UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

Cytoadherence capabilities of *Plasmodium berghei* ANKA and NK65 infected red blood cells in different malaria models

Maria Inês Sousa de Albuquerque

MESTRADO EM MICROBIOLOGIA APLICADA

2011

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

Cytoadherence capabilities of *Plasmodium berghei* ANKA and NK65 infected red blood cells in different malaria models

Dissertação de Mestrado orientada pela Prof. Drª. Ana Margarida Vigário, Instituto de Medicina Molecular, Universidade da Madeira; e pela Prof. Drª. Rita Zilhão, Instituto de Medicina Molecular, Faculdade de Ciências da Universidade de Lisboa

Maria Inês Sousa de Albuquerque

MESTRADO EM MICROBIOLOGIA APLICADA

2011

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

Cytoadherence capabilities of *Plasmodium berghei* ANKA and NK65 infected red blood cells in different malaria models

Dissertação de Mestrado orientada pela Prof. Drª. Ana Margarida Vigário, Instituto de Medicina Molecular, Universidade da Madeira; e pela Prof. Drª. Rita Zilhão, Instituto de Medicina Molecular, Faculdade de Ciências da Universidade de Lisboa

Maria Inês Sousa de Albuquerque

MESTRADO EM MICROBIOLOGIA APLICADA

2011

ACKNOWLEDGEMENTS

To Margarida without whom this would not have been possible. There are not enough words that can thank her enough for her patience (proof-reading!), for her support (even those days when her own work only allows two hours of sleep!) and for her enthusiasm... She was a true mentor and I could not have asked for a better supervisor.

To Prof. Rita Zilhão for her support, her input and proof-reading of the manuscript and for saying "*I always knew it was going to be great*". It meant a lot!

To Prof. Tenreiro, who is one of the reasons I chose to follow Microbiology in the first place.

To Maria and Miguel for giving me such a huge opportunity to keep doing what I love. To Maria, for her input, enthusiasm over Science and for showing a degree of confidence in me that I will do my best to live up to. To Miguel, for always finding new nicknames for me and for that T-shirt that cost me a big chunk of an afternoon discussing the situation in Palestine...

To Vanessa and Fernanda, my two other bosses. Vanessa (my boss#2), for her input, her friendship, the help with the Lumina and the qRT-PCR (and so many other technical stuff I don't need to go on about) and for that car ride! Fernanda, for keeping everything working in the lab, for teaching me how to handle mice and so many other things in the lab, and for being such a great person... What am I doing?! I don't even have to explain why I have to thank you. You two rock!

To Patrícia Meireles (and Miguel) for "Arctic Monkeys" and "The Last Shaddow Puppets". For her friendship, the SBSR, London (we're almost there!), support and enthusiasm over my project. For being a unique, quirky and awesome person.

To Joana Dias, Ana Martins Ribeiro, Joana Carrelha, Patrícia Inácio and Audrey Lopes, the other students (and friends) that have been with me this past year. I can't thank you enough for everything!

To all the others that are part or have been part of UMA since I'm in the lab: Ana Parreira, António Mendes, Bruno Douradinha, Carina Santos, Catarina Marques, Cláudia Cunha, Daniel Carapau, Eliana Real, Filipa Cruz, Ghislain Cabal, Iset Vera, Khirsten Hanson, Liliana Mâncio, Mariana Almeida, Maurice Itoe, Peter Liehl, Sílvia Portugal. Thank you for all the help, input and for receiving me with open arms. Special thanks are in order to António, for helping me with GraphPad and Ilustrator, and to Ana Parreira, my bench-mate!

To Patrícia Almeida, Ana Margarida Bárbaro, Sofia Carvalho, Andreia Marcelino Nunes, Joana Silva and Raquel Jacinto, for taking with me the journey that was Evo-Devo and for being with me ever since. I won't be making big speeches here, because you already know why I have to thank you: you make my life better just for being in it!

To Hélia, for being my great friend these last ten years.

To my parents who have always been there for me and for always having such an incredible faith in me, even when I didn't. For teaching me to do what I love, even though the path might not be the easiest. To my brother, who is always a role-model for me and who actually gets my jokes. I miss you in Portugal! To my aunt Ana, my grandmother Maria, my grandparents Dina and José, my aunts Tina and Antónia, and to my cousins (my other brothers), and to all the rest of my family: I love you all!

To all of you, I dedicate this work. I hope I can make you proud.

ABBREVIATIONS USED

BBB: blood-brain barrier
CM: cerebral malaria
d#p.i.: day *n* post-infection
ECM: experimental cerebral malaria
FI: fluorescence intensity
HCM: human cerebral malaria
i.p.: intra-peritoneal
i.v.: intravenously
MF: mature (intraerythrocytic) forms
MPS: number of merozoites per schizont
NECM: non-experimental cerebral malaria
o.n.: overnight
p.i.: post-infection
PbA: *Plasmodium berghei* ANKA
PbA-GFP: constitutively green-fluorescent protein (GFP) expressing PbA
PbA-LUCI$_{Schiz}$: GFP-LUCIFERASE fusion protein expressing PbA
PbNK65: *Plasmodium berghei* NK65
PbNK65-GFP: constitutively green-fluorescent protein (GFP) expressing PbNK65
PBS: phosphate buffered saline
pRBCs: parasitized red-blood cells
RBCs: red-blood cells
RT: room temperature
WHO: World Health Organization
WT: wild-type

ABSTRACT

Malaria is an infectious disease caused by an intracellular parasite (genus *Plasmodium*). Sequestration of mature erythrocytic forms of *P. falciparum* is a key factor in the development of severe malaria-associated pathologies, namely CM. Brains of patients that died of CM show a preferential sequestration of the MF, and blood-smears of malaria patients at hospital admission show exclusively immature erythrocytic forms. Recently, it was demonstrated that in PbA-infected C576BL/6 mice, an experimental cerebral malaria model, there is accumulation of pRBCs in the brain, as well as $CD8^+$T cells. On the contrary, PbA-infected Balb/C mice, which don't develop ECM, don't accumulate these cell-populations. PbNK65-infected C57BL/6 mice also don't develop ECM, though they accumulate $CD8^+$T cells in the brain, but not infected erythrocytes.

Using these models of malaria, our main goal was to study the blood-stage dynamics and sequestration capabilities of both parasite strains and associate that pattern with the pathology. Results show that MF of both parasite strains are not seen in circulation at a certain point in infection. Also, this disappearance from circulation is concomitant with sequestration of PbA schizonts in the spleen and lungs (C57BL/6 mice), as well as in the fat tissue and liver (both hosts). In addition, we wanted to clarify the importance of parasite *versus* host in cytoadherence of pRBCs to the brain microvasculature. Our results suggest that the absence of PbNK65 sequestration in the brain of C57BL/6 mice is mainly due to differences between this parasite and PbA in its intrinsic cytoadherence capability. Moreover, we observed a significant difference in numbers of merozoites formed per schizont between PbNK65 and PbA infections, which might account for the differences in parasitaemia growth rates and infectivity that have been described for these strains.

Keywords: malaria, *Plasmodium* infection, PbA, PbNK65, sequestration, cytoadherence

RESUMO

A malária é uma doença infecciosa responsável por mais de 225 milhões de infecções e 781 000 mortes por ano, a grande maioria de crianças até aos 5 anos de idade [1]. O seu impacto faz-se sentir não só a nível social, mas também a nível económico, sendo umas das causas da perpetuação do ciclo de pobreza que é vivido nos países afectados por esta doença [2].

A malária é causada por um parasita eucariota intracelular, do género *Plasmodium*, transmitido através da picada de um mosquito do género *Anopheles*, que pode infectar mamíferos, répteis e aves. No Homem, a malária pode ser decorrente da infecção por cinco espécies diferentes: *P. falciparum*, *P. vivax, P. malariae*, *P. ovale* e *P. knowlesi* [3].

Os parasitas do género *Plasmodium* têm um ciclo de vida complexo que se divide entre a fase sexuada, no vector invertebrado, e a fase assexuada, no mamífero. A infecção inicia-se quando o mosquito se alimenta de sangue de um mamífero e deposita na sua derme os esporozoítos [4]. Estes entram na corrente sanguínea, através da qual migram até atingir o fígado, onde irão dar início à fase hepática da infecção, clinicamente silenciosa. Dentro dos hepatócitos, cada esporozoíto irá desenvolver-se, dando origem a milhares de outros parasitas – merozoítos. Estes acabarão por sair da célula-hospedeira em grupos de uma a duas centenas, em vesículas envoltas por membranas do próprio hepatócito [5, 6], chamadas merosomas. Os merosomas entram na corrente sanguínea [5] e tendem a acumular-se a nível dos alvéolos pulmonares [6], onde irão lisar e libertar os merozoítos. Estes infectam eritrócitos, dando início à fase sanguínea da infecção, a fase sintomática da malária [6]. Dentro dos glóbulos vermelhos, o parasita aumenta de tamanho, desenvolvendo-se da forma imatura, em anel, até ao trofozoíto. Nesta fase, o parasita continua a aumentar de tamanho, iniciando-se a replicação de DNA e a produção de cristais de hemozoína, como resultado da digestão da hemoglobina da célula-hospedeira [7]. O momento de passagem a esquizonte dá-se quanto se inicia a esquizogenia. Este processo consiste num conjunto de mitoses assimétricas e assíncronas consecutivas que dão origem a múltiplos parasitas (merozoítos) dentro do eritrócito infectado [8]. Os merozoítos, aquando da lise da célula-hospedeira, libertam-se na corrente sanguínea e infectam outros eritrócitos, dando assim início a um novo ciclo de desenvolvimento sanguíneo. Durante a fase sanguínea, alguns parasitas intraeritrocíticos podem diferenciar-se em gametócitos femininos e masculinos, em resposta a stress ou outros factores. Os gametócitos, ao serem ingeridos por um mosquito numa refeição de sangue, sofrem fusão meiótica e um complexo processo de desenvolvimento que culmina na acumulação de esporozoítos nas glândulas salivares do mosquito. Estes podem ser introduzidos no hospedeiro mamífero na próxima refeição de sangue, assegurando assim a perpetuação da espécie [9].

As formas sanguíneas maturas (FSM) de *P. falciparum* podem ser sequestradas em vários órgãos [10] e esta característica está associada ao grau de severidade da doença [11]. A capacidade de citoaderência tem vindo a ser considerada um factor-chave no desenvolvimento de patologias severas associadas à malária, nomeadamente no desenvolvimento de malária cerebral (MC) [12, 13]. No cérebro de doentes que morreram de MC há sequestração prefencial das FSM, e os parasitas

encontrados nos esfregaços de sangue de pacientes que deram entrada em hospitais com malária severa apresentam exclusivamente formas imaturas em anel [10]. Ratinhos C57BL/6 infectados com *P. berghei* ANKA (PbA) são o modelo de MC mais usado. Foi recentemente demonstrado neste modelo a sequestração de eritrócitos parasitados (EP) no cérebro, assim como de células T $CD8^+$, durante o desenvolvimento da patologia [14]. No entanto, ratinhos Balb/C infectados com o mesmo parasita não desenvolvem malária cerebral experimental (MCE). Foi descrito que no cérebro destes ratinhos não ocorre sequestração, nem de células T $CD8^+$, nem de EP [14]. Curiosamente, ratinhos C57BL/6 infectados com *P. berghei* NK65 também não desenvolvem MCE nem sequestram EP, embora sequestrem células T $CD8^+$ no cérebro [14]. Não é ainda claro se, durante a MCE, as FSM são as únicas que sequestram ou se estão presentes em circulação, tal como na MC humana.

Usando os modelos murinos de malária referidos acima, o objectivo principal deste trabalho é o estudo da dinâmica das formas sanguíneas e a capacidade de citoaderência das duas estirpes de *P. berghei*, e a sua possível relação com sinais de doença observados nestes animais. Os nossos resultados mostram que, nas combinações de parasita-hospedeiro estudadas, as FSM de PbA e de PbNK65 diminuem proporcionalmente em circulação, em determinados fases da infecção *in vivo* e voltam a aumentar em proporção mais tarde na infecção.

Para determinar se o desaparecimento de circulação das FSM de PbA é devido à sua sequestração em algum órgão ou à eliminação do parasita no baço, infectámos ratinhos C57BL/6 ou Balb/C com uma linha de PbA que expressa uma proteína de fusão, a Luciferase-GFP, apenas nas FSM. Em diferentes fases da infecção, e após perfusão para eliminar os parasitas circulantes, determinados órgãos foram extraídos (baço, fígado, pulmões e tecido adiposo) e o conteúdo em luciferase foi determinado por técnicas de bioimagiologia. Os nossos resultados mostram que as FSM de PbA sequestram a nível do baço e dos pulmões (nos ratinhos C57BL/6 infectados), assim como no tecido adiposo e fígado (em ambos os hospedeiros), sequestrando proporcionalmente muito mais no baço e no tecido adiposo. Esta sequestração é concomitante com uma diminuição da proporção destas formas em circulação. É de referir que os baços de ratinhos Balb/C eram mais escuros e de maiores dimensões do que os baços de ratinhos C57BL/6, o que pode indicar um maior conteúdo em hemozoína e uma maior proliferação celular. Isto sugere uma maior eliminação do parasita pelo baço nos ratinhos Balb/C comparativamente com os ratinhos C57BL/6. No entanto, a quantidade de esquizontes viáveis sequestrados é significativamente inferior nos ratinhos Balb/C do que nos ratinhos C57BL/6.

Pretendíamos também esclarecer a importância relativa do parasita *versus* hospedeiro na citoaderência de EP à microvasculatura cerebral, durante o desenvolvimento de MC. Especificamente, pretendíamos perceber se a ausência de sequestração de EP de PbNK65 no cérebro de ratinhos C57BL/6 é devida à incapacidade destes EP aderirem ao endotélio activado, ou se poderão haver diferenças no micro-ambiente gerado no cérebro em resposta ao parasita que possam explicar esse fenótipo. Para isso, co-infectámos ratinhos C57BL/6 com PbNK65-GFP e PbA. Usando PCR-quantitativo em tempo real, medimos a expressão de GFP (e portanto a presença do parasita PbNK65) nos cérebros destes ratinhos, na fase final de MC. Os nossos resultados sugerem

que os EP por PbNK65 não são sequestrados no cérebro de ratinhos C57BL/6, devido à sua incapacidade intrínsica de aderir ao endotélio activado.

Este projecto pretendia clarificar a dinâmica das formas circulantes e a capacidade de citoaderência de duas estirpes de *P. berghei*, e a forma como estes padrões se podem correlacionar com a patologia. Os resultados não são conclusivos e mais experiências têm de ser realizadas para elucidar este assunto. No entanto, consideramos que indiciam diferenças entre os mecanismos de sequestração no cérebro em relação a outros órgãos, de PbA, assim como de PbNK65. Os nossos resultados também sugerem que existem diferenças intrínsecas ao parasita na capacidade de citoaderência de EP por PbA *versus* EP por PbNK65 à microvasculatura cerebral. Além disto, observámos uma diferença significativa no número de merozoítos formados por cada esquizonte, entre a infecção sanguínea por PbA e por PbNK65. Sugerimos que este facto pode estar na origem das diferenças já descritas [15] entre as duas estirpes de *P. berghei* no que diz respeito à taxa de crescimento de parasitémia e infecciosidade.

Palavras-chave: malária, infecção com *Plasmodium*, PbA, PbNK65, citoaderência, sequestração

CONTENTS

Acknowledgements 1
Abbreviations used 2
Abstract 3
Resumo 4
Contents 7
Introduction 8
Malaria 8
Plasmodium life cycle 8
Sequestration and human cerebral malaria 11
Murine models of cerebral malaria 13
The *Plasmodium berghei* parasite 13
Sequestration in ECM – new insights from murine models 14
Aims 15
Materials and Methods 16
Parasites 16
Mice 16
Induction and progression of blood-stage infections 16
Assessment of experimental cerebral malaria onset and progression 16
In vitro synchronization and purification of *Plasmodium berghei* late intraerythrocytic stages 17
Quantification of *Plasmodium berghei* pRBCs by flow-cytometry 18
Quantification of brain parasite load by qRT-PCR 18
Determination of parasite load by *in vivo* imaging techniques 19
Ex vivo cytoadherence assays 19
Violet Cresyl staining for analysis of infected brain sections 20
Statistical analysis 20
Results 21
Dynamics of circulating blood-stage forms of PbA and PbNK65 21
*V*alidation of flow-cytometry gating strategy 21
In vivo dynamics of asexual circulating forms of PbA and PbNK65 23
Assessment of PbA cytoadherence in different organs 26
PbA and PbNK65 cytoadherence capabilities 27
Discussion and future work 32
References 37

INTRODUCTION

Malaria

According to the WHO, malaria alone is responsible for more than 225 million infections and close to 781 000 deaths each year, the vast majority children up to 5 years of age. The number of deaths, if co-infection with AIDS and/or tuberculosis is accounted for, may be close to 3 million, each year [1]. Despite the fact that malaria has accompanied humans since the dawn of civilization – the first records dating back to 2600 BCE, in China and Egypt [16] – it is still one of the most deadly infectious diseases in the world [1]. Moreover malaria is not only a disease with a severe social burden, but also with a serious impact on the economy. In some countries, it accounts for up to 40% of all public health expenditures. If we consider that up to 20% of people who survive the more severe cases of the disease develop *sequelae* (mostly neurological), we must also account for decreasing productivity, which perpetuates the cycle of poverty [2].

Malaria is an infectious disease caused by an eukaryotic parasite from the genus *Plasmodium,* which can infect birds, reptiles and mammals [17], though each *Plasmodium* species infects a limited number of closely related vertebrate species [3]. Five species can cause malaria in humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (a macaque monkeys' parasite that has been recently described by the WHO to infect and cause the disease in humans). *P. falciparum* and *P. vivax* are responsible for the vast majority of clinical cases, the former being responsible for the most severe forms of malaria, which include cerebral malaria (CM) and malaria-associated respiratory distress syndrome (MAARDS). Malaria is transmitted to the vertebrate host by the bite of infected female *Anopheles* mosquitoes, which are part of the most ubiquitous and abundant arthropod genera existent [3].

Plasmodium life cycle

Plasmodium parasites have a complex life-cycle, consisting of the asexual stages, in the vertebrate host, and the sexual stages that take place in the mosquito vector (**Figure 1**).

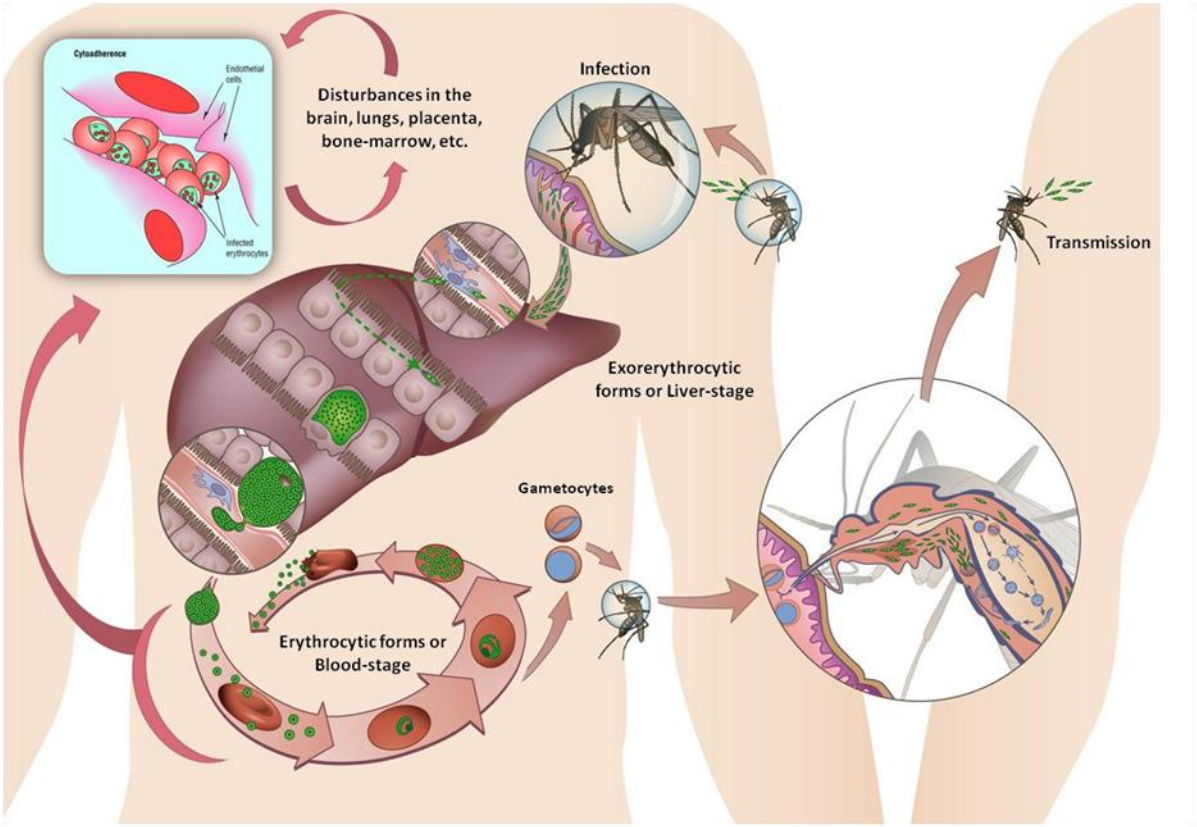


Figure 1. *Plasmodium* life cycle. Adapted from "*Plasmodium* life cycle" from the Malaria Unit, Instituto de Medicina Molecular.

Human malaria infection is initiated when infected female *Anopheles* mosquitoes take a blood-meal and deposit sporozoites in the dermal tissue of the human host [4] (**Figure 1**). Sporozoites then travel through the circulation until they reach the liver. Here they go through an obligatory and clinically silent developmental stage that can last up to 16 days, depending on the *Plasmodium* species (reviewed in [18]). In the liver, each sporozoite traverses a few hepatocytes before developing and replicating in a final one [19], originating thousands of merozoites. The parasite's membrane will collapse, causing the merozoites to be released in the cytosol of the host cell. They will then form aggregates of a couple hundred merozoites that remain enclosed in membranes of host-cell origin [5, 6]. These structures – merosomes – bud out from the infected hepatocytes, enter the bloodstream [5] and tend to accumulate in the lungs microvasculature [6]. Then they will break up and release merozoites into the bloodstream, beginning the erythrocytic/blood-stage, which is the symptomatic stage of the infection [6].

The erythrocytic stage (**Figure 1**, **Figure 2**) begins when a merozoite adheres to and infects a red-blood cell (RBC). Parasite entry into erythrocytes is a complex and dynamic process not fully understood. It likely requires proteins residing on the invasive parasite surface, called merozoite surface proteins, as well as other proteins associated with secretory organelles directed by the parasite toward the junction of invasion (reviewed in [20] for *P. falciparum*). By the end of the recognition and ligation process, the invagination of the erythrocyte membrane leads to the engulfment of the parasite and the establishment of the intracellular ring-stage parasite that is characteristically surrounded by a vacuolar membrane [21].

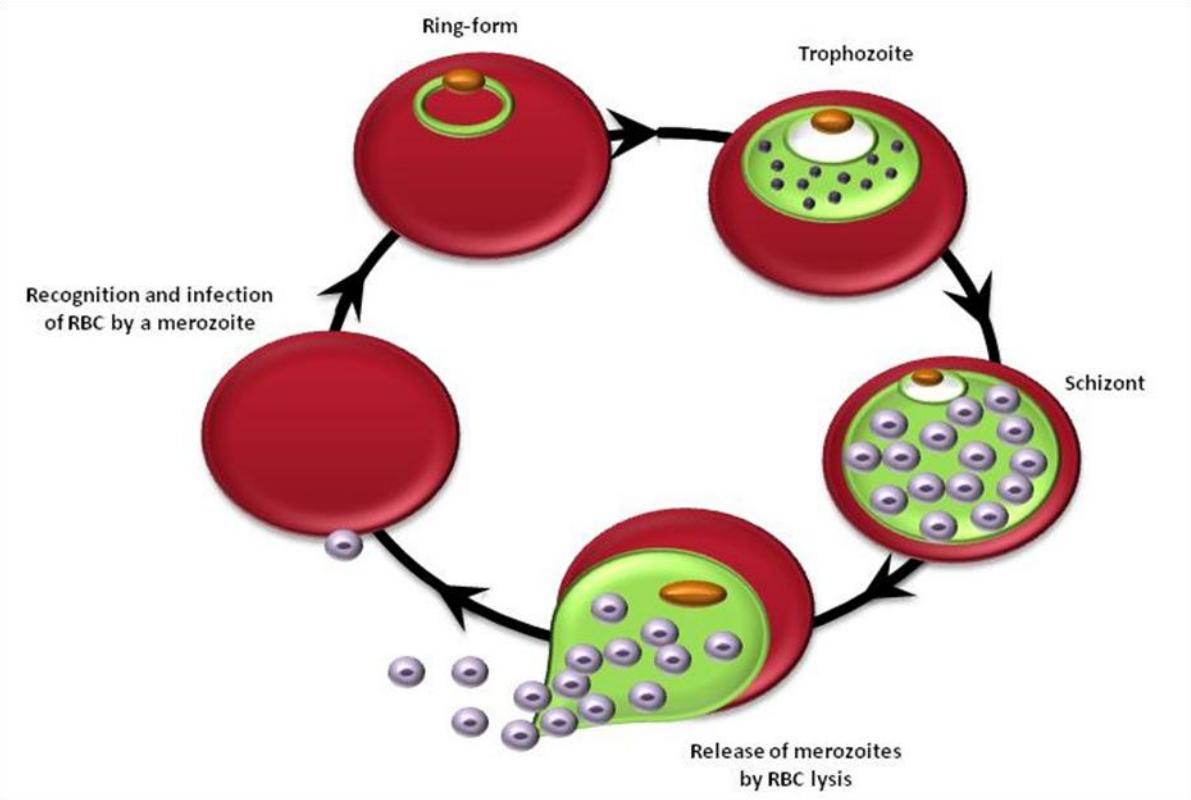


Figure 2. ***Plasmodium*** **intraerythrocytic asexual life cycle.**

The ring-form will grow in size, giving rise to a trophozoite. By this stage, the parasite has exhausted the host-cell's metabolites. Haeme is produced by the trophozoite as a toxic product of haemoglobin digestion and is converted into haemozoin, a pigmented crystal that is characteristic of *Plasmodium* blood-stage infection (reviewed in [7]). Later in this stage, the parasite will continue to grow in size (as well as haemozoin content), starting DNA replication inside its host [7, 8]. Entrance in the schizont stage is defined by schizogeny, i.e. the asymmetric and asynchronous mitotic division that leads to the formation of multiple parasites (merozoites) inside the host RBC [7, 8, 22]. Merozoites will eventually cause the host-cell lysis, being released into the bloodstream. They will quickly invade and infect a new RBC, thus beginning a new cycle of intraerythrocytic development (**Figure 1**, **Figure 2**).

The number of merozoites formed per schizont can be up to 32 and varies between *Plasmodium* species (reviewed in [23]). This multiplication rate is related to the increase in parasite biomass that occurs in each cycle, which is known to be related with disease severity in human patients [24].

Some parasites inside RBCs can differentiate (in response to stress or other cues) into male or female gametocytes. Upon ingestion by a feeding female mosquito, they can fuse, undergo complex sexual development and form infective sporozoites that accumulate in the salivary glands of the mosquito and can be introduced into the human host at the next blood meal, thereby ensuring the perpetuation of the species (reviewed in [9]).

The typical pattern of malaria symptoms and signs – which include fever, nausea and headache, sometimes accompanied by diarrhoea and vomiting – is associated with the release of merozoites by erythrocytes. The time pattern of these signs depends on the length of the erythrocytic cycle (24, 48 or 72 hours, depending on the *Plasmodium* species) and also on its relative synchronization. The fever cycles are due to the synchronous development of *Plasmodium* inside RBCs, their rupture and the coordinated mass-release of merozoites and toxins into the bloodstream (as reviewed in [23]). These symptoms/signs may remain relatively mild, or progress to severe attacks (reviewed in [25]). If left untreated, *P. falciparum* malaria may evolve to severe and often fatal manifestations, such as malaria-associated acute respiratory distress syndrome (MAARDS) or cerebral malaria (CM), partly due to sequestration that is a hallmark of this species.

Sequestration and human cerebral malaria

Cerebral malaria (CM) is a severe form of *P. falciparum* infection that affects 575 000 children a year in Africa with 10-40% lethality (reviewed in [1]). CM is characterized by cerebral complications, such as neuronal damage and coma, often accompanied by fever, convulsions, seizures, metabolic acidosis and hypoglycaemia. A significant proportion of survivors have permanent neurological complications including cognitive and speech disorders, motor abnormalities and cortical blindness (reviewed in [25]).

Although it has been the subject of a number of studies throughout the XXth century [26], the cascade of events leading to CM is still poorly understood [27, 28].

One of the first scientific descriptions of cerebral malaria dates back to 1717 and was written by Giovanni Maria Lancisi. Though his "*De noxiis paludum effluviis, eorumque remediis*" is focused in the possible means of transmission of malaria [29], he was one of the first to describe "the graphite pigmentation of a postmortem (…) brain", i.e. the presence of haemozoin (the malarial pigment present in the blood-stage parasites) in the brains of patients that died of CM.

Nowadays, there are two complementary hypotheses that attempt to explain the causes of human cerebral malaria (HCM): the "mechanical hypothesis" (reviewed in [12]) and the "inflammation hypothesis" (reviewed in [13]).

According to the "mechanical hypothesis", the mechanical pressure or blockage caused by the accumulation of pRBCs, in the brain's microvasculature (due mainly to their decreased deformability), causes a decreased blood-flow in this organ, leading to the blood-brain barrier (BBB) breakdown, which in turn will cause haemorrhages, decreased oxygenation and removal of waste products.

On the other hand, malaria infection causes a generalized inflammatory process directed at controlling the infection. The inflammation process includes the activation of the innate immune response, the production of pro-inflammatory cytokines and the activation of T cells, leading to an up-regulation of adhesion molecules in circulating cells and in the endothelial cells, as well as secretion of chemokines important for leukocyte traffic. According to the "inflammation hypothesis", as a

consequence of this cascade, sequestration of pRBC, platelets and activated immune cells (including $CD8^+$ T cells) would occur in the microvasculature of the host, accounting for the BBB breakdown, haemorrhages and all the neurological disturbances commonly associated with HCM.

Therefore, both hypotheses consider brain sequestration/accumulation of pRBCs as a key factor in the development of HCM. Though both hypotheses are unlikely to mutually exclude each other, the "inflammation hypothesis" has gained more and more evidences to support it in the last years (reviewed in [30] and [31]).

Thus, it has become clear that sequestration in the brain of the later erythrocytic stages (as well as of leukocytes) is a major process in the development of HCM and that it seems to be concomitant with their absence from the circulating blood [10]. This is in accordance with the exclusivity of ring-forms in the blood-smears of *P. falciparum* infected people appearing in hospitals. In other human non- *P. falciparum* malarias, all stages can be present in the circulating blood (as reviewed in [32]).

It is thought that this capability to sequester, which contributes greatly to the virulence of *P. falciparum*, is also a mechanism that favours the development of the parasite by protecting it from the action of the spleen, where the mature erythrocytic stages of *P. falciparum* would be more easily detected and eliminated by the macrophages (reviewed in [11]).

The mechanisms of pRBCs sequestration remain unclear. However, ultrastructural studies have suggested that adherence of parasitized erythrocytes to the vascular endothelium occurs by means of excrescences on the infected erythrocyte membrane that have been called knobs [11]. Others have also pointed out that mature-stage pRBCs express new surface antigens and are significantly less deformable than ring-stage pRBCs (as reviewed in [32]), which may also be a consequence of the presence of knobs. These are cup-shaped structures that underlie protrusions of the erythrocyte membrane, the main component of which is the knob-associated histidine-rich protein (KAHRP), essential for the sequestration phenotype [33].

As the parasite develops inside the erythrocyte, several parasite-encoded proteins become associated with its host-cell cytoskeleton or are inserted into the host cell membrane, via Maurer's clefts. These consist of a convoluted set of membranes, morphologically similar to the Golgi apparatus, located underneath the pRBC membrane, and appear to be involved in trafficking and sorting of proteins destined to be delivered to the erythrocyte's cytosol and membrane (reviewed in [34]). Some proteins, such as the widely studied *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) or protein 3 (PfEMP3), are transported through these clefts in association with knob proteins, like KAHRP, and are necessary to promote cytoadherence and sequestration of pRBCs within the blood-vessels [34]. Proteins associated with Maurer's clefts-like and knob-like structures have been identified in other *Plasmodium* species – such as *P. berghei* and *P. chabaudi* (both infecting rodents) – suggesting that these proteins are conserved among malaria species [35, 36].

Murine models of cerebral malaria

Clinical studies of severe human malaria are limited in terms of the amount of information we can retrieve from them. Though some aspects of the HCM disorder – vascular obstruction and inflammation leading to tissue damage and disordered neurotransmission – are now clear, the exact sequence of events leading to the neurological damage typical of HCM remains unknown. Further knowledge on the different risks and causative factors can only be obtained through a more direct experimental approach, which is clearly unethical in humans. Experimental animal models are therefore required to help us comprehend the processes that lead to malaria associated pathologies (as discussed in [25, 37]).

Different models can be used to mimic different aspects of the disease, i.e. different parasite strains and mouse strains can be combined to study the many manifestations of malaria, such as HCM. Despite recent articles questioning the conclusions that can be drawn from studies in animal models of experimental cerebral malaria (ECM) [38], it is clear the utility of such models in understanding the mechanisms underlying this pathology [25, 37, 39]. Though such mechanisms must be further validated – not only using more than one model of the disease but also in human clinical studies carried out in the field – it is getting more and more obvious the phylogenetic similarities between all the *Plasmodium* species [40, 41] and also the resemblance between HCM and experimental cerebral malaria (ECM) [28].

Infection of C57BL/6 mice with *P. berghei* ANKA (PbA) is the most commonly used model to study ECM [42]. One of the most cited critics against this model of HCM is that it doesn't replicate the human syndrome and therefore conclusions may not be confidently drawn from mechanisms of CM observed in murines [38]. Though parasite sequestration has clearly been shown for *P. falciparum* and is considered essential in the development of HCM (as stated above), it was not demonstrated in *P. berghei* until recently [14]. This new fact shows that the murine CM model shares key features with HCM and that it is relevant to its study.

The *Plasmodium berghei* parasite

Plasmodium berghei is a species of *Plasmodium* capable of infecting rodents that is widely used in experimental studies of malaria.

Plasmodium berghei ANKA has been long used to induce ECM in C57BL/6 mice and to study this pathology experimentally. Eighty to one hundred percent of these infected mice die of ECM at day 5 to 8 after pRBC injection, with relatively low numbers of pRBCs in the blood. On the contrary, close to 100% of Balb/C mice infected with this parasite strain don't develop ECM, but instead go on to develop high levels of pRBCs in the blood and die from anaemia about 3 weeks after blood-stage infection. The latter is the most commonly used non-ECM (NECM) control.

Plasmodium berghei NK65 [43]-infected C57BL/6 mice– though less widely used – also don't develop ECM, but instead die with anaemia and hyperparasitaemia, 2 to 3 weeks after blood-stage infection.

Sequestration in ECM – new insights from murine models

As previously stated, sequestration of pRBCs in the cerebral microvasculature in *P. falciparum*-infected people has been implicated as the major process responsible for the development of HCM. However, the observation of this phenomenon in non-CM cases suggested that other factors are involved [10]. The first evidence of leukocyte involvement in HCM pathogenesis came from the observation that individuals suffering from thymic abnormalities were protected from severe malaria [44]. Moreover, leukocytes were detected in the brains of patients that had died of HCM.

Similarly to what happens in humans, in the murine model there is accumulation of $CD8^+$ T cells in the brains of mice at the onset of ECM. This population of cells was shown to be necessary for the development of this disorder, since their depletion in the 24 hours preceding ECM prevents its development [45]. However, accumulation of $CD8^+$ T cells alone does not account for the development of ECM, since there is also accumulation of these cells in the brains of some non-ECM (NECM) mice models – namely the *P. berghei* NK65 infected C57BL/6 mice model [14].

Recently, it has been clearly shown that there is also accumulation of *P. berghei* infected cells in the brains of ECM mice (but not NECM models) just before they start to show neurological signs, and this process was shown to be essential for the development of ECM [14]. Not only does depletion of $CD8^+$ T cells protect mice from ECM, but also leads to a significant decrease in the pRBC burden in the brain of these mice [14]. Also, if the mice are treated with an anti-malarial drug and the parasite is cleared from circulation, in the 24 hours preceding the onset of ECM, there is no reduction of $CD8^+$ T cells content in the brain, but the mice are protected [14]. This clearly shows that parasite sequestration in the brain is crucial for the development of ECM as of HCM.

However, it is not known whether only the mature erythrocytic forms sequester and whether they disappear from circulation as a result of their sequestration in the brain and/or other organs, as in human malaria.

AIMS

The main goal of this project was to assess the capability and dynamics of *P. berghei* pRBCs sequestration in different malaria models (ECM *versus* NECM), *ex vivo* and *in vivo*.

Whether parasite-features or host-features are more relevant in the development of ECM has always been subject of discussion [46]. Sequestration of pRBCs in the brain is dependent on many factors – including the existence of appropriate parasite ligands in the surface of pRBCs, receptors expressed in the surface of activated endothelial cells, among others – which have to come together inside a relatively small time window. Some argue that the parasite is more preponderant, others that the inflammation process has more influence on the pathogenesis (see above). The sequestration process is essential in the development of ECM and cytoadherence of pRBCs seems to be a key step in the sequence of events leading to the pathology. Using a malaria murine model, namely PbNK65-infected C57BL/6 mice, we wanted to assess whether absence of cytoadherence of pRBCs during *Plasmodium* blood-stage infection may be caused by the lack of an intrinsic factor of the parasite or whether differences in the brain microenvironment generated by the host's immune response may play a role.

Moreover, though sequestration of pRBCs has been established as essential in the development of severe malaria, it is not yet known which erythrocytic forms actually sequester, or if there is differential sequestration. In HCM, the mature forms sequester preferentially, so we wanted to determine if the same occurs in ECM. Therefore, we studied the dynamics of mature intraerythrocytic stages of PbA, and whether or not they are seen in circulating blood before or during the onset of ECM, as opposed to NECM. Additionally, we used an *in vivo* imaging approach to assess if PbA mature schizonts preferentially sequester in certain organs in C57BL/6 or Balb/C mice.

MATERIALS AND METHODS

Parasites

The following Plasmodium berghei ANKA parasites were used: *P. berghei* ANKA "wild-type" line (PbA); *P. berghei* ANKA line expressing GFP under the control of an housekeeping gene (line GFP_{CON} 259cl2; PbA-GFP) [47]; and a parasite line expressing a Luciferase-GFP fusion protein under the control of *ama-1* which is expressed only in the schizonts, during blood-stage infection (line 354cl4; PbA-$LUCI_{Schiz}$) [48]. The following *Plasmodium berghei* NK65 parasites were used: "wild-type" line (PbNK65) and a parasite line expressing GFP under the control of an housekeeping gene (PbNK65-GFP).

P. berghei-infected RBCs are kept in Alsever's solution (at 10^7 pRBCs/ml), stored at -80ºC and defrost immediately before infection.

Mice

All experiments were carried out in male C57BL/6 or Balb/C mice (from Charles River Laboratories), aged 6 to 10 weeks. Animals used for blood passage (see below) were C57BL/6, older than 6 weeks. In each experiment, all animals were matched by age.

All mice were housed at the animal facilities at the Instituto de Medicina Molecular, according to the guidelines of the Animal Care Committee of the IMM. All protocols involving live animals were approved by the aforementioned Committee.

Induction and progression of blood-stage infections

All experimental mice were infected intraperitoneally (i.p.) with either 10^6 fresh pRBCs collected from another mouse, which was infected with a frozen vial (blood passage), or with $2x10^6$ frozen pRBCs (infection with frozen vial).

Concerning the *in vivo* cytoadherence assays, one experimental group was subjected to mixed infections, i.e., was infected i.p. with 10^6 fresh pRBCs of each parasite (PbA and PbNK65-GFP).

Parasitaemia (number of pRBCs / total number of RBCs x 100) was assessed by microscopic evaluation of Giemsa stained tail-blood smears [4], by counting at least 1000 RBCs. Alternatively, when GFP-expressing parasites were used, parasitaemia was evaluated by flow-cytometry. Briefly, a drop of tail-blood was recovered into a solution of 1% fetal bovine serum (FBS) in PBS 1x, and kept at 4ºC until analyzed in a flow cytometer (see below).

Assessment of experimental cerebral malaria onset and progression

In our hands, PbA-infected C57BL/6 mice develop experimental cerebral malaria (ECM) between d5 and d8p.i. Observable signs of the pathology in these mice include: loss of aggressiveness, decrease in activity and body-temperature, loss of strength in both the posterior and anterior limbs,

loss of short-term memory, loss of curiosity, alterations in posture and fur maintenance/hygiene habits, etc.

The neurocognitive state of experimental mice was assessed using a quantitative analytic scale described by Carrol *et al.* [49]. The "Rapid Murine Behaviour and Coma Scale" (RMBCS) consists of a list of ten parameters to which a numerical value is attributed. Parameters tested include gait, curiosity, limb strength, toe pinch and touch escape, and each one can be scored as 0, 1 or 2, adding up to an overall score of 20. The higher the value, the healthier the mouse is, and vice-versa. We considered mice to be in ECM, when their overall score was below 13.

In vitro synchronization and purification of *Plasmodium berghei* late intraerythrocytic stages

Plasmodium berghei is one of the few mammalian parasites whose schizonts don't rupture spontaneously *in vitro.* This feature is the rational support behind the parasite synchronization protocol[50] described below, resulting that after overnight (o.n.) incubation most of the pRBCs are synchronized in the more mature stages [51].

Briefly, infected mice were sacrificed by CO_2 inhalation and the blood collected by cardiac puncture, into 30 ml of PBS 1x. Blood was then centrifuged at 650 g (without brake), for 10 minutes at 4ºC. The supernatant was discarded and the pellet carefully resuspended in 50 ml of the culture medium solution (RPMI 1640, supplemented with 25% FBS, 2mM L-Glutamine, penicillin-streptomycin at 50µg/ml, 1% HEPES, 1% non-essential amino-acids), before being divided by two 25 cm^3 culture flasks. Flasks were gasified with a mixture of 5% CO_2, 90% N_2 and 5% O_2, for 1 minute each, sealed and incubated for 12-16 hours, in the dark, at 37ºC, at low rotational speed. These mature synchronized pRBCs were analysed by flow cytometry and used in the design of gates for GFP-positive sub-populations (*see below*). In addition, a drop of each these suspensions was placed on a microscope slide, allowed to dry before fixating with 4% PFA and staining with a 1:200 DAPI solution (stock solution at 1 mg/ml) for 1 hour. Slides were washed with PBS 1x and mounted with Fluormount-G™ (SouthernBiotech, U.K.).

For the *ex vivo* cytoadherence assays, after the o.n. incubation, mature pRBCs were isolated by a density gradient. Briefly, the RBCs suspensions were carefully resuspended, pipetted on top of 10 ml of a 65% Nicodenz solution and then centrifuged at 550 g, for 30 minutes at 21ºC (without brake). Most of the upper layer was discarded and the interface between the upper layer and the bottom layer was pipetted into a 50 ml Falcon tube and the volume added up to 40 ml of PBS 1x. This cell suspension was centrifuged at 580 g, for 8 minutes (without brake) and the pellet was resuspended in 500 µl of PBS 1x. RBCs were counted in a Neubauer chamber, and a final solution of 10^8 pRBCs / ml was prepared.

Quantification of *Plasmodium berghei* pRBCs by flow-cytometry

For the experiments regarding the *in vivo* parasite dynamics, constitutively expressing GFP parasites were used to infect either C57BL/6 or Balb/C mice. Both parasites used have GFP under the promoter of an housekeeping gene (elongation factor 1 alpha - ef1α), though PbA-GFP has three copies of the GFP construct integrated, while the PbNK65-GFP parasite has only one copy.

Parasitaemia was determined by flow cytometry using a LSRFortessa™ cell analyser (BD Biosciences, USA) and the BD FACSDiva™ software. The GFP-fluorescence intensity of different intraerythrocytic stages was analyzed by gating the GFP-positive RBCs in blood from infected mice, after defining the gates for RBCs based on their forward-scatter (FSC) and side-scatter (SSC) signals. The excitation of cells was performed with an argon ion laser at a wavelength of 488 nm and emission of the green fluorescence was detected using a band pass filter of 530/30 nm. Analysis of flow cytometry data was done with FlowJo™ software v6.4.7 or v9.3.2 (TreeStar Inc., USA).

According to Frank-Fayard *et al.* [52], the asexual intraerythrocytic stages show a linear correlation between developmental stage of the parasite and fluorescence intensity. In their study, late schizonts have 13 to 14 times the fluorescence intensity of ring-forms, which is related to the haploidy of late schizonts, i.e., the number of merozoites existent per late schizont.

In the present work, we used the o.n. synchronized parasites to confirm these values. The number of merozoites per schizont (MPS) was assessed for each combination of parasite-host on DAPI-stained thin blood smears from *in vitro* synchronized pRBCs cultures. MPS values were used to define the gates on flow cytometry for the early pRBCs (mononucleated) and mature parasites (multinucleated) populations. The gates were then applied to the *in vivo* parasite dynamics.

Quantification of brain parasite load by qRT-PCR

Brains were collected after intracardiac perfusion (20 ml of PBS), snap frozen in liquid nitrogen and stored until RNA extraction. Organs were mechanically homogenised in 2 ml of denaturing solution (4 M guanidine thiocyanate; 25 mM sodium citrate pH 7; 0,5% N-Lauroyllsarcosine; and 0,7% of freshly added β-mercaptoethanol, in Diethylpyrocarbonate (DEPC)-treated water). RNA was extracted using a Qiagen RNeasy minikit, according to the manufacturer's instructions.

The synthesis of the first-strand cDNA from the RNA templates was carried out using the AMV Reverse Trancriptase Kit (Roche). The amplification program was as follows: 25ºC (10 min), 55ºC (30 min) and 85ºC (5 min).

cDNA present in the samples was then amplified by qRT-PCR using the Finnzymes F-410XL DyNAmo™ HS SYBR® kit on an ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems), configured with the instrument's standard ramp speed and the following amplification program: 50ºC (2 min) and 95ºC (10 min), followed by 50 cycles of 95ºC (15 sec) and 60ºC (1 min), and one cycle of 95ºC (15 sec), 60ºC (1 min), 95ºC (30 sec) and 60ºC (15 sec). The relative expression of *P. berghei*'s *18s* or *gfp* genes (targeted with primers 5'-AAG CAT TAA ATA AAG CGA ATA CAT CCT

TAC-3', 5'-GGA GAT TGG TTT TGA CGT TTA TGT G-3' and 5'-GTC AGT GGA GAG GGT GAA GG-3', 5'-ACT TCA GCA CGT GTC TTG TAG TTC-3', respectively) was performed using the mouse housekeeping gene hypoxanthine guanine phosphoribosyl transferase (*hprt*) as an endogenous reference (targeted with primers 5'-TTT GCT GAC CTG CTG GAT TAC-3'; 5'-CAA GAC ATT CTT TCC AGT TAA AGT TG-3').

Parasite load was calculated as the relative amount of *P. berghei 18s* cDNA copies against *hprt* cDNA copies or the relative amount of *gfp* cDNA copies against *hprt* cDNA copies.

Determination of parasite load by *in vivo* imaging techniques

C57BL/6 and Balb/C mice were infected with a PbA line expressing a GFP-Luciferase fusion protein, under the control of the promoter of ama-1 (PbA-LUCI$_{SCHI}$), which is expressed only in mature schizonts. The load of circulating schizonts was evaluated at the time of sacrifice, by flow cytometry analysis of GFP (see above), and parasitaemia was calculated by manually counting on Giemsa-stained tail-blood smears (see above).

Five mice per group were sacrificed per time point, in a total of 3 time-points. One mouse of each group (ECM and NECM) was injected i.p. with a solution of D-Luciferin (Promega Luciferase Assay System). After 7 minutes, both mice were sacrificed and perfused with 20ml of PBS before organ extraction. Organs extracted were: liver, lungs, spleen and fat-tissue. Samples were placed in a Petri dish and immediately analyzed using an IVIS Lumina® imaging system (Caliper LifeSciences, Inc.; USA), with 5 min exposure. To calculate relative luminescence units (R.L.U.), total luminescence counts were divided by the area (in pixels) of each organ.

Ex vivo cytoadherence assays

For the *ex vivo* assessment of cytoadherence capabilities of PbNK65 to brain blood-vessels, the Woodruff-Stamper assay [53] was used and optimized similarly to what is described by Neres *et al*. [54].

Brains of naïve or infected mice were collected, after intracardiac perfusion with 20 ml of PBS 1x, and snap frozen in liquid nitrogen. They were then preserved at -80ºC, until cut with a cryostat in 7 µm thick sections (Leica CM 3050S, Leica Microsystems, Germany). Sections were then allowed to dry completely at RT before being used. Each section was delimited with a Dako Pen (Dako, Denmark). Sections were then washed with PBS 1x, for 5 minutes. Sections were then incubated with 50 µl of a 10^8 pRBCs/ml mature schizont-enriched cell suspension, at 37ºC, for 1 hour at low rotational speed, in an opaque humid-chamber. Slides were washed by inversion in a 50 ml Falcon tube with cold PBS 1x, for 5 minutes, and this step was repeated once. Sections were then incubated with paraformaldeyde (PFA) at 2%, for 10 minutes, at RT, and washed with PBS, before incubating with an anti-GFP antibody solution, for 1 hour. Slides were washed and incubated with a DAPI solution for 2 minutes. Slides were again washed by inversion in a 50 ml Falcon tube with PBS 1x, before mounting with Fluormount-G™ (SouthernBiotech, U.K.).

Sections were analysed with a Leica DM5000 B widefield fluorescence microscope (Leica Microsystems, Germany), using 400x magnification, and a monochrome CCD camera for fluorescence image acquisition. For GFP and DAPI detection, excitation of the samples was performed with a laser at a wavelength range of 430-510 nm and 320-400 nm, and emission was detected using a filter of 475-575 nm and 430-510 nm, respectively.

Violet Cresyl staining for analysis of infected brain sections

PbA-infected C57BL/6 mice were sacrificed, by CO_2 inhalation; at ECM score below 8 around day 5 to day 8 p.i. PbNK65-infected C57BL/6 mice were sacrificed at d8p.i. with similar parasitaemias. After intracardiac perfusion with 50 ml of PBS 1x, followed by perfusion with 50 ml of a 4% paraformaldehyde (PFA) solution, brains were collected and kept for 24 hours in 4% PFA, at 4ºC. The 4% PFA solution was replaced by a 30% glucose solution and kept at 4ºC until cryostat sectioning (Cryostat LEICA CM 3050S, Leica Microsystems, USA).

Histological staining was performed on 40 µm thick coronal brain sections, allowed to dry completely before storing at 4ºC until staining.

Before staining, sections were hydrated, for 2 minutes, in dH_2O and for another minute in another dH_2O-filled tank. Sections were then stained for 5 minutes in a Violet Cresyl solution, washed for one minute with dH_2O and passed on a 50% ethanol solution for one minute. Slides were then dipped in an ethanol and glacial acid solution at 70%, for 1 minute, before being dehydrated in 95% and 100% ethanol solutions, as well as xylene (for 1 minute in each tank). Slides were then mounted with DPX (Sigma Aldrich, U.K.), allowed to dry and kept in the dark until observation.

Analysis of stained sections was done using a Leica DMR upright widefield microscope (Leica Microsystems, Germany) and all photographs were acquired with a Leica DC500 colour camera and Photoshop CS2 (Adobe Systems Incorporated, USA).

Statistical analysis

Statistical analysis was performed using the non-parametric Mann-Whitney's test, on GraphPad Prism Software v5.04 (GraphPad Software, USA).

RESULTS

Dynamics of circulating blood-stage forms of PbA and PbNK65

As stated above, in *P. falciparum* malaria the mature forms are absent from circulation and present in the brains of patients suffering from HCM [10] and other organs. In the case of ECM, it has been demonstrated that PbA accumulates in the brains of C57BL/6 mice during the pathology [14], but whether this correlates with a decrease in circulation of schizonts and/or trophozoites in circulation is yet to be shown.

To follow these populations throughout infection and compare their dynamics in ECM and NECM models, a flow-cytometry based assay was used. For this, PbA and PbNK65 lines of GFP-expressing parasites were injected in C57BL/6 and/or Balb/C mice and tail-blood was collected at several time-points for analysis (see above).

*V*alidation of flow-cytometry gating strategy

Franke-Fayard *et al.* [52] have shown that PbA schizonts (in Swiss mice) displayed fluorescence-intensity (FI) values of 13 to 14 times the FI of ring-forms, which correlates with the number of merozoites found per mature schizont (MPS).

We started by analysing samples after *in vitro* synchronization by flow cytometry. This allowed a clear distinction between the populations expressing low levels of GFP and high levels of GFP (peak A and peak B, respectively, in **Figure 3. A**). Considering that the vast majority of pRBCs found in the Giemsa-stained smears obtained for the same samples were maturing schizonts (as expected), it is valid to associate this population with the peak B. Late ring-forms, though much less represented, were also present in these smears. Therefore, we could correlate peak A with these asexual forms. We then used the fluorescence intensity values obtained to calculate the ratio between the FI of B and A, as a measure of the haploidy of mature schizonts, i.e. the number of MPS. To validate this assumption, the number of MPS for each combination of parasite-host was calculated (**Figure 3. B**), by manual counting on DAPI-stained blood smears after *in vitro* synchronization [51]. No statistically significant difference was found between the numbers of MPS measured by both approaches (**Figure 3. B**) which validated the flow cytometry approach. Interestingly, MPS values were similar for PbA-infected blood after o.n. incubation, from both Balb/C and C57BL/6 mice. However, for the PbNK65+C57BL/6 combination this value was significantly reduced (**Figure 3. B**).

By extending peaks A and B, we designed gates G1 and G3, respectively (**Figure 3. C**). Gate G2 was designed to include all intermediate events (**Figure 3. C**). G1 includes ring-forms and some early trophozoites; G2 includes trophozoites; and G3 includes mainly mature schizonts, but also late trophozoites, immature schizonts and possibly male gametes (that express GFP at similar FI as mature schizonts) [52]. The non-gated population with the highest FI included female gametocytes, expressing at least 30 times more GFP than the ring-forms (consistent with data previously published [52]). Since only asexual erythrocytic forms were studied in this project, this population was discarded from further analysis. As was already mentioned, the rationale behind this gating strategy is that there

is a linear correlation between development and green-fluorescence intensity [52], meaning that the higher the amount of parasite-DNA present in the cell, the higher the expression of GFP.

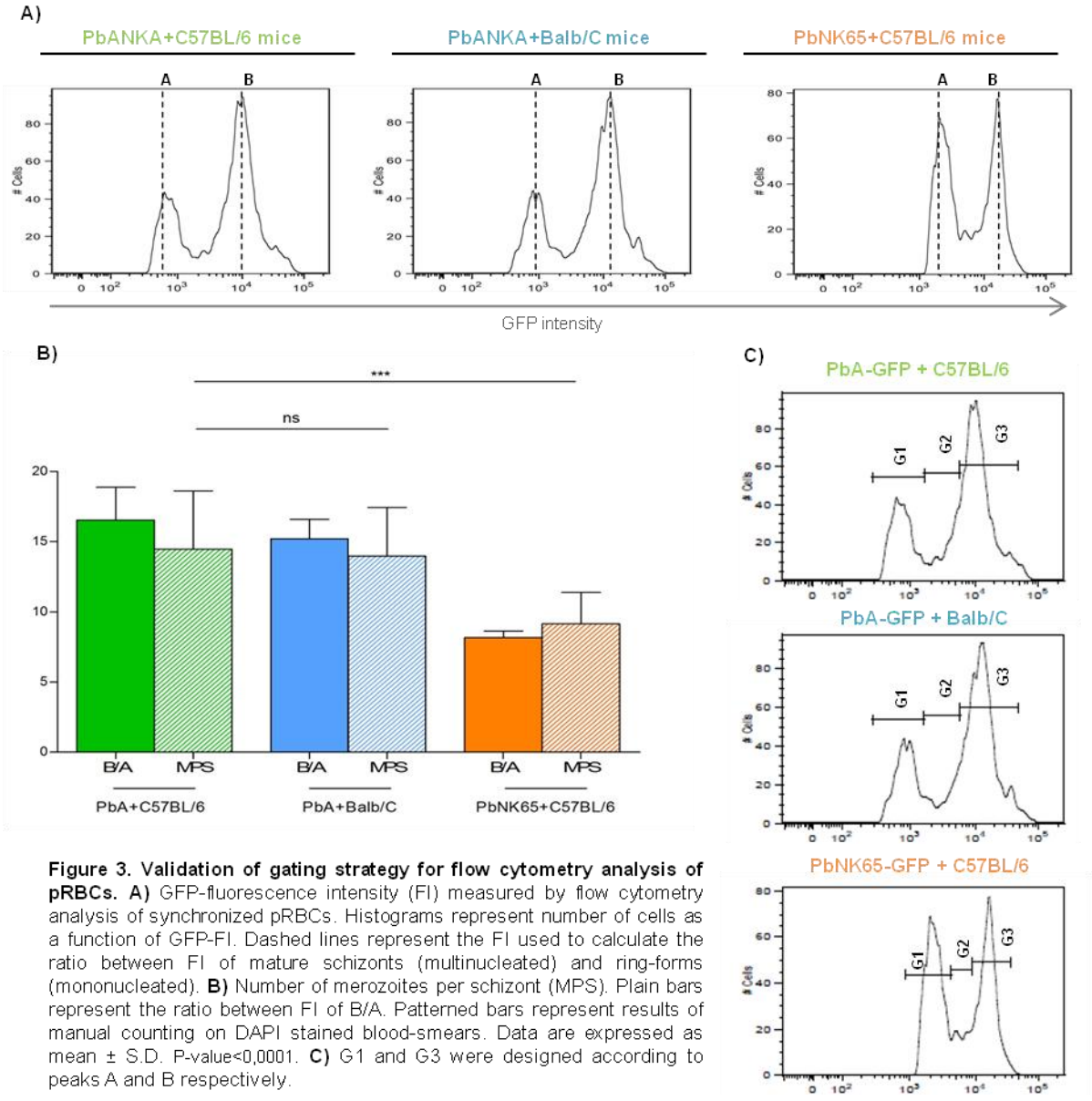


Figure 3. Validation of gating strategy for flow cytometry analysis of pRBCs. A) GFP-fluorescence intensity (FI) measured by flow cytometry analysis of synchronized pRBCs. Histograms represent number of cells as a function of GFP-FI. Dashed lines represent the FI used to calculate the ratio between FI of mature schizonts (multinucleated) and ring-forms (mononucleated). **B)** Number of merozoites per schizont (MPS). Plain bars represent the ratio between FI of B/A. Patterned bars represent results of manual counting on DAPI stained blood-smears. Data are expressed as mean ± S.D. P-value<0,0001. **C)** G1 and G3 were designed according to peaks A and B respectively.

The gates, designed for the analysis of samples after o.n. incubation, were then applied to the study of *in vivo* dynamics of asexual circulating parasites, which was our main objective in these experiments (referred thereafter as “dynamics”). It should be noted that the GFP-expressing PbNK65 line used in this study only has 1 copy of GFP, so it was necessary to use different voltage settings in the acquisition of RBCs infected with this parasite, to be able to separate the GFP-positive from the GFP-negative populations. This also meant designing different gates, which were submitted to the same validation process.

In vivo dynamics of asexual circulating forms of PbA and PbNK65

Three independent experiments were performed to analyse *in vivo* dynamics of PbA in the models already described (two for the PbNK6 model). Mice were injected i.p. with 10^6 pRBCs of GFP-expressing parasites. Blood was then collected (starting on d4 p.i.) at several time-points per day, for flow-cytometry analysis. Gating strategy is described above.

Parasitaemia in early infection was similar for all three combinations of parasite-host (**Figure 4**). However, though parasitaemia in PbA infection quickly increased from d4p.i. (in both hosts), parasitaemia in PbNK65 infection increased much more slowly. In late infection, the difference in parasitaemia between PbA and PbNK65 infections was more evident, with PbNK65 infections at 1/3 the values of parasitaemia of the PbA infections (see legend of **Figure 5. B**).

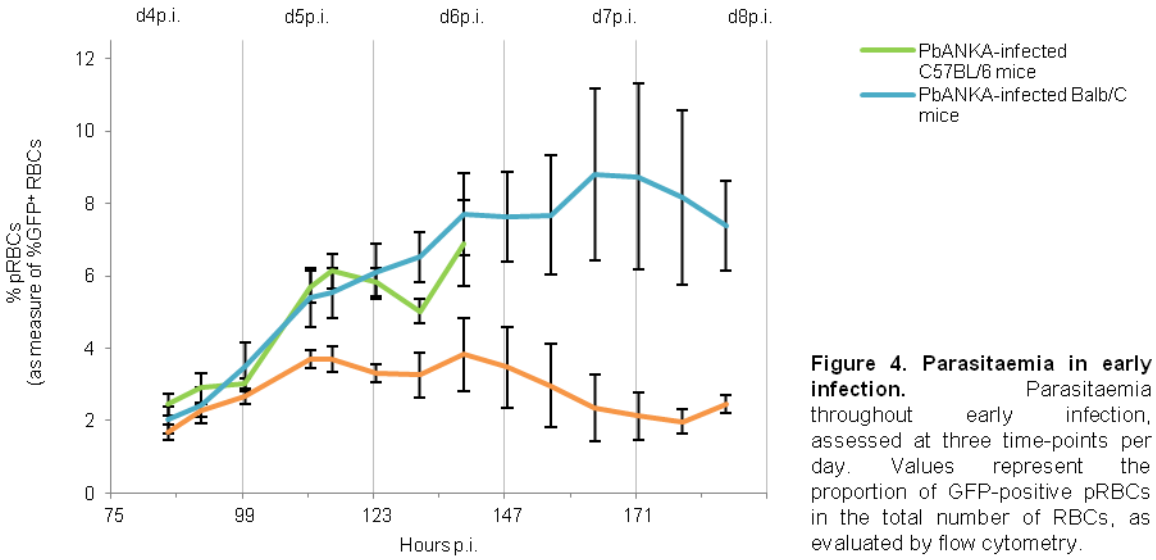


Figure 4. Parasitaemia in early infection. Parasitaemia throughout early infection, assessed at three time-points per day. Values represent the proportion of GFP-positive pRBCs in the total number of RBCs, as evaluated by flow cytometry.

It was observed a striking synchronization in all mice from the same experimental group for the entire duration of the experiments (**Figure 5. A**).

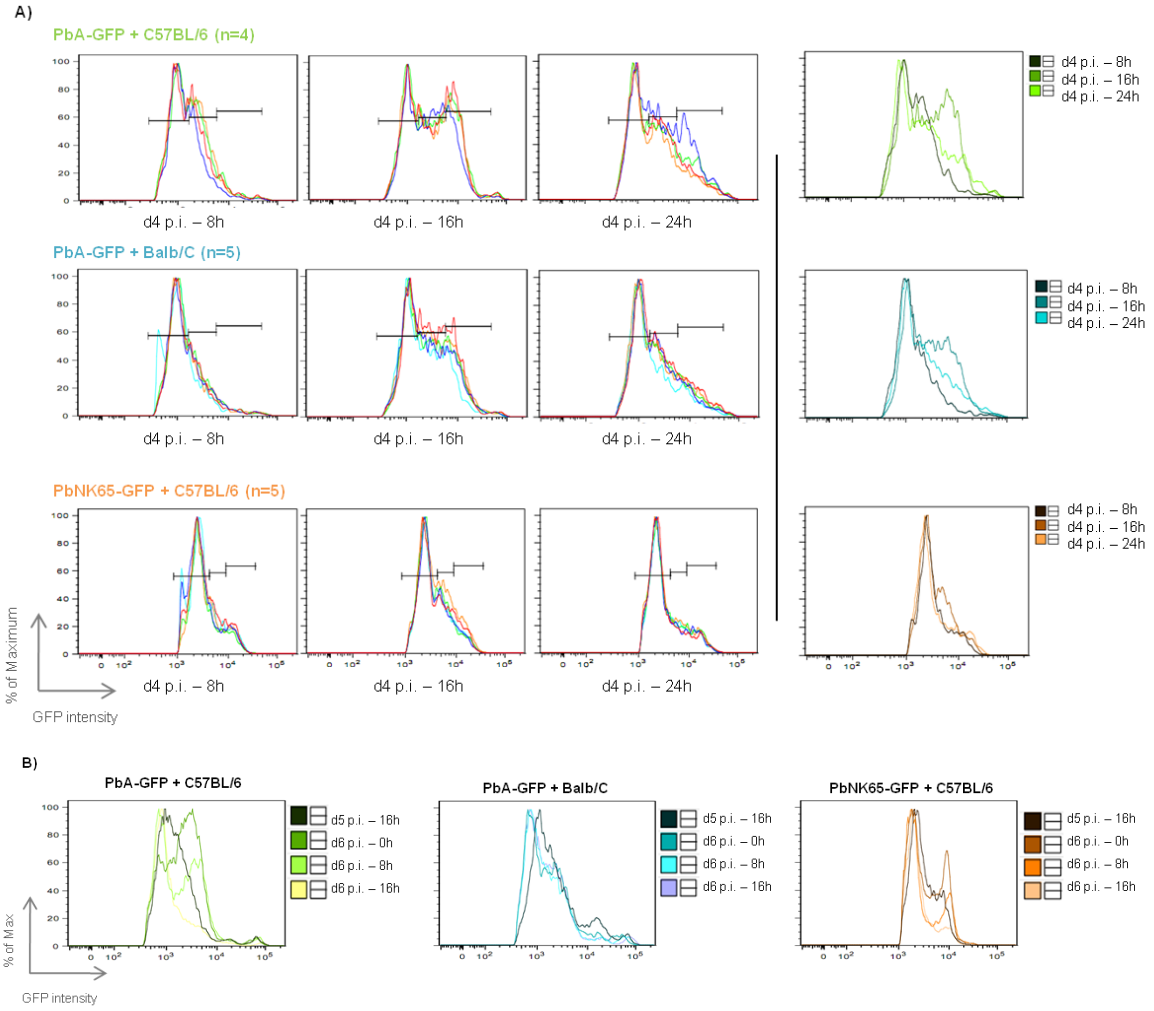


Figure 5. Blood-stage dynamics throughout one day of infection.
A) Left side: graphs represent an overlay of individual mice histograms obtained for each experimental group . Gates are represented by the horizontal black lines. Right side: overlay of the different time-points assed during d4p.i., for each group. Only one histogram per time-point per group is shown.
B) Similar to above, but a period of 24h hours, from d5 p.i. to d6p.i., is shown.

In both hosts, the proportion of PbA's G1 seemed to oscillate cyclically (**Figure 5. B**). Interestingly, in one of the experiments (**Annex A**: **Figure 1. B**), this cycle seemed to be inverted comparing PbA in both hosts, already on d4p.i. In the case of PbNK65, the oscillation in the proportion of G1 *versus* G3 is not as pronounced, but it is also present (**Figure 5. B**).

In the ECM model, PbA's G3 seem to decrease in the circulating blood concomitantly with the onset of the pathology (**Figure 6. B**). However, in the Balb/C host, in spite of this mice not developing ECM, the G3 population seems to also decrease with time (**Figure 6. A**). PbNK65's G3 are seen in lower percentages in the circulation, than in the PbA infection. From d6 p.i. onwards the mature forms (G3) were seen in very small proportions in circulation in all models (**Figure A. A**) and were seen again on the blood at d14 and d15 p.i., at very high percentages (**Figure 6. B**), when mice were showing parasitaemias around 62-72% (PbA) or between 20-25% (PbNK65). At this time-point, the proportion of G3 is particularly high for PbA in Balb/C mice. However, it is most likely that this increase

is due to an increase in multiple infections and not due to an increase in circulating mature blood-stage forms, since multiple infections would also fall in the G3 gate.

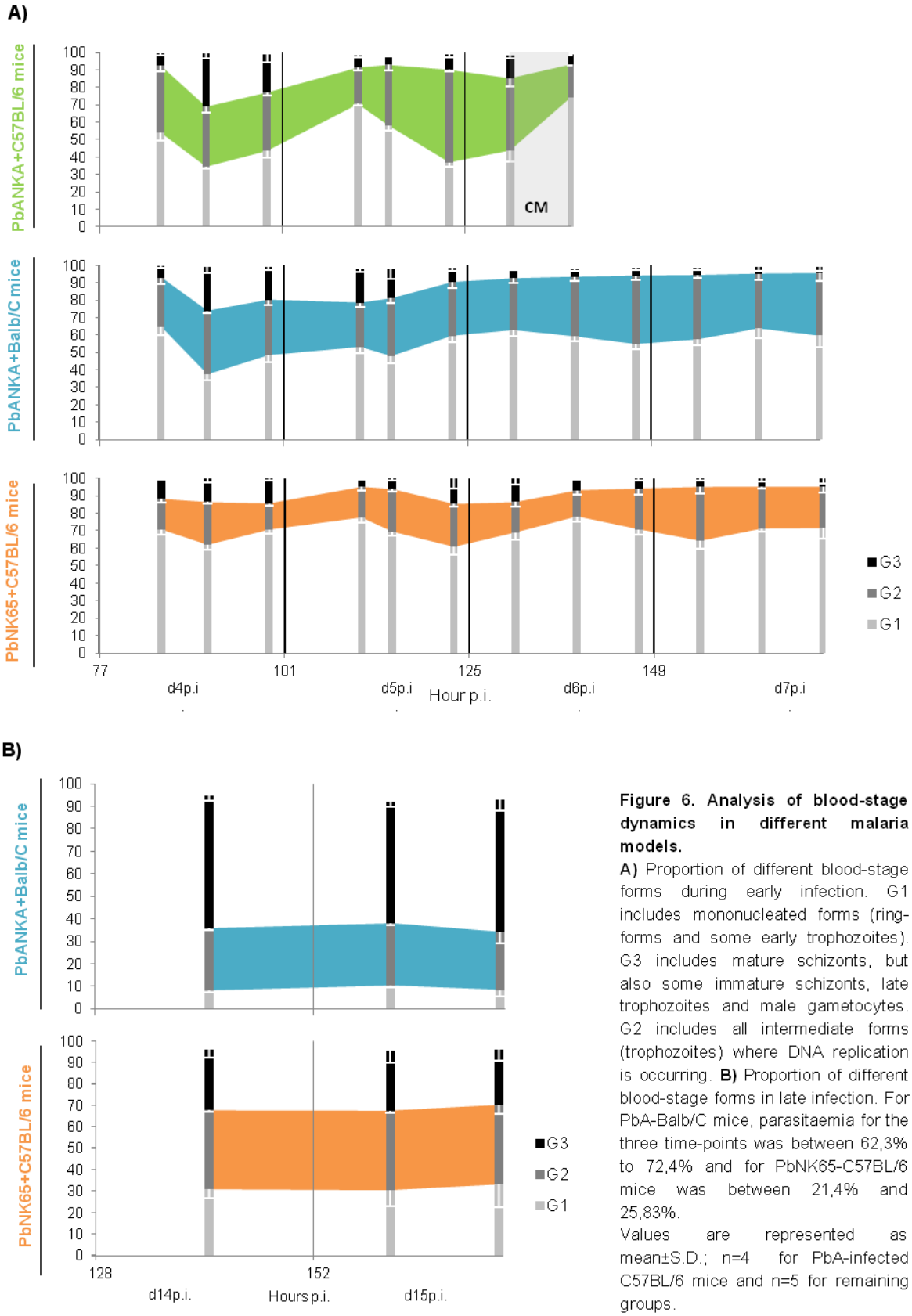


Figure 6. Analysis of blood-stage dynamics in different malaria models.

A) Proportion of different blood-stage forms during early infection. G1 includes mononucleated forms (ring-forms and some early trophozoites). G3 includes mature schizonts, but also some immature schizonts, late trophozoites and male gametocytes. G2 includes all intermediate forms (trophozoites) where DNA replication is occurring. **B)** Proportion of different blood-stage forms in late infection. For PbA-Balb/C mice, parasitaemia for the three time-points was between 62,3% to 72,4% and for PbNK65-C57BL/6 mice was between 21,4% and 25,83%.

Values are represented as mean±S.D.; n=4 for PbA-infected C57BL/6 mice and n=5 for remaining groups.

Interestingly, a decrease in overall ECM score, was not only observed in PbA-infected C57BL/6 mice, but also in PbA-infected Balb/C mice and PbNK65-infected C57BL/6 mice. PbA-infected Balb/C mice presented with scores as low as 14 and PbNK65-infected mice with scores as low as 15, on d6p.i. Though this decrease was not as pronounced as in ECM mice, it was concomitant with the decrease in the proportion of circulating G3 in these mice.

Taken together, these results can suggest that mature schizonts and some late trophozoites of PbA and PbN65 may be able to adhere and sequester in some organs, at a certain moment during infection. However, we can not exclude that these parasite forms were not seen in circulation due to an elimination process mediated by the spleen. In the case of PbA, results suggest that these blood-stage forms may be those sequestering in the brain during ECM, since their proportion in circulation decreases at the onset of the pathology.

Assessment of PbA cytoadherence in different organs

One of the possible explanations for the disappearance from circulation of the MF of asexual blood-stage *Plasmodium* is their sequestration. To test this hypothesis, C57BL/6 or Balb/C mice were infected with PbA expressing a Luciferase-GFP fusion, under the control of the promoter of ama-1 (PbA-LUCI$_{SCHI}$). Since this protein is expressed only in mature schizonts, only those forms will be detected by luminescence.

The circulating blood-stage forms were followed from d4p.i. onwards, both by observation of Giemsa-stained tail-blood smears (for total parasitaemia) and by flow cytometry, to detect the mature forms expressing the luciferase-GFP construct.

Five mice per group were sacrificed at each time point, in a total of 3 time-points. The first time-point (T1) was on d4pi.i, before the onset of ECM, meaning before schizonts disappear from circulation; the second-time point (T2) was at the late stages of ECM for C57BL/6 mice and 24h later for Balb/C, when very few schizonts were observed in circulation on all groups; and the third time-point (T3) at d14p.i., when mature schizonts are detected again in circulation. Only samples from T1 and T2 were analysed until this date.

Our results show for both hosts, at T2, an important sequestration of PbA in the fat tissue (mainly) and, at a lesser extent, in the liver (**Figure 7. B** and **D**). In the case of C57BL/6 mice, there is also a significant increase on the MF remaining in the spleen and the lungs after perfusion, at T2 when compared with T1 (**Figure 7. A** and **C**). It should be noted that the spleens from Balb/C mice at T2 were notoriously darker and enlarged. This might indicate higher haemozoin content, though the amount of viable schizonts was not significant, which suggests increased parasite elimination by the spleen.

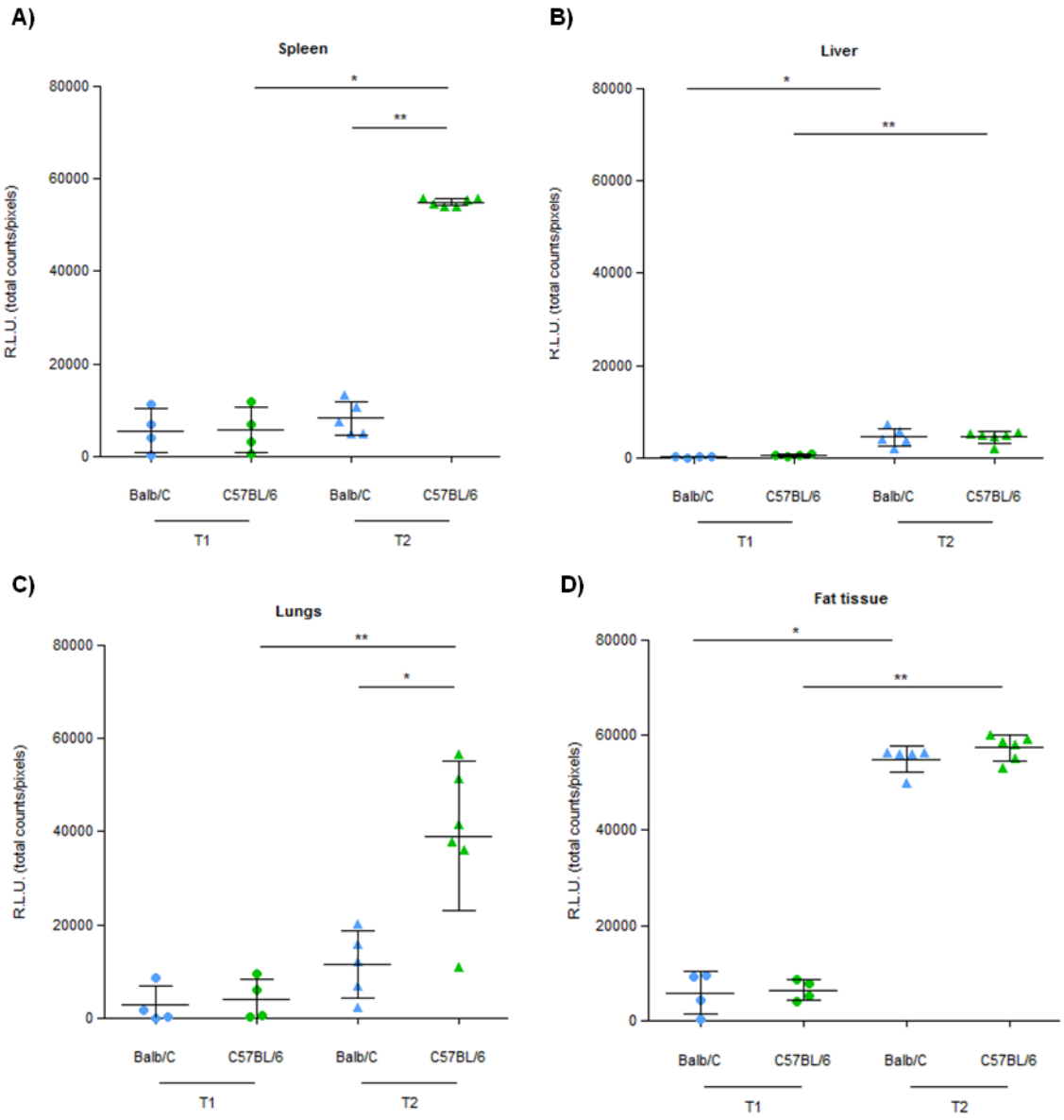


Figure 7. PbA-schizonts detection by luminescence. Balb/C or C57BL/6 mice were infected with PbA-Luci$_{Schiz}$ and sacrificed on d4p.i. (T1) and when C57BL/6 mice were at ECM onset (T2). Sequestration of mature-schizonts was assessed by detection of luminescence (5 minutes exposure). Relative luminescence units (R.L.U.) were calculated for each sample of the following organs: **A)** spleen, **B)** liver, **C)** lungs and **D)** fat tissue.
* p-value<0,05 (two-tailed Mann-Whitney's test) ** p-value<0,01 (two-tailed Mann-Whitney's test)

PbA and PbNK65 cytoadherence capabilities

As previously shown, both CD8$^+$ T cells and PbA accumulate in the brain of C57BL/6 mice during ECM, but not in Balb/C mice [14]. Interestingly, PbNK65 infection leads to accumulation of CD8$^+$ T cells in the brain of C57BL/6 mice, but not to accumulation of pRBCs and does not lead to the development of ECM [14]. This was demonstrated by measuring parasite burden in perfused brain samples, by quantitative real-time PCR.

Brain sections stained with Violet Cresyl were analysed under polarized light to detect haemozoin. As expected from previous studies [14], our results clearly show that haemozoin is present in the brain of mice during ECM, but not in the brain of PbNK65-infected C57BL/6 mice (**Figure 8**). Analysis also revealed an heterogeneous distribution of haemozoin in the brain of ECM mice. No haemorrhages or haemozoin in the brain parenchyma were found in PbNK65-infected brains.

These results show that mature PbNK65 pRBCs do not accumulate in the brain of C57BL/6 mice, since there is no accumulation of haemozoin in this organ.

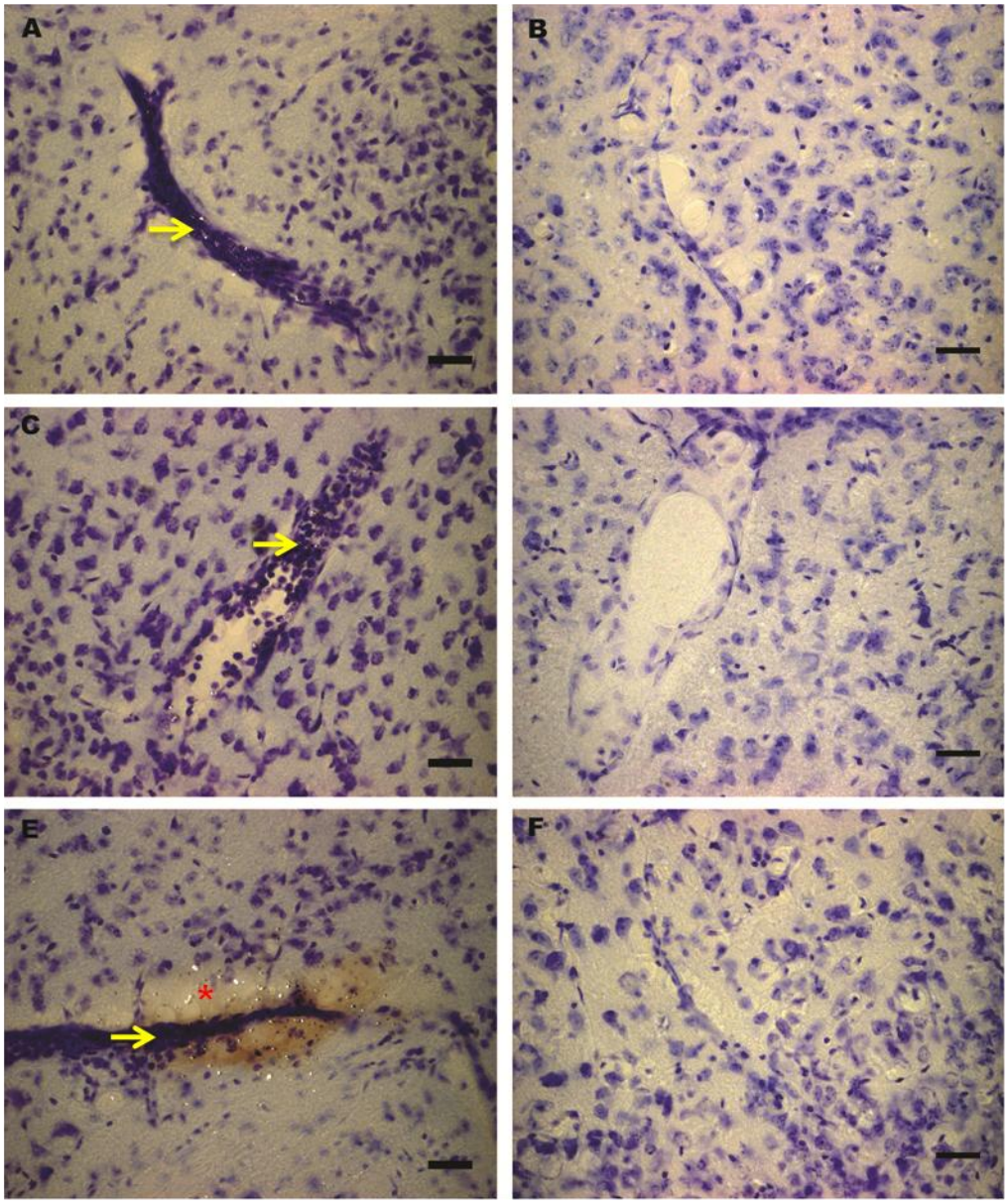


Figure 8. Parasite detection in the brain of C57BL/6 infected mice. Violet Cresyl stained 40 µm-thick brain sections. Mice infected with PbA (A,C,E) or PbNK65 (B,D,F) and sacrificed at ECM score ≤8, or at d8p.i., respectively. Arrows show haemozoin accumulated inside blood-vessels. Asterisks show haemorrhages where haemozoin is also visible. Scale bars represent 30 µm.

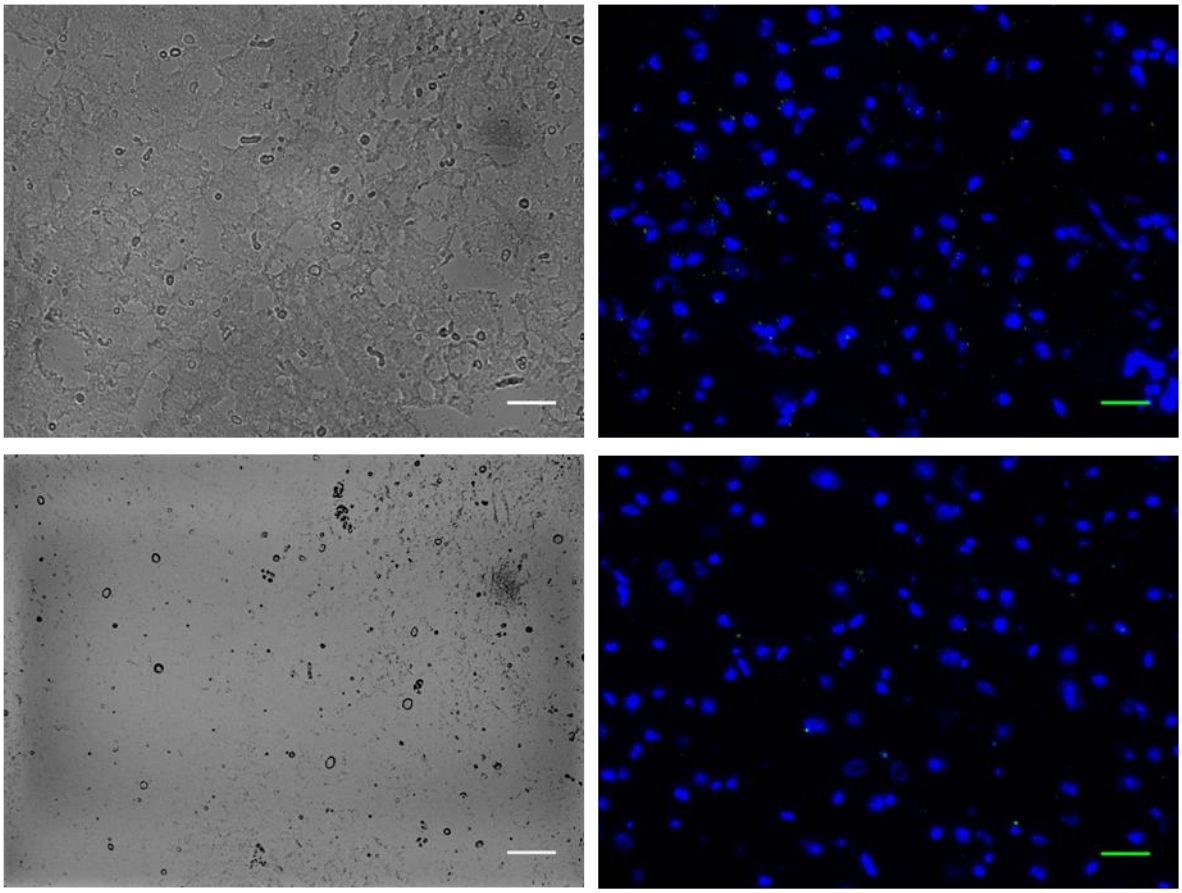
While PbA accumulates in the brain of C57BL/6 mice, PbNK65 does not [14]. One possible explanation that might account for this is that PbNK65 pRBCs don't have the inherent capability to

adhere to the activated brain microvasculature. To test this hypothesis, two experimental approaches were used: an *ex vivo* (a derivation of the Woodruff-Stamper technique) and an *in vivo* approach.

First, brain sections of infected mice were incubated with GFP-expresing pRBCs (see above). Whole blood or schizont-enriched blood were used. As a positive control, brain sections from PbA-infected mice showing signs of ECM were incubated against PbA-GFP infected blood. Since, *in vivo*, PbA pRBCs adhere to the brain microvasculature, if brain tissue and pRBCs maintain their structural status, they should also adhere *ex vivo*.

Multiple variables were tweaked to attempt optimization of this protocol. These included: duration and number of washing steps after incubation with the pRBCs, washing technique, incubation with/without rotation, etc. However, in all experiments the positive control didn't work properly, i.e. after mounting, pRBCs were not found adherent to the brain tissue when analysing the sections under a fluorescence microscope (**Figure 9**). Therefore, no conclusions could be drawn from these experiments.

Figure 9. *Ex vivo* cytoadherence assays. Cells were stained with DAPI and anti-GFP antibody. **A)** PbA-infected brain (C57BL/6 mouse) sections incubated against PbA-GFP infected blood, at 400x magnification. **B)** Brain sections of a naïve mouse incubated against PbA-GFP infected blood.
Left: photo of sample using DIC contrasting technique. Right: same field; merge of GFP and DAPI fluorescence channels. No differences were observed between negative and positive controls.
Scale bars represent 30 µm.



A different approach was then attempted, which involved co-infection of C57BL/6 mice with PbA and PbNK65-GFP. These "mixed infections" mice were sacrificed at final stages of ECM (ECM score below 8) or, in the case of PbNK65-GFP single infection control, 24h later. Relative quantification parasites sequestered in the brain was assessed by quantitative real-time PCR of either *gfp* or parasite *18s* gene expression.

Results showed a statistically significant increase in PbNK65 GFP-expressing parasites in the brains of "*Mixed infections*", comparing with the negative control (mice infected only with PbNK65-GFP). However, PbA had a much greater contribution for the total parasite burden than PbNK65-GFP in these mice (**Figure 9**), considering that there is a much higher GFP expression in the single PbA-GFP infection.

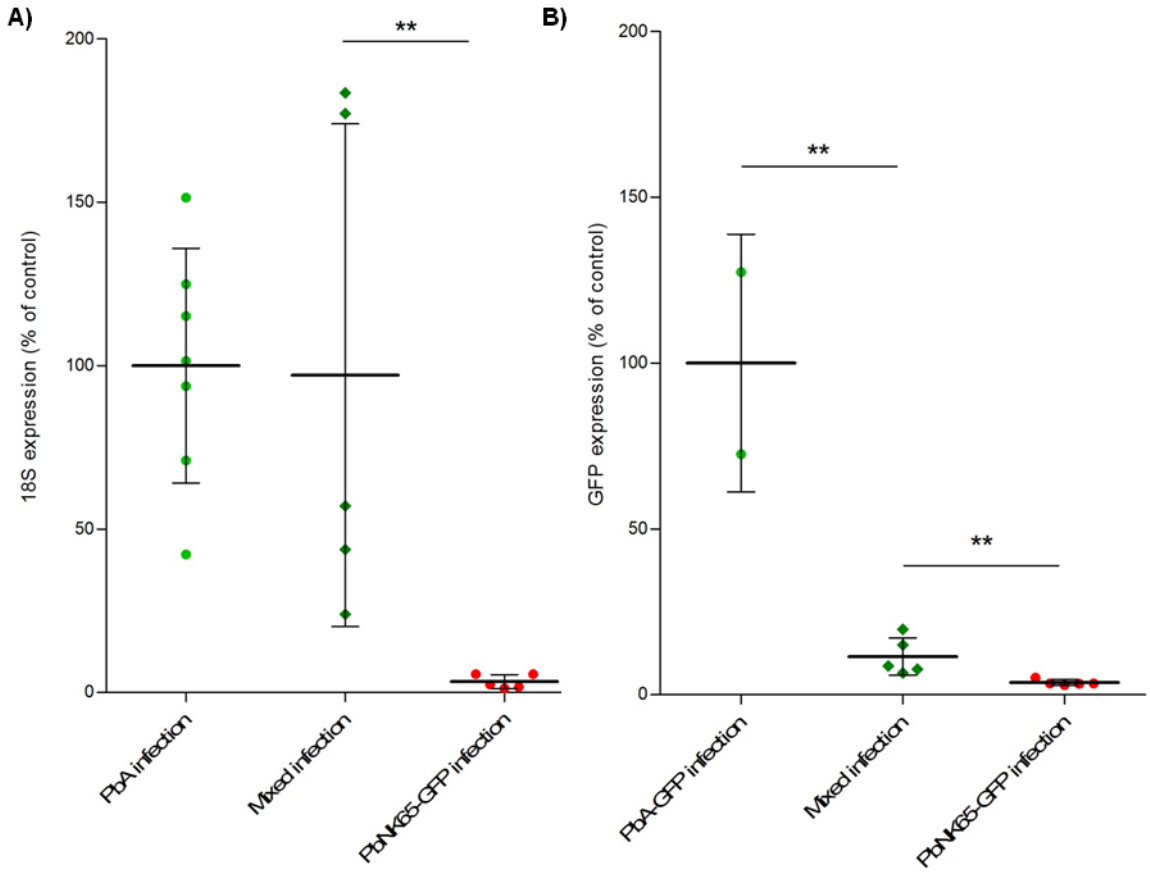


Figure 10. Quantification of accumulation of PbNK65 pRBCs in the brain of C57BL/6 mice co-infected with PbA. Mice were infected with PbA and PbNK65-GFP simultaneously and sacrificed at ECM score ≤8 ("Mixed infection"; n=5). *gfp* and *18s* gene expression in perfused brains was measured by quantitative real-time PCR. Brains of mice infected with PbNK65-GFP were used as a negative control (n=5) and PbA-infected as a positive control. **A)** Total parasite burden by *18s* expression was normalized to expression in PbA-infected C57BL/6 mice sacrificed at ECM score ≤8 (n=7). **B)** Quantification of *gfp* was normalized to expression in PbA-GFP (one copy) infected C57BL/6 mice sacrificed at ECM score ≤8 (n=2). ** p-value<0,05 (one-tailed Mann-Whitney's test)

It has been described that, in a case of co-infection of PbA and PbNK65, total parasitaemia is equivalent to the parasitaemia of each parasite in single infections [55]. In our case, parasitaemias in single PbNK65-infections were always lower (**Figure 5. A**; **Annex A: Table 1**). In the case of "mixed infections", at the day of sacrifice, PbNK65-GFP accounted only for 9-50% of total parasitaemia. Nevertheless, even in the case of the 2 mice that had around 50% of PbNK65-GFP in circulation, the

load of this parasite sequestered in the brain is very low when compared to the single PbA-GFP infections.

These results may suggest that PbNK65 pRBCs do not accumulate in the brain of C57BL/6 mice.

DISCUSSION AND FUTURE WORK

In this project our main goal was to study the blood-stage dynamics and sequestration capabilities of PbA and PbNK65 in different malaria infection models. To reach this goal, we used GFP- or luciferase-expressing parasites to follow blood-stage dynamics.

As stated above, PbNK65 single-infections showed lower parasitaemias throughout blood-stage infection than PbA single-infections. This was more evident in later stages of infection, when parasitaemia in the PbNK65 infection was 1/3 of the parasitaemia in the PbA infection (**Figure 5. C**). Though this phenomenon had already been described [56], the causes behind it are still not known. By analyzing samples of synchronized pRBCs, we were able to show that PbNK65 can produce only half of the number of MPS than PbA. Lower numbers of MPS mean that less new infections are generated per cycle, since less merozoites are comparatively released into the bloodstream. This way, the parasitaemia growth rate may be lower (reviewed in [23]). This results can be an explanation for the lower parasitaemia observed in PbNK65-infected mice. It has been previously described that the rate of multiplication strain of PbNK65 strain is the double in mice with increased reticulocytosis [15]. It is not yet clear what the factors that influence the number of MPS in each parasite strain are, but the size and metabolic state of the host cell likely play a role. PbNK65 has a high affinity for infecting reticulocytes, even higher than PbA [15]. Reticulocytes are immature RBCs, still metabolically active and larger than normocytes. From the evolutionary perspective, if indeed the size and metabolic activity of the host cell play a role in determining the number of MPS, than PbNK65 would gain from preferentially infecting these cells. An interesting experiment, to test the hypothesis that host-cell size and metabolic state influence the number of MPS, would be evaluating this number in mice with increased reticulocytosis *versus* normal mice. We could induce reticulocytosis in mice before infecting with PbNK65-infected blood (by bleeding mice one day prior to infection, or by injecting certain drugs that induce this process) and evaluate the number of MPS in reticulocytes *versus* normocytes. This experiment would allow us to make a direct connection between the parasitaemia growth rate and the number of MPS. We have tried to perform this experiment, but we weren't yet able to increase the level of reticulocytes significantly by simply bleeding the mice 24h prior to infection. We would like to optimize this protocol in the future, perhaps using phenylhydrazine instead of bleeding the mice. This drug is commonly used to induce anaemia in mice, which triggers the production of reticulocytes.

It should be noted that the duration of the erythrocytic cycle for *P. berghei* is described as lasting between 21 and 24h (reviewed in [15]). However, in our hands this cycle lasted close to 32h. For this, we have no explanation, though we can not exclude an interaction between the circadian cycle of the host and the circadian cycle of the parasite.

Concerning the analysis of dynamics of asexual circulating forms, it seems clear that PbA mature schizonts and some late trophozoites are nearly absent from circulation from d6-d7p.i. onwards, in both the NECM and the ECM model. This can be explained by the possibility that these parasite forms are able to adhere and sequester in some organs (other than the brain). Another possibility is that they are being eliminated by the spleen, due to the fact that these blood-stage forms are less deformable,

larger and have increased expression of surface proteins, rendering them more easily detected by the immune system, specifically by splenic macrophages (reviewed in [33]).

To address the first possibility, we used a PbA line expressing a LUCIFERASE-GFP fusion protein in the mature schizonts to infect C57BL/6 mice and Balb/C mice. If our hypothesis was correct and these blood-stage forms were being sequestered, we could detect luminescence in certain organs of infected mice, after perfusion, and correlate that with the disappearance from circulation.

Our results suggest that indeed there is sequestration of PbA-schizonts in multiple organs, when the proportion of these forms decreases in circulation. In the case of C57BL/6 mice, we confirm previously published results [57] that describe sequestration of PbA-schizonts in the spleen, lungs and fat tissue. We also detect a small but significant increase in the load of PbA-schizonts in the liver of C57BL/6 mice. We also describe here, for the first time, sequestration of PbA-schizonts in Balb/C mice, in the fat tissue and the liver. However, there was no significant increase on sequestration of viable schizonts in the spleen of these mice, from T1 to T2. However, due to the darker colour and bigger size of these spleens, it is probable that there is high haemozoin content. The spleens were collected and frozen to quantify haemozoin content (using an haeme detection kit), in the future. This should give us an indication if elimination of PbA by the spleen is higher in Balb/C mice.

In the case of PbA-infected C57BL/6 mice, the proportion of mature stages decreases in circulation at the onset of ECM. Concomitant with this, there is also a small but significant decrease in parasitaemia (p-value=0,0236; one-tailed Mann-Whitney's test) just before the appearance of ECM signs, consistent between all experiments. Taking this together with the presence of haemozoin in the brain microvasculature of these mice, these results suggest the parasite forms that sequester in the brain during ECM are at least in part mature blood-stage forms. This possibility brings this murine model of CM and the human pathology even closer together.

Interestingly, PbNK65 schizonts also appear in low proportions in circulation, from d6p.i. onwards in C57BL/6 mice. However, it has been shown that pRBCs don't accumulate in the brain during ECM [14] (**Figure 7**). If these parasite forms disappeared from circulation due to sequestration in other organs, then the mechanisms responsible for cytoadherence in the brain could be different than those responsible for this process in other organs. This has been shown for PbA (reviewed in [57]), so the same principle might apply for PbNK65 infection. It would have been interesting to also assess sequestration of PbNK65 in C57BL/6 mice, as was done for PbA in Balb/C and C57BL/6 mice. However, a different strategy would have to be used since, to date, there is still not a luciferase-expressing PbNK65 parasite line. The advantage of using a luminescence assay is that this technique allows visualization of viable parasites, because it is a protein that is being quantified instead of a nucleic acid, which may still be present even if the parasite is already being eliminated. This is more problematic when assessing sequestration in the spleen, where the parasite might be going through the process of elimination. One possible alternative would be to infect C57BL/6 mice with PbNK65-GFP and perform a quantitative assay (such as quantitative real time-PCR) to evaluate the presence of GFP positive cells in certain organs, compared with the other two models of malaria used in this

project. We intend to do this in the near future, by detecting expression of *ama-1* instead of expression of *gfp* (to address the fact that this parasite expresses *gfp* constitutively).

For PbA as well as PbNK65, the proportion of mature blood-stages has a huge increase in circulation in later infection. Two possibilities can explain this. First, it might be a result of saturation in the microvasculature, i.e. the organs where mature erythrocytic stages were sequestering are saturated and can no longer accumulate more pRBCs. The second hypothesis is that, as the infection progresses, the microenvironment inside each organ changes and the "new microenvironment" is no longer suitable to promote sequestration.

Concerning the mechanisms of cytoadherence in the brain, one might argue that the fact that PbA adheres in the brain of C57BL/6 mice and not in the brain of Balb/C mice is an indication that the mechanisms responsible for cytoadherence are mostly host-related. However, if PbA pRBCs adhere in the brain of C57BL/6 mice and PbNK65 pRBCs don't, parasite features are probably as important as the host is in this process. If cytoadherence of pRBCs to the brain microvasculature is mostly dependent on the parasite's capability to do so, then in the context of the host-microenvironment of PbA infection, PbNK65 still would not be able to adhere to the brain microvasculature.

To test this hypothesis, our first approach was an *ex vivo* technique, adapted from a previously described protocol, developed by Neres *et al.* [54] to study pregnancy-associated malaria pathologies. In their work, they perform *ex vivo* cytoadherence assays using placentas from infected and non-infected female mice incubated with synchronized PbA-GFP infected blood. In our work, PbA-infected brain sections were incubated with either PbNK65-GFP or PbA-GFP infected blood. No adherent pRBCs were observed in the positive control (PbA-infected brain incubated with PbA-GFP infected blood) which doesn't allow us to take any conclusions from those experiments.

There are three possibilities that might explain why this strategy didn't work in our case, though it was developed for similar models. The first hypothesis is that there is a low probability of finding blood-vessels in a single brain section, so the probability of observing cytoadherence of pRBCs is greatly reduced. To exclude this possibility, we could also use another organ, as positive control, that is more vascularised and for which sequestration is already described, such as the spleen. Another hypothesis is that since the brain tissue used was frozen, instead of fresh, perhaps the receptors important for cytoadherence in the brain are damaged or have the wrong conformation, so proper adherence may not occur. To rule out this possibility, we could use fresh tissue and make sections using a vibratome. The third hypothesis is that the process of purifying synchronized pRBCs may somehow damage these cells, impairing the cytoadherence process in the brain. Using non-purified infected-blood suspensions might allow us to eliminate this possibility. Addressing these hypotheses would allow us to understand how we can optimize this protocol.

In our final approach, we co-infected C57BL/6 mice with PbA and PbNK65-GFP. Since PbA was present, the parasite would sequester in the brain and mice would develop ECM. At later stages of ECM, we observed a small though significant increase in PbNK65-GFP load in the brain of "mixed infections" mice, compared to the negative control (PbNK65-GFP infected mice). However, the GFP

load found in the brains of “mixed infections” was likely due to the fact that perfusion in ECM mice is less efficient than in NECM mice, since there is vasoconstriction in later stages of ECM. Considering this, the GFP load in the brain of “mixed infections” was most probably a residual value. Moreover, compared to the PbA-GFP single-infection control, PbNK65-GFP load in the brain of “mixed infections” was very low. Briefly, our results suggest that PbNK65 pRBCs do not accumulate in the brain of C57BL/6 mice, because they lack the intrinsic capability to do so, and not because the host’s response in the course of infection is not sufficient to generate the appropriate environment for this process to occur.

The present work meant to clarify the dynamics of circulating blood-stage forms in a *P. berghei* infection and how that may correlate with sequestration in multiple organs. Results are not conclusive and, as stated during this discussion, more experiments have to be performed to elucidate this matter. However, we consider that our findings suggest that sequestration mechanisms in the brain differ from those in other organs, in PbA as well as PbNK65. Our results also suggest that PbNK65 pRBCs lack of sequestration in the brain of C57BL/6 mice is mainly due to a difference between this strain and the PbA strain in terms of intrinsic cytoadherence capability. Moreover, this is the first report of a difference in numbers of MPS in PbNK65 infection, which might be an explanation for lower parasitaemia growth rates and infectivity that have already been described for this parasite strain.

ANNEX A

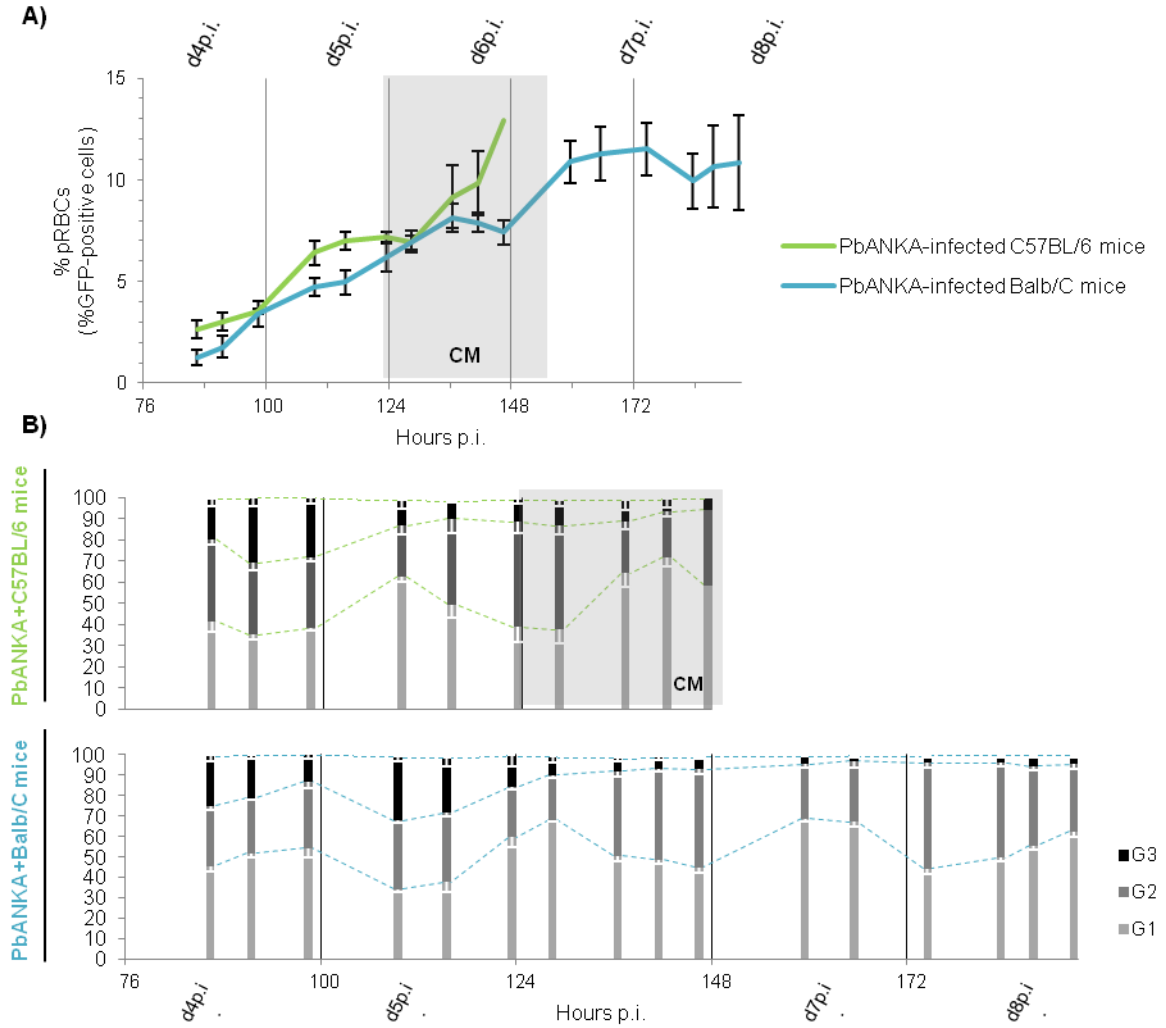


Figure 1. Analysis of PbA blood-stage in different hosts. A) Parasitaemia throughout early infection. Values represent the proportion of GFP-positive pRBCs in the total number of RBCs, as evaluated by flow cytometry. **B)** Proportion of different blood-stage forms during early infection. G1 includes mononucleated forms (ring-forms and some early trophozoites). G3 includes mature schizonts, but also some immature schizonts, late trophozoites and male gametocytes. G2 includes all intermediate forms (trophozoites) where DNA replication is occurring.
Values are represented as mean±S.D.; n=4 per group.

Table 1. Parasitaemias in "Mixed infections" experiments, at the time of sacrifice.

Experimental group	Total parasitaemia	% of GFP-expressing pRBCs	% GFP pRBCs/ total parasitaemia
Mixed Infections	7,21±3,55%	1,63±0,73%	9,78-53,0%
PbA-WT single infections	7,90±2,93%	N/A	N/A
PbNK65-GFP single infections	3,90±0,73%	N/A	N/A

REFERENCES

1. Organization, W.H. (2010) World Malaria Report.
2. Sachs, J., and Malaney, P. (2002) The economic and social burden of malaria. Nature.
3. Matuschewski, K. (2006) Getting infectious: formation and maturation of *Plasmodium* sporozoites in the *Anopheles* vector. Cell Microbiol. 10.1111/j.1462-5822.2006.00778.x.
4. Amino, R., Thiberge, S., Martin, B., Celli, S., Shorte, S., Frischknecht, F., and Menard, R. (2006) Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. Nat Med. 10.1038/nm1350.
5. Sturm, A., Amino, R., van de Sand, C., Regen, T., Retzlaff, S., Rennenberg, A., Krueger, A., Pollok, J.M., Menard, R., and Heussler, V.T. (2006) Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. Science. 10.1126/science.1129720.
6. Baer, K., Klotz, C., Kappe, S.H., Schnieder, T., and Frevert, U. (2007) Release of hepatic *Plasmodium* yoelii merozoites into the pulmonary microvasculature. PLoSPathogens. 10.1371/journal.ppat.0030171.
7. Tilley, L., Dixon, M.W., and Kirk, K. (2011) The *Plasmodium falciparum*-infected red blood cell. International Journal of Biochemistry & Cell Biology. 10.1016/j.biocel.2011.03.012.
8. Arnot, D.E., Ronander, E., and Bengtsson, D.C. (2011) The progression of the intra-erythrocytic cell cycle of *Plasmodium falciparum* and the role of the centriolar plaques in asynchronous mitotic division during schizogony. International Journal of Parasitology. 10.1016/j.ijpara.2010.07.012.
9. Aly, A.S., Vaughan, A.M., and Kappe, S.H. (2009) Malaria parasite development in the mosquito and infection of the mammalian host. Annual Reviews of Microbiology. 10.1146/annurev.micro.091208.073403.
10. MacPherson, G.G., Warrell, M.J., White, N.J., Looareesuwan, S., and Warrell, D.A. (1985) Human cerebral malaria: A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. American Journal of Pathology.
11. David, P.H., Hommel, M., Miller, L.H., Udeinya, I.J., and Oligino, L.D. (1963) Parasite sequestration in *Plasmodium falciparum* malaria spleen and antibody modulation of cytoadherence of infected erythrocytes. Proceeding os the National Academy of Sciences.
12. Berendt, A.R., Ferguson, D.J., Gardner, J., Turner, G., Rowe, A., McCormick, C., Roberts, D., Craig, A., Pinches, R., Elford, B.C., *et al.* (1994) Molecular mechanisms of sequestration in malaria. Parasitology.
13. Clark, I.A., and Rockett, K.A. (1994) The cytokine theory of human cerebral malaria. Parasitology Today.
14. Baptista, F.G., Pamplona, A., Pena, A.C., Mota, M.M., Pied, S., and Vigario, A.M. (2010) Accumulation of *Plasmodium berghei*-infected red blood cells in the brain is crucial for the development of cerebral malaria in mice. Infect Immun. 10.1128/IAI.00079-10.
15. Deharo, E., Coquelin, F., Chabaud, a.G., and Landau, I. (1996) The erythrocytic schizogony of two synchronized strains of *Plasmodium berghei*, NK65 and ANKA, in normocytes and reticulocytes. Parasitology Research.
16. Cox, F. (2002) History of Human Parasitology. Society.
17. Martinsen, E.S., Perkins, S.L., and Schall, J.J. (2008) A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life-history traits and host switches. Molecular phylogenetics and evolution.
18. Prudencio, M., Rodriguez, A., and Mota, M.M. (2006) The silent path to thousands of merozoites: the *Plasmodium* liver stage. Nature Reviews Microbiology. 10.1038/nrmicro1529.
19. Mota, M.M., Pradel, G., Vanderberg, J.P., Hafalla, J.C., Frevert, U., Nussenzweig, R.S., Nussenzweig, V., and Rodriguez, A. (2001) Migration of *Plasmodium* sporozoites through cells before infection. Science. 10.1126/science.291.5501.141.
20. Haldar, K., Murphy, S.C., Milner, D.a., and Taylor, T.E. (2007) Malaria: mechanisms of erythrocytic infection and pathological correlates of severe disease. Annual review of pathology. 10.1146/annurev.pathol.2.010506.091913.
21. Bannister, L.H., Hopkins, J.M., Fowler, R.E., Krishna, S., and Mitchell, G.H. (2000) A Brief Illustrated Guide to the Ultrastructure of *Plasmodium falciparum* Asexual Blood Stages. Parasitology Today.
22. Hanssen, E., McMillan, P.J., and Tilley, L. (2010) Cellular architecture of *Plasmodium falciparum*-infected erythrocytes. International Journal of Parasitology. 10.1016/j.ijpara.2010.04.012.
23. Reilly, H.B., Wang, H., Steuter, J.A., Marx, A.M., and Ferdig, M.T. (2007) Quantitative dissection of clone-specific growth rates in cultured malaria parasites. International Journal of Parasitology. 10.1016/j.ijpara.2007.05.003.

24. Chotivanich, K.U., Rachanee, A. Simpson, Julie, Newton, Paul, and Pukrittayakamee, S.L., Somchai, J. White, Nicholas (2000) Parasite Multiplication Potential and the Severity of *Falciparum* Malaria. Journal of Infectious Diseases.

25. de Souza, J.B., and Riley, E.M. (2002) Cerebral malaria: the contribution of studies in animal models to our understanding of immunopathogenesis. Microbes and Infection.

26. Williams, A.I.O., and MacFarlane, H. (1968) Malarial antigen from human brain. Clinical Experimental Immunology.

27. Lackner, P., Beer, R., Heussler, V., Goebel, G., Rudzki, D., Helbok, R., Tannich, E., and Schmutzhard, E. (2006) Behavioural and histopathological alterations in mice with cerebral malaria. Neuropathol Appl Neurobiol. 10.1111/j.1365-2990.2006.00706.x.

28. Riley, E.M., Couper, K.N., Helmby, H., Hafalla, J.C., de Souza, J.B., Langhorne, J., Jarra, W.B., and Zavala, F. (2010) Neuropathogenesis of human and murine malaria. Trends Parasitol. 10.1016/j.pt.2010.03.002.

29. Cook, G.C., and Webb, A.J. (2000) Perceptions of malaria transmission before Ross' discovery in 1897. Postgraduate Medical Journal.

30. Pamplona, A., Hanscheid, T., Epiphanio, S., Mota, M.M., and Vigario, A.M. (2009) Cerebral malaria and the hemolysis/methemoglobin/heme hypothesis: shedding new light on an old disease. International Journal of Biochemistry & Cell Biology. 10.1016/j.biocel.2008.09.020.

31. Medana, I.M., Geeta, C., Chan-Ling, T., and Hunt, N.H. (2001) Central nervous system in cerebral malaria: 'Innocent bystander' or active participant in the induction of immunopathology? Immunology and Cell Biology.

32. Howard, R.J., and Gilladoga, A.D. (1989) Molecular studies related to the pathogenesis of cerebral malaria. Blood.

33. Wickham, M.E., and Rug, M.R., Stuart A., Klonis, Nectarios, McFadden, Geoffrey I., Tilley, Leann, Cowman, Alan F. (2001) Trafficking and assembly of the cytoadherence complex in *Plasmodium falciparum*-infecred human erythrocytes. The Embo Journal.

34. Tsarukyanova, I., Drazba, J.A., Fujioka, H., Yadav, S.P., and Sam-Yellowe, T.Y. (2009) Proteins of the *Plasmodium falciparum* two transmembrane Maurer's cleft protein family, PfMC-2TM, and the 130 kDa Maurer's cleft protein define different domains of the infected erythrocyte intramembranous network. Parasitol Res. 10.1007/s00436-008-1270-3.

35. Blisnick, T.M.B., Maria Eugenia, Barale, J.-C., Uzureau, P., Berry, L., Desroses, S., Fujioka, H., Mattei, D., and Brau Breton, C. (2000) Pfsbp1, a Maurer's cleft *Plasmodium falciparum* protein, is associated with the erythrocyte skeleton. Mol Biochem Parasitol.

36. Mackenstedt, U., Brockelman, C.R., Mehlhorn, H., and Raether, W. (1989) Comparative morphology of human and animal malaria parasites. Parasitol Res.

37. Taylor-Robinson, A.W. (2010) Validity of Modelling Cerebral Malaria in Mice: Argument and Counter Argument. Journal of Neuroparasitology. 10.4303/jnp/N100601.

38. White, N.J., Turner, G.D., Medana, I.M., Dondorp, A.M., and Day, N.P. (2010) The murine cerebral malaria phenomenon. Trends Parasitol. 10.1016/j.pt.2009.10.007.

39. de Souza, J.B., Hafalla, J.C., Riley, E.M., and Couper, K.N. (2010) Cerebral malaria: why experimental murine models are required to understand the pathogenesis of disease. Parasitology. 10.1017/S0031182009991715.

40. Martinsen, E.S., Perkins, S.L., and Schall, J.J. (2008) A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life-history traits and host switches. Mol Phylogenet Evol. 10.1016/j.ympev.2007.11.012.

41. Liu, W., Li, Y., Learn, G.H., Rudicell, R.S., Robertson, J.D., Keele, B.F., Ndjango, J.B., Sanz, C.M., Morgan, D.B., Locatelli, S., *et al.* (2010) Origin of the human malaria parasite *Plasmodium falciparum* in gorillas. Nature. 10.1038/nature09442.

42. Schofield, L., and Grau, G.E. (2005) Immunological processes in malaria pathogenesis. Nature Reviews Immunology. 10.1038/nri1686.

43. Lanar, D.E. (1990) Sequence of the circumsporozoite gene of *Plasmodium berghei* ANKA clone and NK65 strain. Molecular & Biochemical Parasitology. 10.1016/0166-6851(90)90018-h.

44. Edington, G.M. (1967) Pathology of Malaria in West Africa. British Medical Journal.

45. Belnoue, E., Kayibanda, M., Vigário, A.M., Deschemin, J.C., Van Rooijen, N., Viguier, M., Snounou, G., and Rénia, L. (2002) On the Pathogenic Role of Brain-Sequestered αβ CD8+T Cells in Experimental Cerebral Malaria. Journal of Immunology.

46. van der Heyde, H.C., Nolan, J., Combes, V., Gramaglia, I., and Grau, G.E. (2006) A unified hypothesis for the genesis of cerebral malaria: sequestration, inflammation and hemostasis leading to microcirculatory dysfunction. Trends Parasitol. 10.1016/j.pt.2006.09.002.

47. Franke-Fayard, B., Trueman, H., Ramesar, J., Mendoza, J., van der Keur, M., van der Linden, R., Sinden, R.E., Waters, A.P., and Janse, C.J. (2004) A *Plasmodium berghei* reference line that constitutively expresses GFP at a high level throughout the complete life cycle. Molecular and Biochemical Parasitology. 10.1016/j.molbiopara.2004.04.007.

48. Janse, C.J., Franke-Fayard, B., Mair, G.R., Ramesar, J., Thiel, C., Engelmann, S., Matuschewski, K., van Gemert, G.J., Sauerwein, R.W., and Waters, A.P. (2006) High efficiency transfection of *Plasmodium berghei* facilitates novel selection procedures. Molecular Biochemical Parasitology. 10.1016/j.molbiopara.2005.09.007.

49. Carroll, R.W., Wainwright, M.S., Kim, K.-Y., Kidambi, T., Gómez, N.D., Taylor, T., and Haldar, K. (2010) A Rapid Murine Coma and Behavior Scale for Quantitative Assessment of Murine Cerebral Malaria. PlosOne. 10.1371/journal.pone.0013124.t001.

50. Janse, C., Ramesar, J., and Waters, A. (2004) *Plasmodium berghei*: general parasitological methods.

51. Janse, C.J., and Waters, A.P. (1995) *Plasmodium berghei* the application of cultivation and purification techniques to molecular studies of malaria parasites. Parasitology Today.

52. Franke-Fayard, B., Trueman, H., Ramesar, J., Mendoza, J., van der Keur, M., van der Linden, R., Sinden, R.E., Waters, A.P., and Janse, C.J. (2004) A *Plasmodium berghei* reference line that constitutively expresses GFP at a high level throughout the complete life cycle. Molecular & Biochemical Parasitology. 10.1016/j.molbiopara.2004.04.007.

53. B. Stamper Jr., H., and J. Woodruff, J. (1976) Lymphocyte homing into lymph nodes: in vitro demonstration of the selective affinity of recirculating lymphocytes for high-endothelial venules. Journal of Experimental Medicine.

54. Neres, R., Marinho, C.R., Goncalves, L.A., Catarino, M.B., and Penha-Goncalves, C. (2008) Pregnancy outcome and placenta pathology in *Plasmodium berghei* ANKA infected mice reproduce the pathogenesis of severe malaria in pregnant women. PlosOne. 10.1371/journal.pone.0001608.

55. Voza, T., Vigario, A.M., Belnoue, E., Gruner, A.C., Deschemin, J.C., Kayibanda, M., Delmas, F., Janse, C.J., Franke-Fayard, B., Waters, A.P., *et al.* (2005) Species-specific inhibition of cerebral malaria in mice coinfected with *Plasmodium* spp. Infect Immun. 10.1128/IAI.73.8.4777-4786.2005.

56. Van den Steen, P.E., Geurts, N., Deroost, K., Van Aelst, I., Verhenne, S., Heremans, H., Van Damme, J., and Opdenakker, G. (2010) Immunopathology and dexamethasone therapy in a new model for malaria-associated acute respiratory distress syndrome. Am J Respir Crit Care Med. 10.1164/rccm.200905-0786OC.

57. Franke-Fayard, B., Fonager, J., Braks, A., Khan, S.M., and Janse, C.J. (2010) Sequestration and tissue accumulation of human malaria parasites: can we learn anything from rodent models of malaria? PLoSPathogens. 10.1371/journal.ppat.1001032.