Unraveling maternal and fetal genetic factors protecting from Pregnancy Associated Malaria in the mouse

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Knowledge Creation

Preface

This thesis resulted from the work I developed at Instituto Gulbenkian de Ciência from April 2009 to September 2013, where I was enrolled in the internal Doctoral Program PGD2008 under the supervision and guidance of Dr. Carlos Penha Gonçalves.

All work presented here was carried out at Instituto Gulbenkian de Ciência.

Financial support was provided by Fundação para a Ciência e Tecnologia, Portugal, trough my PhD fellowship grant: SFRH/BD/33566/2008.

The thesis is composed of four chapters:

Chapter one comprises a general introduction providing an overview on malaria with emphasis in Preganancy associated malaria and including a description of human and murine placental structure, a detailed review on the existing PAM mouse models and a summary of TLR4 and IFNAR1 involvement in pregnancy and malaria.

In chapter two presents the work published in 2012 referring to the development of new PAM mouse models.

Chapter three includes a manuscript prepared for publication that dissects maternal and foetal contributions of TLR4 and IFNAR1 to PAM.

Chapter four contains general conclusions and discussion on the work presented in this thesis.

Acknowledgements

I would like to thank Instituto Gulbenkian de Ciência for accepting me into its excellent PhD Program.

To all people providing technical support, for their outstanding professionalism, allowing the successful development of the work presented in this thesis.

To Carlos for taking the risk of being my supervisor. For guiding me through this process with patience and making me grow as scientist and person.

À minha família. Por sempre me terem apoiado, nunca questionarem as minhas escolhas e estarem sempre presentes nos momentos de relativo desespero e partilharem comigo todas as alegrias.

To Bruno, for *we* have trived trought two PhDs.

To all my friends and colleagues for surviving my PhD. For the patience in the lab meetings and pre-institutional seminars periods; for showing me that cassowary exists and dingoes like apples; that pizza in Portugal can be very tasty when eaten in the right company; that football games during dinner time can make it difficult to have "the family" gathered for a meal; that "Chinese chopsticks" are a good way to separate hepatocytes; that there is always someone there to discuss scientific or personal issues; that

very good friends can came out of the working environment.

For all this and other reasons I will remember only after the thesis is printed,

MUITO OBRIGADA!

This dissertation was completed with financial support from Fundação para a Ciência e Tecnologia.

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ABREVIATIONS:

TLR – Toll-Like Receptor

PBLs – Peripheral Blood Lymphocytes

CAM - Chorioamnionitis

PTB – Preterm Birth

LPS – Lypopolisacharide

LBW – Low Birth Weight

IUGR – Intrauterine Growth Reduction

G *n* – Gestational day *n*

E *n* – Embrionic day *n*

IE – Infected Erythrocytes

PM - placental malaria

CM - cerebral malaria

ECM – experimental cerebral malaria

TIR – Toll IL-1 receptor

PRR – Pattern Recognition Receptor

PAMP – Pathogen Associated Molecular Pattern

ICAM-1 – Intercellular Adhesion Molecule 1

CSA – Chondroitin Sulphate A

CSPC – Chondoitin Sulphate Proteoglycan

PAM – Pregnancy Associated Malaria

IFNAR1 – Interferon Type I alpha, beta receptor

SIAP-1 – Sporozoite invasion-associated protein 1

EBL – erythrocyte binding-like

GPI – glycosylphosphatidylinositol

DBL – Duffy binding-like

PBMCs – Peripheral Blood Mononuclear Cells

AMA-1 – Apical Membrane Antigen 1

RON – Rhoptry Neck protein

VSA – Variant Surface Antigens

PfEMP1 – *P. falciparum* Erythrocyte Membrane Protein 1

TSP – Thrombospondin

EGF – Epidermal Growth Factor

TRAP – Thrombospondin-related Anonymous Protein

PTRAMP – *Plasmodium* Thrombospndin-related Apical Merozoite protein

MTRAP – Merozoite TRAP

Abstract

Malaria is one of the most devastating diseases in the world. In *Plasmodium* endemic regions, pregnant women are among the most vulnerable groups. Pregnancy Associated Malaria (PAM) threatens both maternal and foetal lives. Despite differences between human and mouse placentas PAM mouse models recapitulate key pathological features of human PAM. Here we describe new PAM models of mid gestation infection in the C57BL/6 mouse. We demonstrated that infection with *P. berghei* variants NK65, K173 and the mutant ANKAΔ*pm4* reproduce main PAM features such as: increased parasitaemia in pregnant females; elevated number of stillbirths; decreased foetal weight and placental pathology. The NK65 model was used to investigate the role of host factors, namely TLR4 and IFNAR1 in PAM outcomes. Making use of heterogenic pregnancies we dissected the contributions of maternal *versus* foetal TLR4 and IFNAR1 in poor pregnancy outcomes. We demonstrated that TLR4 expression in foetal placenta contributes to foetal viability in infected pregnant females. Accordingly, primary trophoblast cultures showed that foetal TLR4 contributes to the response against Plasmodium-infected erythrocytes. The same genetic mating strategy was used to reveal that maternal but not foetal IFNAR1 contributes to the pathogenesis of PAM namely, to increased levels of

maternal parasitaemia, higher percentage of abortions and low birth weight.

Taken together, the generation of heterogenic pregnancies using this PAM model revealed a dual role of TLR4 and IFNAR1 inflammatory molecules in PAM, showing that maternal cells activation increases disease severity while placental cell responses confers foetal protection. This work provides an experimental system to dissect maternal from foetal components in the pathogenesis of PAM, which may poof useful for the molecular analysis of other pregnancy disturbances such as preeclampsia.

Sumário

O Paludismo é uma das doenças mais devastadoras no mundo. Nas regiões onde o *Plasmodium* é endémico as grávidas estão entre os grupos mais vulneráveis à doença. O Paludismo Associado à Gravidez (PAM para *Pregnancy Associated Malaria*) ameaça tanto a vida da mãe como a do feto. Apesar das diferenças entre as placentas humana e de murganho, modelos de PAM em murganhos recapitulam as principais características patológicas de mulheres grávidas infetadas com *Plasmodium*. Neste trabalho é descrito um novo modelo de PAM onde murganhos da estirpe C57BL/6 são infetados a meio do período de gestação. É demonstrado que a infecção com as variantes NK65, K173 e o mutante ANKAΔ*pm4* do parasita *P. berghei* reproduzem as principais características de PAM, tais como: elevados níveis de parasitémia em fêmeas grávidas; elevado número de nados-mortos; redução no peso dos fetos e patologia da placenta. O modelo com a variante NK65 foi posteriormente utilizado para investigar a contribuição de fatores inflamatórios, nomeadamente de TLR4 e IFNAR1, para a patogénese da doença durante a gravidez. Utilizando cruzamentos genéticos para gerar gravidezes heterogénicas foi dissecada a contribuição do TLR4 e IFNAR1 maternos *vs* fetais para a patologia. Demonstrou-se que a expressão de TLR4 na placenta fetal contribui para a viabilidade fetal em fêmeas grávidas infetadas. Em concordância com estes

resultados, demonstrou-se em culturas primárias de trofoblastos que a expressão de TLR4 contribui para a resposta contra eritrócitos infetados com *Plasmodium*. A mesma estratégia de acasalamento foi utilizada para revelar que o IFNAR1 materno, mas não o IFNAR1 fetal, contribui para a patogénese de PAM nomeadamente, para elevados níveis de parasitémia materna, elevada percentagem de abortos e reduzido peso dos fetos.

Em suma, a geração de gravidezes heterogéneas utilizando este modelo de PAM revelou um efeito dual para as moléculas inflamatórias TLR4 e IFNAR1 em PAM, demonstrando que o estímulo através das células maternas aumenta a severidade da doença ao passo que as células placentárias fetais, quando estimuladas, conferem proteção ao feto. Este trabalho apresenta um modelo experimental que possibilita a dissecção do papel dos componentes fetal e materno para a patogénese da PAM podendo provar-se útil para a análise molecular de outras patologias que ocorrem durante a gravidez, como por exemplo a pré-eclampsia.

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Chapter I

- General Introduction

1.1 Malaria Overview

Malaria is one of the most devastating diseases in the world particularly in tropical countries, accounting for roughly 216 million cases of *Plasmodium* spp. infection and 665.000 deaths from malaria in 2010 [1].

In the late 19th century, the discovery of the malaria parasite and its mode of transmission, allowed the use of focal mosquito control and the wide availability of proper disease diagnosis and treatment led to virtual elimination of malaria in most northern countries in Western Europe. WHO launched the Global Malaria Eradication Programme in 1955, when effective elimination tools as DDT and chloroquine became widely available, for mosquito control and human blood stage parasite treatment. As a result, 37 previously endemic coutries were free of this disease by 1978, including 27 in Europe and the Americas [2].

In other countries, as India and Sri Lanka, the burden of disease and deaths from malaria were greatly reduced. However, failure to sustain the programme led to a resurgence of malaria in many countries leading to increased parasite resistance to chloroquine and its replacements and mosquito resistance to DDT. The adoption of the Global Malaria Control Strategy, in 1992 and in 1998, the Roll Back Malaria initiative,WHO instigated the resurgence of financial investment in

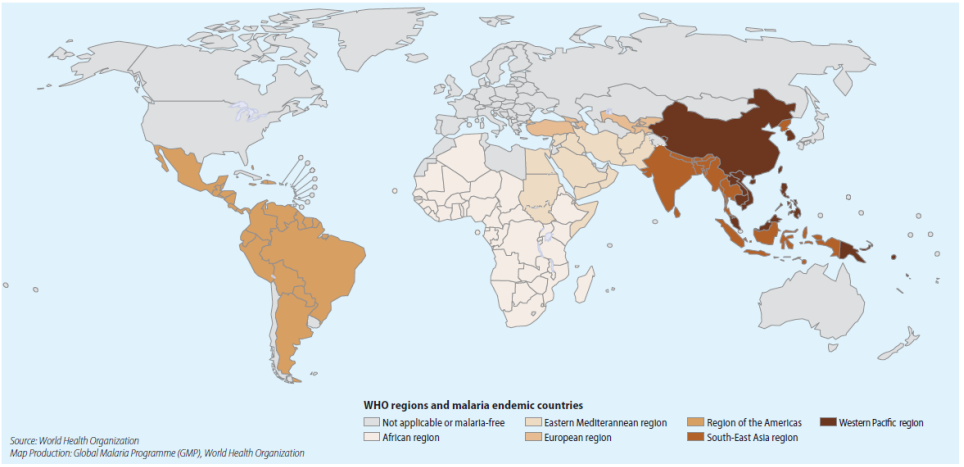

Figure 1.1 - Malaria Endemic Regions and Countries according to WHO official World Malaria Report 2012 [3]

malaria control, resulting in the adoption of artemisinin-based combination therapies for the treatment of malaria patients, the large scale deployment of insecticide-treated nets and, to a lesser extent, house spraying as mosquito control measures [2].Despite recent progresses, malaria is nowadays endemic in 99 countries worldwide, remaining an intractable problem in much of Africa.

Malaria is a disease caused by Apicomplexan pathogens of the genus *Plasmodium*, of which *Plasmodium falciparum* is the most deadly. *Plasmodium* infection usually results in an uncomplicated, mild febrile disease in which intermittent episodes of fever and peaks of parasitemia are controlled by the host's immune system and, eventually, eliminated. In some cases, however, the disease becomes severe and may

lead to death. Severe malaria, induced by *Plasmodium falciparum* in humans, encompasses serious complications including cerebral malaria, respiratory distress, metabolic acidosis and severe anemia.

In malaria endemic regions, pregnant women and children under 5-years of age are the most vulnerable groups. It is estimated that nearly a quarter of all childhood deaths are caused by malaria and with over 10,000 maternal deaths *per annum* mainly attributed to *Plasmodium falciparum* infection [4-7].

Malaria parasites are known for having a significant genetic and genomic plasticity [8], a characteristic that allows the parasite to evade the host immune system and favors the odds for the emergence of drug resistant strains. Although combination therapies with artemisinin derivatives are now highly effective, recent reports on emerging resistance to these compounds [9] call the urgency for new antimalarial drugs [6]. Likewise, vector research will be essential for the development of new insecticides as insecticide resistance is also becoming a major problem [10].

An effective malaria vaccine may ultimately complement available control strategies offering the most cost-effective tool for disease prevention and eradication. In the recent years, considerable effort is being put towards vaccines aiming at significantly reducing *Plasmodium falciparum* induced morbidity and mortality [7]. One of the main global instigators/funders of

this research goal is the Melinda and Bill Gates Foundation that, in 2006 funded the Malaria Vaccine Technology Roadmap [11]. This action has established a global strategy with two important goals for malaria vaccine research: a vaccine that is 50% protective against severe disease and death by 2015, and a vaccine that prevents 80% of clinical malaria episodes by 2025.

Such initiative notably boosted the progress in malaria vaccine in the last decade, with many different approaches being followed. One such research has dominated the advances for malaria vaccination; RTS,S – the first malaria vaccine candidate entering the phase 3 trials, involving 16,000 children in seven African countries [12, 13]. Preliminary data from this trial has shown to reduce clinical malaria episodes, and severe malaria episodes that carry a risk of death, by approximately 50% in groups of children aged 5 to 17 months. Even though protection waned within a few months, and the indications that, in younger children (6–12 weeks) vaccine efficacy is reduced to 36.6% [12, 13], RTS,S continues on the path to licensure due to its anticipated ability to reduce severe morbidity in young children [7].

In face of such results, extra pressure is now in the hands of researchers developing new *P. falciparum* vaccines as they will need to show signifficantly higher protective efficacy than the 30–50% reported

for RTS,S. Furthermore, if malaria eradication is to be achieved, additional attention should be put in vaccine development efforts for species other than *P. falciparum*, especially *Plasmodium vivax* as other countries, such as India, are still facing high malaria endemicity [14].

Despite all the effort around the development of a malaria vaccine, this might unfortunately not be such an easy and hasty goal to achieve. Additionally, the parasites genetic variability and plasticity, together with mosquito's ability to gain insecticide resistance require a constant need of immediate advances in drug and insecticide improvement.

In this context, the growing body of molecular understanding on the host-parasite interactions responsible by disease severity presents as an important tool in the development of new and more effective therapies.

1.2 Parasite life cycle - general overview

The *Plasmodium* spp. is a protist of the phylum Apicomplexa. These parasites need to cycle between an invertebrate vector (hematophagous insect e.g. the genus *Anopheles*, human vector) and a vertebrate host (mammals, birds or reptiles) in order to complete their life cycle that includes several development stages and, sexual and asexual, reproduction phases [15].

1.2.1 *Plasmodium* spp. developmental stages in the invertebrate vector

The *Plasmodium* spp. life cycle in the mosquito begins when a female mosquito takes a blood meal from an infected vertebrate host carrying gametocyte parasite forms. Once in the mosquito, the ingested infected blood goes into the posterior midgut lumen, where male and female gametocytes initiate gametogenesis. This stage is induced by the decrease in temperature, increase in pH and exposure to mosquito derived xanthurenic acid [16, 17]. These stimuli induce activation of a calcium-dependent protein kinase in the male gametocyte (microagametocyte) leading to exflagelation. Exflagelation results in the

differentiation of each male gametocyte into eight haploid gametes, extremely motile due to axoneme assembly. Female gametocytes, or macrogametocytes, produce a single extracellular and non-motile spherical female gamete.

Microgametes and macrogametes rapidly fertilize forming a diploid zygote. Fertilization is followed by endomeiotic replication, producing a single, approximately tetraploid zygote nucleus, assumed to contain four haploid meiotic products [18].

Soon after gamete fusion, the sessile zygote gradually matures into a motile, banana-shaped ookinete. At this stage, a day after the blood meal, trough gliding motility, parasites leave the posterior midgut lumen and penetrate the midgut epithelium. After traversing the midgut epithelium, the ookinete reaches the extracellular space between the midgut epithelium and the overlaying basal lamina. At this site begins its development into a sessile, spherical oocyst. During ookinete maturation, the parasite undergoes meiosis [18-20].

At the basal lamina, a thick extracellular capsule forms around the oocyst and all the molecular apparatus required for cell motility and invasion is reabsorbed. Subsequently, new basal lamina is synthesized beneath the developing oocyst, separating the parasite from the midgut epithelium. DNA

replication and protein synthesis machinery is now upregulated for a massive asexual parasite amplification - sporogony. Multiple nuclear divisions occur in each oocyst resulting in a multinucleated parasite that gradually grows in size. At the same time, the oocyst plasma membrane is folded inwards forming cervices across the cytoplasm, compartmentalizing it into individual sporoblasts. Subsequent sporozoite budding occurs, involving synchronized mobilization of nucleus and other cellular organelles into each budding sporozoite [18, 20]. This culminates into the release of thousands of sporozoites into the hemocoel.

The following migration of sporozoites into the salivary glands is still poorly understood. Some studies suggest this is a passive migration of the parasites, mediated solely by haemocoel circulation, while other studies suggest the role of a chemotaxis process [18, 20]. Recently, the sporozoite invasion-associated protein 1 (SIAP-1) has been implicated in mediating efficient oocyst exit and migration to the salivary glands [21].

Although found throughout the haemocoelic cavity, sporozoites accumulate in the toraxic salivary glands region, more specifically within the distal median and lateral lobes of the salivary gland. This precise localization suggests that there is specificity in the parasite/salivary glands interaction.

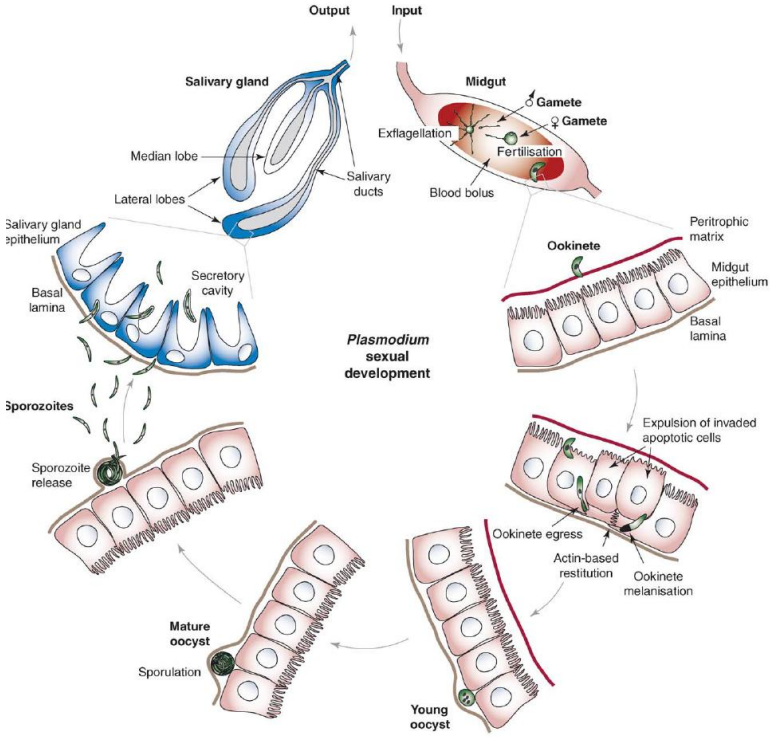

Figure 1.2 - *Plasmodium* sexual development in the invertebrate host. After a female mosquito takes a blood meal on an infected vertebrate host, ingested male and female parasite gametocytes fertilise and form the zygotes which in turn transform into motile ookinetes. Ookinetes traverse the midgut epithelium and, on the basal side of the epithelium, develop to the next parasite stage, the oocyst. Multiple nuclear divisions within each oocyst are followed by membrane partitioning and budding off of several thousand haploid sporozoites into the haemocoel. In the next step, sporozoites will reach and invade the median and distal lateral salivary gland lobes. Invasion of the salivary gland epithelial cells occur through the formation of a parasitophorous vacuole, following the interaction of sporozoites with the basal lamina. A second parasitophorous vacuole is formed around the sporozoites during their escape into the secretory cavity of the glands. Whenever the mosquito goes for a new blood meal, the parasites that have managed to escape this second vacuole and migrated into the

salivary ducts, will be ejected into a new vertebrate host where the asexual development will take place and the parasite life cycle completed (Figure reproduced under permission of authors [20]).

After salivary gland cell invasion, the parasites remain in the cytoplasm only transiently. A second parasitophorous vacuole is thought to surround the sporozoites during their migration into the secretory cavity of the glands. The secretory-cell-derived vacuole then disintegrates, leaving an extracellular sporozoite free within the secretory cavity of the gland. Here, large numbers of sporozoites organize in bundles and remain viable throughout the mosquito life. At each feeding cycle, small numbers of these free sporozoites further migrate, by gliding motility, into the fine secretory ducts [18-20].

When an infected female mosquito seeks for a blood meal, sporozoites present in the saliva are injected into the vertebrate host dermis, initiating the next phase of the parasite life cycle.

1.2.2 A simplified review of the *Plasmodium* spp. developmental stages in the vertebrate host

Infection in the vertebrate host has two phases: an asymptomatic, short lived pre-erythrocytic stage and, an erythrocytic phase where iterative cycles of parasite replication in the host red blood cells take place causing malaria symptoms.

Whenever one infected female mosquito goes for a blood meal, on average, approximately 100 sporozoites [22, 23] present in the saliva of the biting female are deposited in the skin of the bitten vertebrate. In the skin, these forms undergo a significant increase in locomotion ability and trough gliding motility a proportion of these parasites traverses the host capillaries and enters the blood stream or lymphatic system. Once in the blood stream, sporozoites passively migrate to the liver.

1.2.1.1 *Plasmodium* spp liver stage

Interventions at the liver stage present, at the moment, the most promising intervention strategy as this phase precedes the symptomatic blood stage, is clinically silent and represents a bottleneck in the parasite life cycle. In the liver, sporozoites

actively traverse the sinusoidal barrier gaining access to hepatocytes. The liver sinusoid barier is composed of fenestrated endothelial cells and Kupffer cells (resident macrophages). This active traversing ability has been shown essential for successful hepatocyte invasion, as cell traversal mutant parasites have decreased in vivo infectivity, strongly suggesting that Kupffer cells are the main traversing routes [24, 25].

Once a sporozoite has traversed the sinusoidal barrier, it traverses several hepatocytes before the final host hepatocyte where it will replicate and differentiate [26, 27]. During hepatocyte invasion, the parasite is surrounded by a parasiphorous vacuole, the interface between parasite and host, within which it resides during liver stage development. This parasitophorous vacuole membrane, derived from the host, is further remodelled with parasitic proteins and lipids [28].Upon successful hepatocyte invasion and parasitophorous vacuole membrane formation, the sporozoite differentiates from an invasive form to a metabolically active, replicative form; the trophozoite.

The sporozoite initiates its metamorphosis with the disruption of cytoskeleton beneath the plasma membrane, which harbours the invasion motor. This disruption results in a protruding, bulbous area around the nucleus. As this region expands, the two

distal ends of the sporozoite gradually retract, leading to parasite's sphericalization. At the same time, a major intercellular rearrangement takes place with an active exocytic clearance of the organelles involved in invasion. At the completion of metamorphosis, only those organelles necessary for replication within the hepatocyte are retained [29].

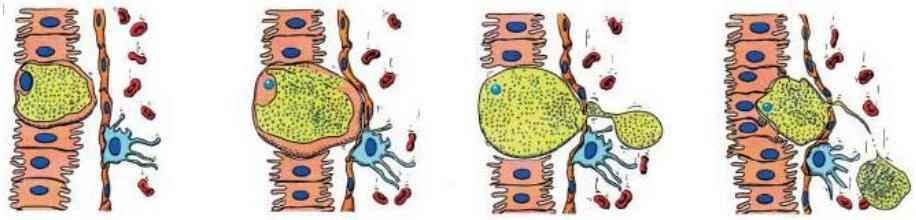

Figure 1.3 - Schematic representation of parasite release from infected hepatocytes into the blood stream. After sporozoite, invasion of hepatocytes and repeated rounds of parasite nuclear division from a single nucleus to thousands of nuclei; breakdown of the parasitophorous vacuole membrane results in the release of thousands of detached daughter merozoites into the host cell. This figure depicts the release of *Plasmodium* merozoite-filled vesicles (merosomes, green) from infected hepatocytes into the blood stream. Red blood cells (red) are separated from hepatocytes by endothelial cells (orange); Kupffer cell is in blue. (Figure reproduced under permission of authors [30])

Once dedifferentiated, the trophozoite undergoes rapid growth, entering schizogony with numerous rounds of DNA and organelle replication. A multinucleated sincytium is thus formed within the infected hepatocyte. As a final step, the organelles undergo morphological and positional changes, before cell

division, finally segregating into individual merozoites [31].

Few to several thousand merozoites are packaged in vesicles, the merosomes [30]. Infected hepatocyte cell death is then induced causing the detachment from the surrounding tissue, followed by the budding of merosomes into the sinusoid lumen. At the same time, parasites inhibit the exposure of phosphatidylserine on the outer leaflet of the dying hepatocytes membranes and merosomes surface. This mechanism ensures hepatocyte-derived merosomes to evade detection and engulfment by Kupffer cells and guarantee safe delivery of merozoites into the bloodstream [30].

Merosomes then travel passively into the bloodstream through the heart and, eventually, into the narrow vasculature of the lung, where they burst and release merozoites into the bloodstream to initiate blood stage infection [32, 33].

1.2.1.2 *Plasmodium* blood stage

Malaria disease occurs during the asexual blood stage of infection, with host erythrocytes being invaded by merozoites. In approximately 48h, the intraerythrocytic parasite grows and divides into a schizont form containing daughter merozoites that are released into the blood stream perpetuating the invasion and replication cycle [34].

Merozoites, deftly adapted to erythrocyte invasion, are the smallest form *Plasmodium* acquires during its lifecycle. Belonging to the Apicomplexa phylum, the merozoite has the conventional organelle repertoire of an invasive protozoan. This repertoire comprises an apical complex of three morphologically distinct secretory organelles - micronemes, rhoptries, and dense granules. These organelles are believed to be required in the process of adhesion (adhesins are released from micronemes) and posterior invasion (invasins) of the host cell. During invasion the organelle content is sequentially released with the final establishment of a parasitophorous vacuole (thought to be mediated by rhoptries) where the next rounds of replication/differentiation will take place.

Mature merozoites are released from infected erythrocytes into the blood stream by rupturing the erythrocyte membrane, rapidly invading uninfected erythrocytes.

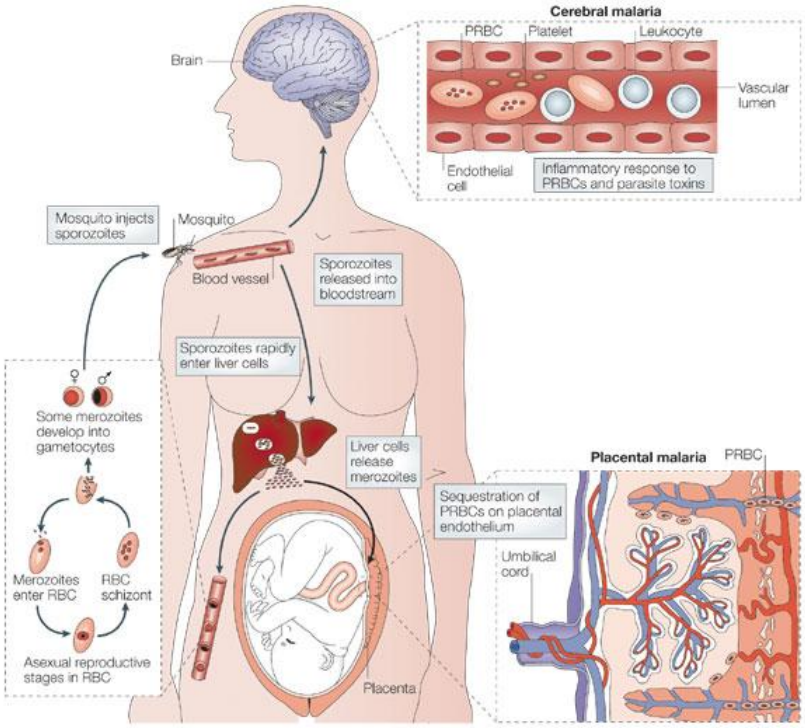

Figure 1.4 – *Plasmodium* life cycle in the human host. When going for a blood meal, an infected female mosquito injects a small number of infectious sporozoites into the human bloodstream while feeding. Shortly after, sporozoites are carried to the liver, where they invade and replicate inside hepatocytes. Later, thousands of daughter merozoites are released back into the bloodstream and enter erythrocytes being carried trough the organism within this cell type. As they mature inside erythrocytes, adhesion ligands are selectively expressed, enabling the maturing parasite to bind receptors expressed by endothelial cells that line the blood vessels in the deep vascular beds of organs such as the brain, lungs and placenta. After each maturation cycle, the parasitized erythrocytes rupture and release more daughter merozoites, thereby perpetuating and promoting the blood-stage cycle. Some merozoites differentiate into gametocytes, which, when taken up by another feeding mosquito, perpetuate the sexual cycle in the insect. (Figure reproduced under permission of authors [35])

The rapid process of *de novo* red blood cell binding and invasion with apical pole reorientation, involves multiple *P. falciparum* proteins.

Once released from an infected erythrocyte (IE), merozoites are exposed to low potassium levels. This condition triggers calcium release activating the secretion of adhesins, one of the classes of proteins governing merozoite invasion functioning as ligands and binding directly to specific receptors on the erythrocyte surface. These proteins are located in both micronemes and rhoptries, and provide *Plasmodium*–erythrocyte specificity. The main adhesins so far identified belong to two protein families that include the EBL and reticulocyte binding–like homologues (PfRh), localized, respectively, to the micronemes and neck of the rhoptries [36]. Initial interaction with erythrocytes involves dramatic movement of the merozoite, with parasite and hosts cell undergoing remarkable changes. The erythrocyte undergoes major surface deformation followed by an apparently active process of reorientation that places the parasite apical end adjacent to the host cell membrane. Commitment to invasion occurs once the merozoite's apical end interacts with the erythrocyte, with the triggering of subsequent events leading to entry.

Some proteins anchored to the merozoite plasma membrane via a glycosylphosphatidylinositol (GPI) anchor and others associated by interaction with

surface proteins have been suggested as main ligands in this initial step although irrefutable proof is still lacking. These proteins are not evenly distributed over the merozoite with some having apical concentrations, suggesting a direct role in invasion. Several proteins include domains suggesting their involvement in protein–protein interactions; as is the case for Duffy binding–like (DBL) or erythrocyte binding– like (EBL) domains that are specific to *Plasmodium* spp. and present in proteins expressed in phases as diverse as invasion, post-invasion remodeling and cytoadherence. Others include EGF and six-cysteine (6-Cys) domains, also implicated in protein–protein interactions.

Invasins are also involved in merozoite invasion. These proteins function in the invasive process but do not necessarily bind directly to receptors on the host cell. Invasins appear to be essential for merozoite invasion being the apical membrane antigen-1 (AMA1) the best characterized of these proteins. AMA1 interacts with a set of rhoptry neck proteins (the RON complex) that comes together at the tight junction during invasion [36].

At this stage, the apical membrane antigen 1 (AMA1) moves to the merozoite surface and binds a segment of rhoptry neck protein 2 (RON2). The RON proteins are secreted into the erythrocyte membrane while a segment of RON2 remains outside the erythrocyte membrane to

bind AMA1. RON2 functions as an anchor in the erythrocyte membrane for RON complex assembly, and as a possible grip that the merozoite uses for invasion.

Formation of the erythrocyte-parasite junction likely triggers the release of the rhoptry bulb, providing proteins and lipids required for the parasitophorous vacuole and its membrane to establish the space into which the merozoite can move as it invades.

Actin filaments also concentrate at this site, presenting a ring like distribution at the tight junction, trailing the RON complex. This likely provides a substrate with which the actin-myosin motor propels the merozoite into the space generated by release of the rhoptries bringing the merozoite into the erythrocyte. As the parasite moves into the erythrocyte, the tight junction is pulled across the surface of the merozoite, drawing with it the erythrocyte membrane until the resealing of the parasitophorous vacuole and the erythrocyte membrane [36].

Now, the internalized parasite is presented in a ring-loke form, undergoes rapid and dramatic transformation/maturation [6, 34, 36-39].

After the differentiation/maturation period, the parasitized/infected erythrocytes (IE) rupture and release more daughter merozoites, thereby feeding and amplifying the blood-stage cycle. It is during this intra-erythrocytic proliferative period that the maturation into male and female gametocytes might take place, by commitment to the sexual pathway [40].

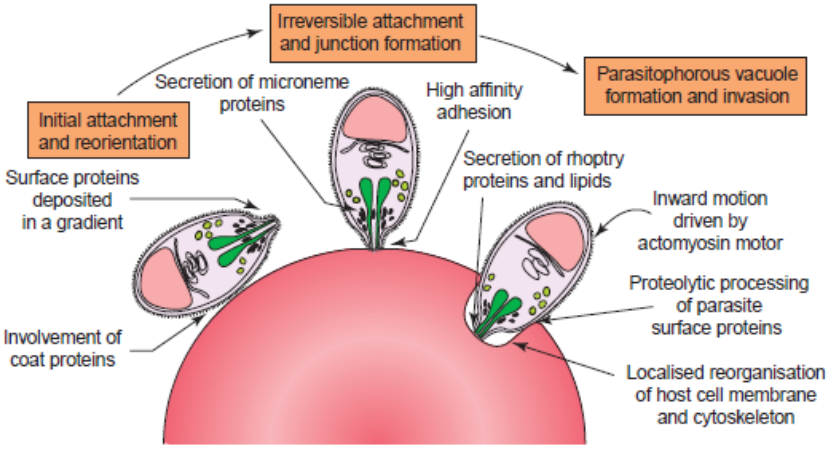

Figure 1.5 - Simplified representation of a *Plasmodium falciparum* merozoite invading an erythrocyte. Depicted is the complex process of invasion with a general overview of the multiple receptor–ligand interactions between the erythrocyte and components of the parasite surface and respective apical secretory organelles. (Figure reproduced under permission of authors [38])

The uptake of these sexual forms during the blood meal of a female mosquito completes the parasite's life cycle.

It is during this blood stage of perpetuated invasion and release of merozoites to and from erythrocytes,

with rapid multiplication, that severe forms of malaria take place with pregnant women and children being the higher risk groups. Corresponding symptoms as fever, anaemia, respiratory distress, lactic acidosis and in some cases coma and death [41, 42] are generally attributed to *P. falciparum* infections, although *P. vivax* infections also have an often neglected contribution to the worldwide disease burden [43-45].

1.3 Human Pregnancy Associated Malaria

Pregnant women are at greater risk of malaria infection and symptomatic disease than the same women before pregnancy or non-infected adult controls [46, 47]. *P. falciparum* and/or *P. vivax* [48-50] infections during pregnancy are a major public health problem, with substantial risks for the mother, foetus and the newborn. In the mother, this form of disease encompasses a large spectrum of clinical manifestations ranging from mild to severe anaemia, pulmonary oedema, cerebral malaria or renal failure and is associated to spontaneous miscarriage, stillbirth, preterm delivery and foetal growth retardation [51, 52].

To face this health problem, the World Health Organization has recommended a package of malaria control interventions which includes promoting usage of insecticide-treated nets, intermittent preventive treatment with sulfadoxine-pyrimethamine during pregnancy and appropriate case management through prompt and effective anti-malaria treatment of pregnant women [53].

Severity of malaria in pregnancy is dependent of many factors including the number of previous pregnancies and local malaria endemicity. Pregnancy Associated

Malaria (PAM) is usually more severe in areas of low endemicity where immunity to infection has not been previously acquired [54, 55]. In these low endemicity conditions, primigravidade or multigravidae do not significantly differ in PAM susceptibility and the severity of disease is a matter of extreme concern [55, 56]. In contrast, in high endemicity areas, less severe manifestations of PAM usually occur. In these regions women had acquired some degree of protection to infection and the effects of PAM in both mother and foetus are less severe [57-59]. These distinct epidemiological features result in contrasting consequences of malaria during pregnancy [60].

PAM severity is also dependent on parity. In areas of unstable malaria transmission, primigravidae are more susceptible to disease [61]. Intermediate outcomes have been registered in meso-endemic regions where secondigravidae present outcomes similar to those of primigravidae in endemic regions [48].

Different hypotheses have been presented as to why pregnant women present higher susceptibility to infection. It has been hypothesized that the immuno-suppressed state due to the pregnancy status would counteract a proper humoral response to the parasite; nevertheless no consensus has been reached as to determining which immunological components are involved in conferring higher susceptibility to malaria during pregnancy [59]. An alternative non-

exclusive explanation is the ability of *P. falciparum* to selectively adhere to the placental tissue. Pregnancy associated malaria often entails marked accumulation of IE in the intervillous space of the placenta, possibly leading to maternal anaemia, low birthweight, prematurity and increased infant mortality [62].

Accumulation of *P. falciparum* infected erythrocytes (IE) in microvasculature endothelium in different organs is believed to be a key factor in malaria associated pathology. This ability is mediated through the insertion, in the IE membrane, of parasite-encoded, clonally-distributed variant surface antigens (VSA). IE sequestration is perceived as an immune evasion strategy to avoid splenic clearance of infected erythrocytes [63-66]. Antigenic variants are coded by the var gene family that enables the expression of a large repertoire of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) variants[67, 68] that mediate adherence to endothelial cells [69]. Endothelial cell receptors such as CD36, thrombospondin (TSP), and intercellular adhesion molecule 1 (ICAM-1) have been identified as major host receptors for mature IE and to be directly involved in the severe malaria forms, as is the case of cerebral malaria [70-75].

The ability of the proteoglycan chondroitin sulphate A (CSA) to mediate IE adhesion was first described *in*

vitro [76, 77] and demonstrated to be the main mechanism of specific adhesion to placenta [66, 78]. It was thus evident that the placenta would constitute a specific and differential niche for an antigenically distinct VSA. This single, uniquely structured *var* gene was first identified in 2003; the *NFAvar2csa* gene [63]. NFAvar2csa is transcribed at higher levels from placental parasite isolates than from peripheral blood isolates of non-pregnant malaria patients [65]. With a particularly unique structure, it is the only *var* gene that does not have an N-terminal DBL-α domain and, as in only three other *var* genes, it lacks a CIDR domain and it does not contain a DBL-γ domain, like eight other 3D7 *var* genes [64].

As to why do *P. falciparum* variants are specifically selected during pregnancy was clarified in 2000 by Achur, R.N. and colleagues [66]. In this study, the chondroitin sulfate proteoglycans (CSPGs) of human placenta were purified, structurally characterized, and the adherence of IE to these CSPGs accessed. Three distinct types of CSPGs were identified. Nevertheless significant quantities of an extracellular low sulphated form uniquely found in the placenta intervillous spaces, was identified as the most efficient in binding IEs [66].

Taken together, the specific presence of CSPG low sulphated form in the placenta, might be the reason why primigravidae are less able mounting an efficient

immune response to the specific cytoadherent forms of the parasite developed during pregnancy, even if they were infected prior pregnancy. This also points to why multigravidae present increased protection to PAM; being previously exposed to the Var2CSA variant renders them with specific IgG antibody levels which have been suggested directly relate to the protective immune response multigravidae present [79].

These observations provide an alternative explanation to the epidemiology of maternal malaria in spite of the changes in the cellular immune system during pregnancy.

In sum, in the search for understanding causes and consequences of increased severity of *Plasmodium* infection during pregnancy, both maternal physiological changes occurring during pregnancy and the parasite variation/adaptation characteristics should be taken into account as they may have a synergistic effect in increasing susceptibility to infection.

Additionally, recent reports have pointed for an active role for the foetal placental tissue in IE uptake upon binding to trophoblasts [76]. These new observations raise the hypothesis that there might be a third, so far unaddressed, player in PAM outcome – the foetus.

One of the main subjects of the work present in this thesis is the putative role of this "third player" during PAM.

1.4 Placentae

The word placenta originates from the Latin *flat cake*, from Greek *plakóenta*, accusative of *plakóeis*, from *plak-plax* "flat surface" [80].

By deffinition, placenta is "*the vascular organ in mammals except monotremes and marsupials that unites the fetus to the maternal uterus and mediates its metabolic exchanges through a more or less intimate association of uterine mucosal with chorionic and usually allantoic tissues*" [80].

1.4.1 Human and murine Placentae - General development and structure

Uterine decidualization, characterized by the transformation of uterine stromal cells into large decidual cells with a secretory phenotype, and the recruitment of specialized macrophages and granulated lymphocytes, have a different timings and triggering mechanisms in humans and mice. While in humans the first signs of decidualization are seen before conception, as early as day 23 of the normal menstrual cycle, in mice, decidualization is only induced upon embryo implantation.

In humans, as early as day 21 of pregnancy, the chorionic villus – definitive functional and

structural unit of the placenta – is already established. Subsequent to blastocist adhesion, trophoblasts rapidly start to proliferate, undergoing posterior fusion, in order to form the multinucleated syncytiotrophoblast, which invades the maternal uterine stroma [81].

The haemotrophic function of the definitive placenta is considered established when all the factors required for physiological exchange between maternal and fetal blood circulations are present. In humans, an effective arterial circulation is completely established around the 12^{th} week of gestation, during which period the human embryo has largely completed the organogenesis stage.

Distinct from the human placenta, the mouse placenta only achieves its definitive structure halfway through gestation. Importantly, murine placentation evolves from an initially choriovitelline pattern to a chorioallantoic pattern at 11.5 days. Specifically, maternal blood is evident in the labyrinth at E10.5, but not at E9.5. Extensive fetal capillary formation is seen by E12.5, but not at E10.5 [81].

By 12.5 days of gestation, the murine definitive chorioallantoic placenta is finally developed [81, 82]. This is reflected in a late trophoblastic invasion leading to a distinct timing on the point at which fetal direct nutrient uptake from circulating

maternal blood by trophoblast cell, when in comparison to human placentas.

Despite the differences in placental development between mice and humans, some similarities can be found between the two species. For example, the trophoblast cell lineage seems to follow the same pathways: an invasive pathway involving extravillous trophoblasts in humans and giant cells and trophoblastic glycogen cells in mice; and an exchange barrier involving the syncytiotrophoblast in both species. Furthermore, also at molecular level, the expression of certain placental genes has been described for both human and mouse [81].

1.4.2 Placental structure in human and mice

1.4.2.1 The human Fetal placenta and the murine Labyrinth

The human fetal placenta is functionally analogous to the murine labyrinth as, in both, fetal and maternal blood circulates in close association for physiological exchange. In both species, the foetal interface is represented by a layer of trophoblasts supported by an extracellular matrix, collectively known as the chorionic plate. The umbilical cord is inserted at the center of the fetal chorionic plate

where the fetal arteries and veins, derived from the allantoic mesenchyme, ramify. These umbilical vessels irradiate to and from the foetus, connecting it to the placenta, allowing fetal blood circulation in the placenta.

From the opposite surface of the chorionic plate arise many tree-like projections known as chorionic villi. The outer-most layer of the chorionic villi is trophoblastic whilst its inner core consists of allantoic mesenchyme and vasculature, continuous with that of the umbilical cord. The allantoic vasculature and its associated blood circulatory system within the fetus itself constitute the feto-placental circulation. The trophoblastic layer of the chorionic villi is directly bathed in maternal blood which is brought to and leaves the fetal placenta/labyrinth via the arterial and venous sinuses from the basal plate/junctional zone and the adjacent uterine tissue, known as the utero-placental circulation.

Based on the overall shape of this region, both species are considered discoid because their placentae resemble a circular cake with a flat surface facing the fetus and an irregular oposite surface adjacent to the uterine wall. The basic architecture similarities between the human fetal placenta and the murine labyrinth classifies them both as chorio-allantoic since the tissue separating circulating maternal from fetal blood consists of chorionic trophoblast and

allantoic mesenchyme and vasculature. Additionally, they both have a haemochorial interface as feto-maternal interactions between the two blood circulations involve direct interaction between maternal blood and chorionic trophoblast.

However, regarding the chorionic architecture, human and mice placentae are differently categorized. Due to their tree-like pattern with innumerable branches the humans are considered to have a villous placenta, while in mice, the interconnected branches of the chorionic projections generate a maze-like pattern, the so called labyrinthine placentae.

The space within which maternal blood circulates in the human fetal placenta is more open and termed as the intervillous space; in mice, this space is named as maternal blood spaces or lacunae. As a result of these differences, the mouse labyrinthine structure allows countercurrent exchanges between maternal and fetal capillaries arranged in parallel to each other. In humans, the multivillous structure, considered to be less efficient, is intermediate between a countercurrent and a parallel-flow [81].

The physiologically important exchange of substances between fetal and maternal blood takes place through foetal-derived cells at the feto-maternal interfaces. In humans, these are the villous trophoblasts. Villous trophoblasts, remain attached to the villous basement membrane, forming a monolayer of epithelial cells.

These cells proliferate and differentiate, to form a syncytiotrophoblast that covers the entire surface of the villus. The syncythiotrophoblast is multifunctional, but its primary functions are absorption, exchanges and specific hormonal functions. The surface of the villous syncytiotrophoblast has numerous microvilli. They are believed to increase the surface area and create areas of relative stasis of maternal blood plasma, thus allowing more time for absorption. In mice, in contrast to the single layered syncytiotrophoblast in humans, the trophoblastic interface is considered to have three layers: two syncytiotrophoblast layers in contact with the fetal endothelium, and one cytotrophoblast layer in contact with maternal blood. This type of placental development is known as haemotrichorial placentation while the human placentation is monochorial. The murine trophoblast layer lining the maternal blood spaces (1^{st} layer), is not a syncytium as in humans, but consists of discontinuous layer of trophoblast cells (cytotrophoblast) that do not show microvilli. The middle and third layers (2^{nd} and 3^{rd} layers respectively) are syncytiotrophoblastic. The maternal surface of the middle layer is loosely attached to the 1^{st} layer and contains irregular invaginations generating spaces between it and the adjacent 1^{st} layer. Importantly, the 1^{st} layer is not the only trophoblast layer that directly contacts with the

maternal blood spaces but also the syncytiotrophoblast, on the 2nd layer.

The anatomy of the rest of the labyrinthine interface is very similar to the human foetal placenta.

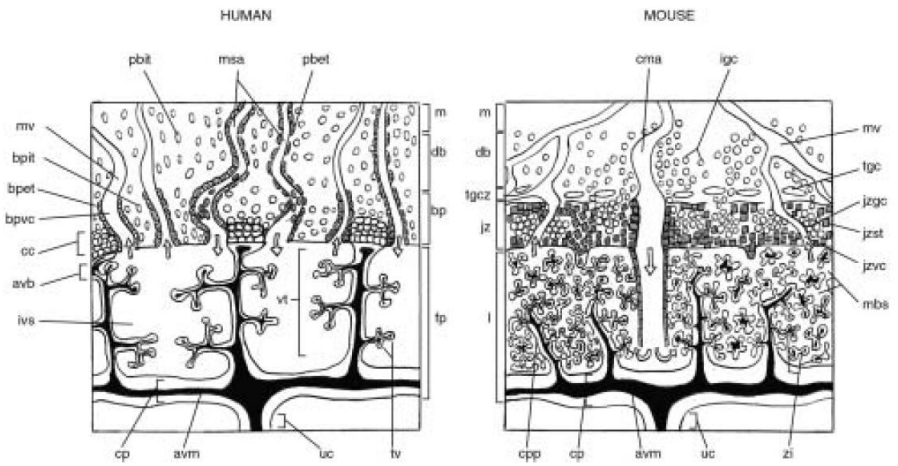

Figure 1.6 – Schematic representation of the main regions and cell types of human and murine placentae at the last third of gestation. At the top half is depicted the maternal side while the bottom half represents the foetal counterpart. Arrows indicate the direction of maternal blood flow. The following constituents are represented in the picture: *bp*- basal plate, *mv*- maternal veins, *zi*- zona intima, *l*- murine labyrinth, *cpp*- chorionic plate projections, *jz*- junctional zone, *cma*- murine central maternal artery, *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 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 and *zi*- zona intima. (Figure reproduced under permission of authors [82])

Thus, analogous cell types among human and murine trophoblasts have been identified, including proliferative trophoblastic cells and cells differentiating into syncytium. In both humans and mice, placentation involves the development of three physiologically and anatomically distinct regions: the human "fetal placenta" or murine labyrinth; the basal plate or junctional zone, for humans and mice, respectively and; the maternal uterine tissue bordering the maternal side of the murine trophoblast giant cell zone or the human basal plate [81, 82]. Furthermore, additional cell types that are neither trophoblasts nor vascular endothelial cells are found in some areas of the villous core and in the mouse placenta labyrinth and include pericytes and macrophages or so-called Hofbauer cells [81, 82].

In conclusion, despite important morphological differences between the human and murine placentas there are similarities in the general placenta architecture and comparable physiological roles at the cellular level that allow using the mouse as a model to study cellular and molecular mechanisms impacting on the placental barrier physiology.

Comparison of pregnancy and placentation in mouse and human

	Mouse	Human
Implantation	Secondarily interstitial	Primarily interstitial
Yolk sac	Inverted yolk sac placenta functions to term	Yolk sac floats free in exocoelom during first trimester
Trophoblast invasion of uterine arteries	Shallow; limited to proximal decidua	Extensive; reaching myometrial vessels
Transformation of uterine arteries	Dependent on maternal factors (uterine natural killer cells)	Dependent on trophoblast
Placental exchange area	Labyrinthine	Villous
Interhaemal barrier	Three trophoblast layers; outer one cellular, inner two syncytial	Single layer of syncytial trophoblast (Langhans layer not part of barrier)
Trophospongium	Extensive	Absent
Placental hormones	Placental lactogens	Chorionic gonadotropin, chorionic somato-mammotrophin, placental growth hormone; major source of progesterone
Gestation	Three weeks	Nine months

Figure 1.7 –.General comparison of mouse and human pacentation and gestation events and timing (Table reproduced under permission of author [83])

1.5 Mouse models

A variety of mouse models of PAM have been established that are instrumental in the investigation of PAM pathogenesis. While acknowledging differences between human and mouse placenta these models take advantage of functional similarities between human and mouse placentas namely in respect to the nature of interhemal barrier. PAM mouse models mainly focus on conditions that best fit low endemicity scenarios where acquired immunity to infection is very low or absent and PAM is severe. Nevertheless, the current mouse models offer a generous variety of infection conditions that range from infection prior to pregnancy to infection early or late in the gestation period, covering different parasite species and mouse strains.

1.5.1 Infection prior gestation

Infection prior gestation has generated mouse models to study parasite recrudescence during pregnancy. The first recrudescence model was established in 1980 by Adriaan A.J.C. van Zon and Winjnand M.C. Eling [84]with the aim to study "recrudescence as a marker for depressed immunity in pregnancy". The recrudescence model arose using the rodent parasite *P.*

berghei K173 in the Swiss, C3H/StZ and B10Lp mouse strains.

This model consists on infecting virgin naïve females and consecutively treating them with sulfadiazine for 31 days, in order to clear parasitaemia. Two days after the treatment is stopped, females are challenged with a new injection of IE in order to confirm acquired immunity. Upon this challenge, only subclinical numbers of persisting parasites were observed with rare spontaneous recrudescence. However, in subsequent pregnancy, 40 to 49% of recrudescence cases were observed (depending on the strain). This model of *P. berghei* K173 recrudescence also revealed malaria-associated prematurity, abortion and death before parturition in 20% of recrudescent pregnant females. Despite the high rate of death in recrudescent females, it was possible to register reduction in the incidence of recrudescence in multigravidae, corresponding to the observations made in pregnant women.

Overall, the *P. berghei* K173 recrudescence mouse model, revealed to be an excellent experimental new tool for the analysis of the mechanism of "depressed malarial immunity" during pregnancy, as it exhibits several similarities with the human disease allowing for manipulation and experimental control that is not amenable when studying human PAM.

Nevertheless, the fact that a high proportion of the mice that recrudesced during their first pregnancy died from the infection impeded as the study of PAM in multigravidae.

This drawback led those authors to address the question from a different angle and test whether the depression in the immune response would be different from the first to subsequent pregnancies. A very interesting finding was made using this alternative approach. Briefly, a group of immune Swiss mice (prone to recrudescence) was treated with chloroquine before mating and supplemented with chloroquine in drinking water for the duration of the first pregnancy. After parturition the mice were reinfected and allowed to mate again. During the second pregnancy 46% (11 mice) exhibited recrudescence, the same percentage as observed in primigravidae. With this result it was shown that fundamental differences in the immunological capacity of the host between the first and a subsequent pregnancy, is unlikely. The authors suggested that the presence of the parasite during pregnancy strengthens immunity that would thereby prevent loss of immunity in a subsequent pregnancy.

In a subsequent publication, Adriaan A.J.C. van Zon *et al.* [85] questioned further how the recrudescent mechanism would act during pregnancy and whether a lower recrudescence rate in multigravidae is the consequence of stronger immunity, how this is

achieved, and what was the role of the parasite as antigenic signal for development of this better immunity. Starting from the observation that mice protected from lethal recrudescence during their first pregnancy usually do not exhibit recrudescence in subsequent pregnancies [86], van Zon and colleagues hypothesized that an enhanced parasite load during the first pregnancy would be necessary to strengthen immunity.

Their first approach was to test if repeated challenges before pregnancy would strengthen immunity and consequently prevent loss of immunity during the first pregnancy. A group of female mice was infected eight times and allowed to mate after the last injection and recrudescence rates were recorded. Obtaining rates of recrudescence in the order of 40%, the authors inferred that prior-gestation challenge would not improve protection to recrudescence during pregnancy. The next step was to verify if improved immunity in subsequent pregnancies was due to enhanced ability to clear parasites (switch from premunity to sterile immunity). To address this, groups of mice were challenged within 1 week after parturition and parasite clearance accessed 2 weeks post challenge. With this experiment, the authors could observe that, in comparison to immune virgin mice, with a clearance rate of 10%, females that presented parasitaemia during a previous pregnancy had now a clearance rate of 82%. On the other hand, if no parasite was detected

during a previous pregnancy the clearance did not improve in comparison to immune virgin mice. These results led to the conclusion that the enhanced clearance observed in recrudescent females requires active infection during the first pregnancy. Furthermore, the authors show that not only the presence of proliferating parasites is required during pregnancy but also that it is essential in the second half of the gestation period to enable pos-gestation parasite clearance. Interestingly, a further experiment showed that the immunity acquired during pregnancy minimizes the loss of immunity when compared with virgin mice [86].

Whether immunization prior pregnancy confers protection to infection during pregnancy was further accessed in lethal *P. yoelii* infections of ICR pregnant mice. In this study, females were hyperimunized with irradiated parasite and subsequently set to mate. When challenged on gestational day 14, these pre-immunized pregnants were able to control parasitaemia to a sub-patent level, with no impact on pregnancy outcome or maternal survival [87]. Of major importance, in this study, the authors show that even in the presence of relevant levels of peripheral blood parasitaemia, close to 40% IE, no parasite passes through the placental barrier to the foetus. When in the presence of IE, *in* vitro, isolated trophoblasts and placental macrophages, presented the same phagocytic activity as peritoneal

macrophages [87]. Although not directly related with recrudescence or the outcome of different gestational times of infection, this observation offers an important clue to why are IEs and parasites not detected in the foetal compartment.

Eighteen years passed before the recrudescence theme was again addressed with two independent studies adding more detailed information on this subject.

In one of these studies, infection of non-pregnant Balb/c females with the K173 strain of the *P. berghei* parasite, followed by sulfadiazine treatment, resulted in subpatent parasitaemias after posterior challenge [88]. Upon mating, 100% of the pre-exposed primigravidae, presented parasite recrudescence leading to intrauterine growth retardation and smaller litter sizes. In accordance with the previous studies, decreased levels of parasitaemia were observed in subsequent pregnancies, correlating with reduction in maternal anemia. As for *P. falciparum* [66], these authors questioned whether the susceptibility to pregnancy-related recrudescence, in mice, could be due to the parasite expression of pregnancy-specific VSAs. Based on an established flow cytometry analysis [89], where IgG levels specific for antigens on IE obtained during pregnancy-related recrudescence are evaluated, they showed that IE from pregnant and non-pregnant mice are partially different. After immunization, virgin females did not present IgG specific for

antigens specific of IE obtained during recrudescence but, recrudescence-specific IgG was posteriorly acquired over the course of several pregnancies and recrudescences [88]. With these observations the authors concluded for a modulator effect of IgG with specificity for recrudescent IEs VSAs on pregnancy-related recrudescence. This study continues with a more detailed analysis on placental IEs. Their analysis shows that there is a preferential accumulation of IE mature stages when compared to peripheral blood IE, where ring-stage parasites are predominant [88].

A more detailed scrutiny on placental sequestration and pathology was published, in the same year, by Marinho and colleagues. In this elegant study, using Balb/c females and the *P. berghei* ANKA parasite, the authors demonstrate that the percentage of recrudescent females, and the associated parasitaemia, significantly decrease with increased parity [90]. As general observation, also in this study a poor pregnancy outcome is observed, characterized by decreased foetal viability and intrauterine growth retardation. Regarding placental pathology, a positive correlation was observed between peripheral parasitaemia and reduction in the vascular spaces. Furthermore, quantification of cell-type specific mRNA expression revealed increased amounts of inflammatory cells as NK, T cells and macrophages, in placentas from recrudescent females. This increase in

inflammatory cells was accompanied by a significant increase in the expression of macrophage chemoatractants MCP-1 and MIP1-α. Expression of vascular stress related molecules, HO-1 and ET-1, were also significantly increased in recrudescent placentas.

As mentioned in the previous study and in human PAM, IE specific sequestration in placenta might be a pathogenic trigger for placental pathology. In this study, IE from non-pregnant males and females were compared to IE from recrudescent females on their ability for placental adhesion. Strikingly, IE from recrudescent females presented a four-fold increase in adhesion to placental sections. This adhesion was partially inhibited in the presence of chondroitinase or CSA suggesting the involvement of these molecules in adhesion and that parasites expanded during recrudescence display an enhanced specificity to placenta [90].

Amongst the recrudescence models we can find those displaying pathological features resembling human PAM in high to mid endemicity regions [84, 85, 87] and others of low endemicity [88, 90].

Overall, recrudescence models are a valuable tool to evaluate acquisition of immunity prior pregnancy and malaria immunity disturbances during pregnancy.

1.5.2 Infection in early pregnancy

Mouse models of infection at the initial stages of gestation allow studying the development of maternal antimalarial immune responses and its impact on malarial infection in early pregnancy prior and during placentation.

Poovassery J. and Moore J.M. [91]established a model on the C57BL/6 background using the *P. chabaudi* AS parasite ($1x10^3$ IE) to infect pregnant females at G0. They observed that pregnant females infected were able to recover from infection with no major differences in peripheral parasite levels when compared with non-pregnant females. Pregnancy was unsuccessful in all cases, although most pregnant mice recovered from infection. A time-course analysis of the pregnant uterus revealed non-viable fetuses and resorptions scars from G12 onwards. This correlated with accumulation of *P. chabaudi* AS IE in the placentae, in significantly higher levels than in the peripheral blood [91, 92].This observation recapitulates the association of placental sequestration of *P. falciparum* with poor fetal outcome in human pregnancy.

Infected pregnant females undergoing abortion had a widespread placental hemorrhage, thinning of the labyrinth and generalized disruption of placental architecture. These pathological features were abrogated by treatment with anti-TNF. In addition maternal blood sinusoids contained fibrin thrombi

possibly related to an increase in the expression of Tissue Factor and other coagulation factors in infected placentas [93, 94]. Taking advantage of the established model, Poovassery J. and Moore J.M. went on to characterize the cytokine response observed in these females upon infection [95].

Analysis of pro-inflammatory citokine profiles in infected pregnant females uncovered that the strong systemic inflammatory response is related with peripheral parasitemia but does not directly correlate with pregnancy outcome and fetal viability.

A robust immune response characterized by increased systemic expression of Inf-γ and IL10 was observed in pregnant and non-pregnant females correlates with similar correlation was observed in non-pregnant females [95]suggesting this response correlated with progression of parasitemia in pregnant females and ability to clear infection. Importantly, on G10, when infected pregnant mice begin to abort, IFN-γ, IL-10 and TNF levels did not differ in aborting and non-aborting mice further suggesting that the systemic inflammatory response was dissociated from the pregnancy outcome.

In contrast, the levels of sTNFRII were assessed and found to be significantly higher in the plasma of infected pregnant mice and further increased in females undergoing abortion than in non-aborting females suggesting a differential inflammatory

response was linked to placental dysfuction and poor pregnancy outcome. In vitro testing of fetoplacental units or ectoplacental cones from infected pregnant females revealed that TNF levels were unexpectedly low but sTNFRII expression was increased[93]. Additionally, treatment of WT infected pregnant with anti-TNF resulted in a significant reduction in the resorption rate, reaching the level of pregnant non-infected females and abrogates main features of placental pathology. Thus, the TNF signaling system appears to play a critical role in the placenta with impact in the pregnancy outcome. These observations suggest that fetal trophoblast cells have the potential to be immunoactive during malarial infection and contribute to a local pathogenic environment in the uterus.

Analysis of *Inf-γ* $^{-/-}$ pregnant females that, despite the higher levels of parasitaemia and although *Inf-γ* $^{-/-}$ pregnant females developed pregnancy associated malaria, significantly less resorptions were observed comparing to WT [93]. This further supports the notion that increased susceptibility to infection in pregnant females does not correlate with severe PAM pathology and pregnancy outcome. Histological examination of the infected placentae revealed massive phagocytosis of iRBCs and hemozoin by trophoblast giant cells resulting, in the absence of maternal IFNγ, in robust TNF production. These findings further support that

the local production of pro-inflammatory factors is a key event in determining placenta pathology.

Analysis of infection in early pregnancy in the A/J mouse further showed that despite increased susceptibility to infection inability to maintain viable pregnancies were comparable to C57BL/6 mice. Peripheral parasitaemia and peripheral levels of TNF and IL-1β was significantly higher in A/J than in B6 mice [92]. But sTNFRII levels in A/J mice show an equally significant increase in plasma levels of sTNFRII as compared to C57BL/6 mice. This data suggest that genetic differences controlling susceptibility to infection in pregnant females do not correlate with increased severity of PAM. Nevertheless, the mechanisms inducing placental pathology appear to be heterogeneous as treatment of A/J mice with anti-TNF did not ameliorate the rate of abortions [92] as observed in the C57BL/6 strain [93].

Thus, the models of early pregnancy infection offer a tool to follow the systemic response to infection and evaluate its relation with placenta pathology and pregnancy outcome. Current analysis suggested that maternal parasitemia and systemic response appear to be dissociated from expression of placenta inflammatory components and that interaction of IE with the placental tissues underlies placental dysfunctions that lead to poor pregnancy outcomes.

1.5.3 Mid-term infection

The relevance of mid-term infections is demonstrated by Vinayak V.K. *et al.* [96] where in a simple experiment they show the need of mid-term infections in order to be able to follow-up delivery. Infection of Swiss albino pregnant females with the *P. berghei* NICD parasite on day 6 of pregnancy resulted on a steep increase of parasitaemia associated with maternal death 6 days post infection (G12), well before parturition. If pregnant mice were infected on day 13 of gestation, parasitaemia curves would follow a kinetic similar to non pregnant mice with death starting on average 9 days post infection allowing for 50% of the pregnant females to reach parturition. For this 50% that reach term pregnancy, fetuses had significantly lower weight than non-infected. From the remaining pregnant, 20% had only reabsorptions and 30% died before delivery. These results indicate that, although 50% of the females reach delivery, foetal weight loss and increased reabsorptions characteristic of pregnancy associated malaria are observed [96].

This experiment strongly indicated that in order to study the effect *Plasmodium* infection might have on fetuses at delivery, infection should occur at least at mid gestation.

This is indeed what was explored in detail in the work by Neres R., *et al.*[97]. In this model of mid-term infection, Balb/c infected with *P. berghei* ANKA were

used to access malaria pregnancy outcome and newborn growth. In this study, it is clearly shown that infection at mid-gestation reproduces many of the features seen in severe human pregnancy associated malaria. This included rampant parasitaemia, reduced maternal survival to infection, high abortion/resorbtion rates and preterm delivery. Similar phenotypes were also reported in IRC pregnant females infected with lethal *P. yoelii* [87]. Consequences of mid-stage infection in the surviving progeny included significantly lower average weight of viable fetuses and growth impairment in the first stages of post-natal life.

A more detailed analysis of the pathological features of this model revealed a significant thickening and disorganization of the placenta labyrinth zone, reduction of the vascular space, distention and disarrangements of perivascular space and presence of IE in the maternal blood space. Some specimens also showed focal fibrinoid necrosis in the placental basal zone, hyperplasia of syncytiotraphoblasts and accumulation of mononuclear cells in the maternal blood space composed mainly of monocytes/macrophages. Additionally, an ex-vivo adhesion assay of IE binding to placental sections strongly suggested, as observed for human placentas, a specific CSA sequestration of IE to the placental tissue [97].

Taken together, the pathophisiology described in this model suggests that mid-gestation infection is a good methodology to study terminal foetal outcome and placental pathology upon *Plasmodium* inoculation in naïve primigravid pregnant females.

After the initial characterization of this experimental PAM model, and shown its similarities with human PAM pathology, further work was developed by a different group focusing on a more specific dissection of which factors could be involved in this pathology. It was shown that placentas from viable but low weight fetuses are associated with dysregulated angiopoietin expression at both transcriptional and protein levels and that this dysregulation preceeds the observed malaria-associated foetal growth restriction. Serum angiopoietin-1 levels were also significantly decreased in infected pregnant mice supporting the hypothesis that malaria infection-associated angiopoietin dysregulation could play a pathophysiological role in growth restriction as seen in human PAM [98]. Recently, potential links between inflammation, angiogenesis alterations, and growth restriction in pregnancy-associated malaria have been investigated [99], using the mid-term, Balb/c - *P. berghei* ANKA infection model [97]. In this work it is proposed that inflammatory cells indirectly generate C5a complement fractions that activate monocytes to produce soluble vascular endothelial growth factor sVEGF, an inhibitor of VEGF. This deregulation of

angiogenic factors is hypothesized to disturb the developing placental vascularization and as a consequence impair placental function and foeatal nurturing.

A different mid-gestation infection model was established by Sharma, L. *et al.*[100] in the Balb/c background. In this model the authors chose to use *P. berghei* NK65 infection at gestational day 10. Coherent with what is expected, also in this model the levels of parasitaemia are significantly higher in pregnant females comparing to non-pregnant females infected in the same day, with placentae showing sinusoids plugged with IE, increased inflammatory cells and malaria pigment [100, 101]. This model was established with main focus on studying the effects oxidative stress may have in placental pathology. It was shown that infected pregnant females present higher levels of Malondialdehyde (MDA, as index of lipid peroxidation) in several organs including placenta [100]. Furthermore, there was a decrease in the activity of catalase while the levels of the antioxidants reduced gluthatione (GSH) and superoxide dismutase (SOD) maintained unaltered during pregnancy [101]. This increase in ROS could explain the significantly higher number of apoptotic cells found in infected placentas while the upregulation of Bax, downregulation of Bcl-2 and increase activity of caspase 3 and 9 suggest the involvement of the mitochondrial pathway in apoptosis induction [101].

The mid-gestation infection model represents a compromise to study placenta inflammation and pathology induced by IE while maintaining maternal survival throughout pregnancy. The model is particularly useful to study IE-placental interactions [76] which is thought to be a key event in triggering placental pathology.

The exploitation of PAM mouse models has helped to experimentally reproduce the severity of placental pathological manifestations and a spectrum of poor pregnancy outcomes. Nevertheless, host cellular and molecular components that control the intensity of the inflammatory response are still poorly investigated.

Despite the multitude of Pregnancy Associated Malaria mouse models, described in this chapter, none in the C57Bl/6 (B6) background allows for the study of gestation outcome. The existing models in this mouse genetic background were characterized for infections at initial gestation, inevitably leading to full abortion by mid-gestation.

Most of the KO mouse strains available are in the C57Bl/6 background, offering a precious tool to study the role of individual molecules in PAM pathogenesis.

1.6 Toll Like Receptors

- General overview and signalling pathways

Toll-like receptors (TLRs) are type 1 transmembrane proteins structurally characterized by extracellular leucin-rich repeat (LRR) motifs and a cytoplasmic signaling domain known as Toll IL-1 receptor (TIR) domain that are joined by a single transmembrane helix [102]. TLR ligation leads to the activation of nuclear factor-kB (NF-kB) and interferon-regulatory factors which lead, respectively, to the production of pro-inflamatory cytokines and type I interferons.

Due to sequence similarity [103, 104], TLRs are named after the *Drosophila melanogaster* Toll gene. This gene was first identified as a regulator of embryonic dorsal-ventral polarity [105] and later was also associated with adult *Drosophlila melanogaster* antifungal response [106]. The association of Toll with innate immune responses in *Drosophila*, led to the important identification of a human homolog of the *Drosophila* Toll, hToll later named TLR4, and its association with the induction of signals for activating both an innate and an adaptive immune response in vertebrates [107].

To date, 10 TLRs have been identified in humans (TLR-1 to TLR-10) and 12 in mice (TLR-1 to TLR-9 and TLR-11 to TLR-13) [104, 107-112].

Toll-like receptors are categorized as pattern recognition receptors (PRRs); germline encoded innate immune receptors that were initially described as sensors for pathogen associated molecular patterns (PAMPs). PRRs can also recognize endogenous molecules that are released in response to stress or tissue damage. Ligands have been identified for all TLRs with the exception of human TLR-10 and mouse TLR-12 and TLR-13. TLR3, TLR7 and 8 and TLR9 are located intracellularly and are mainly involved in antiviral immune responses recognizing dsRNA, ssRNA and CpG DNA and haemozoin, respectively. On the other hand, TLR1, TLR2, TLR4 to 6 and TLR11, reside at the plasma membrane and recognize molecular components located on the surface of pathogens. TLR2 forms heterodimers with TLR1 or TLR6 and in this form recognizes triacylated lipopeptides or diacylated lipopeptides, LTA and zymosan. On its own, TLR2 can recognize peptidoglycan (a major constituent of Gram-positive bacteria), phospholipomannan, tGPI-mucins, haemagglutinin, porins, lipoarabinomannan and glucuronoxylomannan. For TLR5 and TLR11, depolymerized flagellin and profilin are the natural ligands so far identified ([113-120] and reviewed in [121, 122]).

Upon ligand stimulation TLRs signal transduction involves either a MyD88-dependent pathway that leads to the activation of NF-kB resulting in the transcription of pro-inflammatory cytokines and chemokines, or a TIR domain containing adaptor

inducing IFN-β (TRIF)-dependent pathway resulting in the activation of interferon regulatory factor (IRF) leading to the secretion of type 1 interferons. For the MyD88-dependent signaling pathway, MyD88 interacts mith TIRAP (Mal) to recruit members of the IL-1R-associated protein kinases (IRAKs), resulting in an interaction with tumor necrosis factor receptor associated factor 6 (TRAF6). TRAF6 in turn activates the TAK1/TAB1/TAB2/3 complex that subsequently phosphorilates IkB kinase (IKK)-β and MAP kinase (MAPKs). Activation of a complex composed of IKK-α, IKK-β and NEMO (NF-kB essential modulator) results in the degradation of IkB, allowing NF-kB translocation to the nucleus and induction of transcription of NF-kB target genes. Simultaneously, MAP kinase activation is critical for activation of JNK and AP-1, and thus production of pro-inflammatory cytokines as TNF-α, IL-1, IL-6 and chemokines as IL-8. All TLRs with the exception of TLR3 activate the MyD88-dependent pathway.

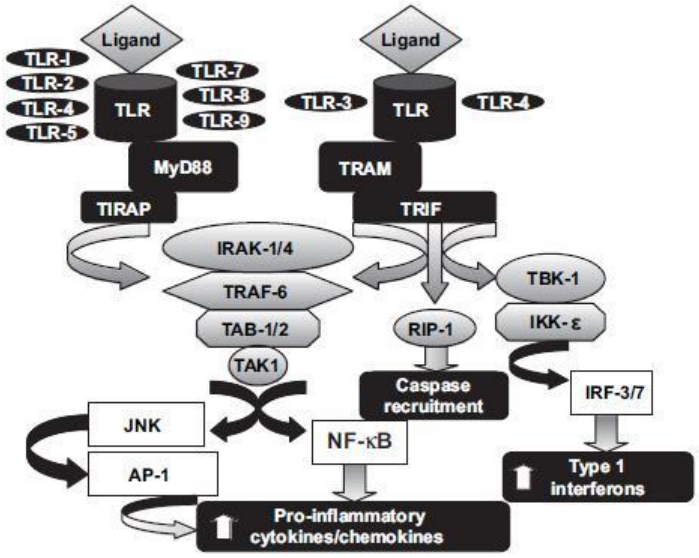

Figure 1.8 – Schematic representation of TLR signalling pathway. MyD88-dependent and TRIF-dependent signalling cascades and resulting cytokine/chemokine secretion are depicted. (Figure reproduced under permission of author [121])

1.7 TLR4

Regarding signaling, TLR4 is unique amongst TLRs as it is the only able to signal trough either MyD88-dependent or TRIF-dependent pathways. As for TLR3, the TLR4 TRIF-dependent pathway requires the TRAM bridging with TRIF resulting in the recruitment of TRAF-6 or RIP-1 to activate NF-kB (similar to the MyD88 pathway). TRIF recruits another signaling complex composed of TRAF-3/TBK-1/IKK to phosphorilate IRF, leading to their nuclear translocation and induction of type I IFN genes [121, 123]. TLR4, as previously mentioned, was the first human homolog to the *Drosophila* Toll to be identified and has been one of the main focuses on TLR research. TLR4 is mainly known for its ability to bind lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria. TLR4 was first identified as an LPS receptor by two independent studies as the gene responsive for LPS hyporesponsiveness in two TLR4 natural mutant mouse strains – C57Bl10/ScCr and C3H/HeJ [124, 125]. Besides LPS, TLR4 recognizes several other pathogen patterns as VSV glycoprotein G [126], RSV fusion protein [127], MMTV envelope protein [128], mannan [129] and glucuronoxylomannan [130]. TLR4 is also known to recognize endogenous molecules normally released upon stress or inflammation such as fibrinogen [131], hyaluronic acid [132], β-defensin 2[133] and HSP60 [134].

At the cell membrane, TLR4 interacts with several proteins that facilitate/hamper the signalling cascade. Having as an example LPS stimulation of mammalian cells, the recruitment of LBP (a soluble shuttle protein which directly binds to LPS), CD14 and MD-2 to interact with TLR4 are responsible for the initiation of the signalling cascade [135].

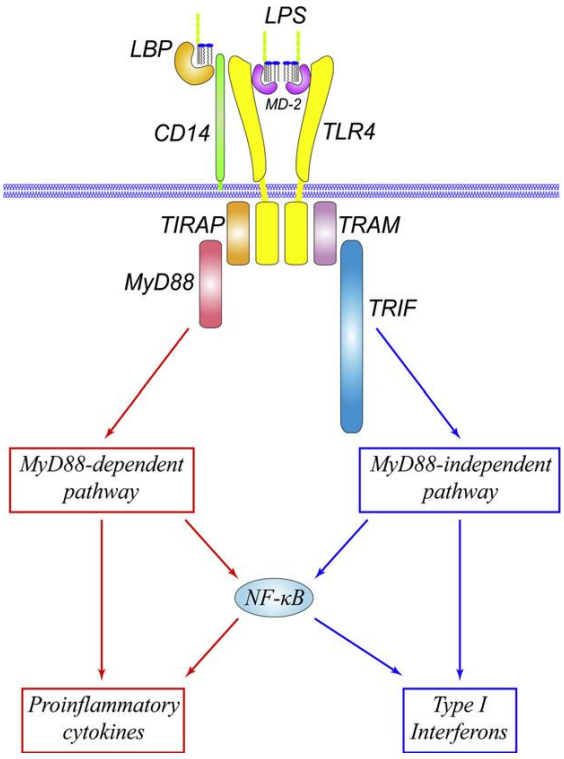

Figure 1.9 – Schematic overview of TLR4 signaling upon LPS recognition. Recognition is mediated by the TLR4/MD-2 receptor complex, assisted by LBP and CD14. LPS mediated TLR4 signalling cascade uses MyD88-dependent and MyD88-independent pathways, which lead to the activation of proinflammatory

cytokines and Type I interferon genes. (Figure reproduced under permission of author [135])

1.7.1 TLR4 and Malaria

In addition to the above mentioned molecules identified as TLR4 stimulators, the glycosylphosphatidylinositol (GPI) anchor glycolipids of the *Plasmodium* parasite [136, 137] have been shown to stimulate macrophages trough TLR4 in a MyD88-dependent fashion (although stimulation trough TLR-2 also occurs) [138]. This stimulation results in an increased secretion of pro-inflammatory cytokines as TNF-α and IL-1 [137-139]. In macrophages and endothelial cells, *P. falciparum* GPIs initiate a protein tyrosine kinase and protein kinase C-mediated signal transduction pathway, regulating inducible NO synthase expression with the participation of NF-KB/c-rel, leading to cell activation and downstream production of NO [139]. Dendritic cells (DCs) can also be directly stimulated by *Plasmodium* IE trough TLR4-MyD88 leading to upregulation of MHC-II, CD86 and CD40, NF-kB phosphorilation and secretion of proinflammatory molecules as TNF-α and IL-12 [140].

Additionally, evaluation of the of impact human TLR4 polymorphisms (TLR4Asp299Gly and TLR4Thr399Ile) in malaria outcome has shown association with increased parasitaemia levels in mild malaria patients from

Kolkota (west Bengal)[141]. In a Brazilian Amazonian population, the TLR4Asp299Gly polymorphism was associated with reduced risk for clinical malaria [142] and, in Tamale (Ghana), TLR4Asp299Gly and TLR4Thr399Ile polymorphisms showed to confer a 1.5 and 2.6 fold increased risk of severe malaria, respectively [143]. As a final remark in the putative role of TLR4 might have in malaria outcome, recent studies aiming at the development o potent malaria vaccines demonstrate the promising adjuvant effect of TLR4 agonists [144, 145]

1.7.2 TLR4 and pregnancy

TLR4 signalling implications in pregnancy outcome have been intensively investigated. Several reports on how bacterial constituents act trough TLR4 leading to inflammation-induced poor pregnancy outcome have been addressed in mouse models; LPS administration or intravenous bacterial infections during pregnancy leads to an increase in foetal death [146, 147], preterm delivery [147] and foetal weight loss [148]. Necrosis and inflammatory infiltrates composed almost entirely of polymorphonuclear leukocytes were apparent in the decidua [147, 148], marginal region, labyrinth and the membranous yolk sac of WT placentas, while significantly reduced in placentas from $TLR4^{-/-}$ pregnant females [147].

In these studies, systemic blockage of TLR4 signalling (either using a TLR4 antagonist [146, 147] or $TLR4^{-/-}$ mice[147]) significantly reduced the preterm delivery percentage [146] and foetal death [146, 147]. Nevertheless, no differences in placental bacterial load were registered [147]. These results can be explained by TLR4 mediated effects on increasing the expression of CD86 co-stimulatory molecule in CD45+ and CD49b+ peripheral blood lymphocytes (PBLs) as well as by increasing the expression of CD69 in CD3+ PBLs [146]. Regarding placental immunocytes, increased CD86 expression on CD45+ cells and increased number of CD49b+ CD45+ and percentage of CD69+ NK cells were observed. These activation profiles were reverted if TLR4 was blocked prior to LPS administration showing the specific effect trough TLR4 [146]. In addition to the peripheral role of TLR4 in poor pregnancy outcome, confocal microscopy and mRNA analysis revealed differences in placental TLR4 expression between bacteria infected and non infected placentas [148].

In humans, it has been shown that peripheral blood mRNA TLR4 is significantly increased in women with an idiopathic preterm labour in comparison to controls. Similarly, the number of TLR4 mRNA copies was 2.2 times more elevated in women with idiopathic preterm labour. Additionally, this increase TLR4 mRNA expression was attributed to overexpression in CD14+ cells in idiopathic preterm labour women [149].

These studies were focused in the role of TLR4 expression in maternal cells and tissues but the TLR4 expression in the trophoblast has been neglected or put apart. In a study by Klaffenbach and colleagues [150] it was shown that highly purified villous trophoblasts were not responsive to LPS as detected by production of IL-8, IL- 10, or IL-6. On the other hand granulocytes, which are closely attached to trophoblasts, were the main cells responsible for the production antimicrobial proteins upon LPS stimulation.

Despite the references to maternal peripheral blood as the exclusive/main participant in TLR4 mediated poor pregnancy outcomes and lack of TLR4 expression in villous trophoblasts, other studies show moderate to strong immunoreactivity for TLR4 protein in extravillous trophoblasts and intermediate trophoblasts independently of coming from term or preterm placentas with or without chorioamnionitis (CAM), while no immunoreactivity was detected in normal villous trophoblasts [151] as also reported by Klaffenbach [150].

Interestingly, in this study, villous Hofbauer cells (placental macrophages) show a weak reactivity in term and preterm placentas without CAM but a moderate to strong reactivity in preterm placentas with CAM indicating that Hofbauer cells in the villi recognize bacterial compounds such as LPS [151].

Knowing that Gram negative bacteria activate TLR4 on immune system cells leading to preterm birth (PTB), A. Bitner and colleagues [152] evaluated the impact of maternal and fetal carriage of TLR4 gene polymorphisms on the risk of PTB. In their study, the frequency of LPS hyporesponsive TLR4 variants (896A>G, 1196C>T) [153] was determined in women who delivered at term (after the 37th week of gestation), in women who delivered preterm (before the 37th week of gestation) and in very-preterm delivery (before the end of the 33rd week of gestation).

It was found a statistically significant difference in the frequency of TLR4 1196C>T polymorphism between mothers who delivered before 33 weeks gestation and those who delivered later.

A further analysis found that the maternal carriage of only one of analyzed polymorphisms inside TLR4 gene (either 896G or 1196T) does not significantly affect the risk of prematurity while simultaneous carriage of both examined polymorphisms (896G and 1196T) was associated with significant reduction in risk of birth before the 33rd week of gestation. Additionally, foetuses born before the 37th week of gestation tended to have lower TLR4 1196T allele frequency than term foetuses[152].

1.7.3 TLR4 and Malaria during pregnancy

TLR4 has also been shown to be involved in pregnancy outcomes in the presence of malaria infection. A study concerning primiparous women, in southern Ghana, examined whether common polymorphisms of TLRs involved in response to *P. falciparum* could influence malaria disease outcome during pregnancy. In non-infected women, the common *TLR4* variants analyzed (Asp299Gly and Thr399Ile) had no influence on anemia, LBW, IUGR, preterm LBW, or preterm delivery.

However, while in *P. falciparum* infected women the TLR4 polymorphisms had no influence on the risk of placental malaria or placental parasite densities, the *TLR4* Asp299Gly polymorphism worsened the clinical outcomes. This polymorphism was associated with a 6-fold-increased risk of LBW in term infants and the risk of anemia was increased almost 5-fold in *P. falciparum*–infected women with *TLR4* Asp299Gly, compared with women with the *TLR4* wild-type allele[154].

Together, the current literature strongly implicates TLR4 expression/signaling in pregnancy outcome, suggesting that TLR4 is implicated in inflammatory responses occurring in the placenta. Nevertheless, to date, a clarification as to the specific contribution of maternal and foetal TLR4 to the observed phenotypes has not been addressed.

1.8 IFNAR1

Interferons was first described by Isaac and Lindenmann in 1957 [155]. After incubation of heat-inactivated influenza virus with chick chorio-allantoic membrane a new factor was released that would *interfere* with the growth of live virus on those membranes.

Since interferons were identified, much research effort has been put into understanding their role in the immune system. Type I interferons are tightly related to viral and tumoral responses, being applied as therapies for those pathologies [156-159]. More recently, some autoimmune manifestations, as is the case of multiple sclerosis and systemic lupus erythematosus, have also been targeted by type I interferon therapies [160, 161].

For long, macrophages have been known as the main source of IFNα/β upon viral infection. Stimulation with dsRNA, microbial pathogens or microbial products such as LPS, strongly induces IFNα/β in this cell type. Other sources of IFNα/β include fibroblasts, NK cells, T cells, dendritic cells and plasmacytoid monocytes [162-164].

Type I interferons are a family of cytokines coded by intronless genes and widely distributed amongst vertebrates. The most studied of this cytokine family are IFNα and IFNβ (IFNα/β). These comprise more than

15 members in humans and mice, with 14 IFNα and one single IFNβ subtypes [163, 165, 166]. The type I IFNs produce differential activation of genes and cellular activities probably due to the differential binding to their receptor or differences in receptor subunit recruitment by the various type I IFNs [167].

IFN α and β exert their function through binding to the type I IFN receptor (IFNAR), activating it. IFNAR is a heteromeric receptor composed of two cloned membrane glycoprotein subunits – IFNAR1 (α) and IFNAR2 (β), ubiquitously expressed in virtually all cell lineages [168, 169].

On its own, IFNAR2 has moderate intrinsic affinity to all type I IFNs, whereas IFNAR1 alone binds type I IFNs weakly. However, IFNAR1 plays an essential role in respect to the final high affinity and differential ligand specificity of the IFNAR complex [167].

Binding of IFN α/β to each IFNAR subunit is followed by a JAK-STAT signalling pathway. Briefly, the activation of the receptor-associated tyrosine kinases Jak1 (Janus kinase 1) and Tyk2 (Tyrosine kinase 2) phosphorylate the receptor chains creating docking sites for the transcription factors STAT 1 and 2 (Signal Transducer and Activator of Transcription 1 and 2). STAT1 and 2 are phosphorylated by the Jaks, and form heterodimers. Together with IRF-9 (Interferon Regulatory Factor 9), this protein complex forms the transcription factor ISGF3 (Interferon-stimulated gene

factor 3) which, in turn binds to DNA at the ISRE (Interferon Stimulated Response Elements) and induces transcription of ISGs (IFN-stimulated genes)[165, 170, 171].

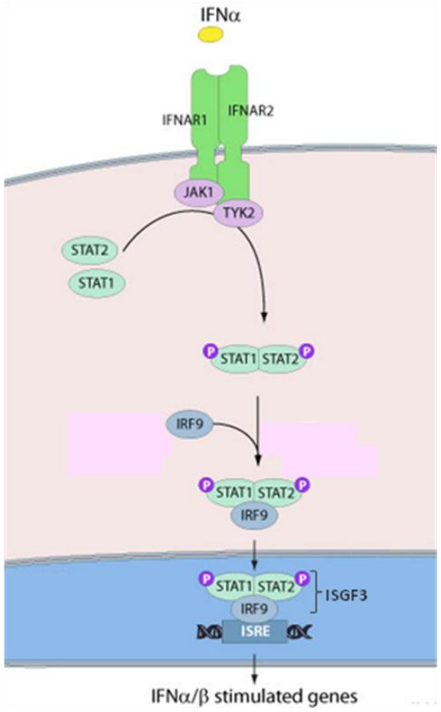

Figure 1.10 –.Schematic representation of IFNAR signaling cascade. (Figure adapted and reproduced under permission of authors [172])

1.8.1 IFNAR1 and Malaria

IFNAR1 has been reported to be involved in determining severity of malaria infection. In humans IFNAR1 polymorphisms have been associated with disease severity and progression to cerebral malaria,

especially in children [73, 173, 174]. In mice, IFNAR1 has been shown to promote sequestration and accumulation of $CD8^+$ cells in the brain tissue. Accordingly, $IFNAR1^{-/-}$ mice show significant protection against the development of experimental cerebral malaria (ECM) with reduced accumulation of $CD8^+$ lymphocytes [73, 175]. Furthermore, it was demonstrated that the specific expression of IFNAR1 in $CD8^+$ cells, *per se*, is a critical mechanism for ECM development [73]. In addition to the reports on the role of IFNAR1 related to adaptive immune responses, recent work has revealed its involvement in innate parasite sensing [176]. In this work, the authors report the existence of a *P. falciparum* DNA sensing pathway alternative to the already well described TLR9 signaling [177-179]. The authors have found the occurence of the AT-rich motif, ATTTTTAC, to be extremely high in *Plasmodium spp.* genome [176]. This motif stimulated potent IFN type I responses on HEK293 cells, mouse macrophages cultures, human PBMC, THP-1, mouse splenocytes, fibroblasts, bone marrow-derived macrophages and splenic DCs [176]. This new TLR9 independent pathway acts through an unknown receptor coupled to the STING, TBK1 and IRF3-IRF7 signalling pathway. Nevertheless, although unknown, this *Plasmodium* sensing pathway is indirectly related to IFNAR1 as mice lacking the type I IFN receptor were resistant to otherwise lethal cerebral malaria [176]. This report involves, for the first time, IFNAR1 in

the innate recognition of *Plasmodium* components opening a new perspective on IFNAR1 role in malaria immunity.

1.8.2 IFNAR1 and Pregnancy

There are numerous studies on the effect IFNα/β might have in pregnancy outcome, mainly due to the clinical applications of such molecules in pregnant women suffering from essential thrombocythemia, chronic myelocytic leukemia, Hepatitis B and C [180] and autoimmune disorders as is the case of multiple sclerosis [181, 182]. In these reports mild to no influence in miscarriages or foetal malformation are detected upon clinical administration of IFNα/β.

Nevertheless, reports regarding a possible role of IFNAR1 during pregnancy in humans and mice are scarce with one report involving IFNAR2 in murine *in utero* development [183].

However, studies in other species indicate a possible role for IFNAR1 in pregnancy. In ruminant angulate species, IFN-τ, a type I IFN structurally related to IFN-α, is secreted by the conceptus trophectoderm before definitive trophoblast attachment and implantation as a signal for maternal recognition. It mediates its effects by acting on the uterine endometrium, where it regulates the normal pulsatile

production of $PGF_2\alpha$, presumably as a result of its binding to type I IFN receptors [184, 185].

In these studies, both IFNAR1 and IFNAR2 mRNA and protein were found to be expressed in endometrial luminal epithelium and superficial glandular epithelium in pregnant and non-pregnant *uteri* [186] with mild temporal variation of IFNAR1 expression in pregnant uteri [184, 187]. Interestingly, IFNAR1 was also identified in the conceptuses trophectoderm and responded to recombinant bovine IFNτ. This led to increased expression of ISGs indicating that IFNτ can also act as an autocrine factor to regulate foetal trophectoderm cell proliferation [185].

Although not expressed in humans, molecular interactions between bovine IFN-τ1c and human IFNAR1 are theoretically possible [188] and ovine IFN-τ has been shown to confer resistance to HIV-1 infection in human macrophages [189].

Taken together such observations allow us to speculate a possible role of foetal IFNAR1 in human and or murine pregnancy and specifically to hypothesize a role for IFNAR1 in pregnancy associated malaria.

Despite no significant attention has been put towards the role of IFNAR1 in pregnancy outcome, PAM and ECM share common features as is the case of IE adhesion/sequestration [190-193], tissue recruitment

of pro-inflammatory cells [73, 194, 195] and tissue damage [196-199].

1.9 Objectives

The overall goal of this thesis was to evaluate the role of specific innate immunity factors in murine pregnancy associated malaria, namely Tlr4 and Ifnar1. To this end the specific objectives were:

1- To establish new mouse models that allow analysis of PAM gestational outcome in the context of mid gestation infection in the C57BL/6 background.

2- To dissect maternal and foetal genetic contributions to PAM outcomes focusing on Tlr4 and Ifnar1.

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Chapter II

Distinct placental malaria pathology caused by different *Plasmodium berghei* lines that fail to induce cerebral malaria in the C57BL/6 mouse.

Distinct placental malaria pathology caused by different *Plasmodium berghei* lines that fail to induce cerebral malaria in the C57BL/6 mouse.

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2.1 Author Contributions

All experiments were designed by me, my supervisor Carlos Penha-Gonçalves and Luciana Vieira de Moraes.

All *Plasmodium berghei* NK54 and ANKA*Δpm4* experiments were performed by me.

All data analysis referring to the above mentioned parasites was performed by me, with the exception of morphometric analysis that was carried out by Renato Barboza and Claudio Romero Farias Marinho, for all parasite lines.

The experiments referring to the *Plasmodium berghei* K173 were performed by Luciana Vieira de Moares.

Manuscript was written by me, Luciana Vieira de Moraes and Carlos Penha-Gonçalves.

Blandine Franke-Fayard and Chris J. Janse provided materials, reviewed and discussed the experimental data and manuscript.

2.2 Abstract

Background: Placental malaria (PM) is one major feature of malaria during pregnancy. A murine model of experimental PM using BALB/c mice infected with Plasmodium berghei ANKA was recently established, but there is need for additional PM models with different parasite/host combinations that allow to interrogate the involvement of specific host genetic factors in the placental inflammatory response to Plasmodium infection.

Methods: A mid-term infection protocol was used to test PM induction by three P. berghei parasite lines, derived from the K173, NK65 and ANKA strains of P. berghei that fail to induce experimental cerebral malaria (ECM) in the susceptible C57BL/6 mice. Parasitaemia course, pregnancy outcome and placenta pathology induced by the three parasite lines were compared.

Results: The three P. berghei lines were able to evoke severe PM pathology and poor pregnancy outcome features.

The results indicate that parasite components required to induce PM are distinct from ECM. Nevertheless, infection with parasites of the ANKAΔpm4 line, which lack expression of plasmepsin 4, displayed milder disease phenotypes associated with a strong innate

immune response as compared to infections with NK65 and K173 parasites.

Conclusions: Infection of pregnant C57BL/6 females with K173, NK65 and ANKAΔpm4 P. berghei parasites provide experimental systems to identify host molecular components involved in PM pathogenesis mechanisms.

Keywords

P. berghei, placental malaria, cerebral malaria, placental pathology, TNF-α, TLR4, TLR2

2.3 Background

Organ pathology evoked by Plasmodium infections often correlates with accumulation of infected erythrocytes in specific organs leading to severe clinical manifestations as is the case of respiratory distress, cerebral malaria (CM) and severe placental malaria (PM) [1]. PM is one major feature of malaria during pregnancy and is usually associated with low birth weight due to intra-uterine growth retardation and/or preterm delivery ([2] and reviewed in [3]), stillbirths, maternal anaemia and mortality [4, 5]. Placental malaria results from accumulation of parasitized erythrocytes that is associated with a prominent monocytic inflammatory response that entails increased IFN-γ and TNF production and enhanced levels of monocyte/macrophage recruiting factors (MIP-1α and MIP-1β) [1, 6]. Placental malaria pathology includes maternal-foetal barrier thickening, disorganization and destruction of placental tissue, proliferation of cytotrophoblastic cells and excessive perivillous fibrinoid deposits usually associated with focal syncytiotrophoblastic necrosis [7-10]. The severity of placental pathological manifestations is associated with a spectrum of severe pregnancy outcomes but the host cellular and molecular components that control the intensity of the inflammatory response are still not well-defined and are difficult to investigate in pregnant women.

An experimental system where *P. berghei* ANKA evokes a syndrome that resembles severe PM in women was established in a experimental cerebral malaria (ECM)-resistant mouse strain (BALB/c) allowing experimental investigation of PM pathogenesis in the mouse. Low foetal viability and increased maternal disease severity correlate with placenta pathology that, in this experimental model, is characterized by thickening of the placental barrier in the labyrinth zone and tissue damage, accumulation of monocyte/macrophages and enhanced expression of pro-inflammatory, apoptosis and oxidative stress factors [11-13]. The use of the *P. berghei* model of malaria for analysis of PM would benefit by development of additional experimental tools. Access to numerous (C57BL/6) mouse mutants would allow interrogating the involvement of host genetic factors in the placental inflammatory response to *Plasmodium* infection. Recently, the C57BL/6 mouse strain in combination with the rodent parasite *Plasmodium chabaudi* has been exploited to study pregnancy malaria pathogenesis with infection initiated early in gestation [14, 15]. Here, different parasite lines derived from the *P. berghei* strains K173, NK65 and ANKA Δ*pm4* [16] that are not able to induce ECM in C57BL/6 mice, were used to establish additional placental malaria experimental models in this mouse strain. The results show that pregnant mice infected with the three lines develop PM indicating that P. berghei parasite factors that are

responsible for inducing ECM in the C57BL/6 mouse are not required to induce placental pathology and poor pregnancy outcome in female mice infected during pregnancy. These experimental systems are valuable tools to study host and foetal genetic factors in the pathogenesis of placental response to Plasmodium infection.

2.4 Methods

2.4.1 Mice and pregnancy monitoring

Eight to twelve week-old C57BL/6 mice were obtained from the animal facility at Instituto Gulbenkian de Ciência. Mice were bred and maintained under specificpathogen free (SPF) conditions. C57BL/6 females were transferred to a cage with one isogenic male (two females: one male) and removed after 48 hours. The day the females were removed was considered gestational day 1 (G1). Pregnancy was monitored every other day by weighing females. Successful fertilization was confirmed between G10 and G13 when animals had an average increase of 3 to 4 g in body weight. Abrupt weight loss after G13 was an indicator of unsuccessful pregnancy. Animal housing and all procedures were in accordance with national regulations on animal experimentation and welfare and

approved by the Instituto Gulbenkian de Ciência Ethics Committee.

2.4.2 Parasites and infection

The following parasite lines were used in this study: i) a reporter parasite line of the K173 strain/isolate of P. berghei which expresses the reporter protein GFPluciferase under the control of the schizont-specific ama-1 promoter. This mutant (line 1272cl1) has been generated in the K173cl1 line [15]. The gfp-luciferase gene has been integrated into the c/d-ssu-rRNA unit by double cross-over integration without a drug selectable marker. Details of this line can be found in the RMgmDB database[17]; ii) a mutant of *P. berghei* ANKA which lacks expression of plasmepsin-4 (ANKAΔ*pm4*; line 1092cl4; RMgmDB-316) and expresses the reporter fusion protein GFP-luciferase under the control of the ama-1 promoter [16]; iii) a parasite line originally derived from the P. berghei isolate NK65 at New York University and kindly provided by Dr Maria Mota (Instituto de Medicina Molecular, Lisbon, Portugal). Infections in Figure 1 were performed by intraperitonial (i.p.) injection of 10^6 infected erythrocytes (IE). Parasitized red blood cell preparations were obtained from one in vivo passage in C57BL/6 mice, when the percentage of infection reached approximately 10%. Pregnant mice were intravenously

(i.v.) injected with 10^6 infected erythrocytes. Infection with ANKAΔ*pm4* at G13 yielded very low parasite burden during pregnancy due to reduced multiplication rate of this parasite [16], but infection at G10 allowed significant parasite expansion within pregnancy time. Thus, infection was performed on G10 (ANKAΔ*pm4* IE) or G13 (*P. berghei* K173 or NK65 IE). Parasitaemia was measured by flow cytometry [18] to detect infected erythrocytes stained with DRAQ5 (Biostatus Limited). The labelling of infected red blood cells with DRAQ5 is an adaptation of the manufacturer's protocol for cell cycle analysis by flow cytometry. Briefly, a drop of blood was collected by tail pinching of infected mice into 400 µl of FACS Buffer (PBS 1x, 2% FBS, sodium azide 0.02%). DRAQ5 was added directly to the collected samples at a final concentration of 1µM. Samples were vortexed to allow an appropriate incorporation of DRAQ5 into the parasite DNA and immediately analysed. Uninfected red blood cells do not stain positive for DRAQ5 as they are devoid of DNA content. Parasitaemia was expressed as % of stained cells within the erythrocyte morphological gate. ECM development was monitored from day 5 post-infection (PI) as including one or more of the following neurological symptoms; head deviations, paralysis, ataxia and convulsions [19].

2.4.3 Pregnancy outcome and foetal survival

Infected pregnant mice were killed by CO2 narcosis and subjected to caesarian section on G18 (K173 or NK65 infection) or G19 (ANKAΔ*pm4* infection) and stillbirths, foetal weight, foetal survival at delivery and placental pathology were evaluated. Foetuses were extracted from their amniotic envelop and viability was immediately evaluated by reactive movement to touching with pliers. The lack of prompt movement indicated that the foetus had recently died. Reabsorptions were identified as small implants with no discernible foetus and placenta, corresponding to embryos that died before complete placenta vascularization. Viable and non-viable foetuses were weighed and counted. Non-viable (dead foetuses plus reabsorptions) foetuses were recorded as stillbirths. Viable foetuses were killed combining hypothermia and CO_2 narcosis. In another set of experiments, infected pregnant mice were allowed to deliver in order to access litter size and newborns viability. Foetuses that have been expelled before the gestational day of analysis were also recorded as dead newborns. Non-infected pregnant mice were used as controls.

2.4.4 Placenta preparations and morphometric analysis

Placentas from infected and non-infected females sacrificed on the same gestational day were equally treated. Each placenta was separated in two halves: one half was fixed in 10% formalin for further histological processing and the other half collected in lysis buffer (RNeasy Mini Kit - Qiagen) 1% β-mercaptoethanol for RNA extraction. Paraffin-embedded non-consecutive placenta sections were stained with hematoxylin-eosin (HE). HE-stained placental sections were analysed for histopathology and vascular space quantification. In each section, three randomly selected microscopic fields in the labyrinthine region (magnification 400x) were acquired at 12 Mpixels resolution, using a colour video camera (AxionCam HRc, Zeiss) connected to a light microscope (Axion Vision, Imager.M2, Zeiss). To quantify vascular spaces only, areas that presented accumulation of glycogen cells, necrosis or thrombi were excluded. An image analysis routine using ImageJ (ImageJ 1.37v, National Institutes of Health) was implemented. Briefly, after acquisition, the images underwent an automated light analysis procedure where noise removal was applied to ensure colour and image quality standardization across sections and specimens. Images were given a colour 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 three nonconsecutive sections. Two independent observers analysed the placentas one of which was unaware of the samples identification. Results were reported as the average of counts obtained by the two observers.

2.4.5 RNA isolation and gene expression analysis

Total RNA from individual placentas was obtained using an RNeasy Mini Kit (Qiagen), following the manufacturer's instructions for animal tissues. One microgram of total RNA was converted to cDNA (Transcriptor First Strand cDNA Synthesis Kit, Roche) using random hexamer primers. Ccl2, Ccl3, Tlr2, Tlr4 and TNF expression was quantified using TaqMan Gene Expression Assays from ABI (Mm00441242_m1, Mm00441258_m1, Mm00442346_m1, Mm00445273_m1, Mm00443258_m1, respectively). For *P. berghei* ANKA quantification specific primers for Taqman were, Forward 5'-CCG ATA ACG AAC GAG ATC TTA ACC T-3', Reverse 5'- CGT CAA AAC CAA TCT CCC AAT AAA GG-3' and Probe 5'- ACT CGC CGC TAA TTA G -3' (FAM/MGB). The endogenous control Gapdh (Mouse GAPD Endogenous

Control, ABI) was used in multiplex PCR with target genes. PCR reactions were performed with ABI Prism 7900HT system. For TaqMan assays, ΔCt was calculated by subtracting the cycle threshold (Ct) of the target gene from the GAPDH and relative quantification was obtained with normalization by GAPDH. Results were plotted as fold change over the respective non-infected controls.

2.4.6 Statistical analysis

Survival curves were compared using the Log-Rank test (Mantel-Cox). Parasitaemia data were presented as mean values +/- SEM. Unpaired t test (Welch´s correction) was performed in comparison of each parasite line with the respective control group. Kruskal-Wallis non-parametric test with Dunn’s post-test was used for comparisons between the three infected groups. Data were considered significant when $p<0.05$.

2.5 Results

2.5.1 Increased parasitemia in pregnant mice

As opposed to the canonical strain *P. berghei* ANKA, parasites of the NK65 isolate and of mutant ANKAΔ*pm4* have been previously described not to cause experimental cerebral malaria (ECM) in the C57BL/6 susceptible strain [13, 14]. Similarly, the K173cl1 line derived from the K173 isolate has lost the capacity to induce ECM [unpublished results, CJJ and BF]. The course of infection by the three parasite variants in non-pregnant C57BL/6 females (Figure 1) showed that differently from wild-type *P. berghei* ANKA the three parasite lines do not induce the characteristic features of ECM. Nevertheless they induced hyperparasitaemia leading to a fatal outcome. *P. berghei* ANKAΔ*pm4* and NK65 exhibited slower growth kinetics when compared to the K173 line and corresponding delayed effects on the survival rate. To ascertain the effect of infection in pregnancy, mice were infected with K173 and NK65 at G13 when placenta vascularisation is established while infection with ANKAΔ*pm4* was performed at G10 due to reduced multiplication rate of this parasite [16]. Pregnant mice did not exhibit symptoms of ECM but showed higher parasitaemia across time as compared to non-pregnant females, reaching 20% at 5 to 9 days after infection (Figure 2) and indicating that parasites of the three

lines show increased ability to cause hyperparasitaemia during pregnancy.

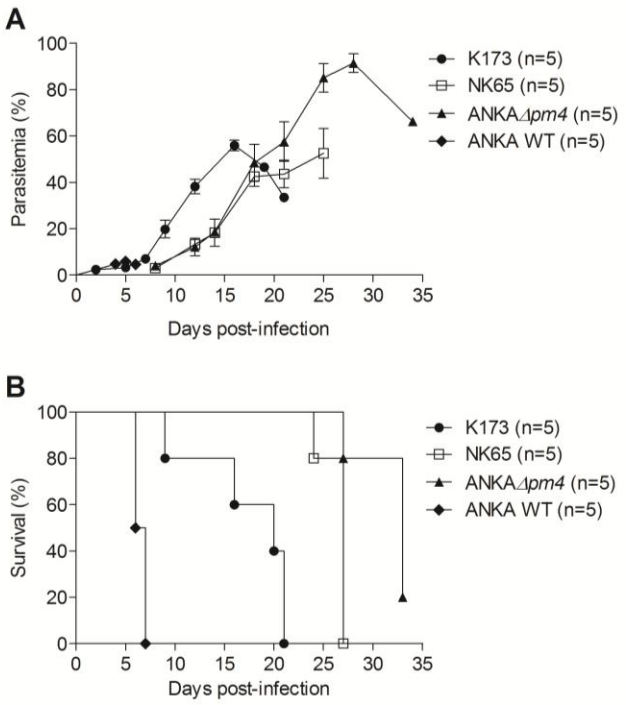

Figure 1- **Susceptibility to infection of C57BL/6 mice.** (A) Timecourse parasitaemia and (B) survival of *P. berghei* K173, NK65 and ANKAΔ*pm4* in C57BL/6 non-pregnant mice. Animals were infected i.p. with 10^6 IE. Parasitaemia of DRAQ-5 labelled samples was followed by FACS. In (B) survival curves were compared using the Log-Rank (Mantel-Cox) test. Statistical significance results were $p<0.01$ when comparing K173 vs NK65 or ANKAΔ*pm4;* $p<0.05$ when comparing NK65 to ANKAΔ*pm4*.

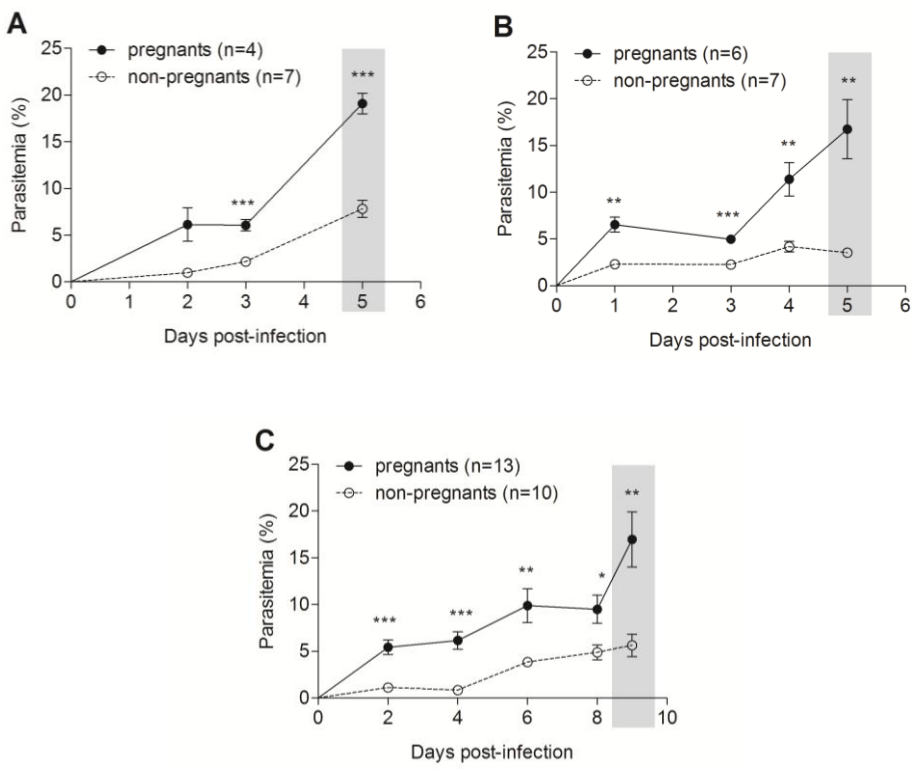

Figure 2- **Maternal susceptibility to infection.** (A) Time-course parasitaemia of *P. berghei* K173 (A), NK65 (B) and ANKAΔ*pm4* (C) in C57BL/6 pregnant and non-pregnant females. Animals were infected i.v. with 10^6 IE at G13 (A, B) or at G10 (C). Highlighted area corresponds to G18 (A, B) or G19 (C). Parasitaemia of DRAQ-5 labelled samples was followed by FACS. Unpaired t test (Welch´s correction) *p<0.05; ** p<0.01; ***p<0.001.

2.5.2 Impaired pregnancy outcome

The impact of infection in the pregnancy outcome was ascertained by the frequency of stillbirths in utero

as well as foetal weight at G18 in pregnant females infected with NK65 and K173 or at G19 in ANKAΔ*pm4* infected mice. Intra-uterine foetal death was increased in pregnancies of NK65-infected mice (69% of the females had a high number of non-viable foetuses),

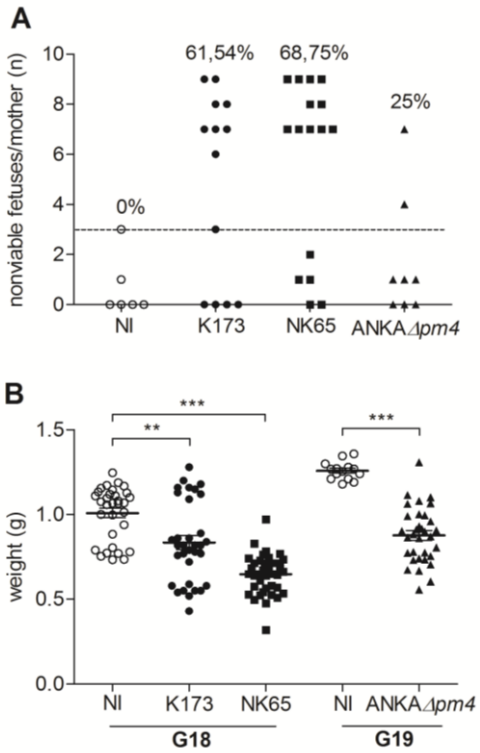

Figure 3- Stillbirths and underweight foetuses as consequence of infection. C57BL/6 pregnants were infected i.v. on G13 (K173 or NK65) or on G10 (ANKAΔ*pm4*) with 10^6 IE. Number of stillbirths (dead foetuses and/or reabsorptions) per mother in utero (A) and viable and non-viable foetuses weight (B) were accessed at G18 or G19. **p<0.01; ***p<0.001; NI: non-

infected. Cut-off for non-viability was established in non-infected pregnant mice (dashed line).

in K173-infected mice (62%) and in ANKAΔ*pm4* infected pregnants (25%) when compared to non-infected controls (Figure 3A and Additional file 1). Viable and non-viable foetuses from NK65, ANKAΔ*pm4* and, to a lesser extent, from K173- infected mothers showed significantly reduced weight as compared to non-infected controls at the same gestational day (Figure 3B). Moreover, newborn viability at delivery was strikingly reduced (Table 1) as compared to non-infected pregnant females. In some instances NK65 infection provoked maternal death during pregnancy (Table 1). These results show that infection of C57BL/6 pregnant females with *P. berghei*-derived parasite lines that fail to induce ECM, recapitulate with different degrees of severity the features of PM

Table I Newborn reduced viability after maternal infection during pregnancy

	NI	K173	NK65	ANKAΔ*pm4*
Number of mothers	5	4	3*	4
Number of newborns	40	24	16	22
Nr. Dead newborns (%)	2 (0.5)	22 (91.6)	16 (100)	15 (68)

NI: non-infected; * Number of surviving mothers from a initial group of 5.

described in BALB/c mice infected *P. berghei* ANKA [11, 12] namely, intrauterine growth retardation and poor pregnancy outcome. Nevertheless, it was clear that infection with ANKAΔ*pm4* had milder effects on pregnancy outcome.

2.5.3 Placental pathology in C57BL/6 mice

Next, infected placentas were examined for pathological features typical of PM including syncytiotrophoblast thickening, tissue destruction, fibrin deposits, thrombi formation and reduction of maternal blood space [12]. HE-stained sections displayed variable degrees of localized trophoblast layer thickening, placental tissue disorganization (Figure 4 B-D) and associated necrosis foci in the labyrinth zone and thrombi (Figure 4E) as compared to non-infected placentas (Figure 4A). Morphometric analysis of the labyrinth zone revealed a significant reduction in the available area for maternal blood circulation in all infected groups when compared tonon-infected controls but NK65-infected mothers showed the highest restriction in blood space area Figure 4F). Thus, the severity of placental pathological alterations evoked by the three parasite lines is distinct which corroborated the observed in pregnancy outcome. Notwithstanding the observed

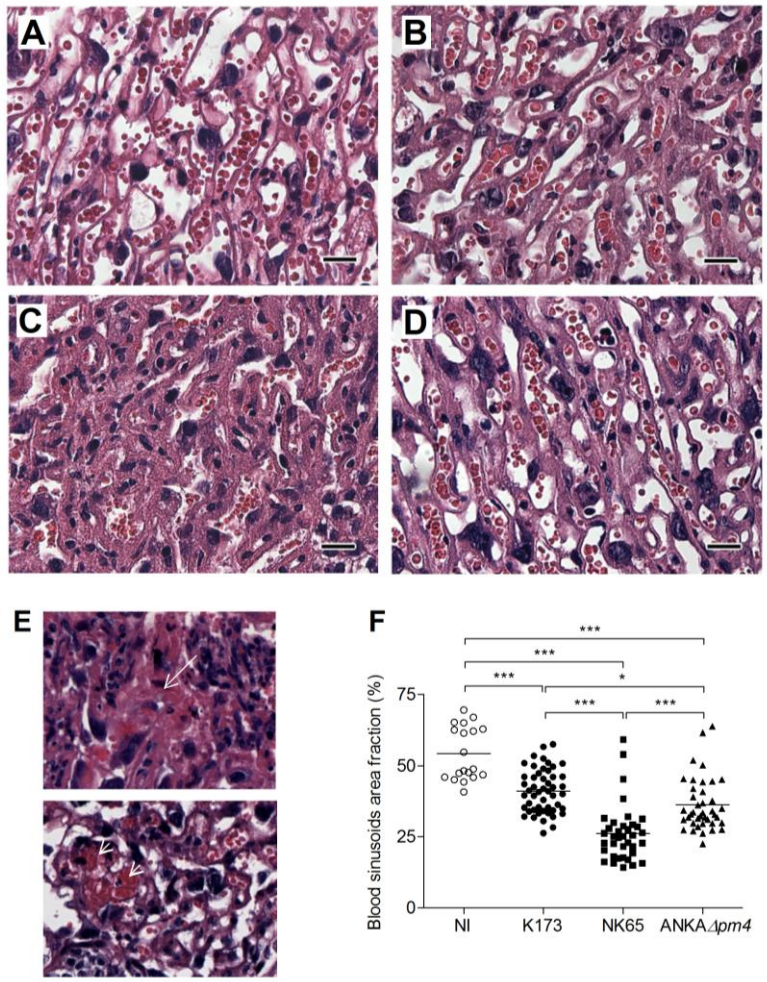

Figure 4. Placental histology of labyrinth zone and morphometric analysis. Representative photomicrograph of H&E stained placental sections of non-infected (NI) (A), K173 (B), NK65 (C) and ANKAΔ*pm4* infected pregnant mice (D). (E) Histological sections show necrotic foci (arrow) and thrombi (arrowhead) in placentas infected with ANKAΔ*pm4* (upper picture) and K173 (lower picture) parasites. (F) Relative quantification of vascular space using an automated morphometric procedure in H&E stained sections evaluated in placental sections. Mice were infected i.v. with 10^6 *P. berghei* K173 or NK65 IE

(G13) or ANKAΔ*pm4* IE (G10); placentas were excised on G18 (K173 and NK65) or G19 (ANKAΔ*pm4*). Scale bar: 20 μm; in (F) line refers to mean; *p<0.05;**p<0.01; ***p<0.001.

differences in pregnancy outcome and placenta pathology, the infection with these three parasite lines did not result in significant differences in placental parasite burden (Figure 5). These results indicate that despite the observed differences in placental pathology and pregnancy outcome, the ability to accumulate in the placenta is not significantly different between the three *P. berghei* lines.

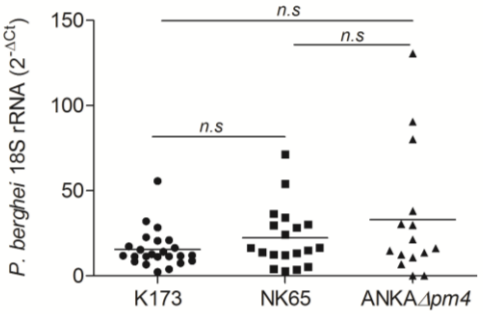

Figure 5- Placental parasite burden. Placentas were collected at G18 (K173 and NK65) or G19 (ANKAΔ*pm4*) and RNA expression of *P. berghei* was evaluated by qReal Time PCR. ΔCT was calculated by subtracting the cycle threshold (Ct) of the target gene from the GAPDH. Dunn´s Multiple Comparison Test, p>0.05 (n.s.).

2.5.4 Differential patterns of placental inflammation

To investigate the differences in pregnancy outcome and placental immune response to infection elicited by the three *P. berghei* lines, gene expression of proinflammatory molecules and informative markers of innate and adaptive immune responses in infected

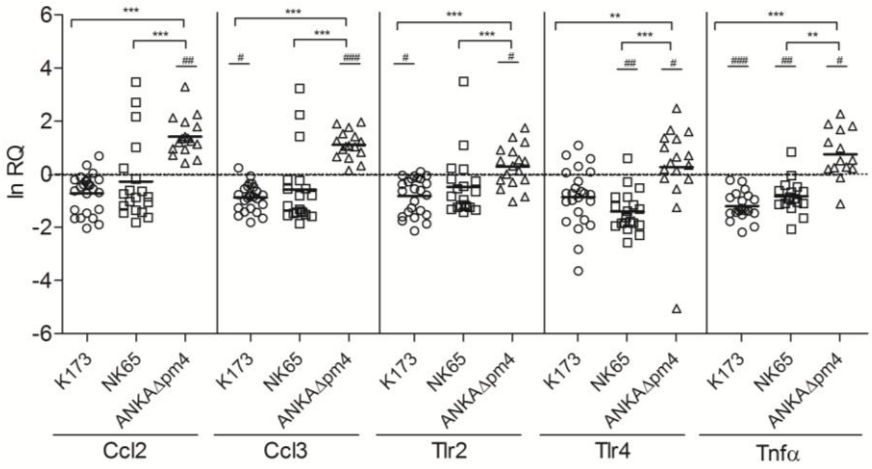

Figure 6- Placental gene expression of inflammatory factors. Placentas were collected at G18 (K173 and NK65) or G19 (ANKAΔ*pm4*) and RNA expression of Ccl2, Ccl3, Tlr2, Tlr4 and Tnfα were evaluated by qReal Time PCR. Relative quantification (RQ) was obtained with normalization by GAPDH. Results are plotted as fold change against non-infected controls collected at the same gestational day (G18 versus K173 or NK65 and G19 versus ANKAΔ*pm4*); Line refers to median values. Kruskal-Wallis Test **p<0.01; ***p<0.001 refers to

differences between different parasite lines; Unpaired t test $^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ compares each parasite line to its respective non-infected control.

relative to non-infected controls were studied (Figure 6 and Additional file 2). Expression of Ccl2 (MCP-1), Ccl3 (MIP-1 α), Tlr (Toll-like receptor) 2 and Tlr4 as well as Tnf was up-regulated in ANKAΔ*pm4*-infected placentas as compared to the other parasite lines. Conversely, placentas of K173- and NK65-infected females exhibited less reactive inflammatory response. This strongly **s**uggests that ANKAΔ*pm4* infection elicits an inflammatory response with a strong innate immunity component, which was not observed with parasites of the K173 and NK65 lines. Taken together, these results suggest that mechanisms involved in PM induction and progression might be different amongst different *P. berghei* lines and that these differences are not associated with the parasite burden in the placenta.

2.6 Discussion

Mouse models amenable to genetic dissection of host factors of PM pathogenesis were established by analyzing the severity of maternal infection, pregnancy outcome, placental pathology and the expression of inflammatory factors following infection with three *P. berghei* lines that do not induce cerebral malaria in the C57BL/6 mouse. The results provide evidence that infection of pregnant females

with these *P. berghei* lines induces PM typical features, strongly suggesting that parasite factors determining cerebral malaria are not required to develop placental infection. Nevertheless, the three parasite strains induced different degrees of placental pathology and impaired pregnancy outcome suggesting that parasite factors could underlie a spectrum of PM manifestations and distinct pathogenesis mechanisms. Thus, NK65 parasites induced the most severe syndrome comprising significantly lower foetal weight and decreased placental vascular area, higher percentage of nonviable foetuses per mother and lower number of live newborns. In addition, NK65 was the only parasite line causing maternal death before delivery (Table 1). Despite similar maternal parasitaemia in NK65 and K173-infected pregnant mice, the latter presented milder effects in placental pathology and in foetal weight loss. On the other hand, ANKAΔ*pm4* infection led to lower stillbirth incidence and increased newborn viability compared to the other strains. These observations support the notion that different *P. berghei* lines show distinct patterns of PM. In fact, an heterogeneous and wide range of clinical manifestations is also observed in women that have malaria during pregnancy, including increased levels of parasitaemia [20-23], increased number of abortions, preterm delivery, intrauterine growth retardation, low birth weight, maternal mortality [24-28] and structural placenta alterations

such as trophoblast thickening and consequent vascular space reduction [7, 29]. Thus, the different *P. berghei* lines represent a fine-tuning resource in constructing experimental systems to study different aspects of pregnancy associated malaria pathogenesis. It is widely accepted that accumulation of IE is a key event in the pathogenesis of severe disease as is the case of respiratory distress, CM and severe PM [1]. The experiments here presented confirmed that PM development was associated with parasite accumulation in the placenta. Nevertheless, the parasite burden in the placenta was not a major determinant of PM severity as the distinct pathology patterns observed in mice infected with NK65, K173 and ANKAΔ*pm4* did not correlate with differences in placenta parasite accumulation. In particular, infection with the ANKAΔ*pm4* line showed a lower impact on foetal viability despite a similar parasite burden in the placenta. An earlier report shows that, ANKAΔ*pm4* parasites failed to induce disease in an ECM model but the resistance phenotype was correlated with lower parasite accumulation in the brain compared to wild-type *P. berghei* ANKA parasites. This virulence attenuated effect was also observed in ECM-resistant mouse strains where self-resolving infection was associated to antibody-mediated response [16]. Nevertheless, this protective effect was not observed in ANKAΔ*pm4*-infected pregnant mice although foetal viability was increased and correlated with a strong

innate immune response. This raises the possibility that the vigorous local innate response in ANKAΔ*pm4* infected placentas deterred the progression of placental tissue disorganization at least for a short period warranting an improved pregnancy outcome. Although expression of pro-inflammatory markers was less stimulated in K173- and NK65-infected placentas at G18 it is not ruled out the possibility that gene expression differences are not exclusively parasite line-related but could also be influenced by differences in parasite kinetics as parasite expansion in pregnant ANKAΔ*pm4* mice was somewhat slower as compared to K173 and NK65. Thus, the observed differences in immune responses might also be influenced by the longer exposure of the maternal immune system to ANKAΔ*pm4* (G10 to G19) as compared to K173 and NK65 parasite lines (G13 to G18). Nevertheless, the PM protracting effects observed in ANKAΔ*pm4* infection offer now interesting research perspectives. This experimental model can be used to (1) discriminate between the effects exerted by foetal- and maternal-derived inflammatory factors in PM pathogenesis and (2) to ascertain whether innate immune responses can be used to provide effective foetal protection in PM.

2.7 Conclusions

The experiments here presented made used of three different *P. berghei* lines and show that parasite components that induce pathology during pregnancy are distinct from those that induce experimental cerebral malaria. In addition, the data indicate that PM pathology in ANKAΔ*pm4* infected mice is associated with an inflammatory response with strong innate immune component, which was not observed in K173- and NK65-infected pregnant mice. The characterization of different experimental systems of PM in the C57BL/6 mouse will allow interrogation of genetically modified mice to ascertain the role of host molecules in PM pathogenesis and to dissect foetal and maternal contributions in placental pathology.

2.8 Acknowledgements

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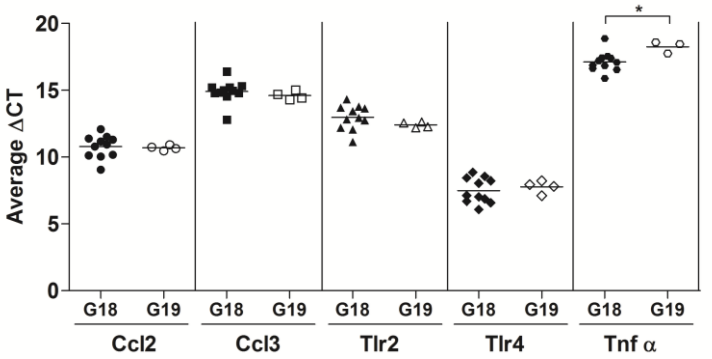
2.9 Supplementary Figures



Supplementary Figure1. Gene expression of inflammatory factors in non-infected placentas. Placentas from healthy pregnant females were collected at G18 or G19 and RNA expression of Ccl2, Ccl3, Tlr2, Tlr4 and Tnfα genes were evaluated by qReal Time PCR. Relative quantification (RQ) was obtained with normalization by GAPDH. *p<0.05.

Supplementary Table 1. Infection during pregnancy increases the percentage of stillbirths

	Number of mothers	**Number of stillbirths**	**% stillbirths /mother**
Non-infected	6	4	0,67
K173	13	64	4,92
NK65	16	91	5,69
ANKAΔ*pm4*	8	14	1,75

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Chapter III

Protective roles of foetal-derived TLR4 and IFNAR1 in experimental Pregnancy-Associated Malaria.

Protective roles of foetal-derived TLR4 and IFNAR1 in experimental Pregnancy-Associated Malaria.

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Manuscript to be submitted.

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3.1 Authors Contributions

All experiments were designed by me and my supervisor Carlos Penha-Gonçalves.

All experiments were performed by me.

All data analysis was performed by me and my supervisor Carlos Penha-Gonçalves.

3.2 Abstract

Pregnancy Associated Malaria is an exquisite form of *Plasmodium* infection that frequently leads to poor pregnancy outcomes. Although innate immunity responses are thought to contribute to the development of placental inflammation, the contribution of foetal derived factors to clinical PAM outcomes have not been addressed. We investigated the role of *Tlr4* and *Ifnar1* genes in a model of mouse PAM using heterogenic pregnancy strategies that allowed the dissection of maternal and foetal-derived contributions to PAM. We found that maternal TLR4 contributes to poor foetal outcomes but does not impact parasite burden. Unexpectedly, foetal TLR4 acted to protect foetal viability and to mediate infected-erythrocytes uptake by trophoblasts in primary cultures but did not influence expression of inflammatory markers expression or pathological features of placental malaria. On the other hand, maternal IFNAR1 contributed to increase peripheral and placental parasitemia and enhanced foetal loss while foetal-derived IFNAR1 conferred resistance to placental infection but did not protect the foetal viability. This work identified maternal *Tlr4* and *Ifnar1* as pathogenesis factors in PAM and uncovered the opposing role of their foetal counterparts in conferring foetal viability protection or placental infection resistance. These findings uncouple placenta parasite

burden and foetal protective mechanisms, highlighting that foetal viability in infected placenta can be controlled by foetal-derived innate immunity factors that may provide new approaches to prevent foetal loss and lessen severe clinical outcomes of PAM.

Keywords

Pregancy-Associated Malaria, Tlr4, Ifnar1, heterogenic pregnancy, foetal viability

3.3 Background

Pregnancy Associated Malaria (PAM) is often linked to severe clinical manifestations including high risk for maternal anemia, foetal abortion, premature delivery and underweight babies [1-5]. Placental Malaria (PM) is a central pathological feature in determining poor pregnancy outcomes and is characterized by the adhesion of infected erythrocytes (IE) to the fetal trophoblast layer [6, 7]. Accumulation of IE in the intra-placental maternal blood spaces correlates with recruitment of inflammatory cells, monocytes and macrophages, and production of pro-inflammatory mediators. Increased IFN-γ and TNF-α production and enhanced levels of monocyte/macrophage recruiting factors (MIP-1α and MIP-1β) have been detected in infected placentas [8, 9]. The local inflammatory process consequently leads to placental tissue disorganization that is often correlated with placental dysfunction and impaired foetal development [8, 10-12].

The severity of placental pathology is associated with a spectrum of severe pregnancy outcomes but the host cellular and molecular components that control the intensity of the inflammatory response are still ill defined. Nevertheless, molecules known to be involved in the host response to the malaria parasite could be involved in placental dysfunction. One such candidate is TLR4. *Plasmodium* glycosylphosphatidylinositol (GPI)

[13, 14] has been shown to stimulate macrophages, dendritic cells and endothelial cells trough TLR4, resulting in increased secretion of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-12 [14-17]. Human genetic studies have associated TLR4 polymorphisms with parasitaemia levels in mild malaria patients [18], risk for clinical malaria [19],risk of severe malaria [20], risk of maternal anemia risk and low birth weight in term infants [21]. Bacterial infections have been shown to cause placental inflammation and poor pregnancy outcomes in a TLR4 dependent manner [22, 23]. Increase foetal death [23, 24], preterm delivery [23] and foetal weight loss [22] was observed in WT but not *Tlr4*$^{-/-}$ females exposed to bacterial infections. In these females, systemic blockage of TLR4 signaling significantly reduced preterm delivery percentage [24] and foetal death [23, 24]induced by bacterial infection. Interestingly, absence of TLR4 expression had no impact on placental bacterial load suggesting that foetal protection mechanisms are independent of infection intensity[23]. Nevertheless bacterial infection favored increased placental TLR4 expression, suggesting that TLR4 signaling participates in the placental response to infection [22]. In addition, it has been shown that peripheral blood mRNA TLR4 is significantly increased in women with idiopathic preterm labor and TLR4 protein is expressed in trophoblasts [25]. TLR4 gene maternal and fetal gene variants were associated to

preterm delivery [26] suggesting a possible role for placental TLR4 expression in pregnancy disturbances.

Recent work has proposed that malaria innate immune responses through TLRs and other putative sensors lead to up-regulation of Type 1 Interferon (IFN-I) stimulatory genes [27]. Strong evidence supports a multi-functional role of IFN-I in during *P. berghei* infection due to the large increase of IFN-stimulated genes and up-regulation of essential gene components of the IFN signaling pathway, such as signal transducers and the IRF family [28].

Interferon type 1 Receptor (IFNAR1) gene polymorphisms have been associated with disease severity and progression to cerebral malaria, especially in children [29-31]. *Ifnar1*$^{-/-}$ mice show significant protection against the development of experimental cerebral malaria (ECM) with reduced accumulation of $CD8^+$ T cells [31, 32]. Furthermore, it was demonstrated that the specific expression of IFNAR1 in $CD8^+$ T cells might, *per se*, be a triggering mechanism for ECM development [31]. ECM and PM share pathogenesis features such as IE adhesion/sequestration [33-36], tissue recruitment of pro-inflammatory cells [12, 31, 37] and tissue damage [38-41]. The involvement of IFNAR1 both in innate and adaptive responses to the malaria parasite warrants the evaluation of its role in PAM.

Here we investigated the role of TLR4 and IFNAR1 in a model of experimental PAM using genetically heterogeneous pregnancies to discern whether the expression of these molecules either in the maternal or foetal compartments is implicated in pregnancy malaria outcomes.

3.4 Methods

3.4.1 Mice and pregnancy monitoring

Eight to twelve week-old C57BL/6(WT), C57BL/6.Tlr4$^{-/-}$ (*Tlr4*$^{-/-}$) and C57BL/6.Ifnar1$^{-/-}$ (*Ifnar1*$^{-/-}$) mice were obtained from the animal facility at Instituto Gulbenkian de Ciência. Mice were bred and maintained under specific pathogen free conditions. Isogenic matings were established as follows: WT, *Tlr4*$^{-/-}$ or *Ifnar1*$^{-/-}$ females were transferred to a cage with one isogenic male (two females: one male). For heterogenic matings, null mutant females were transferred to a cage either with wild-type or null mutant males (two females: one male). In the case of TLR4, an additional mating of null mutant females with heterozygous males was set. In all mating combinations, females were removed after 48 hours, being this considered gestational day 1 (G1). Pregnancy was monitored every other day by weighing females. Successful gestation was confirmed at G13 when females had an increase of 3

to 4 g in body weight. Abrupt weight loss after G13 was an indicator of unsuccessful pregnancy. Animal housing and all procedures were in accordance with national regulations on animal experimentation and welfare and approved by the national animal welfare authorities.

3.4.2 Parasites and infection

In this study we used a parasite line originally derived from the *P. berghei* isolate NK65 at New York University, kindly provided by Dr Maria Mota (Instituto de Medicina Molecular, Lisbon, Portugal). Frozen IE stocks were expanded in C57BL/6 mice prior to infection. Infections in Figure 1 were performed by intra-peritoneal (i.p.) injection of 10^6 infected erythrocytes (IE). For other experiments, pregnant mice and respective non-pregnant female controls were intravenously (i.v.) injected with 10^6 infected erythrocytes. Parasitaemia was measured by flow cytometry [42] to detect infected erythrocytes stained with DRAQ5 (Biostatus Limited). The labeling of infected red blood cells with DRAQ5 is an adaptation of the manufacturer's protocol for cell cycle analysis by flow cytometry. Briefly, a drop of blood was collected by tail pinching of infected mice into 400 µl of FACS Buffer (PBS 1x, 2% FBS, sodium azide 0.02%). DRAQ5 was added directly to the collected samples at a final concentration of 1µM. Samples were mixed by vortex to allow appropriate incorporation of

DRAQ5 into the parasite DNA and were immediately analyzed. Parasitaemia was expressed as the percent of stained cells within the erythrocyte morphological gate. Time of infection and amount of parasite are in accordance to the previously characterized model of PAM in C57BL/6 females infected with *P. berghei* NK65 parasite [43].

3.4.3 Pregnancy outcome and foetal survival

Infected pregnant mice were killed by CO_2 narcosis and subjected to caesarian section on G18 and foetal weight and viability were evaluated. Foetuses were extracted from their amniotic envelop and viability was immediately evaluated by reactive movement to touching with pliers. The lack of prompt movement indicated that the foetus had recently died. Resorptions were identified as small implants with no discernible foetus and placenta. Non-viable foetuses (dead foetuses plus reabsorptions) and foetuses that have been expelled before the gestational day of analysis were recorded as stillbirths. Viable foetuses were killed combining hypothermia and CO_2 narcosis. Weight of viable foetuses was recorded.

3.4.4 Placenta preparations

Placentas from infected and non-infected females sacrificed on the same gestational day separated in

two halves: one half was fixed in 10% formalin for further histological processing and the other half collected in lysis buffer (RNeasy Mini Kit - Qiagen) 1% β-mercaptoethanol for RNA extraction. Paraffin-embedded non-consecutive placenta sections were stained with hematoxylin-eosin (HE).

3.4.5 Isolation of mouse trophoblast

Isogenic matings were established as previously described with the only difference being that females were removed after 24 hours. At G18 females were killed by CO2 narcosis and subjected to caesarian section. Placentas were collected with careful peeling off the decidual tissue. Dissected placentas were kept in a Petri dish containing ice-cold washing buffer (Medum 199 1x – Sigma Aldrich # M9163-500ML, Glutamax - Gibco #35050-038 with a final L-glutamin concentration of 0.1g/L, Sodium Bicarbonate at 0.35g/L – Gibco #25080060 and Hepes Buffer at 20mM – Gibco #15630-056). Trophoblasts were isolated using an adaptation of a previously described protocol[44]. Placentas were sliced into small pieces with a sterile razor blade and collected into a 50 ml conical tube containing an equal volume of 2x concentrated digestion buffer (Colagenase, Sigma Aldrich #C9263-1G at 2mg/ml and DNAse Sigma Aldrich #DN25 at 40mg/ml). Digestion was performed in a water bath at 37°C for 60 minutes with vigorous pipetting every 10 minutes. Undigested material was removed, by passing the

digestion suspension through a 100µm (BD Falcon # #352360) cell strainer followed by a new passage through a 70µm cell strainer (BD Falcon # #352350) and centrifugation at 500g for 5 minutes at 4°C. The resulting pellet was suspended in 10ml washing solution and centrifuged at 500g for 5 min at 4°C. The pellet was suspended in 2ml wash solution and gently overlaid on a Percol® cushion (9.6ml Percol – Sigma Aldrich # P1644-1L, 13.4ml wash solution and 1.1ml 10x Medium 199) in a 50ml tube and centifuge at 30.000g for 30 minutes at 4°C. The trophoblast layer [44] was collected, suspended in RPMI (Gibco #61870-044) complete (10% FBS – PAA # A15-152, 1% HEPES Buffer, 1% Sodium Pyruvate - Gibco® #11360-039, 0.1% 2-Mercaptoethanol – Gibco® #31350-010, 1% Penincilin/Steptomicin - Gibco® #15140-122 and 0.1% Gentamicin – Sigma Aldrich #G1397-10ml) and centrifuged for 5 minutes at 500g at 4°C . Isolated trophoblasts were suspended in RPMI complete and counted.

3.4.6 Parasite synchronization

Infected erythrocytes cells were obtained from infected non-pregnant mice with 10–20% parasitemia. In order to obtain mature blood stage parasite forms (trophozoites/ schizonts), *P. berghei* NK65 infected erythrocytes were synchronized as described elsewhere [45]. Mature forms enrichment yielded over 90% of infected cells. The enriched infected erythrocytes

preparations were suspended in RPMI complete at the desired concentration for posterior stimulation of isolated trophoblasts.

3.4.7 Trophoblasts primary cultures

Isolated trophoblasts from WT or *Tlr4*$^{-/-}$ placentas were plated in 200µl of RPMI complete at the density of 1x10^{6} cells/well in a 96 wells flat bottom plate. Cells were left in culture for 11 days at 37°C 5% CO2. At day 11 the medium was replaced by either RPMI complete only, non-infected or *P. berghei* NK65 synchronized IE at the concentration of 1x10^{6} cells/ml, or purified LPS (Sigma Aldrich #L6511) at the final concentration of 10µg/ml. At 4 and 18 hours after stimulation, cells were washed twice in RPMI complete and harvested by scraping for further analysis to measure RNA expression and to quantify uptake of *P.berghei* parasite by qReal Time PCR.

3.4.8 RNA isolation and gene expression analysis

Total RNA from individual placentas and isolated trophoblasts was obtained using an RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. Equal amounts of each RNA sample were converted to cDNA (Transcriptor First Strand cDNA Synthesis Kit, Roche). *Tlr4, Tnfα, Ifnγ, Il-10, C3, C5a, Md-2 and CD68* expression was quantified using TaqMan Gene

Expression Assays from ABI (Mm00445273_m1, Mm00443258_m1, Mm01168134_m1, Mm00439614_m1, Mm00437838_m1, Mm00439275_m1, Mm01227593_m1 and Mm03047340_m1, respectively). For *P. berghei* quantification, used 18SRNA Taqman assays with specific primers: Forward 5'-CCG ATA ACG AAC GAG ATC TTA ACC T–3', Reverse 5'- CGT CAA AAC CAA TCT CCC AAT AAA GG-3' and Probe 5'– ACT CGC CGC TAA TTA G -3' (FAM/MGB). The endogenous control *Gapdh* (Mouse GAPD Endogenous Control, ABI # 4352339E) was used in multiplex PCR assays with target genes. PCR reactions were performed with ABI Prism 7900HT system. ΔCt was calculated by subtracting the cycle threshold (Ct) of the target gene from *Gapdh* ΔCt. Gene expression results are plotted as fold change over WT non-infected controls (ΔΔCt).

3.4.9 Foetus genotyping

Tlr4$^{-/-}$ and *Tlr4*$^{+/-}$ foetuses were distinguished by tail DNA genotyping. Foetal tails were cut and lysed overnight at 55°C in lysis buffer (100mM Tris HCL pH 8, 5Mm EDTA, 0.2% SDS and 200mM NaCl) with 1% Proteinase K. Genomic DNA was extracted with 2-Propanol and used for TLR4 gene PCR with the following primers: 5' CGT GTA AAC CAG CCA GGT TTT GAA GGC 3' and 5' TGT TGC CCT TCA GTC ACA GAC ACT CTG 3'. PCR products were analyzed in agarose electrophoresis to distinguish mutant and WT alleles. Resorptions tissue was also collected for DNA preparation and genotyping.

3.4.10 Statistical analysis

Survival curves were compared using the Log-Rank test (Mantel-Cox). Non-parametric Mann-Whitney test was applied in pair-wise comparisons of parasitaemia course and Figures 7A and 8B. Kruskal-Wallis non-parametric test with Dunn's multiple comparison test was used for comparisons in peripheral parasitaemia, placental parasite load and foetal wieght. Comparisons of abnormal stillbirth incidence were analyzed with χ^2 Fisher's exact test. Data is considered significant when $p<0.05$. Data regarding parasitaemia in non-pregnant females (Figure 1) and gene expression levels (Figure 7) is presented as mean values +/- standard error of the mean (SEM).

3.5 Results

3.5.1 IFNAR1 but not TLR4 contribute to increased maternal peripheral parasitaemia

The *P. berghei* NK65 parasite has been previously shown to induce death by hyperparasitaemia and pregnancy impairment in C57BL/6 mice [43]. In order to access

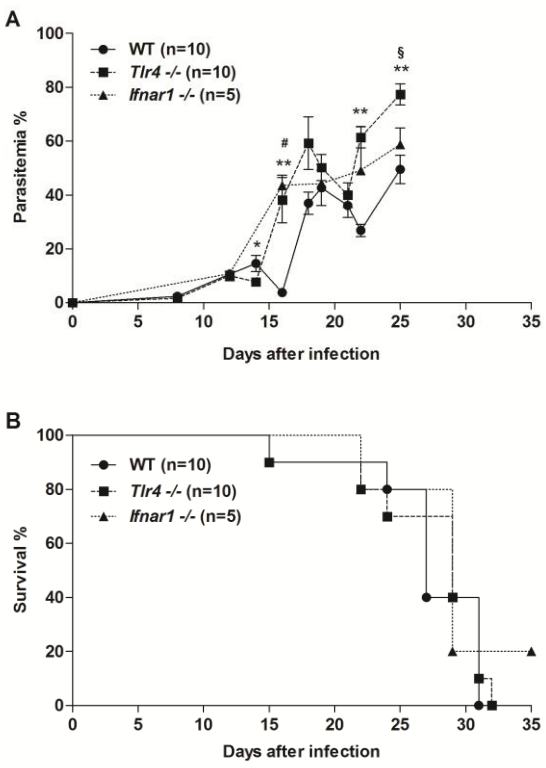

Figure 1. **Susceptibility to *P. berghei* NK65 infection in *Tlr4*$^{-/-}$ and *Ifnar1*$^{-/-}$ females.** (A) Time-course parasitaemia and (B) survival of WT, *Tlr4*$^{-/-}$ and

Ifnar1$^{-/-}$ non-pregnant females infected with 10^6 IE i.p. Parasitaemia was analized by FACS using DRAQ-5 labeled blood samples, at the indicated time points. Genotype pair-wise parasitaemia comparisons used Mann-Whitney test: wild-type *vs* *Tlr4*$^{-/-}$ (*p<0.05 **p<0.01); wild-type *vs* *Ifnar1*$^{-/-}$ ($^{\#}$p<0.05) and *Tlr4*$^{-/-}$ *vs* *Ifnar1*$^{-/-}$ ($^{\$}$p<0.05). Survival curves were compared using the Log-Rank (Mantel-Cox) test (p>0.05, n.s.).

whether the absence of TLR4 or IFNAR1 would influence disease outcome in C57BL/6 (wild-type), *Tlr4*$^{-/-}$ and *Ifnar1*$^{-/-}$ nuliparous females, mice were infected with *P. berghei* NK65 and peripheral parasitaemia and survival were followed over time. The course of infection showed similar parasitaemia profile in the initial infection stages but absence of IFNAR1 or TLR4 slightly anticipated rampant parasitaemia by day 16 post-infection (Figure 1A). No differences were observed in survival rates with most females dying between days 22 and 32 independently of the genotype (Figure 1B). As no detectable differences were observed in initial parasite expansion and infection outcome, this experimental system provided a basis to evaluate the effect of pregnancy in the course of infection in *Tlr4*$^{-/-}$ and *Ifnar1*$^{-/-}$ females. This was not the case in *Rag*$^{-/-}$, *Cd8a*$^{-/-}$ *and* *Tcrβ*$^{-/-}$ females (Supplementary Figure 1A and B) that showed marked differences in infection kinetics as compared to WT mice hampering the evaluation of pregnancy effects in these adaptive immune system impairments. The pregnancy status has been associated to increased susceptibility to *Plasmodium* infection as assessed by

higher levels of peripheral parasitaemia [46-49]. We followed peripheral parasitaemia in *Tlr4*$^{-/-}$ and *Ifnar1*$^{-/-}$ pregnant females infected at G13. All pregnant females presented significantly higher peripheral parasitaemia levels as compared to the respective non-pregnant controls (Figure 2). The absence of Tlr4 in both maternal and foetal compartments or in the maternal compartment alone did not significantly influenced peripheral parasitaemia in pregnant females when compared to WT (Figure 2A). Interestingly, *Ifnar1*$^{-/-}$ pregnant females showed significantly lower levels of peripheral IE when comparing to pregnant wild-type controls (Figure 2C). Furthermore, parasite expansion was also reduced in heterogenic pregnancies where *Ifnar1*$^{-/-}$ pregnant females carry *Ifnar1*$^{+/-}$ fetuses. These results suggest that during pregnancy absence of maternal IFNAR1 confers relative resistance to hyperparasitaemia development.

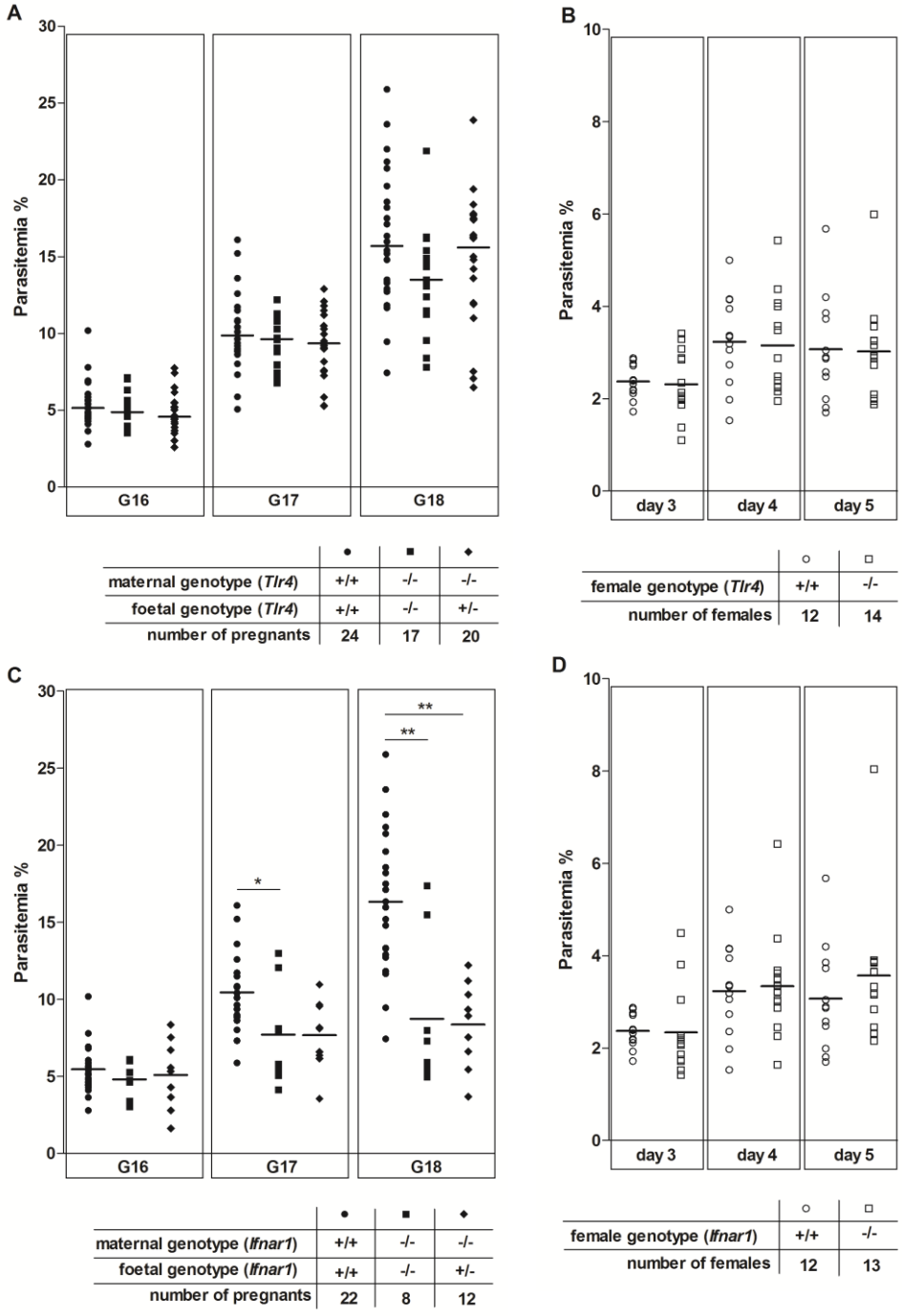

Figure 2. IFNAR1 but not TLR4 contribute to increased maternal peripheral

parasitaemia. Time course parasitaemia of *P. berghei* NK65 in pregnant (A and C) and non-pregnant (B and D) females for the indicated *Tlr4* (A and B) and *Ifnar1* (C and D) maternal/foetal genotype combinations. Animals were infected i.v. with 10^6 IE at G13 for pregnant or day 0 for non-pregnants. Peripheral blood parasitaemia was followed by FACS in DRAQ-5 labelled samples. Kruskal-Wallis with Dunn's multiple comparison test: (A, B and D) $p>0.05$, n.s.; (C) *$p<0.05$; ** $p<0.01$.

3.5.2 TLR4 and IFNAR1 influence foetal weight and stillbirth incidence in pregnancy associated malaria

Experimental PAM is characterized by poor pregnancy outcomes that include low foetal weight and increased stillbirth incidence. The impact of TLR4 and INFAR1 in the pregnancy outcome was ascertained by comparing foetal weight at G18 and stillbirth frequency in infected and non-infected pregnant females of different maternal/foetal genotype combinations. The foetal weight loss induced by infection was less striking in the *Tlr4*$^{-/-}$ pregnant females as compared to WT mice (Figure 3 A). Foetal weight was slightly recovered in *Tlr4*$^{-/-}$ pregnant females that carried foetuses expressing TLR4. Nevertheless, no significant difference was found between the two conditions where the mother lacks TLR4 indicating that foetal TLR4 does not play a significant role in foetal weight loss

(Figure 3A). Likewise, analysis of *Ifnar1* maternal/foetal genotype combinations revealed that independently of foetal genotype, absence of maternal IFNAR1 resulted in significant reduction of foetal weight loss upon infection (Figure 3B).

Interestingly, stillbirths induced by infection were significantly reduced in absence of TLR4. Incidence of abnormal stillbirth was 67% among wild-type infected females against 5% in $Tlr4^{-/-}$ mothers that carried $Tlr4^{+/-}$ foetuses. This dramatic reduction in stillbirth occurrence almost reach stillbirth incidence in non-infected controls. Nevertheless, this result is not fully attributable to absence of maternal TLR4 as $Tlr4^{-/-}$ isogenic pregnancies showed 35% of abnormal stillbirth incidence. This result indicated that the isolated effect of TLR4 in the foetal compartment confers protection against abnormal stillbirth occurrence (Figure 4A). On the other hand, the effect of IFNAR1 in stillbirth incidence was milder and the protective effect appears to be mainly due to the absence of IFNAR1 in the maternal compartment (Figure 4B). Together, these results suggest that maternal IFNAR1 has a role in promoting parasite expansion in pregnant females and contributes to poor pregnancy outcomes. In contrast, TLR4 does not impact on parasitaemia development but expression of foetal TLR4 remarkably contributes to protect foetal viability in context of placental malaria.

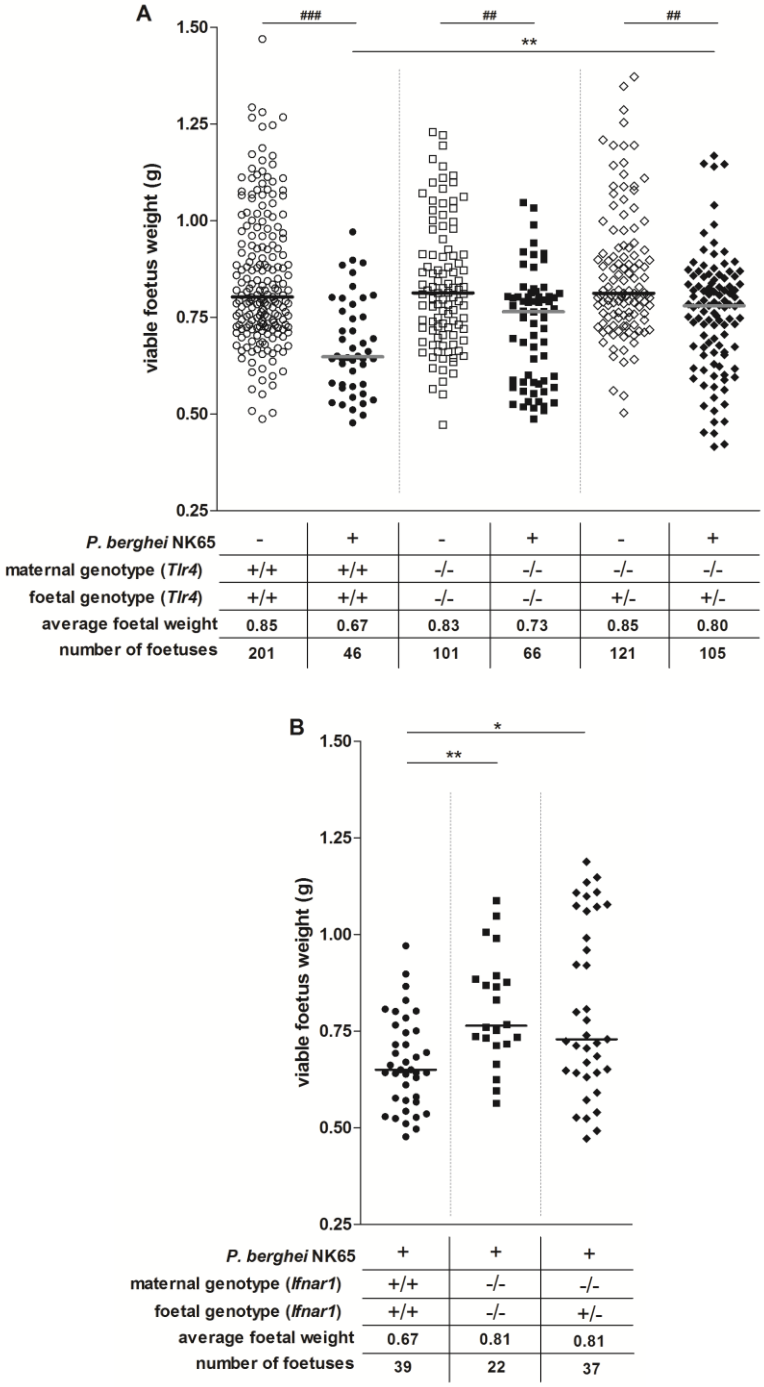

Figure 3. TLR4 and IFNAR1 contribute to low foetal weight in pregnancy

associated malaria. Pregnant females were infected i.v. at G13 with 10^6 IE. Viable foetuses weight was accessed *in utero* at G18 and shown for *Tlr4* (A) or *Ifnar1* (B) in the indicated maternal/foetal genotype combinations. Kruskal-Wallis with Dunn's multiple comparison test (A) infected *vs* infected **p<0.01, non-infected *vs* infected $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 and non-infected *vs* non-infected p>0.05 n.s.; (B) *p<0.05, **p<0.01.

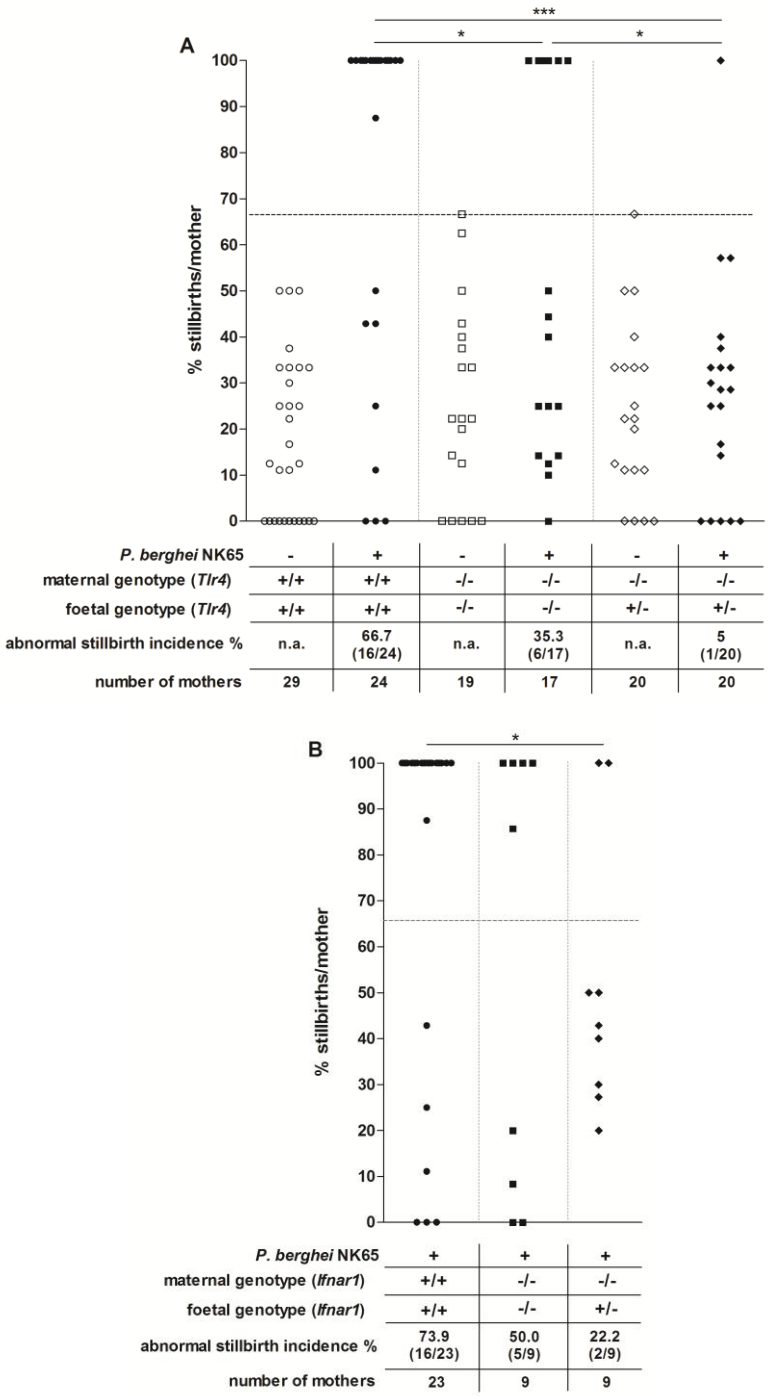

Figure 4. Maternal and foetal contributions of TLR4 or IFNAR1 to

abnormal stillbirth incidence in pregnancy associated malaria. Pregnant females were infected i.v. on G13 with 10^6 IE. Abnormal stillbirth incidence was accessed at G18 and shown for *Tlr4* (A) or *Ifnar1* (B) in the indicated maternal/foetal genotype combinations. Stillbirth percent in individual females was calculated as the number of stillbirths /number of total foetuses X 100. Cut-off for abnormal stillbirth incidence was established considering the maximum number of stillbirths in non-infected pregnant mice (dashed line). χ^2 Fisher's exact test *p<0.05, ***p<0.01.

3.5.3 Placental parasite burden is differentially controlled by maternal and foetal IFNAR1

Pathological features typical of PM include syncytiotrophoblast thickening, tissue destruction, fibrin deposits, thrombi formation and reduction of maternal blood space [10, 38, 39, 43, 50]. Although wild-type placenta appear to present more intense thickening and tissue disorganization (Figure 5C and D), overall sample analysis did not reveal any morphological differences attributable to specific *Tlr4* or *Ifnar1* genotypes (Figure 5C to L). This suggests that the observed differences in parasitaemia and in foetal impairments due to IFNAR1 or TLR4 did not correlate with microscopic alterations in placenta pathology. Likewise, no differences in placental parasite burden were detected in the different maternal/foetal *Tlr4* genotype combinations

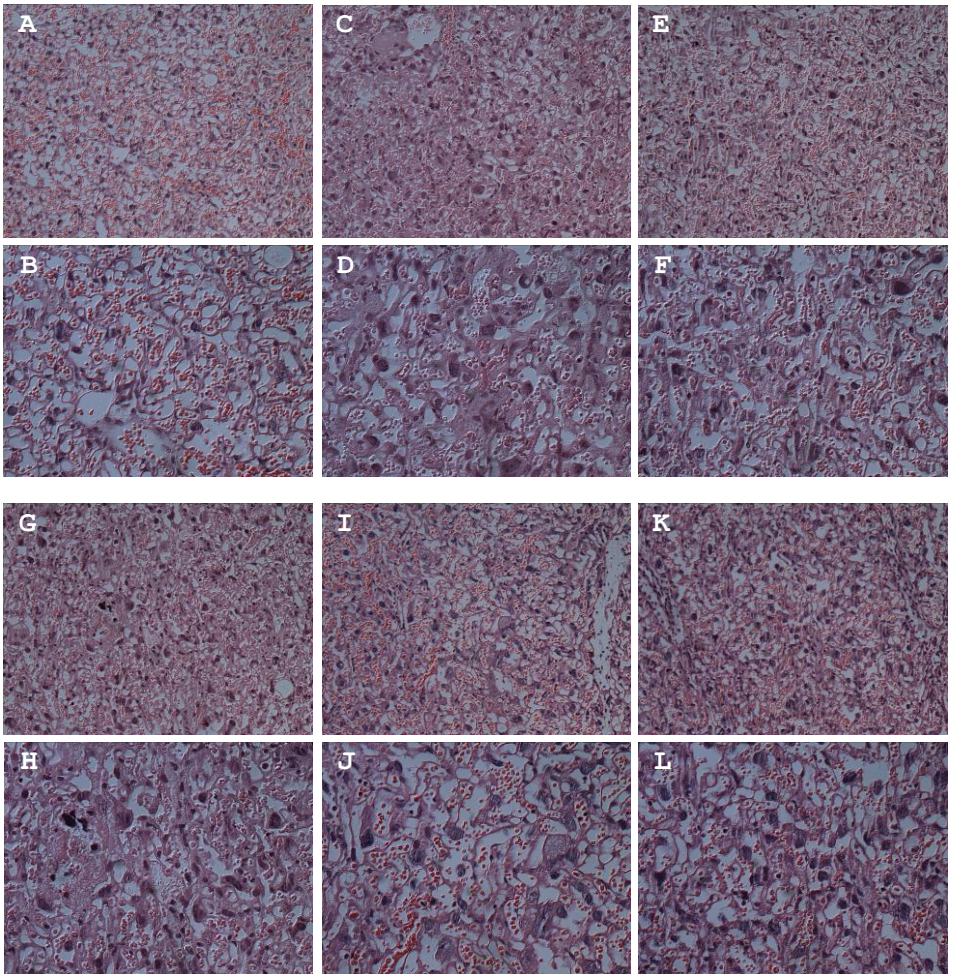

Figure 5. Placental histology of labyrinth zone. Representative photomicrographs of H&E stained placental sections of non-infected (A and B) and infected WT females (C and D), *Tlr4*$^{-/-}$ placenta from *Tlr4*$^{-/-}$ females (E and F), *Tlr4*$^{+/-}$ placenta from *Tlr4*$^{-/-}$ females (G and H), *Ifnar1*$^{-/-}$ placenta from *Ifnar1*$^{-/-}$ females (I and J) and *Ifnar1*$^{+/-}$ placenta from *Ifnar1*$^{-/-}$ females (K and L). Females were infected i.v. with 10^6 *P. berghei* NK65 IE at G13 and placentas were collected on G18. Magnifications: 20x (A,C,E,G,I and K); 40x (B,D,F,H,J and L).

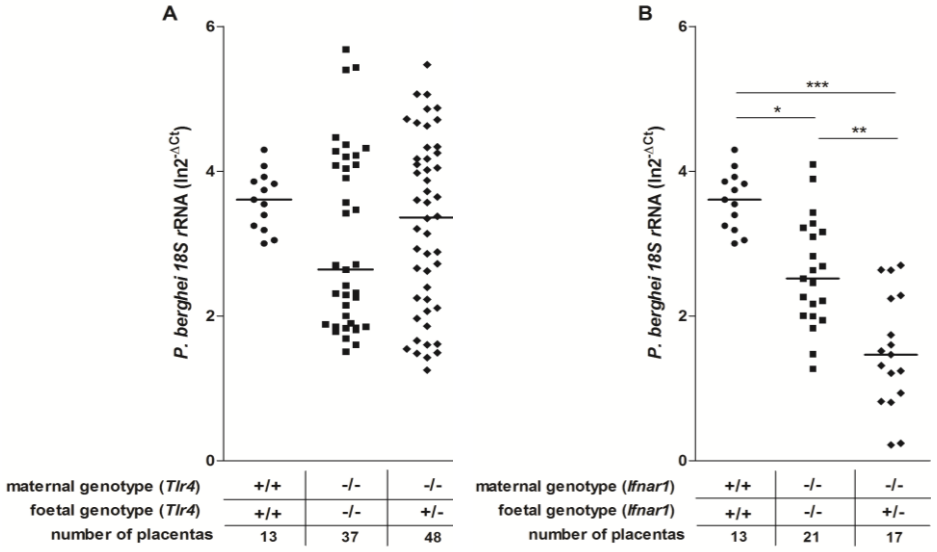

Figure 6. Differential control of placental parasite burden by maternal and foetal IFNAR1. Placentas were collected at G18 and RNA expression of *P. berghei* was evaluated by qReal Time PCR. ΔCT was calculated by subtracting the cycle threshold (Ct) of the target gene from the GAPDH. Kruskal-Wallis with Dunn´s multiple comparison test: (A) $p>0.05$, n.s.; (B) **$p<0.01$; ***$p<0.001$.

(Figure 6A). At contrast, *Ifnar1* genotypic combinations At contrast, *Ifnar1* genotypic combinations revealed that both absence of maternal IFNAR1 and presence of foetal IFNAR1 have a role in reducing placental parasite burden (Figure 6B). These results indicate that maternal and foetal IFNAR1 have opposing effects in placental parasite burden. Interestingly, the observed foetal viability protection conferred by foetal TLR4 expression does not correlate with decreased placental parasite burden, suggesting that protection of fetal viability

by TLR4 was not dependent on controlling intra-placental parasite accumulation.

3.5.4 *Tlr4* and *Tnfα* are downregulated in infected placentas

The indication that foetal TLR4 plays a relevant role in foetal viability upon *Plasmodium* infection, lead us to analyze the molecular inflammatory profile in infected placentas. We found that *Trl4* expression was downregulated in the infected placenta (Figure 7A). On the other hand, no evidence was found for involvement of pro-inflammatory molecules such as *Ifnγ*, *Il-10*, *C3* and *C5a* in placental infection (data not shown). Unexpectedly, we found that *TNFα*, expression in infected placentas was significantly decreased regardless the *Tlr4* genotype (Figure 7B). This analysis suggests that similarly to placenta pathology the inflammatory response in the placenta was not prominently influenced by the *Tlr4* maternal or foetal genoytpes.

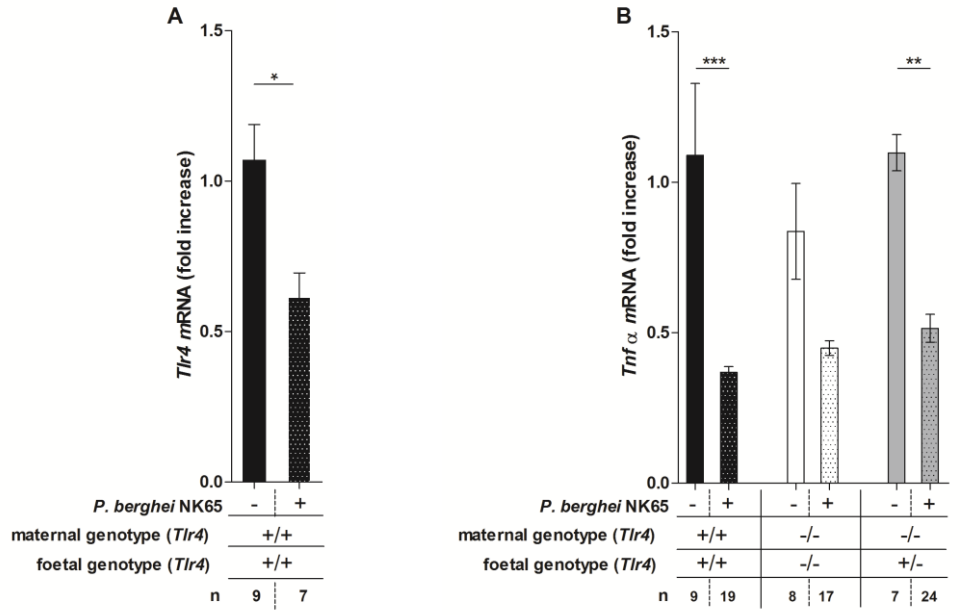

Figure 7. Down-regulation of *Tlr4* and *Tnfα* in infected placentas. Placentas were collected at G18 and RNA expression of *Tlr4* (A) and *Tnfα* (B) were evaluated by qReal Time PCR. Results are plotted as fold change over WT non-infected controls. Represented is the mean +/- SEM. Mann-Whitney test in (A) and Kruskal-Wallis with Dunn's multiple comparison test (B) *p<0.05; **p<0.01; ***p<0.001 compares each mating genetic combination to non-infected WT pregnant females.

3.5.5 Foetal outcome and placenta parasitaemia in heterogenic siblings

To ascertain whether protection of foetal viability afforded by foetal TLR4 was independent of maternal

factors we crossed *Tlr4*$^{-/-}$ females with *Tlr4*$^{+/-}$ males. This heterogenic pregnancies allowed us to compare *Tlr4*$^{-/-}$ and *Tlr4*$^{+/-}$ foetuses and their placentas in single *Tlr4*$^{-/-}$ infected females. In accordance with previous observations in *Tlr4* syngeneic foetus siblings (Figure 3A and 6A), we observed no significant differences in either foetal weight or parasite burden between *Tlr4* heterogenic siblings (Figure 8A and B). We then evaluated stillbirth incidence in the progeny of three females in a total of 17 fetuses. Stillbirth incidence amongst foetuses with *Tlr4*$^{-/-}$ genotype was 75% while *Tlr4*$^{+/-}$ foetuses showed 20% stilbirths (Table 1). Although this result was borderline statistical significance ($p=0.06$) possibly due to small sample size, there was a clear trend towards a local foetal viability protective effect conferred by foetal TLR4 in absence of maternal TLR4.

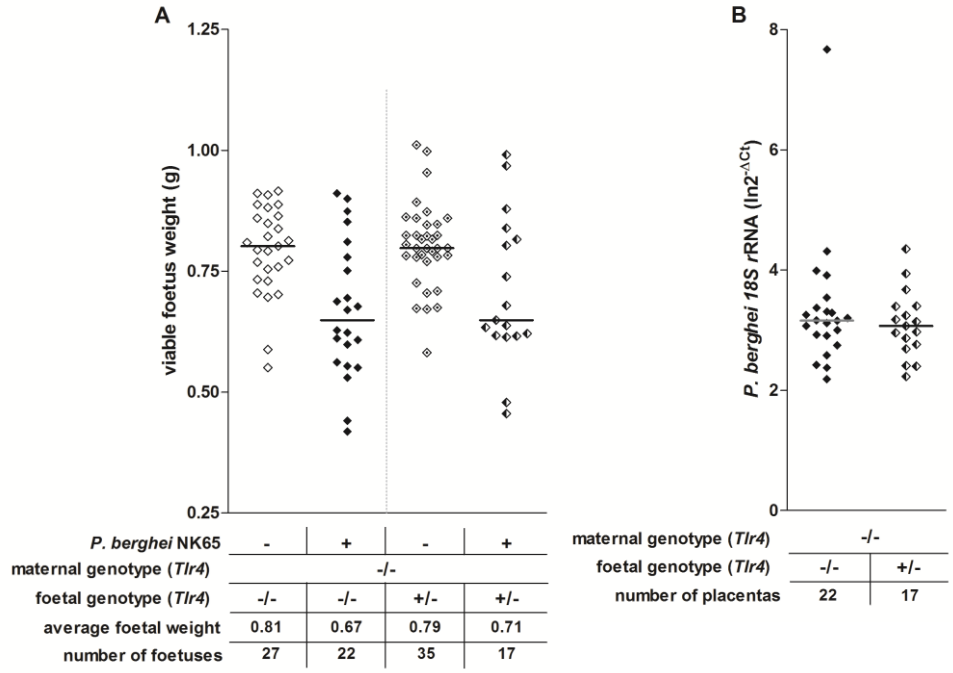

Figure 8. Foetal *Tlr4* genotype, foetal weight and placental parasite burden. *Tlr4*$^{-/-}$ females were mated with *Tlr4*$^{+/-}$ males resulting in pregnant females carrying both *Tlr4*$^{-/-}$ and *Tlr4*$^{+/-}$ fetuses. Pregnant females were infected i.v. on G13 with 10^6 IE. Foetal weight was accessed at G18 (A) and parasite burden was quantified by qReal Time PCR (B). ΔCT was calculated by subtracting the cycle threshold (Ct) of the target gene from the GAPDH. Kruskal-Wallis with Dunn's multiple comparison test in (A) infected *vs* non-infected ($p>0.05$ n.s.) and Mann-Whitney test in (B) *Tlr4*$^{-/-}$ *vs* *Tlr4*$^{+/-}$ ($p>0.05$ n.s.).

Table 1. Differential viability of *Tlr4*$^{-/-}$ and *Tlr4*$^{+/-}$ sibling fetuses from three *Tlr4*$^{-/-}$ pregnant females crossed with WT males.

Foetal genotype	*Tlr4*$^{-/-}$				*Tlr4*$^{+/-}$			
Mother	f1	f2	f3	total	f1	f2	f3	total
Number of stillbirths	4	1	4	9	1	0	0	1
Number of viable foetuses	1	1	1	3	1	1	2	4
Stillbirth incidence %	80%	50%	80%	75%	50%	0%	0%	20%

χ^2 comparison between total stillbirths and viable fetuses between *Tlr4*$^{-/-}$ and *Tlr4*$^{+/-}$ fetuses * $p<0.05$.

3.5.6 TLR4 controls response of isolated trohphoblasts to IE

Trophoblasts are the main cell type of foetal origin that contacts IE in the placental labyrinth. The indication that foetal TLR4 contributes to foetal viability in the infected placenta led us to investigate the role of TLR4 in the placental trophoblasts responses to IE. We isolated wild-type and *Tlr4*$^{-/-}$ trophoblasts from non-infected term placentas and analyzed *in vitro* responses to exposure to *P. berghei* NK65 IE. As for total placenta, no detectable mRNA expression levels were found for pro-inflammatory mediators such as *Ifnγ*, *Il-10*, and *C5a*

(not shown). Likewise, no differences were detected in the levels of *Tnfα* mRNA in *Tlr4*$^{-/-}$ versus WT isolated trophoblasts. Nevertheless, at 4h post IE contact, a significant increase of *Tnfα* mRNA was detected indicating a pro-inflammatory response to parasitized erythrocytes (Figure 9A). The *Tnfα* mRNA falls to levels similar to non-stimulated trophoblasts at 18h post IE contact possibly explaining the observed *in vivo* absence of elevated *Tnfα* levels at G18, 5 days post-infection.

Strikingly, we found that the amount of parasite uptake by *Tlr4*$^{-/-}$ trophoblasts at 4h post IE contact was 3 fold lower as compared to WT trophoblasts suggesting that enhanced IE phagocytosis was mediated by TLR4 expression in trophoblasts. Interestingly, the amount of intra-trophoblast parasite after 18h of IE contact was reduced and indistinguishable in *Tlr4*$^{-/-}$ and WT trophoblasts. These results suggest that TLR4 expression in trophoblasts contributes to uptake of IE but is not impacting the trophoblast production of pro-inflammatory cytokines upon contact with IE.

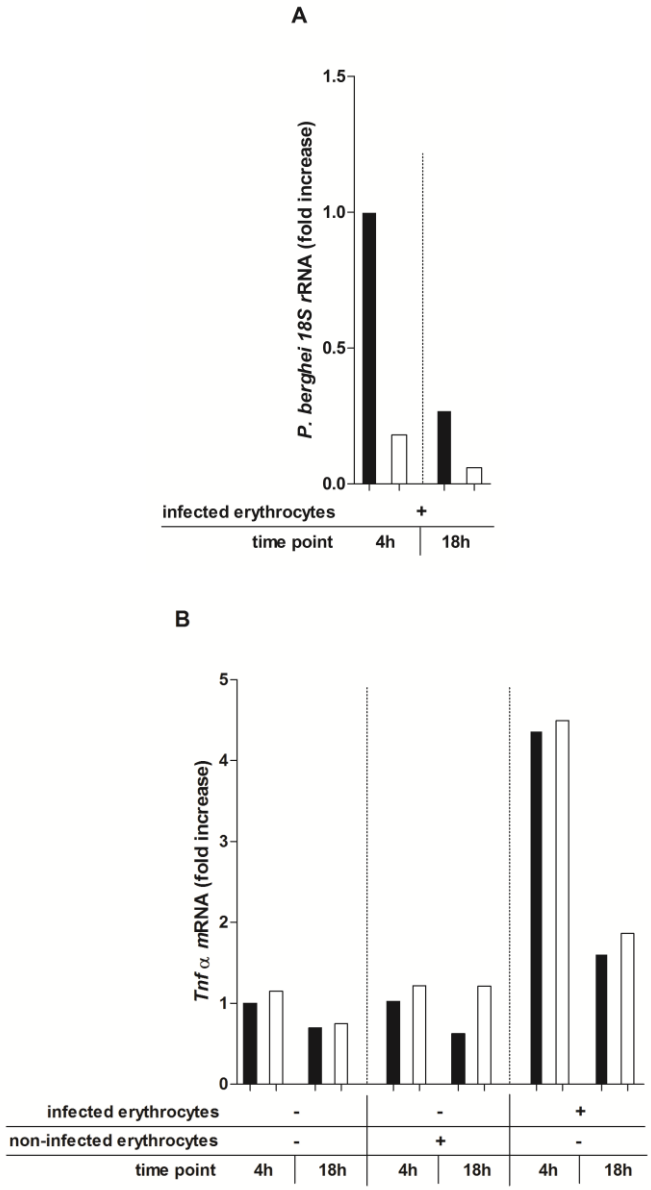

Figure 9. *Tlr4* expression in purified trophoblasts increases *P. berghei* uptake but does not impact *Tnfα* upregulation. Trophoblasts were purified from G18 non-infected placentas. Purified trophoblasts were either left with no stimulus or stimulated with non-infected or *P. berghei* infected erythrocytes and collected

after 4 or 18 hours. Levels of *P. berghei* RNA (A) and *Tnfα* mRNA expression (B) were evaluated by qReal Time PCR. Results are plotted as fold change over 4h WT exposure to IE (A) or 4h no-stimulus WT trophoblasts (B). Representative results from 2 independent experiments are shown. Fold increase is referred to 4h post IE exposure in wild-type trophoblsat (A) or with no stimulus (B).

Together the results suggest that trophoblast TLR4 is involved in parasite clearance in the placenta and that the mechanism of foetal viability protection mediated by foetal TLR4 is not related to impairments in the trophoblast pro-inflammatory response.

3.6 Discussion

We have analyzed the role of TLR4 and INFAR1 in experimental pregnancy-associated malaria with a focus on dissecting contributions of the maternal and foetal compartments to pregnancy outcome and placenta pathology. To this end we compared wild-type, isogenic and heterogenic null-mutation pregnancies in which mother and fetuses carry different genotypes in regard to the gene of interest. This allowed us to analyze the isolated effects of the gene of interest in the foetal compartment in the absence of the same gene in the maternal compartment.

In an experimental system that used the C57BL/6 mouse genetic background and *P. berghei* NK65 infection at mid-gestation, our results provided evidence for a dual role of TLR4 in PAM depending on whether it is expressed in the maternal compartment or in foetal placental tissues. *Tlr4*$^{-/-}$ pregnant females susceptibility to PAM was indistinguishable from WT mice when evaluating maternal disease severity as revealed by increased levels of peripheral parasitaemia. Placental pathology was also prominent regarding placental parasite burden, trophoblast thickening and intravascular space disorganization. Nevertheless, PAM in heterogenic pregnancies was less deleterious to the foetus reducing both weight loss and stillbirth incidence. This suggests that maternal TLR4 plays a pathogenic role in the poor pregnancy outcomes observed in PAM. This observation is in line with the reports on bacterial infections showing that the maternal immune system severely impairs pregnancy outcome in a TLR4 dependent manner [22-24].

Unexpectedly, foetal TLR4 revealed to contribute to protect foetal viability. This indicated that while maternal TLR4 increased stillbirth incidence foetal TLR4 was promoting foetal survival. This effect was only detected when pregnant females lack *Tlr4* suggesting that maternal TLR4 pathogenic effects override foetal TLR4 protection. Analysis of *Tlr4*$^{-/-}$ mothers carrying heterogenic siblings (either *Tlr4*$^{-/-}$ or *Tlr4*$^{+/-}$) confirmed that in the same infection

environment foetal TLR4 did not impact on placental parasite but decreased the occurrence of stillbirths. This finding corroborates the notion that the expression of TLR4 in foetal tissue has an active role in protecting foetal viability in presence of IE.

Interestingly, we found that *P. berghei* infection induced a significant reduction in *Tlr4* mRNA levels (Figure 7A) as opposed to reports of placental TLR4 up-regulation in course of bacterial infection [22]. Furthermore, the amount of parasite uptake in *Tlr4*$^{-/-}$ trophoblast cultures was strikingly lower as compared to WT trophoblasts. This indicated that TLR4 expression in trophoblasts contributes to parasite clearance but is downregulated during infection, suggesting that the parasite counteracts this placental host response. Infection-induced downregulation of TLR4 may in part explain the finding that *"in vitro"* production of pro-inflammatory mediators is not altered in absence of TLR4 expression in trophoblasts.

Although several reports claim that TNFα expression is upregulated upon *Plasmodium* infection [8, 9, 51], there is still controversy to whether this is a hallmark of PAM. In fact, studies have reported low plasma levels of TNF with no differences between aborting and non-aborting females [52]. Our data suggest that the TNFα response in the placenta could be transient and not related to pregnancy outcome. We

concluded that trophoblast pro-inflammatory responses do not provide an explanatory mechanism for the improved foetal viability observed in *Tlr4*$^{+/-}$ *foetuses.* Our findings raise the possibility that *Tlr4* signaling takes part of a trophoblast response to IE that favors foetal viability through a non-inflammation dependent mechanism.

The joint effect of maternal and foetal TLR4 suggests that whenever maternal TLR4 is present, pregnancy outcome will be jeopardized and the foetal TLR4 protection is override. It should be noted that identification of feotal viability protection by foetal TLR4, relies on observations made in hemizygous *Tlr4* expression (*Tlr4*$^{+/-}$ mice and placentas) where TLR4 expression is conferred only by the paternal allele. Therefore, it is conceivable that homozygous expression of foetal *Tlr4* in absence of maternal TLR4 would lead to stronger foetal protection phenotypes.

Contrary to TLR4, a significant increase in peripheral and placental parasite burden is attributable to maternal IFNAR1. This increase in parasite burden correlated with foetal weight loss and increased stillbirth incidence in WT mice as compared to heterogenic pregnancies. This suggests that maternal IFNAR1 contributes to poor foetal outcome in PAM through increasing parasite burden. In opposition, foetal IFNAR1 showed to confer resistance to parasite accumulation in the placenta but does not

significantly influence foetal development and survival. This suggested that reduction of parasite burden in the placenta is not enough to ameliorate PAM outcomes.

Our findings on the involvement of TLR4 and IFNAR1 in PAM highlight that expression of these innate immunity mediators in the maternal compartment has a deleterious role in PAM outcome. Nevertheless, maternal IFNAR1 appears to promote parasite expansion while maternal TLR4 does not impact on parasite burden but possibly impacts on maternal pro-inflammatory response. On the other hand, TLR4 and IFNAR1 foetal counterparts mediate protective responses in the placenta of distinct nature. Foetal IFNAR1 confers relative resistance to placental parasite expansion or accumulation possibly trough enhancing anti-parasite responses but has no effect on poor fetal outcomes. In contrast, foetal TLR4 protects foetal viability but does not influence placenta parasite burden.

Together the data suggests that mechanisms of foetal viability protection mediated by foetal factors are dissociated from responses that control parasite burden in the placenta. Such mechanisms could be of crucial relevance to prevent abortion and stillbirth in PAM. These findings introduce the notion that, regardless of anti-parasite therapeutics, the severe consequences of PAM could be lessened if foetal protective mechanisms were pharmacologically enhanced.

3.7 Acknowledgements

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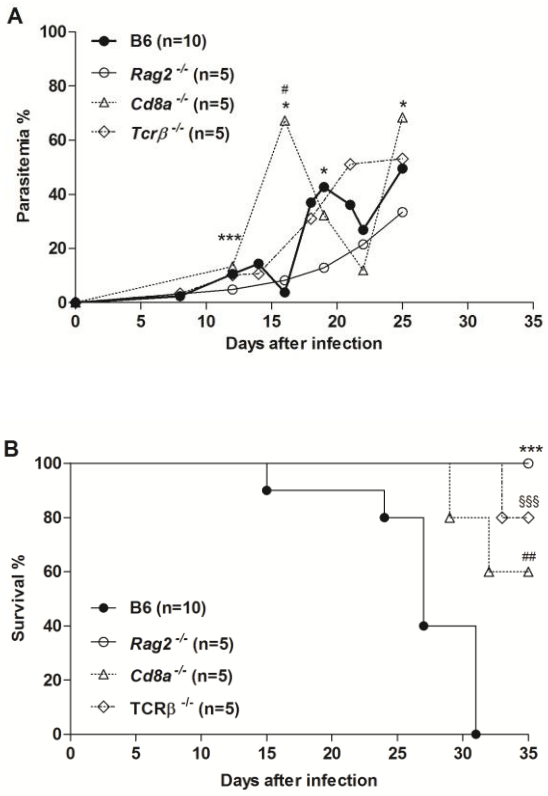
3.8 Supplementary Figure



Supplementary Figure 1. **Susceptibility to infection of B6, *Rag2*$^{-/-}$, *Cd8a*$^{-/-}$ and *Tcrβ*$^{-/-}$ females.** (A) Time-course parasitaemia and (B) survival of *P. berghei* NK65 infected WT, *Rag2*$^{-/-}$, *Cd8a*$^{-/-}$ and *Tcrβ*$^{-/-}$ non-pregnant females. Animals were infected i.p. with 10^6 IE. Parasitaemia of DRAQ-5 labelled samples was followed by FACS. (A) Parasitaemia in the different time points was compared using Mann-Whitney test: WT *vs* *Rag2*$^{-/-}$ (*p<0.05 **p<0.01); WT *vs* *Cd8a*$^{-/-}$ ($^{\#}$p<0.05) and WT *vs* *Tcrβ*$^{-/-}$ (p>0.05, n.s.). (B) Survival curves were compared using the Log-Rank (Mantel-Cox) test: WT *vs* *Rag2*$^{-/-}$ (***p<0.005); WT *vs* *Cd8a*$^{-/-}$ ($^{\#\#}$p<0.01) and WT *vs* *Tcrβ*$^{-/-}$ (§§§p<0.005).

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Chapter IV

Discussion & Conclusions

"The past five years have seen an impressive increase in international funding for malaria prevention, control and elimination. Following the call in 2008 by United Nations Secretary-General, Ban Ki-moon for universal access to malaria interventions, we saw a rapid expansion in the distribution of life-saving commodities in sub-Saharan Africa, the continent with the highest burden of malaria. The concerted effort by endemic country governments, donors and global malaria partners has led to strengthened disease control and visible results on the ground. During the past decade, an estimated 1.1 million malaria deaths were averted, primarily as a result of a scale-up of malaria interventions."

Nevertheless, *"Behind the statistics and graphs lies a great and needless tragedy: malaria - an entirely preventable and treatable disease - still takes the life of an African child every minute. The most vulnerable communities in the world continue to lack sufficient access to long-lasting insecticidal nets, indoor residual spraying, diagnostic testing, and artemisinin-based combination therapies. Unfortunately, only modest increases in access to these interventions were observed between 2010 and 2011 – the first such plateauing in the past 5 years. It is imperative that we act now to ensure that the recent momentum, and its results, are not diminished."*(Quotations from World Malaria Report 2012 [1])

Despite the efforts and clear improvements in malaria elimination, much is yet to be done in order to achieve full disease control and prevent the millions of deaths still registered every year in endemic regions.

Development of an effective vaccine has been one of the main goals of the scientific community and considered the best cost-effective strategy to achieve full protection. Nonetheless, so far, just one vaccine has entered the phase 3 trials with only moderate efficacy in reducing severe malaria episodes in infants [2-4].

Unfortunately, the high degree of *Plasmodium* genetic variability and plasticity poses an exceedingly challenge in the development of a highly effective malaria vaccine. In the context of PAM, the VAR2CSA protein appears as the ideal vaccine candidate. Not only it is expressed on the surface of placenta adhering IEs, as VAR2CSA specific antibodies are prominently acquired by pregnant women and correlate with protection against PAM. Nevertheless, major challenges have been delaying the development of a VAR2CSA vaccine as this is a large and polymorphic protein. The DBL2X N-terminal part of VAR2CSA contains the binding site to placental CSA and is thus currently recognized as the preferential region for vaccine development [5]. Nonetheless, the identification of small epitopes able to induce

adhesion inhibitory antibody responses continues a major challenge for vaccine development [6, 7].

In other perspective, *Plasmodium* infection has also put a strong evolutionary selective force in the human genome. Various genetic host traits, with a direct influence in the severity of infection and disease outcome, are already well documented. Amongst these, the protection afforded by the hereditary red blood cell (RBC) traits as the sickle cell trait [8, 9], Glucose-6-phosphate dehydrogenase deficiency [10] and α and β-talassemia [11]. These RBC genetic traits have all arisen in malaria endemic areas, and their high level of prevalence is thought to result from the significant degrees of protection they confer against *Plasmodium* infection.

In addition to this intense selective pressure with RBC as the prime target for evolutionary adaptation, genetic polymorphisms related to immunity have also been identified.

CXCL10 [12], TLR4 [13], IL-10 and IL-17 [14], ICAM-1 [15], IFNAR-1 [16, 17], TGFB2 [18], IFNGR1 [19], CD40L, IL-1A and IL-13 [20], TNF-α [21] and IL-8 [22, 23] have been shown or strongly suggested to be associated with *Plasmodium* infection manifestations.

In this context, a growing understanding of the molecular basis of host-parasite interactions and genetic factors conferring resistance to the disease

would provide invaluable information on the molecular basis of protective immunity. This type of analysis might soon prove to be the most promising approach in the development of new therapies – such as an effective vaccine - to definitely improve *Plasmodium* infection outcome.

Also during PAM, genetic polymorphism such as KLRK1 and IL-7/IL-7R [24], genes related to the complement system [25], TLR-1 [26], FUT9 [27], TLR-4 and TLR-9 [28] and the TNF2 variant [29] have been indicated to be involved in disease. Nevertheless, despite some genetic polymorphisms have been suggested to be involved in PAM, the genetic analysis of this form of the disease has been neglected when comparing to the amount of data available to other severe forms as is the case of CM.

Interestingly, amongst polymorphisms involved in PM, FLT1 has been demonstrated to have not only a role due to the maternal genetic variant but, also the infant genotype is under selective pressure during infection and influences pregnancy outcome in a parity dependent manner [30].

Taking into account these interesting observations and, intending to further identify genetic factors involved in Pregnancy Associated Malaria, we have decided to dissect maternal from foetal molecular contribution to disease outcome.

While there is growing body of evidence for human genetic factors controlling the outcome of malaria infection, their molecular basis is still poorly understood owing to the ethic and operational limitations. In this context, murine models appear as an excellent alternative genetic tool with comparative mapping studies showing similar genetic-controlled mechanisms of resistance [31].

Although there is a significant offer of murine PAM models, at the beginning of this thesis, none of these models allowed the study of gestation outcome in the C57BL/6 genetic background. Being the goal of this work the study of genetic and molecular basis of PAM and end gestation outcome, I needed to gain access to the multitude of KO strains available in this murine background. In this regard, three new PAM models were established [32] using three *P. berghei* lines that do not induce cerebral malaria in this background. This analysis provides evidence that parasite factors determining cerebral malaria are not required to the development of placental infection and PAM pathology. Interestingly, the heterogeneity in pathology and pregnancy outcome observed with the different *Plasmodium* lines used reflects the wide range of clinical manifestations observed in women that have malaria during pregnancy including increased levels of parasitaemia [33-36], increased number of abortions, preterm delivery, intrauterine growth retardation, low birth weight, maternal mortality [37-41] and

structural placenta alterations such as trophoblast layer thickening and consequent vascular space reduction [42, 43]. Interestingly, parasite burden in the placenta was not a major determinant of PM severity as the distinct pathology patterns observed between infections with NK65, K173 and ANKAΔ*pm4* did not correlate with differences in placenta parasite accumulation. As such, with this work I have developed new PAM models where the different *P. berghei* lines represent a fine-tuning resource in constructing experimental systems to study different aspects of pregnancy associated malaria pathogenesis.

Having established these new experimental models, the next step was to (1) discriminate effects exerted by foetal and maternal-derived inflammatory factors in PAM pathogenesis and (2) to ascertain whether innate immune responses play a role as mechanisms of effective foetal protection in PAM.

To this end, I have analyzed the role of TLR4 and INFAR1 in experimental pregnancy-associated malaria with a focus on pregnancy outcome and placenta pathology. These molecules were chosen due to previous reports showing their involvement either in poor pregnancy outcomes and/or malaria severity. Considering the new PAM models established, NK65 infection was chosen for this part of the work as it induced the most severe syndrome comprising significantly lower foetal weight and decreased

placental vascular area, higher percentage of nonviable foetuses per mother and lower number of live newborns. In addition, NK65 was the only parasite line causing maternal death before delivery.

Focusing on dissecting maternal and foetal TLR4 or IFNAR1 contributions to PAM outcome, wild-type, isogenic and heterogenic null-mutation pregnancies in which mother and fetuses carry different genotypes in regard to the gene of interest were compared. This new approach allowed, for the first time, to analyze the isolated effects of the gene of interest in the foetal and maternal compartments.

With this experimental setup I have provided evidence for a dual role of TLR4 in PAM depending on whether it is expressed in the maternal compartment or in foetal placental tissues. While *Tlr4*$^{-/-}$ pregnant females presented peripheral parasite levels indistinguishable from wild-type, PAM in heterogenic pregnancies was less deleterious to the foetus reducing both weight loss and stillbirth incidence. This suggests that maternal TLR4 plays a pathogenic role in the poor pregnancy outcomes observed in PAM. Unexpectedly, foetal TLR4 revealed to contribute to protect foetal viability. Nevertheless, this effect was only detected when pregnant females lack TLR4 suggesting that maternal TLR4 pathogenic effects override foetal TLR4 protection. The protective role of foetal TLR4 was further confirmed in *Tlr4*$^{-/-}$ mothers carrying

heterogenic siblings (either *Tlr4*$^{-/-}$ or *Tlr4*$^{+/-}$) where foetal TLR4 decreased the occurrence of stillbirths confirming that the expression of TLR4 in foetal tissue has an active role in protecting foetal viability in presence of IE.

Interestingly, we found that *P. berghei* infection induced a significant reduction in *Tlr4* mRNA levels and, the amount of parasite uptake in *Tlr4*$^{-/-}$ trophoblast cultures was strikingly lower as compared to wild-type trophoblasts. Additionally, the expression of TLR4 in trophoblasts does not seem to intervene in production of pro-inflammatory mediators such as TNFα, upon contact with IE.

Together, these data raise the possibility that *Tlr4* signaling takes part of a trophoblast response to IE that favors foetal viability through a mechanism that do not impact the prominent pro-inflammatory response.

Contrary to *Tlr4*, a significant increase in peripheral and placental parasite burden is attributable to maternal *Ifnar1*, correlating with increased foetal weight loss and stillbirths, independently of foetal genotype. On the other hand, foetal IFNAR1 showed to confer resistance to parasite accumulation in the placenta but, contrary to foetal TLR4, does not significantly influence foetal development and survival. This suggested that reduction of parasite burden in the placenta is not enough to ameliorate PAM outcomes.

Overall, this work challenges the commonly accepted pathogenesis model linking placental parasite burden, placental pathology and pregnancy outcome. By showing that the pathogenesis model intervenients can be uncoupled, the current notion that PAM clinical outcomes are determined by placental parasite burden is put into question.

To date, much effort has been put towards the understanding of the maternal immune response during pregnancy. It is well accepted that important immunological changes occur during the gestation period which can influence various diseases outcome.

Nonetheless, there is a growing concern that the maternal immune system is not walking alone on this specific temporal immunological niche [44]. The active role trophoblasts and placental macrophages have during pregnancy is becoming increasingly evident and should definitely not be ignored.

In this work it is presented strong evidence that foetal tissue can significantly interfere in foetal outcome upon malaria infection.

Furthermore, it is shown that mechanisms of foetal viability protection mediated by foetal factors can be dissociated from the mechanistic action of the same molecule in the maternal compartment. Such mechanisms could be of crucial relevance to prevent abortion and stillbirth in PAM.

This work raises the important fact that, regardless of anti-parasite therapeutics, the severe consequences of PAM could be lessened if foetal protective mechanisms were pharmacologically enhanced.

In this sense, if robust therapies are to be applied in preventing poor foetal outcome during PAM, studies where maternal and foetal molecular mechanisms are dissected should be an essential part on the scientific community contribution to disease understanding. Furthermore, our results highlight the relevance of including in epidemiological studies not only maternal genetic analysis but also foetal genetic screening as it might help revealing patterns of genetically-determined clinical outcomes of malaria during pregnancy.

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