blood and positively related to the mean parasite density during the first 2 years of life. In fact, these results show that such antibodies have affinity to *P. falciparum* that cytoadhere in the placenta and this parasite population is only present in pregnant women and not in non-pregnant hosts like children. Therefore the presence of anti-PAM antibodies in cord blood only reflects the past occurrence of a maternal infection and is non-protective in children.

PARASITE-HOST INTERACTIONS IN PREGNANCY-ASSOCIATED MALARIA

P. falciparum infected red blood cells (iRBC) do not generally remain in the peripheral circulation but instead adhere to molecules expressed on the vascular endothelium surface of the capillaries of specific organs of the host. Hence, the parasites find a proper environment for their relatively safe maturation and multiplication, avoiding to be taken by macrophages in the spleen. As a result there is an extensive accumulation of parasites in vital organs and, consequently, high concentration of parasite toxic metabolites and other factors inducing pro-inflammatory responses in the host, leading to vascular damage and organ dysfunction (Miller *et al.*, 2002). The adhesion is mediated by the recognition of parasite proteins expressed on the outer membrane of infected erythrocytes by receptors on the host endothelial cells.

Parasite features determinant for disease establishment

Malaria parasites and disease have several peculiar features and the understanding of how they are achieved is crucial to the knowledge of disease-related mechanisms and for the design of interventions aimed to reduce or eliminate infection. The parasite biology itself presents certain characteristics that appear strategic for immune evasion, ranging from the fact of being an intracellular parasite, to antigenic diversity and antigenic variation, and even to the capacity of being sequestered. Many aspects of PAM epidemiology are explained on the light of some of these parasite features.

Malaria parasites rely on a mosquito vector for a part of their life cycle, which is dependent of suitable moist conditions for its breeding. In areas of highly seasonal transmission with a long dry season, malaria episodes are concentrated in a short period of the year following the rainy season and parasites have to maintain long infections in order to survive. This was imperative to ensure their transmission, since they must survive for 9 or 10 months in their mammalian hosts in order to be transmitted to mosquitoes in the following wet season (Kyes *et al.*, 2001).

Another characteristic of malaria parasites, functioning as a strategy of immune evasion, is evident at RBC invasion by merozoites that evolved a 'just-in-time' mode of invasion (Kats *et al.*, 2008). Merozoites have a polarized morphology and several organelles characteristic of the Phylum *Apycomplexa*, namely an apicoplast (a plastid-like organelle) and an apical complex constituted by three other organelles (rhoptries, micronemes and dense granules), concentrated on the apical end of the parasite that are involved in cellular invasion (Cowman & Crabb, 2006). The apicoplast possibly arose in an ancestor of the apicoplexan parasites that engulfed a eukaryotic alga retaining its plastid. Its genome encodes for 30 proteins and is essential for parasite survival, having a function in the anabolic synthesis of fatty acids, isoprenoids and heme (Gardner *et al.*, 2002). The initial interaction between the merozoite and the RBC appears to occur at any point on the surface of the merozoite, followed by its reorientation in order to juxtapose the apical end of the merozoite with the erythrocyte membrane, allowing a subsequent stronger interaction (Figure 8). To invade a RBC the

merozoite must engage RBC receptors and undergoes apical reorientation. Afterwards, the invasion process is facilitated by released proteins contained within the apical organelles. The fact that these secreted factors are inside an organelle might protect the parasite from recognition, as they are secreted only shortly prior to or at the time of merozoite attachment. This allows the parasite to protect essential ligands from antibody-mediated neutralization by minimizing their exposure to the host immune system. Moreover, the parasite can compartmentalize proteins according to function and deliver them in a temporally-regulated manner. For instance, proteins that are involved in reorientation and tight junction formation are secreted first, followed by proteins necessary for alteration of the host RBC cytoskeleton and parasitophorous vacuole (PV) formation, as the parasite invades the host cell, and finally proteins required for long-term maintenance of the PV are released. Within this PV, derived from the RBC plasma membrane, the parasite creates a frontier to seal itself from the host cell cytoplasm (Kats *et al.*, 2008).

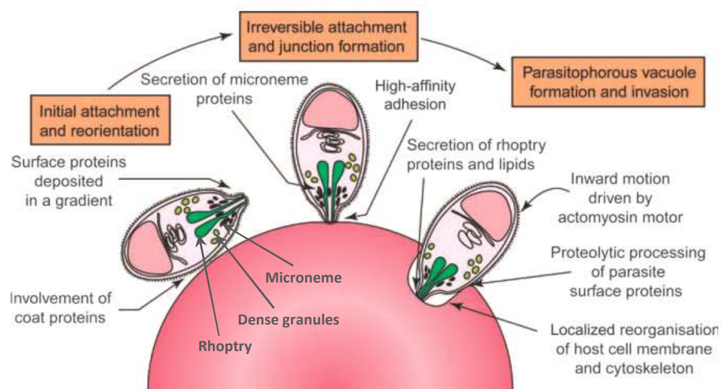


Figure 8. Invasion of a RBC by a *P. falciparum* merozoite. Invasion is a sequence of processes. Initial attachment can occur with the merozoite in any orientation and involves low-affinity interactions between merozoite surface proteins and receptors on the target cell (glycophorin A in *P. falciparum*). Subsequent interactions are facilitated by proteins that are initially hidden within the apical organelles and are secreted only shortly prior to or at the time of attachment. From Kats *et al*. (2008).

Malaria parasites seem to have chosen an immune-privileged site by infecting RBC, which have no surface MHC molecules and no mechanism for antigen presentation. However, the parasite is not invisible to the host immune system, since on the iRBC surface novel proteins appear produced by the parasites. Some of these proteins correspond to highly polymorphic antigens, and can enable the iRBC to cytoadhere to host receptors. In fact, a controlled parasite proliferation may be attained by exposing iRBC to the immune system even without leading to its total elimination. Otherwise, if parasites remained unchecked, proliferation would be unconstrained and the host would be killed before an efficient transmission to a mosquito (Scherf *et al.*, 2008).

Once infection is established in the blood, it continues until either the host dies or the parasite is controlled by drugs or by the immune response. In an untreated individual the typical pattern for parasitemia is to rise to a high level, producing severe clinical symptoms, and then fall, with the symptoms either disappearing or becoming milder. At some later point, parasitemia rises again and clinical symptoms return (Scherf *et al.*, 2008). The clinical pattern of relapses reflects the “strategy” used by the parasite to contour the host immune response

by an antigen switching mechanism. In principle, parasite strains with inherently high switching rates (allowing rapid sequential expression of novel antigens) would be able to cause repeated infections in the same patient and to establish a chronic infection (Kyes *et al.*, 2001).

A further property of some *P. falciparum* isolates is the ability of iRBC to bind to uninfected RBC, leading to the formation of erythrocyte rosettes and this event is associated with an increased risk of severe disease (Udomsangpetch *et al.*, 1989). Parasite sequestration and rosetting are mechanisms that maintain the parasite shielded from destruction by the immune system, favoring its growth and multiplication, but with likely problematic consequences for the host, such as capillary occlusion, organ dysfunction and severe malaria syndromes like cerebral malaria.

Additionally, most immune responses directed to many of these polymorphic antigens may not have any protective function and may confound the immune system. It is noteworthy to remark that *P. falciparum* genome encodes for 5,268 predicted proteins, 31% (1,631) have one or more transmembrane domains, many of which are highly polymorphic (Gardner *et al.*, 2002).

The surface of infected erythrocytes

A normal RBC presents a discoid shape and a diameter of about 7 µm, having a central bi-concave form, enabling it to entry the smallest blood vessels and capillaries. Upon infection of RBC by merozoites, the parasites modify the RBC membrane to enable their survival and proliferation. After RBC invasion, the parasite starts to increase in size and to digest hemoglobin taken from the RBC cytoplasm, depositing the undigested heme residue in a polymerized pigment, the hemozoin (Cooke *et al.*, 2004). Maturation of the parasites causes structural and morphologic changes in the iRBC, which include alterations in cells deformability (loss of the normal discoid shape and acquisition of a spherical appearance), permeability, and perturbations in the mechanical and adhesive characteristics, accomplished essentially by the trafficking of proteins and proteins export to the RBC membrane. Some exported proteins are conserved across plasmodial species and are essential for parasite survival. The parasite develops inside the RBC through the ring, trophozoite and schizont stages and at the termination of this asexual cycle the iRBC needs to be lysed to release merozoites for invading new RBC. This lysis is completed in two steps, firstly the lysis of the internal membrane that surrounds the parasite, the PV membrane, allowing the parasite to enter the RBC cytoplasm, and secondly the lysis of the RBC membrane (Cooke *et al.*, 2004).

The iRBC surface (in trophozoites and schizonts stages) appears punctuated by up to 10.000 distinct knob-like protrusions (with ~100 nm in diameter) (Figure 9) that resulted from the co-localization of parasite proteins and might be associated with altered cellular adhesive properties of the cell (Cooke *et al.*, 2004; Maier *et al.*, 2009). Although knobs are considered necessary for adhesion, some studies show that the presence of knobs does not necessarily lead to sequestration. For example *P. malariae* iRBC show knobs and do not sequester

(Sherman *et al.*, 1995), whereas the rodent parasite *P. chabaudi* iRBC lack knobs and can sequester (Cox *et al.*, 1987) as well as a knobless line of *P. falciparum* (Biggs *et al.*, 1990). Thus, the presence or absence of knobs is not as decisive for cytoadhesion, as the presence of certain parasite proteins on the RBC surface.

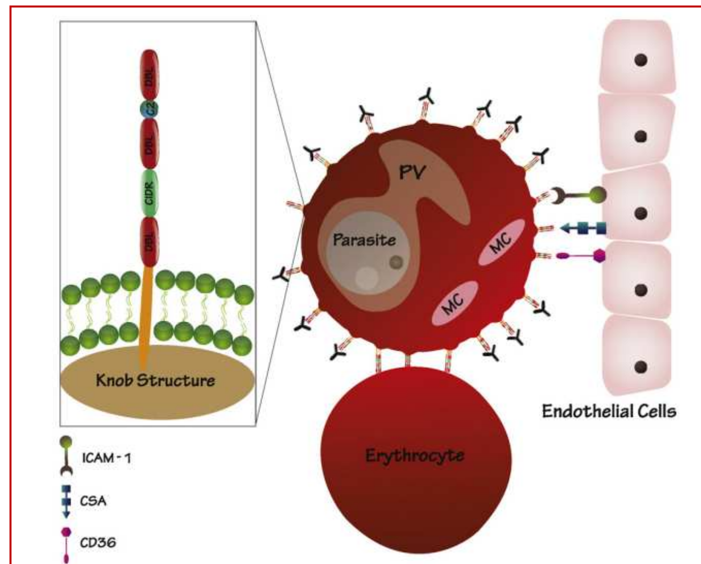


Figure 9. The variant PfEMP1 mediates adhesion of the infected RBC to host receptors. PfEMP1 is expressed by the malaria parasite *P. falciparum* on the knobs formed on the surface of iRBC. The variable extracellular regions of the protein (DBLs and CIDR) mediate adhesion through binding to several tissue receptors such as CD36, ICAM1 and CSA. PfEMP1 also mediates adhesion to uninfected erythrocytes forming rosettes. (PV, parasitophorous vacuole; MC, Maurer's cleft). From Pasternak & Dzikowski (2009).

Antigenic diversity and antigenic variation in P. falciparum

Different populations of *P. falciparum* show antigenic differences, which confer them distinct properties. Antigenic diversity, which reflects polymorphisms in allelic gene products, can be distinguished from antigenic variation, which is a result of the expression of alternative genes in multicopy family genes. This process of antigenic variation prevents the parasite of being attacked by continuously changing some iRBC surface proteins, allowing the extension of the infection period. Antigenic variation in *P. falciparum* has been intensively studied but many underlying molecular mechanisms remain unknown. Significant work in recent years has contributed to the understanding of antigenic variation process and the "state of the art" has been reviewed and summarized by diverse authors (Kyes *et al.*, 2001; Ralph & Scherf, 2005; Kyes *et al.*, 2007; Scherf *et al.*, 2008; Dzikowski & Deitsch, 2009). The process of antigenic variation results from switches in expression between members of a specific gene family, thus altering the form of the surface exposed protein (variant surface antigen or VSA). Four multicopy gene families might be involved in antigenic variation of *P. falciparum*: *var*, *rifin*, *stevor* and Pfmc-2TM. The best characterized is the *var* family, which encodes the *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1), a protein responsible for iRBC sequestration, recognized as the major target of antibodies and clustered on the iRBC knobs (Figure 9). Similarly to the *var* genes, the other gene families also undergo clonal variation, but

the proteins they encode have unknown biological functions (Kyes *et al.*, 2001; Scherf *et al.*, 2008).

The multigene family *var* has approximately 60 genes distributed across all *P. falciparum* genome, each one displaying a distinct repertoire of surface variants for PfEMP1. The switching in transcription from one *var* gene to another appears to rely only in epigenetic changes in the gene locus (Ralph & Scherf, 2005). Each individual parasite expresses a single *var* gene at a time (coding for the dominant neoantigen), maintaining all the other members of the family in a transcriptionally silent state. Therefore, there is a mutually exclusive expression of a single *var* gene member orchestrated by different epigenetic factors that do not require programmed DNA rearrangements. A switch in expression must be coordinated so that activation of one gene coincides with simultaneous silencing of the previously active copy with modifications in chromatin structure clearly playing an important role in determining which *var* gene is active in any given parasite. However, a mechanism of “memory” must exist to maintain the transcription state through subsequent parasite generations, so that the epigenetic marks, namely that the chromatin structure is kept across cell divisions. At present this process is not completely understood. Several other aspects remain to be elucidated, namely the switching sequence process and the clarification on how to maintain an antigen switching rate that allows emergence of a new protein variant without exhausting all the possibilities, and whether the switching follows an order or is a random process.

Studies to clarify those unknown mechanisms are impracticable in humans. For instance, the order and rate at which parasites switch expression from one *var* gene to another is difficult to measure in patients. iRBC VSA switch rates of *P. falciparum* were firstly evaluated in a study where the authors showed that parasites cultured *in vitro*, in absence of immune pressure, switched spontaneously at a rate of 2%, leading to parasites with different antigenic and cytoadherence phenotypes (Roberts *et al.*, 1992). Nevertheless, there is evidence that switching rates *in vivo* are higher (~18%) although the predictions are based in few experiments and on mathematical models founded on assumptions of hypothetical *var* switching mechanisms (Gatton *et al.*, 2003).

Host receptors for malaria parasites

The *P. falciparum* adhesion process, in which most parasites first tether and then roll before becoming firmly secure, is comparable to leukocyte adhesion. Only two receptors, CD36 and chondroitin sulphate A (CSA), have been shown to provide stable stationary iRBC adhesion (Miller *et al.*, 2002). Cooperation between host receptors is known to enhance adhesion of iRBC, which may need to be preceded by tethering and rolling before stabilizing. The type of affinity between iRBC and the host receptors might modulate the final interaction, namely adhesion and/or rosetting properties. Binding studies using recombinant PfEMP1 domains have shown interactions with various host receptors, which include: CD36, the most abundant adhesion receptor and a mediator for most of the clinical isolates and laboratory lines of

P. falciparum; is found in the surface of platelets, monocytes, dendritic cells and microvascular endothelial cells; ICAM-1, expressed in the endothelium, has an important role in the leucocytes adhesion to the endothelium during inflammation and was shown to support rolling of iRBC; thrombospondin, a molecule with anticoagulant properties, that appears on extracellular matrix of the endothelium and syncytiotrophoblast; platelet-endothelial cell adhesion molecule (PECAM); vascular cell adhesion molecule-1 (VCAM1) and the endothelial-cell selectin (E-selectin). The last two receptors are not expressed on resting endothelium but their expression is induced by inflammatory cytokines, such as TNF-α (Sherman *et al.*, 1995; Baruch *et al.*, 2002; Schofield & Grau, 2005).

Rosettes, or aggregates of infected and uninfected RBC, are observed in some *P. falciparum* isolates and appear to involve several RBC surface molecules. Rosetting mediators can include the sulfated glycoconjugates heparin or heparan sulfate, blood groups antigens A and B and the complement receptor (CR1) expressed on uninfected RBC, and also IgM in serum (Kyes *et al.*, 2001). The formation of these aggregates, between parasitized and non-parasitized RBC, might also lead to the obstruction of capillaries in patients with cerebral malaria (Udomsangpetch *et al.*, 1989).

Other host receptors were also found to have affinity to iRBC surface proteins. In 1995, Rogerson *et al*. found that *P. falciparum* laboratory strains can adhere to Chinese hamster ovary (CHO) cells and that the binding is mediated by CSA (Rogerson *et al.*, 1995). In their experiments, CHO cell-adherent iRBC were unable to bind CHO cell mutants lacking CSA expression. Moreover, iRBC binding to CHO cells was inhibited by CSA but not by other glycosaminoglycans and treatment of CHO cells with chondroitinase ABC but not with other enzymes led to a reduction in iRBC binding. Thus CSA was identified as a potential receptor involved in parasite sequestration, a result confirmed a year later by Fried and Duffy. In fact, these authors discovered the biological importance of iRBC adhesion to CSA, by demonstrating that iRBC obtained from human infected placentas binds to uninfected placentas in a CSA-dependent manner (Fried & Duffy, 1996).

Receptors for parasites on human placentas

In human pregnancy-malaria pathogenesis the receptors that are reportedly involved in placental parasite sequestration are glycosaminoglycans (GAG), such as the low-sulphated CSA (Fried & Duffy, 1996; Achur *et al.*, 2000) and possibly the hyaluronic acid (HA) (Beeson *et al.*, 2000; Beeson *et al.*, 2002b; Rasti *et al.*, 2006). Within the placenta, CSA is shown to be distributed throughout the intervillous spaces and at low but significant levels on the syncytiotrophoblast lining (Muthusamy *et al.*, 2004). Studies of placental CSA expression kinetics show that CSA is present in placentas and available for iRBC adhesion during the second and third trimesters of pregnancy (Gowda, 2006). CSA is a GAG composed of repeats of disaccharide units of D-glucuronic acid (GlcA or GlcUA) linked to N-acetyl-D-galactosamine (GalNAc) with a sulfate group at position C4 of GalNAc. CSA molecules from different sources

differ in sulfation patterns, and this property may influence their ability to support iRBC binding in the placenta (Fried *et al.*, 2000). Highly sulfated forms can fail to support adhesion, whereas low-sulfated forms are optimal for binding (Alkhalil *et al.*, 2000).

HA is a controversial candidate as iRBC placental receptor, since it is not present in the intervillous spaces (Achur *et al.*, 2000; Muthusamy *et al.*, 2007) and it is claimed that its presence in the placenta is due to contamination from the umbilical cord where it is abundant (Fried *et al.*, 2006). Previously reported binding to HA could be due to the CSA contaminations in HA preparations. Additionally, since the level of HA is less than 1-2% as compared to other GAG, if it can be a placental receptor, it should not be a main one (Valiyaveettil *et al.*, 2001). Few years ago it was also suggested that non-immune immunoglobulins (of the IgG isotype) can be adsorbed to iRBC surface and this prompted the hypothesis that IgG may act as a bridge for iRBC to bind Fc receptors on the syncytiotrophoblast (Flick *et al.*, 2001; Rasti *et al.*, 2006). Nevertheless, the presence of HA and Fc receptors either in the intervillous space or in the syncytiotrophoblast lining of placentas is still unclear and more data is needed to resolve these controversies.

CSA and other GAG on the intervillous spaces and on the syncytiotrophoblast appear to have several functions. They may play a structural role (similarly to other organs subjected to mechanical deformation like the joints cartilage) in maintaining the shape of the IVS to facilitate maternal blood flow. Additionally, they might constitute chemical barriers in masking fetal antigens from the maternal immune system and they may be involved in adsorbing essential components including metal ions, growth factors, and nutrients from maternal blood and in assembling them in the IVS for effective uptake by fetal villi. The low sulfation of the GAGs is associated with a low charge density of GAG chains that may facilitate the effective transfer of the adsorbed materials by a relatively weak interaction, which would be stronger if the GAGs were to be highly sulfated (Achur *et al.*, 2000). In sum, the accumulation of these molecules with low sulfated GAG chains in the IVS suggests that they play a fundamental role in the placenta, and *P. falciparum* takes advantage of it to sequester and thus survive.

Variant surface antigens associated to PAM

CSA is expressed in large amounts in the placental IVS and on syncytiotrophoblast and, in fact, placental parasite isolates preferentially adhere to CSA *in vitro*, whereas parasites from men and non-pregnant women usually do not (Fried & Duffy, 1996; Beeson *et al.*, 1999). Thus, the conclusion of several experimental observations is that placental parasites are antigenically distinct from those of non pregnancy origin.

PfEMP1 proteins are involved in several malaria disease syndromes, but its role is best understood for PAM (Rogerson *et al.*, 2007). Through switching expression of the different *var* genes, PfEMP1 undergoes antigenic variation and expresses specific VSAs that mediate adhesion to CSA, allowing the parasite to evade former host immune responses (Smith *et al.*, 1995). The gene *var2csa* is transcriptionally upregulated in both placental isolates and

laboratory parasites selected to bind CSA (Salanti *et al.*, 2003), and disruption of *var2csa* causes infected erythrocytes to lose their ability to bind CSA (Viebig *et al.*, 2005; Duffy *et al.*, 2006). In fact, in iRBC with non-CSA-binding abilities the gene *var2csa* is silent and different members of the *var* gene family are transcribed during chronic infection.

During the first pregnancy, women who have previously developed malaria immunity are susceptible to placenta infection. After one or two pregnancies they develop protection to the placental form of the disease and generate antibodies recognizing placental parasites (Fried *et al.*, 1998b; Duffy & Fried, 2003; Staalsoe *et al.*, 2004), suggesting that surface molecules expressed by placental infected erythrocytes may have unique and conserved features. Additionally, Salanti *et al*. show that high levels of anti-VAR2CSA antibodies correlated with a lower risk of delivering LBW neonates (Salanti *et al.*, 2004) and in a different study CSA-binding placental isolates were also significantly associated with LBW children (Tuikue Ndam *et al.*, 2004).

VAR2CSA-PfEMP1 plays a major role in PAM, displaying an extensive polymorphism, but only a limited portion of the variable domain is actively seen by the host immune system (Bockhorst *et al.*, 2007). Thus, placenta-associated parasites have adhesive and antigenic differences between isolates that correspond to *var2csa* polymorphisms, hence stimulating different host responses (Beeson *et al.*, 2006; Trimnell *et al.*, 2006; Kyes *et al.*, 2007). Women exposed to malaria acquire and expand the repertoire of variant-specific antibodies, some of which cross-react with different placental isolates and the extent of reactivity appears to be greater among women who experienced more exposures in pregnancies (Beeson *et al.*, 2006).

Overall, a woman is highly susceptible to placental infection when pregnant for the first time, even if her pre-existing acquired immunity can control non-placental parasitemia. Nevertheless, an interesting question remains: how does a *var* gene that is apparently silent in a woman appear to be the major *var* gene expressed during pregnancy? Nunes and Scherf reviewed this issue recently and considered that there are two hypothetical mechanisms that could lead to *var2csa* activation during pregnancy malaria (Nunes & Scherf, 2007). The first is a selective process by the placenta, which is based on the fact that the placenta expresses a range of new receptors, providing a niche for parasites expressing variant CSA-binding phenotypes (that are circulating in very small numbers in the peripheral blood of the primigravida) to survive and selectively accumulate. The second is an induction mechanism promoted by specific host factors. Pregnancy triggers physiological changes, including serum-specific factors (hormones/cytokines) and locally-released mediators by the syncytiotrophoblast, which may promote the transcription of *var2csa*.

Parasite recrudescence in human PAM

Parasite recrudescence has been observed in course of pregnancy in different situations but is generally difficult to identify. Many *P. falciparum* genes show extensive genetic polymorphism, which can be detected by molecular genotyping studies. Because of this ample polymorphism,

it is highly unlikely for individuals in areas of intense transmission to become newly infected with a parasite having an identical genotype during a disease follow up (this probability is the product of individual allele frequencies of each allele of the total number of genes in study). Therefore, by comparing the genotypes of the target loci across disease relapses, parasite recrudescence can be distinguished from a new infection (Mugittu *et al.*, 2006). A recent study on PAM using a sensitive genotyping method of *P. falciparum* isolates show PAM recrudescent infections in 21% of the women as they had parasite isolates sharing the same antigens without evidence of new infections and with a mean time interval between consecutive malaria-recrudescence episodes of 58 days (Mayor *et al.*, 2009). A case of recrudescence was reported in a pregnant woman who had been absent from endemic regions for a long period of time (4 years) (Giobbia *et al.*, 2005).

It is important to point out that the genotyping of field isolates could be useful in detecting not only the multiplicity of infections (number of concurrent infections) but also in studying infection dynamics in pregnancy. In fact, WHO states that recurrent parasites should be genotyped by polymerase chain reaction (PCR) to distinguish recrudescent from new infections and that these tools are very valuable for studies on drug resistance and for other specialized epidemiological investigations (WHO, 2006). However these tools are not generally available for human PAM studies in malaria endemic areas, where women are continuously exposed to new infections. Identification of recrudescent events in pregnant women by parasite molecular genotyping may improve the understanding of the pathological mechanisms of PAM, the processes of acquired immunity, the evaluation of the placenta-specific parasite antigenic variation, transmission conditions, efficacy of treatments and the genetic basis of drug resistance in pregnancy.

Mechanisms attempts to explain PAM epidemiology

The mechanisms and biological bases of PAM susceptibility and linked recrudescence, as well as protection acquired by multigravida, are still not well understood. Several efforts have been made to explain the epidemiological finding that, especially in areas of stable transmission, malaria is more frequent and severe in first pregnancies with observed reduced susceptibility in women who have had several pregnancies exposed to malaria (Fried & Duffy, 1998).

Some explanatory attempts are based on the higher attractiveness of women for malaria-carrying mosquitoes during pregnancy (Himeidan *et al.*, 2004), due to both physiological and behavioral changes. In fact, pregnant women have a higher production of exhale breath and an increased blood flow / hotter skin than their non-pregnant counterparts, raising the release of volatile products that allow mosquitoes to perceive them more readily (Lindsay *et al.*, 2000). However this can only explain the higher parasite exposure to new infections, which could cause the parasitic load to expand, but not the cases of recrudescence.

Other postulated mechanisms include the hormone-dependent depression of the immune system during pregnancy that would allow the exacerbation of malaria. Indeed, there are few

examples of hormone fluctuations during pregnancy that can modulate the immune system, either by suppressing or stimulating it, and thus modify maternal susceptibility to malaria (Rasheed *et al.*, 1993; Bouyou-Akotet *et al.*, 2005; Mavoungou, 2006). The immunosuppression is mainly sustained by increased blood levels of cortisol that reduces the NK cytolytic effect on *P. falciparum* iRBC (Bouyou-Akotet *et al.*, 2004). Cortisol concentrations were higher in primigravida than in multigravida from the second trimester onwards, and also higher in infected than in uninfected primigravida (Vleugels *et al.*, 1989; Bouyou-Akotet *et al.*, 2005), whereas susceptibility to malaria is higher in the second trimester and then decreases (Brabin, 1983). Conversely, the plasma prolactin levels, a stimulator of the immune system, is higher in multigravida (Bouyou-Akotet *et al.*, 2005). It was proposed that the hypothalamic-pituitary-adrenal (HPA) axis might be modulated by repeated pregnancies/lactations events, which could “desensitize” stress circuits leading to a reduction of cortisol secretion after multiple births (Tu *et al.*, 2006), thus reducing multigravida immunosuppression.

However these hypotheses do not explain the preferential replication of parasites within the placenta. Duffy discussed the immunosuppression hypothesis (Duffy, 2001), stating that malaria could confound cortisol studies in pregnancy, since it makes difficult the distinction between cortisol levels due to pregnancy, due to malaria disease itself, puzzled with malaria-related immunosuppression. Interestingly, the connection between malaria in pregnancy and immunosuppression was first demonstrated in mouse models, showing that development of recrudescent infection leads to increased corticosteroide production, suggesting that cortisol played a regulatory role in malaria during pregnancy (van Zon *et al.*, 1982).

Presently, immunosuppression is not generally acknowledged as an explanation for PAM susceptibility and hypotheses that include acquired immunity to neoantigens, resulting from parasite antigenic variation, are more accepted because they corroborate the epidemiology of the disease. The currently accepted hypothesis emerged after the discovery that *P. falciparum* parasite sub-populations are responsible for maternal malaria (Fried & Duffy, 1996). As specific immunity would start developing towards those parasites, infections could be cleared. Primigravida generally do not have antibodies to placental-binding iRBC, suggesting that these parasites represent novel VSAs to which women have not been previously exposed (Beeson *et al.*, 1999; Ricke *et al.*, 2000). Antibodies to surface antigens expressed by placental isolates and isolates that adhere to CSA are more prevalent in multigravida after exposure to placental malaria (Beeson *et al.*, 1999; Ricke *et al.*, 2000; Beeson *et al.*, 2004) and are associated with a reduced risk of malaria during pregnancy and improved pregnancy outcomes (Staalsoe *et al.*, 2004). This explains why malaria premunition acquired during childhood, by people living in endemic areas, does not include antibodies that prevent CSA-iRBC adhesion and immunity against CSA-adherent parasites. Indeed a placental-parasite related immunity is crucial to control placental malaria and parasite exposure through consecutive pregnancies is required for this immunity to develop and be maintained (Hviid & Staalsoe, 2004).

CONTRIBUTION OF MOUSE MODELS FOR PREGNANCY-ASSOCIATED MALARIA STUDIES

There are still several gaps of knowledge on PAM that constitute priorities of research, both in the parasite side and in the host side, some of which are difficult to perform in vivo for several reasons, including ethical constraints. Animal models can give a contribution in this area of research and some authors mentioned the importance of animal studies in providing new scientific hypotheses (Beeson *et al.*, 2002a; Nosten *et al.*, 2004; Greenwood *et al.*, 2007; Rogerson & Boeuf, 2007).

Malaria exposure is a composite of mosquito and parasite factors including numbers of infective bites, duration of infection and genetic diversity of the parasite population, which are difficult to quantify and to compare. Disease outcomes (duration and density of infection and disease severity) are equally difficult to categorize and measure and are influenced by host factors other than immunity. Animal models can have an added value in this respect, since they allow the tight control of many exposure variables and close monitoring of disease development.

Laboratory animals, such as mice, are suitable models for PAM because of their relatively short gestational period that allows a reasonable experimental time frame, and of the availability of immunological and genetic tools. It is critical to appreciate the degree of similarity/differences of mice and human pregnancy physiology, namely on their immunology, placental structure and function. Desowitz summarizes the possible model systems that can be used to study malaria in pregnancy (Desowitz, 2001) and concludes that the congruency between the mouse and human is enough to allow the use of rodent malaria as a model for human PAM. It is clear that placental malaria is associated with several complications in pregnancy, paralleling analogous features in human and animal pregnancies, including embryonic lethality, fetal growth restriction, pre-eclampsia and the high rates of fetal mortality.

Comparative histology of the human and mouse placentas

Recent studies have provided extensive new data on the anatomy and physiology of the mouse placenta. Although the gross construction of the human and mouse placentas differ somewhat in their details, the overall structures and molecular mechanisms underlying placental development are thought to be quite similar (Rossant & Cross, 2001). Placental development comparison between mouse and human, as well as studies of molecular biology that attempt to localize gene expression patterns, have been described in several other reports (Adamson *et al.*, 2002; Cross *et al.*, 2002; Georgiades *et al.*, 2002; Cross *et al.*, 2003; Cross, 2005; Watson & Cross, 2005).

Both rodents and humans have discoid placentas, with a flat part facing the fetus and a convex opposite surface adjacent to the uterine wall. During implantation and subsequent trophoblast

invasion, fetal trophoblast cells and maternal tissues (endometrium and myometrium in humans but only endometrium in mice) come into intimate contact. Both placentas are classified as hemochorial, because the uterine epithelium is eroded such that maternal blood comes into direct contact with trophoblast villi surface. In a cross-sectional view of both placentas it is possible to observe analogous structures (Figure 10) (Georgiades *et al.*, 2002). Briefly, the three major placental zones have a correspondence in human and mouse: the outer maternal layer (decidua basalis) includes decidua cells of the uterus, as well as the maternal vasculature that brings blood to/from the uterine implantation site; a middle region which attaches the fetal placenta to the uterus and contains trophoblast cells that invade the uterine wall and maternal vessels (known as basal plate in humans and junctional zone in mice); and an inner region formed of highly branched villi where exchanges occur (the fetal placenta (villous tree) in human or labyrinth zone in mouse placenta).

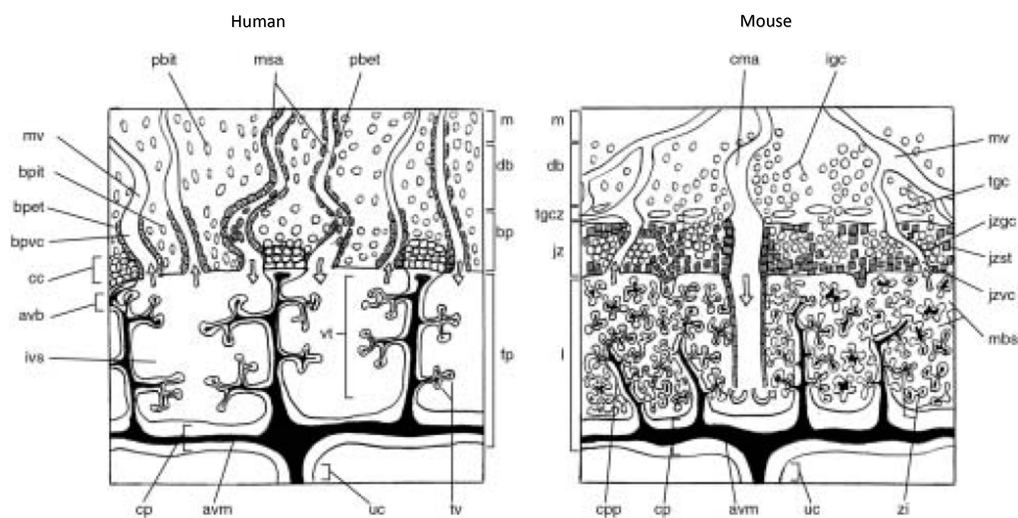


Figure 10. Human and mouse placentas. Schematic representation of the major regions and cell types of the human (left) and mouse (right) placentas, during the last trimester and last fifth of gestation respectively. The placentas are oriented with their maternal side towards the top and that facing the fetus (flat) at the bottom. The plane of sectioning is through the center of the placenta and perpendicular to its flat surface. The major placental zones have a correspondence in human and mouse respectively: decidua basalis (db) in both; basal plate (bp) and junctional zone (jz); fetal placenta (villous tree) (fp) and labyrinth zone (l). Arrows depict the direction of maternal blood flow within the utero-placental circulation. avb, anchoring villous branch; avm, allantoic vasculature and mesenchyme; bp, basal plate; bpet, basal plate endovascular trophoblast; bpit, basal plate interstitial trophoblast; bpvc, basal plate venous channel; cc, cytotrophoblastic cell column; cma, central maternal artery; cp, chorionic plate; cpp, chorionic plate projection; db, decidua basalis; fp, fetal placenta; igc, invading glycogen trophoblast cells; ivs, intervillous space; jz, junctional zone; jzgc, junctional zone glycogen trophoblast cells; jzst, junctional zone spongiotrophoblasts; jzvc, junctional zone venous channel; l, labyrinth; m, myometrium; mbs, maternal blood sinus/spaces; msa, maternal spiral arteries; mv, maternal veins; pbit, placental bed interstitial invasive trophoblast; pbet, placental bed endovascular trophoblast; tgc, trophoblast giant cell; tgcz, trophoblast giant cell zone; tv, terminal villi; uc, umbilical cord; vt, villous tree; zi, zona intima. From Georgiades *et al*. (2002).

The decidua basalis is the zone in contact with the uterus and receive the same name in both species, although in human placentas the assembly of the decidua basalis and the underlying myometrium is known as placental bed (Georgiades *et al.*, 2002).

Between the murine decidua basalis and the junctional zone, is a zone of trophoblast giant cells that appear not to have an analogous zone in human placenta, although a parallel can be

made between the murine trophoblast giant cells and the earlier extravillous cytotrophoblast cells that invade the human decidua basalis (Georgiades *et al.*, 2002). The trophoblast giant cells, so named because of their unusually large size, related to the fact that they are extensively polyploid, mediate implantation and invasion into the uterus and at later stages produce several hormones and cytokines supporting both local and systemic physiological adaptations in the mother (Cross, 2005).

In the junctional zone of the mouse placenta, also known as spongiotrophoblast layer, there are two types of cells, the spongiotrophoblasts and the trophoblast glycogen cells. The function of the spongiotrophoblast layer is unknown, but some of the spongiotrophoblast cells can differentiate into giant cells and are somewhat analogous to the cytotrophoblastic cell columns that anchor the villi of the human placenta. The trophoblast glycogen cells appear within the spongiotrophoblast layer after gestational day (G) 12.5 and later they invade into the uterus in a diffuse interstitial pattern (Cross, 2005). These cells can secrete hormones, contain large amounts of glycogen and under histological observation have large vacuoles given the appearance of a clear cytoplasm (Georgiades *et al.*, 2002). In the basal plate, especially at the begin of gestation, there are also two types of cytotrophoblasts based on the degree of vacuolation and glycogen content and showing a morphological gradient, being the cells closest to decidua basalis more vacuolated and glycogen-rich (distal cells) and the remaining cells (proximal cells) have an eosinophilic cytoplasm and low amounts of glycogen, which allow several analogies between human and mouse (Georgiades *et al.*, 2002).

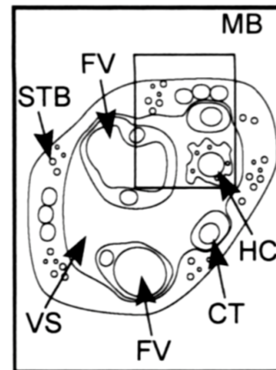
The labyrinth layer of the mouse is completely analogous in function to the chorionic villi (villous tree) of the human placenta and in both the villi are covered by syncytiotrophoblast that lie in direct contact with the maternal blood. These structures differ in terms of the ramification patterns. In human placentas they are of the villous type, maintaining a tree-like pattern with blunt-ended edges. On the contrary, the branches in the murine labyrinth are much more interconnected and generate a maze-like pattern. Consequently the human villi are well separated and the maternal blood space (the IVS) appears as a large open space, whereas in mice the “villi” are found anastomosed together originating tortuous channels in which the maternal blood flows (Cross, personal communication).

All trophoblast cells located outside the placental villi form the extravillous trophoblast (also known as extravillous cytotrophoblast or intermediate trophoblasts). In the basal plate the extravillous trophoblast form clusters of stem cells named cytotrophoblast cell columns, which connect the anchoring villi to the basal plate (Georgiades *et al.*, 2002; Kaufmann *et al.*, 2003).

The “placenta barrier” which constitutes the interface between maternal and fetal bloods, has few differences between both species. Both interfaces include a trophoblastic portion (lining the maternal blood spaces), a basement membrane and the fetal capillary endothelial cells that directly line the circulating fetal blood. The difference resides in the number of cell layers in the syncytiotrophoblast: in humans the trophoblast has a single syncytial layer (monochorial

placenta), whereas in mice it has three layers, namely two syncytial layers and a single mononuclear cell type (trichorial placenta). The unique continuous layer of syncytium in human placenta (Figure 11) is facing the maternal blood and has numerous microvilli to enhanced exchange.

Figure 11. Cross section representation of a terminal chorionic villus in human placenta. Fetal blood enters the placenta by the umbilical cord spiral arteries and arbors into branching chorionic villi that are immersed in maternal blood (MB). This cross section scheme shows the continuous layer of multinucleated syncytiotrophoblasts (STB), a few underlying cytotrophoblasts (CT), and the villous stroma (VS) with fetal vessels (FV) and the macrophage-like Hofbauer cells (HC). From Kristoffersen (2000).



In mice the trilaminar trophoblastic layer (Figure 12) consists of a first layer formed by mononuclear trophoblast cells (layer I), lining the maternal blood sinusoids, which does not have microvilli. The middle and third layers (layers II and III) are syncytiotrophoblastic that surround the fetal blood vessels endothelium.

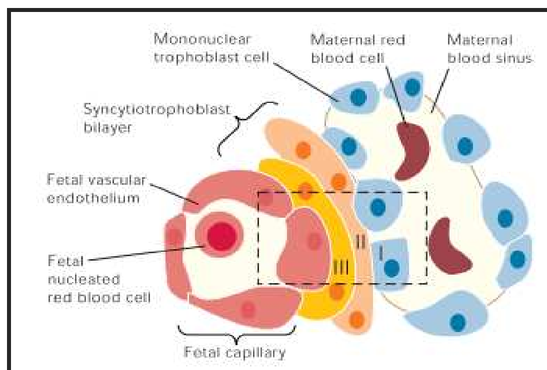


Figure 12. Schematic representation of the trilaminar layer of labyrinth trophoblast cells that separates the maternal and fetal circulations of the mouse placenta. From Watson & Cross (2005).

Placental development in mice and humans

At implantation, which occurs at G4.5 in mice and at G7-10 in humans, a precocious and intimate apposition between the maternal and fetal tissues is established. However, the establishment of a complete maternal circulation only occurs after this point. In fact, according to the effective maternal blood circulation in the placenta, the pregnancy can comprise two contrasting periods. In the first trimester of human pregnancy there is a little maternal blood flow in the placenta, the oxygen tension within the feto-placental unit is low, and the uterine glands may provide much of the nutrient supply (histiotrophic nutrition). At the start of the second trimester (around the 12th week of gestation) the maternal circulation within the intervillous spaces becomes fully established, the oxygen tension rises and the nutrients and gases are supplied by the maternal blood flow (haemotrophic nutrition) (Burton *et al.*, 2001). This stage, correspondent to the full establishment of utero-placental circulation, is seen by

G12.5 in mice. This suggests that the murine definitive placenta becomes functional at or soon after mid-gestation, which indicates that temporarily the end of the first trimester in the human gestation may be equivalent to the time around mid-gestation in the mouse (De Pee *et al.*, 2002; Georgiades *et al.*, 2002).

The change from histiotrophic to haemotrophic nutrition is an important event to take into consideration in the study of diseases such as malaria, whose infectious agent can reach the various tissues through blood circulation. Histiotrophic nutrition, and thus the absence of effective blood flow in the intervillous space during the first pregnancy period, may serve to protect the fetus from excessive high oxygen levels, reducing the damage mediated by free radicals during the sensitive period of organogenesis (Burton *et al.*, 2001). During this phase the metabolism relies on anaerobic glycolysis, with oxygen consumption increasing towards the end of this period. The uterine glands in the endometrium discharge a cocktail of growth factors and carbohydrate-rich secretions into the IVS, which are taken up by syncytiotrophoblast. Moreover, the yolk sac also plays a role in nourishing the developing fetus in the first pregnancy period, by accumulating nutrients and transporting them to the fetus (Burton et al., 2001). The second phase of the pregnancy, starting after the completion of organogenesis and when the definitive placenta becomes functional and fully irrigated by maternal blood, is dominated by fetal growth. The increasing fetal requirements of oxygen arising from its growth are now provided by haemotrophic nutrition. Table 1 summarizes the main characteristics of human and mouse placentation described above.

Table 1. Placentation features in human and mouse. Comparative structural and physiological aspects of the human and mouse placentas.

Human	Mouse
38 weeks of gestation	3 weeks of gestation
Histiotrophic nutrition in the first pregnancy period (3 months)	Histiotrophic nutrition in the first pregnancy period (12.5 days)
Haemotrophic support in the second phase (last 2 trimesters)	Haemotrophic support in the second phase (nearly last half)
Discoid placenta	Discoid placenta (per embryo)
Invasion of endometrium and myometrium during implantation	Invasion of endometrium during implantation
Monochorial placenta (single syncytiotrophoblast layer)	Trichorial placenta (three syncytiotrophoblast layers)
Haemochorial placenta (direct contact between maternal blood and chorionic trophoblast)	Haemochorial placenta (direct contact between maternal blood and chorionic trophoblast)
Chorionic villi with a tree-like pattern	Chorionic villi with a labyrinth or maze-like pattern

Mouse models in immunopathological PAM studies

To date the contribution of mouse models to the placental malaria immunopathogenesis knowledge has been negligible although the development and the exploitation of mouse models for PAM are likely to have a positive impact offering many tools to dissect the immunological and pathological components of pregnancy-associated malaria.

Although differences between mouse and human immunology do exist and have already been reviewed (Mestas & Hughes, 2004), mice constitute an important experimental tool for many immunological studies. Regarding mouse malaria, the course of infection depends on factors inherent both to the host and to the parasite species/clone but is usually acute and results in either death or self-cure. Parasitemia is usually more extreme than in humans and anemia develops rapidly and can be either of short term or lethal.

The first reported studies on mouse PAM were conducted by a Dutch group that used *Plasmodium berghei* K173 to infect outbred (Swiss) and inbred (C3H/StZ and B10LP) mice (van Zon & Eling, 1980a), followed by a chemotherapic treatment with sulfadiazine. They observed peripheral parasitemia recrudescence in about 46% of the pregnant females, but none of the non pregnant controls had recrudescent parasites. The authors noticed that recrudescence in second pregnancy depended on the presence of parasites in the first pregnancy. Therefore, the presence of parasites during pregnancy reinforced immunity, preventing recrudescence in a subsequent pregnancy. In a second report (van Zon & Eling, 1980b) these authors also observed a lower recrudescence rate in multigravida. Moreover, the authors verified that challenges of immunized mice before pregnancy did not reinforce immunity during pregnancy and the presence of parasites before G11 did not act as an antigenic signal, contrarily to the proliferating parasites after the second half of the pregnancy period (van Zon *et al.*, 1985). In these pregnancy-malaria mouse studies, immunosuppression was the most accepted hypothesis to explain the higher malaria vulnerability in pregnancy. Other reports by the same team showed that mice that had recrudescence during pregnancy had significantly higher plasma corticosterone levels and that immune pregnant females without adrenal gland had a reduction of recrudescence rate (van Zon *et al.*, 1982; Van Zon *et al.*, 1986).

Two research groups have used *P.berghei* NK65 in A/J and ICR mice strains (Oduola *et al.*, 1982) and in BALB/c mice (Hioki *et al.*, 1990). In the first report, pregnant females were infected at gestational days 7, 12 and 14 and this resulted in a more severe disease in pregnant females in comparison with non pregnant controls, placenta pathology and reduced birth weight of pups. The second research group observed the fate of pregnant females after infection at several gestational days. Mice infected at G12 or before died before the pregnancy reached term, whereas the other groups (G14 and 16) lived long enough to deliver their litters. All the pregnant infected mice died earlier than non-pregnant controls, which also confirm the higher disease susceptibility during pregnancy.

In other studies, lethal *P. yoelli* challenge of pre-immunized pregnant females, using either the virulent YM or the non-virulent 17X strains, did not cause maternal mortality and led to the delivery of healthy newborns (Pavia & Niederbuhl, 1991). *P. chabaudi* AS was used to evaluate the incidence of abortions/fetal loss, maternal parasitemia and anemia in C57Bl/6 pregnant females infected at conception day (Poovassery & Moore, 2006).

Those reports had the seminal role of revealing disease similarities between mouse and humans, such as the pregnancy-induced loss of pre-existing immunity to malaria and the presence of placental pathologic features. Nevertheless, these observations were not pursued and the PAM pathogenesis mechanisms were not investigated. The availability of sophisticated techniques of analysis, including molecular and genetic approaches, imaging tools, transgenic parasites and mice, and the current knowledge of parasite biology and host immune system, prompt the investigation of many pathological and immunological mechanisms underlying PAM and that remain to be elucidated.

Most of the understanding of the biological basis of PAM is coming from studies conducted with pregnant women living in malaria endemic areas. Consequently the disease mechanisms, including the immunological tools, have been exploited only based in human data and samples, which might constitute a limitation for the disease understanding. In addition, some important questions cannot be addressed due to ethical constraints. An easily manipulable mouse model for malaria in pregnancy could be a precious tool to investigate disease mechanisms that, complemented and validated with human data, would constitute a valuable contribution to resolve unanswered questions in PAM.

In the introduction the current state of knowledge of relevant aspects of PAM in humans was summarized, including currently proposed mechanisms contributing to adverse pregnancy outcomes. In this thesis we set out to establish and characterize immunopathological features of murine models of PAM and we conclude that these models can be useful to address specific questions relevant for human PAM.

AIMS AND PURPOSES

The aim of the work presented in this thesis was firstly to establish an experimental system enabling pregnancy-associated malaria (PAM) studies in mouse models and secondly to characterize the immunological and pathological features in murine pregnancy-associated malaria.

SPECIFIC PURPOSES:

1. To develop a PAM mouse model representing pregnant women with a low premunition status before pregnancy.

2. To establish a PAM mouse model that represents pregnant women carrying a high premunition status before pregnancy.

3. To validate both models by evaluating their capacity of reproducing the main characteristics of the human disease and their limitations.

   The objectives 1, 2 and 3 were the subject of the publications I (PAM Model for Low Premunition) and II (PAM Model for High Premunition). The scope of characterization and validation of the developed PAM models is represented in the following scheme:

<table>
<tr><th></th><th>Infection during Pregnancy Model</th><th>Pre-Exposure Model</th></tr>
<tr><td>Established Model</td><td>Women living in regions with low malaria transmission (low premunition)</td><td>Women living in a region with high malaria transmission (high premunition)</td></tr>
<tr><td rowspan="4">Analysis & Validation</td><td colspan="2">Maternal Disease Severity</td></tr>
<tr><td colspan="2">Pregnancy Outcome: Low Birth Weight / Intrauterine Growth Retardation</td></tr>
<tr><td colspan="2">Placenta Pathology: Parasite-Host Interaction / Adhesion</td></tr>
<tr><td colspan="2">PAM Immunopathology: Molecular and Cellular Components
PAM Immunological Protection: Multigravida Protection and Humoral Response.</td></tr>
</table>

METHODOLOGY

Mice and Parasites

BALB/c mice were bred and maintained in conventional housing and fed with a regular diet. All procedures were in accordance with national regulations on animal experimentation and welfare, authorized by the Instituto Gulbenkian de Ciência animal welfare committee. *P. berghei* ANKA parasites (*P. berghei* ANKA-GFP; 259Cl2 clone) used in infection experiments constitutively express green fluorescent protein (GFP) (Janse *et al.*, 2006a; Janse *et al.*, 2006b). Infected red blood cells (iRBC) were used in experimental infections at the concentration of 10^6 iRBC/animal, either from *in vivo* passage in BALB/c mice, obtained when the percentage of iRBC reached approximately 10%, or from frozen stocks. For the Model of Infection During Pregnancy, pregnant females were infected with iRBC from *in vivo* passage, whereas females of the Model of Pre-Exposure were infected with iRBC from frozen stocks. Animal infections were performed either by intraperitoneal (i.p.) or intravenous (i.v.) injections in accordance with the experiment characteristics. Parasitemia was measured in tail blood using flow cytometry analysis as described elsewhere (Janse & Van Vianen, 1994). Alternatively, parasitemia was evaluated in thin blood films methanol-fixed and Giemsa-stained.

Gestation timing and pregnancy monitoring

BALB/c female and male mice (2:1 or 3:1) were caged together for mating during two to three days and females examined for the presence of vaginal plug every morning. Detection of the vaginal plug and measurement of body weight were jointly used to determine the timing of pregnancy, as described elsewhere (Freyre et al., 2006). The day of finding of the vaginal plug was considered as gestation day one (G1) and pregnancy progression was monitored every other day by weighting the females. Since the presence of vaginal plug was not always followed by pregnancy and in some cases not detectable, successful fertilization was confirmed between G10 and G13 when the animals had an average body weight increase of 3-4 g. Females placed without male did not show a weight fluctuation of more than 1 g for a period of 20 days. Thus, weight gain was taken as sign of pregnancy and abrupt weight loss as indicator of pregnancy disturbance or interruption.

Pregnant females infection (Model of Infection During Pregnancy)

Pregnant mice were infected intravenously (i.v.) between G11 and G13 with 10^6 iRBC from *in vivo* passage in BALB/c mice and parasitemia was recorded every other day. This infection period was determined to be the optimal time point as earlier infections did not allow reaching pregnancy at term (data not shown). Non-pregnant infected females or non-infected pregnant females were used as controls in pregnancy infection experiments as appropriate. Part of the pregnant females (both infected and controls) were allowed to deliver and the progenies were

followed up to weaning. The other pregnant females were subjected to caesarian section at G17-19 for fetal survival evaluation and placenta pathology observation.

Offspring monitoring (Model of Infection During Pregnancy)

As *P. berghei* ANKA-GFP infection is lethal in BALB/c mice, foster mothers were used for newborn post-natal follow-up studies. Hence, both newborns from infected mothers and newborns from control mothers were also transferred to foster mothers to avoid weight bias due to differential maternal nourishment. The newborns were weighted every other day.

Chemotherapic treatment for immunization (Model of Pre-Exposure)

BALB/c females were infected i.p. with 10^6 iRBC obtained from frozen stocks and treated IP with 0.7 mg chloroquine/animal/day for 3 days. Typically the treatment started at day 7 post-infection when parasitemia reaches values of 5 - 10%. Five to ten percent of the female mice exposed to this immunization protocol succumbed but the remaining recovered from the infection and were used in subsequent pregnancy-induced recrudescence experiments.

Pregnancy-induced recrudescence monitoring (Model of Pre-Exposure)

Forty days post-infection, or thereafter, pre-exposed females were put to mate or used as non-pregnant controls. Pregnancy monitoring was performed as described above. Some of the pregnant females were subjected to caesarian section at G19 for placenta pathology studies, while the others were allowed to deliver and to follow to subsequent pregnancies. At delivery, the weight and the number of live newborns were registered. Newborns weight and development was followed up to day 30 after birth. Non-infected pregnant females were used as controls.

Fetal survival evaluation

Females used for *in uterus* pregnancy outcome evaluations were killed by CO_2 narcosis between G17 and G19, the spleens weighted, uterus examined and the number of fetuses and resorptions recorded. Resorptions were identified as small implants with no discernible fetus and placenta, corresponding to embryos that died before complete placenta vascularization. The fetuses were extracted from their amniotic envelop and viability was immediately evaluated by prompted movement reaction to touching with pliers. The lack of reactive movement indicated that the fetus had recently died and was considered an abortion. Macerated pale white fetuses were dead and recorded as abortions. Fetuses and placentas were separately weighted. Non-aborted fetuses were killed combining CO_2 narcosis and hypothermia.

Tissue preparation and histopathological analysis

Placentas from infected and non-infected females were treated in a similar way. Placentas were separated in two halves, one half was fixed either in 10% formalin or, in case they would

follow to immunohistochemistry, in 1.6% paraformaldehyde with 20% sucrose, for further processing and the other half was collected for RNA extraction. Paraffin-embedded non-consecutive placenta sections were stained with hematoxylin-eosin (HE) and examined under a light microscope (Leica DM LB2, Leica Microsystems). For histological and morphometric analysis, placental sections were examined in a blind fashion.

Immunohistochemistry

Fixed placenta samples were washed in PBS with 15% sucrose overnight, soaked in Tissue-Tek® (Sakura) and frozen in dry ice. For immunohistochemistry staining, freshly made frozen sections (6 µm thick) were rinsed in PBS for 30 minutes and blocked with 1% bovine serum albumin (BSA). To enhance parasite GFP signal, we used rabbit polyclonal anti-GFP antibody conjugated with Alexa488 (Molecular Probes). To identify macrophages/monocytes we used anti-CD11b biotinilated antibodies (BD Biosciences, Pharmingen), followed by incubation with Rhodamin-Avidin D (Vector Laboratories). Nuclei were stained with DAPI (Invitrogen) and coverslips were mounted with aqueous mounting media (Mowiol 4-88, Calbiochem). Stained sections were examined under fluorescence microscopy (Leica DMRA2, Leica Microsystems).

Morphometric analysis

HE stained placental sections were analyzed for vascular space quantification. In each section, 5 randomly selected microscopic fields in the labyrinthine region (magnification x40) were acquired at 1280 x 960 resolution, using a color video camera (Evolution ™ MP color, Media Cybernetics) connected to a light microscope (Leica DM LB2, Leica Microsystems). The images were analyzed by a routine implemented in the ImageJ software (ImageJ 1.37v, National Institutes of Health). Briefly, after acquisition, the images underwent an automated light analysis procedure where noise removal was applied to ensure color and image quality standardization across sections and specimens. The images were given a color threshold to cover the area corresponding to blood spaces lumen. The coverage percentage was calculated as the ratio between the number of pixels covered by the area defined by the threshold and the overall number of pixels in the image. The blood vascular area in each placenta was estimated from the analysis of two non-consecutive sections. The reported results correspond to individual pregnant females and represent the average result for 2-3 placentas.

Gene Expression

Total RNA, from individual placentas and viable newborns, was obtained using an RNeasy Mini Kit (Qiagen), following the manufacturer's protocol for animal tissues. One microgram of total RNA was converted to cDNA (Transcriptor First Strand cDNA Synthesis Kit, Roche) using random hexamer primers. MCP-1 (*Ccl2*) and MIP-1α (*Ccl3*) expression was quantified using TaqMan Gene Expression Assays from ABI (Mm00441242_m1 and Mm00441258_m1, respectively) with TaqMan Universal PCR master mix. T lymphocytes (*Cd3e*), natural killer cells (*Klrd1*), macrophages (*Mgl2*), neutrophils (*Ncf2*), cytokines and hemoxygenase-1 (*Hmox-1*)

expression was amplified using primer sequences previously described (Epiphanio *et al.*, 2008). Endothelin-1 (*Edn1*) and β-actin (*Actb*) specific primer sequences were, *Edn1* -5'-ACG CAC AAC CGA GCA CAT TGA CTA C-3'and 5' TCC TGC CCG TCT GAA CAA GAA ACT G-3' and *Actb* - 5' AGC CAT GTA CGT AGC CAT CC-3' and 5'-CTC TCA GCT GTG GTG GTG AA-3'. These qRT-PCR reactions used Applied Biosystems Power SYBR Green PCR Master Mix. The gene expression quantification reactions were performed according to the manufacturers' instructions on an ABI Prism 7900HT system. Relative quantification of specific mRNA was normalized for a mouse housekeeping gene mRNA. To select an appropriate internal control, the expression of the following housekeeping genes: ACTB, GAPDH, TATA box binding protein (TBP), Succcinate dehydrogenase complex, subunit A (SDHA) and Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) was studied. The last three genes have been previously validated in human placental malaria (Boeuf *et al.*, 2008). Due to uneven gene expression, when comparing non-infected and infected placentas, the housekeeping genes TBP, SDHA and YWHAZ were unsuitable for internal controls. Conversely, ACTB and GAPDH expression was maintained under infection conditions.

Synchronization of parasitized erythrocytes

iRBC were collected from infected animals (non-pregnant and pregnant females with recrudescence) with 10-20% parasitemia, by cardiac puncture, suspended in RPMI medium containing 25% of foetal bovine serum (FBS). In order to obtain mature blood stage parasite forms (trophozoites / schizonts), *P. berghei* ANKA-GFP infected erythrocytes were synchronized as described elsewhere (Janse *et al.*, 2006c). Briefly, parasites were maintained *in vitro* at 37^0C for one developmental cycle, which takes about 16 hours. During this period the ring forms and young trophozoites develop into schizonts containing mature merozoites. The schizonts-infected erythrocytes are separated from the uninfected RBC by a Nicodenz-density gradient centrifugation (65% (v/v) Nicodenz/PBS solution), resulting in cell-populations that yield over 90% infected erythrocytes. After mature forms enrichment, infected erythrocytes were suspended in PBS at a concentration of 10^8 iRBC/ml.

iRBC binding assays in placental sections

Placentas from uninfected BALB/c females, obtained at G19, were treated using a previously described protocol (Muthusamy *et al.*, 2004). Briefly, the placentas were fixed in 2% formalin and 0.5% glutaraldehyde for 10 minutes, heated in a microwave oven before being paraffin-embedded, and cut into sections of 5 µm onto glass slides. This fixation protocol aims to preserve the binding capacity of glycosylaminoglycans (GAG) in the placenta IVS (Muthusamy *et al.*, 2004). Tissue sections on the glass slides, after deparaffinized and rehydrated, were delimitated with a DAKO pen. For placenta-receptor cleavage experiments, placental sections were incubated with 0.5 U/ml chondroitinase ABC (from *Proteus vulgaris*, Sigma), with 30 µg/ml hyaluronidase (from bovine tests, Sigma), with heparinase II (from *Flavobacterium heparinum*, Sigma) or with PBS for 2 periods of 2 hours at 37^oC. Both enzyme-treated sections

and non-treated sections were blocked with 1% BSA in PBS at room temperature for 30 minutes. Fifty microliters of synchronized iRBC suspension, at the concentration of 10^8/ ml, were overlaid onto each tissue section for 60 minutes at 37^0C in a humid chamber. After washing the unbound cells, the placental sections were incubated with DAPI. For iRBC-ligand blocking experiments, synchronized iRBC were pre-incubated with the indicated concentrations of chondroitin sulfate A (CSA) from bovine trachea (Sigma), hyaluronic acid (HA) potassium salt from human umbilical cord (Sigma) or colominic acid sodium salt (as negative control) from *E. coli* (Sigma), at 37^0C for 30 minutes with moderate agitation. Of note is that according to Sigma certificate of analysis, HA has less than 5% contamination of chondroitin sulphate. For iRBC-ligand cleavage assays iRBC were treated with trypsin (Gibco), proteinase K (Sigma) or neuraminidase as a negative control (from *Clostridium perfringens*, Sigma). iRBC were pre-incubated with each enzyme at indicated concentrations for 30 minutes at 37^0C. After washing, iRBC were overlaid on placental sections as described above. The slides were mounted with Mowiol and examined under fluorescence microscopy (magnification x40). The number of iRBC adhering placental sections in each experimental condition was determined in a blind fashion, counting 50 fields in each of three independent experiments.

Hemoglobin determination

This procedure is based on the oxidation of hemoglobin to methemoglobin in the presence of alkaline potassium ferricyanide. Methemoglobin reacts with potassium cyanide to form cyanmethemoglobin, which has maximum absorption at 540 nm. The color intensity, measured at 540 nm, is proportional to the total hemoglobin concentration and was quantified by visible spectrophometry using the Drabkin method (Drabkin, 1949; Singh & Shinton, 1965). Briefly, two microliters of tail blood were collected in 500 µl of Drabkin's Reagent and absorbance measured at 540 nm.

P. berghei antigens preparation

Recrudescent parasites were expanded in non-pregnant females following *in vitro* culture for parasite synchronization and parasite mature forms enrichment (about 95% of late stages). A crude preparation of blood stage *P. berghei* components was obtained from the mature forms that were freeze-thawed six times, sonicated and ultra-centrifuged. The protein was quantified and aliquots stored in liquid nitrogen.

ELISA

For the determination of parasite - specific antibodies, 96 well plates (NUNC MaxiSorp) were coated with *P. berghei*-iRBC proteins extract (50 µl/well at the concentration of 5 µg protein/ml) and incubated overnight (ON) at 4ºC. The unbound antigen was removed by washing with 0.05% Tween-20 in PBS (PBST). Possible residual free sites were saturated by treatment with PBS 1% BSA for 1 h at RT and the plates washed five times with PBST. Fifty µl of serum serially diluted samples (diluted from $1:2^7$= 1:128 to $1:2^{14}$= 1:16384) were incubated for 3 h at 37ºC. The plates were washed five times with PBST and developed using either

antibodies AP-conjugated diluted 1:1500 (for the classes IgG, IgM or IgA) (Southern Biotech) or antibodies horseradish peroxidase (HRP)-conjugated diluted 1:4000 (for the IgG isotypes IgG1, IgG2a or IgG3) (Southern Biotech) and incubated for 1.5 h at 37°C. Plates were revealed to detect bound immunocomplexes by adding, to each group, PA buffer/PNPP or TMB buffer (BD), respectively. When PA conjugated was used the absorbance was read at 405 nm wavelength filter (Bio-Rad plate reader) after 5-15 minutes of development. When using HRP conjugated, the enzymatic reaction, developed for 10 minutes, was blocked with sulfuric acid (0.1 M, 50 µl per well) and the OD was read at 450 nm. The anti-*P. berghei* antibody titers were expressed as log2 of the reciprocal serum dilution giving an absorbance value of 30% of the saturation level, as previously described (de Moraes *et al.*, 2006).

Visualization of luciferase activity in whole body and dissected organs

A pregnant female was infected with a *Plasmodium berghei* line expressing a GFP-Luciferase (676m1cl1 clone) fusion protein, under the control of the eef1a-promoter, at G13 and at G18 the parasites accumulation was observed through the determination of luciferase activity previously described (Franke-Fayard et al., 2005). Briefly, luciferase activity was visualized through whole-body imaging or dissected organs with an intensified-charge-coupled device (I-CCD) video camera of the in vivo Imaging System (IVIS 100, Xenogen). The pregnant mouse was injected i.p. with d-luciferin dissolved in PBS (100 mg/kg of body weight; Synchem, Kassel, Germany). After 10 minutes the female was killed by CO_2 narcosis and bioluminescence imaging was acquired with a 15-cm FOV, a medium binning factor and exposure times of 10–60 s. Individual organs and fetuses were obtained by dissection and placed in a Petri dish and imaged with a 10-cm FOV, a medium binning factor, and exposure times of 10–60 s.

Cytokines quantification in serum

IL-4, IL-6 and IL-10 levels were determined by Cytometric Bead Array (CBA) (Becton Dickinson Biosciences, San Diego, CA, USA) assay, according to manufacturer’s recommendations. Briefly, 50 µl of the mixed capture beads were added to 50 µl plasma diluted 1:4 of each animal from the different groups in their respective tubes and mixed. After 1 hour of room temperature (RT) incubation, 50 µl mouse-phycoerythrin (PE) (Positive Control Detector) were added, followed by another incubation of 1 hour at RT. Standards were ran simultaneously for each cytokine, and were mixed with capture beads and detection reagent conjugated with PE. Samples were incubated for 1 h at RT once they had been mixed and protected from exposure to light. The samples were washed and centrifuged at 200 × *g* for 5 min to remove unbound detection antibody and then analyzed by flow cytometry (FACScan, BD). Cytokine concentration (pg/ml) was calculated by a standards regression curve.

Statistical analysis

Statistical differences between groups of mice used in this study were evaluated by the Student’s t test, Mann-Whitney test or Kruskal-Wallis, and Log Rank test for survival curves. Chi-square or Pearson tests were used for association or correlation analysis, respectively.

RESULTS

EXPERIMENTAL SYSTEMS

This work aimed to establish mouse models for Pregnancy-Associated Malaria studies taking in consideration that different mouse strains are differentially susceptible to malaria and different parasite species/strains can lead to different disease manifestations in the same mouse strain. In this section we describe the preliminary experiments that were performed in order to choose suitable experimental systems that would allow investigating PAM development and pathological outcomes.

Parasite species and mouse strain selection

Several preliminary tests were performed, using *Plasmodium berghei* ANKA, *P. yoelli* 17XL and *P. chabaudi chabaudi*, and the C57Bl/6, BALB/c and DBA-2 mouse strains. When pregnant females were infected during pregnancy the three parasite species caused pregnancy disturbances in all tested mouse strains, both in the mother and in the offspring. However, when females were pre-exposed to each parasite species, only *P. berghei* ANKA parasites relapsed during pregnancy. Thus, *P. berghei* ANKA was the selected parasite to pursue our model set-up. It is worth to remark that this choice would take advantage of a *P. berghei* transgenic line expressing the green fluorescent protein (GFP).

P. berghei ANKA caused severe malaria syndromes causing high lethality soon after infection in all the tested mouse strains except in BALB/c mice that showed progression to hyperparasitemia and survived for longer periods (20 days in average). As the other strains could not survive long enough to evaluate the typical manifestations of malaria in pregnancy, the BALB/c strain was chosen as the host model.

Infection protocol establishment

Several preliminary pre-exposure protocols were tested aiming to avoid anti-parasite chemotherapy, namely the use of irradiated parasites and RBC transfers.

Blood stage parasites were irradiated in order to abolish their infective capacity while maintaining their antigenicity. In order to identify an irradiation dose suitable to trigger protective immunity, iRBC were irradiated with 0, 12, 15, 18, 20 and 23 krad and injected in six groups of animals. The results show that parasites irradiated with 12 rad kept their viability and lethality while parasites irradiated with 15 rad or higher did not have parasitemic infection but did not induce protection against a challenging infection. Thus, we did not find an iRBC irradiation regimen that would provide the needed protection to infection in non-pregnant females (Figure 13).

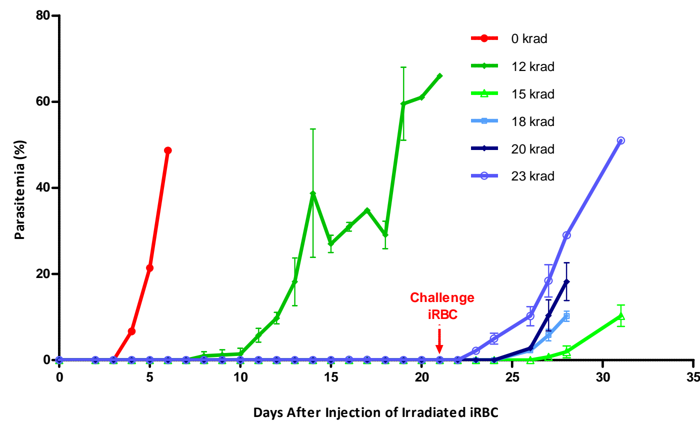


Figure 13. Irradiated *P. berghei*-iRBC were not inducers of a protective status. Blood stage parasites were irradiated according to the irradiation doses indicated (0, 12, 15, 18, 20 and 23 krad) and 5 x 10^6 iRBC were injected i.v. in C57Bl/6 mice at day 0 (5 animals). The mousethat received non-irradiated parasites (0 krad) died at day 8. The parasitemia of the group injected with 12 krad irradiated - iRBC was delayed by day 21 all the animals were dead. The four remaining animal groups, corresponding to higher doses of irradiation, did not revealed parasitemia and were challenged with non-irradiated iRBC at day 21. The parasitemia curves show a dosis dependent-kinetics, but all animals succumbed. Each point represents mean ± s.e.m.

Next, we tried to prolong the course of infection expecting to obtain a protective immune response. We replaced the erythrocytes that were destroyed by the infection through repeated blood transfusions from non-infected adult females. Several experiments were performed with slight modifications in the protocol, but all of them failed to confer protection. In one of these experiments, thirty-one BALB/c females were infected with 10^5 infected red blood cells (iRBC) intraperitonealy (i.p.). Seven days after infection each infected female started to receive 100 µl of non-infected blood by intravenous injection (i.v.), and this treatment was repeated within an interval of two to four days (Figure 14).

This transfusion regimen reduced parasitemia almost collectively, but the mice were never completely cured and the initial weight was never recovered. The mice start dying on day 14 post-infection and at day 60 post-infection all the mice succumbed. To discard any detrimental effect caused by blood transfusion, five non-infected females were also treated on the same way and no weight fluctuation was observed (Figure 2, lower graph, black line). This attempt did not result in a protective response implying that prolonging the blood stage of infection is not enough to generate an immune response that resolves the infection and confers ulterior protection.

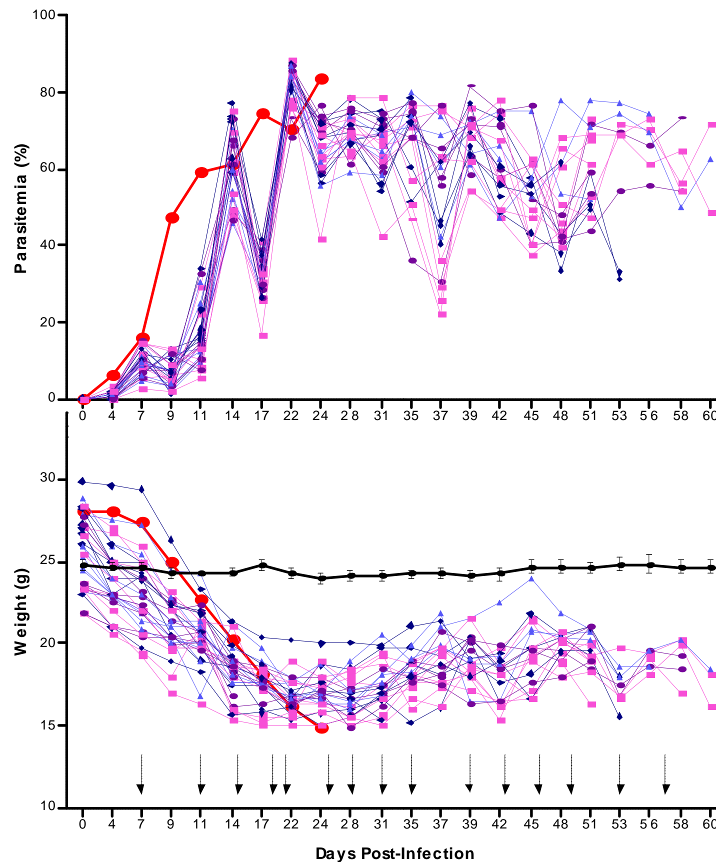


Figure 14. RBC replacement failed to control infection. Blood transfusion treatment of BALB/c *P. berghei* infected females. Parasitemia (upper plot) and weight (lower plot) of thirty one females that were infected i.p. at day 0 with 10^5 *P. berghei* infected red blood cells (iRBC) and treated with exogenous blood in the indicated days (arrows in the lower plot). In each blood transfusion treatment every infected female received 100 µl of non-infected blood, starting seven days after infection, and repeated with an interval between two and four days. By day 60 all the treated females were dead. Red line represents a female infected and not treated. Black line (lower graph) corresponds to the weight (mean ± s.e.m) of five non-infected BALB/c females that also received blood transfusions.

Finally, the pre-exposure protocol chosen was based in treating chemotherapeutically infected females to promote their premunition. Chemotherapic treatments have been used with the purpose of generating malaria protected mice (Poels *et al.*, 1977). Since *P. berghei* ANKA GFP is not sensitive to drugs such as pyrimethamine (Franke-Fayard *et al.*, 2004), we used chloroquine to perform the immunization protocol. Females immunized under this protocol, as described in Methodology section (Figure 15), were the players of the pre-exposure model. Thus, the immunization was attained using chloroquine therapy that interferes with the parasite metabolism inhibiting the enzymatic reaction of hemozoin synthesis. Briefly, the malaria parasite takes up hemoglobin from the host RBC and degrades it to heme (toxic for the parasite) and aminoacids. A parasite enzyme – heme polymerase – converts the potentially harmful heme into hemozoin (non-toxic storage form) in a reaction that can be blocked by chloroquine (Hunt & Stocker, 2007).

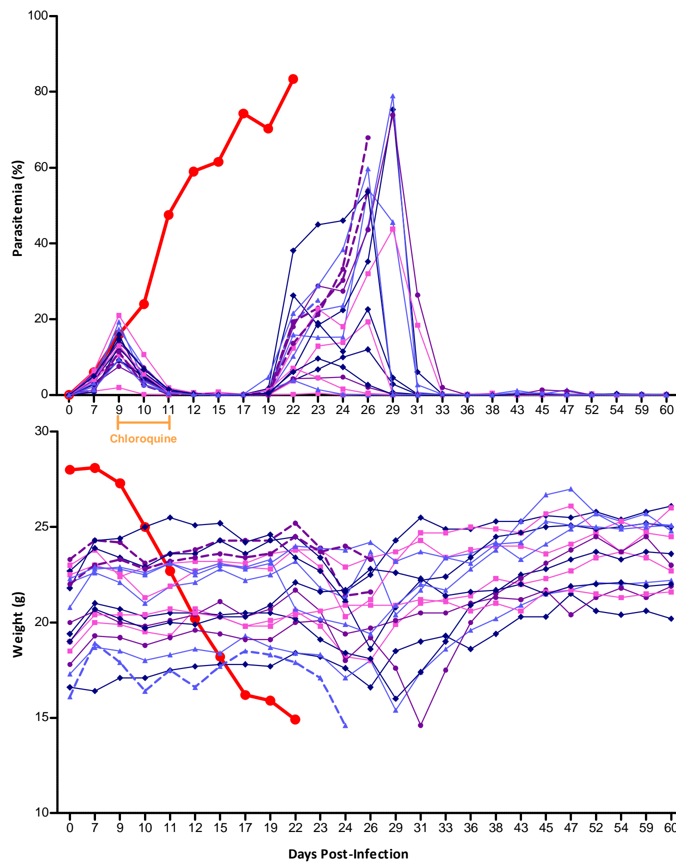


Figure 15. Subpatent infection is acquired with chemotherapy. Parasitemia and weight follow-up of eighteen females infected with 10^6 *P. berghei*-iRBC at day 0 and treated with chloroquine at days 9, 10 and 11. Typically the parasitemia is controlled after the treatment, raised around day 20 and reaches a second peak, which the mice likely self-clean, and thereafter the parasitemia will never come up again. In this experiment three females did not resist to the second parasitemia peak (represented by dashed lines) and died. Red line corresponds to a female infected and not treated.

The data plotted in Figure 15 correspond to one of the several pre-exposure experiments that were performed, but in all of them the patterns of parasitemia and weight were very similar. Usually about 5-10% of the treated females died during the second peak but never before, suggesting they were not able to mount an immune response capable of controlling the infection. Females that survive after controlling the second peak did not experienced parasitemia relapses, unless they became pregnant. Thus, following day 40 after infection the females acquired a silent infection and had already recovered their physical condition, as judged by the weight recovery, and thus were prepared to be used in the pre-exposure PAM model.

MODEL OF INFECTION DURING PREGNANCY

This mouse model aims to represent acute malaria in pregnancy, which enables experimental evaluation of human PAM when women have no acquired immunity, a characteristic of regions with unstable malaria transmission.

To set up an experimental model that recapitulates the typical pathology features of severe malaria in pregnancy, we took in consideration that poor pregnancy outcomes and fetal growth impairments are critically dependent of the gestational day (G) chosen for infection. In fact, infection at early stages led to premature pregnancy interruption, while infection around mid-pregnancy (G13) when blood flow has already full access to the placenta (Figure 16), allowed pregnancy to proceed to later stages, and fetal and placenta pathology became apparent resembling human PAM.

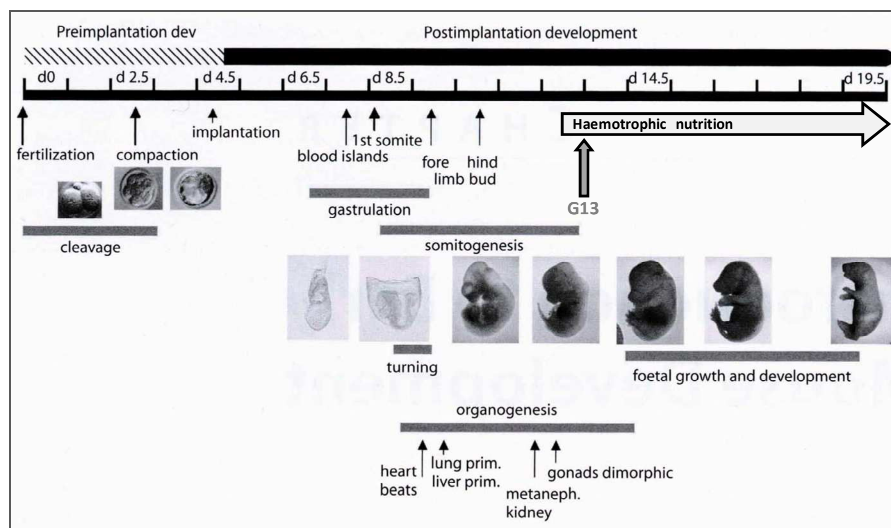


Figure 16. Schematic representation of mouse development *in uterus*. The pre- and post-implantation phases are shown above the time line. Bellow, the critical events and processes are indicated. After G12.5 the placenta is already functional and the maternal circulation in the placenta is fully established. Adapted from Kispert & Gossler (2004).

Effects of malaria infection on the pregnant females

Comparison of *P. berghei*-GFP course of infection in pregnant and non-pregnant females confirmed earlier findings that pregnancy in mice confers an increased susceptibility to malaria showing that pregnant mice experienced faster increase in parasitemia as compared to non-pregnant females (Oduola *et al.*, 1982; Hioki *et al.*, 1990; Pathak *et al.*, 1990). Parasitemia in pregnant mice was 55.41 ± 5.44 % (mean ± SE) on day 7 post-infection as compared to 33.83 ± 3.47 % in non-pregnant mice (*P*-value = 0.007) (Figure 17A). In addition, survival to infection was reduced in pregnant mice, with all deaths occurring between day 5 and day 10 post-infection (Figure 17B). In contrast, the majority of non-pregnant infected females survived until day 20 post-infection and by day 30 all had succumbed to infection (data not shown). Average survival time for pregnant and non-pregnant infected mice was 7.5 and 20.5 days, respectively.

These results suggest that, similarly to humans, pregnant mice show increased susceptibility to malaria infection which may affect their progeny or compromise pregnancy.

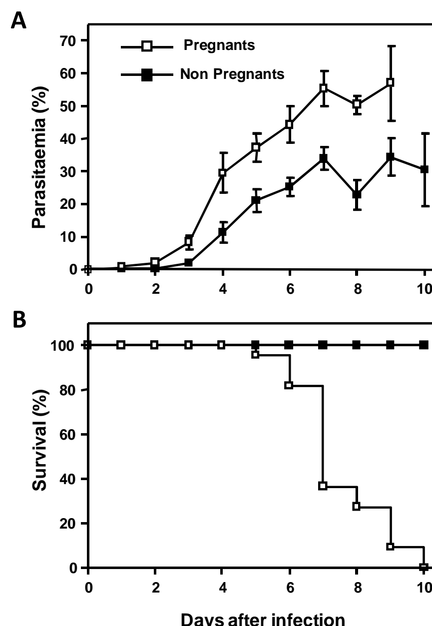


Figure 17. Increased disease susceptibility in pregnant BALB/c mice infected with *P. berghei*-GFP. BALB/c pregnant females were infected on G13 by IV injection of 10^6 iRBC and non-pregnant females were simultaneously infected. The plots represent cumulative results of three independent experiments in a total of 32 pregnant and 16 non-pregnant females. (A) Parasitemia curves where data points represent mean ± s.e.m. From day 3 post-infection onwards parasitemia was significantly higher in pregnant females (*P*-value < 0.05). (B) Survival curves up to 10 days after infection show that survival time of pregnant female mice are significantly lower than in controls (*P*–value < 0.0001). Non-pregnant females died at a later stage with hyperparasitemia.

Effects of maternal malaria on the progeny

- **Unsuccessful pregnancy and impaired post-natal growth**

We followed-up the pregnancy outcome in 22 infected females and found out that malaria had a strong negative effect in pregnancy success (Table 2). Approximately two-thirds of infected pregnant females (14 out of 22) did not give rise to viable pups due to maternal death before parturition (8 cases) or to preterm delivery/abortions (6 cases). The remaining mothers carried out pregnancy to term giving rise to 27 viable newborns. The progeny of 2 infected mothers, out of 8 that gave birth, died after birth between day 2 and day 21, indicating that malaria during pregnancy increases newborns mortality.

Table 2. Effect of *Plasmodium berghei* infection during pregnancy on reproductive outcome and fetus development [(a)]

P. berghei exposure	No. of Pregnant females	Gestational period (days)[(b)]	Birth weight (g)[(b)]	Weight day10 (g)[(b)]	No. Successful fetus[(b)]	No. Unsuccessful pregnancies[(c)]
Infected	22	19.8	1.3	3.4	5	14(8/6)
Uninfected	14	20.7	1.4	5.6	6	0
p- value[(d)]	__	0.05	0.03	< 0.0001	0.39	__

[(a)] BALB/c mothers were infected on G13 with *P.berghei* by IV injection of 10^6 iRBC and were allowed to give birth at term.
[(b)] Average values.
[(c)] Number of unsuccessful pregnancies (mother dead pregnant / preterm delivery or abortion).
[(d)] Student's *t* test.

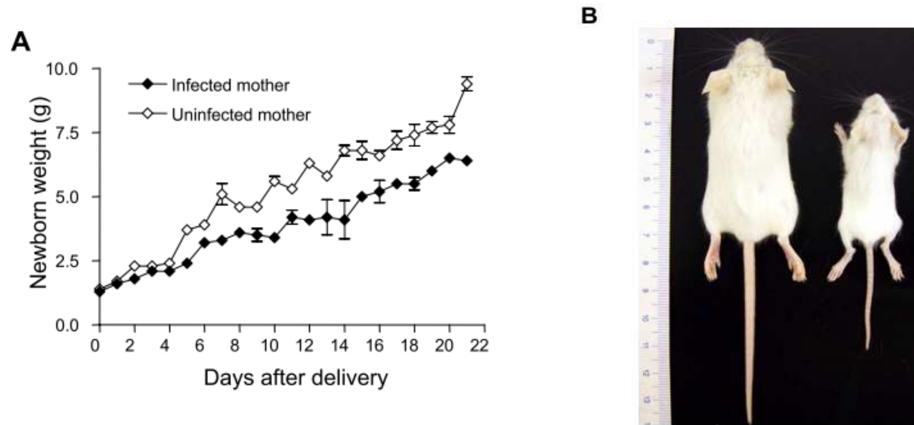


Figure 18. Reduced growth rate in progenies of *P. berghei*-GFP infected mothers. BALB/c pregnant females were infected on G13 by IV injection of 10^6 iRBC. After delivery newborns were transferred to a foster mother and their body weight was followed up to weaning (A). Example of body size difference at day 21 of age is shown in (B), mouse born from non-infected (left side) and from infected mother (right side).

- **Fetal survival and intrauterine growth retardation**

We evaluated the effects of malaria in pregnancy on fetal survival and fetal growth at late pregnancy stages (G18) by analyzing fetuses from 28 pregnant females infected at G13 and from 9 non-infected pregnant females (Table 3). Uterus collected at G18 from infected pregnancies frequently showed macroscopic abnormalities, as compared to controls, corresponding to the presence of aborted fetuses (Figure 19A). In fact, infected mothers had significantly lower number of viable fetuses as compared to non-infected mothers ($p = 0.01$) and had higher number of aborted fetuses ($p = 0.002$) (Table 3).

Figure 19. *P. berghei*-GFP infection impairs pregnancy outcome and fetus development. (A) Representative uterus at G18 from BALB/c pregnant females uninfected (upper) and infected on G13 with *P. berghei*-GFP by IV injection of 10^6 iRBC (bottom). The arrowheads indicate abortions. (B) Fetus from uninfected (left) and from infected mother (right). In detail, mouse placenta from an uninfected (C) and infected mother (D). Lack of blood circulation is noticeable in the placenta, paws and tail in panel (D). Scale bar represents 1 cm in A-B and 0.5 cm in C-D.

We searched for intrauterine signs of fetal impaired development. Fetuses from uninfected healthy mothers showed pink coloration, had translucent skin with visible blood flow in the blood vessels and the placentas were replenished with blood (Figure 19B left and 19C). In contrast, many fetuses from infected mothers appeared abnormal having remarkable reduced

size, pale tone with poor blood vessel replenishment and placentas with reduced blood content (Figure 19B right and 19D). It is worth to remark (Table 3) that average weight of viable fetus at G18 was significantly lower in infected mothers (0.55 ± 0.034 g) as compared to non-infected mothers (0.9 ± 0.053 g). Together, these data strongly suggest that fetuses from infected mothers suffer IUGR and have decreased viability due to placenta blood flow impairment, recapitulating pathological features of severe malaria manifestations typically observed in pregnant women from low malaria transmission regions.

Table 3. Pregnancy outcome obtained at caesarean section on G18 after *Plasmodium berghei* infection during pregnancy [(a)]

***P. berghei*-GFP exposure**	**No. of pregnant females[(b)]**	**Mother's spleen weight (mg)[(c)]**	**Fetus weight (g)[(c)]**	**No. Abortions[(c)]**	**No. Resorptions[(c)]**	**No. Successful fetus[(c)]**
Infected	28	521	0.6	2.4	1.7	3.7
Uninfected	9	102	1.0	0.2	0.7	7.7
p-value[(d)]	__	0.02	< 0.0001	0.002	0.11	0.01

[(a)] BALB/c mothers were infected G13 with *P.berghei* by IV injection of 10^6 iRBC.
[(b)] Pregnant females sacrificed at G18.
[(c)] Average values.
[(d)] Student's *t* test.

Placental pathology

- **Placenta is a site of parasite accumulation**

It has been described that *P. falciparum* iRBC have the capacity of being sequestered in several organs, including the placenta (Brabin *et al.*, 2004b). Parasite-placenta interaction studies in humans are based on *ex-vivo* assays (placental tissues) and on *in vitro* assays (cell cultures and immobilized candidate receptors). To date *in vivo* placental sequestration studies have not been performed in animal models and there is no direct evidence of *P. berghei*-iRBC dynamics inside pregnant hosts. Infection of BALB/c pregnant females at G13 with *P. berghei* luciferase-GFP allowed the subsequent visualization of the parasite distribution at G18, confirming that the placentas are predilection sites of *P. berghei* parasites accumulation (Figure 20), similarly to organs like lungs and spleen. The observed parasite accumulation strongly suggests that placentas are indeed a parasite preferential target. An intriguing observation was that parasites accumulated asymmetrically within the uterus.

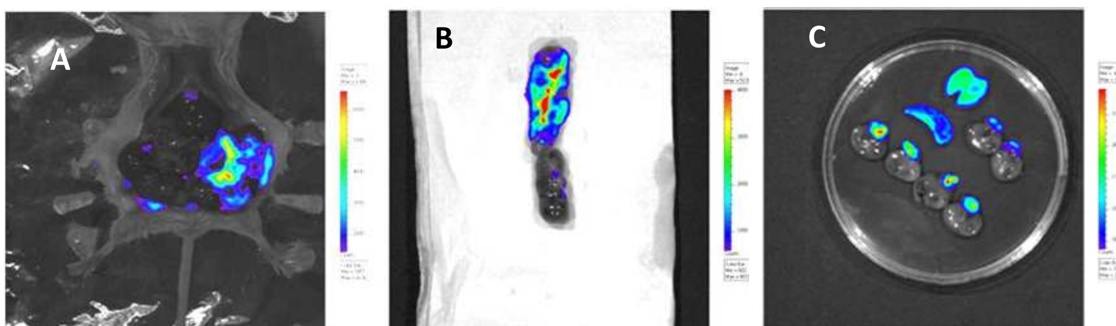


Figure 20. Placentas are target destination of *P. berghei* parasites. One pregnant female was infected with 10^6 *P. berghei* GFP-luciferase-iRBC at G13 and parasites accumulation was revealed by measuring luciferase activity in the whole pregnant female at G18 and in dissected organs. Rainbow scale of the total photon counts show the relative level of luciferase activity, ranging from low (violet, blue) to high (red). (A) Whole body acquisition, where parasite groups can be visualized in several points distributed on the right side (placentas, fat, lungs) and a strong parasite accumulation in the left side. This corresponds to the zone of the spleen and to the uterus left horn, as it is illustrated in (B) where it is visualized the asymmetric parasite distribution in the uterus. In (C) dissected organs: lungs (top), spleen (bellow) and two groups of fetus with the respective placentas. The two placentas of the fetuses from the rifgt horn of the uterus show a lower luciferase activity, whereas the four placentas from the left horn of the uterus have a higher parasite accumulation.

- **Placental pathology and inflammation**

Placenta represents the interface between mother and fetus, playing a critical role in fetal growth and development and thus any modification on its structure or function can have consequences for the pregnancy outcome. Placental tissue of infected pregnant females revealed a number of abnormalities in comparison to non-infected controls (Figure 21).

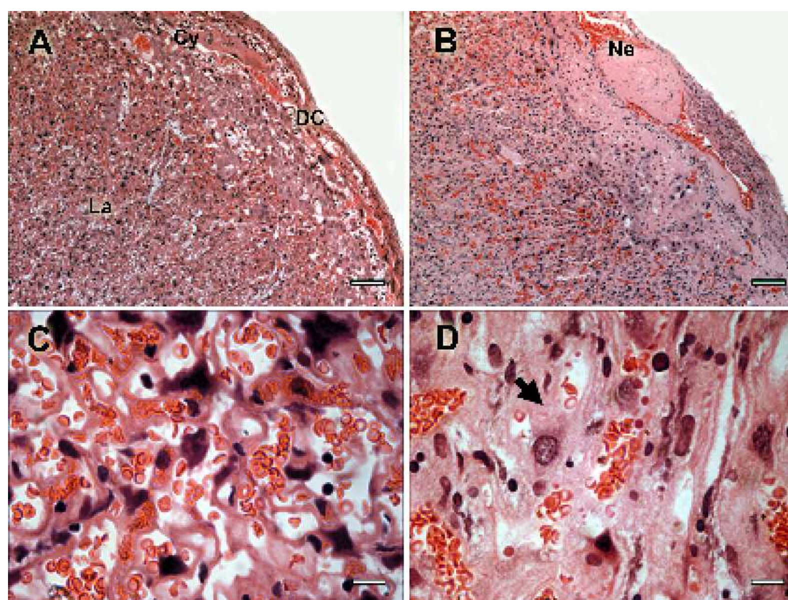


Figure 21. Placenta pathology in infected pregnant mice. Histology of infected placentas collected at G18. HE stained sections from non-infected mice (panels A and C) and infected (panels B and D) are depicted. Different cell types are identified in panel A as (DC) decidual cells, (Cy) cytotrophoblastic cells and (La) labyrinth region. Fibrinoid necrosis areas (Ne) are indicated in panel B. Arrowhead in D shows tissue thickening. Scale bar represents 100 µm in (A-B), and 10 µm in (C-D).

We repeatedly observed significant thickening and disorganization in the labyrinthine zone, distension and disarrangements of perivascular space (Figure 21D), as well as presence of

parasitized red blood cells in the maternal blood space (Figure 22A). Hemozoin, the malaria pigment, was observed in most of the infected placentas (Figure 22B). Fetal blood circulation often contains larger amount of erythroblasts (Figure 22D) but they never presented any sign of parasites or hemozoin.

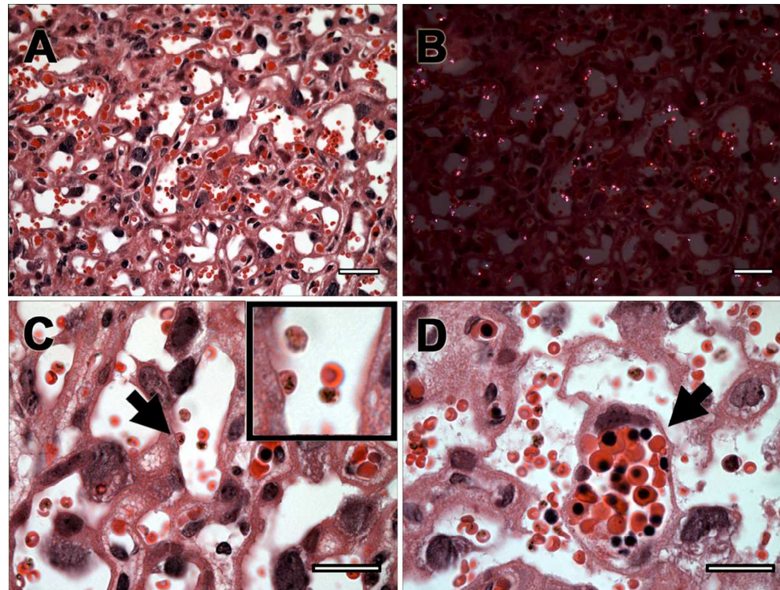


Figure 22. Placental malaria features. HE stained placentas from BALB/c females infected with *P. berghei* and collected at G18. (A) Image from severely infected placenta with high number of parasitized maternal erythrocytes. (B) The same field as (A) under polarization microscopy revealing hemozoin. (C) Arrow and insert show an infected erythrocyte adhered to the syncytiotrophoblast layer. (D) Placental section with infected erythrocytes in the maternal blood and fetal erythroblasts (arrow). Scale bar represents 30 µm in (A, B and D) and 20 µm in (C).

Some specimens show focal fibrinoid necrosis in the placenta basal zone (Figure 21B), hyperplasia of syncytiotrophoblastic cells (Figure 21D) and accumulation of mononuclear cells in the maternal blood space as revealed by immunofluorescence staining (Figure 23A). The accumulation of CD11b expressing cells, suggested that the infiltrate was predominantly composed by monocytes/macrophages. This result prompted us to measure the expression of macrophages attracting chemokines MIP-1α and MCP-1 in the placenta. RNA quantification revealed that MIP-1α gene expression was significantly increased in the infected placenta (Figure 23B) providing support for the notion that cell and molecular components of the innate immune system participate in the host response to the placenta malaria infection.

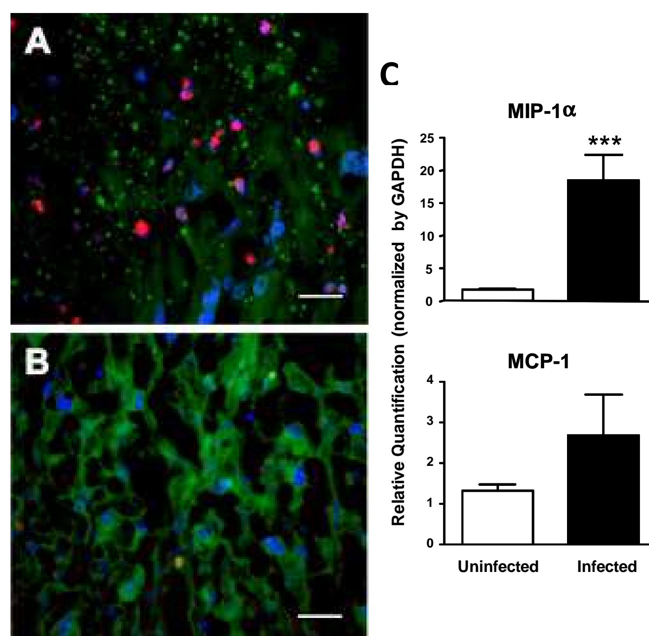


Figure 23. Inflammatory infiltration and macrophage/monocyte attractant chemokine expression in malaria infected placenta. (A) Immunohistochemistry analysis of placentas from BALB/c females infected on G13 with *P. berghei*-GFP iRBC and collected at G18 that were stained with anti-GFP (green) and anti-CD11b (red) revealing the presence of parasites on vascular walls and monocytes/macrophages infiltration, respectively. The (B) panel represents sections of non infected placentas. The cell nuclei were stained with DAPI (blue). Scale bar represents 30 µm. (C) RNA expression of MIP-1α and MCP-1 genes was quantified in 30 infected and 8 uninfected BALB/c placentas collected on G18. Relative quantification was obtained by normalization for GAPDH expression. Each bar represents the mean ± s.e.m. of individual values. *P*-value = 0.0002 is represented by ***.

- **Placental vascular space reduction**

The alterations in tissue organization observed in the infected placenta suggested that the maternal blood flow could be reduced in pregnancy malaria. Thus, we used a computerized morphometric method to quantify cross-sectional areas of blood sinusoids in placental labyrinthine region. Morphometric analysis was performed as described in methods section and confirmed that the blood sinusoids areas differed significantly between infected and non-infected placentas. The average blood sinusoid area was 52.0 ± 4.0 (mean ± SD, arbitrary units) in the control group and it dropped to 34.7 ± 7.5 in the infected pregnant group (Figure 24). The blood sinusoids area was measured in five different regions of the labyrinthine zone and in all of them the area decreased in similar degree, indicating that this phenomenon is spread across the placenta rather than restricted to specific areas.

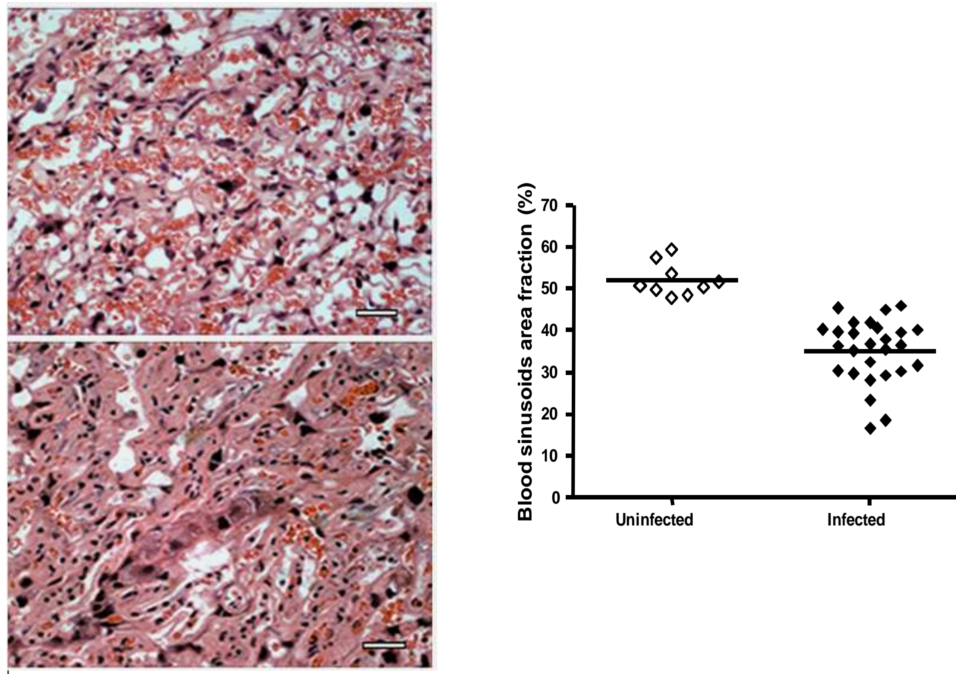


Figure 24. **Reduction of placental vascular space in infected pregnant mice**. The available area for blood circulation at G18 is reduced in infected placentas (lower photo) in comparison with non-infected placentas (upper photo). Scale bar represents 25 µm. The placental area occupied by blood sinusoids was quantified in relation to the total placental area (plot) in non-infected and infected placentas using an automated morphometric procedure, as described in Methodology section (P – value < 0.001).

Together, the data suggest that alterations of pregnancy outcomes observed in mice infected with *P*. *berghei*-GFP correlate with pathological alterations of the placenta tissue, involving inflammation, tissue disorganization, reduction of vascular spaces and consequent reduction in blood supply.

EXPOSURE PRIOR PREGNANCY

The aim of this model was to establish and analyze a system recapitulating the specificities of PAM protection observed in women with premunition and allowing investigations on the host and parasite components of PAM protection. Non-pregnant women living in regions with intense malaria transmission typically display a good protection against clinical malaria, but when they become pregnant this scenario of resistance likely changes.

Malaria outcome in multigravida pre-exposed females

- **Pregnancy-induced malaria recrudescence**

In total, more than one hundred female mice, distributed by several experiments, were infected with *P. berghei*-parasitized red blood cells and subsequently treated with chloroquine. These mice typically showed a transient parasitemia peak, which eventually resolved (see Figure 15). In the absence of pregnancy, parasitemia remained essentially sub-patent and was never observed for the rest of lifespan (Figure 25, upper graph). In contrast, parasite recrudescence was frequently observed when malaria-treated females become pregnant (Figure 25, under graph), more often after gestation day 14 (G14) but never before G12. We followed the first pregnancy of eighty-four pre-exposed females and found out that forty-nine (58%) showed parasite recrudescence induced by pregnancy. Twenty-nine recrudescent females were followed to the end of pregnancy and we observed uncontrolled parasitemia leading to severe malaria and eventually to maternal death in nine of those females (31%), while the remaining controlled the parasitemia peak and were apparently cured. These results confirm the hypothesis that sub-patent *P. berghei* infection is exacerbated by pregnancy.

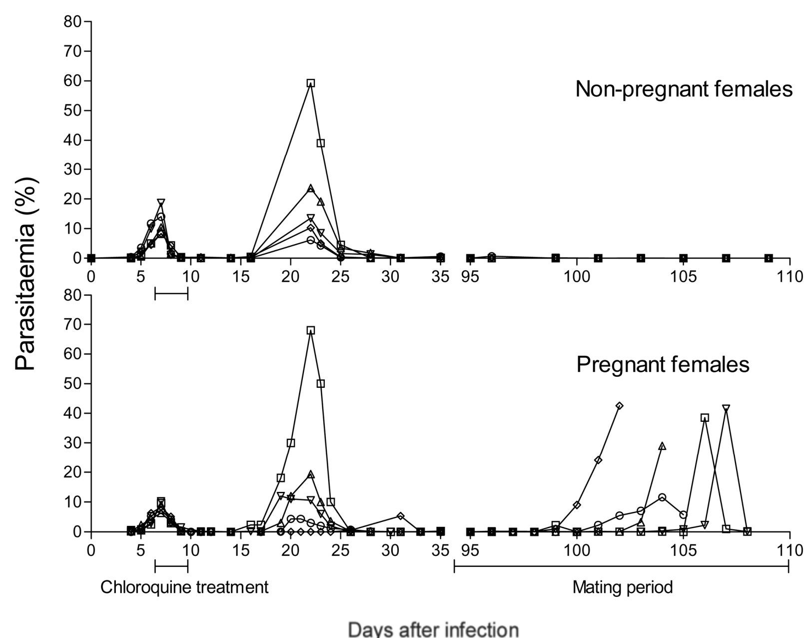


Figure 25. **Malaria susceptibility is increased during pregnancy.** Representative parasitemia curves of BALB/c females infected with *P.berghei* (day 0) and treated with chloroquine for 3 days starting at day 7. Parasitemias of females maintained without male (non-pregnant) are represented in the upper plot. The lower plot shows 5 typical parasitemia curves of recrudescent primigravida.

We investigated whether pregnancy related factors such as reticulocytosis, hormonal balance and imnunosuppression could *per se* trigger malaria recrudescence in pregnancy. Reticulocytosis is very frequent during pregnancy and it has been described that *P. berghei* preferentially infects reticulocytes (Cromer *et al.*, 2006). To ascertain if reticulocytosis could elicit parasite recrudescence, reticulocytes production was stimulated using phenylhydrazine. Phenylhydrazine is an oxidant drug that destroys RBC by denaturation of hemoglobin with little evidence of toxicity to other tissues. Consequently, the treatment induces a sudden erythropenia followed by an increase in erythropoiesis, as evidenced by the increased number of reticulocytes into the blood circulation (Flanagan & Lessler, 1970). This treatment is commonly used to increase *P. berghei* parasitemia level in mice due to its preference by reticulocytes. We treat seven pre-exposed and two non-infected females with phenylhydrazine (2.5 mg/20 g of body weight, i.p. in one single dose) which were monitored during the subsequent month. The treatment evoked extensive reticulocytosis that persisted for eight days and weight loss was evident in the immediate subsequent days (Figure 26), without a single parasite observation in the peripheral blood. Reticulocytes were depicted in Giemsa blood smears as showing stained granules. All the mice were able to recover the weight loss.

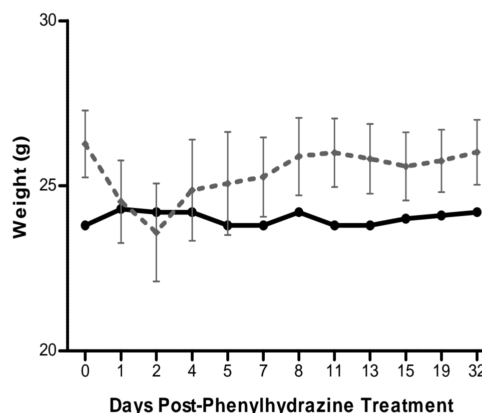


Figure 26. **Effect of phenylhydrazine treatment in pre-exposed mice.** Phenylhydrazine treatment (at day 0) provoked reticulocytosis in six pre-exposed females treated. Blood smears and weight were monitored to identify any detrimental effect of the drug. No parasite was found in the blood but an exuberant presence of reticulocytes was observed immediately after the drug administration. The weight of the treated pre-exposed females suffers a decrease on the days following the drug administration but it was recovered (dashed line, mean ± s.e.m.). Continuous line corresponds to the weight fluctuation of a naïve female not treated.

The mice treated with phenylhydrazine were challenged three weeks later with *P. berghei* and also more than four months after the treatment, controlling the parasitemia, which reveals that protection was still active. However, we cannot guarantee that the phenylhydrazine treatment is not toxic for the parasite, since none of the treated females that become pregnant revealed pregnancy-associated recrudescence. Collectively, these results cannot exclude that reticulocytosis has an effect on eliciting parasite recrudescence during pregnancy.

On the other hand, hormonal immunoregulation occurring in pregnancy induces a level of immunosuppression that was suggested to be implicated in the higher malaria susceptibility during pregnancy in humans (Vleugels *et al.*, 1989) and also in animal models (van Zon *et al.*, 1982). We made use of *P. berghei* pre-exposed females to test this possibility in two ways. First, we mimicked the pregnancy hormonal balance in malaria pre-exposed females through inducing pseudo-pregnancy by mating them with vasectomized males during 30 days. We did not detect any parasite recrudescence after mating in any of the females that exhibit vaginal

plug. However, when the same females became pregnant after mating with normal males, ten out of fifteen (60%) showed recrudescence (data not shown). It is possible that the hormonal modifications in pseudo-pregnant females, although sufficient to sustain embryonic development in embryonic transfers, were inadequate to induce immunosuppression and therefore we further tested whether a direct immunosuppressive treatment would evoke parasite recrudescence (Alvarez *et al.*, 1991). Thus, a group of pre-exposed females was treated with a single dose of cyclophosphamide (200 mg/Kg of body weight, i.p.) and was monitored for parasitemia in the following two weeks, but again no recrudescence was detected (data not shown). These results do not discard that pregnancy-induced immunosuppression could play a role in the breakdown of malaria protection but they strongly suggest that other mechanisms are implicated in malaria recrudescence during pregnancy and suggest that as the placenta is absent in pseudo-pregnant females it could play a role in inducing parasite recrudescence in pregnancy.

- **Disease severity and pregnancy-induced recrudescence are reduced in multigravida**

Analysis focused on recrudescent females revealed that maternal mortality associated to recrudescence decreases with parity (Figure 27) suggesting a decrease in disease severity. Conversely, we noted that among non-recrudescent females, irrespective of parity, about ten percent died during pregnancy or shortly after delivery.

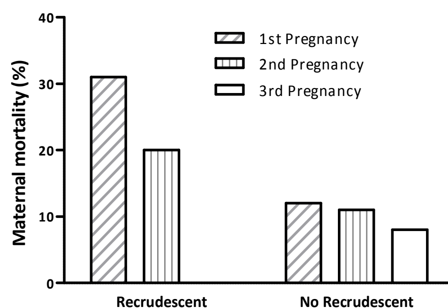


Figure 27. Exposure to pregnancy-induced recrudescent parasites reduces maternal mortality in subsequent pregnancies. Maternal mortality rate is plotted according to parity and recrudescence occurrence.

To test whether pregnant females were able to develop PAM protection upon exposure to the recrudescent parasite, we followed up the fate of thirty-two primigravida in subsequent pregnancies. The pregnancy-induced peripheral parasitemia peak was graded as high recrudescence, if higher than 5%, and as patency if between 1% and 5 %. The pregnant females with less than 1% of iRBC as detected by FACS analysis were declared non-recrudescent. We found that the aggregate incidence of high recrudescence and patency significantly decays from the first (59%) to the second (41%) and third pregnancy (22%) (Figure 28A). The reduced incidence of cases with high pregnancy parasitemia peaks (more than 5% of iRBC) was particularly striking and close to a four-fold reduction from the first (44%) to the second pregnancy (12%). Accordingly, the level of parasitemia also decreased significantly when comparing first (14.5 % ± 19.2 %), second (3.9 % ± 9.4 %) and third pregnancy (2.0 % ± 4.1 %) (Figure 28B).

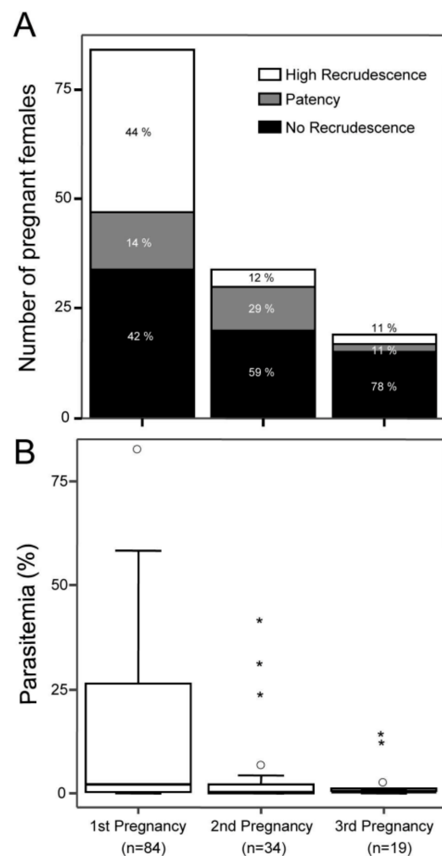


Figure 28. Recrudescence incidence and peripheral parasitemia are decreased in multigravida. (A) Frequency of females with high recrudescence (above 5% parasitemia), patency (parasitemia between 1% and 5%) and no recrudescence (parasitemia < 1%) according to parity. Recrudescence incidence is significantly associated with parity (*P*-value = 0.001, Chi-square test). (B) Box-plots illustrate the range of the peripheral parasitemia peak according to parity. The parasitemia peak in the first pregnancy was significantly different from the second (*P*-value = 0.004) and third pregnancies (*P*-value = 0.006). Box-plots show medians (middle line in the box), central 50% of data (box), data range (whiskers) and ^{0} and * represent outliers and extremes, respectively.

These results indicate that females that are repeatedly exposed to recrudescent parasites during pregnancy develop a protective response that tends to control parasite recrudescence and placental malaria during subsequent pregnancies.

PAM protection in multigravida is not attributable to the age of the pregnant females as we observed that pregnancy-induced recrudescence incidence in primigravida was not reduced at older ages. In particular, females infected under 20 weeks of age presented about 55% of recrudescence and in the group of older females, with more than 20 weeks of age, the recrudescence was about 65%. Furthermore, pregnancy-induced recrudescence seems to be uncorrelated with the period between infection and the first pregnancy, since we were able to observe primigravida recrudescence 40 weeks after infection. These data suggest that both the age of the mother and the duration of subpatent parasitemia are not determining factors in triggering parasite recrudescence or in malaria protection in multigravida.

- **Susceptibility to pregnancy-induced anemia in pre-exposed mice is associated with parasite recrudescence**

Malaria-induced anemia in pregnancy is a major concern in endemic regions. Our data support the idea that hemoglobin levels are strongly correlated with parasite density in the peripheral blood. We observed that hemoglobin levels decrease with parasitemia rise, both in infected or challenged mice (Figure 29A and 29B). In the last case, hemoglobin levels recover after parasite clearance. Corresponding correlations were observed with respect to levels of

parasitemia and hemoglobin concentration (Figure 29C), both in infected or challenged mice. Of note is the fact that infecting naïve mice induces faster hemoglobin decay than in challenged mice, as indicated by the respective correlation slopes, - 0.253 and - 0.217. Besides, we observed that both P. berghei pre-exposure and pregnancy-associated *P. berghei* recrudescence caused a significant reduction in hemoglobin levels (Figure 29D) in comparison with non-exposed and non-recrudescent pregnant females (*P*-value < 0.001).

Taken together, these results confirm that *P. berghei* parasitemia adversely affects hemoglobin levels and, consequently, maternal anemia is a likely clinical complication of parasite recrudescence during pregnancy. Still, maternal acquired and cumulative immunity reduces recrudescent parasitemias, thereby partially protecting the pregnant mice from death by severe anemia.

Offspring of multigravida pre-exposed females

- **Poor pregnancy outcome is associated with pregnancy-induced malaria recrudescence but convalesces in multigravida**

To evaluate the effect of pregnancy-induced parasite recrudescence in the pregnancy outcome we monitored the offspring of recrudescent females. The twenty-nine recrudescent primigravida had significantly smaller litter sizes (average of 1.9 newborns/litter) as compared to twenty non-infected females (5.9 newborns/litter in average) (Table 4). Likewise, the average birth weight of newborns from recrudescent mothers (1.1 g) was significantly lower when compared to the newborns from non-infected mothers (1.4 g). These findings indicate that recrudescent primigravida females show poor pregnancy outcome that is characterized by decreased fetal viability and intra-uterine growth retardation.

Table 4. Disease severity and pregnancy outcome in *P.berghei* recrudescent females according to parity

Parity	Pregnant females	Average parasitemia peak (%)	Maternal mortality (%)	Litter size [a]	Newborns Birth weight (g) [a]
Primigravida	29	22.7	31	1.9 ± 3.0 (29)	1.1 ± 0.2 (6)
Second Pregnancy	9	10.7	20	5.6 ± 2.1 (9)	1.3 ± 0.2 (7)
Third Pregnancy	3	3.4	0	6.7 ± 1.2 (3)	1.3 ± 0.1 (3)
Non-Infected	20	___	0	5.9 ± 2.2 (20)	1.4 ± 0.2 (20)

[a] Mean ± stdev (number of litters analyzed)

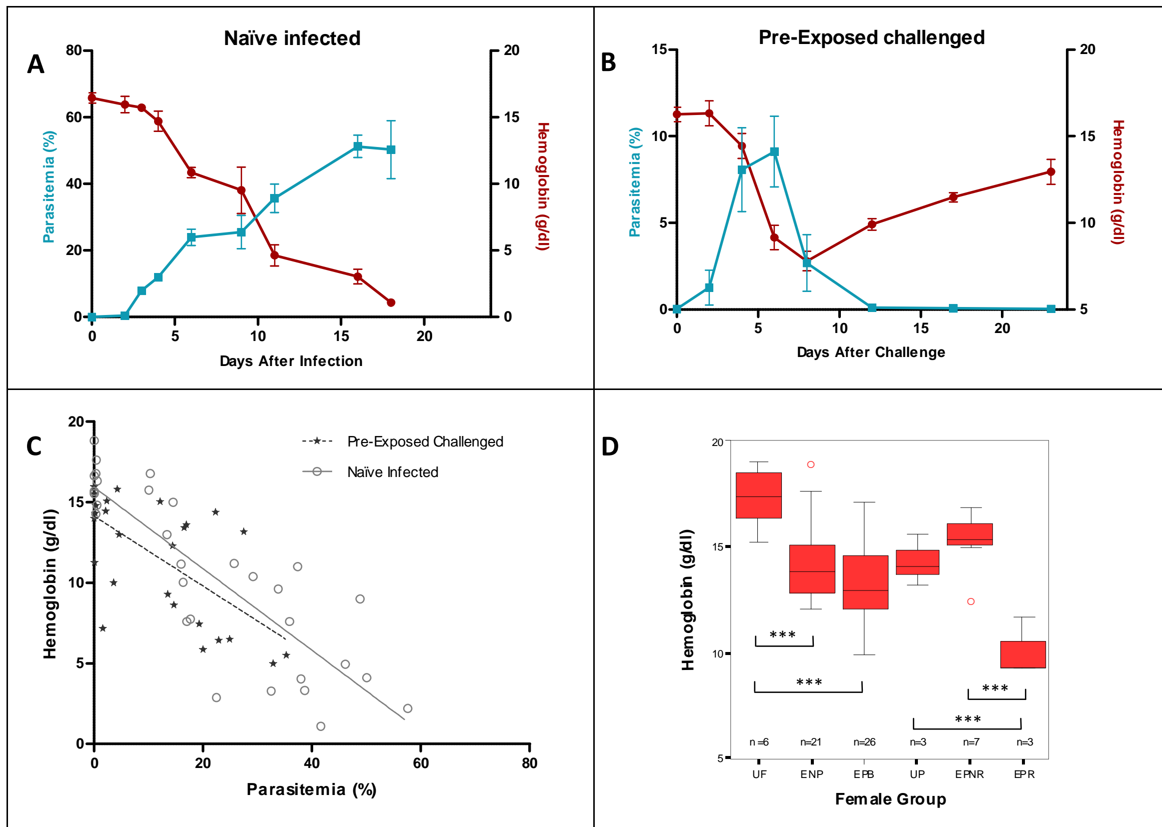


Figure 29. Hemoglobinemia is strongly correlated with parasitemia. Peripheral blood hemoglobin and parasitemia were followed after infection in eight naïve BALB/c females (A) and after challenge in seven pre-exposed (B). In (C) scatter plot of parasitemia versus hemoglobin with adjusted linear curves for each group of mice: naïve infected (r^2 = 0.718, *P*-value < 0.0001, 31 observations) and pre-exposed challenged collected during the parasitemia peak (r^2 = 0.418, *P*-value = 0.0003, 27 observations). Hemoglobinemia is represented in (D) for different groups of females. The first three boxes correspond to non-pregnant females: uninfected females (UF), pre-exposed never-pregnant (ENP), pre-exposed pregnant-before (EPB); the others correspond to pregnant females: uninfected pregnant females (UP), pre-exposed pregnant females with no recrudescence (EPNR), pre-exposed pregnant females with recrudescence (EPR). In pregnant mice hemoglobin was measured between G18 and delivery. Box-plots (D) show medians (middle line in the box), central 50% of data (box), data range (whiskers) and outliers (dots). The number of mice (*n*) per group is indicated. (***, *P*-value < 0.001).

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Non-Infected	20	___	0	5.9 ± 2.2 (20)	1.4 ± 0.2 (20)

[a] Mean ± stdev (number of litters analyzed)

The litter size and the newborn birth weight were lower in the first pregnancy but recovered and approximated normal levels in subsequent pregnancies (Figure 30).

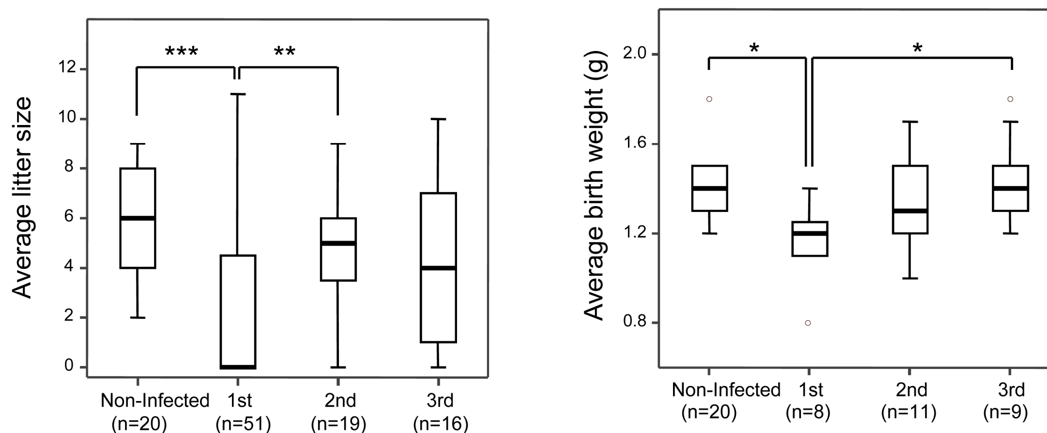

Figure 30. **Reduced adverse pregnancy outcomes in multigravida.** Box-plots of the average litter size (left) and average newborn birth weight (right) according to parity (first, second and third pregnancy). Pregnancy outcome was significantly different in primigravida as compared to multigravida and non-infected pregnant females (***, *P*-value < 0.001; **, *P*-value < 0.01; *, *P*-value < 0.05).

- **Progeny costs in absence of Maternal Recrudescence**

Progeny derived from non-recrudescent pregnant females, irrespective of parity, displayed a litter size below normal levels, even though the mean birth weight appears not to be affected (Table 5). This data indicate that in absence of peripheral parasitemia, pre-exposed pregnant females may display a degree of placental malaria leading to a slight but significant loss of fetuses.

Table 5. Disease severity and pregnancy outcome in non-recrudescent females according to parity

Parity	Pregnant females	Maternal mortality (%)	Litter size [a]	Newborns Birth weight (g) [a]
Primigravida	22	12	3.2 ± 3.3 (22)	1.4 ± 0.2 (2)
Second Pregnancy	10	11	3.8 ± 2.4 (10)	1.4 ± 0.3 (4)
Third Pregnancy	13	8	3.5 ± 0.2 (13)	1.5 ± 0.7 (6)
Non-Infected	20	0	5.9 ± 2.2 (20)	1.4 ± 0.2 (20)

[a] Mean ± stdev (number of litters analyzed)

Effects of maternal pre-exposure on placental immunopathology

- **Parasitemia recrudescence correlates with placenta pathology**

The poor pregnancy outcome in females infected during pregnancy is associated with a placental inflammatory response that leads to marked tissue disorganization, and the presence of maternal iRBC at different stages of maturation in the placenta. In recrudescent primigravida the intensity of peripheral parasitemia was quantitatively correlated with the reduction of the placental vascular spaces (*P*-value = 0.0012) (Figure 31A).

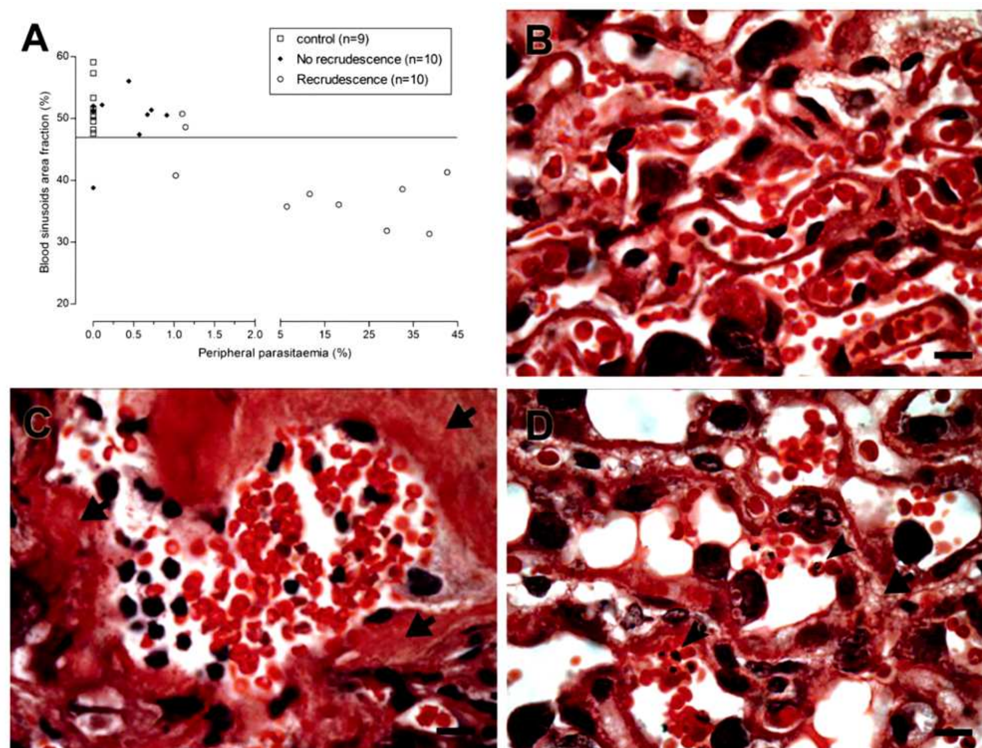

Figure 31. Peripheral parasitemia correlates with the reduction of placental blood sinusoids area. (A) The blood sinusoidal area is plotted against the peripheral parasitemia peak observed in primigravida. The area of placental blood sinusoids, expressed as a fraction of the total placental area, was obtained using an automated morphometric procedure as described in the Methodology section. In recrudescent females, the degree of parasitemia was correlated with sinusoidal area reduction (correlation coefficient for recrudescent females is 0.45, *P*-value = 0.0012). Representative photomicrograph of placental sections HE stained from non-infected (B) and recrudescent (C-D) mothers. Accumulation of inflammatory cells (C), trophoblast thickening (arrows) and presence of iRBC (D) in blood sinusoids (arrowheads) are evidenced in placenta tissue from recrudescent mothers. Scale bars represent 15 µm in (B-D).

In particular, recrudescent females with high parasitemia showed an increased reduction of vascular spaces. These results strongly suggest that malaria recrudescence correlates with placental tissue damage (Figure 31C and 31D) that possibly underlies the observed poor pregnancy outcomes. In addition, the expression analysis of cell-type specific genes in placentas from females with recrudescence, revealed increased amounts of inflammatory cells, particularly natural killer (NK) cells, T cells and macrophages (Figure 32A) and up-regulation of macrophages attractant chemokines (MCP-1 and MIP-1α) (Figure 32B).

We also found that the expression of several molecules related to vascular stress, namely hemoxygenase-1 (HO-1) and endothelin-1 (ET-1), was increased in placentas of recrudescent females (Figure 32D). TNF-α expression showed a trend to increase in infected placentas and the balance of the immuno-modulatory molecules IL-12 and IL-10 expression denoted an anti-inflammatory response in the course of the placenta malaria pathogenesis (Figure 32C). In fact, IL-10 expression was mostly increased in placentas where pathology was more intense (Figure 32E).

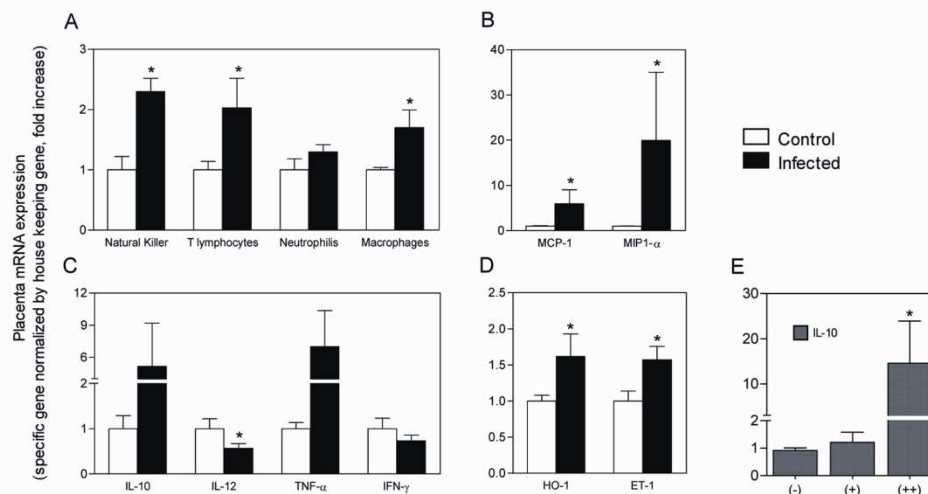

Figure 32. Placenta pathology is associated with altered gene expression of inflammation markers. qRT-PCR of placenta tissue was used to detect the expression of cell type–specific genes indicating infiltration of inflammatory cells: *Klrd1* gene for Natural Killer cells, *Cd3e* gene for T cells, *Ncf2* gene for neutrophils and *Mgl2* for macrophages (A). Placental gene expression was quantified for relevant markers of monocyte/macrophage chemotaxy (B), inflammation mediators (C) and vascular stress (D). RNA expression was quantified in 15 placentas from recrudescent primiparous BALB/c females and in 8 uninfected placentas, collected on G19. In (E) placental *IL10* mRNA expression was separately analyzed in 5 placentas showing moderate pathology (+) and 4 placentas showing severe (++) pathology. Relative quantification was obtained with normalization by ß-actin for (A), (C), (D) and (E) and by GAPDH for (B). In (E) results are plotted as fold change over the respective non-infected controls. Each bar represents the mean ± s.e.m. (*, P-value < 0.05).

It is worth noting that, regardless the number of previous pregnancies, the placentas from recrudescent females typically showed iRBC in the maternal blood spaces, inflammatory infiltrates, erythroblast accumulation in the fetal blood, placenta architecture disruption and trophoblast basal membrane thickening. Together these data suggest that placental malaria is an inflammatory syndrome likely driven by parasite expansion and accumulation in the placental blood spaces.

- **Modified levels of cytokines in sera**

The type of cytokines response may modulate the effective immune response and can have a role in tailoring the pregnancy outcome. Using a Cytokine Beads Array we intended to capture in several animal groups, variations in serum levels of IL-6 as well as IL-4 and IL-10 that are typically involved in B cell activation and anti-inflammatory responses (Figure 33).

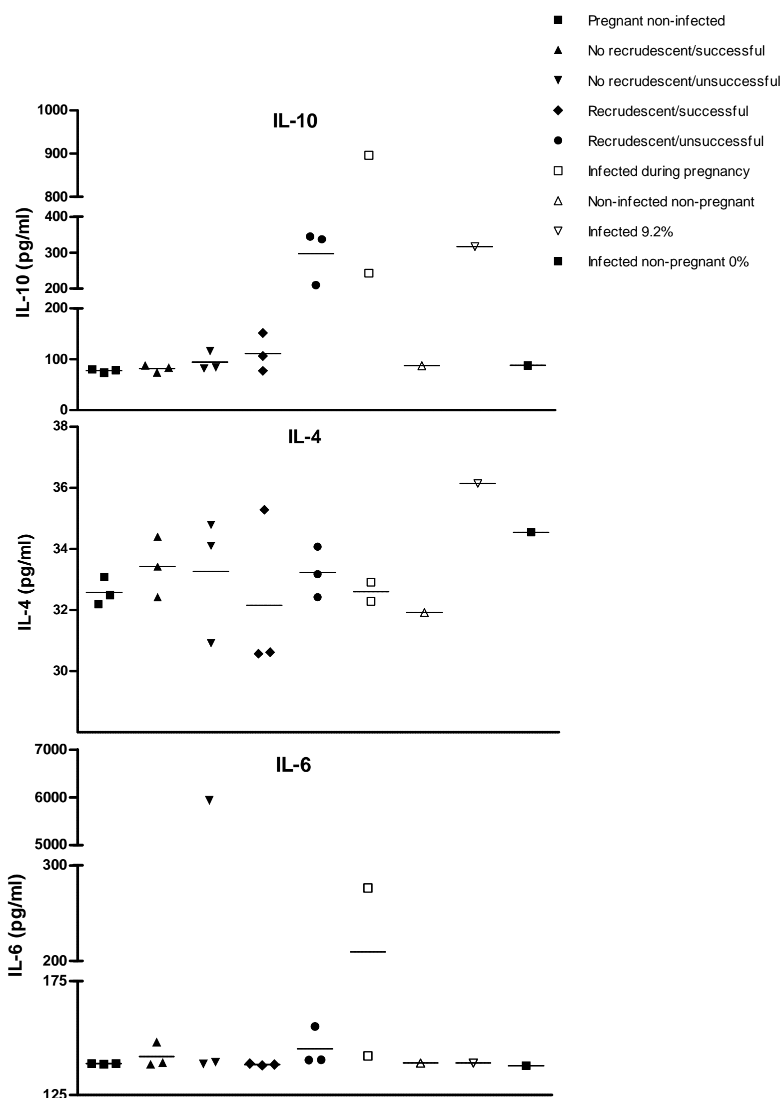

Figure 33. Cytokines evaluation. IL-4, IL-6 and IL-10 cytokines levels in various groups of malaria infected mice. Using the Cytokine Bead Assay technique the cytokines were measured in sera from individual mice either non-pregnant in the indicated conditions or at G18 of pregnancy.

IL-10 higher levels are consistently associated with the presence of parasites probably as part of a response to compensate the exacerbated inflammation elicited by the active infection (Figure 33, upper graph). Among the pre-exposed pregnant, the recrudescent with unsuccessful pregnancy show higher IL-10 levels, whereas the recrudescent that succeed in delivering offspring only manifest a slight increase. These results are concordant with the overexpression of IL-10 in the placenta (Figure 32). IL-4 levels have a more heterogeneous pattern but a common characteristic is that all the pre-exposed females are more likely to have increased levels of this cytokine, independently of the presence of the parasite (Figure 33, middle graph). This corroborates with the fact that IL-4 is associated with the control of the disease in later phases by providing B cell help on the generation of specific IgG antibodies (Malaguarnera & Musumeci, 2002). IL-6 is a cytokine that can have both a pro-inflammatory and anti-inflammatory role, and like IL-1 and TNF-α, is involved in the induction of fever and

the acute phase response (Robinson *et al.*, 2009). High levels of this cytokine may compromise pregnancy success (Margni & Zenclussen, 2001). Interestingly, increased levels of this cytokine were observed in unsuccessful cases of pregnancy (Figure 33, lower graph). These results suggest that IL-10 and IL-6 in sera of pregnant females with malaria are associated with poor pregnancy outcomes.

- **Sporadic placental parasites are associated with pathologic events in non-recrudescent mothers and adverse pregnancy outcomes**

The analysis of non-recrudescent placentas in some cases revealed the presence of iRBC and tissue lesions resembling the pathology observed in recrudescent females (Figure 34). We quantified *P. berghei* parasites by qRT-PCR in 24 placentas from six non-recrudescent mice, and observed very small amount of parasites of about seventeen-fold less in average as compared to placentas from recrudescent mice (data not shown). Since it is possible that small parasite numbers are circulating in the peripheral blood of non-recrudescent mothers, we carried out an isodiagnosis test in a group of non-recrudescent pregnant females, by injecting a blood drop diluted in PBS in naïve animals and observing the parasitemia occurrence. Among the 14 non-recrudescent pregnant females tested, 7 had positive isodiagnosis, meaning that the parasite was present in the blood. These data suggest that albeit at low frequency, placental malaria occurs in absence of detectable peripheral parasitemia recrudescence.

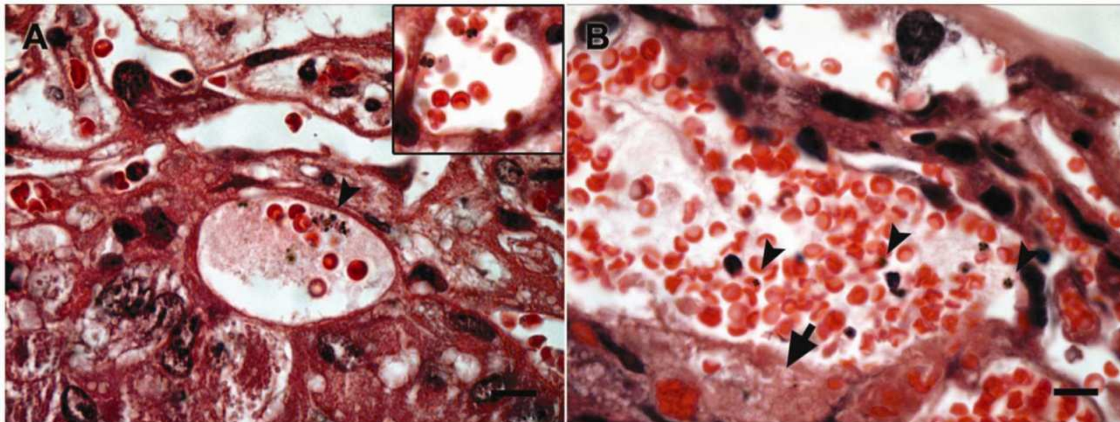

Figure 34. Occasional placenta pathology in non-recrudescent pregnant females. Photomicrographs of HE-stained placental sections of sporadic cases of placental pathology in non-recrudescent females. The figure shows presence of iRBC adhered to the syncytiotrophoblast layer (A, insert) and in blood sinusoids (arrowheads) as well as trophoblast thickening (arrow). Scale bar represents 15 µm.

Together, the data suggest that pregnancy-associated malaria evoked by recrudescent *P. berghei* is attributable to parasites, which on one hand are able to trigger an inflammatory response of the placental tissue and, on the other hand, induce a cumulative protective response in multigravida that had experienced recurrent infection relapses induced by pregnancy.

PARASITE AND PLACENTA INTERACTION

iRBC binding to placental sections

In histological observations it is common to find iRBC attached to syncytiotrophoblast or in the labyrinth vascular space of placentas both from pregnant females infected during pregnancy and recrudescent females (Figure 35). To characterize the nature of the interaction of the *P. berghei*-iRBC and mouse placenta tissue, we investigated the role of receptors that have been suggested to mediate *P. falciparum* cytoadhesion and sequestration to the human placenta.

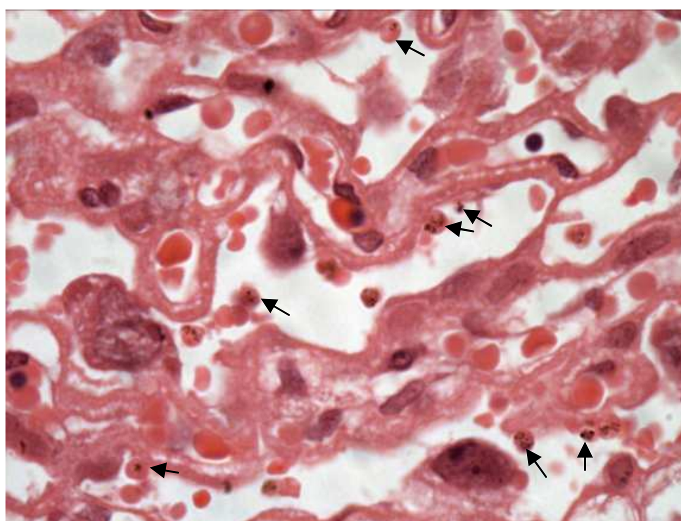

Figure 35. Placental section of a recrudescent pregnant female. Of note, iRBC on syncytiotrophoblast surface and in the IVS (arrows). Erythroblasts and tissue thickening are also evident. Pre-exposed pregnant female with 29% of recrudescence.

Parasite molecules that likely mediate the interaction parasite-placental are surface membrane proteins expressed by mature iRBC, the trophozoites and schizonts. Thus, for the adhesion/inhibition assays, we used *P. berghei*-iRBC preparations that were enriched for mature forms of parasites prepared by selection, after parasite synchronization (Figure 36), as described in the Methodology section.

With the aim of demonstrating that adhesion properties of iRBC were dependent on the presence of surface proteins, we pre-treated iRBC with two proteolytic enzymes (trypsin and proteinase K) and a non-proteolic enzyme (neuraminidase). Proteolitic depletion of iRBC surface proteins showed to reduce adhesion capacity to placental sections in a concentration-dependent manner (Figure 37).

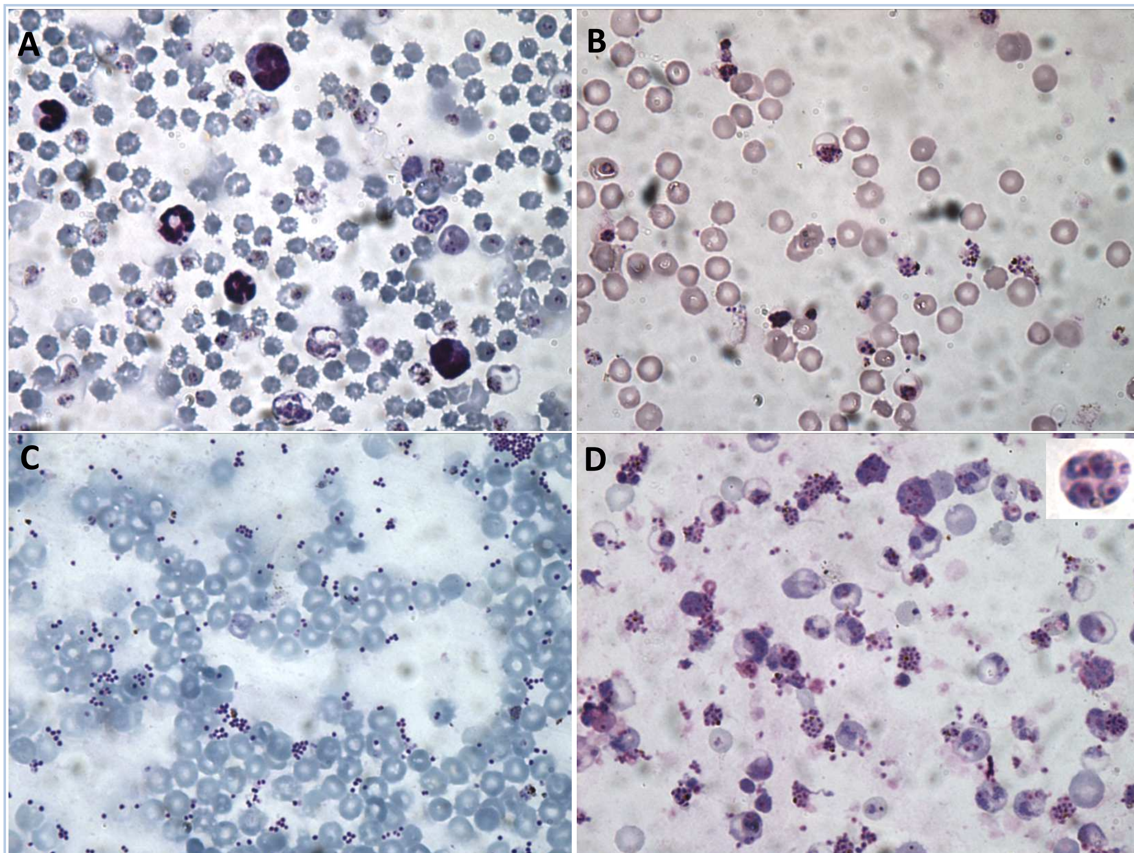

Figure 36. Synchronization of blood stage parasites and schizonts enrichment. Blood stage parasites were incubated to develop into mature forms as described in the methodology section. In (A) a thin blood smear Giemsa-stained from a recrudescent pregnant female with 20% parasitemia. Of note is the presence of polymorphonucleated cells and echinocytes (modified RBC with small knob-like surface projections evenly spaced and uniform in shape that are poorly deformable and usually impart high viscosity to the blood, interfering with capillary blood flow; the mechanisms of echinocytes formation are diverse but include erythrocytes dehydration as a result of electrolyte imbalance; these RBC were frequently observed in blood smears from infected animals). After 16 h of synchronization, blood stage parasites have developed essentially into mature forms, trophozoites and schizonts (B). By a gradient selection it is possible to separate the cultured cell essentially in two groups: one group (found in the pellet) formed mainly by non infected RBC and free merozoites (C) and the other group (obtained in the ring on the gradient), formed mostly by blood stage mature forms (D), usually enriched in more than 90% of iRBC. Inset (D) showing a mature schizont with differentiated merozoites.

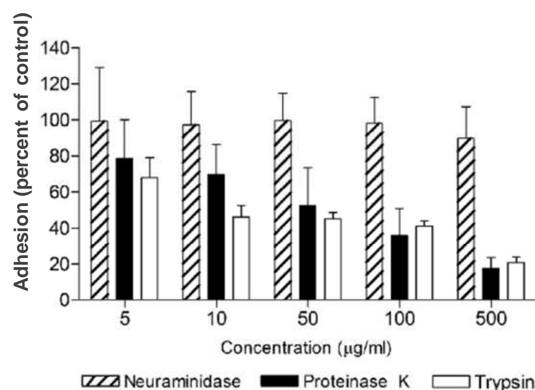

Figure 37. Protein-like molecules have a role in iRBC-placenta interaction. Intact iRBC were treated with neuraminidase, proteinase K and trypsin prior incubation with the placental tissue. All data represent the proportion of bound iRBC expressed as a percentage of control (mean ± s.e.m. for three experiments).

Candidate placental receptors modulate iRBC adhesion

We analyzed the involvement of two placental candidate mediators of iRBC binding, chosen on the basis of human trials. The first one is a generally accepted receptor for parasites sequestration in human placentas (CSA), whereas the second (HA) is a controversial candidate. Our results showed evidence that CSA and HA are involved in specific interactions of *P. berghei*-GFP iRBC of non-placental origin with the placental tissue (Figure 38). In fact, iRBC adherence was significantly reduced if parasite mature forms were previously incubated with CSA (Figure 38B) or HA (Figure 38C).

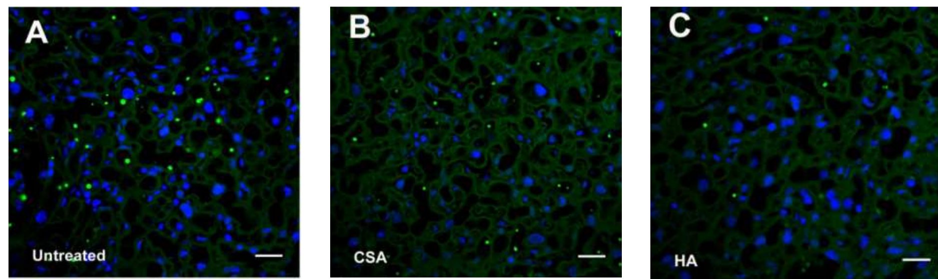

Figure 38. *Ex vivo* adhesion of *P. berghei*-GFP iRBC to mouse placenta. (A) Typical microscopic image of adhesion assays showing iRBC adhered in the intervillous space and to syncytiotrophoblast cell layer (A). Representative images of blocking adherence assays where iRBC were pre-incubated with 2 mg/ml of CSA (B) or HA (C).

Adhesion was competitively inhibited in a dose-dependent fashion by both CSA (69% reduction at 1mg/ml) and HA (80% reduction at 1mg/ml), but not with colominic acid (Figure 39, upper graph). In addition, iRBC adhesion also registered a significant reduction on tissue sections pre-treated with chondroitinase (66% reduction) or hyaluronidase (74% reduction), but heparinase had no effect on the iRBC adhesion (Figure 39, lower graph).

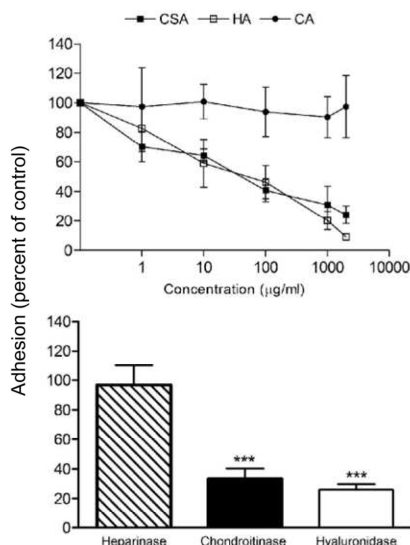

Figure39. **Adhesion-inhibition assays regarding CSA and HA as candidate receptors.** IRBC were pre-incubated with increasing concentrations of HA, CSA and colominic acid and then used in binding assays (upper graph) as described in Methodology section. Adhesion of iRBC to uninfected placental tissue was partially abolished by pretreatment of the placental sections with chondroitinase and hyaluronidase but not with heparinase (lower graph). All data represent the proportion of bound iRBC expressed as a percentage of control (non-preincubated iRBC or non-treated placentas, in upper and lower plots, respectively). Points and bars represent mean ± s.e.m. for three experiments. (***, *P*-value < 0.001).

These findings strongly suggested that CSA and HA in the mouse placental tissue participate in adhesion of iRBC, since *P. berghei* iRBC adhesion is partially dependent on the presence of CSA and HA receptors in the placenta and is inhibited by blocking their putative ligands in *P. berghei*-GFP iRBC or after their cleavage on the placentas.

Pregnancy-induced recrudescent *P. berghei* show enhanced affinity to placenta

iRBC sequestration appears to be the pathogenic trigger of the placenta pathology observed in pregnant women. In the previous section, we have shown that *P. berghei* iRBC from non-pregnancy origin has the ability to specifically adhere to the mouse placenta tissue. To evaluate the adhesion properties of the recrudescent *P. berghei* we performed adhesion assays on placental sections, that compared the adhesion properties of iRBC collected from recrudescent primiparous females with iRBC isolated from infected males and non-pregnant females. Strikingly, the amount of iRBC adhering to the placenta sections was four-fold increased in the samples from recrudescent primigravida (Figure 40A). The adhesion of the recrudescent parasite was also partially inhibited when the placental sections were treated with chondroitinase (70%) or hyaluronidase (43%), as well as when the iRBC were pre-incubated with CSA (56%) or HA (76%) (Figure 40B).

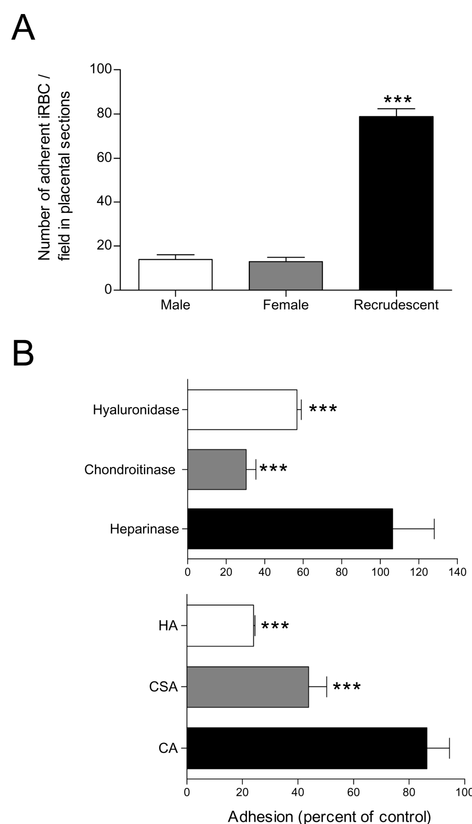

Figure 40. *P. berghei* iRBC from recrudescent females show enhanced adhesion to placenta. (A) iRBC from males, non-pregnant females and recrudescent females were incubated on uninfected placental sections and the adherent parasitized cells were counted as described in methodology section. (B, upper plot) Adhesion assays were also performed after pre-treatment of placental sections with chondroitinase ABC, hyaluronidase or heparinase (negative control). (B, lower plot) Adhesion inhibition assays were carried out by pre-incubating iRBC from recrudescent females with 1 mg/ml concentrations of HA, CSA or CA (negative control). In panel B the proportion of bound iRBC is expressed as a percentage of the control (non-treated placentas or non-preincubated iRBC, in upper and lower plots, respectively). Error bars represent the mean ± s.e.m. of three independent experiments. (***, *P*-value < 0.001).

These results suggest that the recrudescent *P. berghei* expanding during pregnancy display enhanced specificity to the placenta and consequently may induce a specific host response to the pregnancy-associated parasite, namely the triggering of placenta pathology events associated to PAM. Additionally, our data on mouse PAM indicate that both CSA and HA might be candidate mediators for recrudescent parasite adhesion to the placenta. Further methodologies to study binding of recrudescent parasites *in vivo* would have to be applied to certify the candidacy of these receptors. Moreover, research is needed to determine the GAGs composition on mouse placenta, namely of the chondroitin family, the presence of HA and their distribution within the placenta vascular spaces throughout the course of pregnancy.

ACQUIRED IMMUNITY IN PRE-EXPOSED FEMALES

Anti-*P. berghei* antibody response in pre-exposed females

We investigated the involvement of components of the acquired immune system in pre-exposed pregnant females and the persistence of the anti-*P. berghei* antibody response. We were interested in understanding if there is any association between the higher susceptibility to malaria in primigravida and the level of specific immunoglobulins.

- **Adaptive immunity appears crucial for malaria control**

It has been reported that mice lacking adaptive immune system cannot clear the malaria infection (Couper *et al.*, 2007; Nunes *et al.*, 2009) and we have confirmed these findings. Thus, immunocompetent BALB/c infected with *P. berghei* and treated with chloroquine are capable of controlling parasitemia while BALB/c RAG2 KO mice, which lack lymphocytes, die with hyperparasitemia (Figure 41) and are unable to control the disease even under chloroquine treatment. Infected BALB/c RAG2 KO mice respond to chloroquine treatment reducing the level of parasitemia but after a short period the parasitemia rises again and this sequence continues until the animal eventually dies.

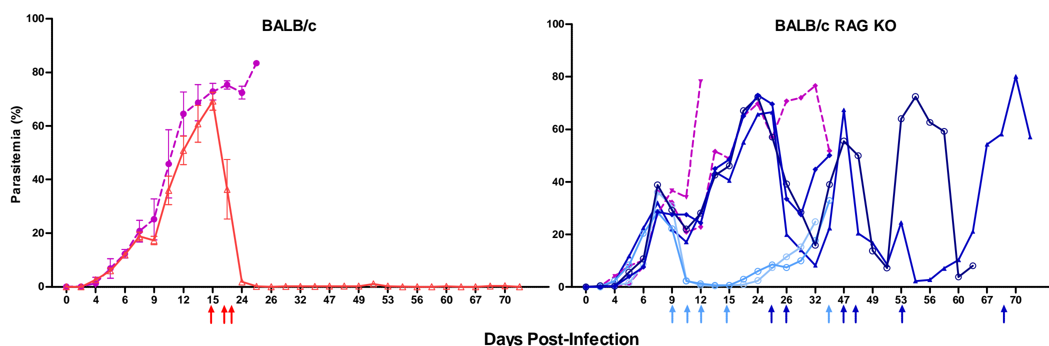

Figure 41. Mice lacking B and T cells are unable to control malaria when chloroquine treated. Course of infection as measured by parasitemia is represented for BALB/c grouped mice (6 animals per group) and individual BALB/c RAG2 KO (7 animals) infected with *P. berghei*. Solid lines represent treated mice while dashed purple lines correspond to untreated animals. BALB/c mice were treated with chloroquine in the days indicated by the red arrows. Conversely, BALB/c RAG2 KO mice were treated with chloroquine in the days indicated by the blue arrows but all died without controlling the infection. Light blue lines correspond to females receiving early chloroquine treatment (indicated by the light blue arrows) while dark blue represents animals treated lately. Untreated BALB/c and BALB/c RAG2 KO (dashed purple lines) died with hyperparasitemia. Red lines correspond to BALB/c. Arrows indicate chloroquine treatment with colors matching animal lines.

These results illustrate the requirement of an adaptive immune response to control *P. berghei* infection. Thus, we analyzed the course of the adaptive immune response in pre-exposed females by monitoring anti-parasite antibodies in the serum. The humoral response, as measured by anti-*P. berghei* IgG, starts around 2 weeks after infection (Figure 42). The anti-*P. berghei* IgM production appears to start slightly earlier but overall follows the same kinetics as IgG.

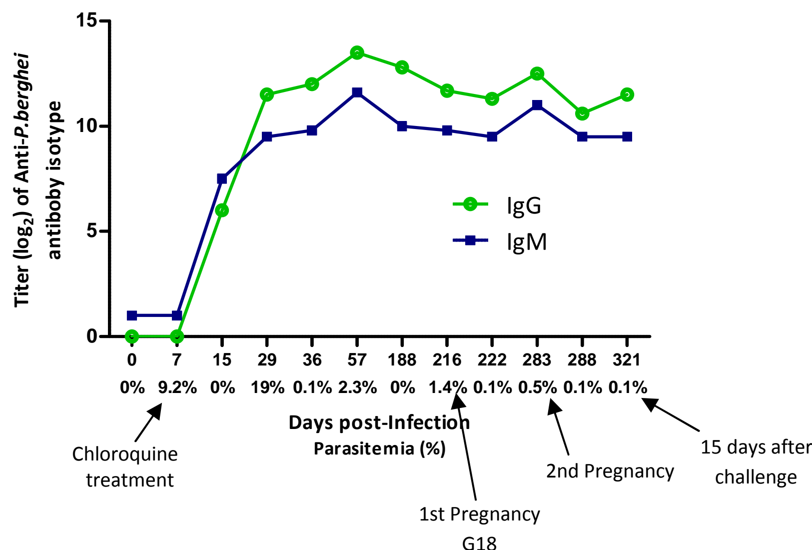

Figure 42. Representative kinetics of anti-*P. berghei* IgG and IgM antibody production. IgG and IgM titers for a BALB/c female, infected on day 0, who further experienced two pregnancies and a challenge as indicated. Serum antibodies were measured by class-specific ELISA.

Pre-exposed aparasitemic mice maintain a long term production of IgG (Figure 43) and can maintain IgG titers for their life time. What keeps this long-term response it is not known but it could be due to a persistent unapparent infection.

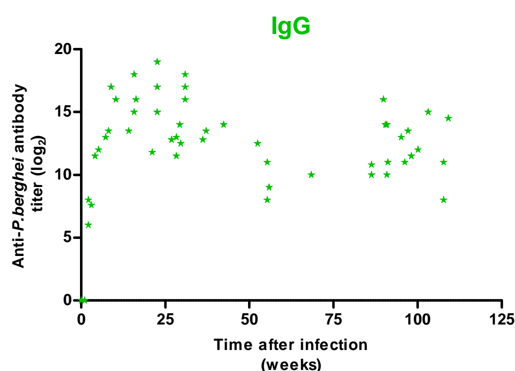

Figure 43. Long lasting IgG production in pre-exposed females. Anti - *P. berghei* IgG antibody production of BALB/c females infected on day 0, followed by chloroquine treatment (days 7-10 post infection). Antibodies were measured by IgG-specific ELISA in serum.

Different lines of evidence suggest that long term subclinical infections are sustained in pre-exposed mice. Isodiagnosis using organs of pre-exposed BALB/c allow to verify that the parasite can be "hidden" and hence causing a sub-patent infection. Briefly, several organs of eight pre-exposed animals were perfused or macerated and the collected material was separately injected in naïve animals. The materials originated from liver and lungs led to five positive isodiagnosis, the spleen and kidneys to three, the bone marrow to two and the fat and lymph nodes to one. Overall, isodiagnosis was positive for six out of eight pre-exposed animals. Furthermore, the isodiagnosis of blood from non-recrudescent pregnant females was positive for 7 out of 14 of the cases analyzed, with the period after infection ranging from 110 to 450 days for the positive cases. In line with these findings we have observed that pre-exposed pregnant females can have parasite recrudescence a long time after being infected (more than

30 weeks after infection, data not shown). These results indicate that parasites may persist for long periods in different organs of pre-exposed and protected mice, suggesting that these parasites provide the antigenic stimulation that sustains the immunological protective response.

Acquired protection in pregnant females

P. berghei pre-exposed mice that had not experienced pregnancy respond to parasite challenge (with 10^6 iRBC of non-pregnancy origin/animal, i.p.) with a parasitemia peak that typically rises up to 2-10% and eventually resolved (Figure 44). Conversely, pre-exposed females that have experienced a former pregnancy, with or without recrudescence, are likely to develop, upon challenge uncontrolled parasitemia with a high mortality rate (60%), dying normally up to two weeks after challenge. This outcome appears to be independent of the age and period of sub-patent infection.

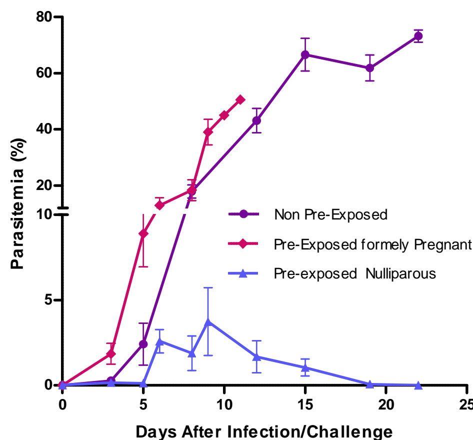

Figure 44. Effect of pregnancy on the malaria protection status of pre-exposed females. Parasite challenge was used to test protection conferred by pre-exposure in non-pregnant nulliparous females (*n*=12) and non-pregnant females that formerly have experienced at least one pregnancy (*n*=10). Non pre-exposed females (*n*= 5) died with hyperparasitemia up to day 25 after infection. Parasitemias of females after recovering were excluded. Data points and error bars represent average values and s.e.m., respectively.

Following challenge of pre-exposed that did not experienced pregnancy, IgG production showed a slight increase but later suffered a reduction and maintained a steady state (Figure 45A). Interestingly, the observed breakage of malaria protection in females that have experienced a pregnancy is paradoxically accompanied by a humoral response of anti-parasite antibodies that did not differ from the response of protected pre-exposed females (Figure 45B). Within each group in Figure 45B (those that recovered and those that succumbed after challenge) it was not possible to establish a common previous history, since in both groups there were females with and without recrudescence and with different duration periods of sub-patent infection.

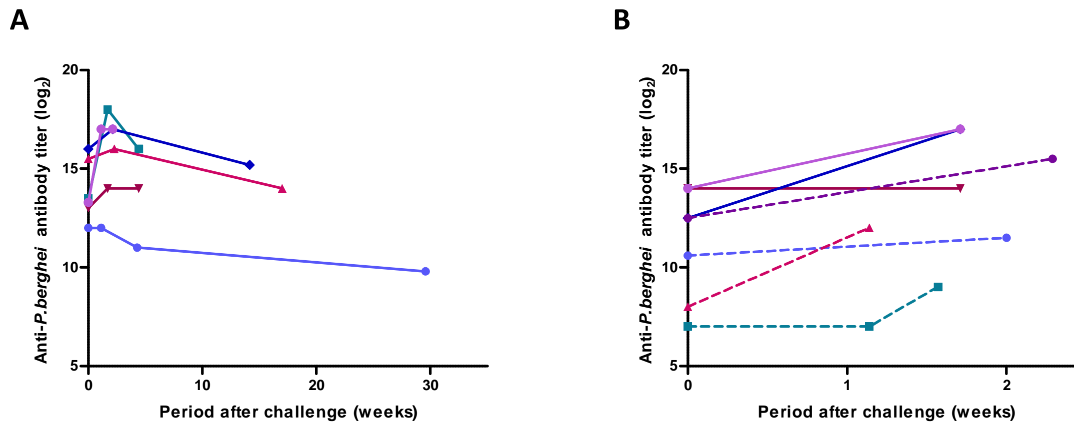

Figure 45. Influence of challenge on IgG production. Typical curves of anti-*P. berghei* IgG production after challenge on day 0 of six pre-exposed BALB/c females never pregnant before (A) and of seven females previously pregnant (B). On panel B dashed lines correspond to females that have succumbed after re-infection. Antibodies were measured in serum by IgG-specific ELISA.

Pre-exposed females previously pregnant challenged with non-pregnancy parasite during pregnancy showed poor control of parasitemia (Figure 44), and display a high mortality rate, regardless the slight increase in IgG production after challenge (data not shown). These data strongly suggest that pregnancy abrogates the malaria protection status conferred by pre-exposure and induces a state of long term susceptibility to non-pregnancy-associated *P. berghei*.

Anti-*P. berghei* antibody response in recrudescent females

We investigated whether the anti-*P. berghei* antibody response correlated with parasite recrudescence during pregnancy. Analysis of *P. berghei*-specific IgG in multigravida showed that the titers of anti-*P. berghei* IgG antibodies in sera of pregnant females with recrudescence were significantly higher than from pregnant females without recrudescence, independently of parity (Figure 46). This suggests that emergence of recrudescent parasite during pregnancy elicits a strong specific-antibody response to pregnancy-associated parasite.

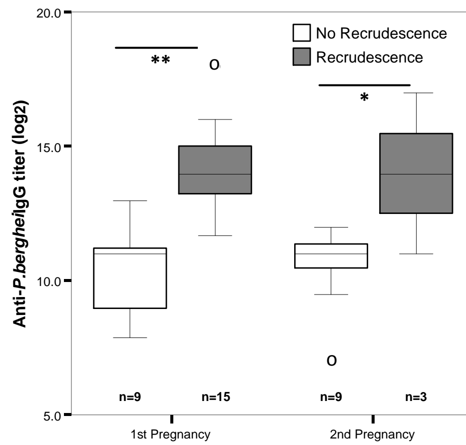

Figure 46. Anti–*P. berghei* IgG in serum samples from pregnant females grouped according to parity and recrudescence occurrence in pregnancy. Data are shown as medians (middle line in the box), central 50% of data (box), data range (whiskers) and outliers (o). The number of mice per group is indicated (*n*). Antigen preparations used in the ELISA was obtained from iRBCs of recrudescent females. Significant differences observed between antibody titers of individuals with and without recrudescence in each parity (**, *P-value* < 0.01; *, *P*-value < 0.05).

We next evaluated whether the immune response to malaria in pregnancy would favor particular IgG subclasses. Again, the antibody response was parity independent and was enhanced in presence of recrudescent parasite (Figure 47). IgG2a was significantly increased in sera from recrudescent pregnant females irrespective of parity, whereas IgG1 and IgG3 response appeared to be more relevant in recrudescent primigravida.

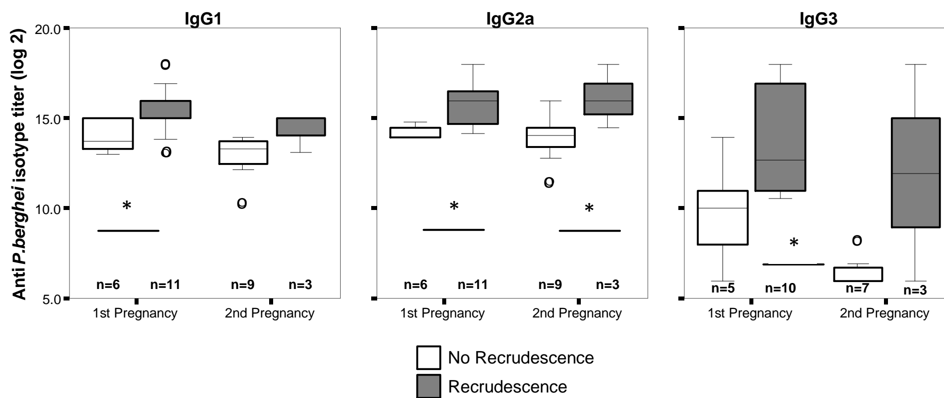

Figure 47. Anti-*P. berghei* IgG isotypes in serum samples from pregnant females grouped according to the parity and recrudescence occurrence in pregnancy. Data are shown as medians (middle line in the box), central 50% of data (box), data range (whiskers) and outliers (o). The number of mice per group is indicated (*n*). Significant differences observed between antibody titers are indicated (*, *P-value* < 0.05).

The IgG1/IgG2a ratios indicated a tendency for a relative increase in IgG2a, and thus towards a Th1 response, which appears to be more relevant in second pregnancies with recrudescence (Figure 48).

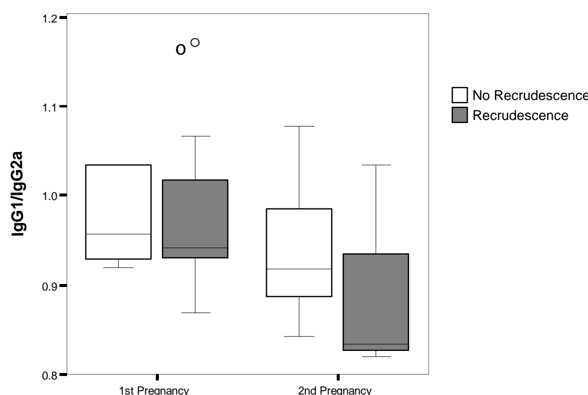

Figure 48. **IgG1/IgG2a antibody ratios**. IgG1/IgG2a ratios for pregnant females grouped according to the parity and recrudescence occurrence in pregnancy. Data is represented as in Figure 47.

- **Serum from *P.berghei*-immune mice does not confer protection against malaria in naïve individuals**

To investigate if malaria-specific antibodies elicited by PAM and non-PAM parasite cross-protected against blood-stage malaria infection, we transferred immune serum from one immune male and one pre-exposed multigravida into naïve mice. As a control, a group of mice received non-immunized serum. On the day after, mice that received the serum were injected with 10^5 iRBC using a group of pre-exposed immune mice as a control. No difference was observed in the parasitemia curves of the naïve mice receiving serum and disease protection was not observed in any of the serum-recipient naïve mice (Figure 49).

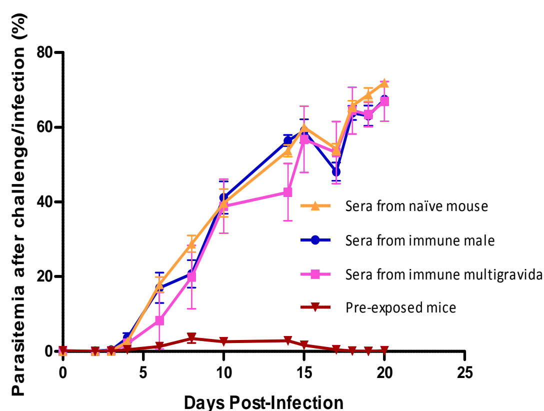

Figure 49. Passive transfer of immune sera to naïve mice is not protective. Serum samples were collected either from non-immune or immune mice (male and multigravida). Serum was injected i.p. into naïve BALB/c recipients (3 animals per group). On the day after passively immunized mice and pre-exposed mice were challenged with 10^5 iRBC from an infected male and parasitemia was followed as indicated.

While these results raise the possibility that serum transfer is not protective we cannot exclude that the observed lack of protection could be due insufficient antibody transfer. Four days after sera transfer, we detected low titers of specific-*P. berghei* IgG antibodies, in sera of three out of six recipient mice (data not shown). Future experiments using higher amounts of transferred antibody will be needed to test whether protection conferred by parasites recrudescing during pregnancy is specific to PAM or is also effective against blood stage infection in naïve mice.

DISCUSSION

The proposed goals of this thesis were focused on mouse models of pregnancy-associated malaria (PAM). Firstly, comments will be tailored on both established models and on their validation by paralleling them with the human disease. Secondly, the few immunopathological contributions attained with those models will be described. Finally, the potential applications and future directions of using mouse models to study PAM will be highlighted.

PAM MURINE MODELS

The first aim of this work was to establish and validate mouse experimental systems that represent congruent models representation of human PAM. According to Desowitz (Desowitz, 2001), an experimental model that represents malaria in pregnancy should comprise several attributes. Firstly, the maternal disease should show higher parasitemia and virulence as compared to non pregnant controls and reflect hematological changes, such as anemia. Secondly, the pregnancy outcome (offspring) should be affected by fetal abortions, low birth weight, impaired postnatal growth and/or reduced litter size. Finally, the placentas should manifest inflammatory/ histopathological defects and show reasonable pathogenesis congruency to human disease characteristics, including placental sequestration/cytoadherence phenomena.

The mouse models established in this work appear consistent with the main features of the PAM disease seen in women and their attributes are discussed in the context of standard indicators of detrimental outcomes of malaria in human pregnancy, namely:

- Mortality - maternal, fetal and infant (reflects the worst effects of malaria)
- Intrauterine growth retardation, low birth weight (used as a proxy measure of infant mortality; epidemiological marker for the impact of malaria in pregnancy)
- Peripheral parasitemias and placental parasites (used to detect malaria in pregnancy; it is recognized that peripheral parasitemias may remain below the level of microscopical detection while parasites are harbored in the placenta)
- Placental histology (histological examination of the placenta provides one of the most sensitive indicators of maternal infection especially in women with high premunition)
- Maternal anemia (important indicator to describe the impact of malaria in pregnancy)

Maternal and Fetal Consequences

In areas where malaria transmission is low or unstable, the levels of immunization are weak or inexistent and PAM clinical outcomes seem to be more severe for both to the mother and the fetus. The PAM experimental model here established, by infecting non-immune BALB/c mice during pregnancy, displayed enhanced disease severity and led to impaired fetal viability and post-natal growth. Thus, pregnant mice were more susceptible to *P. berghei* infection as they experienced faster increase in parasitemia and earlier death by hyperparasitemia as compared to non-pregnant mice. A significant proportion of the infected pregnant females most of the times abort or even die before parturition, without allowing the progeny observation (Table 2).

The mechanism by which maternal malaria contributes to IUGR and LBW is not fully understood. In human PAM the increased *P. falciparum* parasite density in the placenta and in maternal peripheral blood at delivery, is frequently associated with infant anemia and, consequently, child development and survival are at risk (le Cessie *et al.*, 2002; Brabin *et al.*, 2004a). Our experimental data showed a strong correlatyion between peripheral parasitemia and hemoglobin levels, both in pregnancy-induced recrudescence and in infected females. The infant risk of having low hemoglobin when birth weight was normal (> 2500 g) was greater in anemic mother than in non-anemic mothers. Moreover, infants born to anemic mothers have low iron stores, even when they are born at term with normal weight, and are more likely to develop anemia (De Pee *et al.*, 2002). In addition, anemia, pathologic disorders may complicate oxygen delivery to the fetus by interfering with the intrinsic respiratory capacity of the placenta at any given gestational age (Salafia *et al.*, 1995). Therefore, maternal anemia lead to insufficient *in uterus* hemoglobin/iron/oxygen availability, inducing a compensatory increase in the number of fetal circulating erythroblasts that we also observed in our experimental systems.

Many common diseases of pregnancy, including recurrent early miscarriage, pre-eclampsia and eclampsia, are characterized by inflammation in the fetal-maternal interface and/or systemic manifestations. Hypertensive disorders in pregnancy are estimated to cause 10-15% of maternal deaths. Pre-eclampsia, a pregnancy-induced hypertension in association with significant amounts of protein in the urine (protenuria), is the most frequently reported disorder and is more common in primigravida, both in malarious and in non-malarious areas (Brabin & Johnson, 2005). The placenta is essential for the pathogenesis of pre-eclampsia and it appears that the secretion of inflammatory mediators can activate the placental vascular endothelium leading to increased levels of endothelial markers, many of which are vasoactivators and procoagulant promoters, resulting in microtrombi formation. This leads to a vascularization restriction and high-flow blood flow across placental villi, with consequences for the mother and the fetus (Brabin & Johnson, 2005). In placental malaria, the parasites can stimulate host inflammatory mediators that directly activate endothelial cells which can lead to maternal hypertensive disorders or pre-eclampsia. Reduced placental perfusion and loss of endothelial integrity are common features of placental malaria and pre-eclampsia. However an

important distinction between both conditions is the frequent accumulation of maternal leucocytes in placental malaria and its absence in exclusively pre-eclampsia. This issue has recently received more attention since there are evidences that pre-eclampsia and malaria can be associated (Duffy, 2007). In a study from Tanzania, placental malaria was associated with hypertension in young first-time mothers who present histological features of disease but not in older or multigravida women (Muehlenbachs *et al.*, 2006). More longitudinal studies are needed to evaluate the interaction between placental malaria and pre-eclampsia.

The pre-exposure model, in which the females were immunized before pregnancy, corresponds to a mouse model of pregnancy-induced parasite recrudescence that recapitulates epidemiological observations of PAM occurring in pre-immune women that live in regions with high-endemicity. There is solid epidemiological evidence from high-endemicity malaria regions that the incidence of PAM in women is parity-dependent (Rogerson *et al.*, 2007), since resistance to pregnancy malaria is acquired over successive pregnancies. In the pre-exposure PAM model we have found that disease incidence and severity decreased with parity. Furthermore, the adverse pregnancy outcomes from recrudescent mothers were also reduced with parity, since we observed an increased recovering of both the litter size and birth weight. Nevertheless, we did not find a correlation between the intensity of the parasitemia peak in the pre-mating period and the occurrence of pregnancy-induced recrudescence in the first pregnancy, implying that such previous exposure did not confer PAM protection. Remarkable was the observation of parasites in placentas and associated pathology in the absence of maternal peripheral parasitemia. This event is especially common in pregnant women from high transmission regions (Dorman & Shulman, 2000).

Our experiments confirmed that PAM in pre-exposed individuals does not require re-infection and suggest that malaria recrudescence during pregnancy requires pregnancy-specific factors. More specifically, parasite recrudescence was never detected before G12, and most frequently parasitemia arose after G14. These observations converge to the notion that the vascularization of placenta occurring at G12.5 plays a critical role in murine PAM development, possibly having a role on promoting parasite recrudescence.

All together, our findings appear to parallel human PAM characteristics in terms of pregnancy outcome and placental damage and inflammation, which are underlying the clinical manifestations observed in humans (Nosten *et al.*, 1991; Rogerson *et al.*, 2003b). Our observations also support the hypothesis that PAM protection in the mouse is progressively acquired through repeated exposure to malaria in pregnancy which is in line with a recent report on cumulative immunity to PAM over several pregnancies (Megnekou *et al.*, 2009).

Placental Pathology Caused by *P. berghei*

In the course of *P. falciparum* infections, the placenta can harbor a striking density of parasites, macrophages, hemozoin and excess of fibrinoid deposits associated to morphologic alterations, such as necrosis and trophoblast basement membrane thickening (Walter *et al.*,

1982) that would be harmful to the developing fetus, as the placental exchanges of respiratory gases and nutrients became difficult and reduced. Monocytic/macrophagic infiltrate has been considered a hallmark of *Plasmodium*-infected placentas (Rogerson *et al.*, 2003b; Diouf *et al.*, 2004).

P. berghei infected placentas showed general tissue architecture disorganization with prominent thickening of the trophoblast basement membrane and fibrinoid deposits. This may result in part from fibrosis which has been proposed to arise from the reparative process stimulated by the response to infection (Oduola *et al.*, 1986). However, extensive fibrinoid necrosis and fibrinoid deposition are abnormal and typical of malaria infected placentas (Davison *et al.*, 2000). It is noteworthy that in mouse placentas we observed low degree of massive chronic intervillositis as compared to reported observations in infected human placentas (Ordi *et al.*, 1998). Possibly this difference is related to the short pregnancy time span that may condition the inflammatory process in the mouse as compared to human pregnancy. We hypothesize that accumulation of *P. berghei*-GFP iRBC in the placenta may evoke the inflammatory response that resembles the placental malaria pathology attributed to *P. falciparum*. We firstly demonstrate that these characteristics can be observed in the model system using non-immune BALB/c mice. A striking pathological finding in infected placentas was the reduction of blood sinusoids space, which is attributable to placental tissue thickening that presumably compressed available blood vascular space. Furthermore, in recrudescent placentas the reduction of the blood sinusoidal space is highly dependent on the parasitemia level, reinforcing the notion that the parasite has a pivotal role in the genesis of the placental pathology.

Interestingly, we found that in some non-recrudescent females the placentas could harbor a very low density of iRBC, which nevertheless seemed high enough to trigger placenta pathology. We speculate that these pathologic mechanisms would explain the death of pregnant females that do not show peripheral parasitemia but exhibit placenta pathology. Cytoadherence of *P. berghei*–infected erythrocytes to receptors expressed on the syncytiotrophoblast surface is considered to contribute to the described placental disorders, but might not be a sufficient condition for pathogenesis, as well as in cerebral malaria syndrome (Schofield, 2007). Placental malaria studies propose that the observed intervillositis is mostly an immunopathologic process, due to cytokines and chemokines production, leading to the activation of the syncytiotrophoblast (Fievet *et al.*, 2001; Lucchi *et al.*, 2008).

Although we have found important pathological changes in both basal and labyrinthine zones of mouse placenta, parasites and hemozoin were never visualized in the fetal circulation and positive parasitemia was never recorded in newborns from infected mothers. The absence of evidence for congenital infection, despite the presence of numerous iRBC in the placental maternal blood, points to the efficacy of the placental trophoblastic layer to block parasite traversing to fetal blood. The mechanism by which the trophoblastic cells prevent fetal infection is poorly understood, but several trophoblast defense mechanisms have been

described, including its capacity of producing immune cells chemoattractants (Guleria & Pollard, 2000) or its selective phagocytic ability allowing the removal of infectious agents from the maternal-fetal interface (Amarante-Paffaro *et al.*, 2004).

The vascular control in the placenta is dependent in large part on locally produced vasoactive compounds and the loss of main vasodilator properties can have significant consequences on the intraplacental perfusion possibly intensifying local areas of hypoxia (Bainbridge & Smith, 2005) and on the triggering of pre-eclampsia (Brabin & Johnson, 2005). We could not detect a significant increase in Hypoxia-Inducible Factor-1alpha (HIF-1α) gene expression in infected placentas (data not shown). It is likely that tissue stress responses induced by the alterations in placenta blood circulation could play a role in placental physio-pathology. Placenta microcirculation is in part controlled through a fine balance between different vasoconstrictors such as ET-1, and vasodilators like HO-1 enzyme (Bourgeois *et al.*, 1997; Bainbridge & Smith, 2005). Recrudescent placentas showed an increase in both ET-1 and HO-1 mRNA expression, suggesting that the placenta vasculature is exposed to an abnormal vasoactive regulation.

Adhesion of *P.berghei*–iRBC to Mouse Placenta

One of the currently proposed roles for the placenta in *P. falciparum* PAM pathogenesis is to provide new ligands that are recognized by the iRBC. The adhesion mechanisms of iRBC in *P. falciparum* infected placentas remains controversial, but the main placental candidate receptors and their cognate parasite ligands participating in iRBC adhesion have been identified. Our data show that CSA and HA can be important candidate adhesion receptors in mouse placentas. Additionally, iRBC collected from recrudescent females displayed a marked enhancement of CSA and HA binding properties, and thus we raise the hypothesis that *P. berghei* expanding during PAM is positively selected by the ability to bind placental ligands. This hypothesis is highly supported by recent work showing that pregnant mice acquire immunity specific to the recrudescent parasite (Megnekou *et al.*, 2009). Overall, the experimental data suggest that cytoadherence of *P. berghei*-GFP in the placenta may involve CSA and HA as receptors and raises the hypothesis that human and murine malaria in pregnancy have similar pathogenesis basis.

In human PAM specific *P. falciparum* parasites expressing variants of the PfEMP1 such as the molecule encoded by the *var* gene *var2csa*, are probably expanded via the increased cytoadherence of the iRBC to the placental receptors, prominently CSA (Duffy *et al.*, 2005). Although PfEMP1 orthologues were not yet found in *P. berghei*, our findings raise the interesting possibility that the receptors mediating adhesion in the mouse placenta could have in *P. berghei*-iRBC cognate ligands. Even though antigenic variation had been shown in other murine malaria species, such as *P. chabaudi* AS (Phillips *et al.*, 1997; Janssen *et al.*, 2002), it is noteworthy that *P. berghei* VSAs were not described so far, although an ancient study (Cox, 1959) show that *P. berghei* relapsed parasites (relapsing 90 or more days after infection followed by chemotherapic treatment) led to an increased virulence when compared to stock

parasites. Nevertheless, our data opens the possibility that murine PAM entails an overrepresentation of *P. berghei* - iRBC displaying parasite components that mediate the iRBC-placenta interactions.

IMUNOPATHOLOGY IN MOUSE PAM

The second aim of our investigation was to show that PAM mouse models presented in this thesis could contribute to a better understanding of the immunological basis of the protection/susceptibility to malaria shown in pregnancy and of the underlying molecular mechanisms.

Placental inflammatory response to infection in pregnancy

Systemic immunity to malaria in non-pregnant mice has been subject of intense investigation and is very well documented. The early response mainly involves the innate system and is dominated by Th1-type cytokines, namely IL-12 and IFN-γ. In mice, blood-stage *P. berghei* XAT infection induces IL-12 production, important for the development of host resistance via IFN-γ production, which promote anti-parasitic properties, at least in part by generating high levels of TNF-α and NO (Yoshimoto *et al.*, 1998). However, during pregnancy there may be a bias towards a Th2-type response and the placenta may synthesize anti-inflammatory cytokines to antagonize pro-inflammatory responses that could otherwise be harmful to the fetus.

Our data show that, in mice infected during pregnancy, the malaria infection rapidly becomes established in the placenta, as revealed by the placental accumulation of luciferase-expressing *P. berghei* parasites. In the placenta, the parasite could take advantage of a propitious environment for its survival, due to the absence of inflammatory mediators and dominated by Th2-like microenvironment necessary to the pregnancy success. Nevertheless, the presence of parasites in the placenta can activate the trophoblast that has a role in shaping the local immunological milieu (Lucchi *et al.*, 2008) and synthesize chemoattractants that recruit monocytes/macrophages to this site of infection. Hofbauer cells, the placental resident macrophages, can also be stimulated to produce β-chemokines chemotactic for monocytes/macrophages. This is in line with our observation that the chemokine MIP-1α is up-regulated in infected placentas. MIP-1α is produced by monocytes, macrophages, lymphocytes and other cells. Such type of inflammatory triggering can explain the observed recruitment of a mononuclear infiltrate that predominate in maternal blood spaces of the labyrinthine zone. Expression analysis of cell-type specific markers indicates that in the case of placentas from recrudescent mice the mononuclear infiltration includes NK cells, T lymphocytes and macrophages ad is associated with increased expression of the chemokines MCP-1 and MIP-1α (Figure 32A and 32B). Interestingly, in our model, IL-12 expression in the placenta was significantly decreased (Figure 32C), while the expression of TNF-α and IL-10 was increased. This down-regulation of IL-12 production was suggested to be due to the inhibitory effects of hemozoin.

We noted that iRBC were in intimate contact with placental tissue components and that hemozoin was widely spread in maternal blood spaces of infected placentas. In fact, hemozoin can accumulate in tissue and within macrophages, remaining for several months after parasite clearance, leading to placental function impairment and having inhibitory effects on tissue monocytes and macrophages (Sullivan *et al.*, 2000; Schwarzer *et al.*, 2001). Early events in the cell-mediated immune response required for protection against malaria are initiated by the release of IL-12 from monocytes/macrophages, dendritic cells and neutrophils. However, it was shown that the constitutive production of IL-12 by monocytes is inhibited following phagocytosis of small amounts of hemozoin. Ingestion of hemozoin may possibly have differential effects on cytokines production, namely by enhancing IL-10 production and suppressing IL-12 release (Luty *et al.*, 2000). These observations allow the speculation that the strong local inflammatory environment generated by the iRBC adhesion is counteracted by a systemic anti-inflammatory response. In fact, we noted that the up-regulation of *IL10* expression was correlated with the severity of placenta pathology (Figure 32E). Coincidently, IL-10 levels in the serum were associated with poor pregnancy outcomes and this cytokine has been suggested as a biomarker for placenta inflammation in pregnant women (Kabyemela *et al.*, 2008).

It remains to be resolved whether T cell infiltration corresponds to activated effector T cells or to T regulatory cells as part of a placenta anti-inflammatory response. IL-12, produced by activated macrophages, DCs, B lymphocytes and neutrophils, has an important role in inducing NK cells to produce IFN-γ. However, in recrudescent placentas the IL-12 expression decreases and IFN-γ levels did not increase substantially, despite the presence of more NK cells and T cells. Similarly TNF-α expression was not substantially increased even though the increased number of recruited macrophages to the placenta. These overall results may be a consequence of induction of regulatory cytokines that act to suppress synthesis of inflammatory cytokines. In fact, placental IL-10 expression was substantially increased in placentas with higher parasitic load. In sum, *P. berghei* clearly induces a Th1/Th2 disequilibrium in the placenta favoring inflammatory responses; IL-10 is also increased and should remain effective in protecting the placenta by controlling the negative effects of Th1-type cytokines. This is of utmost importance as it allows the mother to keep nurturing and protecting the fetus. However, at delivery numerous placentas remain infected, suggesting that the parasite-induced response is not effective enough to clear placental infection. An additional increase in Th1 response could be efficient in clearing infection but could endanger the fetus, given the detrimental effects of TNF-α and IFN-γ on pregnancy.

Classically, BALB/c mice show a natural bias towards Th2 responses (Hansen *et al.*, 2003). When infected with *Leishmania major*, also a protozoan, produce low levels of IFN-γ and high levels of IL-4 and IL-5 (Scott *et al.*, 1989). Conversely, C57Bl/6 that has a higher Th1 response is capable of self-healing the infection, while BALB/c develop a non-healing and fatal infection. Therefore, it would be interesting to evaluate if mice with a stronger inflammatory response

(characterized by elevated levels of IFN-γ, for example) and showing a more efficient initial malaria attack would have less deleterious placental consequences in terms of parasite accumulation and pathology.

Adaptive immune response in mouse pregnancy

Pregnant women develop antibodies that are pan-reactive towards placental parasites isolates from different geographic areas (Fried *et al.*, 1998b; Lekana Douki *et al.*, 2002). This has suggested that the antigenicity of the placental parasite drives a humoral specific response that contributes to protect mutigravida from severe forms of PAM.

We observed that pre-exposed aparasitemic mice display long-lasting high titers of anti-parasite IgG (Figure 43) and we also observed likewise that the parasites may persist for long periods in different organs of pre-exposed mice. Such mice are protected of severe disease, as they develop a strong response upon parasites challenge that is revealed by a transient parasitemia peak that resolves to apparent cure. These observations sustain the interpretation that residual and persistent infection supports a continued humoral anti-parasite response that would contribute to a long-term malaria protection state.

We also observed that pre-exposed females that experience pregnancy maintain high titers of antibodies against parasites derived from placenta, implying that these mice were immunocompetent towards *P. berghei*. This was particularly visible in recrudescent females irrespective of parity (Figure 46), which display higher levels of IgG (and its subclasses) as compared to their non-recrudescent counterparts. These results are suggestive that residual *P. berghei* in pre-exposed females elicits further antigenic stimulation during pregnancy particularly when the parasite recrudescence is apparent.

Surprisingly, we found out that pre-exposed females that are pregnant or that experienced pregnancy, loose strong malaria protection upon challenge with parasite of non-placental origin (see Figure 44). These data strongly suggest that pregnancy abrogates the malaria protection status conferred by pre-exposure and induces a state of long term susceptibility to non-pregnancy-associated *P. berghei*.

This breakage of malaria protection does not correlate with inability to mount an anti-parasite humoral response and contrasts with the increased premunition against recrudescent *P. berghei* acquired by pregnancy-experienced females. Thus, the immune response that confers protection to pregnancy–associated *P. berghei* appears not to protect from non-placental *P. berghei*. This implies that the immunogenicity of *P. berghei* involved in PAM differs from regular *P. berghei*. This is line in with a report demonstrating differential specificities in the humoral response to *P. berghei* parasites collected from pregnant females (Megnekou *et al.*, 2009) and raises the possibility that antigenic specificities are critical to confer protective immune response to the malaria parasites.

Notwithstanding, it is intriguing that pre-exposed females that are pregnant or have experienced pregnancy not only to gain PAM-specific premunition but sharply loose the previous protection against non-placental parasite. This also suggests that recrudescent *P. berghei* in pregnancy drives an immune response that abrogates the protection conferred by previous exposures to parasites that possibly show different antigenicity. This abrogation of previous immunological protection could be a general mechanism by which malaria parasites escape to immunological control in successive infections, as is frequently observed in high-transmission regions.

An alternative explanation for the lack of correlation of the high-antibody titers and disease protection could be that the humoral response is not a relevant contributor to the effectiveness protection against malaria. This hypothesis would explain the failure of hyperimmune serum transfers in protecting from infection (Figure 49). In this context it remains to be analyzed the possibility that the effectiveness of previous humoral response to malaria parasite is compromised as the proportion of asymmetric antibodies raises in the pregnant females. Presumably, this could lead to a repertoire that is able to recognize the parasite antigens but would not be able to elicit the effector phase of the immune response.

CONCLUDING REMARKS

This thesis describes two PAM mouse models that are based on experimental placental infection by *P. berghei*. These PAM mouse models were scrutinized for pathological and immunological criteria that are relevant for human PAM. We conclude that experimental PAM constitutes a lever for new approaches in studying PAM pathogenesis and identifying PAM protection mechanisms.

The experimental model established in non-immune mice showed enhanced disease severity and magnified pathology phenotypes as compared to the human disease. The prominent histological alterations in mouse placenta heavily infected with *P. berghei* resemble those described for acute *P. falciparum* malaria in humans. Such phenotypic exacerbation allowed us to identify trophoblast thickening and vascular space reduction as hallmarks of placenta malaria induced by the *P.berghei* and to hypothesize that placenta immunopathology results from an exacerbated inflammatory response to the presence of adherent *P. berghei*. In the pre-exposure (pregnancy-induced malaria recrudescence) mouse model the intensity of parasite recrudescence showed to be quantitatively correlated with the placenta pathology while the recrudescence incidence and adverse pregnancy outcomes decreased with parity.

P. berghei-GFP adhesion experiments indicate that iRBC express ligands for different receptors in the mouse placenta. iRBC from recrudescent females displayed enhanced adhesion to the placenta suggesting that *P. berghei* parasites mediating PAM have increased specificity for placenta receptors.

We observed that pre-exposed females showed residual but persistent *P. berghei* infection that could explain the long-lasting anti-parasite humoral response that would contribute to a long-term malaria protection state. Pregnancy abrogates this malaria protection status strongly indicating that the host mechanisms that confer protection to pregnancy–associated *P. berghei* appear not to protect from non-placental *P. berghei*. This observation compelled us to hypothesize that the immunogenicity of *P. berghei* involved in PAM differs from regular *P. berghei* in an analogy with the *P. falciparum* variants involved in human PAM.

The data provided in this thesis demonstrate that the experimental systems based on *P. berghei*-BALB/c mouse are valid models to study the pathogenesis of placenta malaria, the adhesion of placenta parasites and the parasite-placenta interaction and the mechanisms of PAM protection elicited during pregnancy.

FUTURE PERSPECTIVES AND DIRECTIONS

The experimental systems presented in this thesis could prove useful in drawing hypothesis and testing analogies on the factors and mechanisms that are considered relevant for human PAM. The data presented in this work suggests a number of research avenues that could be followed for the detailed characterization of the parasite-placenta interaction in the mouse, the dissection of the inflammatory components of placenta malaria and the analysis of the antigenic specificities that are involved in PAM acquired protection (see Table 6). Many of these questions remained unsolved in human PAM and these investigations in experimental systems could provide new hypothesis and solutions to be tested in human PAM.

Table 6. **Exploring PAM mouse models**

Parasite-Host Interaction Characteristic	**Approach**
Antigenic specificity of placental *P. berghei*	• Immunologic and molecular studies and gene expression analysis of antigens of recrudescent parasites
***P. berghei* ligands mediating placental sequestration**	• Ligand-specific binding assays and analyze knockout parasites for candidate ligands
Placenta receptors for iRBC	• Purify and test individual mouse placental potential receptors for iRBC (GAGs), setting up *in vitro* cell culture systems
Dynamics of placenta sequestration	• *In vivo* imaging of recrudescent and non-recrudescent parasite in pregnant females
Host inflammatory components in pregnancy-induced recrudescence	• Make use of knockout mice to identify several factors involved in PAM immunopathology • Investigate the existence of soluble peripheral markers that quantitatively reflect placental disease
Protective immunity in multiparity	• Identify antibodies and T cell responses specific for the placental *P. berghei* • Assess the role of asymmetric antibodies in PAM
Vertical transmission of malaria and protection	• Analyze the cellular response of newborns to malaria antigens • Evaluate the transmission of protection by anti-parasite antibodies

There are a plethora of other unknown mechanisms worthy of further investigation using PAM models. Mouse models of PAM can even be used in more generic studies concerning malaria in pregnancy, namely:

- Perform drug trials in pregnancy. The identification of safety/toxicity and effectiveness of novel anti-malarials for pregnancy is urgent (WHO, 2006) and it would be appropriate to evaluate the mouse models contribution in this respect. Anti-malarial drugs have been facing problems of parasite resistance and recrudescence after drug treatment is more common during pregnancy. The spread of drug-resistant parasites has eroded the value of the few drugs considered safe in pregnancy.
- Contribute to PAM vaccine studies to prevent pregnancy malaria by identifying in *P. berghei* proteins targeted by the PAM protective immune responses. This would provide additional vaccine targets to be tested in *P. falciparum* to complement current efforts to produce a VAR2CSA-based vaccine.
- Analyze how placental malaria and pre-eclampsia interplay. Pre-eclampsia, the disease with the highest pregnancy related maternal lethality in western countries, it is caused by impaired trophoblast invasion and placental vascularization alterations, which can be caused by inflammation. Recent research interests are focusing in studying the interaction and overlap of malaria and pre-eclampsia and the experimental systems presented in this thesis provide a useful tool for this research.

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