

**Universidade de Lisboa**  
Faculdade de Medicina  
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## **INTERACTIONS OF *PLASMODIUM* BLOOD AND LIVER STAGES WITHIN A SINGLE HOST**

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A dissertation for the degree of Doctor of Philosophy in Biomedical Sciences  
Specialization in Biopathological Sciences

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As opiniões expressas nesta publicação são da responsabilidade do seu autor.



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## Preface

This dissertation assembles data obtained during my Ph.D research project, developed at Faculdade de Medicina da Universidade de Lisboa, Instituto de Medicina Molecular, Unidade de Malaria, under the supervision of Prof. Maria M. Mota, from October 2006 to April 2010.

This thesis is structured in 5 chapters, preceded by a summary both in Portuguese and English, outlining the aims, results and outcomes of this project.

The first chapter provides an insight on previous malaria knowledge, and the aims of this work.

The second chapter contains the description of the methods and materials employed to carry out the present work.

The third chapter contains the original data regarding this project.

Chapter four encloses an overall discussion and conclusions of the studies performed, together with a section where future perspectives of the work developed are described.

In the Appendix is included a table with the information obtained with a microarray analysis performed during the course of this work.

The data described in this dissertation is the result of my own work. This work has never been previously submitted for any degree at this or any other university



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## Resumo

A infecção pelo agente causador da malária, o parasita *Plasmodium*, encontra-se ainda hoje disseminada pelas populações de 108 países no mundo. A malária produz sintomas que variam entre febres ligeiras até ao coma, anemia severa, síndrome respiratória aguda ou malária cerebral. Só em 2008 esta infecção foi responsável por mais de 800000 mortes, das quais a maioria se ficou a dever ao parasita *Plasmodium falciparum*. Adicionalmente a malária é ainda responsável por uma redução de 1.3% no crescimento económico dos países de maior endemicidade.

O parasita *Plasmodium*, pertence ao filo Apicomplexa e partilha com o Homem a presença na Terra desde o próprio aparecimento da espécie humana, tendo a adaptação parasita-hospedeiro vindo a evoluir ao longo do tempo, procurando um balanço entre a transmissão do parasita e a sobrevivência do hospedeiro.

É com a picada de uma fêmea de mosquito *Anopheles* que o parasita chega ao hospedeiro mamífero. Depois de uma passagem pela pele, os esporozoítos migram via corrente sanguínea até ao fígado onde depois de atravessar vários hepatócitos, invadem um último com a formação de um vacúolo parasitário. No hepatócito dá-se um processo de crescimento e replicação que no homem demora vários dias até à formação de vários milhares de merozoítos que serão libertados de novo na corrente sanguínea. Uma vez no sangue os merozoítos entram numa nova fase de reprodução assexuada, com sucessivos ciclos de invasão de eritrócitos, replicação e libertação para a corrente sanguínea e nova invasão de eritrócitos. É nesta fase da infecção que todos os sintomas associados à malária surgem.

Ocasionalmente, o ciclo de reprodução assexuada dentro dos eritrócitos para formação de novos merozoítos é substituído pela formação de gametócitos femininos ou masculinos que poderão ser aspirados durante uma nova picada de mosquito. É no hemocélio deste vector que se dá a fase sexual do parasita da malária com a formação de um zigoto e subsequente oocineto que migra para a lâmina basal onde se transforma num oocisto. A partir da esquizogonia dos oocistos surgem novos esporozoítos que uma vez chegados às glândulas salivares do mosquitos estão prontos para que se inicie uma nova infecção.

Em zonas de alta transmissão de *Plasmodium* é provável a ocorrência de sobreposição das fases hepática e sanguínea num só hospedeiro, bastando para isso que uma picada infecciosa ocorra num indivíduo que alberga já uma infecção circulante proveniente de uma picada anterior. Apesar de potencialmente importantes, as possíveis interacções entre as diferentes fases de desenvolvimento do parasita e o hospedeiro que os acolhe nunca foram objecto de estudo.

O objectivo desta tese prende-se precisamente com o estudo das interacções que surgirão desta situação a que damos o nome de re-infecção. Quão efectivos serão os esporozoítos na infecção do fígado de indivíduos com *Plasmodium* no sangue, quando comparados com a infecção do fígado de indivíduos sem qualquer parasita de malária presente?

Fazendo uso de modelos animais previamente estabelecidos e diferentes clones de parasitas, que nos permitiram distinguir as infecções hepática e sanguínea nos animais re-infectados, verificámos uma redução fortíssima na infecção no fígado de animais re-infectados. A capacidade dos esporozoítos para infectar hepatócitos de ratinhos com infecção sanguínea a decorrer apareceu altamente limitada quando comparada com a mesma capacidade para infectar hepatócitos de ratinhos naive.

O estudo detalhado deste fenómeno permitiu-nos relacionar a redução da infecção hepática com a diminuição tanto do número como do desenvolvimento das formas exo-eritrocíticas (EEF no original) no fígado. Esta redução verificou-se independente do nível da parasitemia da infecção primária, desde que esta se encontrasse acima de um valor que se mostrou ser baixo e rapidamente atingido. Mais ainda, verificou-se que a administração de um tratamento anti-malárico aos ratinhos infectados com *Plasmodium* no sangue antes da re-infecção, resulta na perda deste efeito protector.

Várias hipóteses, baseadas em trabalhos anteriores a este e num estudo de expressão genética que realizámos para melhor compreender as alterações hepáticas em resposta à presença de *Plasmodium* no sangue, foram colocadas na tentativa de entender o mecanismo pelo qual se observa tamanha redução na infecção de fígado de ratinhos re-infectados. Diversas moléculas associadas ao sistema imunitário, à inflamação e à apoptose no fígado foram testadas por meio de ratinhos transgénicos, ou pelo uso de anticorpos depletantes ou drogas bloqueantes sem que nenhum dos factores testados indicasse ser relevante.

A infecção de hepatócitos co-cultivados com eritrócitos infectados com *Plasmodium*, não mostrou ser eficiente em produzir o mesmo tipo de redução, afastando a hipótese de um factor solúvel libertado pelo eritrócito infectado.

Ao avaliar alterações no fígado relacionadas com a disponibilidade de nutrientes verificámos que o gene codificante da hormona reguladora do ferro, hepcidina se encontrava sobre-expresso no fígado de animais com parasitas no sangue. Sabendo que a disponibilidade de ferro pode limitar o crescimento de vários patogénios incluindo o *Plasmodium*, perguntámo-nos se seria esse o cerne da redução na infecção verificada.

A expressão do gene codificante da hepcidina provou estar intimamente ligada à presença de eritrócitos infectados no sangue de ratinhos, subindo rapidamente com poucos ciclos replicativos de *Plasmodium* no sangue, e voltando rapidamente a valores basais com o tratamento anti-malárico, o que rapidamente reduz a parasitemia para zero permitindo então que a infecção hepática se processe como em ratinhos naive. Além disso, pôde verificar-se uma redistribuição de ferro no fígado, perdendo-se parte do conteúdo nos hepatócitos para incrementar o conteúdo em macrófagos residentes e infiltrados no fígado.

Adicionalmente, mostrou-se que a hepcidina *per se* pode reduzir a infecção hepática. A administração a ratinhos de um adenovírus expressando o gene codificante desta hormona reguladora de ferro confirmou que este componente promovido pelo parasita no sangue, mesmo na sua ausência, pode actuar limitando a infecção por esporozoítos.

Ao tentar perceber a implicação destes resultados na malária humana, deparámo-nos com dados epidemiológicos há muito conhecidos mas parcamente explicados. Em áreas altamente endémicas é conhecido consistentemente que a incidência da infecção aumenta inicialmente com a idade das crianças para depois decrescer, possivelmente pela acção da imunidade adquirida. Ao mesmo tempo, a complexidade da infecção, em número de clones de parasitas diferentes no sangue de indivíduos, aumenta à medida que as crianças crescem em idade.

Divisando um modelo baseado unicamente na existência de um valor mínimo de parasitémia que inibisse o estabelecimento da infecção hepática, tal como foi observado no decurso desta tese, e assumindo o há muito estabelecido, que a densidade de parasitas no sangue decresce com a idade dos indivíduos. Institui-se então que a probabilidade de um picada infeciosa produzir infecção depende do nível de parasitémia no momento

da infecção e da história clínica do hospedeiro no que concerne a episódios prévios. Sob estas assumpções mínimas o modelo criado prevê correctamente um aumento inicial de infecções nas crianças seguindo-se um declínio à medida que os indivíduos adquirem imunidade devido a repetidas infecções. Quer isto dizer que uma densidade mínima de parasitas no sangue, da qual dependa a inibição de novas infecções de *Plasmodium* no fígado, pode por si justificar o aumento do risco de infecção e crescente complexidade das mesmas em crianças novas.

A aplicação do modelo com os resultados esperados é mais proeminentemente observada quando testado segundo áreas com taxas de transmissão moderadas ou elevadas, e permite explicar ainda algumas diferenças de incidência da doença ao longo da idade das crianças em áreas com diferentes taxas de transmissão.

O ciclo de vida do *Plasmodium* tem vindo a desenvolver-se ao longo de milhões de anos de co-evolução das interacções hospedeiro-parasita, com implicações importantes para a saúde humana. A infecção de eritrócitos acima de uma densidade mínima eleva a produção da hormona reguladora do ferro, hepcidina, que redistribuindo o ferro protege o nicho do parasita existente, inibindo o estabelecimento de uma infecção secundária, prevenindo assim a superinfecção. Este fenómeno actua independentemente e de forma complementar à imunidade adquirida e vem aclarar observações epidemiológicas prévias, podendo ainda ter implicações em futuras intervenções na luta contra a malária.

**Palavras-chave:** malária, re-infecção, superinfecção, hepcidina, ferro





## Summary

In regions of high malaria transmission, infected individuals are constantly exposed to potential re-infection. Mosquito bites transmit liver-tropic sporozoites into subjects who already have blood-stage parasitaemia. How these two stages of the *Plasmodium* life cycle interact within their host is unknown. Here, using a rodent model, we show ongoing blood stage infections impair the growth of subsequently inoculated sporozoites. Secondary infections are arrested in liver hepatocytes and fail to compete for colonization of red blood cells. This protection of the erythrocyte niche only occurs beyond a certain threshold of blood parasite density, and so is phenotypically akin to quorum sensing. We eliminate *Plasmodium*-secreted factors, host cell survival and innate or adaptive immunity as explanations for this observation. Instead, we find parasitized erythrocytes induce expression of the host iron regulatory hormone hepcidin, which by diverting iron from hepatocytes to macrophages, limits *Plasmodium* growth in the liver. Presuming a similar interaction between malaria and the human host we demonstrate how parasite threshold-density dependent growth inhibition alone can explain the epidemiological patterns of age-related risk and complexity of infections in young children. Our findings thus have broad implications for malaria and have general relevance for understanding host-pathogen interactions.



## Top 10 Abbreviations

CQ	Chloroquine
EEF	Exo-Erythrocytic Forms
EIR	Entomological Inoculation Rate
<i>gfp</i>	Green Fluorescent Protein
<i>hamp</i>	Hepcidin
<i>hprt</i>	Hypoxanthine Guanine Phosphoribosyltransferase
iRBC	Infected Red Blood Cell
PbA	<i>P. berghei</i> ANKA
PbNK	<i>P. berghei</i> NK65 iRBCs
RBC	Red Blood Cell



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## Introduction





*From the beginning...*

## **Malaria**

Descriptions of malaria appear in history prior to its name in several ancestral civilizations. Ancient Chinese manuscripts dating as early as 2700 BC have descriptions of patients with fever accompanied by enlarged spleen (reviewed in <sup>1</sup>). Later, Indian and Egyptian manuscripts also recorded indications of malaria infection. Furthermore, DNA of one of the species causing malaria, *Plasmodium falciparum* was recently isolated from an approximately 4000 year old Egyptian mummy <sup>2</sup> and *Plasmodium falciparum* DNA was also found in the mummy of Egyptian King Tutankhamun and three members of his family <sup>3</sup>. Reports by Hippocrates in 400 BC described for the first time the various periodic malaria fevers. In the *Corpus Hippocraticum*, he distinguished the intermittent malarial fever from the continuous fever of other infectious diseases, and also noted the daily, every-other-day, and every-third-day increase in body temperature (reviewed in <sup>4</sup>).



Fig. I1. **La Mal'aria** (1850-1851), oil over canvas by the French artist Antoine Auguste Ernest Hébert (1817-1908). Reproduction rights requested to Musée d'Orsay and Musée Hébert.

The name Malaria appears later from the Italian *mal'aria*, translating into *bad air*, as in Rome, where the disease raged for centuries, it was commonly believed until the 20th century that swamp and marshes produced the illness (reviewed in <sup>5</sup>).

In world history, the disentanglement of the Greek Empire is attributed to malaria and the disease is also believed to have stopped armies of European and Asian empires on different occasions <sup>1</sup>. Now as then, malaria still limits civilizations. Indeed, in the second half of the 20<sup>th</sup> century, countries with intensive malaria showed an average increase of gross domestic product (GDP) 5 times lower than that observed for non-malarial countries <sup>6</sup>.

The clinical features of malaria infection, which made it so recognisable throughout centuries, occur only during the asexual cycles of *Plasmodium* parasite inside red blood cells (RBCs). In humans, symptoms range from mild fever to coma, severe anaemia, respiratory distress or cerebral malaria <sup>7</sup>. The 2009 World Malaria Report of the World Health Organization (WHO) states that 108 countries were endemic for malaria in 2009, and that 863000 deaths were attributable to malaria in 2008, the majority of which were African children under 5 years of age <sup>8</sup>. Moreover, the disease is estimated to be responsible for an annual average reduction of 1.3% in the economic growth for those countries with the highest burden <sup>9</sup>.

*From the very beginning...*

### **Plasmodium phylogeny and its co-evolution with man**

*Plasmodium* is the causative agent of malaria and belongs to the Apicomplexa phylum. Apicomplexans have probably been present on earth since the earliest animals appeared and modern *Plasmodium* species descend from apicomplexans that interacted and adapted to increasingly sophisticated hosts over hundreds of millions of years <sup>10</sup>. Species of the genus *Plasmodium* have co-existed with humankind throughout its

evolution. *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* are the species that systematically infect humans. In addition, a fifth one, *P. knowlesi*, a natural parasite of macaque monkeys in southeast Asia, was recently reported to infect considerable numbers of humans <sup>11</sup>, besides several reports on isolated cases in several Asian regions <sup>12,13</sup>. Likewise, recent work reports that *P. falciparum* infections are not rare in gorillas <sup>14</sup>. These species represent only a glimpse of the systematic and ecological diversity of *Plasmodium* and related genera of Apicomplexa parasites. Several hundreds of malaria parasite species use squamat reptiles, birds, and mammals as vertebrate hosts along with many genera of dipteran vectors <sup>15</sup>, but the evolutionary and ecological events that led to this diversification and success remain poorly resolved. Recent genetic data challenged the classical systematic classification of malaria parasites, both within the *Plasmodium* genus, and in its relations among the currently recognized genera of the order Haemosporidia of Apicomplexa parasites. A study recovering multi-gene phylogeny of malaria parasites, including the analysis of sequencing data from 4 genes, indicated that parasites identified as *Plasmodium* species fall in two major clades, each clade being associated with a unique vector family, one clade containing parasites of mammals, and other of lizards and birds. Within the mammalian parasite clade, there are 4 monophyletic lineages: the human parasite, *P. falciparum*, a “primate” lineage that contains *P. vivax* and *P. knowlesi*, a lineage of parasites that infect African rodents, and the three samples of *Hepatocystis* isolated from bats <sup>16</sup>.

Evolutionarily, *Plasmodium* emergence was accompanied by a major change in parasite’s life cycle that differed from other genera. In addition to an initial round of asexual replication in fixed tissues that was general for all parasites, the *Plasmodium* life cycle includes additional rounds of asexual reproduction in blood cells, revealing increasing complexity when compared to ancestral parasites. There was also a shift to the use of

mosquito vectors, which seems to have made the exploitation of a greater variety of vertebrate taxa as hosts possible <sup>16</sup>.

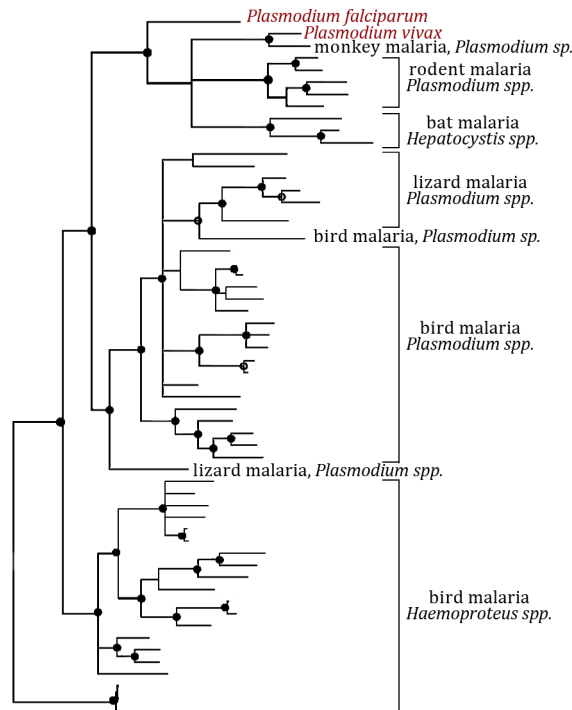


Fig. I2. **Phylogenetic tree of malaria causing parasites** Adapted from a multi-gene analysis study performed by Ellen S. Martinsen and collaborators, using 11 mammalian parasite species, 7 species that infect lizards, and 39 lineages from avian hosts.

It has been established that *Plasmodium* entered mammals only once, coincident with a switch from Culicine to Anopheline mosquitoes <sup>17</sup>. All known vectors of mammalian-infecting *Plasmodia* belong to the *Anopheles* genus <sup>18</sup>, indicating that the shift of *Plasmodium* into mammals was associated with specialization to anopheline vectors. It is well accepted that the closest identified sister taxon of *P. falciparum* is *P. reichenowi* whose host is the chimpanzee <sup>19</sup>. It was recently proposed that extant *P. falciparum* populations originated from *P. reichenowi* as late as 1.5 million years ago,

likely by a single host transfer <sup>20</sup>, an event that appears to have happened frequently between primates, including humans <sup>19</sup>. Others believe that the appearance of *P. falciparum* occurred even later during the initiation of agriculture, ten thousand years ago <sup>21</sup>. Still, there is some controversy around these proposals and the relationship between *P. falciparum* and *P. reichenowi* is not yet completely clear.

Nevertheless, it is clear that *Plasmodium* (or its ancestors) and their hosts had millions of years to co-evolve and co-adapt, leading to a balance between parasite replication and host survival enabling transmission to other hosts. A possible explanation for *P. falciparum* being the most virulent species infecting humans is the fact that, being much more recent than other *Plasmodium* species, it has not yet had the necessary time to fine-tune its effects on the host population.

Despite the significant burden of malaria-related mortality only a small proportion of infections lead to severe disease or death. *Plasmodium* infections in malaria endemic regions most often go unnoticed even in children, who are the most affected sector of the population. In children, around half of all infectious bites produce no infection, about a quarter leads to asymptomatic infections, roughly the other quarter gives rise to uncomplicated malaria with mild symptoms, and only a very small proportion of the infective bites will induce severe malaria with manifestations such as coma, cerebral malaria, respiratory distress, or severe anaemia <sup>22</sup> that can ultimately lead to death. Still, the prevalence of *P. falciparum* is high enough to kill more than half a million children every year in sub-Saharan-Africa.

Evidence for the adaptation of *Plasmodium* to man, and man's strategies to circumvent malaria infection can be seen in how the infection has modulated certain human genes in affected populations, selecting for resistance. As Haldane hypothesised and Allison demonstrated <sup>23</sup>, malaria is

known to be a strong evolutionary force of selection in the recent history of the human genome. Sickle-cell disease, thalassemia, and glucose-6-phosphatase deficiency, among other erythrocyte defects that together comprise the most common Mendelian human diseases, are under *Plasmodium* infection selection force, and can aid understanding the genetic basis of resistance shown by some exposed populations. Indeed, different resistant alleles have been identified for sickle cell trait in different areas and at different times, suggesting convergent evolution (reviewed in <sup>24</sup>).

*Plasmodium modus operandi...*

### **The *Plasmodium* life cycle in mammals**

Alfonse Laveran identified the exflagelating malaria parasite in human blood in 1880 <sup>25</sup>. Eight years later, using an avian model, Ronald Ross attributed the transmission of malaria disease to the anopheline mosquito <sup>26</sup>. The first observation of the pre-erythrocytic stage parasite was described later in 1948 by H. E. Short and P. C. Garnham in the liver of a simian model <sup>27</sup>. The three stages of malaria infection were then described, and from there on research tries to fill in the gaps, and understand the exact details of the entire life cycle, what the parasite's needs are, and how host and vector molecules contribute to or fight infection.

During its life cycle the malaria parasite takes many forms, shifting between invasive and replicative stages both in the vertebrate host and in the anopheline vector.

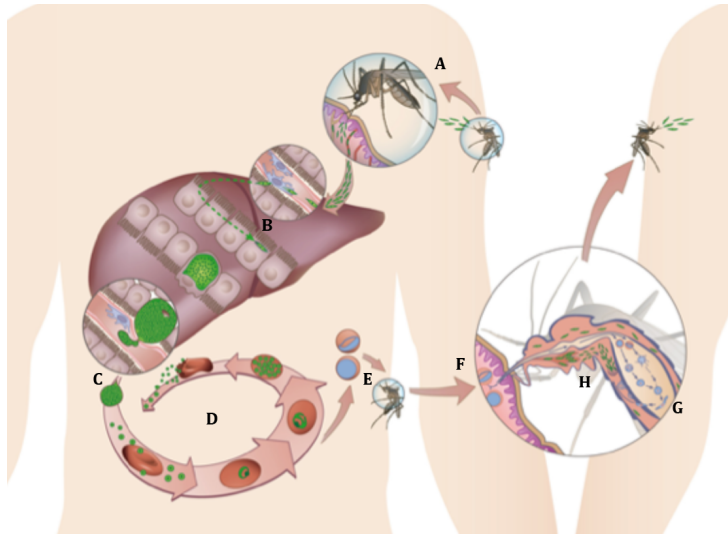


Fig. 13. ***Plasmodium* life cycle** (A) During a blood meal an anopheline mosquito injects *Plasmodium* sporozoites into the host dermis. (B) After reaching a blood vessel, sporozoites will travel to the liver where after traversing several hepatocytes, sporozoites invade a final one. (C) After asexual replication and development inside a hepatocyte, merozoites are released into the blood stream. (D) Merozoites infect red blood cells during cycles of asexual replication. (E) Occasionally replication cycles will originate female and male gametocytes. (F) Through another blood meal, a mosquito ingests gametocytes into its midgut. (G) Fertilization of gametes occurs in the mosquito midgut with the formation of ookinetes and later the oocysts. (H) Sporozoites released from the oocyst migrate to the salivary gland of the mosquito awaiting the next blood meal.

**Deposition in the skin.** All natural human malaria infections start with the deposition of *Plasmodium* sporozoites by a female anopheline mosquito in the host's dermis, during a blood meal. Each sporozoite is approximately 10  $\mu\text{m}$  long and 1  $\mu\text{m}$  wide, and once in the dermis, one of four possible fates will determine its future: (i) it can invade a blood vessel and reach the liver<sup>27</sup>, (ii) it can invade a lymphatic vessel and accumulate in the draining lymph node<sup>28</sup>, (iii) it can remain in the dermis<sup>29</sup>, or (iv) it can be re-ingested into the mosquito gut during the blood meal<sup>30</sup>. Only the first of these fates allows infection to proceed.

In the avascular tissues of the dermis, sporozoites actively glide forward in random directions. This movement however, seems to be specific to its substrate, as it differs from the one observed in salivary gland ducts and from that exhibited *in vitro* <sup>28</sup>. Like other apicomplexan parasites, *Plasmodium* sporozoites display a unique kind of locomotion, known as gliding motility, defined by the lack of obvious modification in the moving cell shape, and the need for a supporting substrate <sup>31</sup>. The parasite glides both to achieve motility and to invade cells, and the two mechanisms are made possible by the same acto-myosin motor located in the cortical space in between the plasma membrane and the inner membrane complex (IMC), a continuous layer of flattened vesicles underneath the plasma membrane <sup>31-34</sup>. In this space, trans-membrane proteins displaying adhesive extracellular domains and anchored to the motor trigger either forward locomotion or penetration into the host cells <sup>31,35,36</sup>. One such molecule of particular importance is the thrombospondin-related anonymous protein (TRAP), whose expression is restricted to *Plasmodium* sporozoites <sup>37,38</sup>.

Most sporozoites remain in the dermis for at least 5 minutes, as the removal of the biting site 5 minutes after the bite greatly reduces the number of infections in mice. However, a similar removal 15 minutes after the mosquito bite does not cause any significant alteration in the outcome of liver infection <sup>39</sup>, showing that during this timeframe following inoculation a significant and sufficient proportion of the sporozoites leave the biting site to find a blood vessel, which will later lead to infection of hepatocytes. Nevertheless, it takes one hour for half of the inoculated sporozoites to leave the dermis, either into blood or lymphatic vessels, and sporozoites have been found in the dermis up to 7 hours after injection <sup>40</sup>. During this period in the skin, sporozoites might trigger the first line of immune response.



To leave the dermis sporozoites may make use of their ability to traverse cells <sup>41</sup>, disrupting membranes of endothelial cells and penetrating vessels. Several proteins have been identified as essential for cell traversal, such as the sporozoite microneme protein essential for cell traversal (SPECT) <sup>42</sup>, SPECT2 <sup>43</sup>, cell traversal protein for ookinetes and sporozoites (CelTOS) <sup>44</sup> or phospholipase (PL) <sup>45</sup>. Indeed, sporozoites lacking SPECT are unable to reach blood vessels <sup>41</sup>.

Close to one third of sporozoites inoculated into mice through mosquito bite are later found in lymphatic vessels <sup>28</sup>. From there, only a few sporozoites can be rescued back to blood circulation, while the rest are drained to the closest lymph node. Once there, some sporozoites have been observed to develop into young and small Exo-Erythrocytic Forms (EEFs) during the first hours. This initial development is not continued and, instead, these small EEFs are then cleared, which seems to contribute to the initiation of an immune response <sup>46</sup>.

The mechanism of invasion of the blood vessels is still not fully understood. It has been described that gliding speed is decreased when sporozoites are in contact with the vessels and it is known that the entry process lasts less than a minute <sup>47</sup>.

**The liver stage.** Once inside the vessel, sporozoites are carried in the blood flow at the same speed as erythrocytes <sup>40</sup>, and will later be arrested in the liver by not completely understood mechanisms. Circumsporozoite protein (CSP), a protein encoded by a single copy gene, and the major coat protein of *Plasmodium* sporozoites <sup>48</sup>, has been shown to be involved in this process. Indeed recombinant CSP binds specifically to sulfated glycoconjugates <sup>49</sup>. It has been proposed that heparan sulfate proteoglycans (HSPGs) of stellate cells extending through endothelial fenestrations to the sinusoidal lumen, can attract and arrest sporozoites. These cells synthesize proteoglycans that are eight times more sulphated than those of hepatocytes and incorporate

twice the amount sulphate into heparan sulphate, known to be preferentially attractive to sporozoites <sup>50</sup>. Being unable to pass through fenestrations of the liver sinusoid, sporozoites must migrate through cells, mostly Kupffer cells, using their capacity to traverse barriers and finally access the liver parenchyma <sup>51</sup>. Still, this is a matter of controversy and some authors have proposed that the passage through Kupffer cells occurs by a mechanism akin to phagocytosis <sup>52</sup>. Once having gained free access to the liver parenchyma, sporozoites traverse several hepatocytes, disrupting their plasma membranes to get in and out of cells <sup>53</sup>, until they ultimately invade a final hepatocyte with formation of a parasitophorous vacuole (PV). Different studies have shown hepatocyte traversal makes sporozoites prone for final hepatocyte infection, either by activating sporozoites to form a PV <sup>54,55</sup> or by leading to the release of hepatocyte growth factor, which sustains host cell viability <sup>56</sup>. Importantly, although demonstrated to be beneficial for infection, traversal ability of *Plasmodium* sporozoites has been shown not to be essential for hepatocyte invasion *in vitro*, since parasites lacking essential proteins for cell migration as SPECT, CelTOS or PL still invade hepatocytes and complete liver stage development similarly to wild type parasites <sup>42-44</sup>. Not much is known about the signal to stop migration and invade, but mouse studies indicate that sporozoites are activated to initiate the invasion process in the presence of cells expressing high levels of sulfated HSPGs <sup>50,54</sup>.

To invade hepatocytes, *Plasmodium* sporozoites, like other apicomplexan parasites, release proteins from apical organelles termed rhoptries, micronemes and dense granules, and attach to host cells using cell-adhesive domains of some of these proteins. TRAP seems to be essential to this process <sup>57</sup>. Although adhesion to the host cell is initially reversible, it later becomes irreversible, forming tight junctions that will allow the parasite to move forward propelled by its acto-myosin motor, allowing it to enter the

cell. With the completion of the tight junctions, the sporozoite is included inside the host cell surrounded by the PV membrane <sup>58</sup>.

Inside hepatocytes, sporozoites develop and replicate originating thousands of merozoites that ultimately will proceed to blood infection. The astonishing replication rate observed most certainly implies strong cellular and molecular host-parasite interactions. Although little is known about the intra-hepatic development of *Plasmodium* parasites, recent studies have provided a new and more comprehensive insight into gene expression and protein abundance profiles of *Plasmodium* liver stage <sup>59</sup>. *Plasmodium* sporozoite and liver stage asparagine-rich protein (SLARP) has been observed to be essential for initiation of EEF development, as parasites mutant for this protein are arrested in very early development <sup>60</sup>. Other parasite proteins have been shown to be essential for development inside hepatocytes. Parasites deficient for the microneme protein P36p present reduced invasion and impaired development <sup>61</sup>, and up-regulated in infective sporozoites genes 3 and 4 (UIS3)- or (UIS4)-deficient parasites present impaired development <sup>62</sup>. *Plasmodium* Fatty Acid Synthesis II pathway enzymes were found to be modulated during infection, and later functional studies revealed that type 2 fatty acid synthesis is important for liver stage development <sup>63</sup> and, more specifically, essential for the late steps of liver stage development <sup>64</sup>. Another recent study showed that cGMP dependent protein kinase (PbPKG) disruption in sporozoites leads to an arrest of liver stages <sup>65</sup>.

Host molecules have also proven to be important for complete development of the *Plasmodium* parasite inside hepatocytes. The expression of several host genes is modulated during liver infection, as shown recently in a microarray study comparing infected and non-infected cells patterns of gene expression over the time course of infection <sup>66</sup>. In functional studies, tetraspanin CD81 <sup>67</sup> and scavenger receptor B1 <sup>68</sup> have been shown to be

important for liver stage development. Their absence reduces liver infection to very low levels in rodent models. Yet another functional screen, aimed at the host kinome, has linked an efficient parasite infection with several human kinases <sup>69</sup>.

During the liver stage, as will later be shown for the blood stage, *Plasmodium* parasites seem to export proteins beyond the PV to the cytosol of the hepatocyte or even to the hepatocyte nucleus. Up to now this has been shown for CSP, and mutant parasites incapable of this export movement show impaired development <sup>70</sup>.

By the end of exoerythrocytic stage, merozoites are released in the blood-stream within large vesicles known as merozoites <sup>71</sup>. These budding structures full of merozoites squeeze through endothelial cells, and are initially hidden from the host's innate immune system by being covered with a hepatocyte-derived membrane.

**The blood stage.** After reaching circulation, merozoites release the merozoites into the blood-stream. This seems to occur only when merozoites reach the lung microvasculature. Although the reason behind is not known, it has been speculated that low macrophage density and reduced blood velocity with reduced shear forces within the lung microvasculature enhances the ability of merozoites to invade erythrocytes, since once merozoites are free in circulation they quickly have to find a red blood cell to infect. <sup>72</sup>.

Merozoites invade RBCs, highly differentiated cells, whose main purpose is oxygen distribution throughout the body. However, RBCs lack several cellular functions as they are anucleated, transcriptionally and translationally inactive, lack any secretory apparatus, have only a limited repertoire of solute and iron transporters, and are readily removed from circulation when damaged (reviewed in <sup>73</sup>).

Invasion of RBCs follows sequential steps: cell recognition, merozoite binding, reorientation and deformation of the RBC, junction formation and parasite entry. In *P. falciparum* alone, more than 50 surface and secreted proteins have been identified as playing a role in these events <sup>74</sup>. Initial host-parasite contact is believed to be random but, once it has happened, merozoite surface proteins (MSP) immediately mediate adhesion to the host cell <sup>75</sup>. The merozoite's surface is covered in glycosylphosphatidylinositol (GPI)-anchored proteins and their partners <sup>76</sup>. Many of these proteins seem to act as ligands for RBCs. Besides sharing GPI-anchors, surface proteins of the merozoite also share with sporozoites and gametes the cysteine-rich domains that have been shown to potentiate adherence <sup>77</sup> of *Plasmodium* species. As such, it is believed they play similar roles in the cytoadherence during the blood stage of infection. In order to invade RBCs, merozoites make use of complex mechanisms, evidencing great adaptation to their host. Some parasite strains that depend on sialic-acid receptors, for example, can even shift their invasion pathway to a sialic-acid independent entry process <sup>78</sup>.

After this initial binding, there is a reorientation of the merozoite in order to position its apical and secretory organelles (rhoptries, micronemes and dense granules) in contact with the RBC membrane. An indentation is then formed in the contact zone. A number of proteins of the apical organelles bind to specific erythrocyte receptors. Apical membrane antigen 1 (AMA-1) is known to establish the apical interaction through parasite adhesins initially located at the neck of the rhoptries and in the micronemes <sup>79</sup>. Recently, parasites with green fluorescent protein (GFP)-tagged AMA-1 were used in live imaging studies that revealed this protein's crucial function <sup>80</sup>.

The actual invasion process involves two major protein families, the Duffy binding-like (DBL) protein family <sup>81</sup>, and the *P. falciparum* reticulocyte

binding protein homolog (PfRh) family <sup>82</sup>. DBL and PfRh proteins are important for merozoite invasion of RBCs, but are not considered essential, as gene disruption for each of them in different *P. falciparum* lines still show normal blood-stage growth rates <sup>83-87</sup>. This might be due to complementation between different family members or to a great level of adaptation of *Plasmodium* to its host cell. When the apical interaction is formed, the merozoite establishes a tight junction, which is accompanied by the release of more proteins from the microneme and rhoptry organelles. When attachment is finally achieved, the merozoite is hypothesised to discharge mediators into the RBC, and although these have not yet been identified, visualization of merozoite invasion by electron micrographs suggests material transfer <sup>88</sup>. Entry is then mediated by activation of the acto-myosin motor in the pellicle of the invading merozoite <sup>89</sup>. At the end of the invasion process, the RBC bilayer envelops the invading merozoite in a new vacuole <sup>90</sup>, which lacks normal proteins of the RBC membrane. Proteins like Band 3 are cleaved by proteolytic enzymes secreted by the parasite <sup>91</sup> and are replaced by recruitment of detergent-resistant membrane (DRM) raft proteins, making the PV richer in lipid rafts and its proteins <sup>90</sup>.

Inside the normally quiescent environment of a RBC, the merozoite undergoes some rounds of nuclear division, transforming sequentially in ring, trophozoite and schizont stages. It eventually egresses and releases 16 to 32 new merozoites that will follow the same sequence of events. In *P. falciparum*, the ring stage lasts approximately 24h, accounting for half of the entire erythrocytic cycle <sup>92</sup>.

Following entry, and throughout all the developmental and replication processes, the parasite modifies the permeability and adhesive properties of its host cell, possibly to promote its own survival.

The ring stage is followed by the trophozoite stage, during which most of the cytoplasm of the RBC is consumed, a process that leads to the

degradation of 60 to 80% of the haemoglobin present in the RBC <sup>93</sup>. This is followed by the schizont stage, during which 4 to 5 rounds of binary replication take place, originating merozoites that will later invade other RBCs. The most common and concentrated molecule inside RBCs is haemoglobin that is degraded by *Plasmodium*. Haemoglobin proteolysis yields haem besides amino acids as end products. Haeme is not metabolized or recycled by the parasite. Instead, it is stored as hemozoin, a polymer that confers the characteristic pigmentation to the organs of infected individuals <sup>94,95</sup>. The amino acids of the haemoglobin polypeptide chain are used in the synthesis of parasite's proteins <sup>96</sup> and appear to be used as an energy source <sup>95</sup>. The recovery of the amino acids is essential for the parasite as it has limited capacity of *de novo* synthesis <sup>94</sup>. However, the recycling of amino acids originating from haemoglobin degradation is not sufficient for parasite maintenance as haemoglobin lacks some essential amino acids, which the parasite is able to withdraw from the host plasma <sup>97</sup>. The feeding of the parasite in the RBC occurs through a cytostome, an invagination of the PV membrane and the parasite plasma membrane, which ingests small packets of haemoglobin from the host cytosol. Budding vesicles full of haemoglobin are transported and fused to an acidic digestive vacuole (DV) where haemoglobin is degraded and haeme is detoxified by the action of several proteases <sup>98</sup> acting like haemoglobinases. Data suggest that this degradation process is ordered and requires, among other reactions, an initial aspartic protease-mediated cleavage, followed by secondary aspartic protease and cysteine protease cleavages. The aspartic proteases have been shown to be Plasmepsin I and II and the cysteine protease has been identified as Falcipain <sup>99,100</sup>. Different patterns of gene expression are observed during the erythrocytic cycle, suggesting that their functions are not exactly the same. Several other Plasmepsin proteins were later identified both inside and outside the DV. Their functional redundancy was

shown when independent disruption of Plasmepsin I, II, IV or histidine aspartic protease (HAP) was obtained and very little effect on the parasite growth was observed <sup>101,102</sup>.

*Plasmodium* has the ability to export hundreds of its own proteins beyond its plasma membrane and the PV to the cytosol of the erythrocyte <sup>103</sup>. This represents 5% of its whole genome. Although the function of many of these proteins remains unknown, some of them have been associated with virulence, promoting cell adhesion and/or rigidity of the erythrocyte <sup>104,105</sup>. To be exported, proteins must first enter the secretory pathway <sup>106</sup> showing a recessed amino-terminal hydrophobic endoplasmic reticulum (ER) signal sequence that allows transport across the plasma membrane of the parasite but not the PV membrane. Most proteins exported across the PV membrane require an additional sequence element known as *Plasmodium* export element (PEXEL), or a vacuolar transport sign (VTS) that is found downstream of the ER signal sequence <sup>107</sup>. This export movement has recently been proposed to be performed by a proteinaceous translocon within the PV membrane, an ATP-powered complex containing heat shock protein 101, a novel protein PTEX150 and a known parasite protein identified as exported protein 2 (EXP2) that potentially works as the common portal through which most or all exported proteins must pass <sup>108</sup>. Moreover Plasmepsin V was recently shown to be the protease responsible for cleavage of the PEXEL motif of proteins to be exported, thereby allowing their traversal of the PV and trafficking to the RBC surface <sup>109,110</sup>. Following this export via this translocon at the PV membrane, exported proteins have to reach different destinations in the host RBC, such as the cytoplasm or the plasma membrane. Because RBCs have no secretory system, the parasite builds its own to allow its proteins to reach the host cell plasma membrane. Maurer's cleft is a central structural component of this "extracellular" protein export system <sup>111</sup>. These stacks of flattened lamellae of long slender



membranes with a translucent lumen were identified more than a century ago and are located below the erythrocyte plasma membrane <sup>112,113</sup>. Several parasite proteins are synthesized in the parasite's ER and then transferred to the cleft <sup>114,115</sup>. It is still not completely clear whether the Maurer's cleft is part of a continuous network that connects the PV to the erythrocyte cell surface like some imaging reconstructions of thin sections seem to suggest <sup>116</sup>, or if the cleft is a well-defined structure continuously supplied with vesicles budding from the membrane lining the PV. Subsequently, vesicles bud out from the cleft and migrate towards the erythrocyte cell surface, with secretion of their contents into the medium and incorporation of some proteins in the erythrocyte plasma membrane <sup>74</sup>.

The very strong remodelling of the host RBC eventually leads to its distortion, with positively charged knobby protrusions. Using atomic force microscopy, the knob was shown to consist of two subunits that might be central to the phenomenon of cyto-adherence in *P. falciparum* malaria <sup>117</sup>. Indeed, in *P. falciparum* infections, schizonts are known to barely circulate <sup>118</sup>. Instead, they are sequestered in different organs by adhering to the endothelial cells of the vessels. Knobs are created by the deposition of parasite proteins, such as knob-associated histidine-rich protein (KAHRP) and the adhesion protein *P. falciparum* erythrocyte membrane 1 (PfEMP1), which are inserted in the RBC membrane.

PfEMP1 is restricted to *P. falciparum*, and undergoes clonal antigenic variation switching, changing its antigen type at high frequencies during intra-erythrocytic cycles <sup>119,120</sup>. PfEMP1 belongs to a large polymorphic gene family called *var*, in which each individual gene encodes a different form of the protein, and only one is expressed at a time through a mutually exclusive mechanism. It comprises three regions: the cytoplasmatic or acidic terminal segment (ATS) that is anchored to the knobs; the transmembrane regions that are inside the RBC membrane; and the

ectodomain or N-terminal segment followed by the DBL domains and the Cys-rich interdomain regions (CIDR) (reviewed in <sup>74</sup>) that interact with endothelial cell receptors, leading to sequestration and thus preventing destruction of infected RBC (iRBC) in the spleen <sup>121</sup>. PfEMP1 is highly related to virulence as it is believed that the adherence it promotes triggers much of the associated pathology, including cerebral malaria and pregnancy-associated malaria (reviewed in <sup>122</sup>).

Following this period (whose length varies between different *Plasmodium* species) where *Plasmodium* replicates with the consumption of the RBC cytoplasm, parasites leave the RBC by a process called egress that happens very fast and must be tightly regulated. Several hypothetical models explain egress. Although these vary by disagreeing on whether the PV membrane or the RBC membrane disintegrates first, all the models accept that proteases play a critical role. Protease inhibitors have been shown to prevent *P. falciparum* iRBC rupture, and to promote accumulation of mature schizonts *in vitro* due to egress blocking <sup>123</sup>, which was later attributed to prevention of the proteolytic effect of serine repeat antigen (SERA)-5 <sup>124</sup>. Further studies showed that different protease inhibitors have different effects on egress <sup>125</sup>. Still, the mechanism by which egress is regulated is only beginning to be understood. It has been recently reported that just prior to egress essential serine protease PfSUB1 is discharged from *Plasmodium* organelles called exonemes, from the merozoite into the PV <sup>126</sup>, where it mediates the proteolytic maturation of members of SERA family that have been previously implicated in egress <sup>127</sup>. Furthermore PfSUB1 has been shown to directly mediate primary proteolytic processing of MSP1, MSP6 and MSP7. PfSUB1 seems to prepare not only the merozoite for release from the erythrocyte but also to ensure that, once in circulation, merozoites get inside a new cell without delay <sup>128</sup>, avoiding exposure to the host response.

**Shifting to sexual stages.** Differentiation and sexual commitment happen prior to schizogony during asexual replication cycles inside RBCs. All siblings of a schizont are either asexual, female gametocytes or male gametocytes <sup>129</sup>, but the mechanism that determines which of these possibilities occurs remains unknown. Translational repression and mRNA turnover have been shown to be key players in determining stage-specific gene expression in *Plasmodium*. More specifically, development of zygote inhibited RNA helicase (DOZI) was identified in the female gametocyte and shown to have a central role in the silencing and maintenance of steady-state levels of a population of gametocyte-specific transcripts, allowing the coordinated production of essential proteins for the further development and establishment of infection inside the mosquito <sup>130</sup>.

**Inside the mosquito.** Mature gametocytes in the blood can later be engorged during a mosquito blood meal. The abrupt environmental change inside the mosquito triggers gametocytes to round up and emerge from the RBC within minutes <sup>131</sup>. The presence of xanthurenic acid allows the male gametocyte to transform into eight motile microgametes after three rounds of exceedingly fast genome replication followed by nuclear division and axoneme assembly, and the female emerges from the erythrocyte as a roundshaped non-motile gamete <sup>132</sup>. Exflagellation of the male gametocyte and the vibratory movements in waves of its microgametes allow it to penetrate into the female gamete, and fertilization to occur <sup>133</sup>. The resulting zygote will be the only parasite survivor in the aggressive environment of the mosquito gut, and further develops into a tetraploid <sup>134</sup> motile ookinete that is the only invasive form of the whole cycle that is not originated by replication. There is a great loss of ookinetes during traversal of the midgut epithelial cells to reach the basal lamina, due to host protective mechanisms <sup>135</sup>. Once in the basal lamina, ookinetes become sessile and, after meiosis <sup>136</sup>, transform into oocysts that are the only extracellular developmental stage

of the whole cycle. Oocysts make use of their capsule to recruit nutrients from the hemolymph <sup>137</sup> to grow, 50 to 60  $\mu\text{m}$  in diameter, and originate sporozoites. CSP, the protein that will cover all of sporozoites, starts to be expressed and accumulate in the oocyst plasma membrane even prior to sporozoite formation <sup>138</sup>. With the retraction on the oocyst plasma membrane, several lobes called sporoblasts appear in this form <sup>139</sup>. CSP is essential for this formation <sup>140</sup>, and also seems to be essential for the organization of the microtubule organizing centres (MTOCs) <sup>138</sup> that will later lead to the formation of the apical complex and nuclei positioning in the daughter sporozoites.

Sporozoite release into the hemolymph occurs asynchronously <sup>141</sup> and was shown to be dependent on proteinase activity, including that of egress cysteine protease 1 (ECP1) <sup>142</sup>. Once released in the hemocoel, sporozoites can be spread in the whole mosquito body but only recognize specific host receptors in the basal lamina of the salivary glands <sup>139</sup>. Invasion occurs only in this organ and several parasite ligands, like CSP <sup>143</sup>, TRAP <sup>144</sup> or MAEBL <sup>145</sup>, have been identified as being important for recognition and invasion. After attaching, sporozoites breach the basal lamina and invade through the basal membrane of salivary secretory acinar cells with the brief formation of a vacuole that will allow them to emerge from the apical side of these same cells into the salivary gland duct <sup>146</sup>.

Sporozoites are now ready to infect a new mammalian host when the infected mosquito takes another blood meal.

*Malaria outcome...*

### **Malaria transmission and naturally acquired immunity**

Measuring malaria endemicity or *Plasmodium* prevalence in a geographical area has been the subject of an active debate for decades. Surveys of

splenomegaly across a population <sup>147</sup>, examination of peripheral blood for asexual malaria parasites <sup>148</sup>, stability/instability of transmission, entomological inoculation rates (EIR) <sup>149</sup> or haemoglobin measurements <sup>150</sup> were, and are, some of the metrics to establish classes of endemicity in which a given population should fall. Considering Africa alone, the existing endemic populations show rates of transmission that can differ in intensity by 100-fold.

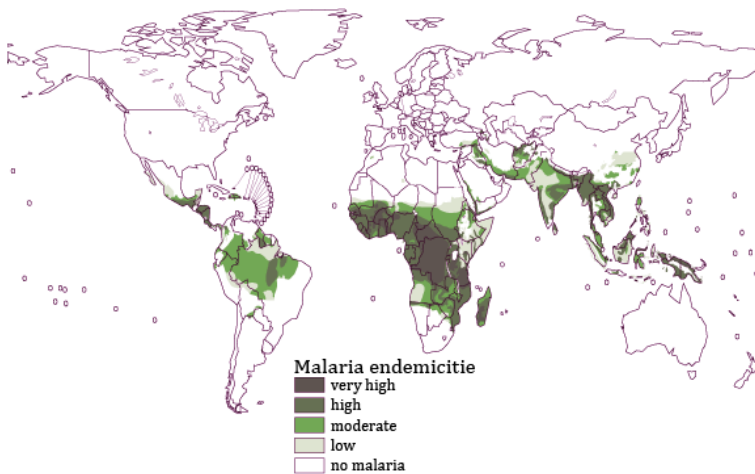


Fig.14. **Global distribution of malaria transmission risk.** Adaptdted from WHO World Malaria Report 2005.

Clinical presentations of *P. falciparum* malaria vary according to different transmission intensities, but the biological interactions promoting these different outcomes are not easily understood. Studies across fifty years of field research in areas with different rates of transmission, in several communities with different access to health systems or treatment, and with variations in the prevalence of infection occurring from year to year in a given place, allow all sorts of interpretations concerning how mortality and morbidity vary with transmissions rates. There is, however, a consensus in

accepting that the mean age of severe malaria disease decreases as *P. falciparum* transmission increases. The frequency of cerebral malaria declines with increasing transmission rates while anaemia takes over the clinical burden (reviewed in <sup>151</sup>). It is generally accepted that in low to moderate transmission settings, the incidence of severe disease grows after the first year of age, while in high transmission areas the peak in severe disease happens between the fifth and the seventh month of age, after which it declines significantly before the first birthday (reviewed <sup>152</sup>). Common to all settings is the fact that for the first 3 months of age the incidence of severe disease is very low, most likely due to the presence of maternal antibodies <sup>153</sup>. In addition, the much lower risk of severe disease after 5 years of age indicates that some degree of immunity is acquired early in life. However, under very low transmission regions the risk of clinical malaria is extended until adulthood <sup>154</sup>.

An extensive study in an area with decreasing parasite prevalence over 16 years showed that although transmissions decreased significantly in the first ten years, there were no major alterations in clinical cases reported. Only later, when parasite prevalence fell below a certain threshold, could a drop in hospital admissions be observed. At the same time, as parasite prevalence dropped, the mean age of slide-positive children increased <sup>155</sup>. Moreover in cross-sectional studies with increasing malaria transmission, there is an initial increase in the rate of hospital admissions with malaria, but thereafter, the risk of hospitalization rises either more slowly or plateaus at intermediate ranges of transmission intensity and may decrease slightly in areas of very high transmission <sup>156</sup>.

Very strong evidence that protection against malaria increases with age more than with any other factors comes from a study performed in Mali where it was observed that older children treated with chloroquine were better able to clear chloroquine-resistant *P. falciparum* parasites than

younger children treated in a similar way <sup>157</sup>, again pointing towards an effective acquired immune response.

However, although in endemic settings older children and adults are resistant to severe morbidity and death, they are still susceptible to infection <sup>158</sup>, showing that only a certain level of immunity can be developed against malaria. This natural immunity is acquired at the cost of very high early mortality and is still defective, as multiple infections are required before clinical protection is achieved, and persistent infection is typical. Indeed, back in 1900, Robert Koch compared areas of different endemicities and deduced that there was the need for heavy and continued exposure to the parasite in order to acquire protection against malaria. Later, in 1920, the essential features of naturally acquired immunity against *Plasmodium* were described: it was accepted that natural immunity was (i) effective in adults after uninterrupted lifelong heavy exposure, (ii) lost upon cessation of exposure, (iii) species specific, (iv) somewhat stage specific, and (v) acquired at a rate which was dependent upon the degree of exposure (reviewed in <sup>159</sup>).

In spite of these premises, very little is known about the mechanisms through which immunity against malaria is acquired. Early studies with humans, showed that antibodies can protect against malaria infection <sup>160</sup>, and several antigens have later been shown to be associated with malaria protection. Antibodies against MSP1 <sup>161</sup>, MSP2 <sup>162</sup> or MSP3 <sup>163</sup> were found significantly elevated in the sera of protected individuals in different endemic areas and are thought to correlate with protection. These associations vary between studies, and antigens encountered until now show considerable polymorphisms through the existence of alternative allelic forms <sup>164</sup> and through antigenic variation <sup>165</sup>, limiting their use as possible targets for vaccination and treatment. Also, the antibody response to the most common merozoite antigens is believed to be short-lived <sup>166</sup>,

peaking one week after the malaria episode and rapidly decaying to very low levels within 6 to 8 weeks. The reason for such a short half-life is still not understood. It might be that the catabolic half-life of these antibodies is just shorter, but other hypothesis have been raised such as the predilection towards IgG3 which is known to be short lived <sup>167</sup>, or the poor development of memory or long-lived plasma cells <sup>168</sup>. Independent of the mechanism behind it, this short half-life of anti-malaria antibodies continues to create problems when planning an intervention strategy toward an effective vaccine against malaria.

Liver stage naturally acquired immunity is not believed to be strong enough or acquired fast enough to provide any kind of protection in endemic populations. Still, it cannot be said that natural immunity against *Plasmodium* pre-erythrocytic stage is not involved in the final outcome observed. Indeed, infection of both humans and mice with *Plasmodium* sporozoites followed by chloroquine treatment that abrogates the establishment of blood stages protects individuals from subsequent infections <sup>169,170</sup>. It is true, however, that early studies performed in humans, bypassing the hepatic phase of the infection through direct blood stage infection, showed that immunity could be maintained (reviewed in <sup>162</sup>). However, vaccine development efforts have invested a significant effort in pre-erythrocytic immunization and proven efficient in rendering protection for variable periods of time, probably because of the very high numbers of attenuated sporozoites used <sup>171</sup>, which are very different from the very low numbers of sporozoites that mosquitoes inject during a blood meal <sup>172</sup>. Both attenuated forms of the parasite <sup>173</sup> and live sporozoites <sup>170</sup> can completely protect humans, making them resistant to infection. However, so far the most advanced vaccine candidate against *Plasmodium falciparum* is RTS,S. It is a recombinant, yeast-expressed subunit vaccine using hepatitis B virus



surface antigens carrying epitopes from *P. falciparum* CSP<sup>174</sup> the major coat protein of sporozoites.

In areas with very high EIRs, as children get older malaria attacks become less frequent after an initial peak at young age, and parasite levels between manifestations steadily decrease. Although the child seems to be acquiring immunity, they remain parasitized, often with higher parasitaemias than other children at greater risk of clinical disease from areas with lower EIRs<sup>175</sup>. This protection against clinical disease is the product of acquired immunity that is able to control parasitaemia but does not fully abrogate infection, producing only non-sterilizing immunity<sup>176</sup>. Several reports of *Plasmodium* superinfection describe single individuals hosting more than one *Plasmodium* species, or different genotypes of the same *Plasmodium* species infecting RBCs<sup>177-179</sup>. However, these observations have been much more frequent among asymptomatic carriers than in clinical cases<sup>180</sup>, and have been shown to depend on the age of individuals in a population<sup>175,181-183</sup>.

Premunition or concomitant immunity is the protection against infections and clinical disease that associates with persistence of multiple infections, providing protection against new infections by maintaining a low-grade and generally asymptomatic parasitaemia and high levels of antibodies (reviewed in<sup>159,184</sup>). A better understanding of mechanisms behind premunition is clearly central to the comprehension of naturally acquired immunity to malaria. Periodic blood-stage infection presumably serves to boost a pre-existing immune response and maintain high frequencies of effector cells in areas of high transmission. Exposure to a greater variety or antigens of families with associated variance increases the repertoire that each individual recognises<sup>185</sup>.

Antigenic variation creates distinct waves of parasitemia that must be chased by different antibody responses, on the one hand promoting long

infections that might endanger the host, while on the other hand allowing eventual acquisition of disease-controlling immunity. Effective antiparasitic immunity is, if ever, achieved only after very many and frequent infections<sup>149,186</sup>.

So, the doubt remains, whether reducing the risk of *Plasmodium* infection will always lead to a decrease in the threat of severe malaria throughout life. This is a concern that was raised 50 years ago, when wondering if altering the natural risk of exposure to parasite, by reducing vector or transmission itself, would change the epidemiology of severe disease<sup>187</sup>. Knowing that increasing exposure to *Plasmodium* parasites is reflected in increasingly rapid acquisition of immune responses that limits life-threatening effects of malaria, the full understanding of the transmission/immunity associations is of major importance in defining malaria control interventions.

### **Aims**

*Plasmodium* passage through the skin followed by infection of liver hepatocytes and, later, of blood erythrocytes, are natural and sequential steps of *Plasmodium* life cycle in the mammalian host and should not be seen as independent entities. The sophistication and complexity of *Plasmodium* life cycle are evidence for co-evolution with man, and are reflected in the complicated nature of relationships between transmission rates, immunity and disease severity in endemic areas. Importantly, liver and blood stages of *Plasmodium* infection may frequently occur simultaneously in the same individual where transmission rates allow it.

Indeed, after infecting the hepatocyte for approximately a week, *P. falciparum* can infect RBCs for weeks or even months after that<sup>188</sup>. Thus, a new infection initiated by a mosquito bite may occur while the parasites from a previous one are still replicating inside erythrocytes. Very little is known about the interactions between the two different stages of a malaria

infection. So far it has been shown that malaria blood-stage can be immunosuppressive <sup>189</sup>, but it is clear that very close interactions are established between parasite and host throughout the parasite's life cycle.

The objective of the work presented here arises from intending to clarify effects of one stage on another in individuals dually infected with blood and posterior liver stage malaria, and try to understand how re-infection fits what is known so far regarding different patterns of infection in different EIR settings.

To access *Plasmodium* blood stage/liver stage interactions new tools were used to outwit past limitations, we have made use of wild-type *Plasmodium* parasites to start blood stage infections and of *gfp*-expressing or luciferase-expressing *Plasmodium* parasites to initiate liver stage infections. Using transgenic parasites we were able to distinguish the two infections circumventing the lack of specific liver stage markers. Because the GFP or luciferase genes are under the control of a *Plasmodium* housekeeping gene promoter, their expression correlates with *Plasmodium* liver load.

The study of the temporal coincidence of *Plasmodium* blood and liver stages in a single host at a given time was mostly performed in rodent models of infection to answer 4 major questions:

What would be the impact of an ongoing blood stage infection on the establishment of a secondary sporozoite infection in the liver?

By what molecular mechanisms do any interactive effects between blood and liver stages occur?

What, if any are the consequences of such interaction for the re-infected individual?

How such interactions adjust to established relations between patterns of infection and transmission rates in different endemic settings?



## Materials and Methods



**Mice.** C57BL/6, BALB/c, Balb-SCID as well as mice deficient in RagII, IL-10, caspase 3, MyD88 or Kit W-sh/W-sh deficient were bred in the specific pathogen-free facilities of the Instituto de Gulbenkian de Ciência (Oeiras, Portugal). RAGII/ $\gamma$ c deficient mice were kindly provided by James Di Santo (Cytokines and Lymphoid Development unit, Institut Pasteur, Paris, France). NOS2- (B6.129P2-Nos2tm1Lau/J), TCRdelta- (B6.129P2-Tcrdtm1Mom/J), and C5a- (B10.D2-Hc0.H2d.H2-T18c/oSnJ) deficient mice were purchased at The Jackson Laboratory along with their respective wild type littermates. All mice were housed in the Instituto de Medicina Molecular (IMM) facilities and the IMM Animal Care Committee approved all protocols. IFN- $\gamma$  deficient mice were kindly provided by Rui Appelberg (Microbiology and Immunology of Infection laboratory, Instituto de Biologia Molecular e Celular-IBMC, Porto, Portugal). Experiments with IFN- $\gamma$  deficient mice were performed at IBMC, and IBMC Animal Care Committee approved all protocols. All transgenic mice were genotyped by tail genomic DNA PCR to confirm their respective mutations.

***Plasmodium* blood infection.** Primary blood stage infection of mice was achieved by 30 minutes exposure of mice to 15 *Anopheles* mosquitoes infected with *P. berghei* ANKA (parasite line GFPcon259cl2), or by intraperitoneal inoculation of the designated quantity of red blood cells infected with *P. berghei* ANKA (1.49L), *P. berghei* NK65, *P. yoelii* 17X NL or *P. chabaudi chabaudi* AS. Peripheral blood parasitaemia was determined by Giemsa staining followed by microscopic counting of iRBCs and results are expressed as percentage of RBCs. On Fig. 1 C parasitemia was verified by real time *in vivo* imaging using the *in vivo* IVIS® Lumina Imaging System<sup>190</sup> as described bellow. And on Fig. 1 E parasitemia was determined by flow cytometry, measuring red blood cells infected with *gfp*-expressing *P. berghei* ANKA. The results are expressed as percentage of infected red blood cells, as previously described<sup>191</sup>.

**Plasmodium liver infection.** Green fluorescent protein (*gfp*)-expressing *P. berghei* ANKA (parasite line GFPcon259cl2) or *gfp*-expressing *P. yoelii* sporozoites<sup>191,192</sup> were obtained by dissection of *Anopheles stephensi* infected mosquitoes bred in the insectarium of IMM. Mice were infected by intra-venous inoculation of the designated quantity of GFP sporozoites, or by 30 minutes exposure of mice to 15 *Anopheles* mosquitoes infected with *P. berghei* ANKA. Parasite liver load was quantified 40, 48 or 72h post-infection.

**Real time *in vivo* imaging luminescent *Plasmodium*.** Naïve mice (control) and blood stage infected (PbA in Fig. 1A and PbNK in Fig. 1B and Fig. 1C), were intra-peritoneally injected with 200µl of anaesthesia mixture (80 mg/kg Ketamine and 10 mg/kg Xylazine) diluted in PBS in order to allow each mouse exposure to 15 *Anopheles* mosquitoes infected with luciferase expressing *P. berghei* (parasite line 354cl4). After 30 minutes of mosquito bites, mice were shaved in the abdomen. Forty h post-infection for Fig. 1A and 1B and 6 days post-infection for Fig. 1C, D-luciferin dissolved in PBS (150 mg/kg; Caliper Life Sciences, USA) was injected subcutaneously in the neck. Animals were anesthetized again as described above for the whole duration of measurements (performed within 5 to 10 minutes after the injection of D-luciferin). Bioluminescence imaging was acquired with a 12.5 cm field of view (FOV), medium binning factor and an exposure time of 180 seconds. Luciferase activity in animals was visualized through imaging of whole bodies using the *in vivo* IVIS® Lumina Imaging System<sup>190</sup>.

**qReal-Time PCR quantification of liver infection.** Livers were collected and homogenized in denaturing solution (4 M guanidine thiocyanate; 25mM sodium citrate pH 7, 0.5% N-Lauroylsarcosine and, 0.7% β-Mercaptoethanol in DEPC-treated water). Total RNA was extracted using RNeasy Mini kit (Qiagen), and then reverse transcribed into cDNA using Transcriptor First



Strand cDNA Synthesis kit (Roche), according to the manufacturers' protocols. Infection load in the liver was determined as previously described by qRT-PCR using classic PbA 18S *rRNA*<sup>193</sup> specific primers or *gfp* specific primers (*gfp* expression correlates with PbA 18S *rRNA*, fig. S1). qRT-PCR reactions used Power SYBR Green PCR Master Mix (Applied Biosystems) and were performed according to the manufacturer's instructions on an ABI Prism 7000 system (Applied Biosystems). Relative amounts of PbA 18S *rRNA* and *gfp* mRNA were calculated against the Hypoxanthine Guanine Phosphoribosyltransferase (*hprt*) housekeeping gene, following a prime denaturation of 10 minutes at 95°C, then 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. PbA 18S *rRNA*, *gfp* and *hprt* specific primer sequences were: 5'-CGG CTT AAT TTG ACT CAA CAC G-3' and 5'-TTA GCA TGC CAG AGT CTC GTT C-3' for PbA 18S *rRNA*, 5'- GTC AGT GGA GAG GGT GAA GG- 3' and 5' - ACT TCA GCA CGT GTC TTG TAG TTC - 3' for *gfp* and 5' - TGC TCGAGA TGT GAT GAA GG - 3' and 5' - TCC CCT GTT GAC TGG TCA TT - 3' for mouse *hprt*. External standardization was performed using plasmids encoding the full-length genes cDNA cloned in TOPO TA (Invitrogen).

**Chloroquine treatment.** Mice infected 4 days previously with *P. berghei* NK65 and control mice received 0.8 mg of chloroquine (CQ) by intra-peritoneal injection for 1 or 2 days before re-infection, a protocol leading to decrease in peripheral blood parasitaemia until zero by day 2 of treatment.

**Liver slice histopathology, morphometric analysis and immunofluorescence.** Liver tissues were harvested from control or re-infected mice 40 h after sporozoite infection. Tissues were fixed in 4% paraformaldehyde for 15 minutes, washed three times in PBS and then sliced into 50 µm sections using a vibratome (VT1000S, Leica). Sections were later permeabilized and blocked overnight in 0.3% Triton X-100

(Calbiochem) and 1% Bovine Serum Albumin (Sigma) to avoid non-specific reactivity. Sections were then incubated overnight at 4°C in the same solution containing anti-GFP IgG Alexa fluor 488 conjugate antibody (Invitrogen), Alexa 594 phalloidin (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI, Sigma). After mounting 15 to 20 sections on slides, the areas of the sections were measured using a scope, the EEFs were counted using the Leica DM5000B Widefield Fluorescence Microscope, and the sizes of 20 randomly chosen EEFs of each mouse were measured using a Zeiss LSM 510 META Point Scanning Confocal Microscope. All images of immunofluorescence-stained sections were analysed using the ImageJ 1.42b software. Areas and numbers of EEF were normalized to the total area observed.

**Cells.** Huh7 cells, a human hepatoma cell line, were cultured in RPMI (Gibco/Invitrogen) medium supplemented with 10% fetal calf serum (FCS, Gibco/Invitrogen), 1% penicillin/streptomycin (pen/strep, Gibco/Invitrogen), 1% glutamine (Gibco/Invitrogen) at pH 7 and maintained at 37°C with 5% CO<sub>2</sub>.

**Isolation of murine primary hepatocytes.** Mouse primary hepatocytes were isolated as previously described <sup>194</sup>. Briefly, cells were initially obtained by perfusion of mouse liver lobules with liver perfusion medium and liver digest medium (Gibco/Invitrogen) at 37°C using a peristaltic pump. Hepatocytes were then purified using a 1.12 g/ml, 1.08 g/ml and 1.06g/ml Percoll gradient. Cells were cultured in William's E medium containing 4% FCS, 1% pen/strep in 24-well plates coated with 0.2% Gelatine in PBS. Cells were maintained in culture at 37°C and 5% CO<sub>2</sub>.

**Sporozoite infection and development in contact with infected blood.** Mouse primary hepatocytes and Huh7 cells, a human hepatoma cell line, were cultured as described above in complete William's E or RPMI

(Gibco/Invitrogen) in transwell system plates (COSTAR/Corning). In the lower chamber, liver-derived cells were infected with 20,000 *P. berghei* ANKA sporozoites, and allowed to share medium with upper chambers containing serum (CTRL), non-infected blood (NI) or blood containing  $6 \times 10^5$  *P. berghei* NK65 iRBCs (PbNK). After 36h of co-culture, hepatoma cells were collected and treated for FACS analysis and primary hepatocytes were fixed in 4% paraformaldehyde for 10 minutes, washed three times in PBS, and later permeabilized and blocked 1h in 0.3% Triton X-100 (Calbiochem) and 1% Bovine Serum Albumin (Sigma) to avoid non-specific reactivity. Coverslips were then incubated 1h at 4°C in the same solution containing anti-GFP IgG Alexa flour 488 conjugate antibody (Invitrogen), Alexa 594 phalloidin (Invitrogen) and DAPI (Sigma). After mounting coverslips on slides, EEFs were counted using the Leica DM5000B Widefield Fluorescence Microscope, and the sizes of 20 randomly chosen EEFs of each coverslip were measured using a Zeiss LSM 510 META Point Scanning Confocal Microscope. All immunofluorescence-stained images were analysed using the ImageJ 1.42b software.

**Fluorescence activated cell sorting (FACS) analysis of sporozoite infection.** FACS analysis of sporozoite-infected Huh7 hepatoma cell cultures at 36h post-sporozoite addition was performed to determine the percentage of parasite-containing cells and parasite-GFP intensity within infected cells. Cell samples for FACS analysis were processed as previously described <sup>195</sup>.

**Transcription profiling.** Total RNA from livers of naïve mice (control group), mice infected with  $10^6$  *P. berghei* NK65 iRBCs for 7 days (Blood stage group, BS), mice infected with  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoites for 40h (Liver stage group, LS), and mice infected with  $10^6$  *P. berghei* NK65 iRBCs for 7 days then re-infected with  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoites for 40h (Reinfection group, Reinf) was extracted using RNeasy

Mini kit (Qiagen), following the manufacturer's instructions. Three biological replicates were analysed for each group. RNA quality and quantity was assessed on a Bioanalyser nanochip (Agilent). Total RNA was reverse transcribed and end-labelled as cRNA using the GeneChip WT Amplified Double-Stranded cDNA Synthesis Kit and the GeneChip WT Terminal Labeling Kit as recommended by Affymetrix. Hybridisation to an Affymetrix Mouse Gene 1.0 ST Array was carried out at 45°C for 16h under rotation (60 rpm). Arrays were washed on an Affymetrix FS450 and scanned using an Affymetrix Genechip Scanner 3000 7G. Fluorescence intensities were background adjusted, quantile normalised and median polished into expression values using the robust multi-array averaging program RMA<sup>196</sup> in R/Bioconductor suite<sup>197</sup> (software package: affy<sup>198</sup>) To assess significance and differential expression (DE), each infected group was compared to the non-infected group, and out of 28815 main probesets represented on the array, 6273 transcript clusters were classified as significant (F-test,  $p < 0.05$ ) with 1704 being DE at a fold change of 2 (software packages: limma<sup>199</sup> and gplots). Each cluster separated from the non-hierarchical clustering was subjected to a Gene Ontology Biological Process enrichment analysis using a hypergeometric test (software package: GOstats<sup>200</sup>). Original data was submitted to Array Express and is accessible in the reviewer's user account: Username: Reviewer\_E-TABM-839, and Password: 1257429875724.

**Depletions using monoclonal antibodies.** (i) IL-6. Mice infected with *P. berghei* NK65 and control mice received 1 mg of IL-6 specific monoclonal antibody (clone MP520F3, Rat IgG1) 3h, 3 days, and 5 days after inoculation with infected blood, a protocol leading to IL-6 depletion at the time of sporozoite infection, 7 days post- *P. berghei* NK65 infection.

(ii) IFN $\gamma$ . Mice infected with *P. berghei* NK65 and control mice received 0.4 mg of IFN $\gamma$  specific monoclonal antibody (clone R46A2, Rat IgG1) 1, 2 and 3 days, after inoculation with infected blood, a protocol leading IFN $\gamma$  depletion at the time of sporozoite infection, 5 days post-*P. berghei* NK65 infection.

(iii) TNF $\alpha$ . Mice infected with *P. berghei* NK65 and control mice received 0.4 mg of TNF specific monoclonal antibody (clone XT3.11, Rat IgG1) 4 and 6 days after inoculation of infected blood, a protocol leading to TNF $\alpha$  depletion at the time of sporozoite infection, 5 days post-*P. berghei* NK65 infection.

**N $\omega$ -Nitro-L-arginine methyl ester hydrochloride treatment.** Mice infected with *P. berghei* NK65 and control mice received 50mg/kg of N $\omega$ -Nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma) 3, 4, 5 and 6 days after inoculation of infected blood, a protocol leading to NOS inhibition at the time of sporozoite infection, 7 days post- *P. berghei* NK65 infection.

**S-methyl-isothiourea treatment.** Mice infected with *P. berghei* NK65 and control mice received 100  $\mu$ g of S-methyl-isothiourea (SMT, Sigma) 3, 4, 5 and 6 days after inoculation of infected blood, a protocol leading to iNOS inhibition at the time of sporozoite infection, 7 days post- *P. berghei* NK65 infection.

**C3a receptor antagonist treatment.** C5a deficient (B10.D2-Hc0.H2d.H2-T18c/oSn) naïve or *P. berghei* NK65 infected mice received 0.5 mg of C3a receptor antagonist (SB290157, Calbiochem) 4h before sporozoite re-infection, 5 days post-*P. berghei* NK65 infection.

**Kupffer cell depletion using clodronate liposomes** <sup>201,202</sup>. Kupffer cells were eliminated from naïve and *P. berghei* NK65 infected mice by injection of 200  $\mu$ l clodronate liposomes (liposome-encapsulated dichloromethylene

diphos-phonate; clodLip) daily, from day one after *P. berghei* NK65 infection until the day of sporozoite infection (4 days post-blood stage infection). As controls, the same volume of PBS containing liposomes was injected into naïve or *P. berghei* NK65 infected mice. Clodronate was a gift from Roche Diagnostics GmbH (Mannheim, Germany). It was encapsulated into liposomes, as previously described<sup>203</sup>. Kupffer cell depletion was verified by immunofluorescence microscopy. Livers were collected and 50 µm sections obtained and fixed, as described above. Sections were then incubated overnight at 4°C in a solution containing rat anti-mouse pan tissue fixed macrophages antibody (Fitzgerald) and Alexa 660 phalloidin (Invitrogen). After washing twice with PBS, sections were incubated for 6h with secondary Alexa fluor 595 donkey anti-rat IgG (Invitrogen) and DAPI (Sigma). After mounting sections on slides, sections were imaged on a Zeiss LSM 510 META Point Scanning Confocal Microscope. Images of immunofluorescence-stained sections were analysed using the ImageJ 1.42b software.

**Splenectomy.** Intra-peritoneal injection of 250 µl of anaesthesia mixture (80 mg/kg Ketamine and 10 mg/kg Xylazine) was administered to mice 10 to 15 min before surgery. After observing the animals for response to anaesthesia, the animals were laid on their right side; and the fur removed with a razor. A 1.5 to 2 cm long skin incision was made in the left hypochondrium, the connective tissue under the skin was loosened and another incision in the peritoneal wall was made. Gently the spleen was pulled onto the exterior surface of the peritoneum. The artery, attached to the hilum of the spleen, close to the stomach was tied off with a single knot using 3-0 plain CATGUT suture. The efferent venule attached at the other end of the spleen was tied off similarly by a single knot using 3-0 plain CATGUT. Cutting away the mesentery and connective tissue, the spleen was removed. Peritoneal wall and underlying muscles were closed with plain

CATGUT (3-0) and the skin incisions closed with wound clips. Mice with accessory spleen were excluded from the study. The control group underwent a sham surgery and they were maintained in the same conditions.

**Liver hepcidin (*hamp1*) quantification.** After RNA extraction and cDNA conversion as described above, hepcidin (*hamp1*) mRNA expression in the liver was determined by TaqMan qRT-PCR using TaqMan Gene Expression Master Mix (Applied Biosystems) and Applied Biosystems inventoried gene expression assays for *hamp1* (ABI TaqMan Gene Expression Assay probe/primer set Mm00519025\_m1) and *gapdh* (Glyceraldehyde-3-phosphate dehydrogenase) (ABI TaqMan Gene Expression Assay probe/primer set Mm99999915\_g1), according to the manufacturer's instructions. qRT-PCR was carried out using an ABI Prism 7500 FAST system (Applied Biosystems). Changes in *hepcidin* mRNA expression between control and re-infected mouse livers were calculated using the  $2^{-\Delta\Delta C_t}$  method with *gapdh* as the endogenous control gene.

**Ferritin staining.** Livers were collected and 50  $\mu\text{m}$  sections were obtained and fixed as described above. Sections were then incubated overnight at 4°C in a solution containing rabbit anti-mouse heavy and light ferritin chain antibody (a kind gift by Paolo Arosio, Faculty of Medicine, Brescia, Italy) and Alexa 660 phalloidin (Invitrogen). After washing twice with PBS, sections were incubated for 6h with secondary Alexa fluor 595 donkey anti-rat IgG (Invitrogen) and DAPI (Sigma). Sections were imaged on a Zeiss LSM 510 META Point Scanning Confocal Microscope. Images of immunofluorescence-stained sections were analysed using the ImageJ 1.42b software.

**Total non-heme iron quantification.** Total non-heme iron was measured as described previously <sup>204</sup>, in brief after drying samples (spleens and primary hepatocytes) for 48h at 45°C, tissues were weighed, and digested

for 48h in 10% trichloroacetic acid/10% HCl at 65°C. Two hundred microliters of the extract were then added to 1 ml of chromogen solution (0.01% bathophenanthroline-disulfonic acid, 0.1% thioglycolic acid, 2.5 M sodium acetate) and after 15 minutes of incubation, absorbance was measured at 535 nm. A standard curve performed for known iron concentrations between 10 and 500 µg/ml revealed a linearity of response with a slope of ~1. Samples were diluted appropriately to fall within the linear range. Values were expressed as % of control, in micrograms of iron per gram of dry weight.

**HAMP adenovirus *in vivo* infection.** Mice were infected with adenoviral  $5 \times 10^{11}$  pu of pAd.CMV.GFP (control) or pAd.CMV.Hamp.ires.GFP.Wpre (HAMP.adV) kindly provided by Stefano Rivella for (Weill Cornell Medical College, USA) 48h prior to sporozoite infection, hepcidin expression was 2 fold upregulated by the Hamp.adv compared to uninfected and GFP.adv control groups. All mice were then infected with  $2 \times 10^4$  *P. berghei* ANKA (1.49L) sporozoites, and 40h later livers were collected and liver infection was determined by expression of PbA 18S *rRNA*.

**Iron treatments.** (i) *In vitro*: Ferric Ammonium Citrate (FAC, Sigma) bathophenanthrolinedisulfonate (BPS, Acros) and Desferrioxamine (DFO, Sigma) solutions were prepared in at the designated concentrations. Huh7 cells were incubated with different concentrations of FAC, BPS or DFO, using water as control for FAC and PBS as control for BPS and DFO respectively, 24h prior to sporozoite infection and for 36h post-infection, after which cells were collected for FACS analysis.

(ii) *In vivo*: DFO (Sigma) solution was prepared in PBS at the designated concentration. Mice were inoculated intra-peritoneally with 250 mg/Kg of DFO using PBS as control 24h prior to sporozoite infection.



**Tunel detection of apoptosis.** Liver tissues were harvested from wild type and caspase 3-deficient mice infected with *P. berghei* NK65 for 8 days along with non-infected wild type mice. Tissues were frozen in OCT freezing medium and then sliced into 16  $\mu\text{m}$  sections using a cryostat. Analysis of DNA fragmentation by transferase-mediated dUTP nick-end labelling (TUNEL) was performed using the In Situ Cell Death Detection kit, POD and DAB Substrate kits (Roche, USA) following the manufacturer's instructions, and later stained with DAPI (Sigma) for general nuclei count. Sections were imaged on a Zeiss LSM 510 META Point Scanning Confocal Microscope. Quantification of positive cells was determined counting a total of 1000 cells/section and calculating the proportion of stained nuclei. Images of immunofluorescence-stained sections were analysed using the ImageJ 1.42b software.

**Cardiac puncture, blood collection and serum production.** Mice were maintained under general inhalant anesthesia, a needle was inserted through intact skin and between ribs and up to 1ml of blood was quickly withdrawn from heart using a 1ml plastic syringe with 25ga. needle. Mice were euthanized by cervical dislocation. The blood was allowed to clot for a while, after what the cloth is removed and the remaining liquid centrifuged for 20min, 3000rpms. Supernatant was collected and injected to mice intra-venously.

**Cholesterol measurements.** (i) Serum. Total cholesterol, HDL- and LDL-cholesterol in the serum were biochemically determined by DNAtch (INETI, Portugal).

(ii) Liver. The amounts of cholesterol in a sample of liver collected after perfusion with PBS were measured using the Amplex Red cholesterol assay kit (Invitrogen), according to the manufacturer's instructions. Briefly, cholesterol is oxidised by cholesterol oxidase to yield hydrogen peroxide

and ketones. The hydrogen peroxide reacts with 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red reagent) to produce highly fluorescent resorufin, which is measured by excitation at 550 nm and emission detection at 590 nm. A standard curve was performed with serial dilutions with a linearity of response with a slope of  $\sim 1$ . Samples were diluted appropriately to fall within the linear range. Values were expressed as % of control.

***Toxoplasma* infection.** *Toxoplasma gondii* tachyzoites (Toxo-GFP/LacZ kindly provided by Markus Meissner, Department of Parasitology, University Hospital Heidelberg, Germany) were grown in VERO cells and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% glutamine and 20  $\mu\text{g}/\text{ml}$  gentamicin.  $10^4$  freshly released tachyzoites were intra-peritoneally injected into mice 4 days prior to *Plasmodium* sporozoite challenge.

**Model description.** A stochastic, individual-based model was used to follow a cohort of  $N$  individuals for up to 20 years in a low, medium and high transmission setting (with according annual EIR of 2, 20 and 200, respectively). At each time point  $t_i$  the probability of an individual becoming infected,  $P$ , is given as the product of the probability of being bitten by an infected mosquito,  $p_{bite}$  ( $=1-1/\text{EIR}$ ), times the probability of this bite leading to successful blood stage infection which itself is dependent on the immune history of the host,  $p_{imm}$ , and the parasite density of a possible ongoing infection,  $p_{dens}$ . That is,  $P(\text{infection}) = P(p_{bite} \cap p_{imm} \cap p_{dens})$ . We assumed that the average parasite density per infection decreases with age<sup>175,181,205</sup> given as  $\pi_{age} = \pi_{\max} (1 + k \exp(-age))$ , and that mean parasitaemia level also decreases over the course of infection<sup>206</sup>; for simplicity we assumed this to be linear although the actual functional form does not change the results presented here. We further assumed that there exists a threshold parasite

density,  $p_{crit}$ , above which the host is mostly protected from super-infections. This threshold density then defines the probability of a successful inoculation as a function of infection length and is given as

$$p_{dens} = \prod_i \left( 1 + \exp(\tau_i - \Delta t_i)^\beta \right)^{-1},$$

where  $i$  is the number of concurrent infections,  $\Delta t_i$  is the time since infection  $i$ , and  $\tau_i = T_i \left( 1 - \pi_{crit} / \pi_{max} \right)$  is the time when the probability of a new blood stage infection is approximately half;  $1/T_i$  is the clearance rate of infection  $i$ . For reasons of simplicity and because we are here more concerned with the inhibitory effect on new liver-stage infections, we do not take into account the full complexity of super-infections (see e.g. <sup>178</sup>) and assume infections and their respective clearance rates to be independent. A graphical representation of the relationship between parasitaemia and probability of infection is given in Supplementary Information. Finally we assumed acquired immunity to be driven by exposure, or rather the number of life-time blood stage infections. We chose this to be non-linear to take into account antigenic diversity of and immunity against the various life-stages of the parasite; again, the exact functional form does not have a significant effect on the results presented. The probability of an infection not being blocked by immunity is then given

$$\text{as } p_{imm} = \prod_{i=1..n} \sqrt{\frac{D-i}{D}},$$

where  $n$  is the life-time number of blood stage infections and  $D$  can be understood as a measure of antigenic diversity and which defines the rate of acquired protection with infection history (see SI). The parameter values used for the simulations are as follows:  $N=500$ ,  $p_0=5000$ ,  $k=2$ ,  $b=2$ ,  $p_{crit}=3000$ ,  $T \sim \text{normal}(100)$  and  $D=500$ . Note, our results are robust to a wide range of parameter values; however, we do not attempt to fit the outcome to actual epidemiological data but here are more concerned with the qualitative behaviour of the model.

**Statistical analysis.** All experiments were performed with a minimum number of 5 mice per group, and all shown results are one representative of at least three independent experiments, or the cumulative result of at least three independent experiments. In all bar graphs, bars represent average and error bars represent standard deviation. Significance is indicated by \* and its value is identified in every graph legend. For samples in which  $n > 5$ , statistical analysis was performed using unpaired Student t or variance (ANOVA) parametric tests. Normal distributions were confirmed using the Kolmogorov-Smirnov test.

## Results



## **Blood stage *Plasmodium* parasites suppress co-infection in the liver**

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**In regions of high malaria transmission, infected individuals are constantly exposed to potential re-infection. Mosquito bites transmit liver-tropic sporozoites into subjects who already have blood-stage parasitaemia. How these two stages of the *Plasmodium* life-cycle interact within their host is unknown. Here, we show that ongoing blood stage infections in a rodent model impair the growth of subsequently inoculated sporozoites. Secondary infections are arrested in liver hepatocytes and fail to compete for colonization of red blood cells. This protection of the erythrocyte niche only occurs beyond a certain threshold of blood parasite density. Parasitized erythrocytes induce expression of the host iron regulatory hormone hepcidin, which, by diverting iron away from hepatocytes, limits *Plasmodium* growth in the liver. We show that this effect of parasite threshold-density dependent growth inhibition could explain the epidemiological patterns of age-related risk and complexity of infections in young children.**

results

*Plasmodium* sporozoites, initially deposited in the skin by *Anopheles* female mosquitoes, subsequently travel to the liver where they infect hepatocytes. Once there, each sporozoite replicates into thousands of merozoites, which then infect red blood cells (RBCs) and cause malaria associated pathology<sup>58,207</sup>. In human malaria, apart from hypnozoites, hepatocytes remain infected for one to two weeks<sup>58</sup>, whereas infection of RBCs continues for months<sup>188</sup>. We asked what the impact would be of an ongoing blood stage infection on a subsequent *Plasmodium* sporozoite liver infection. We infected mice via mosquito bite with a wild-type *P. berghei* parasite and as soon as parasites appeared in the bloodstream (peripheral blood parasitemia  $0.99 \pm 0.03\%$ ), we challenged the mice by mosquito bite of a luciferase-expressing *P. berghei*. Liver stage development was monitored relative to controls by light emission from the liver 40h later and found to be dramatically inhibited (Fig. 1A). Identical results were obtained if mice were first infected with blood stage parasites and later challenged via mosquito bite with luciferase expressing *P. berghei* (Fig. 1B) and most strikingly, luciferase-labelled parasites never reached detectable levels in the blood (Fig. 1C). To quantify this effect we challenged infected mice with sporozoites constitutively expressing *gfp* and used qRT-PCR of *gfp* mRNA as readout (fig. S1). A potent and significant reduction in *P. berghei* liver load was confirmed in mice with an ongoing blood stage infection, when compared to naïve controls (Fig. 1D). Once again, no *gfp*-expressing parasites were ever found in blood (Fig. 1E). Mice carrying blood stage infections with *P. berghei* NK65, *P. berghei* ANKA, *P. chabaudi chabaudi* AS or *P. yoelii* 17X parasites and later challenged with *gfp*-expressing *P. berghei* ANKA sporozoites, all showed a very strong reduction in *Plasmodium* liver infection when compared with control mice ( $P < 0.01$ ; Fig. 1D). Likewise, *P. berghei* NK65 blood stage infection caused a strong reduction in liver

infection by *gfp*-expressing *P. yoelii* 17X sporozoites ( $P<0.05$ ; Fig. 1F). This effect was thus neither species- nor strain-specific.

Besides, this decrease in liver parasite load was consistently observed from day 3 to day 15, regardless of differences in peripheral blood parasitaemia ( $1.24\pm 0.24 - 15.53\pm 3.80\%$ ;  $P<0.05$ ; Fig. 1G). However, if an ongoing blood stage infection was treated with the antimalarial drug chloroquine then inhibition of liver stage development was abrogated (Fig. 1H).

To investigate in more detail the nature of the inhibition we examined thick liver sections from control and re-infected mice and observed that both the number and size of exoerythrocytic forms (EEFs) were decreased in the presence of blood stage parasites. (Fig. 2A-D). No EEFs were present 72hr after sporozoite infection in mice with a blood stage infection (fig. S2).

We thus conclude from these data that the inhibition of EEF development is independent of species, transient and results in very small EEFs that disappear and never give rise to blood stage infection. The data are consistent with a critical density of blood stage parasites inhibiting the development of the liver stage, resembling a quorum sensing mechanism described for bacteria <sup>208</sup>, by which the blood stage is able to protect its niche from the threat of superinfection. What then is the mechanism by which this occurs?

We ruled out the involvement of a soluble factor secreted in the blood stage, since infection of both mouse primary hepatocytes and hepatoma cells by *P. berghei* sporozoites is not affected when co-cultured with iRBCs (fig. S3). In addition and contrary to a previous report <sup>209</sup>, we found that sporozoite infection was similar in primary hepatocytes isolated either from blood-infected or naïve mice (fig. S3). We next examined the differences in liver stage transcription between naïve mice (NI), sporozoite infected mice (LS),

mice carrying a blood stage infection alone (BS) or after secondary sporozoite infection (Reinf) by genome wide microarray analysis. Analysis of these data showed first that immune-related genes were upregulated in BS or Reinf mice, and that genes associated with a defense response were upregulated in the latter (fig. S4 and table S1). We therefore hypothesized that impaired liver stage development could be caused by a blood stage initiated immune response. However, using either splenectomised mice, genetically deficient mice or depletion methods, we showed that the impairment of *Plasmodium* liver infection is independent of T or B cells; liver macrophages, NK or  $\gamma\delta$  T cells; IFN- $\gamma$ , TNF- $\alpha$ , NOS, IL-10, IL-6, TLR/MyD88 signalling; the complement system or mastocytes (Fig. 3A and fig. S5). This set of experiments provided no support for a role of adaptive immunity or aspects of innate immunity in the phenotype observed.

*Plasmodium* blood stages increase apoptosis in hepatocytes <sup>210</sup> and our microarray data showed a robust pro-apoptotic stimulus in the livers of mice with an ongoing blood stage infection (highlighted in table S1). However, although a significantly reduced level of apoptosis was observed in *P. berghei* NK65 blood stage infected caspase-3 deficient mice (fig. S6), blood stage infection in these mice still impairs a secondary *P. berghei* ANKA liver infection ( $P<0.05$ ; Fig. 3B). Thus, hepatocyte apoptosis does not explain the observed protection.

The fact that both the number and size of EEF are reduced in the presence of a blood infection (Fig. 2) suggested that some nutrient or growth factor might be limiting liver stage development. Our microarray data showed that *hamp*, the gene encoding the iron regulatory hormone hepcidin, is significantly overexpressed in mouse livers with ongoing blood stage infection (2.7-fold increase in Reinf,  $P<0.05$ , highlighted in table S1). To confirm this, we compared *hamp* expression levels in mice infected with

*Plasmodium* sporozoites and in re-infected mice. Mice with an ongoing blood stage infection show a 7-fold increase in *hamp* expression levels, when compared to controls ( $P<0.05$ ; Fig. 3C), consistent with previous findings of increased hepcidin in human blood stage infection<sup>211,212</sup>. Hepcidin regulates iron homeostasis by triggering the degradation of the iron exporter protein ferroportin, which is strongly expressed by enterocytes and macrophages<sup>213,214</sup>. We found that increased *hamp* expression during blood stage infection re-distributes iron, promoting its sequestration inside macrophages in the liver (Fig. 3E) and in the spleen (Fig. 3F) and that should happen in enterocytes as well. In addition, the level of iron in hepatocytes is reduced (Fig. 3G). The increase in hepcidin is immediately reversed to normal levels after treatment of infected mice with chloroquine ( $P<0.05$ ; Fig. 3D), which also allows the liver stage infection to grow (Fig. 1F).

Previous reports make a connection between iron availability and *Plasmodium* infection of both red blood cells<sup>215</sup> and hepatocytes.<sup>216-218</sup> In addition, a recently performed microarray analysis shows that, in *Plasmodium*-infected hepatoma cells, while the expression of the iron exporter protein ferroportin is significantly reduced ( $47.0\pm 0.2\%$  reduction,  $P<0.0001$ ), that of divalent metal transporter 1 (DMT1) is significantly increased ( $2.0\pm 0.2$  fold increase,  $P<0.0001$ )<sup>66</sup>. This suggests that iron acquisition and retention might be essential for complete *Plasmodium* development inside its host cells. Reducing iron availability (using iron chelators bathophenanthrolinedisulfonate (BPS) and desferrioxamine (DFO)) in host cells led to a significant and dose-dependent reduction of *Plasmodium* EEF development (Fig. 3G, H and fig. S7) whereas iron supplementation (using Ferric Ammonium Citrate (FAC)) increased development (Fig. 3H and I and fig. S7). These effects can be reproduced in mice infected with *P. berghei* sporozoites (Fig. 3J). Most importantly,

hepcidin *per se* (introduced by the administration of *hamp*-expressing adenovirus to mice), prior to sporozoite infection, can on its own reduce significantly liver stage infection (Fig. 3K).

Altogether, our data shows that an ongoing blood stage infection leads to a rapid increase in liver hepcidin levels, which results in a redistribution of iron in infected mice. This limits iron availability necessary for *Plasmodium* EEF development/replication inside hepatocytes.

What are the possible implications of our findings for human malaria? Epidemiological studies from highly endemic areas consistently show that the incidence of infection first increases with age in young children before it declines presumably as a result of acquired immunity (e.g. <sup>175,183</sup>). At the same time, the complexity of infection, i.e. the average number of clones per infection, also increases as hosts get older <sup>175,181-183</sup>. A previous attempt to explain these observations relied on the biting preference of mosquitoes towards older individuals <sup>219</sup>. Here we propose an alternative solution depending only on a threshold-density dependent inhibitory effect reported in this work. We devised a simple agent-based model to simulate infection histories in a number of individuals which we followed over time whilst recording the average annual infection rates as well as the number of co-infecting clones (see Materials and Methods). According to epidemiological observations the model assumes that the average parasite density per infection decreases with age <sup>175, 181, 205</sup>. The probability of an infectious bite leading to a new infection then depends on the level of a current blood-stage infection as well as the host's infection history. Under these minimal assumptions the model correctly predicts an initial increase in infection rates, which subsequently declines as individuals acquire immune protection through repeated exposure (Fig. 4A and C). As a result of a higher risk of infection and reduced inhibitory effect in older individuals we also

observe an increase in the multiplicity of infection (Fig. 4B and D). We tested the model under various assumptions regarding the relationship between an ongoing blood-stage infection and the probability of superinfection and find that no other inhibitory effect (including acquired immunity) can explain the observed data (Fig. 4A and B). This means that threshold-density dependent inhibition of new liver-stage infections alone can account for the increase in infection risk and complexity of infections in young individuals. Crucially, however, our results show that this effect is strongly dependent on the transmission intensity and most prominently observed under moderate to high transmission settings (Fig. 4C and D). Our findings therefore provide an explanation for some of the differences in age-dependent risk of infection within the endemicity spectrum. A recent clinical trial of iron supplements to children under five years of age showed a statistically significant increase in the number of malaria infections in those receiving iron <sup>220</sup>. It may be that altered iron availability due to dietary factors could also modify the threshold-density dependent inhibitory effect modelled in Fig. 4.

The sophisticated lifecycle of *Plasmodium* has resulted from millions of years of parasite/host co-evolution and communication, having broad implications for evolution and human health. *Plasmodium* infection of RBCs, above a critical threshold, leads to an increase in the level of the host hormone hepcidin that, by re-distributing iron, protects the parasite niche (and thus the host) from the risk of superinfection. This phenomenon, which acts independently of and in addition to acquired immunity, explains many previous epidemiological observations and has important implications for future anti-malarial interventions.



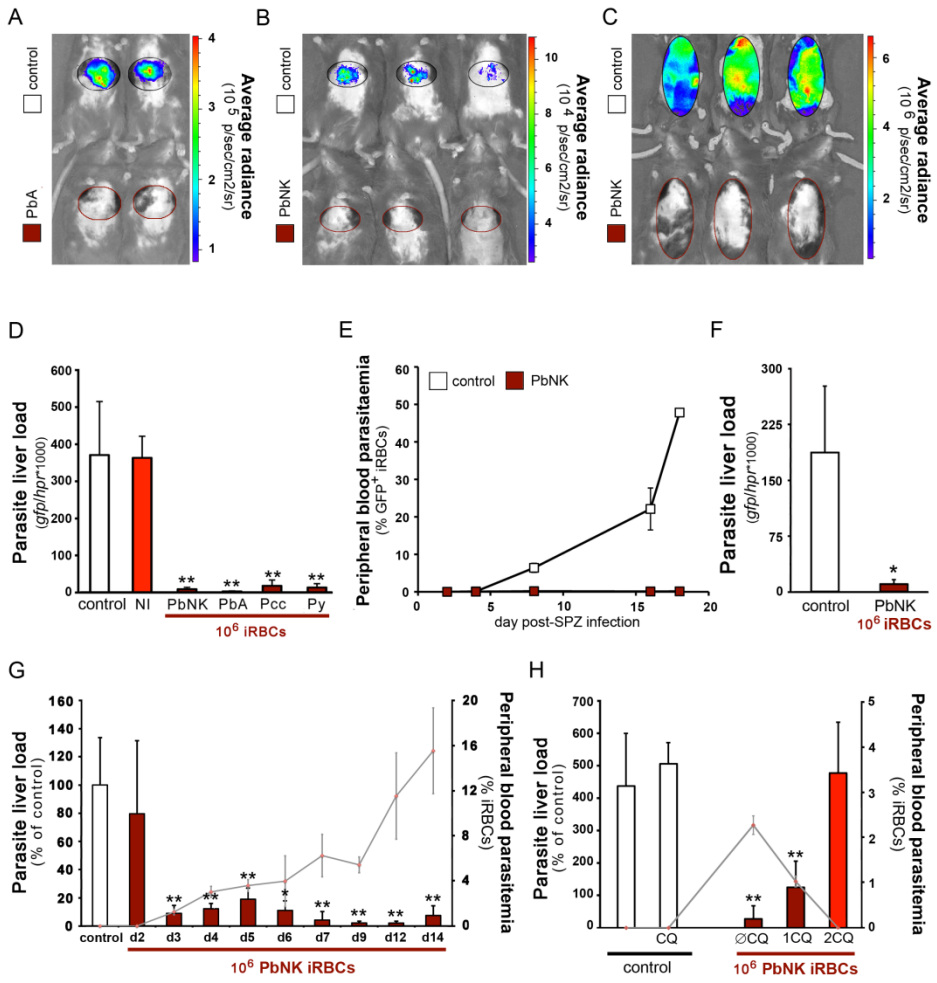
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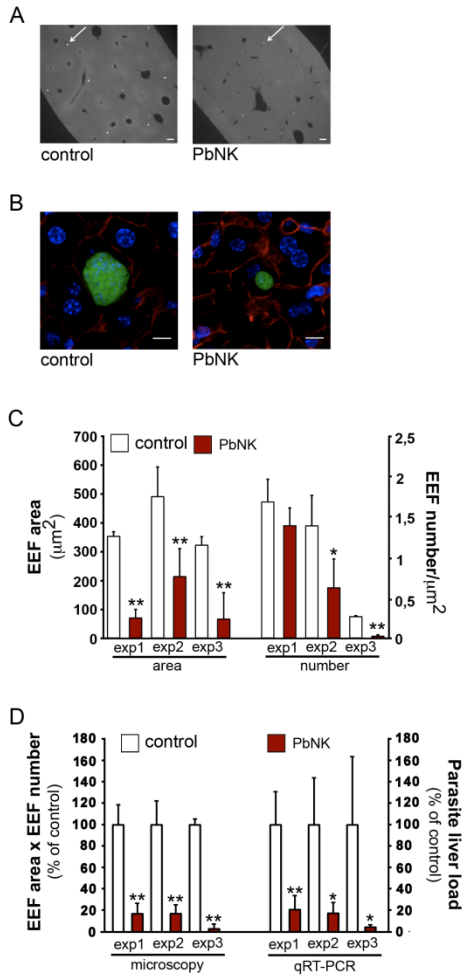
**Fig. 1. Malaria blood-stage infection confers protection against a secondary *Plasmodium* liver infection.** (A) *Plasmodium* liver load measured by radiance signal 40h post-infection by mosquito bite of 15 *Anopheles* mosquitoes infected with luciferase-expressing *P. berghei* sporozoites in the livers of naïve mice (control) and mice that had been infected 6 days previously by mosquito bite of 15 *Anopheles* mosquitoes infected with *P. berghei* ANKA (PbA), imaged in the IVIS® Lumina Imaging System, 5-10 minutes after injecting luciferin into mice. (B) *Plasmodium* liver load measured by radiance signal 40h post-infection by mosquito bite of 15 *Anopheles* mosquitoes infected with luciferase-expressing *P. berghei* sporozoites in the livers of naïve mice (control) and mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK), imaged in the IVIS® Lumina Imaging System, 5-10 minutes after injecting luciferin into mice. (C) *Plasmodium* load measured by radiance signal in the whole body of naïve mice (control) and mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK), 5 days post infection by mosquito bite of 15 *Anopheles* mosquitoes infected with luciferase-expressing *P. berghei* sporozoites, imaged in the IVIS® Lumina Imaging System, 5-10 minutes after injecting luciferin into mice. (D) *Plasmodium* liver load 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), mice transfused 5 days previously with non-infected blood (NI) or mice infected 5 days previously with  $10^6$  *P. berghei* ANKA (PbA), *P. berghei* NK65 (PbNK), *P. chabaudi* AS (Pcc) or *P. yoelii* 17X (Py) iRBCs respectively. (E) Peripheral blood parasitaemia after injection of  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite into naïve mice (control) and mice infected 5 days previously with  $10^6$  *P. berghei* NK65 (PbNK), measured by FACS, counting only GFP positive RBCs. (F) *Plasmodium* liver load 40h post  $5 \times 10^4$  *gfp*-expressing *P. yoelii* 17X sporozoite injection into naïve mice (control) or mice infected 5 days previously with  $10^6$  *P. berghei* NK65 (PbNK) iRBCs. (G) *Plasmodium* liver load (bars) 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), and mice infected 2-14 days previously with  $10^6$  *P. berghei* NK65 iRBCs; gray line represents the peripheral blood parasitaemia at the time of re-infection by *Plasmodium* sporozoites. (H) *Plasmodium* liver load 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), mice treated with chloroquine for 2 days before sporozoite injection (CQ control), mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs (CQ PbNK), or mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs and treated with chloroquine for 1 or 2 days before *Plasmodium* sporozoite injection (d1CQ or d2CQ respectively); gray line represents the peripheral blood parasitaemia at the time of re-infection by *Plasmodium* sporozoites. (\* $P < 0.05$ ; \*\* $P < 0.01$ , Ttest). Results are expressed as the mean  $\pm$  s.d. of 3 independent infections each counting with a minimum of 5 mice per group.

Fig. 1



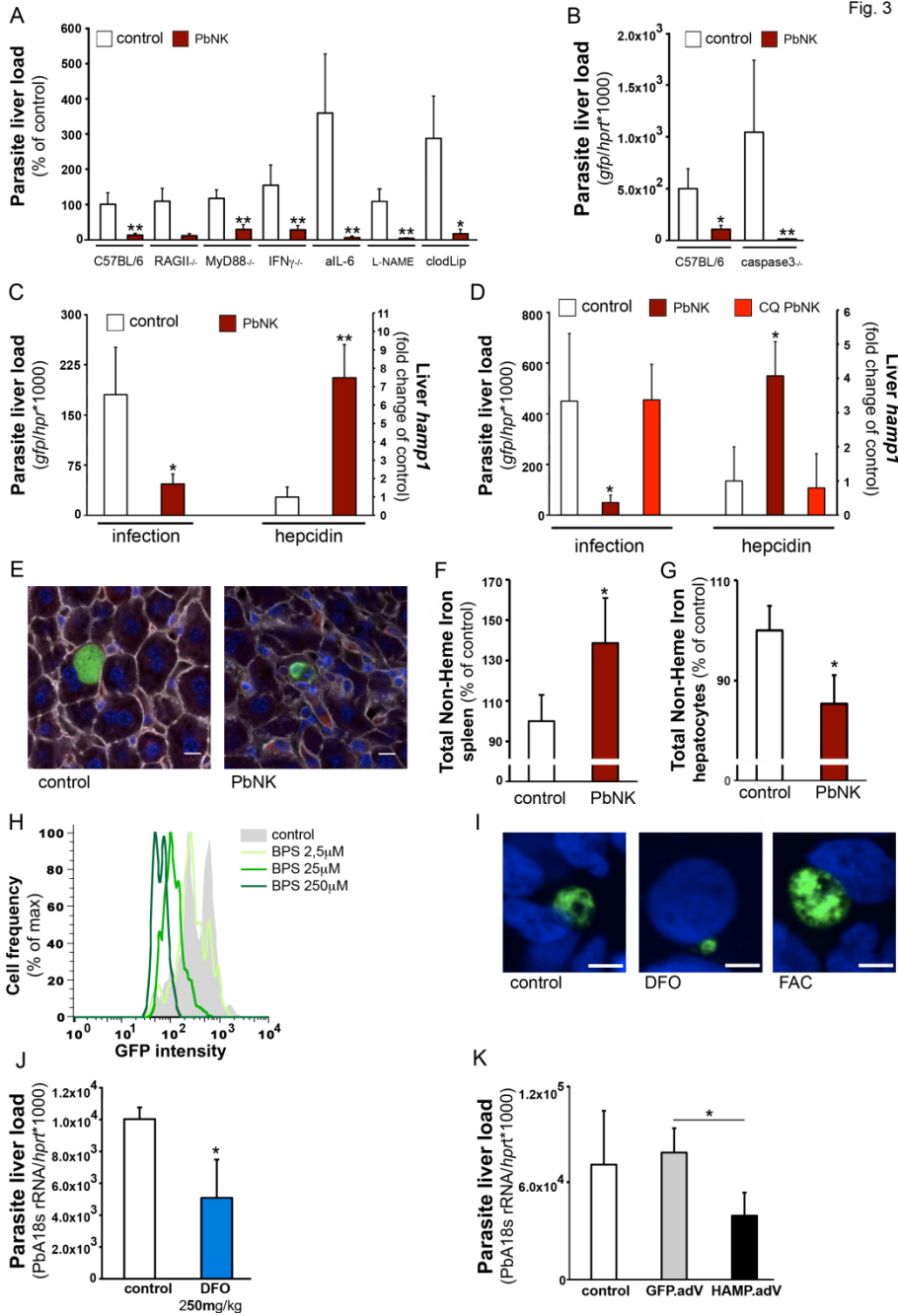
**Fig. 2. Ongoing malaria-blood stage infection causes reduction in the number and development of *Plasmodium* EEFs in hepatocytes.** (A) Fifty  $\mu\text{m}$  liver sections, 40h after *P. berghei* ANKA-GFP infection of naïve mice (control) and mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK); EEFs shown as white spots exemplified by arrows. Bar =  $100\mu\text{m}$ . (B) Representative EEF in liver sections of naïve mice (control) or mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK), 40h post  $5 \times 10^4$  *P. berghei* ANKA- *gfp*-expressing sporozoite infection. (Blue = DNA, red = polymerized actin, and green = GFP (EEF); Bar =  $10\mu\text{m}$ .). (C) *P. berghei* ANKA-GFP size (area) and density (number) in liver sections of naïve mice (control) or mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK), 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite infection, in three independent experiments. (D) Comparison of microscopy and qRT-PCR quantification of infection in the liver 40h post  $5 \times 10^4$  *P. berghei* ANKA-*gfp*-expressing sporozoite injection in naïve mice (control) or mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK), in three independent experiments. (\* $P < 0.05$ ; \*\* $P < 0.01$ , Ttest). Results are expressed as the mean  $\pm$  s.d. of 3 independent infections each counting with a minimum of 5 mice per group.

Fig. 2



**Fig. 3. An increase in hepcidin levels during blood stage infection leads to a redistribution of iron in infected mice, which restricts *Plasmodium* sporozoite development/replication.** (A) *Plasmodium* liver load 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), and mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK), using mice with different genotypes (RAGII<sup>-/-</sup>), (MyD88<sup>-/-</sup>) and (IFN $\gamma$ <sup>-/-</sup>), after monoclonal antibody treatment (anti-IL-6), or after N $\omega$ -Nitro-L-arginine methyl ester hydrochloride (L-NAME) and clodronate liposome treatment (clodLip), all compared to wild-type C57BL/6 mice. (B) *Plasmodium* liver load 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), and mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK), using wild-type (C57BL/6) and caspase3 deficient (caspase3<sup>-/-</sup>) mice. (C) *Plasmodium* liver load (left axis), and relative hepcidin expression levels (right axis), 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control) or mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK), measured by qRT-PCR. (D) *Plasmodium* liver load (left axis), and relative hepcidin expression levels (right axis), 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK) and mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs and treated with chloroquine for 2 days before *Plasmodium* sporozoite injection (CQ PbNK), measured by qRT-PCR. (E) EEF and ferritin distribution in liver sections of naïve mice (control) or mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK), 40h post  $5 \times 10^4$  *P. berghei* ANKA- *gfp*-expressing sporozoite infection. (Blue = DNA, green = GFP (EEF), red = ferritin, white = polymerized actin; bar = 10 $\mu$ m.). (F) Spleen total non-heme iron quantification 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control) or mice previously infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK). (G) Primary hepatocyte total non-heme iron quantification of primary hepatocytes isolated from naïve mice (control) and mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK). (H) Effect of iron chelator bathophenanthrolinedisulfonate (BPS) titration on *P. berghei* ANKA-GFP EEF development inside Huh7 hepatoma cells, measured by flow cytometry 36h after sporozoite addition. (I) Representative EEFs in Huh7 hepatoma cells 36h post- $2 \times 10^4$  *P. berghei* ANKA- *gfp*-expressing sporozoite infection of untreated cells (control), cells treated 24h before infection with iron chelator Desferrioxamine (DFO) and cells treated 24h before infection with Ferric Ammonium Citrate (FAC). (Blue = DNA, and green = GFP (EEF); bar = 10 $\mu$ m.). (J) *Plasmodium* liver load 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control) or mice treated 24h previously with 250 mg/kg of iron chelator Desferrioxamine (DFO). (K) *Plasmodium* liver load 40h post  $5 \times 10^4$  *P. berghei* ANKA sporozoite injection into mice infected 48h previously with  $5 \times 10^{11}$  units of *gfp* adenovirus (GFP.adV) or  $5 \times 10^{11}$  units of *hamp* expressing adenovirus (HAMP.adV) compared with non previously infected mice (control). (\* $P$  < 0.05; \*\* $P$  < 0.01, Ttest). Results are expressed as the mean  $\pm$  s.d. of 3 independent infections each counting with a minimum of 5 mice per group.

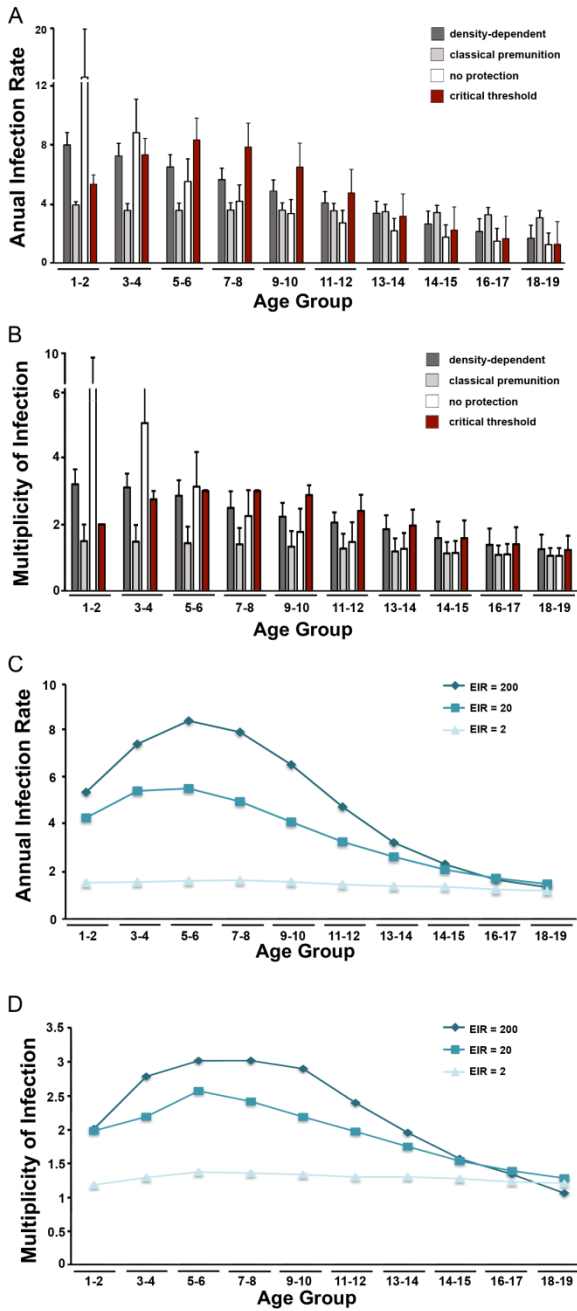
Fig. 3



**Figure 4. Model predictions for age-related incidence and multiplicity of infections.** (A and B) We tested four different assumptions regarding a possible inhibitory effect of a blood-stage infection: (i) density-dependent, where the probability of infection directly correlates with parasite density; (ii) classical premunition, where a new infection can only be established after an ongoing infection is essentially cleared; (iii) no protection, where the probability of infection is independent of an ongoing infection; and (iv) critical threshold dependent, where a new infection can only be established once the blood-stage parasite density falls below a certain level. Only the presence of a critical threshold density results in an initial increase in both infection rate and multiplicity of infection, which then decline due to acquired immunity. (C and D) The results reveal a strong dependency on transmission intensities and predict that the observed age-dependency in the rates of infection and multiplicity will be lost under low levels of transmission.

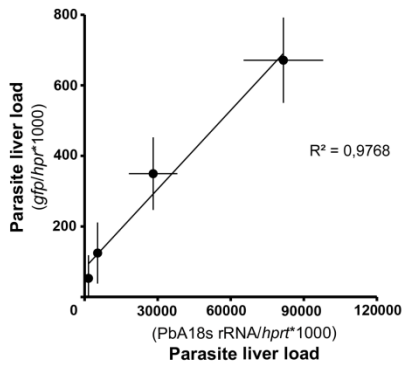


Fig. 4



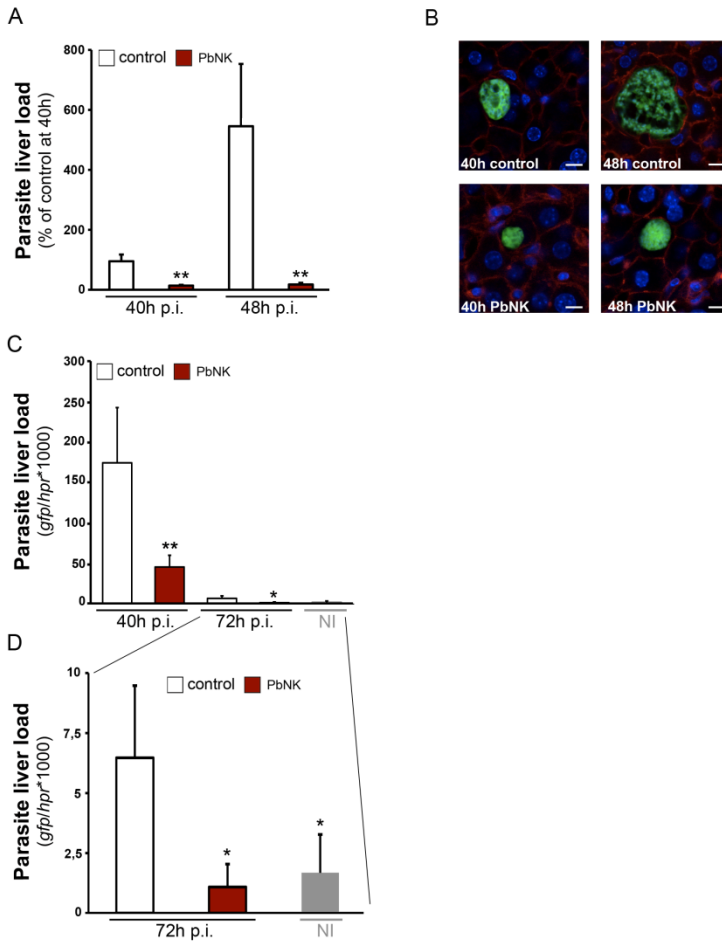
**fig. S1.** Correlation between expression levels of 18S *rRNA* and *gfp* cDNA in mouse livers, 40h post  $5 \times 10^3$ ,  $2 \times 10^4$ ,  $5 \times 10^4$  and  $1.5 \times 10^5$  *P. berghei* ANKA-GFP sporozoite injection. Dots show the average expression of 4 mice and the black line represents the positive correlation between the two parameters measured.

fig. S1



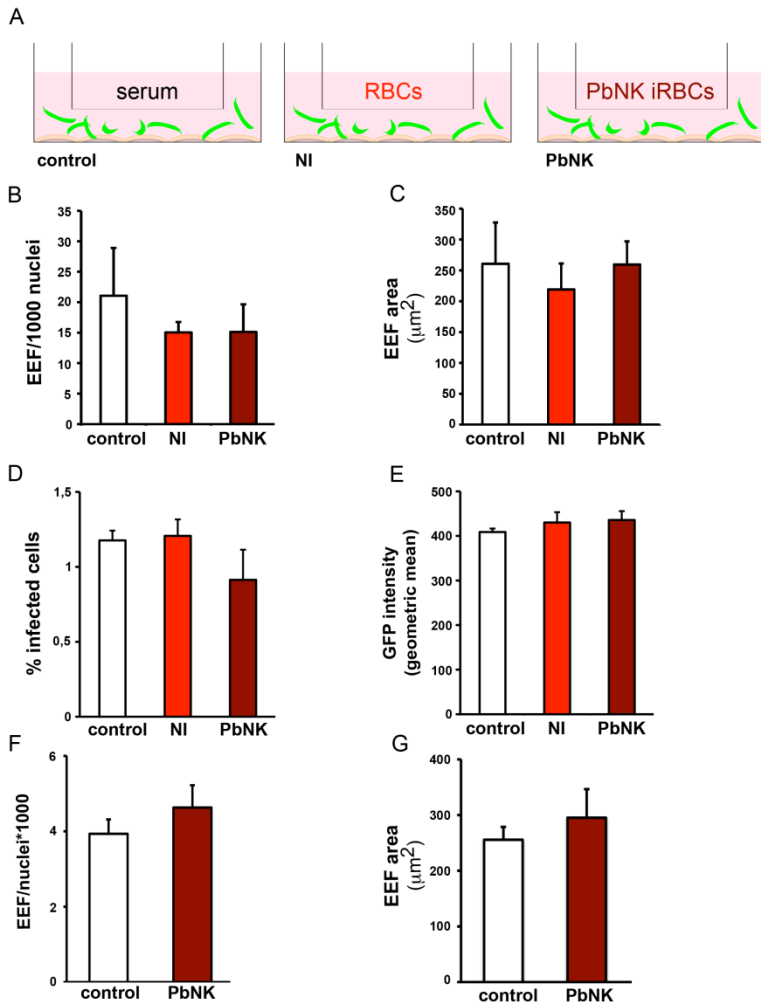
**fig. S2.** (A) *Plasmodium* liver load 40 and 48h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control) and mice infected 5 days previously with  $10^6$  *P. berghei* NK65 (PbNK). (B) Representative EEF in liver sections of naïve mice (control) or mice infected 5 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK), 40 and 48h post  $5 \times 10^4$  *P. berghei* ANKA *gfp*-expressing sporozoite infection. (Blue = DNA, red = polymerized actin, and green = GFP (EEF); Bar =  $10\mu\text{m}$ .). (C) *Plasmodium* liver load 40 and 72h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control) and mice infected 5 days previously with  $10^6$  *P. berghei* NK65 (PbNK), compared to a non-infected group (NI). (D) Magnification of 72h post infection of  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control) and mice infected 5 days previously with  $10^6$  *P. berghei* NK65 (PbNK), and non infected control group shown in (C). (\* $P < 0.05$ ; \*\* $P < 0.01$ , Ttest). Results are expressed as mean  $\pm$  s.d. of 3 independent infections each counting with a minimum of 5 mice per group.

fig. S2



**fig. S3.** (A) Effect of *Plasmodium* iRBC supernatants on sporozoite hepatocyte infection. Hepatocytes grown in the lower chamber of a transwell system where infection by sporozoites (green crescents) takes place, contacting with mouse serum (control), non-infected blood (NI) or blood containing  $6 \times 10^5$  *P. berghei* NK65 iRBCs (PbNK) within the upper chamber. (B) Sporozoite infection (% infected cells) of mouse primary hepatocytes in the three conditions described above 40h post- $2 \times 10^4$  *P. berghei* ANKA *gfp*-expressing sporozoite addition, analysed by wide field microscopy. (C) EEF development (EEF area) in mouse primary hepatocytes in the three conditions described above 40h post- $2 \times 10^4$  *P. berghei* ANKA *gfp*-expressing sporozoite addition, analysed by confocal microscopy. (D) Sporozoite infection (% infected cells) of Huh7 cells, in the three conditions described above 36h post- $2 \times 10^4$  *P. berghei* ANKA *gfp*-expressing sporozoite addition, measured by FACS. (E) EEF development (GFP intensity) in Huh7 cells, in the three conditions described above 36h post- $2 \times 10^4$  *P. berghei* ANKA *gfp*-expressing sporozoite addition, quantified by FACS. (F) Sporozoite infection (EEF/1000 nuclei) of mouse primary hepatocytes isolated from naive mice (control) and mice infected 5 to 7 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK), 48h post- $2 \times 10^4$  *P. berghei* ANKA *gfp*-expressing sporozoite addition, analysed by wide field microscopy. (G) EEF development (EEF area) in mouse primary hepatocytes isolated from naive mice (control) and mice infected 5 to 7 days previously with  $10^6$  *P. berghei* NK65 iRBCs (PbNK), 48h post- $2 \times 10^4$  *P. berghei* ANKA *gfp*-expressing sporozoite addition, analysed by confocal microscopy.

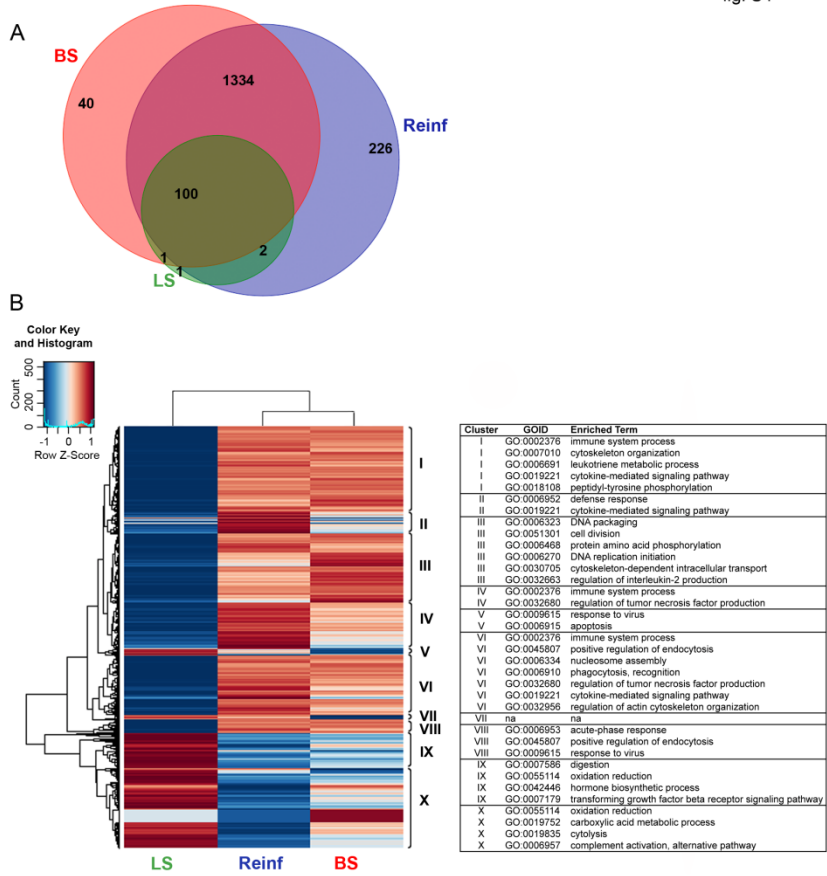
fig. S3



**fig. S4.** Microarray data analysis of liver RNA and targeting of hit molecules. **(A)** Venn diagram showing the proportion of differentially expressed (DE) transcripts found in our analysis. Numbers are indicated for each group. **(B)** Heatmap generated from the group of DE transcripts, reflecting the log ratios of each infected category compared to non-infected mice, as indicated at the bottom. Gene Ontology enrichment analysis results for each identified cluster are indicated on the table, ( $P < 0.05$ , Fisher Exact Test One Tail). (BS: blood stage, LS: liver stage, Reinf: re-infection).

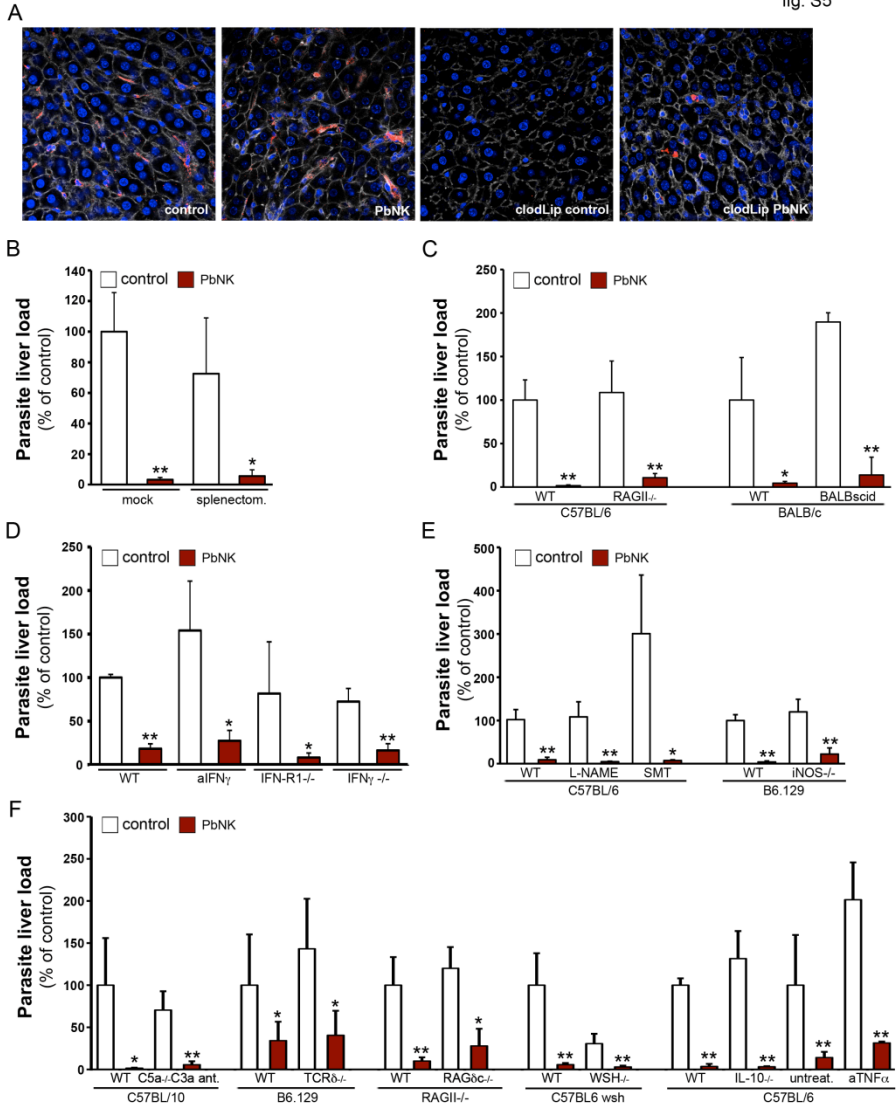


fig. S4



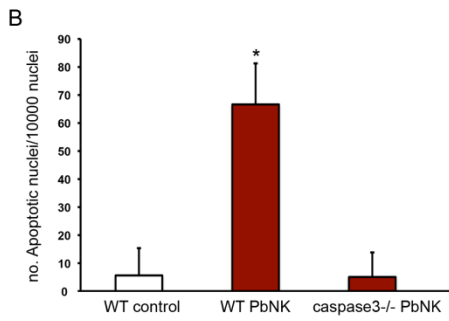
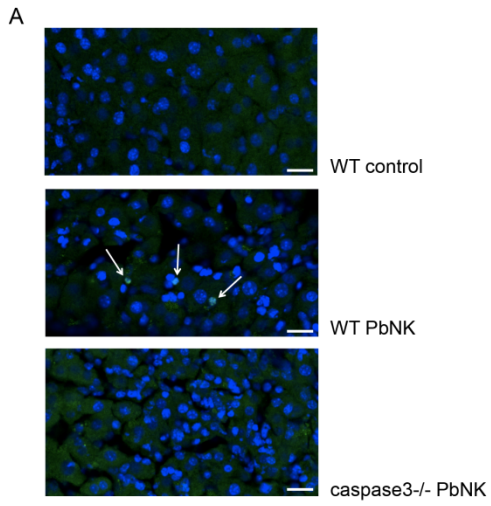
**fig. S5.** (A) Macrophage and monocyte staining in 50 $\mu$ m liver section 40h post 5 x 10<sup>4</sup> *P. berghei* ANKA *gfp*-expressing sporozoite infection, of naïve mice (control), mice 5 days previously infected with 10<sup>6</sup> iRBCs of *P. berghei* NK65 (PbNK), mice treated with clodronate liposomes 48h prior to sporozoite infection (clodLip control) and mice infected with 10<sup>6</sup> iRBCs of *P. berghei* NK65 and treated with clodronate liposomes 48h prior to sporozoite infection (clodLip PbNK), (Blue = DNA, white = polymerized actin, and red = pan macrophage marker. Bar = 10 $\mu$ m.) (B) *Plasmodium* liver load 40h post 5 x 10<sup>4</sup> *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), and mice infected 5 days previously with 10<sup>6</sup> iRBCs of *P. berghei* NK65 iRBCs (PbNK), preceded 15 days by mock surgery (mock) or splenectomy (splenectom.). (C) *Plasmodium* liver load 40h post 5 x 10<sup>4</sup> *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), and mice infected 11 days previously with 10<sup>6</sup> iRBCs of *P. berghei* NK65 iRBCs (PbNK) using RAGII deficient mice in C57BL/6 background, and naïve mice (control), and mice infected 14 days previously with 10<sup>6</sup> iRBCs of *P. berghei* NK65 iRBCs (PbNK) using BALBscid mice in BALB/c background. (D) *Plasmodium* liver load 40h post 5 x 10<sup>4</sup> *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), and mice infected 5 days previously with 10<sup>6</sup> iRBCs of *P. berghei* NK65 iRBCs (PbNK), using untreated WT mice, mice treated with IFN $\gamma$  monoclonal antibody 24h prior to sporozoite infection (aIFN $\gamma$ ), mice deficient for IFN $\gamma$  receptor 1 (IFN-R1-/-), and mice deficient for IFN $\gamma$  (IFN $\gamma$ -/-); all in C57BL/6 background. (E) *Plasmodium* liver load 40h post 5 x 10<sup>4</sup> *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), and mice infected 5 days previously with 10<sup>6</sup> iRBCs of *P. berghei* NK65 iRBCs (PbNK), using untreated mice, mice treated with L-NAME or SMT every day for the whole extent of the experiment, all mice in C57BL/6 background, and using WT mice and NOS2 deficient mice (iNOS-/-) in B6.126 background. (F) *Plasmodium* liver load 40h post 5 x 10<sup>4</sup> *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), and mice infected 5 days previously with 10<sup>6</sup> iRBCs of *P. berghei* NK65 iRBCs (PbNK), using mice with different genotypes, C5a deficient mice treated with C3a antagonist SB 290157 (C5a-/- C3a ant.) in C57BL/10 background, (TCR $\delta$ -/-) in B6.129 background, RAG $\gamma$ c deficient compared to RAGII (RAG $\gamma$ c), mastocyte deficient in (*wsh*-/-), IL-10 deficient (IL-10-/-) and after monoclonal antibody treatment (aTNF $\alpha$ ) in C57BL/6 background, all compared to respective background littermates. (\*P < 0.05, \*\*P < 0.01, Ttest) Results are expressed as the mean  $\pm$  s.d. of 3 independent infections each counting with a minimum of 5 mice per group.

fig. S5



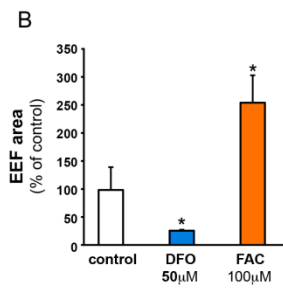
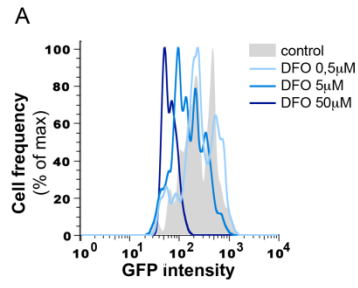
**fig. S6.** Blood stage induced apoptosis in caspase 3-deficient mice. **(A)** Apoptosis in liver sections (16 $\mu$ m) of naïve wild-type C57BL/6 mice (WT control), wild-type C57BL/6 mice infected 6 days previously with 10<sup>6</sup> *P. berghei* NK65 iRBCs (WT PbNK) and caspase3-deficient mice infected 6 days previously with 10<sup>6</sup> *P. berghei* NK65 iRBCs (caspase 3<sup>-/-</sup> PbNK). (Blue = DNA and green = apoptotic nuclei; bar =20 $\mu$ m). **(B)** Quantification of apoptotic nuclei in liver sections (16 $\mu$ m) of naïve wild-type C57BL/6 mice (WT control), wild-type C57BL/6 mice infected 6 days previously with 10<sup>6</sup> *P. berghei* NK65 iRBCs (WT PbNK) and caspase3 deficient mice infected 6 days previously with 10<sup>6</sup> *P. berghei* NK65 iRBCs (caspase 3<sup>-/-</sup> PbNK) (\*P <0.01, Ttest). Results are expressed as the mean  $\pm$  s.d. of 3 independent infections each counting with a minimum of 5 mice per group.

fig. S6



**fig. S7.** Iron availability and infection. **(A)** Effect of various concentrations of Desferrioxamine (DFO) on *P. berghei* ANKA-GFP EEF development inside Huh7 hepatoma cells, measured by flow cytometry 36h after- $2 \times 10^4$  *P. berghei* ANKA *gfp*-expressing sporozoite addition. The graphs show one representative data set of triplicate samples. **(B)** Area of EEFs inside Huh7 hepatoma cells 36h post- $2 \times 10^4$  *P. berghei* ANKA-GFP sporozoite infection of untreated cells (control), cells treated 24h before infection with DFO and cells treated 24h before infection with Ferric Ammonium Citrate (FAC), (\* $P < 0.01$ , Ttest). Results are expressed as the mean  $\pm$  s.d. of 3 independent infections, each measuring the area of 12 EEFs per group.

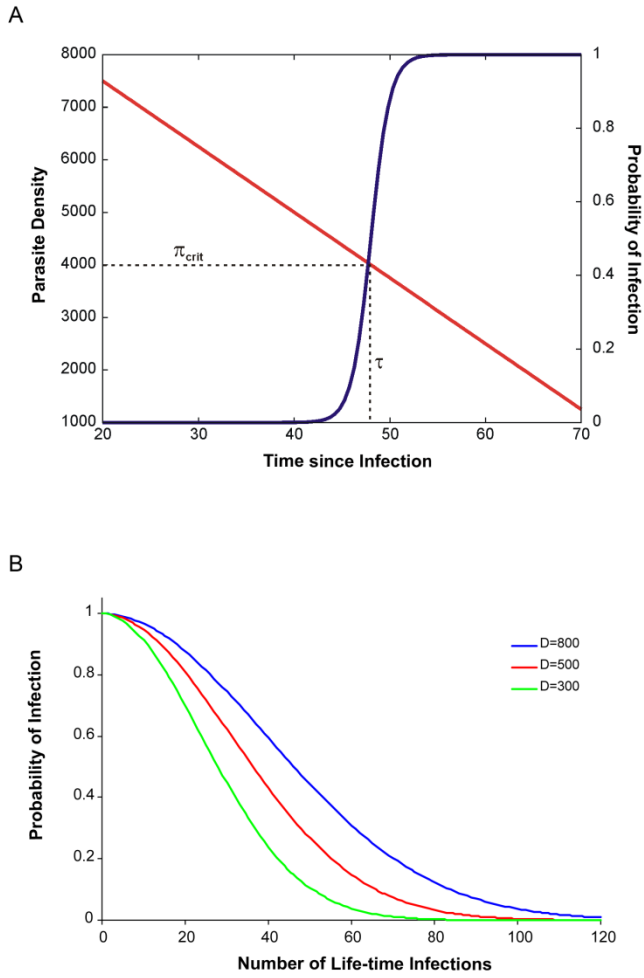
fig. S7



**fig. S8.** (A) Functional relationship between parasite density and probability of infection. As the level of parasitaemia of an ongoing infection (red line) remains above a critical threshold density,  $\pi_{\text{crit}}$ , the probability of establishing a new blood stage infection (blue line) is extremely low but significantly increases as soon as the parasitaemia level falls below this threshold. (B) Acquired immunity as a function of infection history. The probability of infection is dependent on the total number of blood-stage infections experienced by the host and  $D$ , which can generally be understood as a proxy for antigenic diversity, where greater  $D$  requires more infections to gain protection.

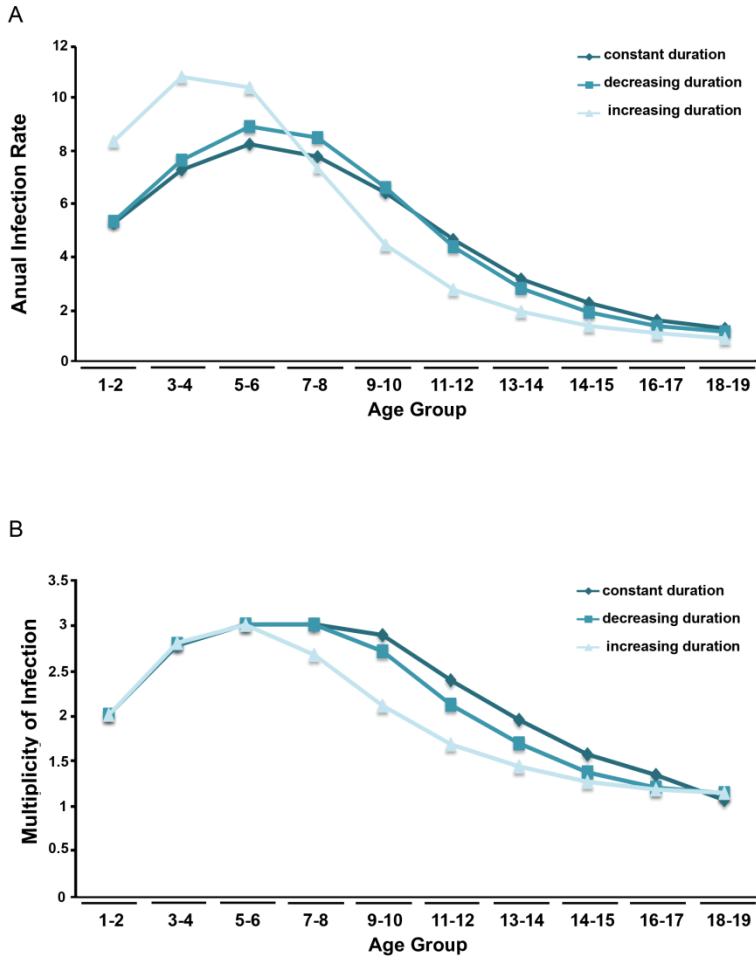


fig. S8



**fig. S9.** Model behaviour under changes in duration of infections. The model was tested under three different assumptions concerning the duration of infection: (i) constant over age ( $T=100$ ), (ii) decreasing with age ( $T=100-2*\text{age}$ ), or (iii) increasing with age ( $T=60+2*\text{age}$ ). Shown are the age-related annual infection rates (**A**) and multiplicity of infections (**B**) under these assumptions, clearly demonstrating a qualitative invariance of the model's behaviour to the underlying assumption about the period of infection.

fig. S9



results

## Additional Results

*Our data strongly points that the observed impairment of secondary liver infection in Plasmodium blood infected mice is promoted by iron redistribution in the liver induced by hepcidin upregulation. However, many other theories were investigated during the course of this work that ultimately led us to propose the iron redistribution mechanism.*

*Besides the hypothesis mentioned in the main body of the Results chapter (see Results section **Blood stage Plasmodium parasites suppress co-infection in the liver**) we have tested several others that we projected during the course of this work. The results presented here serve to further explain the conclusions drawn, and although not included in the previous section were discussed later on this thesis and are of some importance to the conclusions made.*

**Serum transfers.** Considering the possibility that *Plasmodium* molecules released by iRBCs could affect hepatocytes towards impairment of EEF development, we designed experiments to investigate if the serum of blood infected mice transferred into naïve ones, prior to sporozoite infection, would protect these mice from sporozoite challenge.

Two groups of mice were infected with  $10^6$  *P. berghei* NK65 iRBCs. One of these groups was then re-infected on day 6 post blood infection (PbNK) and the rest of the mice were sacrificed on the same day to collect blood and produce serum. Serum of every two donor mice was injected to a recipient naïf one (serumPbNK), 2 to 12h prior to  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite challenge. Expression of *gfp* was determined to assess liver infection, 40h post sporozoite injection, and compared to control naïve non-transferred mice (control).

It was never observed any effect of the transferred serum on liver infection. Indeed no reduction was observed in the mice that were injected serum

from mice with *Plasmodium* blood stage infection prior to sporozoite challenge (fig. A1).

**Cholesterolemia.** Numerous molecules involved in lipid metabolism appeared modulated in the liver microarray study performed during this thesis and described in the previous section. Of those molecules several related to cholesterol metabolism, in that context we designed experiments to address cholesterol involvement in the observed reduction of sporozoite infection in re-infected mice.

Blood and livers were collected 40h after  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite of naïve mice (control) and mice 4 days previously infected with  $10^6$  *P. berghei* NK65 iRBCs (PbNK).

Serum was then collected and total cholesterol, HDL- and LDL-cholesterol biochemically determined by DNAtch (INETI, Portugal). No significant differences in either of these values were found between control and re-infected group (fig. A2).

In addition, livers were also collected after perfusion with PBS and a section was used for quantification of cholesterol by fluorometric method with The Amplex® Red Cholesterol Kit Assay (Invitrogen). No significant difference was observed in cholesterol levels control and re-infected mice (fig. A2).

***Toxoplasma* infection effect over *Plasmodium* sporozoite liver infection.** In order to address the dependence and specificity of *Plasmodium* blood stage infection to impose protection from secondary liver challenge by sporozoites, we substituted the primary blood infection by an infection of a related parasite.

Mice bearing a 4-day infection with  $10^4$  *Toxoplasma gondii* tachizoites (TOXO) or  $10^6$  *P. berghei* NK65 iRBCs (PbNK) were challenged with  $5 \times 10^4$  *P. berghei* ANKA sporozoites, in parallel with naïve mice (control).

*Plasmodium* liver infection was monitored 40h post-sporozoite infection by qRT-PCR.

A strong and similar reduction of *Plasmodium* liver infection was found in all previous infected mice compared to naïve ones, regardless of the parasite used for primary infection (fig. A3).

Hepcidin quantification was performed to these same samples and a 4-fold increase was observed in all previous infected mice compared to naïve ones (fig. A3).

*All these results are also considered in the general discussion of this thesis.*

### **Effect of secondary sporozoite infection over primary blood infection.**

These experiments aimed at further investigating possible interactions of *Plasmodium* blood and liver stages within a single host. We verified what would be the impact of the secondary sporozoite infection over the primary blood stage infection already established at the time of re-infection.

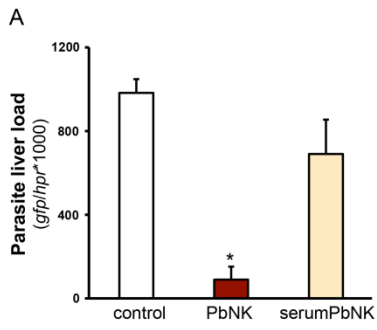
After  $5 \times 10^4$  *P. berghei* *gfp*-expressing sporozoite infection of mice 4 days previously infected with  $10^6$  *P. berghei* NK65 iRBCs (Reinf), blood parasitemias were followed by Giemsa staining of thin blood films. Non-reinfected mice 4 days previously infected with *P. berghei* NK65 infected were used as a control group (BS). While non-reinfected mice (BS) died of hyper-parasitemia on day 18 post- *P. berghei* NK65 iRBCs infection, re-infected mice showed increasing parasitaemias until day 20 post- *P. berghei* NK65 iRBCs infection, time when peripheral blood parasites began to decrease and reach zero between days 30 and 35 post infection. Parasitaemias were kept undetectable until day fifty post infection, when all surviving mice were sacrificed by cervical dislocation (fig. A4).

*These last results are considered in The next in line section where future perspectives are proposed.*

**fig. A1.** *Plasmodium* liver load 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), mice infected 5 days previously with  $10^6$  iRBCs of *P. berghei* NK65 iRBCs (PbNK), and mice injected serum collected from mice infected 5 days previously with  $10^6$  iRBCs of *P. berghei* NK65 iRBCs, 6h prior to sporozoite infection (serumPbNK). (\* $P < 0.01$ , Ttest) Results are expressed as the mean  $\pm$  s.d. of 3 independent infections each counting with a minimum of 5 mice per group.

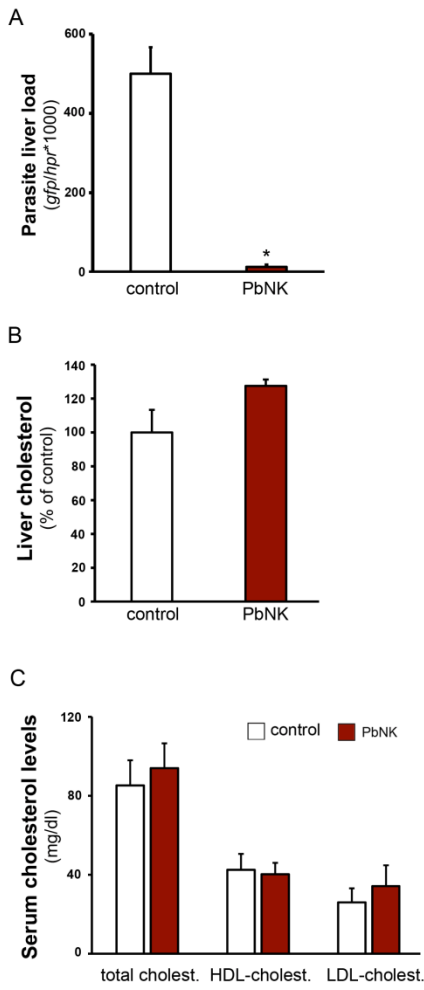


fig. A1



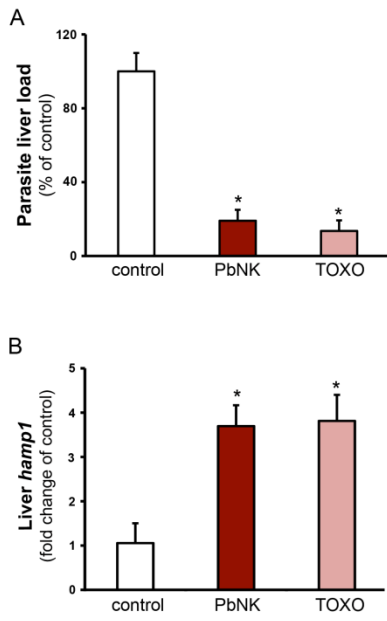
**fig. A2.** (A) *Plasmodium* liver load 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), and mice infected 5 days previously with  $10^6$  iRBCs of *P. berghei* NK65 iRBCs (PbNK). (B) Liver cholesterol quantification 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), and mice infected 5 days previously with  $10^6$  iRBCs of *P. berghei* NK65 iRBCs (PbNK) measured by a fluorometric method with The Amplex® Red Cholesterol Kit Assay. (C) Serum cholesterol quantification 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), and mice infected 5 days previously with  $10^6$  iRBCs of *P. berghei* NK65 iRBCs (PbNK) by a biochemically method performed at DNAtch (INETI, Portugal) measuring total cholesterol, HDL- and LDL-cholesterol respectively. Results are expressed as the mean  $\pm$  s.d. of 3 independent infections each counting with a minimum of 5 mice per group.

fig. A2



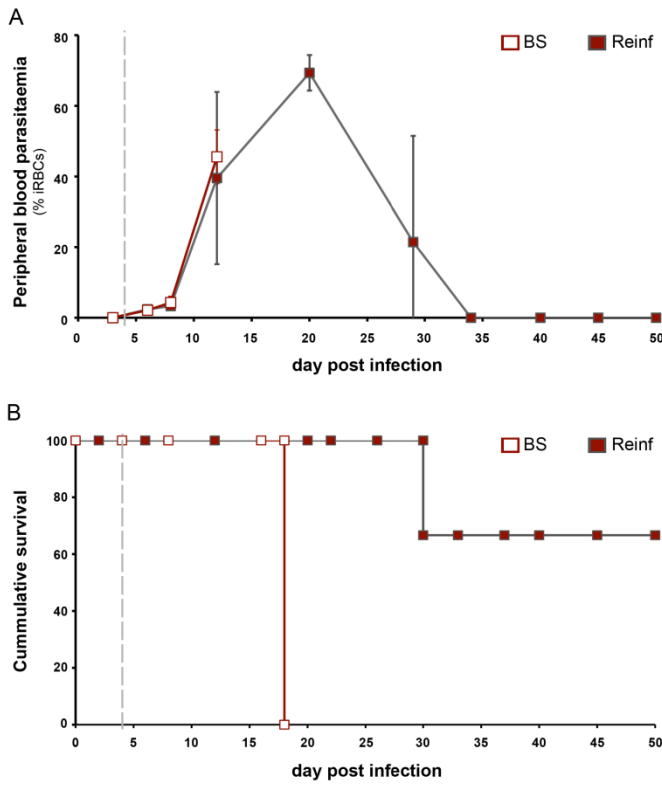
**fig. A3.** (A) *Plasmodium* liver load 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), mice infected 4 days previously with  $10^6$  iRBCs of *P. berghei* NK65 iRBCs (PbNK), and mice infected 4 days previously with  $10^4$  tachizoites of *Toxoplasma gondii* (TOXO), measured by qRT-PCR. (B) Relative hepcidin expression levels in the liver 40h post  $5 \times 10^4$  *P. berghei* ANKA-GFP sporozoite injection into naïve mice (control), mice infected 4 days previously with  $10^6$  iRBCs of *P. berghei* NK65 iRBCs (PbNK), or mice infected 4 days previously with  $10^4$  tachizoites of *Toxoplasma gondii* (TOXO), measured by qRT-PCR. (\* $P < 0.01$ , Ttest) Results are expressed as the mean  $\pm$  s.d. of 3 independent infections each counting with a minimum of 5 mice per group.

fig. A3



**fig. A4.** (A) Peripheral blood parasitaemia after infection with  $10^6$  iRBCs of *P. berghei* NK65 of non-re-infected mice (BS) and mice re-infected 4 days later with  $5 \times 10^4$  *P. berghei* ANKA *gfp*-expressing sporozoite (Reinf), light grey line shows the time when re-infection occurred. (B) Survival curve after infection with  $10^6$  iRBCs of *P. berghei* NK65 of non-re-infected mice (BS) and mice re-infected 4 days later with  $5 \times 10^4$  *P. berghei* ANKA *gfp*-expressing sporozoite (Reinf), light grey intermitent line shows the time when re-infection occurred. Results represent 1 of 3 independent infections counting with a minimum of 3 mice per group.

fig. A4







## Discussion



## General discussion and conclusions

*Although malaria infection is still a major scourge throughout the world, globally extraordinary moves are being made to tackle malaria, involving research, governments, funding agencies and donors, private companies, and civil society towards the common goal of malaria eradication, proposed by Melinda Gates in 2007.*

*In the 1950's, during the first Global Malaria Eradication Program, there was a significant decline in the number of areas at risk of Plasmodium transmission <sup>221</sup>. Later, with the emergence of both drug and insecticide resistance in parasite and vector respectively, there was a rebound in the regions where final elimination was not achieved <sup>222</sup>.*

*Nowadays, with renewed efforts to fight Plasmodium infection, some goals are being accomplished. Indeed, the latest Malaria Report from WHO states that the number of deaths in Africa decreased by 34000 since the previous report one year before. However, this reduction was primarily attributed to a reduction in the total number of deaths from all causes among children under 5 years of age, mostly as a result of the profound effect on health systems that so many interventions produce <sup>8</sup>. Importantly, the most promising vaccine candidate so far, RTS,S/AS01 has shown around 50% efficacy against clinical malaria disease in a phase III trial in 5 to 17 months old children <sup>223</sup>, which although very positive, is still far from the level required to achieve eradication.*

*It remains of great importance to learn more about the biology of parasite-host interactions, the determinants of endemicity and transmission dynamics, the social, economic and cultural implications of malaria at household, community and national levels, and the demands made upon health systems in endemic countries. Acknowledging the present situation and increasing our*

*understanding of the biological features of Plasmodium infection will allow the development of better future control or eradication strategies.*

*Plasmodium blood stage of infection is the primary target of most therapeutic interventions. New insights on its biology and biochemical pathways provide new ways to target the apicoplast organelles, the invasion or egress processes, the remodelling of the iRBC, or aim at a blood stage vaccine. On the other hand, the liver stage has not been as extensively studied, and as such new discoveries might offer targets for new drugs and vaccine development.*

*The aim of the present thesis was to analyse potential interactions between the blood and liver stages of the Plasmodium parasite within a single host. Indeed, the sequence of events from mosquito salivary glands towards man's liver and, later, to the blood are likely to be perturbed in high transmission areas, with new infections occurring in individuals harbouring blood-stage infections. Understanding possible host-pathogen interactions can lead into new insights on Plasmodium liver infection, as well as enabling better comprehending of the heterogeneity amongst malaria transmissions when extrapolating to human malaria. The study presented in this thesis focused on the effect of Plasmodium iRBCs on the establishment and development of a secondary infection of the liver of a murine model by Plasmodium sporozoites, seeking to clarify inter-stage interactions within a single host. What would the impact of such interactions for the re-infected individual be? Furthermore, assuming similar interactions occurred in human malaria, what would their influence at the population-level in regions where transmission rates impose Plasmodium re-infection be?*

*Not much research has been done on the interactions of the two stages of Plasmodium infection occurring within the mammalian host, although one early attempt of addressing this question was initiated in 1939 in a human study <sup>224</sup>. Later, while one mouse study proposes the existence of cross stage protection of blood over liver stage, without existing concomitant infection <sup>169</sup>,*

a distinct study indicates that *Plasmodium* blood stage infection has an immuno-suppressive effect over the establishment of CD8 mediated immunity against sporozoite infection in mice immunized with irradiated sporozoites<sup>189</sup>. Also, a previous report observes the influence of blood stage *Plasmodium* on sporozoite infection of mouse primary hepatocytes in vitro<sup>209</sup>.

Facing the limitation of few publications of previous related work, the present thesis attempted to discuss the obtained results in the light of numerous observations made for each of the two independent stages of *Plasmodium* infection.

**Malaria Superinfections.** In its endemic settings, *Plasmodium* may often co-exist with other pathogens within the same host. Different pathogens can lead to different outcomes of plasmodial infection, some being part of an antagonistic and others a synergistic interaction with *Plasmodium* (reviewed in<sup>225</sup>). Nevertheless, in regions of high malaria endemicity, a very likely co-infection is unquestionably malaria.

Several reports of *Plasmodium* superinfection describe single individuals hosting more than one *Plasmodium* species, or different genotypes of the same *Plasmodium* species infecting RBCs<sup>177-179</sup>. These mixed infections can be originated from a single infectious bite from one mosquito infected with more than one *Plasmodium* species or genotype, or from consecutive infectious bites<sup>226</sup>. Still, it is well established that superinfection occurs more often in areas of high transmission and, generally superinfections are more frequently seen in older children than in younger ones<sup>175,181-183</sup>.

A number of studies focusing on mixed *Plasmodium* infections, both in human populations and rodent models often present ambiguous results<sup>179</sup>. Nonetheless, common themes discussed are the suppression of mixed infections where one blood parasite will prevail over the others, the oscillatory peaks of infection with no coincidence of the species, or the

general proposal that *P. vivax* helps reduce the severity of *P. falciparum* malaria<sup>179,227,228</sup>. Indeed, until now, studies concerning superinfection have concentrated on interactions between different genotypes or species of *Plasmodium* infecting RBCs. The fact is, however, that in an endemic area any infection starts by an infectious bite, which likely often occurs in individuals already bearing *Plasmodium* blood stage parasites from a previous infection. This event presents an opportunity for interactions between the two stages of *Plasmodium*, inside the same host.

**Observations.** In order to distinguish *Plasmodium* blood and liver stage parasites, in a single mouse we made use of parasites genetically tagged with GFP or luciferase expression for the sporozoite secondary infection and non-tagged parasites for the primary blood infection.

The present thesis shows that the presence of *Plasmodium* peripheral blood parasitaemia above a certain threshold protects mice from secondary liver infection. Regardless of whether blood stage infection was initiated by blood transfusion or by mosquito bite, a reduction in liver stage infection was always observed in re-infected mice. Regardless of the strain or species of *Plasmodium* used for primary infection, secondary sporozoite infection was always impaired in the liver. Furthermore, this impaired secondary liver infection never produced visible peripheral blood parasitemia, again regardless of the species of *Plasmodium* used for secondary infection and of the number of sporozoites inoculated, either by mosquito bites or through intra-venous injection. These results prompted a number of questions concerning the mechanism leading to this inhibition and its consequences for the individual and the population.

More specific investigations of the phenotype revealed that both the number and the size of the developing liver stages or EEFs were decreased in re-infected mouse livers. The reduction of secondary liver infection was

also dependent on the presence of iRBCs at the time of re-infection. Clearance of RBCs with an anti-malarial drug prior to re-infection abolished the impairment of secondary infection. Moreover, the decrease in liver parasite load was consistently observed from the third day post-blood stage infection, in spite of differences in peripheral blood parasitaemia, indicating that the reduction mechanism was initiated beyond a threshold of iRBCs that was rapidly achieved after primary blood infection.

**Quorum sensing and bacteria.** Host-pathogen interactions have evolved towards a balance between the roles of both elements that, ideally, ensures the survival of the host and the successful transmission of the pathogen. Many relevant findings on host-pathogen interactions have been obtained for bacterial infections, and host factors were shown to be significant in controlling infections <sup>229,230</sup>. Moreover, bacteria are capable of monitoring their own population by a process called *quorum sensing* through which their density is maintained below potential harmful levels. The same mechanism can be used to control competing bacterial infections in the same host and so protect its niche and ensure transmission. Bacteria sense the accumulation of specific molecules in the environment that increase in the surroundings as their density grows. Beyond a certain threshold, individual bacteria will sense these molecules and trigger gene expression accordingly, promoting a concerted group response through several distinct strategies that include biofilm formation, secretion of virulence factors, motility, conjugation, sporulation, and bioluminescence expression, among others (reviewed in <sup>231-233</sup>).

**Could the interaction between *Plasmodium* blood and liver stages be mediated by a quorum sensing-like mechanism?** The data presented in this thesis shows that the presence of *Plasmodium* peripheral blood

parasitaemia above a certain threshold protects mice from secondary liver infections in a parasite-dependent fashion. Thus, in a process somewhat akin to *quorum sensing*, liver stage parasites could be responding to molecules released by *Plasmodium* iRBCs and thereby aborting their development. However, the involvement of a possible soluble factor secreted by the blood stage parasites was discarded, as infection of both mouse primary hepatocytes and hepatoma cells by *Plasmodium* sporozoites was not affected when they were co-cultured with iRBCs. Additionally, and contrary to a previous report <sup>209</sup>, sporozoite infection was repeatedly found to be similar in primary hepatocytes isolated either from blood-infected or naïve mice, indicating that possible alterations occurring in hepatocytes during blood stage infection reverted when these primary cells were cultured *ex vivo*.

Nevertheless, the complete rejection of the hypothesis that the observed impairment in *Plasmodium* liver infection by malaria blood stage is due to a released molecule by iRBCs is more complex than can be shown by co-culture experiments. *Plasmodium* asexual replication inside the RBC leads to important changes in its shape and content, promoting secretion of a vast array of molecules and iRBC adhesion to the vasculature of certain organs, including the liver <sup>234,235</sup>. Thus, one could hypothesize that the constant secretion of certain molecules would be necessary for the observed protective effect. These molecules might exist in the circulating serum or be concentrated in certain areas of circulatory system, where iRBCs sequester. Still, during the course of this work, several serum transfer experiments were performed under various conditions without ever recapitulating the reduction in infection observed in re-infected mice. Altogether, the present data seems to exclude that a soluble factor released by iRBCs acts directly on hepatocytes preventing full development of sporozoite liver infection.



**Liver gene expression during *Plasmodium* blood infection.** Since secreted parasite molecules appear not to be directly involved in reduction of liver infection, host molecules were then investigated. Examining the differences in liver transcription between naïve mice (NI), sporozoite infected mice (LS), mice carrying a blood stage infection alone (BS) or after secondary sporozoite infection (Reinf) by genome wide microarray analysis, most of the 1704 transcripts identified were found similarly differentially expressed between the BS and the Reinf groups, while only a limited number of genes were differentially expressed in the LS group. Gene ontology enrichment analysis highlighted immune system processes as up-regulated in both BS and Reinf, while genes involved in oxidation-redox were down-regulated in those two groups. Moreover, genes specifically over-expressed in Reinf appear predominantly to play a role in defence response and TNF regulation. Altogether, this analysis showed that major host alterations occur in gene expression in the liver during malaria blood stage infection.

It is possible that the observed alterations concur to explain the impairment of *Plasmodium* liver stage during an ongoing blood stage infection. However, testing synergies would be complex. Nevertheless inflammation, immune, apoptotic and metabolic genes were found to be differentially expressed in mice with an ongoing blood stage infection, recapitulating numerous previous studies where host responses to *Plasmodium* blood infection have been described (reviewed in <sup>159,210,236-238</sup>).

**Immunity.** Protection from infection is consensually attributed to immunity, and malaria is not an exception, with strong clinical evidence that *Plasmodium* infections are fought by the immune system. Through different cellular populations and by action of cytokines and chemokines produced by immune cells, the *Plasmodium* host can reduce and even neutralize

infection<sup>159,238,239</sup>. Also, *Plasmodium* is known to manipulate host immunity for its own benefit or to circumvent its actions<sup>240</sup> and thereby prevent its own annihilation, evidencing an extreme adaptation to its host<sup>10</sup>. Hence, part of this work aimed at establishing what the role would be, if any, of immune factors in the observed protection. To that end, we used the presented microarray analysis, which showed great alterations occurring in immunity during blood stage malaria infection, to select the best candidates to be tested.

Infection of erythrocytes by *Plasmodium* parasites is known to have repercussions in several of the host's organs. Bone marrow, spleen, brain, placenta and lungs show cytokine and chemokine enhancement of infiltrates, composed of macrophages, neutrophils, natural killer (NK) cells, gamma/delta ( $\gamma\delta$ ) T cells and both CD4(+) and CD8(+) effector T cells. These changes lead to local vascular and organ disturbances showing that, in addition to parasite-mediated pathology, the host inflammatory response contributes to the development of disease (reviewed in<sup>241</sup>). In addition, the liver is strongly affected by blood stage malaria. The liver is known to enlarge and accumulate hemozoin pigment during *Plasmodium* infection, and it has been reported that it plays an active role in controlling blood-stage infection, as a site of parasite destruction<sup>242</sup>. Also, several cellular changes occur in the liver during blood-stage infection. The livers of *Plasmodium* blood stage-infected mice are characterised by increased numbers of lymphocytes, blasts, monocytes and granulocytes, as well as an increase in cell numbers in the sinusoids and periportal region specifically, completely modifying the architecture of the liver, as we too observed. Additionally, these livers present an increase on both numbers of liver macrophages and their phosphatase activity<sup>234</sup>.

Hypothesizing that impaired liver stage development of re-infected mice could be caused by a blood stage-initiated immune response, several

features of the immune system were tested in an attempt to identify any involved.

The protection of re-infected mice was observed to be acquired very rapidly. Protection is achieved in just three days, and the level of protection is maintained, independently of the increasing parasitaemia and time of exposure to blood stage infection. This rapid effect implies that the involvement of the adaptive immunity in the observed protection is unlikely, as production of antibodies or of effector T cells would not be possible in such a short period of time. Furthermore, protection is also quite transient, disappearing straight away after clearance with anti-malarial drugs. Still, the involvement of the adaptive immune system in reducing secondary liver infection was assessed using splenectomised mice, and mice deficient for B and T cells. Expectedly, these experiments provided no support for any role of adaptive immunity in the phenotype observed; re-infected mice were protected from secondary infection regardless of surgery or adaptive immune cell deficiency.

The inflammatory response and the innate immune system were targeted using either genetically deficient mice, depletion methods and/or drug treatments. The results from such experiments show that the impairment of *Plasmodium* liver infection was independent of Kupffer cells and macrophages in the liver, NK or  $\gamma\delta$  T cells; IFN- $\gamma$ , TNF- $\alpha$ , NOS, IL-10, IL-6, TLR/MyD88 signalling; the complement system or mastocytes. All the experiments performed, testing a range of immune mediators, provided no support for a role of features of innate immunity or inflammation to the phenotype observed.

Co-operation and redundancy among the innate germline and its encoded receptors allow synergistic responses to infection, allowing appropriate defences against microbes and protecting the host against immune evasion by parasites <sup>243</sup>. Still, it is hard to consider that impairment of liver stage

infection would be under such a synergistic process that would never even produce an intermediate phenotype in at least some of the mouse lines tested, which exhibited deficiency in more than one cellular population (e.g. RAGII/ $\gamma$ c mice lack T and B cells as well as NK cells and as such produce very low levels of certain cytokines including IFN- $\gamma$ ).

**Host cell survival.** Developing sporozoites in the liver are known to modulate cell death of infected hepatocytes. Initially during early development, parasites inhibit apoptosis<sup>244</sup>, and later, to egress and colonize the blood, EEFs promote cell death to ease their way out of the hepatocyte<sup>245</sup>. It is also known that *Plasmodium* blood stages increase apoptosis in the liver<sup>210</sup>. Accordingly, the microarray data showed a robust pro-apoptotic stimulus in the livers of mice with an ongoing blood stage infection. This raised the question of whether alterations in the apoptotic status of the liver, due to the presence of blood infection, could impair secondary liver infection. Using caspase 3-deficient mice, we showed that malaria blood stage infection impaired *Plasmodium* liver stage even in the absence of a significantly increased level of apoptosis in the liver. Thus, hepatocyte apoptosis does not explain the observed protection. The stimulus reducing secondary liver infection seems to be stronger than that of caspase 3 deficiency allowing sporozoites to infect better due to the absence of apoptosis.

**Nutrient depletion.** Prevention from liver secondary infection could be promoted by the absence or deficiency of a nutrient resulting from the ongoing primary blood stage infection. The fact that not only the number, but also the size of EEFs was reduced in the presence of a *Plasmodium* blood infection suggests that some nutrient or growth factor could be limiting *Plasmodium* development in hepatocytes.

The extensive *Plasmodium* proliferation rate inside hepatocytes certainly requires the availability of nutrients for its metabolism and growth, besides sufficient lipids for the synthesis of the large amounts of membranes that must surround the newly formed merozoites at the end of liver stage development. Thus, it was hypothesized that altered nutrient resources in hepatocytes during blood stage infection could be responsible for the impairment of EEF development. Malaria blood stage infection would cause the removal of resources that would in turn impair survival or replication of *Plasmodium* in the liver.

As previously stated, in the enrichment analysis of the microarray data several clusters of differentially expressed genes stood out related to immunity and defence mechanisms. To a lesser extent, metabolic processes, such as the carboxylic acid metabolic process, were highlighted too. In addition, numerous genes involved in metabolic processes were found differentially expressed. These include over 40 genes concerning iron homeostasis and almost 80 genes related with lipid metabolism.

It is known that malaria patients exhibit significant lipid profile abnormalities, such as decreased total cholesterol levels, decreased low (LDL) and high (HDL) density lipoprotein-cholesterol, elevated lactate dehydrogenase, and moderately elevated triglycerides <sup>236,237</sup>, strongly suggesting lipid metabolism alterations, possibly caused by malaria blood stage infection. However, what was observed during the course of this work was that the low parasitaemias that were able to induce protection against secondary sporozoite infection produced no changes in cholesterolemia (including LDL-cholesterol and HDL-cholesterol levels), in the serum or in the liver of blood stage-infected mice.

Nevertheless, the possibility that alterations in lipid metabolism during blood stage infection might limit to a certain extent the development of EEFs could not be completely ruled out. More complex experiments would

be needed to verify the involvement of other lipid-related molecules. For instance, transplanting livers from blood-infected to naïve mice just prior to sporozoite infection would theoretically be ideal. Of course other consequences would arise from such an experiment; for example, the inflammatory response associated to the transplant technique could easily mask the object of the experiment.

Iron has received great interest as a nutrient that is vital for pathogen infection of development inside cells (reviewed in <sup>246</sup>). Moreover, a recent clinical trial of iron supplementation to children below 5 years of age showed a statistically significant increase in the number of malaria infections in those receiving iron <sup>220</sup>. This observation can be attributed to the known effect of iron on iRBCs <sup>215</sup>, but a possible effect on the liver stage of infection cannot be excluded.

Regarding iron related genes that were found altered in the microarray analysis, one stood out for its key role in iron homeostasis. *Hamp1* encodes for hepcidin, a peptide hormone produced in the liver that represents the master regulator of iron homeostasis in humans and other mammals <sup>247</sup>.

When hepcidin levels in primarily or secondarily sporozoite-infected mice were analysed, a strong correlation was observed between hepcidin expression in the liver during the blood-stage and protection from secondary liver infection. The increase in hepcidin was shown to be tightly dependent on the presence of iRBCs, promptly raising its levels after a few division cycles of *Plasmodium* in the blood, and being immediately reversed to normal levels after treatment of infected mice with an anti-malarial drug, thereby allowing liver stage infection to grow. Hepcidin was therefore investigated in greater detail as a candidate for explaining the reduced liver infection in re-infected mice.

Hepcidin regulates iron homeostasis by triggering the degradation of the iron exporter protein ferroportin, which is strongly expressed by

enterocytes and macrophages and to a lesser extent, hepatocytes <sup>213,214</sup>. An increase in hepcidin levels therefore inhibits iron uptake through duodenal enterocytes and leads to iron redistribution into macrophages, consequently promoting relative iron depletion in hepatocytes.

Connections between iron availability and *Plasmodium* infection of both red blood cells <sup>215</sup> and hepatocytes <sup>216-218</sup> have previously been reported. This was also observed *in vitro* and *in vivo* for the work gathered in this thesis. Reducing iron availability with iron chelators led to a significant and dose-dependent reduction of *Plasmodium* EEF development whereas iron supplementation increased EEF development. This indicates that if blood stage infection is indeed reducing iron availability inside hepatocytes, then this reduction could account for the reduced liver infection of re-infected mice, and the whole mechanism could be mediated by *hamp* upregulation in the liver.

Liver staining of re-infected mice for ferritin chains, the major intracellular iron storage protein, showed noticeable redistribution of iron to reticuloendothelial cells, consistent with hepcidin activity <sup>214</sup>. Moreover, it was found that increased *hamp* expression during blood stage infection redistributes iron, promoting its sequestration inside macrophages in the liver and in the spleen. In addition, the level of iron in hepatocytes of blood-infected mice was shown to be reduced, as hypothesized.

Interestingly, a recently performed microarray analysis was consistent with iron availability being important in *Plasmodium* liver stage infection. In *Plasmodium*-infected hepatoma cells, while the expression of the iron exporter protein ferroportin, which is degraded by hepcidin, was significantly reduced, that of the iron receptor DMT1 was significantly increased <sup>66</sup>, suggesting that iron acquisition and retention might be important for complete *Plasmodium* development inside its host cells, and manipulated by the infecting liver stage parasite, possibly to potentiate its

resources. This work proposes, on the other hand, that during a blood stage infection liver parasites are no longer able to modulate the iron exporter and receptor due to the presence of a much stronger stimulus induced by the iRBCs. Even only three days post-blood stage infection, hepcidin mRNA levels in the liver are already quite high, so the level of iron in circulation should already be low, promoting depletion of iron from hepatocytes.

Nevertheless, if hepcidin is to be attributed the functional role of decreasing liver infection in re-infected mice, it should affect liver stage infection when introduced in the absence of a blood stage infection. Crucially, hepcidin introduced into mice by administration of *hamp*-expressing adenovirus, prior to sporozoite infection, could alone significantly reduce liver stage infection. This demonstrated that sporozoite infection is impaired by an increase in the iron regulatory hormone, to the same extent that limiting infection through iron negative regulation in hepatocytes was proven in this study and by previous work of others<sup>216</sup>. Thus, increased hepcidin alone is sufficient to protect against the liver stage, even in the absence of the blood stage infection.

Altogether, the presented data showed that an ongoing blood stage infection leads to a rapid increase in liver hepcidin levels, which results in a redistribution of iron in infected mice. This limits iron availability in hepatocytes necessary for *Plasmodium* EEF development/replication inside hepatocytes, preventing EEFs from fully developing and therefore preventing the release of new merozoites into the blood-stream, thereby interrupting the cycle of secondary infection and preventing superinfection.

***Plasmodium* co-infection with other pathogens.** In *Plasmodium* endemic areas, people often bear more than one infectious agent at a given time<sup>248</sup>. In order to determine whether other pathogens would lead to similar results as those observed for *Plasmodium* blood stage infection, we used



*Toxoplasma*, an apicomplexan parasite closely related to *Plasmodium*, for the primary infection.

When challenging mice bearing an infection with *Toxoplasma gondii* tachyzoites with *Plasmodium* sporozoites, in parallel with *Plasmodium*-infected mice and compared with control naïve mice we monitored the effect of this other parasite on liver stage malaria infection. Indeed, mice with an ongoing *Toxoplasma* infection exhibited the same level of protection as *Plasmodium* iRBC-infected ones. This result provides evidence that our observation of impaired liver stage development is not genus specific, but rather consistent with induction of hepcidin. Mice infected with *Toxoplasma* show an increase in hepcidin expression in the liver to a similar extent as that observed on *Plasmodium*-infected mice. Iron redistribution should therefore happen for the *Toxoplasma* infection similarly to what was observed for the *Plasmodium* blood stage.

It is likely that some, but not all, infections have a similar effect on establishment and development of secondary liver infection as long as the primary infection leads to an increase of hepcidin expression. However, some pathogens, such as Hepatitis C virus (HCV), are known to suppress hepcidin<sup>249-251</sup>. A non-hepcidin inducing pathogen would be ideal to test and use as a negative control in our study. Murine models for HCV would be of useful to clarify the impact on a non-hepcidin increasing infection over a secondary *Plasmodium* sporozoite infection; unfortunately they are not currently available.

We hypothesize that different outcomes may arise depending on how much hepcidin is induced by any particular type of infection, and/or by the balance of hepcidin induction *versus* other influencing factors. Co-infections probably do not all have the identical effects and, moreover, in regions where malaria is endemic, one of the most likely co-infections is actually malaria itself.

**Outcome for the individual.** The scenario of secondary malaria challenges in individuals already harbouring blood-stage infections, modelled using a murine system in this thesis, must frequently occur in malaria endemic populations. If the findings for the mouse model were extended to human malaria, would be expected that new liver infections initiated during previous blood ones would be interrupted prompting protection from superinfection. This should prevent infected people with peripheral blood parasitaemias above a certain threshold from having a second circulating parasite to combat. Furthermore, abrogating competition between two *Plasmodium* blood stages sharing the same host, primary *Plasmodium* in the blood protects its niche and tries to ensure transmission to the next infection on a *first come, first served* basis. Or if any merozoite reaches the bloodstream it does so in such a low number or viability status that is never able to compete with the already settled iRBCs population. The host immune system will then have to fight only one circulating parasite, raising its chances of eliminating the circulating iRBCs and clearing infection, thereby increasing its chances of survival. Moreover, it avoids competition between two different parasites in the blood, promoting transmission of the first colonizer. Both host and pathogen gain from the interaction, only the third part, the secondary liver infection is condemned not to succeed.

The speculation that peripheral blood parasitaemia above a threshold prevents successful re-infections and thus superinfection is not in disagreement with the numerous observations of people bearing mixed plasmodium infections, as in humans the liver stage development takes around a week and early very low levels of parasitaemia should not affect secondary liver infections, giving plenty of time for successful re-infections to occur and thus promote the observed superinfections.

*P. falciparum* iRBCs were shown in the work of others to raise hepcidin levels in the urine and serum of naturally and experimentally *Plasmodium* infected humans<sup>212,252,253</sup> and to uphold hepcidin *mRNA* induction in human leukocytes *in vitro*<sup>211</sup>. Therefore, a possible side effect of increased levels of hepcidin induced by blood stage malaria infection could be a significant contribution to acute anaemia associated with clinical malaria and the chronic decrease in haemoglobin levels that occur in individuals living in malaria endemic areas<sup>211,212</sup>.

Iron is also known to influence immunity. Specifically, some cells involved in innate immunity have been described to be iron-modulated in the presence of infections, and hepcidin has been implicated in several proposed mechanisms (reviewed in<sup>254</sup>). Therefore, it is tempting to speculate that the effect observed is induced by hepcidin, as demonstrated in the results presented during this work, still without totally excluding some possible involvement of immune response that could also be responding to iron restrictions, although we could not, during this work, demonstrate any role of immune cells in the observed reduction.

**Outcome in populations.** Re-infections occurring during ongoing blood stage malaria are certainly frequent in endemic settings for *Plasmodium*. Mouse models, although not perfect, have been extensively used in *Plasmodium* research producing a great deal of important discoveries. If it is assumed that the observations produced in this work and presented in this thesis can be extrapolated to what occurs in human malaria, several questions and hypotheses regarding the possible implications of these findings for endemic populations can be raised.

Epidemiological studies from highly endemic areas consistently show that the incidence of infection first increases with age in young children before it declines later presumably as a result of acquired immunity (e.g.<sup>175,183</sup>). At

the same time, the complexity of infection, expressed by the average number of clones per infection, also increases as hosts get older <sup>175,181-183</sup>. Although well known, these facts were never completely elucidated or justified. A previous attempt to explain these observations relied on an apparent biting preference of mosquitoes towards older individuals, where the risk factor would be the increase in body area exposed to anopheline biting as children grow older <sup>219</sup>.

The model proposed in this work to explain the same epidemiological observations provides a more parsimonious solution, depending only on the threshold density-dependent inhibitory effect reported here. A simple agent-based model was devised to simulate infection histories in a number of individuals, which were followed over time whilst recording the average annual infection rates as well as the number of co-infecting clones to enable comparison with the well known epidemiological data.

Based on several epidemiological observations, the model assumes that the average parasite density per infection decreases with age <sup>175,181,205</sup>. The probability of an infectious bite leading to a new infection then depends on the level of a current blood-stage infection as well as the host's infection history. Under these minimal assumptions the model correctly predicts an initial increase in infection rates, which subsequently declines as individuals acquire immune protection through repeated exposure, fitting to the expected curves previously observed in several endemic regions. As a result of a higher risk of infection and reduced inhibitory effect in older individuals, the model also predicted an increase in the multiplicity of infection with age, as is observed in endemic sites.

The model was tested under various assumptions regarding the relationship between ongoing blood-stage infection and the probability of superinfection, and was found that no other inhibitory effect, including acquired immunity or premunition could explain the observed data as the

threshold density-dependent inhibitory effect does. Altogether, this means that threshold density-dependent inhibition of new liver stage infections alone can account for the increase in infection risk and complexity of infections in young individuals. Crucially, the results show that this effect is strongly dependent on the transmission intensity and most prominently observed under moderate to high transmission settings, and this too fits malaria field data. Our findings therefore provide an explanation for some of the differences in age-dependent risk of infection within the endemicity spectrum.

This is of particular importance in the first years of age and then might fade away as parasitaemias decline below a certain threshold, allowing superinfection to happen more frequently later in life when adaptive immunity or premunition conferred by a number of infections should already protect the young, at least from severe forms of disease. Indeed, besides knowing that superinfection varies throughout the age of individuals in a population <sup>175,181-183</sup>, it has been reported that superinfections have been much more frequently observed among asymptomatic carriers who exhibit lower peripheral blood parasitaemias than in clinical cases <sup>180</sup>, and are thus probably below the threshold of protection.

Re-infection of blood-infected individuals is of course much more probable in areas where transmission is medium to high, and in these settings people are known to acquire immunity faster than in areas where transmission rates are low <sup>151,152</sup>. In the light of the present work it is tempting to speculate that re-infection too can play a role in naturally acquired immunity.

Naturally acquired immunity to malaria has been largely debated and described for almost one hundred years. It is known that it is dependent on constant exposure to *Plasmodium* and on the degree of exposure. Still the re-infection situation and the great impact that an ongoing blood stage

infection has on a subsequent sporozoite infection has never been taken in consideration to assist explaining it. Developmental problems in EEF growth of re-infected individual might naturally be acting as a live attenuated vaccine, promoting protection in endemic populations. Also, the number of liver infections would be higher than the number of episodes of blood stage infection, possibly explaining why high *Plasmodium* transmission rates associate with faster acquisition of immunity and partial protection from severe disease.

Furthermore, one of the previous studies showing interaction between blood and liver stages presents the possibility that blood stages might suppress immune responses <sup>189</sup>. This result is not in disagreement with the work presented in this thesis, as we show no striking involvement of immune cells in reducing liver infection. Nevertheless, we do not know what would happen after several infections, when acquired immunity would play a stronger role and a possible different interaction could happen between these two stages of *Plasmodium* infection.

The sophisticated lifecycle of *Plasmodium* has resulted from millions of years of parasite/host co-evolution and communication, having broad implications for evolution and human health. Above a critical threshold, *Plasmodium* infection of red blood cells leads to an increase in the level of the host hormone hepcidin, which, by re-distributing iron, protects the parasite niche (and thus the host) from the risk of superinfection. This phenomenon, which acts independently of and in addition to acquired immunity, explains previous epidemiological observations and has important implications for future anti-malarial interventions.

The old concern remains of whether reducing the risk of *Plasmodium* infection will always lead to a decrease in the threat of severe malaria throughout life <sup>187</sup>. Would decreasing the possibility of re-infections in an

endemic area solely favour the community, or would potentially harmful consequences arise from interventions that, rather than eliminating transmission, would decrease it to levels where protection induced by re-infections would no longer exist? It is still not totally clear, but results from the trial of the pre-erythrocytic vaccine trial, RTS,S provide evidence that reducing *Plasmodium* liver load in children led to a significant decrease in severity of disease <sup>223</sup>.

Further work towards fully understanding associations between transmission and immunity will be of major importance in defining malaria control interventions and prediction of their outcome over time.

*To conclude, during this work a new interaction between Plasmodium blood and liver stages and its shared host has been identified. Bearing a malaria blood stage infection above a certain threshold prevents the appearance of a de novo infection in mice. Plasmodium liver stage is impaired and the new infection does not compete for blood infection.*

*Our observation that infection of RBCs by Plasmodium impairs the parasite development inside hepatocytes, mediated by iron deficiency is, per se, a significant contribution to understanding the dynamics of acquired protection against malaria infection. Increasing our knowledge of the molecular mechanisms behind the achieved protection in endemic settings will help to better design actions and predict the outcomes from such interventions. .*

discussion



## The next in line

*The work presented in this thesis showed for the first time the impairment that an ongoing Plasmodium blood stage infection imposes on a secondary liver infection. Besides being an interesting observation on its own and having possible implications on malaria interventions in the field should the same be proved for human Plasmodium infection, the results gathered here pave the way for future lines of work, as new questions arise.*

One of the questions raised during the course of this work concerns quorum sensing mechanisms. Would another of its kind impair a sporozoite infection? Liver infection in humans lasts for 5 to 13 days <sup>58</sup>, and in highly endemic areas an infected person is likely to be secondarily infected. Although the murine model offers a liver infection of solely 48h, the re-infection scheme could still be attempted. Using once more parasites tagged with different markers, we could observe what effect, if any, would an ongoing liver stage have on a secondary one. While no significant alteration was found in hepcidin expressions levels, in a recently performed microarray analysis in *Plasmodium*-infected hepatoma cells, the expression of the iron exporter protein ferroportin, which is degraded by hepcidin, was significantly reduced and that of the iron receptor DMT1 was significantly increased <sup>66</sup>. This suggests that iron acquisition and retention are manipulated by the infecting liver stage parasite, possibly to potentiate its resources. Therefore, it would be interesting to investigate whether any interactions would arise between competing liver forms and to what extent the mechanisms observed during the work in this thesis would be important for any possible interaction.

On the parasite side, it would be interesting to investigate how iron is required for *Plasmodium* metabolism. In studies with bacteria, iron has been

identified to work as a co-factor for several enzymes (reviewed in<sup>246</sup>). During *Plasmodium* infection, iron has been shown to be of relevance during the blood stage of infection in its iron-sulfur cluster form<sup>255</sup>. Numerous proteins that are central to several cellular processes, such as redox-actions, metabolic catalysis, and sensing of iron and oxygen levels, use these clusters as co-factors. The Apicoplast genome has been involved in assembly of such clusters, but not much is known regarding where the iron-sulfur clusters are used or if they are exported to the RBC cytoplasm or surface. Also during the blood stage of infection, iron is intimately connected to *Plasmodium* through the vast haemoglobin degradation. However, the haemoglobin-derived iron seems not to be required for the parasite heme synthesis (reviewed in<sup>256</sup>).

Not much information is available on the literature regarding the requirements of iron during liver stage infection, although a transcriptome analysis points that iron-sulphur subunit of succinate dehydrogenase is upregulated in sporozoites<sup>257</sup>. Understanding the pathways through which *Plasmodium* utilises iron could provide a basis for testing new prophylactic actions to combat malaria prior to its symptoms or to its radical cure, including combating hypnozoites.

On a different perspective of blood and liver stage interactions within a single host, we preliminarily observed that not only the blood primary infection has an effect on secondary liver challenge, but also the challenge produces alterations on the course of the primary blood infection. Indeed, some experiments were performed in which blood parasitemia from primary infection was followed, in an attempt to identify iRBCs originated from the secondary infection. Although secondary parasitaemia was not detected, we have observed that, in a few situations, primary blood infections were prevented from increasing after the mice were challenged

with sporozoites. Parasitaemias from the primary infection were found to decrease over time until mice fully cleared iRBCs. This result seemed to depend on the level of primary parasitemia at the time of re-infection, probably because the path towards hyper-parasitemia and death could no longer be interrupted if re-infection happened at already high primary parasitaemias. The inter-stage interactions between blood and liver *Plasmodium* within a single host could therefore continue to be studied and provide possible new insights concerning malaria transmission dynamics and disease protection in endemic settings.



## Appendix

**table S1.** Differentially expressed (DE) transcripts organised as toptable (sorted by log odds that a transcript is DE = B column). Cluster Nb corresponds to the clusters found in the non-hierarchical clustering and used for GO enrichment analysis. GO annotation for each transcript is also added, as well as if the transcript detected is a full length or partial or even if it's an EST (expressed sequence tag). All transcripts are significant at p.value adjusted for multiple testing  $p < 0.05$ . Transcript Cluster ID is the Affymetrix accession number. logFC is the log base of the fold change. Grey highlighted rows correspond to genes mentioned in the text.



Table with columns: Affymetrix ID, Transcript, BS/Non.inf., logFC, L5/Non.inf., logFC, BS/Non.inf., logFC, L5/Non.inf., Average expression, Cluster Number, Gene Symbol, Gene Title, Gene Ontology (GO), Transcript Classification. The table lists various gene transcripts and their associated biological and clinical data.



Table with columns: Affymetrix ID, Transcript, BS/Non Inf., L5/Non Inf., ReInf/Non Inf., Average expression, Cluster Number, Gene Symbol, Gene, Gene Ontology (GO), Description, Transcript Classification. The table contains a large number of rows, each representing a transcript with associated data across these columns.



















Table with columns: Affymetrix\_ID, BS/Non Inf., LogFC, L5/Non Inf., LogFC, LS/Non Inf., B, B, Average expression, Cluster Number, Gene Symbol, Gene Title, Gene Ontology (GO), Description, Transcript Classification.







Affymetrix ID	BS/Non Inf.	logFC	B	LS/Non Inf.	logFC	B	RE/Non Inf.	Average expression	Cluster Number	Gene Symbol	Gene Title	Gene Description	Gene Ontology (GO)	Transcript Classification
10513384	-2.9564486101	-0.344831351	-4.805185009	-1.097474683	1.267175736	1.267175736	7.723190895	transducin-like enhancer of split 1, negative regulation of transcription from RNA polymerase II promoter	Tie1	Tie1	transducin-like enhancer of split 1, negative regulation of transcription from RNA polymerase II promoter	transducin-like enhancer of split 1, negative regulation of transcription from RNA polymerase II promoter	full-length	
10514763	-0.634423018	-0.100967011	-6.390556991	-1.812128689	1.858303039	1.858303039	8.156933399	complement component B, alpha	C18a	C18a	complement component B, alpha	molecular_function // extracellular region // full-length		
10514912	-1.317011623	-0.016984484	-6.391350580	-1.800609574	3.472423852	3.472423852	11.963344694	deiodinase, iodothyronine, type I	C10x1	Dio1	thyroxine 5'-deiodinase activity // endoplasmic reticulum	full-length		
10515187	-1.243074871	-2.390161726	-5.990012881	-0.956789494	4.181687463	4.181687463	12.02133561	cyclochrome P450, family 4, subfamily 1, member 4	Cyp4b14	Cyp4b14	monooxygenase activity // iron ion binding // full-length			
10515201	-0.996936631	-0.180022879	-5.386539476	-1.058183399	3.31539584	3.31539584	7.389424209	cyclochrome P450, family 4, subfamily 1, member 4	Cyp4b14	Cyp4b14	monooxygenase activity // iron ion binding // full-length			
10515257	-1.465222473	-2.299666831	-6.86521003	-0.993509464	4.74349973	4.74349973	5.418414643	RAD54-like (S. cerevisiae)	Rad54l	Rad54l	nucleotide binding // double-strand break repair // full-length			
10515336	2.3792924664	6.022861466	-6.389410103	0.950705695	-1.519888942	-1.519888942	5.345771783	kinesin family member 2C	Kif2c2	Kif2c2	nucleotide binding // motor activity // microtubule binding // full-length			
10515343	0.864970913	0.047033327	-0.294000008	-0.790340268	4.474301413	4.474301413	5.7965994912	cydine B1	Cybs1	Cybs1	in vivo embryonic development // protein binding // full-length			
10515620	1.694396867	1.302439984	-0.176353347	-5.348707385	4.432272761	4.432272761	6.3948185974	lymphocyte protein tyrosine kinase	Ctks	Ctks	catalytic activity // CTP synthase activity // full-length			
10516736	0.844941679	0.025137936	-0.547222706	1.037536235	9.010068544	9.010068544	7.903686222	lymphocyte protein tyrosine kinase	Ctks	Ctks	catalytic activity // CTP synthase activity // full-length			
10516789	0.741901365	0.660694765	0.759444836	0.119493106	4.42913267	4.42913267	6.3948185974	lymphocyte protein tyrosine kinase	Ctks	Ctks	catalytic activity // CTP synthase activity // full-length			
10516996	0.698998745	0.599879399	0.652643701	-0.632949998	9.010068544	9.010068544	7.903686222	lymphocyte protein tyrosine kinase	Ctks	Ctks	catalytic activity // CTP synthase activity // full-length			
10517141	2.243295791	0.599879399	0.599879399	0.632949998	9.010068544	9.010068544	7.903686222	lymphocyte protein tyrosine kinase	Ctks	Ctks	catalytic activity // CTP synthase activity // full-length			
10517165	2.187924802	0.366749011	0.219309129	-6.5411325978	0.939460328	0.939460328	3.2545150911	high mobility group nucleosome	Hmg20	Hmg20	high mobility group nucleosome	full-length		
10517169	2.193709969	0.320559468	0.406297269	-4.5278493826	11.80269499	11.80269499	8.258739156	CD52 antigen group nucleosomal	Cd52	Cd52	CD52 antigen group nucleosomal	full-length		
10517508	1.651673624	2.805014639	0.235731943	-6.129431345	2.018337369	2.018337369	11.80269499	CD52 antigen group nucleosomal	Cd52	Cd52	CD52 antigen group nucleosomal	full-length		
10517513	1.50213702	0.806654411	0.052136359	-6.16437191	1.292978497	1.292978497	9.789895194	SH3 domain binding glutamic acid	Sh3b3	Sh3b3	SH3 domain binding glutamic acid	full-length		
10517573	-4.020076475	-1.0665265038	-2.22952128	-0.858394224	1.022916741	1.022916741	6.621416685	elastase 2A	Elas2a	Elas2a	serum-type endopeptidase activity // extracellular matrix metalloprotease activity // full-length			
10518050	-3.462694361	-0.800181744	-2.786466163	-1.073853751	-6.639056473	-6.639056473	7.239456988	EF hand domain containing 2	Efh2	Efh2	EF hand domain containing 2	full-length		
10518109	1.279084737	0.83301824	0.066612817	-6.612196172	8.522600698	8.522600698	9.351403993	high mobility group box 2	Hmg2b	Hmg2b	DNA binding // nucleus // chromosome	full-length		
10518352	2.260347026	2.360347026	0.088737347	-6.242364556	2.129224781	2.129224781	7.879624932	DNA binding // nucleus // chromosome	Hmg2b	Hmg2b	DNA binding // nucleus // chromosome	full-length		
10518455	1.149324052	1.098963719	1.158885267	-4.978083181	1.141937937	1.141937937	9.583810891	angiotensin II, type I receptor	Agtr1	Agtr1	receptor activity // angiotensin type II receptor	full-length		
10518570	1.019036746	1.043540111	-6.239309302	0.934764796	9.766586059	9.766586059	9.276266547	phosphotyrosinase	Ptdg	Ptdg	phosphotyrosinase	full-length		
10518686	1.598785802	6.732289463	0.157290799	-5.954636732	6.972624099	6.972624099	6.135767054	potassium voltage-gated channel	Pkc3cd	Pkc3cd	B cell homeostasis // ionostol or potassium voltage-gated channel	full-length		
10519060	1.260194989	5.704153807	0.276476088	-4.789489348	1.594007211	1.594007211	9.377134244	neurocatalytic factor receptor	Kna2bd	Kna2bd	receptor activity // protein binding // external side	full-length		
10519497	1.162284779	4.610943813	-0.16506683	-6.087135569	2.168188272	2.168188272	11.483130367	ATP-binding cassette, sub-family B	Trnsr14	Trnsr14				



















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