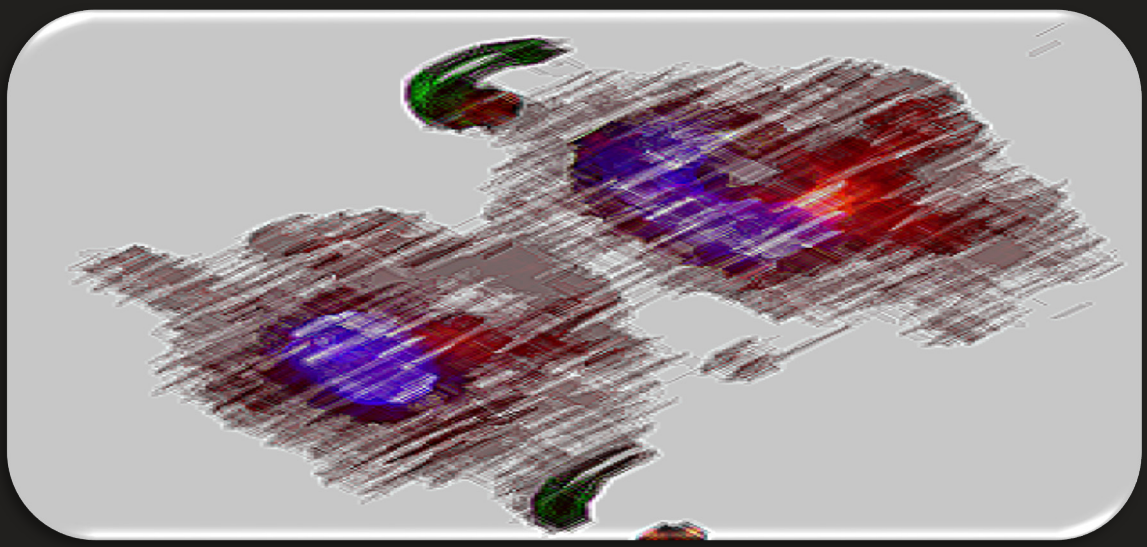
Host genetic factors in mouse malaria liver stage infection

Lígia Antunes Gonçalves



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
December, 2014

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Cover illustration: © Lígia A. Gonçalves [Kupffer Cells (nucleus in blue) and *Plasmodium* sporozoites (green)]

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It does not matter how slowly you go
as long as you do not stop.

Confucius

PREFACE

This dissertation assembles research performed at Instituto Gulbenkian de Ciência, Oeiras, under the supervision of Prof. Carlos Penha Gonçalves to obtain the Ph.D. degree in Biology specialization in Host-Pathogen Interactions at Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa.

This thesis dissertation is structured in five chapters, preceded by a summary in both Portuguese and English that outlines the aims, results and outcomes of the project.

Chapter 1 compromises an introduction to malaria, with a focus on the *Plasmodium* liver stage infection and parasite-host interactions. It includes an updated description of murine genetic variation that impact in the infection outcome. It describes the aims and objectives of this thesis.

Chapter 2 presents a published paper that characterizes the identification of a malaria liver stage susceptibility locus in the mouse genome.

Chapter 3 presents a published paper reporting the fine mapping of the locus described in Chapter 2.

Chapter 4 comprises a manuscript prepared for publication that characterizes the genetics of hepatocyte apoptosis upon *Plasmodium* liver stage infection.

Chapter 5 consists of a general conclusion of the results obtained and their relevance.

RESUMO

A fase hepática da malária é o estadio inicial e assintomático da expansão do *Plasmodium* no hospedeiro vertebrado, representando um alvo preferencial para o desenvolvimento de vacinas e terapias preventivas. Esta tese aborda a identificação e análise de factores genéticos do hospedeiro que controlam a fase hepática da malária após infecção experimental de murganhos com esporozoítos de *Plasmodium berghei*.

Foram utilizadas técnicas de mapeamento genético para identificar QTL (quantitative trait loci) que controlam a carga de parasita no fígado, tendo revelado que numa região de 28 Mb no Cromossoma 17 mapeia um locus que foi denominado *Belr1*. Seguidamente, foi gerado e analisado um grupo de dez linhas subcongénicas de murganhos que permitiram isolar dois efeitos genéticos distintos (o *Belr1* e o locus de *H2),* ambos controlando a carga parasitária do fígado. O locus *Belr1* foi finamente mapeado numa região de 4 Mb distal ao H2, que contém trinta e quatro genes codificantes. A análise quantitativa de mRNA hepático de estirpes congénicas informativas evidenciou o gene Trem2 como o único nesta região cuja regulação de transcrição era coerente com a carga parasitária do fígado. Em concordância com estes resultados, a ablação genética do *Trem2* resultou no aumento da carga parasitária da fase hepática após infecção *in vivo*.

Observou-se que as células de Kupffer são funcionalmente activadas *in vitro* por esporozoítos de *P. berghei* de uma forma Trem2-dependente. O contacto directo da célula de Kupffer com os hepatócitos revelou um mecanismo de controlo da carga parasitária que é comprometido na ausência de expressão de TREM2 nas células de Kupffer. Estas observações sugerem que TREM2 actua na interface esporozoíto/célula de Kupffer/hepatócito mediando o

reconhecimento do parasita e subsequente activação das células de Kupffer, de que resultam funções efectoras que controlam a carga parasitária nos hepatócitos. Também observámos que após exposição *in vitro* a esporozoítos, as células de Kupffer secretam quantidades significativas de HGF, que por sua vez é indutor das vias de apoptose independente da via mitocondrial, nas fases tardias da infecção hepatocitária. Estes resultados revelam um diálogo inesperado entre células de Kupffer e hepatócitos que proporciona o controlo da infecção dos hepatócitos pelo *Plasmodium* e também demonstram que a expressão de TREM2 e HGF em células de Kupffer contribuem para a resposta do hospedeiro contra hepatócitos infectados com o parasita da malária.

Por outro lado, observou-se que a redução espontânea de células infectadas em culturas primárias de hepatócitos ocorre através da modulação da via de sinalização do Fas, a qual desencadeia nos estadios iniciais da infecção vias apoptóticas mitocondrio-dependentes. Os nossos dados também sugerem que na presença de infecção, a expressão de FasL em hepatócitos não infectados actua por via parácrina para reduzir a eficácia da infecção em hepatócitos vizinhos.

No seu conjunto, este trabalho proporciona a visão de que tanto as células de Kupffer como os hepatócitos participam na resposta do hospedeiro contra formas hepatocitárias da malária utilizando diferentes vias de sinalização para reduzir o sucesso da infecção intra-hepatocitária pelo parasita da malária.

SUMMARY

Malaria liver stage infection is the initial and asymptomatic phase of parasite expansion in the vertebrate host and represents a preferred target for vaccine development and preventive therapies. This thesis focuses on the identification and analysis of host genetic factors controlling malaria liver stage upon mouse experimental infection with *P. berghe*i sporozoites.

Genetic mapping techniques used to search for QTL (quantitative trait loci) identified a locus controlling liver stage parasite burden within 28 Mb region in Chromosome 17. Subsequent generation and analysis of a panel of ten subcongenic mouse strains dissected the genetic control of liver parasite burden conferred by Chromosome 17 in two loci, the *Belr1* and the H2 locus. The *Belr1* locus was narrowed down to a 4 Mb interval distal to the H2 locus, which encompasses thirty-four protein coding genes. Quantitative mRNA analysis of informative subcongenic strains highlighted *Trem2* as the only gene in the *Belr1* region with transcriptional regulation congruent with liver stage parasite burden. Consistently, *Trem2* genetic ablation determined increased liver stage parasite burden after in vivo infection.

Additionally, we observed that Kupffer cells are functionally activated *in vitro* by *P. berghei* sporozoites in a *Trem2* dependent manner. Kupffer cell-hepatocyte direct contact offered *in vitro* control of parasite yield that was impaired when TREM2 is ablated in Kupffer cells. This unveiled that TREM2 operates in sporozoite/Kupffer cell/hepatocyte interfaces by mediating parasite recognition and subsequent activation of Kupffer cells, which induces effector functions able to control parasite yield in hepatocytes. In addition, upon *in vitro* exposure to sporozoites, Kupffer cells secrete significant amounts of HGF that in turn sensitize infected hepatocytes to undergo mitochondrial-independent

apoptosis at late stages of infection. These findings implicate an unexpected Kupffer–hepatocyte crosstalk as a host mechanism to control *Plasmodium* liver stage infection and demonstrate that expression of TREM2 and HGF in Kupffer cells are operators of host responses against malaria-infected hepatocytes.

Conversely, we show that spontaneous reduction of infected cells *in vitro* occur through induction of hepatocyte-autonomous Fas signaling pathway that triggers mitochondrial-dependent apoptosis in early stages of hepatocyte infection. Our data strongly suggest that FasL expressed in non-infected hepatocytes acts in a paracrine fashion to reduce the establishment of a productive infection in neighboring hepatocytes.

This work provides evidence that the host senses malaria liver stage infection and responds by activating distinct signaling pathways in Kupffer cells and hepatocytes that operate to reduce successful intra-hepatocytic parasite expansion.

ABBREVIATIONS

AMA1 - apical membrane antigen 1

Berr – berghei resistance locus

cAMP - cyclic adenosyl monophosphate

CelTOS - cell transversal protein for ookinetes and sporozoites

Char – chabaudi resistance locus

cM - centimorgan

CM – cerebral malaria

CSP - circumsporozoite protein

EBL – erythrocyte binding-like

ECM – experimental cerebral malaria

EEF - exo-erythrocytic form

HGF - hepatocyte growth factor

HSPG - heparan sulfate proteoglycans

ICP – cysteine protease

iRBC – infected red blood cell

KAHRP - knob-associated histidine-rich protein

KC – Kupffer cell

L-FABP - liver fatty acid synthesis

LISP - liver specific protein

LSA1 – liver-stage antigen 1

LSEC – liver sinusoidal endothelial cell

Mb - megabase

MHC – major histocompatibility complex

MSP – merozoite surface protein

NPC – non-parenchymal liver cell

pDCs – plasmacytoid dendritic cell

PfEMP1 - *P. falciparum* erythrocyte membrane protein 1

$PI(3,5)P_2$ - phosphatidylinositol 3,5-biphosphate

PI3Ks - phosphoinositide 3-kinases

PV - parasitophorous vacuole

PVM - parasitophorous vacuole membrane

QTL – quantitative trait loci

RBC - red blood cell

RBL – reticulocyte binding-like

RCS – recombinant congenic strains

ROM1 – rhomboid 1

RON - rhoptry neck

SAP1 - sporozoite asparagine-rich protein 1

SERA - serine repeat antigens

SLARP - sporozoite and liver stage asparagine-rich protein

SR-BI - scavenger receptor type B I

SPECT - sporozoite microneme protein essential for cell transversal

SUB1 – subtilisin-like protease 1

TRAP - thrombospondin related anonymous protein

TREM - Triggering Receptor Expressed on Myeloid cells

UIS - upregulated in infectious sporozoites

VSA – variant surface antigen

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CHAPTER 1

General Introduction

Malaria Overview

Malaria is a poverty-related infectious disease with an outstanding global death toll and a history that extends into antiquity. The oldest records of the typical malaria periodic fevers and spleen enlargement date back to 2700 BC in old Chinese manuscripts and the earliest detailed account occurred in 500 BC by Hippocrates. The name malaria derives from the Latin "*malus aria*" (bad air) as Ancient Romans associated the illness to the bad fumes from swamps [1,2].

Malaria is a parasitic disease caused by protozoa of the genus *Plasmodia* that belongs to the *Apicomplexa phylum* and is transmitted to the vertebrate host through the bite of an infected mosquito vector. There are five *Plasmodium* species known to infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and the more recently described, *P. knowlesi* [3,4]. Each of these species shows distinct clinical outcomes, regional prevalence and geographic distribution [5,6].

Nowadays, malaria remains as a major global health problem, but the big efforts made to eradicate this plague in the last decade, correlated with a reduction in the death rate by 47% since 2000. Nevertheless, 97 countries still show ongoing transmission with an estimation of 3.2 billion people being at risk of infection and developing disease of which 1.2 billion are at high risk (Figure I-1) [7]. In 2013 were reported a total estimation of 198 million clinical cases and 584 000 deaths from malaria. More than 80% of the clinical cases and 90% of the deaths occur in sub-Saharan Africa, mostly (78%) in children under five years [7]. Malaria clinical symptoms range from mild fevers to hypoglycemia, severe anemia, acute respiratory syndrome and cerebral malaria [8,9]. This disease impacts on premature mortality, population growth, work productivity and absenteeism, representing a serious social-economic burden for malaria endemic countries [10].

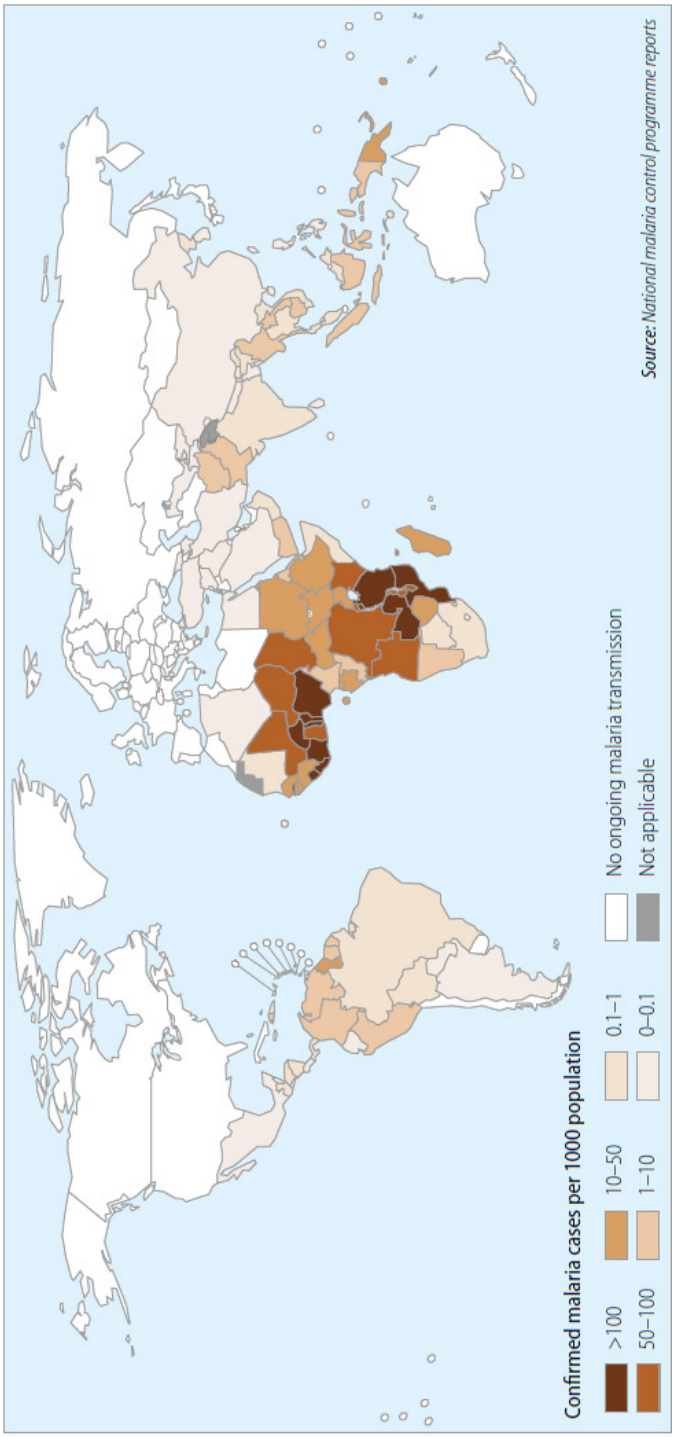

Figure I-1. Countries with ongoing transmission of malaria in 2013 according to World Health Organization. [7]

Plasmodium Life Cycle in Mammals

Plasmodium is a large genus of parasitic protozoa with more than 200 species described. The parasite life cycle comprises two obligatory hosts: a mosquito vector of the *Anopheles* genus and a vertebrate host. Apart from the five species infecting humans, other species infect a large range of mammals and other animals, including birds and reptiles [11].

The *Plasmodium* parasite acquires a series of different stages across its life cycle (Figure I-2). The sporozoite, the mammalian infective form, is carried in the salivary glands of the *Anopheles* female mosquito and is deposited into the skin. The mosquito proboscis ejects saliva to prevent blood coagulation while probing for a blood meal, which provides an opportunity to release the parasite in the dermis of the vertebrate host [12,13]. In experimental conditions, it is estimated that the mosquito injects on average 100 sporozoites [14,15] whereas in the wild, infected mosquitoes hold lower numbers of parasites and probably inoculate on average fewer than 50 sporozoites per bite [16]. Although some sporozoites rapidly leave the injection site, many take hours to reach the bloodstream. The majority of the injected sporozoites (~60%) will remain at the biting site while *circa* 15% invade the lymphatic vessels and 25% reach the blood circulation. In the dermis avascular tissues, sporozoites actively glide forward at 1-2µm/s in random directions until they contact endothelial cells of blood or lymphatic vessels [17]. The sporozoites that enter the lymphatic circulation, are captured in the draining lymph nodes [17,18] where most are internalized by dendritic cells, some of which are able to develop partially into exo-erythrocytic forms (EEFs) but do not progress in the cycle [17].

The sporozoites that enter the blood vessels travel passively to the liver sinusoids. Liver sinusoids are unique low-pressure capillaries with a highly fenestrated thin endothelium, populated by abundant Kupffer cells

(KCs), liver resident macrophages that delimit the space of Disse [19]. Sporozoites cross the sinusoidal barrier and infect hepatocytes, their initial site of replication for cycle progression in the mammalian host [20].

Sporozoite arresting in the liver is a not yet completely understood mechanism. It is believed that the parasite is attracted and arrested in

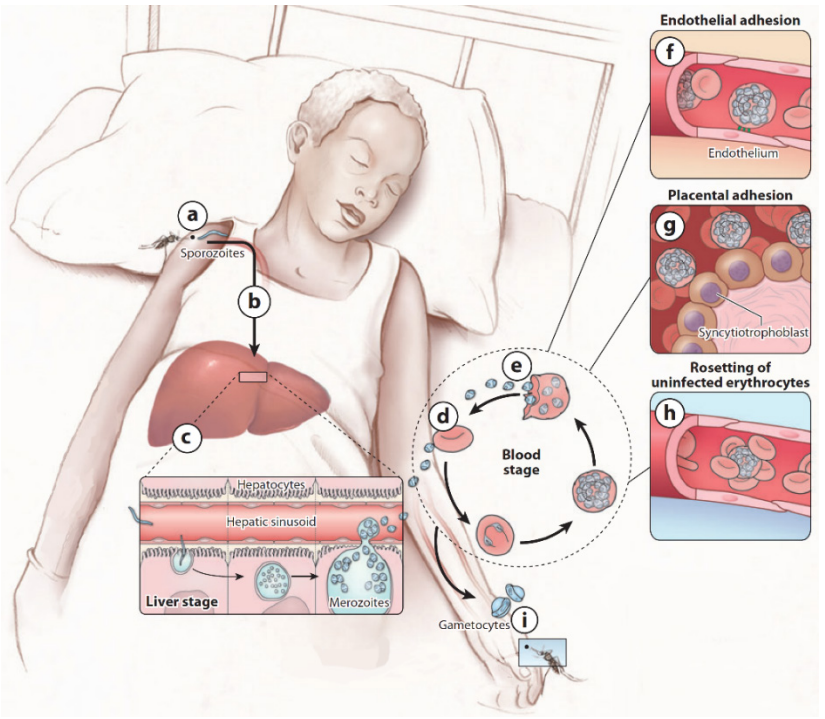

Figure I-2. *Plasmodium* life cycle in humans.

Infection begins when a female *Anopheles* mosquito injects sporozoites into the skin **(a)**. The sporozoites migrate to the liver and start the clinically silent liver stage **(b)**. Each sporozoite invades a hepatocyte, giving rise to tens of thousands of merozoites **(c)**. When merozoites are fully developed (~one week) exit the liver into the bloodstream and begin a 48 h cycle of red blood cell (RBC) invasion**(d)**, replication, RBC rupture, and merozoite release **(e)** starting the clinical phase of malaria. Inside the RBCs, the parasite exports variant surface antigens (VSAs) to the RBC surface. The VSAs mediate binding of iRBCs to the microvascular endothelium of various organs **(f)**. This leads to local inflammation and circulatory obstruction associated with clinical syndromes of severe malaria, including cerebral malaria and pregnancy-associated malaria **(g)**. VSA-mediated rosetting of iRBCs to uninfected RBCs may also contribute to disease and severe anemia **(h)**. A small number of blood-stage parasites differentiate into sexual gametocytes, which are taken up by mosquitoes subsequent blood meals **(i).** (adapted from Crompton et al 2014 [307])

the liver through two major sporozoite surface proteins, the circumsporozoite protein (CSP) [21] and the thrombospondin-related anonymous protein (TRAP) [22]. These parasite proteins specifically interact with the heparan sulfate proteoglycans (HSPGs) present in the extracellular matrix of KCs and stellate cells [23–27]. Although HSPGs are present in several mammalian tissues, their degree of sulfation in the liver is markedly higher [28], possibly explaining the extraordinarily selective targeting of *Plasmodium* sporozoites to the liver.

To proceed to hepatocyte infection, sporozoites must actively cross the liver sinusoidal layer to access the liver parenchyma and invade hepatocytes. The molecular mechanisms used by the parasite have been intensively studied using rodent models of liver stage infection, but are still a matter of controversy. Different lines of experimental evidence favor the hypothesis that sporozoites cross the sinusoidal layer exclusively by traversing KCs and not endothelial cells [29–33]. This hypothesis was strongly supported by *in vivo* infections showing that KCs depletion abrogated the lower infectivity of parasites defective for cell traversal (lacking SPECT 1 or 2 proteins) [34]. These findings led to the formulation of the gateway model formulation [35]. However, it was recently shown that KCs are not mandatory for sporozoites to cross the liver sinusoidal barrier, as intravital imaging revealed a multiplicity of sinusoidal crossing mechanisms, associated or not with cell traversal activity and targeting either KCs or endothelial cells [36].

Once a sporozoite reaches free access to the liver parenchyma it traverses several hepatocytes before invading one that serves as a host cell [37]. How the sporozoites switch from migratory to invasive mode is still unclear. Until recently, it was believed that initiation of the invasion process was not dependent on liver-specific factors, but rather prompted after several hepatocytes were traversed exposing the sporozoite to the intra-hepatocytic environment [38]. This includes the

release of hepatocyte growth factor (HGF) [39] or high concentrations of intracellular potassium [40] that were shown to increase infectivity. On the other hand, parasite mutants deficient for cell traversal, like SPECT, CelTOS or PL, fully retain the capacity to invade hepatocytes, therefore, opposing the hypothesis that traversal is required to activate sporozoites for infection [34,41,42]. However, it was shown more recently that cell traversal is a commonly observed sporozoite behavior and that once in contact with highly sulfated-heparan sulfate proteoglycans (hsHSPGs) in hepatocytes, the sporozoite stop migrating and switch to an invasive mode [43]. The sporozoite interaction with hepatocyte hsHSPGs leads to the cleavage of the CSP N-terminus, exposing the type I thrombospondin repeat (TSR) domain [44], a cell-adhesive domain thought to be critical for hepatocyte invasion [45].

The hepatocyte invasion by *Plasmodium* sporozoites occurs through a process of invagination of the host-cell plasma membrane, forming the parasitophorous vacuole (PV) [46]. The cell invasion process by Apicomplexa zoites relies on the gliding motility machinery [47], which requires coordinated and ordered release of molecules from apical specialized organelles, called microneme and rhoptries [48]. The exported proteins contain cell-adhesive domains required to attach and establish tight-junctions with the host cell. Such junctions will allow the parasite to move, propelled by the actin-myosin motor that translocate these junctions posteriorly along the parasite surface, resulting in a forward movement into the host cell [47,49]. Once the parasite is completely within the cell, the junctions that are now at the posterior end of the parasite seal off and complete PV formation. Few parasite proteins have been implicated in PV formation in the *Plasmodia* genus. One such protein, crucial to cell invasion is TRAP exported from micronemes at the sporozoites' anterior end [50,51]. More recently, the rhoptry neck 4 (RON4) protein was identified as having an essential role in the formation of tight-junctions between sporozoites

and hepatocytes [52]. In addition, some host factors are critical for invasion of hepatocytes namely, tetraspanin cluster of differentiation 81 (CD81) [53] and scavenger receptor BI (SR-BI) [54,55].

The parasite resides throughout the liver stage development within the established PV. The PV membrane (PVM) is the boundary between the parasite and the hepatocyte, and guarantees the sustained supply of host molecules to support parasite expansion, implicating that the parasite actively remodels the PVM to fulfill its needs [56]. Upon the formation of the PVM, the sporozoite dedifferentiate from an invasion-competent elongated sporozoite to a metabolically active, replicative and round trophozoite, morphologically characterized by a central spherical bulb region with two distal ends that gradually retract [57]. Genetic manipulation in *Plasmodium* revealed that knockout mutants for several proteins are unable to form or remodel the PVM and/or are arrested early in liver stage development. Targeting P52 (termed P36p in *P. berghei*) and B9, proteins of the *Plasmodium*-specific 6-Cys family, results in failure to develop the PVM and in development arrest [58–61]. Deletion of secreted rhomboid 1 (ROM1) protease, was also found to be crucial for the PV formation, aborting development at 12h of infection [62]. Genetic deficiency of sporozoite and liver stage asparagine-rich protein (SLARP) in *P. berghei* and its *P. yoelii* ortholog, sporozoite asparagine-rich protein 1 (SAP1), allow hepatocyte invasion and PV formation, but impair PVM remodeling leading to parasite development failure [63–65]. Moreover, parasite mutants for PVM-resident proteins coded by up-regulated in infective sporozoites 3 and 4 genes (UIS3 and UIS4) were unable to develop beyond trophozoite stage [66–68].

Following the trophozoite stage, starts the schizogony phase that generates thousands of merozoites [69–71] capable of establishing infection within red blood cells (RBCs). This process occurs through

repeated rounds of replication of DNA and cellular organelles, resulting in a massive syncytium that grows within the hepatocyte to an extent that can surpass the size of uninfected hepatocytes. In rodent-infective *Plasmodium* species, one trophozoite can generate up to 30 000 merozoites, in just 30 hours [72]. This remarkable replication rate requires abundant energy, which implies a strong host-parasite interaction to scavenger hepatocyte nutrients such as glucose [73,74], cholesterol [75] and fatty acids [76] to generate membranes and lipid bodies. Recently, it was shown that *P. berghei* PV is surrounded by host cytoplasmic vesicles of the late endocytic pathway that when disrupted impair schizogony, suggesting they are a required as a nutrient source [77]. It was that PVM fuses the late endocytic vesicles in a PI(3,5)P_2-dependent manner [78]. In addition, the *Plasmodium*-infected hepatocyte transcriptome revealed that infection modulates the host carbohydrate and fatty acids biosynthetic pathways [79]. Furthermore, *Plasmodium* engages in endogenous production of fatty acids via the type II fatty acid biosynthesis pathway (FAS-II) that is needed for merozoite formation, as shown by disruption of *Plasmodium* FABI [80] and FAB/F or FABZ [81] genes that encode FAS-II enzymes. Moreover, other liver stage specific proteins have been shown to be crucial during merozoite development. Conditionally disrupting *P. berghei* sporozoite cGMP-dependent protein kinase allows development until late liver stage, but blocks merozoite release [82]. Upon loss-of-function mutation of *Plasmodium*-specific apicoplast protein for liver merozoite formation (PALM,) merozoite segregation is strongly impaired [83]. Also, *P. falciparum* mutants for liver-stage antigen-1 (LSA-1) do not differentiate into mature merozoites, as LSA-1 is essential to form a matrix that embeds merozoites during maturation [84]. Disruption of liver-specific protein 2 (LISP2) that is exported to the hepatocyte causes defects in merozoite formation at the final phase of development [85]. Likewise, parasites lacking the subtilisin-like protease 1 (SUB1) was shown to

exhibit a developmental defect prior to egress [86]. Nevertheless, the development program of parasite liver forms is largely unknown, and further studies are needed to unveil how these and other proteins are implicated in the subtle mechanisms of hepatic merozoite formation and segregation.

The release of merozoites into the bloodstream is a well-orchestrated multistep process. While merozoites develop, parasite proteins begin to leak into the hepatocyte cytosol, thus showing that PVM becomes increasingly permeable before it is completely disrupted. The PVM breakdown is dependent on cysteine proteases, as the cysteine protease inhibitor E64 was shown to block this process [87]. Likely, serine repeat antigen (SERA) proteases, PbSERA-1 [88] and PbSERA-3 [89], that leak into host cell cytoplasm are mediators of PVM rupture. Similarly to blood stage egress [90], *Plasmodium* SUB1 was shown recently to play a role during PVM destruction and subsequent parasite egress [86,91]. Moreover, *P. berghei* deficient for the liver-specific protein 1 (LISP1), which localizes in the PVM, do not break the PVM and remain trapped inside hepatocytes [92]. After PVM disintegration, the infected hepatocyte membrane remains intact [87,93] and it is not clear how parasite eludes pro-apoptotic signals in hepatocyte during merozoites release. One possible player is the parasite inhibitor of cysteine protease (ICP) (termed falstatin in *P. falciparum*) that is leaked into the cell host cytosol upon PVM rupture, and was shown to block hepatocyte apoptosis after *P. berghei* merozoite release [94]. *In vitro*, hepatocytes that contain free merozoites usually detach from its surroundings towards the end of liver stage infection [93]. Subsequent to PVM rupture, merozoites are held in hepatocyte-derived membrane vesicles [93] termed merosomes, that hold up to thousands of motile merozoites [87]. The merosomes bud-off to reach the liver sinusoids [87] and may act as a protective barrier allowing merozoites to evade detection and engulfment by KCs lining the liver sinusoids. Once the

merosomes reach the bloodstream, they travel passively through the right side of the heart before accumulating in the pulmonary capillaries where their cargo of infectious merozoites is released [95].

The merozoite is exquisitely adapted to invade circulating red blood cells (RBCs) [96], initiating the malaria blood stage, which is responsible for malaria clinical manifestations. Once the merozoite is free, it takes, on average, less than two minutes to invade an RBC [97]. The speed and efficiency of invasion may help merozoites to evade host defense mechanisms as extracellular merozoite surface antigens are easily recognized by the immune system. After RBC invasion, *P. falciparum* merozoites sequentially develop into the ring stage (0-24h), followed by the trophozoite stage (24-36h) and finally into the replicative schizont stage (40-48h) [98]; this development process dramatically remodels the RBC which acquires propensity to adhere to a variety of host cells.

The merozoite invasion is a complex process mediated by several surface proteins [99,100] and comprises recognition, attachment, reorientation and finally invasion of RBC. When the merozoite contacts an RBC, it binds and reorientates to bring its apical prominence in contact with the host cell [101]. The initial contact elicits a dynamic RBC deformation, allowing the merozoite to reorientate to its apical tip [97,102]. This initial binding is thought to be mediated by merozoite surface proteins (MSPs) [103] since there is evidence that MSP-1 and it associated protein MSP-7 are essential for binding to RBCs as invasion is impeded by antibodies against MSP-1 fragments and when MSP-7 is deleted [104–107]. In addition, MSP-1 was shown to bind directly to erythrocyte surface band 3 [106].

Merozoite reorientation and RBC invasion also involve several *Plasmodium* proteins that unlike surface proteins are released just prior or during invasion [108,109]. These proteins fall into two categories: adhesins, which function as ligands that bind directly to RBCs receptors

and; invasins, which mediate invasion. The adhesins belong to the erythrocyte binding-like (EBL) and reticulocyte binding-like (RBL or PfRh) protein families that are located in both micronemes and rhoptries [110–117]. Its apical localization indicates that these proteins can be involved in reorientation or apical anchoring to the RBC, which is required for development of the moving junction. The EBL proteins comprise the EBA-175, EBA-181 and EBA-140 [108,116,118–120] that specifically bind RBC glycophorin A, B and C, respectively [121–124]. On the other hand, the RBCs receptors for the PfRh family [114,125–128] remain unknown, except for PfRh4 and PfRh5 ligands: PfRh4 binds to erythrocyte complement receptor 1 (CR1) [129] and PfRh5 binds to erythrocyte CD147 or basignin [130]. These proteins establish an irreversible attachment to RBC, which commits merozoite to invasion and triggers subsequent events that lead to RBC entry [108,109]. On the other hand, invasins play an essential role in the merozoite invasion and the best characterized of these proteins, the apical membrane antigen-1 (AMA1) [131] was shown to be required for invasion [52,132–136]. AMA1 is a micronemal protein present at the merozoite surface that interacts with rhoptry neck proteins (the RON complex) [137–142]. AMA1 couples with RON2, which acts as anchor [143], therefore forming a bond between the RBC and the merozoite [109,141,144] that prompts the formation of the moving junction and release of the rhoptry contents into the host cell [109]. Upon the formation of the tight junction between the parasite and the RBC, the entry is mediated by the activation of the actin-myosin motor, using a similar mechanism as described above for hepatocyte invasion [145,146]. At this point, the merozoite is capable of creating the PV, the space inside the RBC in which it can move and further develop.

Succeeding the entry, at early ring stage development, the parasite starts to dramatically remodel the RBC through proteins that are exported into the cytoplasm and membrane of the RBC [98,147]. One of these proteins is the *P. falciparum* erythrocyte membrane protein 1

(PfEMP1) [148] that is expressed at the RBC membrane and contributes to cytoadherence properties of *P. falciparum*-infected RBCs (iRBCs) [149–151]. The PfEMP1 protein family is encoded by about 60 *var* (variable) genes [152] with monoallelic expression [153] that underpin an enormous sequence diversity and host cell-binding selectivity. These characteristics contribute to the wide range of clinical manifestations resulting from *P. falciparum* infection [154]. The export of these proteins is dependent on the establishment of an extraparasitic trafficking network in the host cell that comprises extraparasite organelles such as Maurer's clefts [155]. These are large, flat and disc-shaped mobile structures in the RBC cytoplasm [156–159] that traffic parasite proteins [156,158,160]. These structures are known to be responsible for the transport of the knob-associated histidine-rich protein (KAHRP) soon after merozoite invasion of the RBC. KAHRP is the main component of *P. falciparum* iRBCs knobs [160–163] that play an important role in the cytoadherence complex. At later stages, Maurer's clefts traffic the PfEMP1 into the formed knobs on the RBC surface [164,165]. Once the parasite reaches the schizont stage, the cytoadherence complex with the knobby structures are fully formed, and the RBC elasticity is reduced [166]. This promotes iRBC adherence to host receptors in the endothelia, which prevents the destruction of iRBC in the spleen and contributes to malaria pathogenesis. When the schizont is completely mature, the RBC ruptures to allow the egress of the newly developed merozoites that immediately invade a new RBC. This is a highly regulated event, critical for disease progression and involving multiple parasite and host-derived molecules [167]. Just before the egress of *P. falciparum* merozoites, the subtilisin-like serine protease SUB1 is discharged into the PV [168–170]. SUB1 is involved in the proteolytic maturation of members of the SERA family, which are implicated in the egress process [171]. In addition, SUB1 is also involved in the proteolytic process of MSP1, MSP6 and MSP7, indicating a role in merozoite preparation for the next RBC invasion [102].

A subset of the blood stage parasites differentiates into gametocytes towards the *Plasmodium* sexual stage pathway. The gametocytes mediate the transition between the vertebrate host and the mosquito [172–175]. The gametocytogenesis is the shift from asexual blood stage parasites into intraerythrocytes gametocytes and is initiated 7-15 days after the beginning of the blood stage. *P. falciparum* gametocytogenesis takes around ten days, while in other species this maturation process can occur in less than two days [176]. Up to now, little is known about the genes that regulate the gametogenesis and how the parasite is committed to leave the asexual cycle and enter the sexual stage. It is believed that gametocytogenesis can be triggered by environmental signals during infection [172–174]. The gametocytes maturation occur in five morphological stages [177], forcing the conformation of the RBC into the crescent shape of the gametocyte. Moreover, the gametocytes maturation also promotes significant changes at the molecular level that allow the parasite to adapt rapidly to the mosquito midgut [178]. The gametocytes gender determination is established when the schizont is committed to gametocytogenesis [179], with a ratio of one male for about five female gametocytes [180]. It is thought that this difference is due to the fact that each male gametocyte forms approximately eight microgametes in the mosquito midgut [172,180]. Across the duration of the first four morphological stages the gametocytes carrying-RBCs are sequestered in the bone marrow or in the spleen and are released into the bloodstream at the stage five [181,182], only becoming infectious to the mosquito after 2-3 days [183,184]. Subsequently and upon uptake of gametocytes by a female mosquito during a blood meal, occurs the gametes formation that initiates the *Plasmodium* sexual cycle in the invertebrate host.

Clinical Forms of Malaria

Malaria infection has a broad spectrum of clinical forms ranging from subclinical infection to life-threatening syndromes. Most frequently, clinical malaria symptoms include vague and non-specific symptoms like headache, fatigue, muscle aches, abdominal discomfort, and also nausea, vomiting and orthostatic hypotension. The majority of patients with uncomplicated infection show few clinical signs other than fever, mild anemia and splenomegaly [6,8]. Nevertheless, infection may evolve into complicated and life-threatening clinical forms, known as severe malaria, which encompasses a number of clinical syndromes, prominently, cerebral malaria, respiratory distress, and severe anemia [185]. These myriad of clinical manifestations often co-occur in the same individual and result from direct action and proliferation of parasite as well as exacerbated host inflammatory and immune responses that damage tissue and impair organ function. Key pathogenic events combine to cause severe disease, namely, rapid expansion of infected RBC mass, destruction of both infected and non-infected RBC, adhesion of iRBCs to endothelia, microvascular obstruction and inflammatory processes [9].

Severe anemia is the most frequent manifestation of severe malaria in young children in areas of high transmission and results from the combinatory effect of accelerated RBCs destruction in the spleen and ineffective erythropoiesis. Additionally, interactions between malaria infection with other parasite infections and nutritional deficiencies may favor anemia progression [186].

The main clinical manifestation of cerebral malaria is coma. Untreated CM is probably universally fatal and 3-15% of children who survive have long life neurological sequelae. Nevertheless, there is not a unified pathological picture of cerebral malaria (CM) - in some cases obstruction of cerebral capillaries and venules with iRBCs are

paramount while other cases evidence cerebral inflammation and breakdown of the blood−brain barrier [6].

Metabolic disturbances related to acidosis are a pathophysiological feature in many forms of clinical malaria, which can lead to respiratory distress syndrome and acute kidney injury [187,188]. In children with severe malaria, lactic acidosis is attributed to several causes, namely, production of lactic acid by the parasite, decreased clearance by the liver and other factors that reduce oxygen delivery to tissues, including microvascular obstruction, anemia and reduced blood flow [189–191]. In adults, the respiratory distress syndrome has been associated with inflammation-mediated endothelia damage and parasite sequestration in pulmonary vessels [192,193]. Also, acute kidney injury in adults is caused by tubular necrosis and is frequently associated with dysfunction of other vital organs, leading to multi-organ failure and high mortality [194,195].

The current understanding of malaria pathogenesis points to a complex correlation between clinical and pathogenic processes. Indeed, multiple pathogenic mechanisms have been implicated in specific clinical forms of malaria. At individual level, the clinical outcome of the malaria infection from a multifactorial etiological complex that includes parasite, environmental and host genetic components.

Host Genetics

Malaria human genetics

The notion that malaria parasites act as a selective pressure on the human genome has been supported by compelling evidence associating specific traits found in exposed populations with risk of malaria infection and resistance to severe malaria. The hypothesis that occurrence of certain hemoglobin mutations were associated with the geographical distribution of *Plasmodium* infection was firstly formulated by Haldane in 1949 [196]. Since then genetic epidemiology research has collected growing evidence for ethnic differences in susceptibility to malaria and for genetic adaptations to *Plasmodium* infection. It is estimated that 25% of the risk for developing severe malaria is attributable human genetic factors [197]. These findings provide a rationale to study the genetics of malaria susceptibility and to identify host genetic factors that control infection and severe disease phenotypes. Host genetic studies on malaria repeatedly found two main grounds of genetic variance associated to malaria resistance: genetic polymorphisms of RBCs genes and genes related inflammatory and immune responses.

Red blood Cell polymorphisms. Red blood cells (RBCs) are the elected site of *Plasmodium* infection in the clinical phase of malaria and RBCs genes are targets of the selective pressure exerted by malaria. The gene coding for hemoglobin β chain (HBB) represents a striking example with three different variants that correlate with malaria prevalence (HbS [198,199], HbC [200,201] and HbE [202,203]). These variants have very different geographical and ethnic distribution within malaria endemic regions, suggesting that independent genetic adaptations to malaria converged in the HBB gene. Genetic epidemiology evidence is suggestive of other malaria-related RBCs genetic traits, including alpha-thalassemia [204], G6PD deficiency [205], ovalocytosis [206] as well as

the Duffy-negative [207] and ABO [208] blood groups. This reinforces the notion that genetic variation in RBCs genes is successful in conferring genetic resistance to clinical malaria.

Inflammation and Immunity genes. Sequestration of infected RBCs in small blood vessels is a critical event in initiating tissue and organ damage driven by malaria infection. Development of severe malaria has been associated to polymorphisms in host receptors for infected RBCs adhesion, but the protective role of specific alleles often failed to replicate across populations. Numerous reports on CD36 [209], ICAM1 [210], PECAM1 [211] and CR1 polymorphisms [212] suggest that genetic variance in adhesion receptors impacts on cytoadherence of infected RBCs and might be relevant in natural resistance to disease. Nevertheless, current research does not yet provide a coherent picture on the mechanistic role of these genetic variants in severe malaria susceptibility.

Genetic investigations on immunity-related genes aim to provide insights on mechanisms of protective immunity in naturally exposed individuals that have profound implications for vaccine efficacy. Research in inflammatory genes has been focused in cytokines and cell receptors such as TNF-alpha, IFNG, IL4, IL12, IL1, IL10 [213], IFNAR1 [214], NOS2a [215] and TGFB2 [216]. In addition, genetic associations of severe malaria or parasitemia levels have been found with the genes coding CD40L and FCGR2A that control antibody response by B cells as well as HLA genes related to T cell responses specificity [213].

Genetic studies on human populations are hindered by allelic heterogeneity across ethnic groups and differential patterns of linkage disequilibrium, heterogeneity of the disease outcome as well as by confounding factors related to diversity in *Plasmodium* parasite and *Anopheles* mosquito factors and their interactions with the human host. Circumventing these challenges requires significant efforts to engage in genome-wide studies of large sample collections with detail

information of samples and high-quality clinical information. While these conditions might to be difficult to meet in malaria endemic regions the use of experimental models of genetic resistance to malaria may provide useful insights in the pathogenesis mechanisms and protective immunological responses to *Plasmodium* infections.

Malaria mouse genetics

Mouse malaria revealed to be a precious source of prototypical experimental systems to study malaria pathogenesis, overcoming the limitations of human malaria studies [217]. Human and mice have a very similar genome, more than 90% of the genomes can be partitioned into corresponding regions of conserved synteny and ~80% of mouse genes have orthologues in the human genome [218]. Additionally, it is well established that different mouse strains also exhibit distinct degrees of susceptibility to malarial infection [219]. These features set the ground to use mouse strains to identify host genetic components involved in *Plasmodium* infections.

In the mouse, two major genetic approaches have been used to identify genes and host molecular pathways that affect the response to *Plasmodium* infection. The reverse genetics approach tests the role of a particular candidate gene by infecting gene-manipulated mice that usually carry a loss-of-function mutation. The forward genetics approach represents an unbiased search for major gene effects that control the differential susceptibility to infection in genetic crosses of inbred mouse strains (reviewed by Schofield et al. [220]). Both reverse and forward genetic approaches have led to the identification of genetic factors involved in malaria resistance and severity.

The choice of experimental models with specific combinations of rodent *Plasmodium* species and mouse strains, is based on displayed phenotypes that usually mimic a given human malaria pathology. Up

to now, more than 30 inbred mouse strains have been tested against available rodent *Plasmodium* species, and the vast majority show some degree of susceptibility to infection [221–224]. Mouse malaria laboratory models use one of four rodent *Plasmodium* species - *P. berghei*, *P. yoelii*, *P. vinckei* or *P. chabaudi* that were first isolated from African wild rodents [225].

Usually, *P. chabaudi* infection is used to study malaria immune responses, blood-stage antigens and RBCs invasion [226]. The infection courses with a range of clinical signs such as anemia, splenomegaly, hepatomegaly, sequestration of infected erythrocytes in the liver and spleen that resemble typical *P. falciparum* malaria features [227]. However, *P. chabaudi* does not induce cerebral malaria [227]. *P. berghei* ANKA is the most used parasite to study experimental cerebral malaria (ECM), which is characterized by of neurological symptoms, including ataxia, hind limb paralysis, deviation of the head and coma that leads to death [228,229]. In mouse strains resistant to ECM, *P. berghei* is used to study other severe pathologies, namely, severe anemia, acute lung injury and placental malaria [230–232]. Decades ago *P. yoelii* was used to study ECM, but currently is extensively employed to investigate immune responses and in the development of vaccine candidates [233]. *P. vinckei* is the least characterized rodent *Plasmodium*, being mostly employed in antimalarial drug research [234,235]. Table I-1 summarizes the degree of susceptibility to malaria of commonly used laboratory mouse strains against different *Plasmodium* species.

The initial descriptions of mouse genetic resistance to *Plasmodium* infection date back to the 50's. These reports used genetic crosses of inbred mouse strains to characterize the effect of the genetic background on the response to the infection [219,236]. Since then, an impressive number of reports described and characterized more than twenty genetic loci (Table I-2) linked to rodent malaria resistance. The

Table I-1. Phenotype of inbred mouse strains after infection with rodent *Plasmodium*

Inbred Mouse Strain	*P. chabaudi chabaudi AS*		*P. berghei ANKA*		*P. yoelii yoelii 17XL*		*P. vinckei vinckei*	
	Surv.	Pathol.	Surv.	Pathol.	Surv.	Pathol.	Surv.	Pathol.
A/J	50% F; 0% M	SMA	0%	CM	-	-	-	-
BALB/c	100% F; 0% M	-	0%	HP&A	No	HP&A	No	-
C3H/HeJ	0%	-	0%	CM	-	-	-	-
C57BL/6	100%	A	0%	CM	No	HP&A	No	-
CBA	60% F; 40% M	-	0%	-	Yes	-	No	HP&A
DBA/2	100% F; 0% M	LP & SMA	0%	ALI;HP &A	Yes	None	-	-

F – female; M – male; Surv. – survival; Pathol. – pathology; A – anemia; SMA – severe malaria anemia; CM – cerebral malaria; HP – hyperparasitemia; LP – liver pathology; ALI – acute lung injury. (Source: Lamb et al. 2006 [221], Bopp et al. 2010 [222], Laroque et al. 2012 [223], Mouse Phenome Database [224] and MalariaImDB [308])

complexity of the genetic architecture behind disease resistance is commonly dissected by making use of crosses between genetically well-defined mouse strains that allow whole-genome phenotype-genotype segregation analysis and the mapping of resistance loci [237]. Other than survival analysis, the phenotype of interest is often a continuous variable related to disease (e.g. level of parasitemia). The identification of loci controlling such traits (quantitative trait loci, QTL) in a typical genetic experiment does not offer resolution to pinpoint a particular, but rather ascribes a genetic effect on chromosomal regions that might contain multiple genes [238]. Therefore, further analyzes are required to identify plausible candidate genes. Strategies for QTL fine mapping include genetic derivation and selection of mouse strains assisted by positional genetic markers such as microsatellites or single nucleotide polymorphisms (SNPs). These methods aim to obtain informative congenic and subcongenic strains, consomic strains, recombinant inbred lines and advanced intercross lines [239]. Using forward genetic approaches, eleven loci associated with

Table I-2. Genetic loci associated to murine malaria resistance.

Plasmodium Specie	Locus	Chr.	Phen.	Parasite Strain	Mouse Cross	Linkage	Candidate Genes	Reference
P. chabaudi	*Char1*	9	PP	adami DS	(SJL/J X C57BL/6J)F2	LOD = 6.6	*Trf, Rbp1, Rbp2*	Foote 1997 [242]
					(C3H/He X C57BL/6J)F2	LOD = 9.91		
				chabaudi AS	(SM/J X C57BL/6J)F2	LOD = 7.4	*Slc25a20, Prkar2a, Ip6k2, Nckipsd, Celsr3, Slc26a6, Uqcrc1, Col7a1, Ucn2, Pfkfb4, Shisa5, Trex1, Atrip*	Laroque 2012 [223]
	Char2	8	PP	adami DS	(C3H/He X C57BL/6J)F2	LOD = 8.83	*Hp, Ea1*	Foote 1997 [242] Burt 2002 [244] Lin 2006 [245]
				chabaudi AS	(A/J X C57BL/6J)F1 X A/J and (A/J X C57BL/6J)F2	LOD = 3.73	*Ea1, GypA, Il15, Erp1, Msr1*	Fortin 1997 [243]
				chabaudi 54X	F11 AIL (A/J X C57BL/6J)F1	LOD = 3.13	*Cspg3, Il12rb1*	Hernandez-Valladares 2004 [246]
	Char3	17	P	adami DS	(C3H/He X C57BL/6J)F2	LOD = 5.0	*H2 complex*	Burt 1999 [247]
				chabaudi 54X	F11 AIL (A/J X C57BL/6J)F1	LOD = 5.71	*Lta, Ltb, Tnf, Tnfsf, Ifi15, Thy2*	Hernandez-Valladares 2004 [246]
	Char4	3	PP	chabaudi AS	CS (A/J X C57BL/6) - AcB	LOD = 6.57	*Pklr*	Fortin 2001 [250] Min-Oo 2003 [251] Min-Oo 2007 [309]
	Char5	5	P	chabaudi 54X	F11 AIL (A/J X C57BL/6J)F1	LOD = 2.16	*Ache, Epo, Hspb1*	Hernandez-Valladares 2004 [246]
	Char6	5	P	chabaudi 54X	F11 AIL (A/J X C57BL/6J)F1	LOD = 2.05	*Ncf1, Act1*	Hernandez-Valladares 2004 [246]
	Char7	17	P	chabaudi 54X	F11 AIL (A/J X C57BL/6J)F1	LOD = 5.71	*C3, Il25, Ir5*	Hernandez-Valladares 2004 [246]
	Char8	11	P	chabaudi 54X	F11 AIL (A/J X C57BL/6J)F1	LOD = 1.9	*Il3, Il4, Il5, Il12b, Il13, Irf1, Csf2, Gdf9, Hspa4, Tcmp1, Tcf7, itk, Tim1, Tim3*	Hernandez-Valladares 2004 [256]
	Char9	10	PP	chabaudi AS	CS (A/J X C57BL/6)-AcB	LOD = 4.74	*Vnn1, Vnn3*	Min-Oo 2007 [257] Min-Oo 2011 [258]
	Char10	9	PP	chabaudi AS	AcB62 ($Pklrl^{90N}$) X CBA-Pkslc ($Pklr^{G338D}$)	LOD = 7.24	*Adam10, CD276/B7-H3, Csk, Pias1, Pml*	Min-Oo 2010 [259]
	Char11	X	PP	chabaudi AS	(SM/J X C56BL/6J)F2	LOD = 4.26	-	Laroque 2012 [223]

Table I-2. Genetic loci associated to murine malaria resistance. (Cont.)

Plasmodium Specie	Locus	Chr.	Phen.	Parasite Strain	Mouse Cross	Linkage	Candidate Genes	Reference
P. berghei	*Esmr*	18	Surv.	ANKA	(C57BL6 X DBA/2)F2	χ^2 =30.1 p=4.1x10^{-8}	*Csf1r, Pdgfrb, Cd14, Ii*	Nagayasu 2002 [262]
	Berr1	1	ECM	ANKA	(C57BL/6J X WLA) F1X C57BL/6J	χ^2 =18.98 p=1.3 x 10^{-5}	*Tgfb2, Tlr5, Tnfr5*	Bagot 2002 [265] Campino 2005 [269]
	Berr2	11	ECM	ANKA	(C57BL/6J X WLA) F1X C57BL/6J	χ^2 =16.51 p=4.8x10^{-5}	–	Bagot 2002 [265]
	Cmsc	17	ECM	ANKA	(CBA/N X DBA/2)F1 × CBA/N	χ^2 =26.18 p<0.0001	*Tnf, Lt, MHC classes I (H2-K), II (H2-IA, H2-IE)*	Ohno 2004 [268]
	Berr3	9	Surv	ANKA	(C57BL/6J X WLA) F2	LOD = 4.9	*Ccr1–5, Cxcr6, Ccr9, MyD88*	Campino 2005 [269]
	Berr4	4	HP	ANKA	(C57BL/6J X WLA) F2	LOD = 3.42	*Tlr4, Csf3r, C8b*	Campino 2005 [269]
	Berr5	19	Surv.	ANKA	(C57BL/6J X BALB/c) F2	LOD = 4.69	*Ifit1, Ifit2, Ifit3, Fas*	Berghout 2010 [271]
	Berr6	6	Surv.	ANKA	Genome wide of 32 mouse strains	LOD = 4.77	*Ppar-γ, Tsen2*	Bopp 2010 [222]
	Berr7	1	Surv.	ANKA	ENU (C57BL/6 X 129Si)	LOD = 4.03	–	Torre 2013 [278]
	Berr8	4	Surv.	ANKA	ENU (C57BL/6 X 129Si)	LOD = 6.7	–	Torre 2013 [278]
P. yoelli	*Mlrrq*	1	P	17X	BALB/c X BALB.D2	–	*Mtv-7* locus	Swardson 1997 [267]
	Pymr	9	P	17XL	[(NC/Jic×129Si/SvJ)× NC/Jic]	LOD = 4.4	*Trf, Cish, Cmkbr4*	Ohno 2001 [270]

Chr. – Mouse Chromosome; Phen. – Phenotype; PP – Peak Parasitemia; P – Parasitemia; Surv. – Survival; ECM – Experimental Cerebral Malaria; HP – Hyperparasitemia; – data not avalable

P. chabaudi infection, ten loci linked to *P. berghei* and two loci associated with *P. yoelii* infection have been reported (Table I-2).

Genetics of host resistance to *P. chabaudi*.

All mouse strains tested with *P. chabaudi* develop productive infections. However, there is significant variability in the kinetics of parasite growth and the infection outcome among inbred mouse strains [240]. Furthermore, susceptibility to infection is often gender dependent [240,241]. This implies that the outcome of *P. chabaudi* infection is controlled by host genetic factors. In 1997, two independent groups published the first two mouse loci controlling resistance *P. chabaudi* infection [242,243]. Linkage analysis of F2 progenies obtained from crossing susceptible strains, SJL and C3H/He with the resistant strain C57BL/6 identified the *chabaudi* resistance locus (*Char*) 1 and 2 [242] upon infection with *P. chabaudi adami* DS.

Char1 was mapped on Chromosome 9 in both crosses as a QTL that control peak parasitemia [242]. Further, an independent study in an F2 progeny generated from SM/J and C57BL/6 mouse strains infected with *P. chabaudi chabaudi* AS, confirmed the *Char1* locus as a key regulator of parasite density [223]. *Char2* mapped on Chromosome 8 was reported to control peak parasitemia in a (C3H/He X C57BL/6) F2 progeny [242] and parasitemia levels in both backcross and intercross progenies of susceptible A/J with C57BL/6 [243]. Subsequently, congenic [244] and subcongenic [245] mice for the *Char2* locus were generated and uncovered the considerable complexity of this locus, identifying in Chromosome 8 two controlling regions, one proximal locus located within the 35 - 42.6 cM interval and a locus in distal part of the chromosome (68.9 – 75.4 cM) [245]. Advanced intercross lines were derived from (A/J X C57BL/6) F1 progeny and confirmed this QTL and

refined the position of *Char2* on the distal part of the Chromosome 8, which apparently act from early stages of parasite infection [246].

A distinct locus was identified on Chromosome 17, *Char3*, from an F2 progeny of a (C3H/He X C57BL/6) cross that controls the time of peak parasitemia [247]. It was hypothesized that *Char3* controls the rate of parasite clearance. This locus was closely linked to the mouse major histocompatibility complex (H2), which had been previously linked to *P. chabaud*i infection resistance in mice [248]. More recently, this locus was dissected into two QTLs, with *Char3* being mapped on the proximal region of Chromosome 17 closely linked to the H2 [246].

A set of 37 recombinant congenic strains (RCS) between the susceptible A/J (A) and resistant C57BL/6 (B) parental mice [249] used to narrow down the *Char1* and *Char2* loci unveiled a new *P. chabaudi* resistance locus on Chromosome 3, *Char4* [250]. One of the RCS, AcB55, was highly resistant to the infection despite carrying the susceptible alleles at *Char1* and *Char2*. Subsequent linkage analysis of an F2 progeny of an (AcB55 X A/J) cross mapped the peak parasitemia determinant to *Char4* [250]. Further analysis of two resistant RCS, AcB55 and AcB61 (the later carrying the susceptible alleles at *Char 1* and *Char2*, but not the resistant allele at *Char4*) and subsequent sequencing of candidate genes detected a mutation that leads to an isoleucine-to-asparagine substitution at amino acid 90 of the *pklr* protein (pyruvate kinase), in both AcB55 and AcB61 congenic strains [251]. This remarkable finding exemplifies the power and applicability of mouse genetic studies to identify key genes in malaria host responses. Additional human genetics studies found associations of pyruvate kinase polymorphisms with resistance against *P. falciparum* infection [252–255].

Furthermore, the analysis of F11 progeny advanced intercross lines from A/J and C57BL/6 parents allowed the identification of four novel *Char*

loci and confirmed previously described loci as referred above [246]. The *Char5* and *Char6* loci, on Chromosome 5, showed opposite additive effects and seemed to act in the highest parasitemia days [246]. The *Char7* locus, a second locus mapped on Chromosome 17, distal to the H2 was proposed to control the parasitemia by acting in the early stages of the challenge [246]. The *Char8* locus, mapping on Chromosome 11 was involved in blood parasite clearance [256].

Recently, *Char9* and *Char10* were identified using RCS previously derived to fine map the *Char1* and *Char2* loci. *Char9* was mapped to Chromosome 10 through linkage analysis of an F2 progeny of (AcB55 X A/J) cross and was shown to regulate the blood-stage replication independently of *Pklr* [257]. Alterations of gene expression suggested the *Vanin* genes (*Vnn1*/*Vnn3*) as *Char9* candidate genes. The *Vanin* genes code for a pantetheinase that is involved in the production of cysteamine and the absence of the protein activity in tissues are associated with susceptibility to malaria [257]. Furthermore, when cystamine is administered *in vivo* not only displays anti-malarial activity but also dramatically potentiates the artemisinin antimalarial activity [257,258]. *Char10* was mapped on Chromosome 9 through analysis of an F2 progeny derived from (AcB62 X CBA-Pk^{slc}). The RCS AcB62 is susceptible to *P. chabaudi* infection although it carries the Pklr^{90N} mutation. This locus is associated with the modulation of the malaria severity in the context of pyruvate kinase deficiency [259].

The latest locus suggested to control *P. chabaudi* infection was mapped on Chromosome X by phenotyping an F2 progeny of a (SM/J X C57BL/6) cross. The SM/J allele(s) are associated with lower parasitemia, and this effect was exacerbated in males [223]. Additionally, several studies using mouse mutants for specific candidate genes revealed the involvement of many host genes in different clinical outcomes of *P. chabaudi* infection: the majority of these genes are

involved in immune system pathways, as is the case of Toll-like receptor 7 (*TLR7*) [260] and Macrophage migration inhibitory factor (*MIF*) [261].

Genetics of host resistance to *P. berghei* ANKA.

Mouse strains tested for infection with *P. berghei* ANKA (PbA) present distinct pathological features, but all succumb to infection. Interestingly, some mouse strains, such as C57BL/6, develop acute terminal neurological damages and are considered susceptible to ECM whereas strains resistant to ECM, such as BALB/c, die at later stages of severe anemia (Table I-1). By using either the occurrence of ECM or the time of death after PbA infection, several QTLs were identified in different chromosomal regions.

The first linkage analysis, identified a significant locus in the middle region of Chromosome 18 as a major determinant of resistance to ECM using an F2 progeny derived from C57BL/6 (susceptible) and DBA/2 (resistant) mouse strains [262]. Since the authors did not attribute a name to this locus, recently, it was named *Esmr* for experimental severe malaria resistance [263].

While laboratory mouse strains represent a limited genetic pool, in search for new genetic models for ECM, six out of twelve wild-derived mouse strains were found to resist to ECM [264]. One resistant strains (WLA) was crossed with C57BL/6 to identify genetic factors associated with ECM resistance. The analysis of informative backcrosses detected significant linkage in the distal regions of Chromosome 1 and 11 that control the appearance of ECM and were named *berghei resistance* (*Berr*) locus 1 and 2, respectively [265]. The highest association on *Berr1* was over marker D1Mit221, localized at 0.5 cM from the *Tgfb2* gene. The association strength of the *Tgfb2* gene suggested it as a plausible candidate gene [265] for CM resistance that was subsequently confirmed in Angolan children [216]. Interestingly, it had been shown that mouse

strains carrying the endogenous mouse mammary tumor virus-7 (*Mtv-7*) integrated on the distal part of the Chromosome 1 are resistant to ECM [266]. Moreover, this same locus was previously described as having a protective effect on disease outcome when mice carrying *Mtv-7* were infected with *P. yoelii* 17X [267].

A study that used a backcross progeny derived from CBA (ECM susceptible) and DBA/2 (ECM resistant) mouse strains mapped a locus near the H2-region, Chromosome 17, that control the ECM susceptibility. This locus was named by *Cmsc* (cerebral malaria susceptibility) [268]. Like the *Char3*, the *Cmsc* locus mapped proximal to the H2-region, close to *Lta* and *Tnf* genes. Suggesting that this locus represents a resistance factor common to *P. chabaudi* and *P. berghei* infections.

Furthermore, individuals from the F2 progeny generated by intercrossing the wild-derived WLA and C57BL/6 mouse strains presented spontaneous cure after infection with PbA [264]. A QTL analysis of the F2 progeny exploited the natural genetic factors behind this unexpected and unique phenotype. The segregation analysis revealed three loci on chromosomes 1, 4 and 9 differentially controlling survival time and parasitemia kinetics [269]. The WLA allele at Chromosome 1 locus co-localizes with *Berr1* and is associated to early death resistance. The C57BL/6 allele at the novel locus on Chromosome 9, *Berr3*, enhanced the probability of ECM cure and subsequent cure of hyperparasitemia. The WLA allele at the locus on Chromosome 4, *Berr4*, conferred resistance to hyperparasitemia [269]. Interestingly, *Berr3* mapped near *Char1*, involved in resistance to *P. chabaudi* infection [242] in a region that was also found to control survival and parasitemia after infection with *P. yoelii* 17XL [270]. The *Pymr* (*Plasmodium yoelii malaria resistance*) was revealed in a linkage analysis of a backcross progeny from (NC/Jic X 129/SvJ) cross [270]. The

co-localization of the *Berr3*, *Char1* and *Pymr* may provide an insight of the pervasive role of this region on Chromosome 9 in conferring resistance to *Plasmodia* infection.

Recently, two new loci associated with survival time upon PbA infections were identified. The *Berr5* locus that was mapped on Chromosome 19 using an F2 progeny derived from a C57BL/6 and BALB/c cross, which partially controls the differential response between C57BL/6 and BALB/c mouse strains [271]. The BALB/c allele in the *Berr5* locus increased survival through the cerebral phase [271]. *Berr5* co-localizes with three previously mapped loci affecting host response to other infectious or inflammatory stimuli: the *Trl-4*, tuberculosis resistance 4 locus [272]; the *Tsiq2*, T-cell secretion of IL-4 locus [273]; and the *Eae19*, experimental allergic encephalitis 19 locus [274].

The other locus, *Berr6*, was identified through a different genetic approach [222]. First 32 different inbred mouse strains were characterized after PbA infection, being the survival time the best trait that allowed forming three clusters: the susceptible cluster including strains with mean survival of 6.7 days; the intermediate cluster with mean survival of 9.6 days and the resistant cluster with mean survival of 13 days. The genome-wide analysis made use of 297,674 informative SNPs [222] and employed the haplotype associated mapping [275] and efficient mixed-model for association [276] to infer haplotypes controlling time of survival. This strategy allowed mapping the *Berr6* on Chromosome 6 in a region that contains only two genes, the *Tsen2,* and *Ppar-γ*, which shown to have differentially regulated expression in various tissues upon infection [222]. Interestingly, the *Ppar-γ* gene was previously linked to host response to malaria [277].

The latest loci identified that control ECM upon PbA infection was mapped on chromosomes 1 and 4 [278]. A genome-wide screen in N-ethyl-N-nitrosourea-mutagenized (ENU) mice revealed a strong

interaction of C57BL/6 and 129Si/SvImJ genomes in modulating the ECM resistance. The two new loci were mapped on the proximal part of the Chromosome 1, *Berr7*, and on the central region of Chromosome 4, *Berr8*. At *Berr7*, resistance to ECM is conferred by129Si alleles in a dosage-dependent manner while at *Berr8,* the C57BL/6 alleles are associated with resistance and is inherited in a co-dominant manner. Further, when mice are heterozygous for *Berr8* the resistance to ECM is modulated by the *Berr7* [278], denoting a mechanism of genetic interaction.

For most *Berr* loci identified up to now, the association spans large chromosomal regions. Though there are attractive candidate genes within their confidence intervals, the causative genes and polymorphisms underlying the ECM outcome in *P. berghei*-infected mice have not been identified. Nevertheless, several studies in mouse mutants highlighted the role of early pro-inflammatory responses in determining host-driven mechanisms in ECM pathogenesis.

Strikingly, vast majority of studies on host genetics in malaria relate to the blood stage infection, but little attention has been paid to host genetic factors controlling malaria liver stage infection.

The Liver as a Site of Pre-Erythrocytic Infection

The liver is the largest solid organ in the human body receiving about 80% of the blood supply from the gut, through the portal vein, and only 20% arterial blood supplied by the hepatic artery. This particular positioning in the circulatory system favors liver metabolization of absorbed nutrients but also exposes the liver tissue to microbial products, environmental toxins and food antigens that leak through the intestinal barrier. To face this exposure, the liver tissue is equipped to perform a broad range of inflammatory defensive responses and is considered an organ with specialized immunologic functions.

Most liver cell mass is composed of hepatocytes (70-80%) that perform the liver metabolic functions, while the non-parenchymal cells include sinusoidal endothelial cells (LSECs), stellate cells, dendritic cells, KCs and lymphocytes. This important component of immune-related cells plays a crucial role in the response against antigens and pathogens that reach the liver. The reticuloendothelial system in the liver acts through LSECs to remove soluble macromolecules and colloidal waste by endocytosis, whereas the KCs, which constitute 80-90% of the body tissue macrophages [279], eliminate microorganisms and insoluble waste by phagocytosis.

The liver resident lymphoid cells include natural killer cells (NK), natural killer T cells (NKT) and gamma-delta T cells (γδ T), located in the liver sinusoids and responsible for initiating local immunological reactions that may amplify into systemic immune responses. NK cells represent 50% of the liver lymphocytes and when activated are induced to synthesize IFN-γ and to perform perforin-dependent cytotoxic responses. Liver NKT cells are abundant both in mice [280] and humans [281], and their activation leads to cytotoxic effector functions and to abundant secretion of cytokines that interact with other cells in the liver [282]. These CD1d-reactive T cells recognize glycolipid antigens that are

conserved on bacterial cell walls. Antigen presentation in the liver can be performed by plasmacytoid dendritic cells (pDCs), KCs, LSECs, hepatocytes and possibly by stellate cells. Thus, the liver has a high potential for antigen presentation activity, allowing for local priming of T cells, which is helped by the distinctive architecture of hepatic sinusoids that permits circulating T cells to make direct contact with LSECs and KCs.

Nevertheless, the immunological responses triggered in the liver often leads to immunosuppression or tolerance induction. It has been argued that continuous exposure of liver cells to toxins and antigens derived from intestinal flora could be a driving mechanism in inducing liver tolerogenic responses. Such exposure would promote the expression of anti-inflammatory cytokines and the down-regulation of co-stimulatory signals in liver antigen-presenting cells that would lead to inactivation of antigen-specific T cells and/or induction of immune-regulatory mechanisms [283].

Thus, the liver provides a local environment that promotes T cell tolerance, which may offer opportunities to well-adapted pathogens to invade hepatocytes and establish infection while protected from the adaptive immune system. In this context, innate immunity responses acquire a central role in the liver defense against pathogens, which must adapt to circumvent these host responses and survive. As an example, hepatitis C virus (HCV) show mechanisms of innate immunity escape that target the toll-like receptor 3 (TLR3) pathway and the retinoic acid-inducible gene 1 (RIG-I) pathway, subverting NF-κB activation and IFN-β secretion [284,285]. A similar strategy of RIG-I pathway targeting is observed in hepatitis A virus (HAV) infection [286].

Likewise, it would be expected that the malaria parasite is able to evade innate immunity responses in the liver while progressing through liver stage development. We still lack a comprehensive view on how

Plasmodium liver stage forms escape the surveillance mechanisms in the liver. Nevertheless, a number of host genes and pathways were shown to impact on *Plasmodium* liver stage infection.

Host factors in *Plasmodium* liver stage infection

Successful liver infection by an individual sporozoite relies on a delicate balance of a multitude of host-parasite interactions that allows parasite expansion inside one hepatocyte and the production of thousands of merozoites, which eventually burst into the bloodstream. Across all steps of liver infection, the parasite needs to circumvent, block or subvert a variety of host factors and mechanisms that are mounted to impede initiation and spreading of liver infection.

Migration from the bloodstream into the liver parenchyma is a first step in liver infection where interaction with host factors may decide the fate of sporozoites. Extracellular matrix components and possible cell surface receptors play a critical role in sporozoite arrest in hepatic sinusoids. Highly sulfated heparan proteoglycans (HSPGs) expressed on hepatocytes, and stellate cells were shown to bind CSP and TRAP proteins from *P. falciparum,* as well as *P. berghei* sporozoites. On the other hand, KCs chondroitin proteoglycans bind to *P. falciparum* CSP and *P. berghei* sporozoites, while stellate cells chondroitin proteoglycans bind *P. falciparum* CSP and TRAP [26]. The molecular interactions occur between highly sulfated heparin-like oligosaccharides of HSPGs and two CSP domains, a thrombospondin-like cell-adhesive region II-plus at the C-terminus and a positively charged motif upstream of the conserved region I [287–289].

Little is known on how the parasite is allowed to cross the sinusoidal barrier. Low-density lipoprotein receptor-related protein 1 (LRP-1) expressed on KCs was shown to recognize CSP inducing a cAMP/EPAC-dependent signal transduction pathway that suppresses the respiratory

burst in KCs [290]. Furthermore, it has been demonstrated that KCs exposure to *P. yoelii* results in a non-inflammatory activation profile and may induce KCs apoptosis [291]. Thus, it is plausible that other surface molecules on KCs may interact with the parasite, impeding activation or inducing the suppression of innate immunity responses.

Once a sporozoite crosses the sinusoidal barrier it retains the traversal mode and crosses several hepatocytes [37]. It has been claimed that shifting to the invasive mode requires the action of host factors released during hepatocyte traversing. HGF signaling has been shown to sensitize hepatoma cells to *P. berghei* sporozoite infection [39], possibly by conferring resistance to induce cell death [292]. Nevertheless, experimental infection of different hepatoma cell lines showed that the induction of HGF signaling did not improve *P. falciparum* invasion [293] and was not crucial for infection by *P. falciparum* or *P. yoelii* [294], suggesting that other factors are involved in determining hepatocyte permissiveness to infection. Cell traversal causes the release of cytosolic factors that activate NF-κB [295], a central regulator of host regulatory inflammatory responses, resulting in a reduction of the infection load. Conversely, inhibition of NF-κB increases infection rates suggesting that a host inflammatory response is elicited during the hepatocyte traversal process. Toll-like receptors appear to play a significant role in this response, as MyD88 deficiency leads to inability to activate NF-κB upon membrane rupture resulting in increased infection [295].

In contrast to cell traversing, parasite invasion and PV formation seem to require specific interaction between hepatocyte cell surface and sporozoite components. CD81 expression at cell surface has shown to be necessary for PV formation by *P. yoelii* and *P. falciparum* sporozoites [53], but was not sufficient to confer susceptibility to *P. falciparum* infection [296] and not required for *P. berghei* invasion [297]. Although these

differences may reflect requirements of particular experimental systems, they provide evidence that host-pathogen molecular interactions are critical to parasite invasion and PV formation. In fact, it was recently shown that CD81 was required to rhoptry neck protein discharge during invasion [298]. CD81 co-localizes in cholesterol-rich microdomains on the cell surface [299] along with SR-BI, a high-density lipoprotein receptor that is needed for efficient sporozoite invasion [54,55]. The identification of CD81 and SR-BI are likely to represent a first glance into a complex of host-pathogen interactions that determine infection permissiveness.

Upon PV formation, the sporozoite undergoes differentiation and development into the merozoite stage [69,70], a process that involves intensive parasite replication into thousands of parasites called EEFs, representing a prominent cellular insult. In hepatoma cell lines and primary cultures of hepatocytes only a reduced number of EEFs reach maturity as compared to the number of sporozoites that are allowed to invade hepatocytes [300]. It is plausible that hepatocytes react to the invasion and strive to abort the infection through autonomous mechanisms of induced cell-death. The apoptosis pathways elicited by parasite infection are still poorly understood but is has been shown that parasite survival depends on inhibition of hepatocyte apoptosis mechanisms, including p53 down-regulation and inhibition of mitochondrial-dependent induced cell death [301,302]. Nevertheless, it is not clear whether neighbor non-infected cells are involved in the reaction against infected hepatocytes or if they participate in delivering apoptosis signals.

The outcome of liver stage infection results from balancing host inflammatory responses and parasite mechanisms to modulate such responses. *Plasmodium* infection has shown to induce expression of anti-inflammatory factors, as is the case of heme oxygenase-1 (HO-1).

Livers of sporozoite-infected mice have shown up-regulation of HO-1, which is required for the progression to blood stage infection. Conversely, over-expression of HO-1 resulted in lower liver parasite burden and lower production of pro-inflammatory cytokines [303]. On the other hand, it was recently shown that liver stage *Plasmodium* infection triggers a type I interferon response that mediates an inflammatory reaction in the liver. Intracellular *Plasmodium* RNA was claimed to work as a ligand of the cytosolic pattern recognition receptor Mda5, which activates a type I IFN response via the adaptor molecules Mavs and the transcription factors Irf3 and Irf7 [304]. This response decreases liver parasite load and affects the dynamics of liver stage infection, possibly because type I IFN signaling in myeloid cells contributes to the recruitment of immune cells to the inflammatory foci around infected hepatocytes [304]. In fact, recruitment of NKT cells in sporozoites-infected livers was shown to be dependent on the expression of interferon-alpha/beta receptor alpha chain (IFNAR1) [305]. Together, these works provide mechanistic evidence on molecular pathways through which the host recognizes sporozoite infection in the liver at very early stages and reacts by mounting inflammatory responses to subdue the parasite. Nevertheless, it is likely that other host factors and molecular pathways are involved in sensing and reacting to *Plasmodium* during liver stage infection.

Research in liver stage infection has highlighted specific host-parasite interactions at molecular level that allow the parasite to target the liver, invade the hepatocytes and establishes productive infections. Likely, this research will bring in the near future a clearer picture on how the parasite overcome host defense mechanisms in the liver to guarantee the passage to the next step of development (blood stage forms). It is reasonable to anticipate that investigating the complex molecular responses of different liver cell types when facing *Plasmodium* sporozoites and liver exo-erythrocytic forms may provide critical

information on potential targets for driving the liver system to halt liver stage infection.

Aims and Objectives

Many malaria-related traits have been analyzed, but the role of genetic host factors in controlling liver infection has seldom been addressed. Early observations suggested that natural genetic variance among mouse strains may underlie differences in susceptibility to infection with *Plasmodium* sporozoites [306].

The overall goal of this thesis was to identify host genetic factors that represent natural-occurring mechanisms of resistance to hepatic infection by *P. berghei*. The work plan comprised the following objectives:

- To identify and map genetic loci associated to malaria liver resistance phenotypes.
- Genetic fine mapping of liver resistance phenotype loci.
- To identify hepatic cellular components that mediate resistance to infection.

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CHAPTER 2

Malaria Liver Stage Susceptibility Locus Identified on Mouse Chromosome 17 by Congenic Mapping.

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Author Contributions

All experiments were conceived and designed by me, Carlos Penha-Gonçalves and Maria M. Mota, and executed by myself.

The data analysis was performed by me and Paulo Almeida.

Maria M. Mota contributed with reagents, materials and analysis tools.

The manuscript was written by Carlos Penha-Gonçalves and myself.

Summary

Host genetic variants are known to confer resistance to Plasmodium blood stage infection and to control malaria severity both in humans and mice. This work describes the genetic mapping of a locus for resistance to liver stage parasite in the mouse. First, we show that decreased susceptibility to the liver stage of *Plasmodium berghei* in the BALB/c mouse strain is attributable to intra-hepatic factors and impacts on the initial phase of blood stage infection. We used QTL mapping techniques to identify a locus controlling this susceptibility phenotype (LOD score 4.2) on mouse chromosome 17 (*belr1* locus). Furthermore, analysis of congenic mouse strains delimited the *belr1* locus boundaries distally to the H2 region. Quantification of parasites in the liver of infected congenic mice strongly suggested that the *belr1* locus represents a genetic factor controlling the expansion of *P. berghei* in the hepatic tissue. The mapping of *belr1* locus raises the hypothesis that host gene variation is able to control the progression of *Plasmodium* liver stage infection and opens the possibility that the human genomic region orthologue to *belr1* may contain genes that confer resistance to the human malaria liver stage.

Introduction

Malaria is caused by the hematoprotozoan of the genus *Plasmodium* and provides one of the best examples of how positive selective pressure upon host genetic variants may confer resistance to disease. For example, the mutation underlying sickle cell anemia conferred resistance to malaria (cited in [1]) other erythrocyte traits such as G6PD deficiency, alpha-thalassemia and hemoglobin C are thought to have beneficial effects by reducing parasite invasion or growth in the erythrocytes or by facilitating the elimination of infected erythrocytes (reviewed in [2]). More recently, genome-wide analysis using inbred mouse strains has revealed that a considerable number of chromosomal regions that enhance control of infection with different *Plasmodium* species (reviewed in [3]).

Genetic analysis of infection with *P. chabaudi* infection has identified nine loci that contribute to control of the parasitemia (*char1–9*) [3,4]. Analysis of recombinant mouse strains allowed the dissection of multipartite loci in *char2* [5,6], *char3* [7] and *char4* [8] regions and to the identification of positional candidate genes for the *char9* locus [9]. In particular, a mutation in the pyruvate kinase gene has been identified as mediating a protective mechanism to *P. chabaudi* infection involving increased splenic clearance of erythrocytes through hemolysis [10,11].

Similarly, resistance to cerebral malaria caused by *P. berghei* infection was mapped to four loci (*berr1*, *berr2*, *cmsc* and a locus on chromosome 18) [12–14]. In addition, genetic analysis of the course of *P. berghei* infection identified a locus that controls the resistance to lethal infection (*berr3*) and a locus enhancing survival time (*berr4*) [15]. Parasite clearance and survival to *P. berghei* infection was attributed to a combinatorial effect of *berr1* and *berr3* loci, illustrating that resistance

to infection may arise from genetic interaction among host resistance factors [15,16].

Many malaria-related traits have been analyzed, but the role of genetic variants in controlling liver infection has not been addressed. During the initial liver stage of infection, individual sporozoites infect hepatocytes and grow to large schizonts that finally differentiate into 10,000–40,000 merozoites that are released in the bloodstream and infect the red blood cells. A major obstacle to genetic studies in man is the fact that the liver stage infection is largely asymptomatic. Genetic studies in the mouse were initially focused on evaluating the role of MHC alleles in conferring protection induced by irradiated sporozoites [17]. More recently, analysis of knockout mouse models has proved the involvement of host genes like CD81 in the malaria liver stage [18]. As early observations suggested that natural genetic variance between mouse strains may underlie differences in susceptibility to infection with Plasmodium sporozoites [19] we have conducted a genetic mapping study to identify genetic factors controlling the liver stage of infection. We report that a quantitative trait locus (*belr1*) mapping to mouse chromosome 17 partially controls expansion of *P. berghei* sporozoites during liver infection.

Results

Course of liver stage infection

Early reports suggest that infection of BALB/c mice with *P. berghei* sporozoites resulted in low level of hepatocyte infection at the end of the liver stage (44 h post-infection) [19]. We quantified *P. berghei* 18S rRNA in the liver during the course of infection in BALB/c and C57BL/6 mice, after intravenous sporozoite injection. No difference was observed between the two mouse strains during the initial phase of infection but from 24 h up to 40 h post-infection the parasite burden in BALB/c mice was significantly lower (Figure II-1). Given that sporozoites invade the

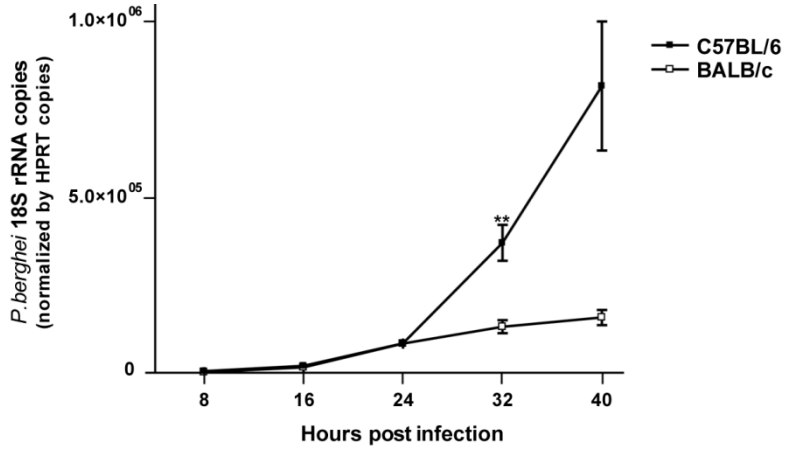

Figure II-1. Reduced expansion of *P. berghei* in BALB/c liver after intravenous injection.

P. berghei 18S rRNA was quantified by real-time PCR in the liver of C57BL/6 and BALB/c males at the indicated times after intravenous injection of 10^4 *P. berghei* sporozoites. Each data point represents the average value and standard deviation of parasite quantification in the liver of 5-10 mice.

liver in less than 1 h, this result suggested that the low parasite expansion in BALB/c mice was controlled within the liver. Next, we quantified the parasite burden in the liver of BALB/c and C57BL/6 mice infected by intra-hepatic injection (Figure II-2). Once again, the parasite burden

was lower in BALB/c mice confirming that the poor expansion of *P. berghei* in BALB/c mice was attributable to intra-hepatic factors.

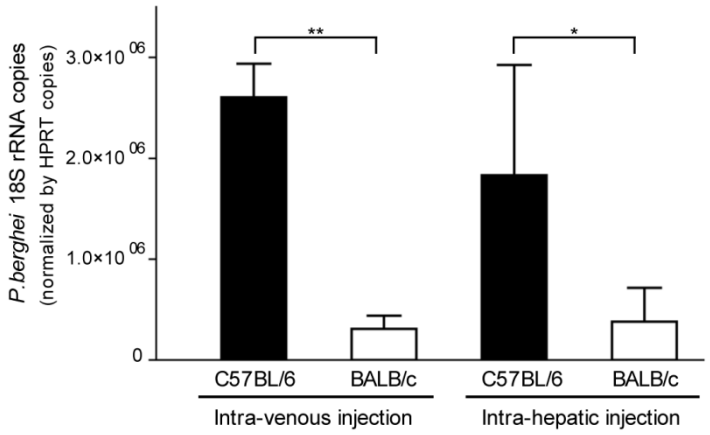

Figure II-2. Reduced expansion of *P. berghei* in BALB/c liver after intra-hepatic injection.

C57BL/6 and BALB/c mice were surgically injected under the liver capsule with 10^4 *P. berghei* sporozoites and compared with mice injected intravenously. *P. berghei* 18S rRNA was quantified by real-time PCR at 40 hours after injection. Average values and standard deviations for each experimental group are represented. Differences between groups were evaluated by unpaired t-test (* $p < 0.05$, ** $p < 0.01$).

Liver stage susceptibility phenotype

Blood smear analysis of parasitemia rising after sporozoite injection showed that BALB/c mice displayed lower parasitemia than C57BL/6 mice on days 4 and 5 after infection (Figure II-3), suggesting that the decreased parasite expansion in the BALB/c delays the development of blood stage infection. Thus, we used parasitemia at day 5 post-infection as an assay to assess the previously occurring parasite expansion in the liver. We studied the segregation of the day-5 parasitemia phenotype in genetic crosses of BALB/c and C57BL/6 mice and we observed that the first generation crosses showed an intermediate phenotype (Figure II-3) and that the phenotypic spectrum of the second generation progeny indicated that alleles

controlling the phenotype were segregating in the (C57BL/6 X BALB/c) F2 cross. The phenotype in the 115 F2 progeny approached a normal distribution suggesting that could be analyzed as a quantitative trait (Figure II-4).

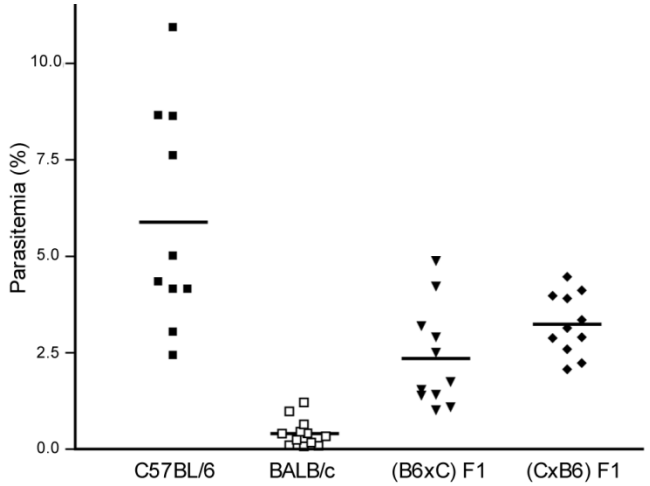

Figure II-3. The liver stage susceptibility trait.

Parasitemia was measured at day 5 post-infection with 10^4 *P. berghei* sporozoites in 10 C57BL/6 (dark squares), 12 BALB/c (white squares), 11 (C57BL/6 X BALB/c) F1 (inverted triangles) and 11 (BALB/c X C57BL/6) F1 (diamonds). Group averages are shown as horizontal bars.

Genetic mapping of liver susceptibility phenotype

In order to map the gene(s) controlling the parasite expansion in the liver, we used 93 microsatellite markers across the whole genome and scanned for quantitative trait loci (QTL) in the 115 (C57BL/6 X BALB/c) F2 progeny. Using QTL statistical analysis, significant linkage was found to the medial region of chromosome 17, where the highest linked marker D17Mit20 reached a LOD score of 4.2 (Figure II-5). No other region in the genome reached the genome-wide level of significance, but markers on distal chromosome 1 have shown suggestive linkage (LOD score 2.73), raising the possibility that a second locus mapping in this region may also contribute to the phenotype. The mapped locus in

chromosome 17 was named *belr1* locus (berghei liver resistance) and the genetic effect of the highest linked marker (D17Mit20) was estimated to explain 15.4% of phenotypic variance observed in the F2 progeny.

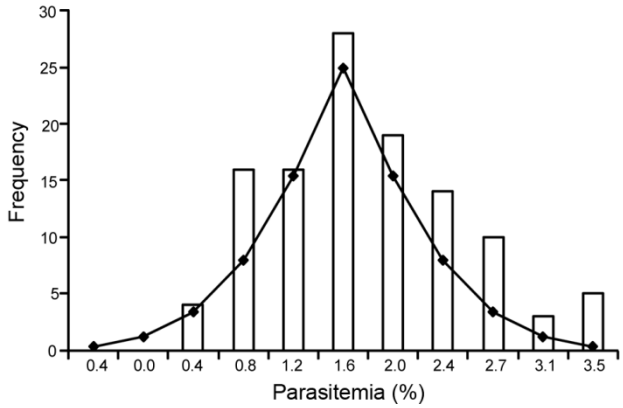

Figure II-4. Variance of liver stage susceptibility trait.

Frequency distribution of parasitemia at day 5 post-infection in the 115 (C57BL/6 X BALB/c) F2 genotyped in this study. The observed frequencies are represented in bars, and the predicted t-distribution curve is superimposed.

Congenic mapping of *belr1* locus

To confirm the genetic mapping of the *belr1* locus we analyzed the decreased susceptibility to liver infection in congenic strains containing defined chromosome 17 segments of one parental strain introgressed in the background of the other parental strain. The B6.C-H2d congenic strain carries a 38 Mb segment of BALB/c chromosome 17 encompassing the H2 locus in the genetic background of the C57BL/6 strain. Conversely, the C.B10-H2b strain carries the H2b haplotype in the BALB/c genetic background. We found that the B6.C-H2d strain showed decreased parasite burden in the liver, indicating that the genetic factor mediating the *belr1* effect is contained in this congenic region (Figure II-6A). In contrast, C.B10-H2b mice showed liver-stage resistance similar to BALB/c mice (Figure II-6B). These results demonstrate that the H2b haplotype *per se* is not controlling the liver

burden phenotype, and strongly suggest that the *belr1* locus is mapping distally to the H2 locus. The combined analysis of the congenic strains confines the *belr1* locus to a region of 28 Mb on

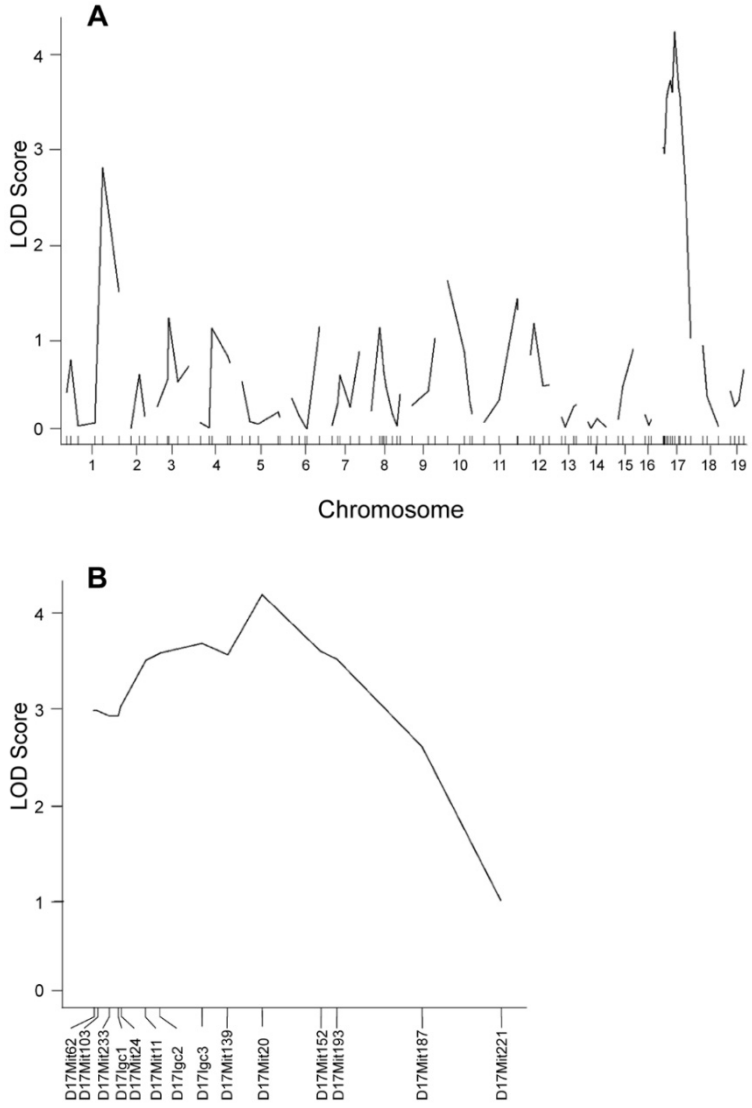

Figure II-5. Genome-wide mapping of liver stage susceptibility trait.

LOD score curves representing the likelihood for linkage of parasite burden trait. Genome-wide linkage is significant for LOD score above 3.3. (A) Shows LOD score curves for 19 mouse autosomes. (B) Shows LOD score curve for chromosome 17 where the X-axis ticks represent the relative position of microsatellite markers. Markers from left to right: D17Mit62; D17Mit103; D17Mit233; D17Igc1; D17Mit24; D17Mit11; D17Igc2; D17Igc3; D17Mit139; D17Mit20; D17mit152; D17Mit193; D17Mit187; D17Mit221.

chromosome 17 distal to the H2 locus (Figure II-7). As an alternative approach, we performed a conditional analysis in the F2 progeny that fixed the H2 genetic effect by using the D17Mit233 marker as a co-variate in the QTL analysis. Under this analysis, the LOD score curve still persisted on the *belr1* locus albeit with lower values. These results suggest that the *belr1* genetic effect is independent of the H2 locus, but do raise the possibility that genes in the H2d haplotype present in

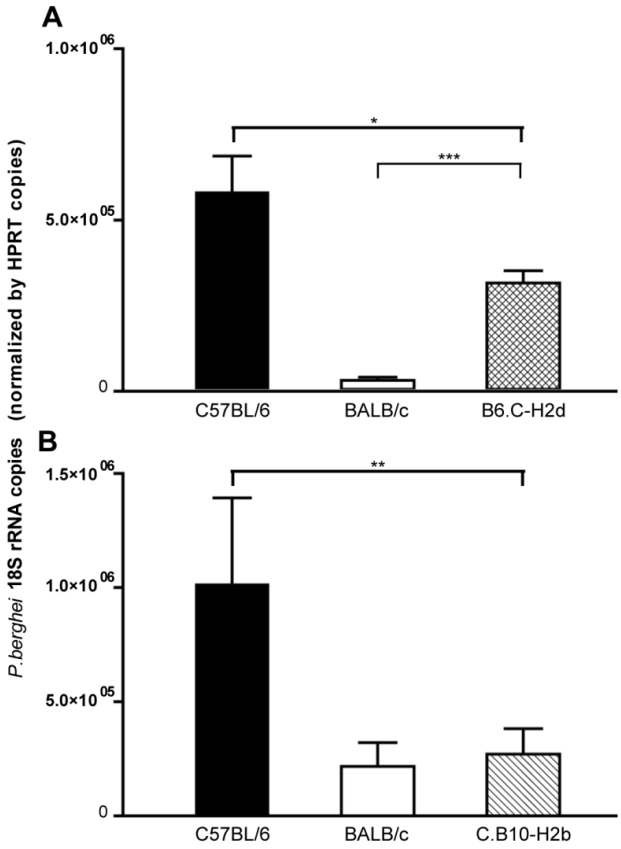

Figure II-6. The *belr1* locus controls *Plasmodium* liver burden.

P. berghei 18S rRNA was quantified by real-time PCR both in the livers of parental mouse strains (C57BL/6 and BALB/c) and in B6.C-H2d congenic mice (A) or in C.B10-H2b congenic mice (B) 40 hours after infection with 10^4 *P. berghei* sporozoites. The observed difference between C57BL/6 and B6.C-H2d is attributable to the congenic region in chromosome 17 of B6.C-H2d mice while the chromosome 17 congenic region in the C.B10-H2b shows no control over malaria resistance phenotype. Statistical significance was evaluated by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

the B6.C-H2d mouse strain may also contribute to the malaria liver-stage resistance.

Collectively, the results indicate that a genetic factor(s) controlling susceptibility to liver infection by *P. berghei* is located in the *belr1* locus and acts at the intra-hepatic level to control parasite liver stage expansion.

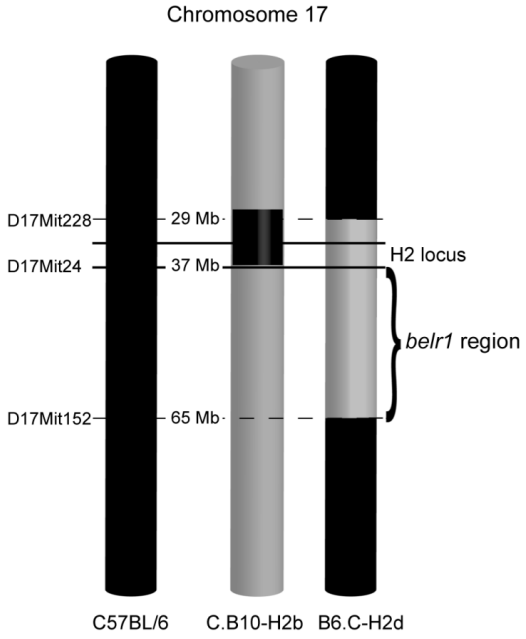

Figure II-7. Location of *belr1* locus in chromosome 17.

The diagram represents the physical map of chromosome 17 congenic regions in B6.C-H2d and C.B10-H2b mouse strains compared to the C57BL/6 strain. Delimitation of *belr1* locus is represented taking into account the malaria resistance phenotypes in the congenic strains.

Discussion

The key observation in this work is the demonstration that the *belr1* locus controls the expansion of *P. berghei* in the liver and that impacts on the level of subsequent blood parasitemia. Using mouse congenic strains the locus controlling parasite expansion in the liver was delimited within an interval of 28 Mb on mouse chromosome 17 close to the *MHC* locus previously linked to malaria resistance in both man and mouse. Although this analysis indicated that the major genetic effect on chromosome 17 maps outside the H2 region, it is still possible that the H2 locus could contribute to the observed phenotype. In addition, genetic markers in distal chromosome 1 suggest that a second locus could also be involved in the genetic control of this phenotype. Incidentally, this region coincides with a locus (*berr1*), that we have previously shown to control cerebral malaria and parasitemia clearance following *P. berghei* ANKA infection [12,15]. To further dissect such multiple genetic effects will include the generation and analysis of subcongenic strains carrying different congenic intervals derived from the B6.C–H2d mouse strain.

The possible involvement of the MHC region in the genetic control of resistance to malaria resistance in humans has stimulated a search for the contribution of the MHC and TNF molecules to the pathogenesis of malaria. For example, a significant number of reports claim genetic linkage and allelic association of these genes with severe forms of disease and re-infection risk [20–24]. At present, it is difficult to determine whether the genetic effects presented in these studies could result from genetic factors mapping just outside the MHC region like the *belr1* locus. It is of immediate interest to evaluate the human orthologue to *belr1* region to malaria genetic resistance in humans through unbiased association mapping studies.

Previous genetic analysis determined that mouse chromosome 17 contains loci exerting weak control of resistance to *P. chabaudi* parasitemia [25], in particular the H2-linked locus, *char3*, which mediates delayed parasitemia kinetics [26]. Further work suggested that these genetic effects were possibly contributed by an additional locus, *char7*, mapping distally to the H2 in a region that co-localizes with *belr1* locus [7]. Thus, it remains to be determined whether the locus described in this report coincides with *char7* locus, thereby representing a genetic factor controlling malaria infection at both liver and blood stages and having a relevant role in the disease caused by different murine *Plasmodium* species.

The *belr1* region contains 384 genes and includes known genes, predicted genes and other ORFs. Certainly, some genes are readily excluded from the liver resistance phenotype, as is the case of 49 genes coding for olfactory receptors. However, the presence of at least 80 unknown genes precludes the identification of plausible candidates. Subcongenic mapping will be performed to narrow down the number of credible positional candidate genes which will then be subjected to functional and structural analysis. The identification of candidate genes for the *belr1* locus may lead to the characterization of similar human genetic factors playing a role in malaria resistance by through their impact on asymptomatic liver infection.

It is likely that cell and molecular components that are involved in the observed host resistance exert their effect by limiting the expansion of the parasite liver stages in the hepatic tissue, raising the question of the underlying resistance mechanism. One possibility is suggested by the work with *P. berghei* [19] where the blockage of liver-stage parasite development in BALB/c liver has been correlated with the immediate liver inflammatory response [27]. However, the relevant effectors and target cells have not been defined. One attractive hypothesis is that,

liver resident cell types, such as Kupffer cells or NK and NKT cells act on the parasite, in either extracellular or intracellular forms.

Alternatively, the observed resistance to *Plasmodium* expansion could be mediated by a hepatocyte factor that controls the survival of infected hepatocytes, for example, sporozoite mediated inhibition of hepatocyte apoptosis. In fact, release of hepatocyte growth factor (HGF) [28] and signaling through the HGF/c-Met pathway [29] are considered as part of a chain of events that leads to apoptosis resistance of *P. berghei*-infected hepatocytes [30]. It has been shown that the initial protection of the host cell from spontaneous apoptosis mediated by HGF/c-Met signaling is extended in time possibly by induction of additional apoptosis resistance mechanisms [31]. We are currently testing this possibility by studying the induction of hepatocyte apoptosis in the presence of *P. berghei* sporozoites.

Searching for such cellular phenotypes in relevant congenic strains is part of a strategy to identify the cell type(s) mediating the *belr1* effect. If successful, this approach will accelerate the identification of the *belr1* gene(s).

Material and Methods

Mice. Parental mouse strains used in this study were C57BL/6, BALB/c, (BALB/c X C57BL/6) F1 and (C57BL/6 X BALB/c) F1. (C57BL/6 X BALB/c) F1 mice were used to generate the (C57BL/6 X BALB/c) F2 progeny of 115 males. Two H2 congenic mouse strains were obtained from the Jackson Laboratory (Maine, USA) and maintained at IGC. B6.C-H2^{d}/bByJ carries a BALB/c-derived congenic segment of 37.9 Mb on chromosome 17, flanked by markers D17Mit198 and D17Mit152, on C57BL/6 genetic background and is here referred to as B6.C-H2d. C.B10-H2^{b}/ LilMcdJ carries a C57Bl/10-derived segment of 10.4 Mb on chromosome 17, flanked by markers D17Mit80 and D17Mit232, on BALB/c genetic background and is here referred to as C.B10-H2b. The haplotype b of H2 is common to strains C57BL/6 and C57BL/10. All mice were bred and maintained in conventional housing facilities at the Instituto Gulbenkian de Ciência. All experiments used male mice with 8 to 15 weeks of age. All procedures were in accordance with national regulations on animal experimentation and welfare.

Infection. *Plasmodium berghei* ANKA sporozoites were obtained from dissection of salivary glands from infected female *Anopheles stephensi* mosquitoes. Sporozoites suspensions in RPMI medium were injected i.v. in 100 µl inocula of 10^{4} sporozoites per mouse.
To perform the intra-hepatic injection, the mice were anesthetized with Ketamine by i.p. injection. The abdominal cavity was surgically accessed through the medial line, partially exposing the left liver lobe. Sporozoites were injected under the liver capsule in a 50 µl inoculum. Then, the abdominal wall muscles and skin were sutured, and the animals were maintained until 40 h post-infection.

Parasite quantification. Parasitemia progression was measured at day 5 post-infection by blood smears with Giemsa. Liver parasite 18S rRNA was quantified by real-time PCR. Livers were collected at 40 h post-

infection, immediately homogenized in denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl and 0.7% b-mercaptoethanol in DEPC treated water) and total RNA was obtained using RNeasy Mini Kit (Qiagen). One microgram of total RNA was converted to cDNA (Transcriptor First Strand cDNA Synthesis Kit, Roche) and cDNA specific to *P. berghei* 18S rRNA was amplified with primers NYU-Pb1 5'-AAG CAT TAA ATA AAG CGA ATA CAT CCT TAC-3' and NYU- Pb2 5'-GGA GAT TGG TTT TGA CGT TTA TGT-3'. The real-time PCR reactions were performed in ABI Prism 7900HT system using ABI Power SYBR Green PCR Master Mix. Absolute *P. berghei* 18S rRNA estimates were normalized for mRNA of Hypoxanthine Guanine Phosphoribosyl-Transferase (HPRT), a mouse housekeeping gene.

Genotyping. Genomic DNA was prepared from mouse tails before infection, using standard techniques. The 115 (C57BL/6 X BALB/c) F2 progeny mice were genotyped by using conventional PCR protocols for 93 microsatellite markers obtained from the Whitehead/MIT Center for Genome Research collection (www. genome.wi.mit.edu/cgi-bin/mouse/index). Primers for Igc markers were: D17Igc1 For-GGG AGT GGG AAT TCT TTT ATT TTA and Rev-TGC TTT CTT CTG GTG TCT CTG AA; D17Igc2 For-GCT CAC TTT TTC CTA GCA TCA TC and Rev-GCC ATG GGA AGA AGT TAT ATG TC; D17Igc3 For-GAT AAG TTT GGA GTC AGG CCT AA and Rev-ACT TAT TCA CTC CTG AGC CTT GT. Individual mouse genotypes were scored using established agarose gel electrophoresis protocols.

Statistical Analysis. Quantitative trait locus analysis was performed using the R/QTL software [32]. This program calculates logarithm of odds (LOD) scores over intervals between linked markers, generating LOD score curves representing the likelihood of genetic linkage of quantitative phenotypes with markers along the chromosome. The level of statistical significance was empirically determined by

permutation tests (5000), and genome-wide significant linkage was considered when LOD ≥3.3 ($p<0.05$). Comparisons between groups of animals were considered statistical significant when the p-value of the unpaired t-test was <0.05.

Acknowledgements

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CHAPTER 3

TREM2 Governs Kupffer Cell Activation and Explains *belr1* Genetic Resistance to Malaria Liver Stage Infection.

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Author Contributions

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

The experiments were executed by me, Lurdes Rodrigues-Duarte, Joana Rodo and Luciana de Moraes.

The data analysis was performed by me, Lurdes Rodrigues-Duarte, Joana Rodo, Luciana de Moraes, Isabel Marques and Carlos Penha-Gonçalves.

The manuscript was written by Carlos Penha-Gonçalves and myself.

Summary

Plasmodium liver stage infection is a target of interest for the treatment of and vaccination against malaria. Here we used forward genetics to search for mechanisms underlying natural host resistance to infection and identified triggering receptor expressed on myeloid cells 2 (TREM2) and MHC class II molecules as determinants of *Plasmodium berghei* liver stage infection in mice. Locus *belr1* confers resistance to malaria liver stage infection. The use of newly derived subcongenic mouse lines allowed to map *belr1* to a 4-Mb interval on mouse chromosome 17 that contains the *Trem2* gene. We show that *Trem2* expression in the non-parenchymal liver cells closely correlates with resistance to liver stage infection, implicating TREM2 as a mediator of the *belr1* genetic effect. *Trem2*-deficient mice are more susceptible to liver stage infection than their WT counterparts. We found that Kupffer cells are the principal cells expressing TREM2 in the liver, and that *Trem2*−/− Kupffer cells display altered functional activation on exposure to *P. berghei* sporozoites. TREM2 expression in Kupffer cells contributes to the limitation of parasite expansion in isolated hepatocytes in vitro, potentially explaining the increased susceptibility of *Trem2*−/− mice to liver stage infection. The MHC locus was also found to control liver parasite burden, possibly owing to the expression of MHC class II molecules in hepatocytes. Our findings implicate unexpected Kupffer–hepatocyte crosstalk in the control of *Plasmodium* liver stage infection and demonstrate that TREM2 is involved in host responses against the malaria parasite.

Introduction

Malaria liver stage infection is asymptomatic but is absolutely required in the progression of *Plasmodium* infection in the vertebrate host, preceding propagation of parasites in the blood and clinical manifestations of malaria [1,2]. Current efforts in therapy and vaccine development include strategies aimed at deterring infection at the liver stage, preventing subsequent clinical complications and malaria transmission [3,4]. During liver stage infection, one *Plasmodium* sporozoite develops into thousands of merozoites inside each infected hepatocyte [5]. Identification of host genetic factors that control liver parasite expansion may help elucidate response mechanisms operating during liver stage infection.

Gene deficiency models and gene expression studies focusing on hepatocyte infection have highlighted genes that control hepatocyte invasion and intra-hepatocyte parasite expansion (e.g., CD81 [6], SR-B1 [7,8]), but the mechanisms of host response to liver stage infection remain elusive. It has been proposed that sporozoites' ability to traverse liver macrophages [9] and/or hepatocytes [10] in the course of liver stage infection may favor the release of pro-inflammatory factors at liver sites of sporozoite expansion [11,12]. Innate immune mechanisms might be involved in sensing *Plasmodium* sporozoites and in controlling liver stage infection [13].

Mouse models of liver stage infection suggest that sporozoites induce an innate inflammatory response associated with liver macrophage activation [14] and formation of inflammatory foci in the liver [15,16]. In mice, the severity of the inflammatory response depends on the genetic background, with, for example, BALB/c mice showing reduced sporozoite expansion in the liver and enhanced local inflammatory responses during malaria liver stage infection compared with C57BL/6 mice [17,18]. Little is known about host genetic factors and cell

mechanisms that control the natural resistance to liver stage *Plasmodium* infection, however.

In the present study, we aimed to dissect the resistance to liver stage *P. berghei* ANKA infection in mice. We previously reported that partial resistance to liver stage *P. berghei* infection in the BALB/c strain could be attributed to intra-hepatic factors. Genetic crosses with susceptible C57BL/6 strain mice revealed the contribution of mouse chromosome 17 and mapped the *belr1* resistance locus distally to the H2 locus [19]. Here we report the analysis of a newly generated panel of chromosome 17 subcongenic mouse strains that allowed us to unravel mechanisms of resistance to malaria liver stage infection mediated by the surface receptor TREM2 and MHC class II molecules.

Results

We used a subcongenic fine-mapping strategy to reveal the genetic and cellular basis of relative resistance to malaria liver stage infection observed in B6.C-H2d mice, a C57BL/6 congenic strain that carries both the belr1 locus and the H2 region within a 37.9-Mb chromosome 17 segment of BALB/c origin [19]. Ten subcongenic mouse lines were generated by backcrossing B6.C-H2d mice on the C57BL/6 mouse strain and then selected for recombination using a high-density set of region-specific genetic markers (Figure III-S1). Quantification of *P. berghei* ANKA rRNA at the end of the liver stage infection [at 40 h post-infection (p.i.)] served as a proxy of resistance against malaria liver stage infection in the subcongenic lines and allowed the dissection of two distinct controlling regions.

Trem2 expression correlates with the *belr1* resistance phenotype

Two double-recombinant subcongenic mouse strains that carried the MHC locus of C57BL/6 origin (R64 and R90) were instrumental in fine-mapping malaria liver stage resistance conferred by the *belr1* locus. This narrowed down to *belr1* within a 4.04-Mb region delimited by the upper recombination boundary of R64 (D17Igc5) and the upper boundary of R90 (D17Igc6) (Figure III-1A and B). Analysis of parasite expansion in hepatocyte primary cultures indicated that the resistance phenotype observed in the R64 subcongenic line was not attributable to hepatocyte factors (Figure III-1C); strongly suggesting that other cell types in the liver mediated the *belr1* effect.

Thirty-four protein-coding genes map within the *belr1* interval (D17Igc5–D17Igc6), including nine genes belonging to the Triggering Receptor Expressed on Myeloid cells (TREM) gene family (Table III-S1), orthologous to the TREM gene cluster on human chromosome 6 [20]. To correlate the

expression of genes in this interval with the liver stage resistance phenotype, we analyzed mRNA expression of all 34 protein-coding genes in isolated non-parenchymal liver cells (NPCs) from infected and

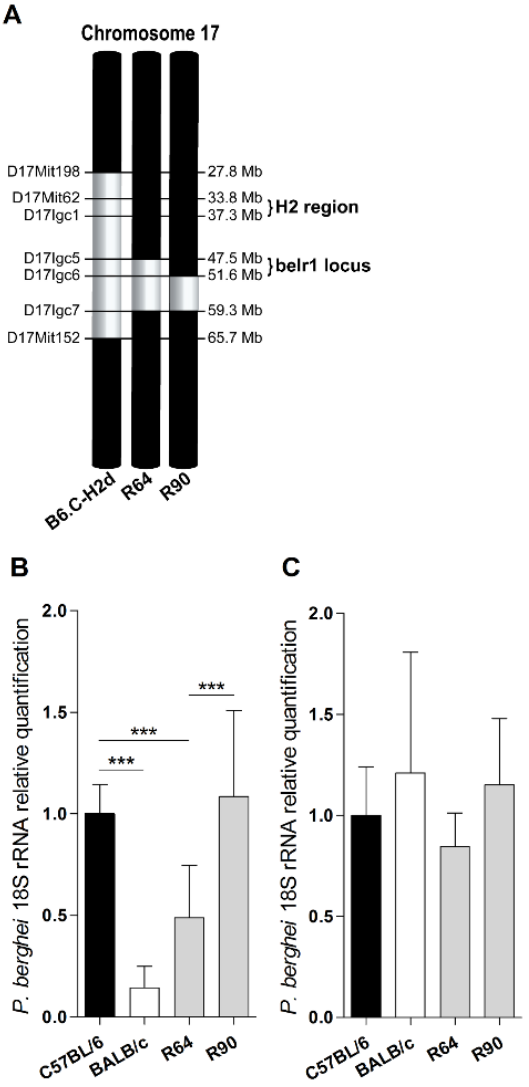

Figure III-1. *belr1* maps within a 4.04-Mb region of mouse chromosome 17.

(A) Diagram of BALB/c-derived congenic regions (in white) in R64 and R90 double-recombinant subcongenic lines with C57BL/6 background (in black). (B) Liver parasite burden in subcongenic lines at 40 h p.i. measured by rel- ative quantification of P. berghei 18S rRNA using a parental C57BL/6 strain as the calibrator. Data are mean ± SD; n = 10. (C) In vitro parasite yield in infected primary hepatocyte cultures from C57BL/6, BALB/c, R64, and R90 mice. Data are mean ± SD of triplicate cultures. *** $p < 0.0001$.

non-infected parental strains and subcongenic lines R64 and R90. Nineteen genes, including five TREM genes, were expressed in NPCs (Figure III-2 and Figure III-S2). Only *Trem2* mRNA up-regulation from non-infected NPCs correlated closely with the liver stage resistance phenotype in the subcongenic lines that define the *belr1* locus (Figure

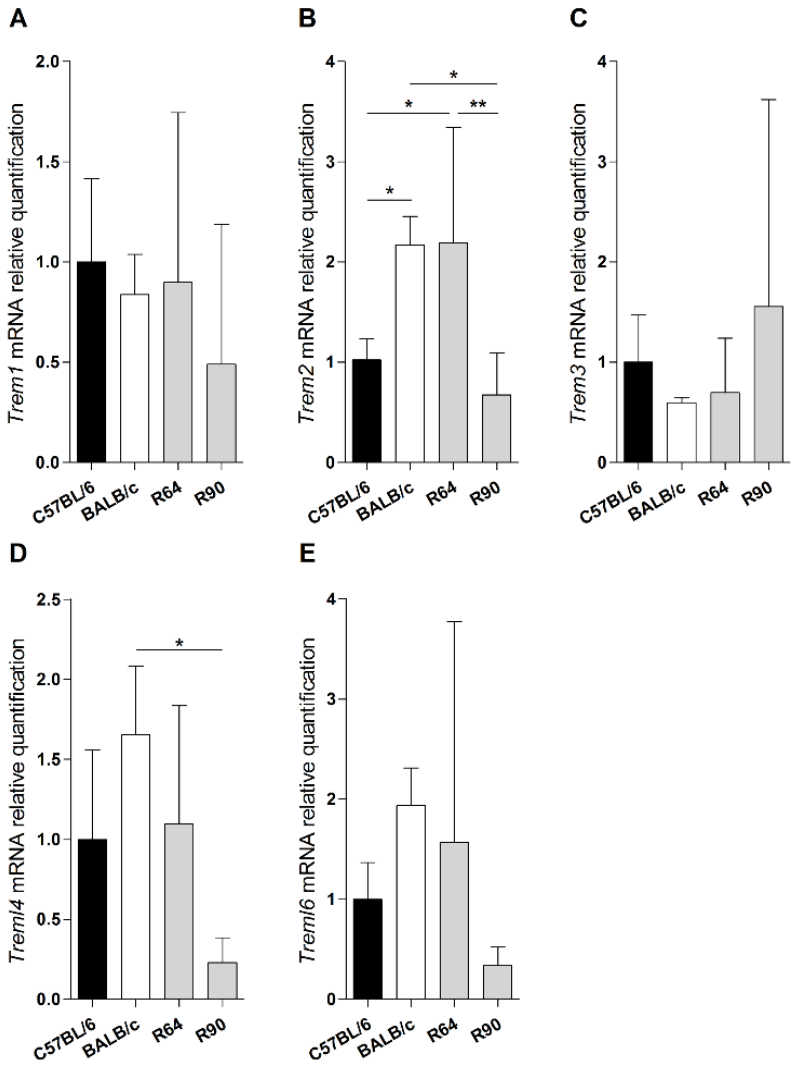

Figure III-2. *Trem2* mRNA expression correlates with resistance to liver stage infection.

mRNA quantification of Trem1 (A), Trem2 (B), Trem3 (C), Treml4 (D), and Treml6 (E) in NPCs isolated from noninfected R64, R90, and BALB/c mice, relative to the C57BL/6 strain. Data are mean ± SD; n > 6. * $p < 0.05$; ** $p \leq 0.001$.

III-2B). Given that TREM genes are expressed mainly in cells of monocyte/ macrophage lineage [21], these results suggest *Trem2* as a *belr1* positional and functional candidate gene.

Trem2 controls liver stage infection and polarization of Kupffer cells

To investigate whether TREM2 is involved in resistance to malaria liver stage infection, we analyzed *P. berghei*-infected *Trem2*−/− mice. We found that in the absence of TREM2 expression, parasite expansion in the liver was increased (Figure III-3A). We further analyzed TREM2 expression in NPCs from C57B/6 mice by FACS analysis and identified Kupffer cells (KCs) as the principal NPC type expressing TREM2 (Figure III-3B). Flow cytometry analysis of TREM2 protein expression in KCs from subcongenic and parental strains in C57BL/6 and R90 susceptible mice showed that a sizeable fraction of $F4/80^{+}$ cells expressed low to intermediate levels of TREM2 (Figure III-3C). A large majority of KCs from the resistant strains BALB/c and R64 expressed high levels of TREM2, indicating that surface expression of TREM2 in KCs correlates with the liver resistance phenotype conferred by the *belr1* locus (Figure III-3D).

Because liver stage parasite expansion occurs inside hepatocytes, we investigated whether KCs are able to control hepatocyte infection, using in vitro infection in co-culture assays. We found a reduced parasite burden when primary hepatocytes were co-cultured in direct contact with sort-purified WT KCs, but not with *Trem2*−/− KCs (Figure III-3E). This finding explains the increased susceptibility of *Trem2*−/− mice and indicates that TREM2 takes part in a mechanism enabling KCs to control the yield of hepatocyte infection.

We next used a Transwell culture system to examine whether direct KC–hepatocyte contact is required for the control of hepatocyte infection. We found no TREM2-dependent effects on parasite burden when hepatocytes were infected in the presence of soluble mediators

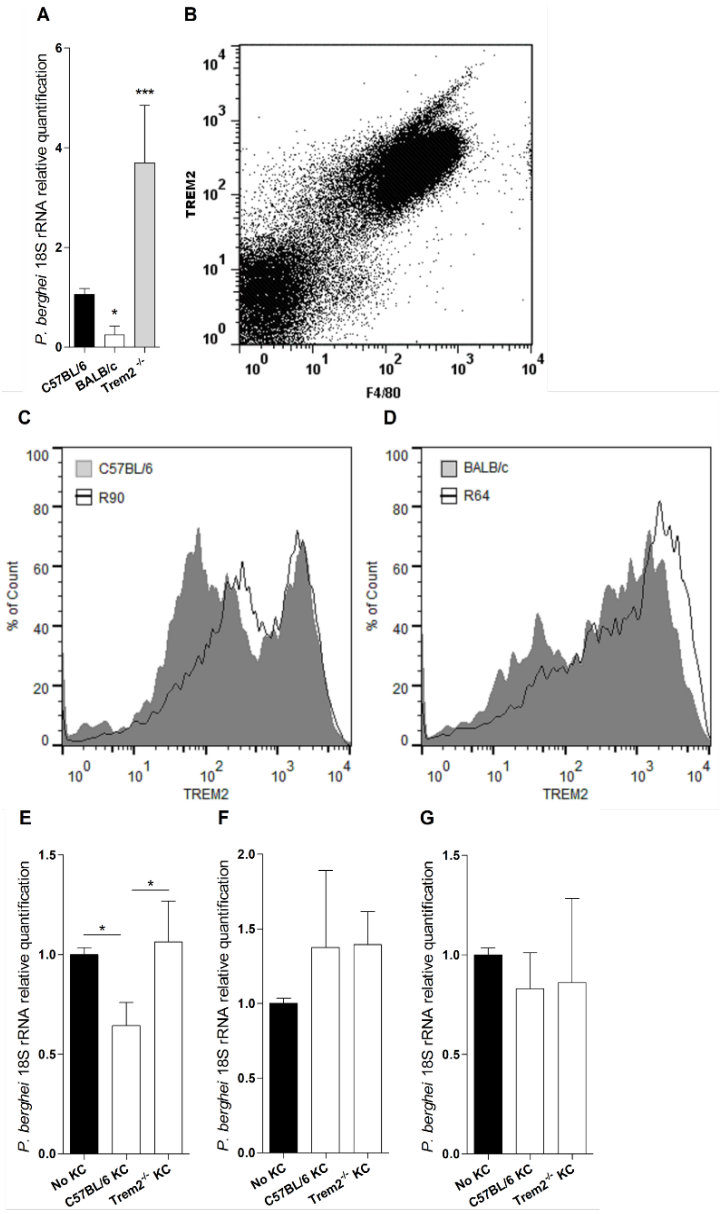

Figure III-3. *Trem2* deficiency dictates enhanced susceptibility to liver stage infection and reveals KCs' control of hepatocyte infection.

(A) Liver parasite burden in C57BL/6, BALB/c, and *Trem2*−/− mice at 40 h p.i. was quantified as described in Fig. 1. Data are mean ± SD; n = 10. (B) FACS plot of TREM2 and F4/ 80 surface expression in NPC from C57BL/6 mice. (C and D) FACS histograms of TREM2 expression in F4/80+ NPCs were obtained from C57BL/6 and R90 mice (C) or BALB/c and R64 mice (D). (E) Parasite yield at 40 h p.i. in isolated C57BL/6 hepatocytes or direct contact co-cultures with sorted-purified F4/80+ NPCs from C57BL/6 or *Trem2*−/− mice. Data are mean ± SD of triplicate cultures. (F and G) Parasite yield at 40 h p.i. in C57BL/6 hepatocytes cultured in the lower chamber of a Transwell system with or without sort-purified F4/80+ liver cells from C57BL/6 or *Trem2*−/− mice in the upper chamber (mean ± SD of triplicate cultures) using a 4 μM Transwell filter and infection in the upper chamber only (F) or using a 0.3 μM Transwell filter and infection in both the upper and lower chambers (G). * $p < 0.05$; *** $p < 0.0001$.

derived from WT or *Trem2−/−* KCs exposed to *P. berghei* sporozoites (Figure III-3G), or any differences when sporozoites were exposed to WT or *Trem2−/−* KCs before migrating to infect hepatocytes in the lower Transwell chamber (Figure III-3F). These results suggest that KC exposure to sporozoites does not induce the production of soluble macrophage-derived factors that influence the efficacy of liver stage infection, or affect sporozoite infectivity. Taken together, these *in vitro* results suggest that *Trem2* expression in KCs plays a key role in sporozoite-induced KC activation, which on direct KC–hepatocyte contact leads to decreased efficacy of liver stage infection.

Trem2 conditions the polarization of Kupffer cells by _P. berghei_

To test whether TREM2 expression has an impact on functional activation of KCs by *P. berghei* sporozoites, we profiled parasite-exposed cells measuring gene expression of activation markers associated to inflammatory effectors (M1 profile) or to anti-inflammatory properties (M2 profile) [22]. Sort-purified *Trem2−/−* KCs exposed to *P. berghei* sporozoites for 40 h expressed high levels of *Arg1* mRNA and low levels of *Il6*, *Il1b*, *Tnf*, and *CD68* (Figure III-4). In the absence of TREM2, exposure to *P. berghei* sporozoites determines KC anti-inflammatory activation, indicating that TREM2 is involved in functional polarization of liver macrophages. Although the TREM2 ligands remain unknown, these findings suggest that contact with parasite components impinge on TREM2 signaling, raising the possibility that the effect of TREM2 on inflammation during malaria infection is also exerted in subsequent phases of malaria pathogenesis. We found that *Trem2−/−* mice infected with *P. berghei* sporozoites developed blood parasite levels comparable to those seen in WT mice, but were highly resistant to cerebral malaria, a neuroinflammatory syndrome to which C75BL/6 mice are highly susceptible (Figure III-5).

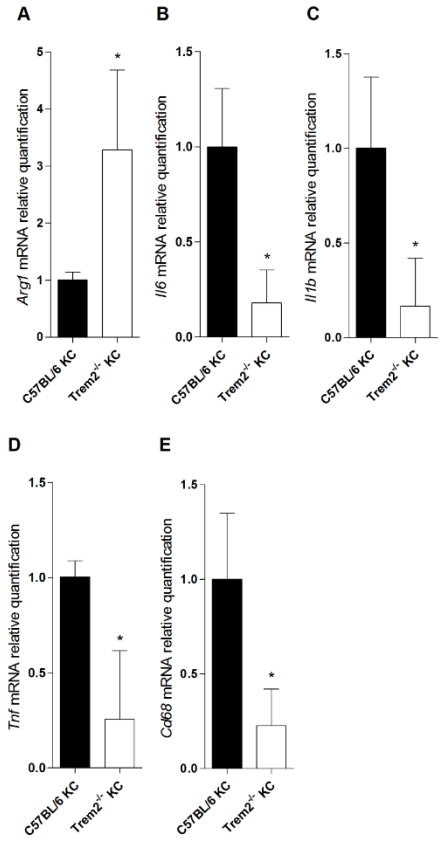

Figure III-4. *Trem2* determines KC functional polarization.

mRNA quantification of Arg1 (A), Il6 (B), Ilb1 (C), Tnf (D), and Cd68 (E) in cultured, sort-purified KCs of Trem2−/− mice relative to C57BL/6, after a 40-h exposure to P. berghei ANKA sporozoites. Data are mean ± SD of triplicate cultures. * $p < 0.05$.

Role for MHC class II molecules in hepatocyte resistance to infection

We further analyzed eight B6.C-H2d single-recombinant subcongenic lines and identified a 5.9-Mb region proximal to the *belr1* locus that contributes to resistance to malaria liver stage infection (Figure III-S3). Subcongenic lines R13 and R7 delimit this region within the D17Mit228–D17Igc1 interval, encompassing the H2 locus. This finding implies a role of the H2 locus, along with *belr1*, in controlling the resistance to liver stage infection in the B6.C-H-2d strain [19]. Mice deficient in MHC class II genes had a reduced liver parasite burden compared with WT mice.

Lack of expression of MHC class II molecules in purified hepatocytes decreased the in vitro expansion of liver stage parasites (Figure III-S3), suggesting that the effect of H2 is independent of antigen presentation functions.

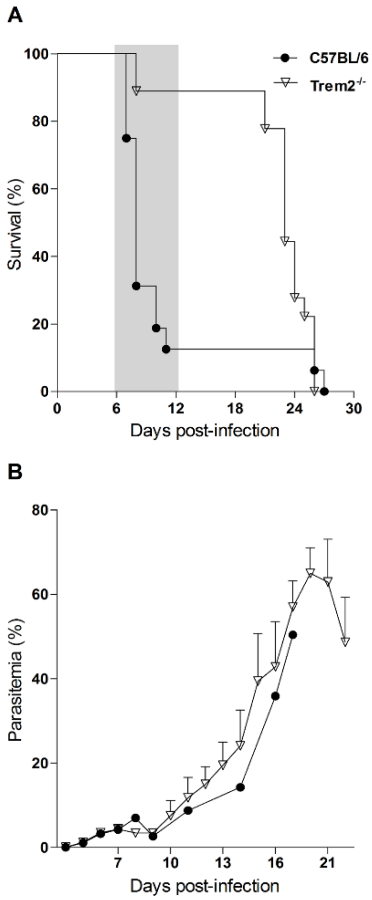

Figure III-5. *Trem2* deficiency confers resistance to cerebral malaria.

(A) Kaplan–Meyer survival curves after infection with P. berghei sporozoites of Trem2−/− and C57BL/6 mice (n = 10 per group). The time window of fatal cerebral malaria development in C57BL/6 mice is shaded while death at a later stage is due to hyperparasitemia (P = 0.010, log-rank test). (B) Parasitemia was followed from day 4 to day 18 p.i.. Data are mean ± SD for each group.

belr1 and H2 did not demonstrate an additive effect on the malaria liver resistance phenotype. Mouse lines containing both regions of BALB/c origin (i.e., R108 and R41) did not differ from lines carrying only

one of the two controlling loci (i.e., R13 or R64). These results indicate that natural resistance to liver stage infection, previously ascribed to mouse chromosome 17 [19], can occur via one of two routes, dependent on either MHC class II expression in hepatocytes or on TREM2 expression in KCs.

Discussion

The study of malaria liver stage infection has focused primarily on *Plasmodium*–hepatocyte interactions during invasion, intracellular parasite growth, and parasite egress [2,23,24]. *Plasmodium* sporozoites have been found to directly contact liver macrophages in the liver [9], modulating their cytokine profile [12]. This work identifies TREM2 as underlying the *belr1* genetic effect and implicates the functional activation of KCs by *Plasmodium* sporozoites in governing the resistance to liver stage infection.

Our findings imply that the C57B/6 and BALB/c parental strains carry different TREM2 genetic variants. Comparison of the published TREM2 genomic sequence in BALB/c and C57BL/6 mouse strains revealed only three single nucleotide differences: rs107941689 upstream of TREM2, rs49686564 in intron 2, and rs479921182 downstream of TREM2. rs107941689 maps 4.5 Kb upstream of the TREM2 protein coding sequence within a putative sequence regulatory element, suggesting a possible role in TREM2 transcriptional regulation (Ensembl, release GRC m38). This is in line with the observation that the level of TREM2 surface expression in KCs correlates with the liver resistance phenotype in the congenic strains that define the *belr1* locus (Figure III-3). Although our data suggest a role for *Trem2* in malaria liver stage infection, we cannot completely exclude the possibility that other genetic factors in the *belr1* region could contribute to the liver stage resistance phenotype. mRNA expression analysis of protein-coding genes mapping within the *belr1* region did not yield any other candidates.

Our data suggest that KCs play a role in liver stage infection at two stages: first, through activation by *Plasmodium* sporozoites in the course of their migration from sinusoidal vessels to invade the hepatocytes, and second, in the crosstalk with infected hepatocytes, leading to reduced intra-hepatocytic parasite expansion. The idea that KC

activation by *Plasmodium* sporozoites decreases the success of hepatocyte infection is corroborated by previous reports of activation on contact with sporozoites [12] and involvement in parasite clearance [25]. The precise mechanism of KC crosstalk with infected hepatocytes requires further study to identify molecular pathways involved in this natural control mechanism of liver stage infection.

Expression of TREM2 in liver macrophages is in line with reports of its expression in resident macrophages in other tissues [21]. TREM2 signaling was initially associated with negative regulation of macrophage activation [26], but multiple independent observations of TREM2 pro-inflammatory effects on macrophage activation in bacterial infections have been reported [27,28]. This suggests that cellular functions of TREM2 may be context-dependent. Our findings indicate that TREM2 is involved in determining pro-inflammatory macrophage activation on contact with the malaria parasite, similar to the profile recently described in microglia cells [29]. Indirect evidence suggests expression of TREM2 ligands by both yeast and bacteria [30], supporting a role for TREM2 as a pathogen sensor [31]. Our results indicate that *P. berghei* sporozoites activate macrophages in a TREM2-dependent fashion, suggesting that *Plasmodium* expresses as-yet unidentified TREM2 ligands.

TREM2 has been shown to contribute to both inflammatory and phagocytic responses to infectious agents [31,32]. Our in vitro experiments suggest that the triggering of TREM2 in macrophages is part of a decisive effector signal that enables a degree of control of parasite expansion inside hepatocytes, possibly by promoting killing/phagocytosis of infected hepatocytes and decreasing the yield of mature parasites. Histological examination of sporozoite-infected livers revealed no significant difference in the number of inflammatory foci between *Trem2−/−* and WT mice. Analysis of *P. berghei* infection

over time in *Trem2*−/− mice led us to conclude that TREM2 plays a dual role in disease progression, favoring resistance against malaria liver stage infection while also promoting tissue-damaging responses elicited by infected erythrocytes (e.g., in the brain), further supporting a pro-inflammatory role for TREM2 in malaria infection. TREM1 has been implicated as a biomarker of macrophage activation in human malaria patients [33]. TREM family genes are expressed mainly in the monocytic/macrophage lineage [34]; our results support the idea that TREM genes control macrophage functional activation and thus intervene in innate immune responses, raising the possibility that TREM genes control effector functions that ultimately have impact on disease outcome in infections such as malaria.

The genetic dissection of two malaria liver stage resistance loci in mouse chromosome 17 (*belr1* and *H2*) underlies the polygenic nature of the phenotypic difference between the BALB/c and C57BL/6 mouse strains. Although our observations pertain to *P. berghei*, it is possible that these genetic factors may control liver stage resistance against other *Plasmodium* species as well.

Our findings in this study demonstrate that the identification of genetic factors underlying natural resistance to malaria liver stage infection provides insights into the host response to *Plasmodium* infection. Although multiple parasite–hepatocyte interactions are likely involved in successful infection of hepatocytes by the malaria parasite, we propose that KC functional activation is an effective mechanism for controlling the host response to malaria liver stage infection.

Material and Methods

Mice. All procedures involving laboratory mice were performed in accordance with national (Portaria 1005/92) and European regulations (European Directive 86/609/CEE) on animal experimentation and were approved by the Instituto Gulbenkian de Ciência's Ethics Committee and the Direcção-Geral de Veterinária. Mice were bred and maintained in conventional housing facilities at the Instituto Gulbenkian de Ciência. H2-Ab1−/− and B6.C-H2^{d}/bByJ mice were obtained from the Jackson Laboratory. B6.C-H2^{d}/bByJ is a congenic mouse strain that carries a BALB/c-derived congenic segment of 37.9 Mb on chromosome 17 in a C57BL/6 genetic background and is referred to herein as B6.C-H2d. Trem2-deficient mice [35] were kindly provided by Marco Colonna, Washington University School of Medicine, St. Louis, MO. To generate subcongenic lines [36], B6.C-H2d male mice were backcrossed to C57BL/6 females. In the second backcross generation, chromosome 17 recombinants were selected from among 187 progeny by individual genotyping with a high-density set of microsatellite markers covering the B6.C-H2d congenic region (Figure III-S1). Markers were selected from the Whitehead/ MIT Center for Genome Research collection (now at http://www.informatics. jax.org) or identified as polymorphic between C57BL/6 and BALB/c and termed Igc markers (Table III-S2). Ten recombinants (eight single and two double) were selected and expanded in a third backcross generation and bred to homozygosity by brother–sister mating in the fourth generation. All experiments were conducted using male mice age 8–15 weeks.

Genotyping. Tail genomic DNA was isolated, and genotyping was performed, applying conventional PCR and electrophoresis protocols for amplification and detection of polymorphic microsatellite markers

(Figure III-S1). Primers for the markers identified in this study are listed in Table III-S2.

Parasites and Infection. GFP-expressing *P. berghei* ANKA sporozoites [37] were obtained by dissection of infected salivary glands from *Anopheles stephensi* mosquitoes bred in the insectarium at the Instituto de Medicina Molecular, Lisbon, Portugal. Sporozoite suspensions in RPMI medium were injected intravenous (i.v.) in 100 µl of inocula containing10^4 sporozoites per mouse. Livers were collected at 40 h p.i. or survival, and parasitemia was followed for 28 days (d). For experimental cerebral malaria scoring, neurologic symptoms were monitored from day 5 p.i. Hepatocyte primary cultures were infected with 4×10^4 *P. berghei* ANKA sporozoites, and non-infected controls were mock- infected with salivary glands from non-infected mosquitoes.

Liver Cell Preparation. Primary mouse hepatocyte cultures were prepared as described previously [38]. In brief, liver lobes were perfused, and hepatocytes were dissociated and separated using 1.12 g/ml, 1.08 g/ml, and 1.06 g/ml Percoll gradients (GE Healthcare). Hepatocytes were harvested from the gradient and cultured in Gibco Williams' E complete medium (Life Sciences). NPCs were obtained as described previously with modifications [39]. In brief, liver lobes were removed and perfused with liver perfusion medium (Life Sciences) supplemented with 750 mg/l of Collagenase H (Roche) at 37 °C. The resulting suspension was filtered through a 100 µm cell strainer (BD Falcon; BD Biosciences), and cells were suspended in RPMI complete medium (Life Sciences) and then mixed with Percoll solution (GE Healthcare) to a final concentration of 30% Percoll, followed by centrifugation at 850 × g for 10 min. The cell pellet was resuspended in RPMI and carefully layered on 30% Percoll solution, then centrifuged at 850 × g for 10 min. The cell pellet was washed and resuspended in ACK (NH4Cl 0.15 M, KHCO3 10

mM, Na2EDTA 2H2O 0.1 mM; pH 7.2) for 3 min to lyse remaining erythrocytes. Cells were washed and centrifuged at 153 × g for 20 s to discard the remaining hepatocytes. The supernatant was recovered, and NPCs were collected at 478 × g for 5 min.

Flow Cytometry and KC Sorting. NPC preparations were stained with F4/80 APC (A3-1; Serotec) and anti-TREM2 PE antibodies (LifeSpan Bioscience) at 4 °C. A FACScalibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star) were used for FACS analysis. Purified KCs were obtained by high- speed cell sorting using a FACSAria cell sorter (BD Biosciences) after staining of NPC preparations with F4/80 APC (Serotec).

Hepatocyte-KC Cultures. Two culture systems were used. Sorted-purified KCs (from WT or Trem2−/− mice) were added to WT primary hepatocyte cultures that had been plated 24 h previously. The culture setups allowed modulation of the degree of crosstalk between cell types: in direct contact or separated by filters of different pore size. KCs in direct contact with hepatocytes were seeded at a 3:1 ratio and infected with 4 × 10^4 *P. berghei* ANKA sporozoites after 12 h. In filter-separated culture, KCs were seeded in the upper chamber of a Transwell culture system (Millipore). Two different filter systems were used. The first system consisted of a 0.4 μm filter that allows the exchange of soluble factors, but not parasites, between KCs (upper chamber) and hepatocytes; both chambers were infected with sporozoites. In the second system, to test changes in sporozoite infectivity on exposure to KCs, we used a 3 μm filters and infected only the upper chamber, allowing sporozoites to contact KCs and migrate through the filter to invade hepatocytes in the lower chamber. Hepatocytes were collected for parasite quantification at 40 h p.i. For KC activation studies, we allowed FACS-sorted KC cells to contact *P. berghei* sporozoites (1:1) for 40 h before collection for RNA analysis.

Parasite and Gene Expression Quantification. Livers were collected at 40 h p.i. and immediately homogenized, and total RNA was extracted using the RNeasy Mini Kit (Qiagen). Then 1 µg of total RNA was converted to cDNA (Transcriptor First-Strand cDNA Synthesis Kit; Roche). Cultured cells were collected at 40 h p.i. Cell lysis and reverse-transcriptase reactions were performed using the TaqMan Gene Expression Cell-to-CT kit (Ambion). cDNA specific to *P. berghei* 18S rRNA was amplified with TaqMan-specific primers: forward, 5′-CCG ATA ACG AAC GAG ATC TTA ACC T-3′; reverse, 5′-CGT CAA AAC CAA TCT CCC AAT AAA GG-3′; probe, 5′-ACT CGC CGC TAA TTA G-3′ (FAM/MGB). Trem1, Trem2, Trem3, Treml4, Treml6, Arg1, Il6, Il1b, Tnf and Cd68 expression was quantified using best-coverage TaqMan Gene Expression Assays (Applied Biosystems). Endogenous control GAPDH (Mouse GAPD Endogenous Control; Applied Biosystems) was used in multiplex PCR reactions (Prism 7900HT; Applied Biosystems). Relative quantities were calculated by the ΔΔCt method.

Sample Size and Statistical Analysis. All cell experiments were performed on a minimum of triplicate samples. Reported data are representative of at least three independent experiments. Two groups of samples were compared using the Mann–Whitney U test. Comparisons of more than two groups were performed by multiple comparisons using the Tukey–Kramer test. Survival curves were compared using the log-rank test. Between-group differences were considered statistically significant at a P value < 0.05.

Acknowledgements

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Supplementary Information

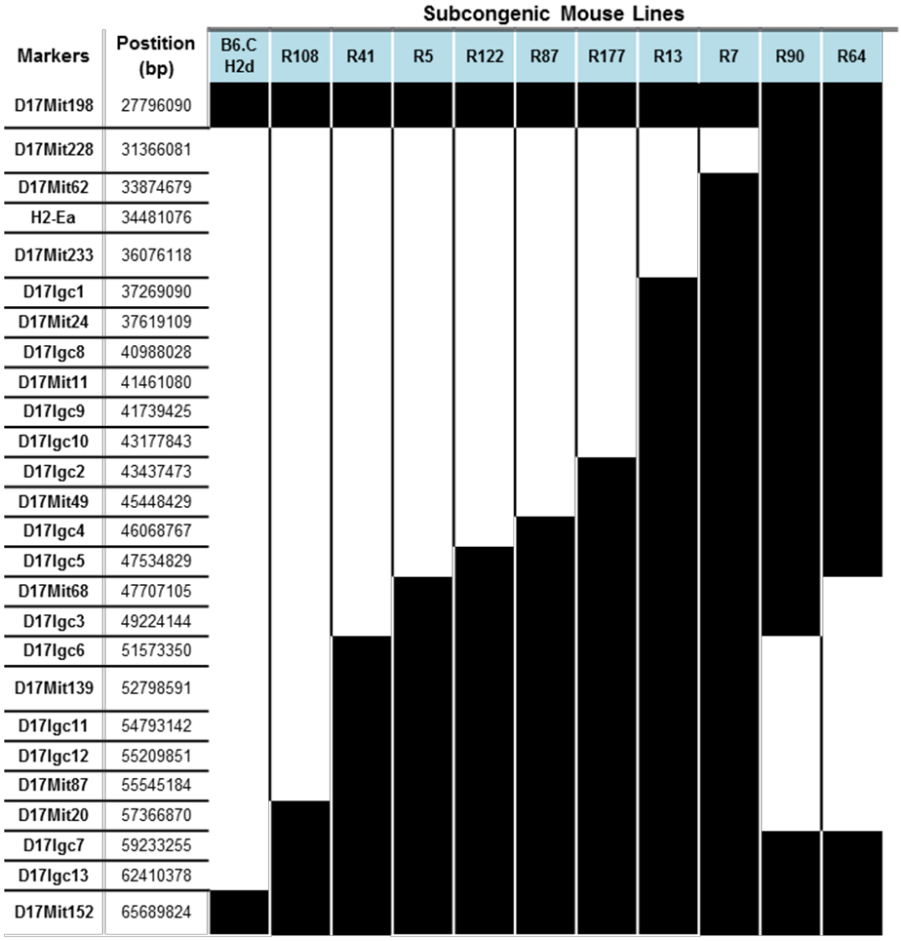

Figure III-S1. Chromosome 17 genotype maps of 10 subcongenic lines selected from the B6.C-H2d × C57BL/6 backcross progeny using 24 polymorphic microsatellites.

Genotypes of BALB/c origin are in white, and those of C57BL/6 are in black.

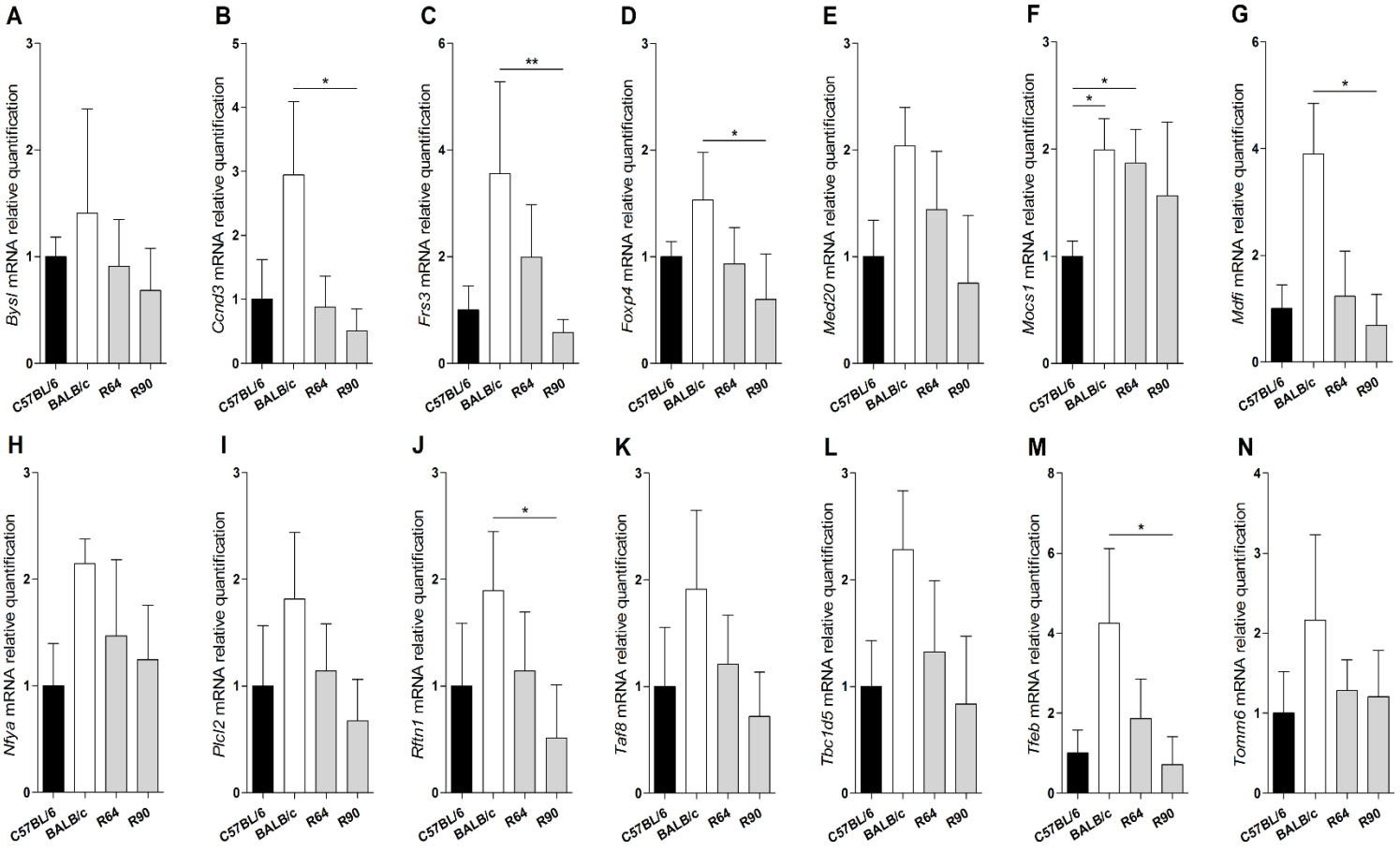

Figure III-S2. NPC expression of non-TREM genes mapping within the belr1 interval.

mRNA of the indicated genes was quantified in NPCs isolated from non- infected R64, R90, and BALB/c mice relative to C57BL/6 mice (A, *Bysl*; B, *Ccnd3*; C, *Frs3*; D, *Foxp4*; E, *Med20*; F, *Mocs1*; G, *Mdfi*; H, *Nfya*; I, *Plcl2*; J, *Rftn1*; K, *Taf8*; L, *Tbc1d5*; M, *Tfeb*; N, *Tomm6*). Data are mean ± SD; n > 6. *P < 0.05; **P < 0.01. mRNA expression of indicated genes did not correlate with the liver stage resistance phenotype in the tested strains.

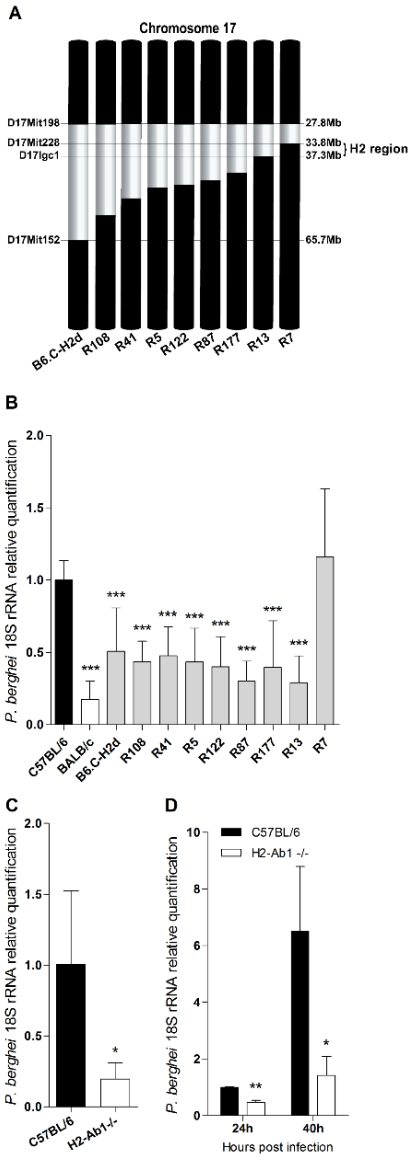

Figure III-S3. The H2 region and class II genes contribute to malaria liver stage resistance.

(A) Diagram representing the relative size of BALB/c-derived congenic regions (in white) in the B6.C-H2d strain and eight single recombinant subcongenic lines in the C57BL/6 background (in black). The R13 congenic region encompasses the H2 locus within 5.9 Mb. (B) Liver parasite burden in subcongenic lines at 40 h postinfection (p.i.) was ascertained as described in Fig. 1. Data are mean ± SD; n = 10. (C) Liver parasite burden in H2-Ab1−/− mice at 40 h p.i. Data are mean ± SD. (D) Parasite yield in infected primary cultures of H2-Ab1− deficient and C57BL/6 hepatocytes measured at 24 h and 40 h p.i. Data are mean ± SD of triplicate cultures. * $p < 0.05$; ** $p \leq 0.001$; *** $p < 0.0001$.

Table III-S1. *belr1* gene content. Protein coding genes mapping on mouse chromosome 17 between markers D17Igc5 and D17Igc6.

Gene Symbol	Gene Start, bp	Gene Name
Apobec2	48 558 556	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 2
Bysl	47 73 6280	bystin-like
Ccnd3	47 642 000	cyclin D3
Dazl	50 418 719	deleted in azoospermia-like
Daam2	49 595 347	dishevelled associated activator of morphogenesis 2
Frs3	47 825 979	fibroblast growth factor receptor substrate 3
Foxp4	48 004 082	forkhead box P4
Guca1a	47 531 509	guanylate cyclase activator 1a (retina)
Kif6	49 754 461	kinesin family member 6
Lrfn2	49 071 704	leucine rich repeat and fibronectin type III domain containing 2
Med20	47 748 531	mediator complex subunit 20
Mocs1	49 567 687	molybdenum cofactor synthesis 1
Mdfi	47 952 277	MyoD family inhibitor
Nfya	48 526 210	nuclear transcription factor-Y alpha
Plcl2	50 648 872	phospholipase C-like 2
Prickle4	47 823 688	prickle homolog 4 (Drosophila)
Pgc	47 863 791	progastricsin (pepsinogen C)
Rftn1	50 131 582	raftlin lipid raft linker 1
Trem5	48 262 555	triggering receptor expressed on myeloid cells 5
Trem4	48 288 846	triggering receptor expressed on myeloid cells 4
Treml6	48 455 466	triggering receptor expressed on myeloid cells-like 6
Taf8	47 624 999	TAF8 RNA polymerase II, TATA box binding protein (TBP)-associated factor 8
Tbc1d5	50 872 452	TBC1 domain family, member 5
Tfeb	47 873 979	transcription factor EB
Tomm6	47 823 595	translocase of outer mitochondrial membrane 6 homolog (yeast)
Tspo2	48 587 281	translocator protein 2
Trem1	48 371 903	triggering receptor expressed on myeloid cells 1
Trem2	48 485 726	triggering receptor expressed on myeloid cells 2
Trem3	48 386 944	triggering receptor expressed on myeloid cells 3
Treml1	48 499 241	triggering receptor expressed on myeloid cells-like 1
Treml2	48 438 864	triggering receptor expressed on myeloid cells-like 2
Treml4	48 403 659	triggering receptor expressed on myeloid cells-like 4
Usp49	47 767 639	ubiquitin specific peptidase 49
Unc5cl	48 594 226	unc-5 homolog C (C. elegans)-like

Table III-S2. *belr1* fine mapping: Polymorphic microsatellite markers Marker.

Marker	Start position, bp	Fragment size*	Forward	Reverse
D17Igc1	37 269 090	132	GGGAGTGGGAATTCTTTTATTTTA	TGCTTTCTTCTGGTGTCTCTGAA
D17Igc2	43 437 473	135	GCTCACTTTTTCCTAGCATCATC	GCCATGGGAAGAAGTTATATGTC
D17Igc3	49 224 144	146	GATAAGTTTGGAGTCAGGCCTAA	ACTTATTCACTCCTGAGCCTTGT
D17Igc4	46 068 767	158	TAGGGCCTTTGCCTGTTTAA	AGCTCACAATCACCTGTAACTCTT
D17Igc5	47 534 829	120	TGAGATGTGAAATGGATTCCC	TCAACCTGGGCTGTATAATGAGA
D17Igc6	51 573 350	139	ATACCCCACACACAATTCCAT	GGGAAAGTATTTGGTGATCAGAGA
D17Igc7	59 233 255	301	TATTTTGCCAGCCTCCAAGA	ACATGCCTATTTTTGTGGGC
D17Igc8	40 988 028	134	CCAAAGCATGTTAGAGGTAAGACA	TGAAGCCAATAAACATCCTAGGTT
D17Igc9	41 739 425	116	TGTACAGTGTTTTTGTTTGTGCA	TGAAGGTATGAACACTCAAGCAA
D17Igc10	43 177 843	115	GAATCTTGCTTTTGGCTTTGT	GGATTGGGTGAGATTCTAAACATT
D17Igc11	54 793 142	137	CTCAAAGTTTGTTTGGGTGTGT	AGGGTGAATGAAATAAAGTTCCA
D17Igc12	55 209 851	128	CTAATTCTAAGGCGGGTTTCTATC	TAAAGCACAAGGCAGGTATAGGA
D17Igc13	62 410 378	313	AAGTTTGAGAGACTGGCTTCAGA	TTCTTGGTACTTTAGGTTGGGAG

*Referred to the C57BL/6 allele.

Gene expression quantification supplementary material and methods

mRNA from isolated non-parenchymal cells were similarly purified as described in material and methods section from manuscript. cDNA specific to *Bysl*, *Ccnd3*, *Frs3*, *Foxp4*, *Med20*, *Mocs1*, *Mdfi*, *Nfya*, *Plcl2*, *Rftn1*, *Taf8*, *Tbc1d5*, *Tfeb* and *Tomm6* expression was quantified using TaqMan Gene Expression Assays from ABI (Mm00479473_m1, Mm01612362_m1, Mm00460745_m1, Mm00466364_m1, Mm00498409_m1, Mm01273749_m1, Mm00521984_m1, Mm00477820_m1, Mm01277344_m1, Mm01281622_m1, Mm01273585_m1, Mm00813934_m1, Mm00448968_m1 and Mm00481240_m1, respectively). Endogenous control *Gapdh* (Mouse GAPD Endogenous Control, ABI) was used in multiplex PCR reactions performed in ABI Prism 7900HT and relative quantification was calculated using ΔΔCt method.

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CHAPTER 4

Fas Pathway Signaling and Kupffer Cells-Derived HGF Induce Hepatocyte Apoptosis in Response to *P. berghei* Liver Stage Infection.

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Author Contributions

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The data analysis was performed by me, Joana Rodo, Lurdes Rodrigues-Duarte and Carlos Penha-Gonçalves.

Maria M. Mota contributed with scientific input and critical reading of the manuscript.

The manuscript was written by Carlos Penha-Gonçalves and myself.

Summary

Malaria liver stage represents a parasite population bottleneck in *Plasmodium* infections, providing a target for disease protection strategies based on blocking or suppressing parasite life cycle progression. The establishment of the parasite in hepatocytes represents a gross cellular insult that is likely to induce host responses that counteract the full development of intra-hepatocytic parasites, leading to reduced efficacy of liver stage infection. Here, we made use of hepatocyte primary cultures to investigate whether infection by *P. berghei* sporozoites triggers hepatocyte cell death mechanisms, particularly Fas-mediated apoptosis that thereby reduce the success of liver stage infection. We found that spontaneous reduction of infected cells in primary cultures is due to increased hepatocyte apoptosis that was partially dependent, and modulated, by Fas pathway signaling that triggers the Type II apoptosis pathway, as epitomized by prominent Bid up-regulation. Furthermore, we observed that HGF signaling in infected hepatocyte primary cultures promotes a shift from Type II to Type I apoptosis that results in a potent reduction of parasite yield by specifically sensitizing hepatocytes carrying early liver schizont to undergo apoptosis. Unexpectedly, we found that HGF is secreted by infected liver Kupffer cells at levels that enable parasite yield reduction in infected cultures. This work provides evidence that Fas and HGF signaling pathways orchestrate an apoptosis response during liver stage infection and implicates paracrine signals from Kupffer cells and non-infected hepatocytes in directing infected hepatocyte suicidal.

Introduction

Malaria liver stage represents a feeble life cycle bottleneck prior *Plasmodium* expansion in the mammalian host so that attempts aiming at reduction of liver stage efficacy provide a promising strategy towards disease protection [1,2]. *Plasmodium* parasites need to overcome a challenging and obligatory hepatic pre-patent phase of malaria infection during which allegedly low numbers of infecting sporozoites lead to the inexorable generation of blood stage forms that are responsible for symptomatic malaria. Through a high-efficacy process of pre-erythrocytic schizogony, one sporozoite develops into a liver stage form that undergoes exponential growth entrenching thousands of merozoites inside the parasitophorous vacuole in each infected hepatocyte [3].

Success of parasite development and growth inside individual hepatocytes is dependent on the ability to sustain host cell survival until late stages of infection [4]. Experiments using genetically modified rodent malaria parasites (e.g. P36p$^{-/-}$ and UIS4$^{-/-}$) suggest that parasite molecules located at parasite-host interface are involved in impeding hepatocyte apoptosis [5,6]. Accordingly, hepatocytes acquire resistance to apoptosis signals during infection as revealed by *in vitro* peroxide treatment or serum deprivation and by *in vivo* TNF treatment, possibly as a result of PI3K apoptosis resistance pathway engagement [7,8]. Also, recently it was shown that the expression of the pro-death protein p53 was reduced in infected hepatocytes [9]. This strategy is thought to warrant liver stage parasite survival and progression to blood stage infection.

Notwithstanding, parasite invasion and accumulation of exo-erythrocytic forms (EEFs) inside hepatocytes represent a gross cellular insult that plausibly elicits host protection mechanisms. Little is known about host strategies to counteract EEFs development that could result

in decreased efficacy of the liver stage infection. Incidentally, it has been reported that a considerable number of sporozoites infecting hepatocytes in culture do not succeed in accomplishing full EEF development, but the causes of such aborted infection events have not been identified [10,11]. Thus, it is tempting to investigate whether specific apoptosis pathways acting in infected hepatocytes provide an early mechanism to respond to *Plasmodium* infection.

Hepatocytes are particularly sensitive to Fas-mediated apoptosis, a prominent pathway to induce hepatocyte death in course of viral infections, alcoholic exposure and other cellular stress factors [12,13]. Fas is a TNF-family receptor constitutively expressed in hepatocytes at levels functionally sufficient to trigger cell apoptosis upon binding to Fas Ligand (FasL) in autocrine or paracrine fashion [13]. As opposed to other cell types, Fas-induced apoptosis in hepatocyte is restricted to a typical Type II cell mechanism that couples the Fas pathway to the mitochondrial pathway via the activation of Bid, a pro-death Bcl-2 family protein [14].

On the other hand, it has been shown that high doses of Hepatocyte Growth Factor (HGF) signaling in combination with Fas stimulation induces a shift from Type II to Type I cell death enabling mitochondria-independent hepatocyte apoptosis [15]. Additionally, exposure to HGF was shown to prevent apoptosis and increase *Plasmodium* yield in infected hepatoma cell lines [8,16]. These observations prompted us to investigate the possibility that Fas and HGF pathways are involved in apoptosis responses of infected hepatocyte and determine the efficacy of liver stage infection.

Here, we tested the hypothesis that *Plasmodium berghei* sporozoites infection triggers early hepatocyte apoptosis mechanisms leading to the unsuccessful infection of individual hepatocytes. By investigating the Fas and HGF signaling pathways, we found that a successful

infection escapes a combination of intracellular and paracrine signals that determined the fate of infected hepatocytes.

Results

Involvement of Fas-mediated apoptosis in *Plasmodium* liver stage infection

To ascertain the success of *P. berghei* sporozoites infection in hepatocyte primary cultures we performed time course counting of infected hepatocytes that showed that less than 50% of the initial infected cells attained EEF full development (Figure IV-1A). To investigate whether the aborted EEFs were related to apoptosis induction in *P. berghei* infected cultures we analyzed the expression of apoptosis genes in hepatocyte primary cultures and found out that Fas pathway signaling-related genes were significantly altered at 24h p.i. A strong up-regulation of *Bid* coupled with a slight up-regulation of other pro-apoptotic genes and down-regulation of the anti-apoptotic *Cflar*, represent a signature that Fas pathway signaling was induced in infected primary cultures (Figure IV-1B), presumably executing a Type II apoptotic response that requires mitochondrial participation [14]. To test whether Fas pathway signaling was detrimental to successful hepatocyte infection we analyzed primary hepatocytes from lpr mice (Fas null mutant) which demonstrated that by the end of the infection (40h) the parasite yield was significantly increased in absence of Fas pathway signaling, an effect that was also observed by blocking Fas pathway signaling with anti-Fas Ligand antibodies (Figure IV-1C). Furthermore, the absence of Fas pathway signaling improved the number of successfully infected hepatocytes (Figure IV-1D), possibly because apoptosis induced by infection was partially dependent on Fas pathway signaling (Figure IV-1E) and impact the viability of both infected and non-infected hepatocytes. Accordingly, immunofluorescence microscopy of infected cultures revealed prominent FasL expression in non-infected hepatocytes as compared to infected hepatocytes (Figure IV-1F), indicating that induction of FasL

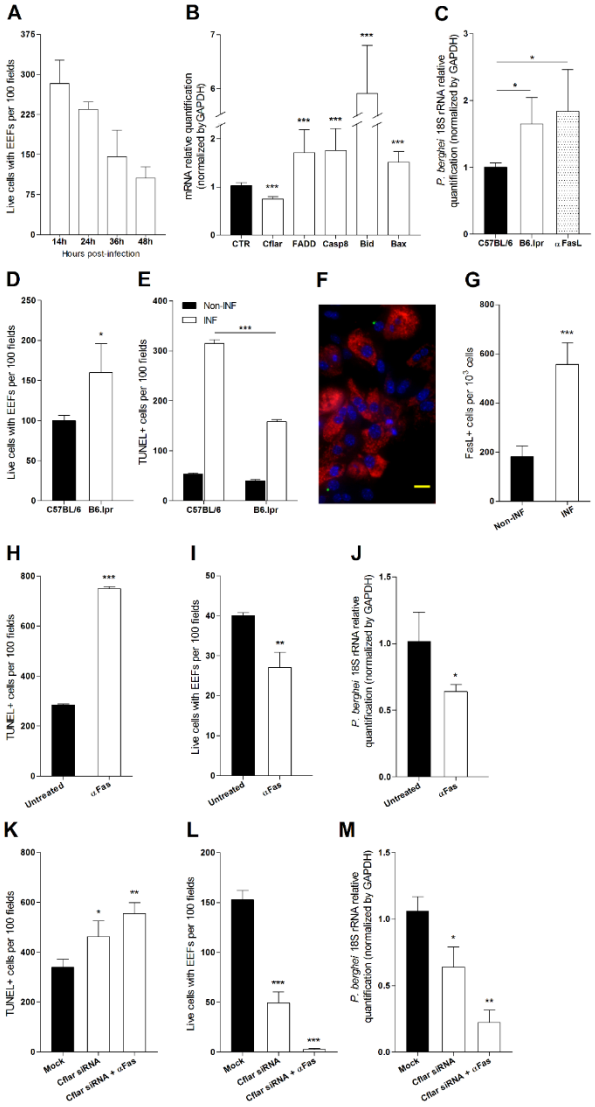

Figure IV-1. Fas-mediated hepatocyte apoptosis impairs the success of *P. berghei* infection.

(A) EEF-containing viable hepatocytes were counted at indicated times in infected primary cultures. (B) mRNA quantification of Fas pathway genes at 24h p.i., relative to non-infected control (CTR) hepatocyte cultures. (C) Parasite yield in infected cultures of Fas-deficient hepatocytes from B6.lpr mice or after blocking Fas pathway with anti-FasL antibody was measured by qRT-PCR of *P. berghei* 18S rRNA at 40h p.i. (D) EEF-containing viable hepatocytes of B6.lpr and C57BL/6 mice were counted and (E) the number of apoptotic cells was determined by TUNEL staining in infected and non-infected cultures at 24h p.i. (F) Representative immunofluorescence image of non-infected hepatocyte expressing FasL (red) in the vicinity of *P. berghei*-gfp (green) infected hepatocytes (nuclei stained with DAPI (blue)), scale bar - 20µm and (G) counting of FasL expressing cells in infected and non-infected cultures at 24h p.i. (H and K) number of apoptotic cells (TUNEL positive), (I and L) number of viable hepatocytes containing EEFs and (J and M) parasite yield were measured in primary hepatocyte cultures in presence or absence of signaling anti-Fas antibody and/or *Cflar* siRNA. (unpaired t-test, $p<0.05$ (*), $p\leq0.001$ (**) and $p\leq0.0001$ (***)). Data are represented as mean±SD.

expression by *P. berghei* infection (Figure IV-1G) promotes hepatocyte apoptosis. Conversely, we observed that increasing Fas pathway signaling in hepatocytes either by adding anti-Fas antibodies or by knocking down *Cflar*, a potent Fas pathway inhibitor [17], increased apoptosis susceptibility in infected hepatocyte cultures (Figure IV-1H and K) leading to decreased number of hepatocytes containing EEFs (Figure IV-1I and L) and reduced parasite yield (Figure IV-1J and M). The effects of *Cflar* knock-down were exacerbated by anti-Fas stimulation (Figure IV-1K-M) and strongly suggest that *Cflar* inhibition sensitized hepatocytes to undergo apoptosis through the Fas pathway. These experiments provide evidence that apoptosis signals evoked by the parasite favor Fas-dependent hepatocyte apoptosis in infected cultures leading to a decrease in number of infected primary hepatocytes that support full parasite development.

HGF promotes apoptosis of EEF-carrying hepatocytes

Given that the combination of Fas and HGF signaling induce in hepatocytes a shift from Type II to Type I apoptosis response [15] and that HGF interferes with liver stage infection [8,16], we characterized the apoptosis response elicited by HGF in infected hepatocytes. Firstly, we observed that pre-treatment with HGF one hour prior infection impair parasite yield and has a cumulative effect with anti-Fas antibody signaling (Figure IV-2A). By probing the expression of key informative genes we found that HGF induces down-regulation of *PI3K*, a component of HGF/c-Met pathway that is involved in apoptosis protection (Figure IV-2B) [18,19]; up-regulated *Caspase 3* (Figure IV-2C), but decreased the expression of *Bid* (Figure IV-2D), which suggests that type II apoptosis is not operating and that apoptosis signals conferred by HGF follow the alternative type I apoptosis response [15] in *Plasmodium* infected hepatocytes. Thus, infected primary hepatocytes

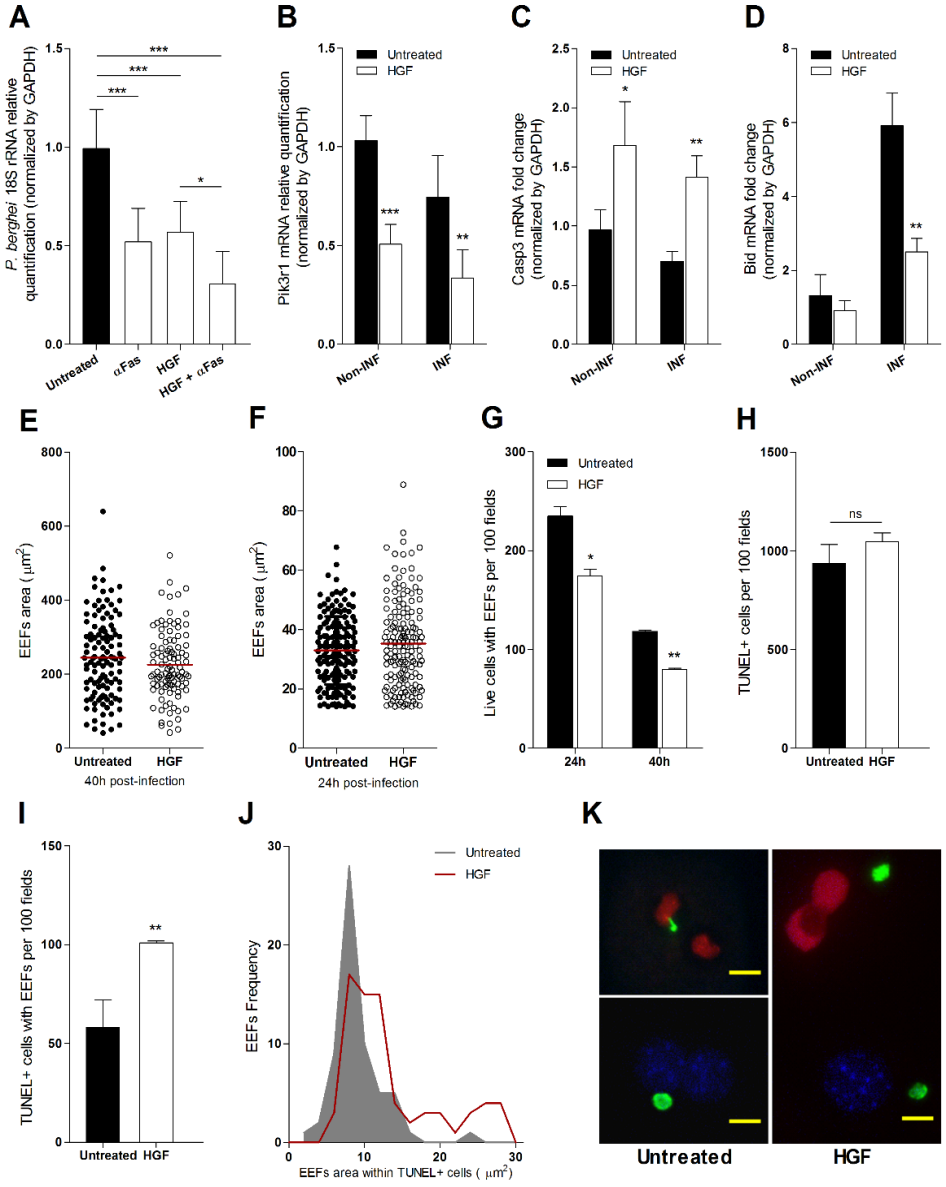

Figure IV-2. HGF-conditioning specifically induces type I apoptosis of parasitized hepatocyte.

(A) Cumulative effect of HGF pre-treatment and Fas pathway signaling in parasite yield. mRNA quantification of apoptosis markers, (B) *Pik3r1*, (C) *Caspase 3* and (D) *Bid* at 24h p.i., relative to non-infected control (CTR) hepatocyte cultures. Primary hepatocyte cultures untreated or pre-treated with HGF were analyzed for EEFs size (E) at 40h and (F) at 24h p.i.; (G) viable hepatocytes containing EEFs. (H) Total apoptotic cells (TUNEL positive), (I) apoptotic cells containing EEFs and (J) EEFs distribution size inside apoptotic cells were measured at 24h p.i. (K) Immunofluorescence representative images of apoptotic (upper left and right) and non-apoptotic (lower left) hepatocytes carrying EEFs at 24h p.i. with or without HGF, evidencing larger EEFs in TUNEL+ cells after HGF pre-treatment (red -TUNEL; green – P.berghei-GFP; blue – DAPI), scale bar - 10μm. (unpaired t-test, $p<0.05$ (*), $p\leq0.001$ (**) and $p\leq0.0001$ (***)). Data are represented as mean±SD.

appear to respond to HGF treatment following a mitochondrial independent apoptosis mechanism. Accordingly, reduction of parasite yield by HGF was not associated with altered EEFs size either at the end of the liver infection (40h) (Figure IV-2E) or at earlier stages (24h) (Figure IV-2F), but was attributable to the reduced number of successfully developed EEFs (Figure IV-2G), showing already a striking reduction at 24h post-infection. Although infected hepatocyte cultures pre-treated with HGF did not show increased bulk apoptosis (Figure IV-2H), we noted that parasitized hepatocytes were significantly more susceptible to apoptosis (Figure IV-2I). The size distribution of EEFs inside apoptotic hepatocytes was significantly deviated to higher values in pre-treated cultures (p value <0.0001) (Figure IV-2J). This result suggests that spontaneous apoptosis of infected hepatocytes in absence of HGF, targeted mainly cells containing underdeveloped parasite forms, but remarkably pre-treatment with HGF allowed apoptosis induction in hepatocytes carrying early schizonts (Figure IV-2J and K). These results show that HGF-conditioning promotes apoptosis induction of parasitized hepatocytes that carry EEFs in a development stage that otherwise would reach successful maturation.

Kupffer cells impact the efficiency of primary hepatocyte infection

Cultured primary hepatocytes do not express detectable levels of HGF irrespective to infection with *P. berghei* (data not shown). In search for a cellular source of HGF that could mediate this apoptosis effect in vivo, we analyzed HGF gene expression in isolated non-parenchymal liver cells and found that HGF mRNA expression was highly increased after *in vivo* infection as compared to basal levels (Figure IV-3A). Furthermore, FACS analysis revealed that F4/80$^+$ cells representing Kupffer cells (KCs), expressed intracellular HGF as opposed to other non-parenchymal cells, e.g. CD11c$^+$ (dendritic cells) (Figure IV-3B). To

ascertain whether KCs-derived soluble factors were able to impair parasite growth *in vitro*, we performed Transwell experiments using

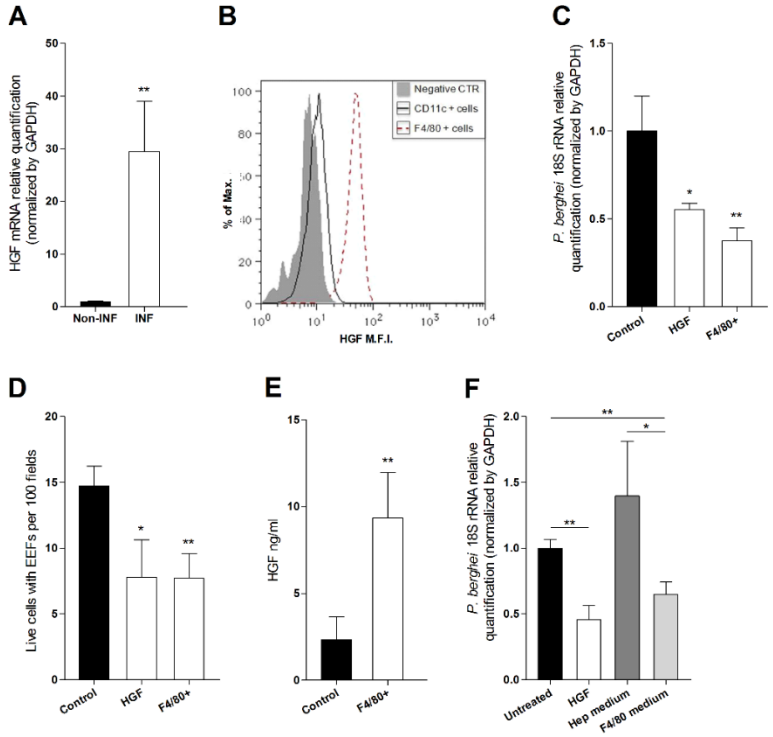

Figure IV-3. Kupffer cells secret HGF and control efficacy of primary hepatocyte infection.

(A) HGF mRNA quantification and (B) FACS analysis of intracellular HGF in F4/80$^+$ cells and CD11c$^+$ cells, measured in liver non-parenchymal cells isolated from C57BL/6 mice at 40h p.i. with *P. berghei* sporozoites. (C) The parasite yield, (D) the number of viable hepatocytes containing EEFs and (E) secreted HGF measured in the supernatant after co-culture with F4/80$^+$ cells in transwell. (F) Parasite yield in hepatocyte cultures treated with HGF or incubated with culture supernatant from single hepatocyte culture or from hepatocyte-F4/80$^+$ cells co-culture in transwell. (unpaired t-test, $p<0.05$ (*) and $p\leq0.001$ (**)). Data are represented as mean±SD.

FACS sorted F4/80$^+$ cells from infected livers and naïve primary hepatocytes. Remarkably, parasite yield and the number of infected hepatocytes were reduced in presence of KCs, to an extent comparable to cultures pre-treated with HGF (Figure IV-3C and D), indicating that this effect is attributable to soluble factors derived from KCs, the liver resident macrophages. Moreover, HGF was detected in

the supernatants of KCs/hepatocyte cultures (Figure IV-3E) that when used as media in simple hepatocyte cultures were able to induce parasite yield reduction (Figure IV-3F). These results strongly suggest that HGF secreted by Kupffer cells is able to reduce successful hepatocyte infection.

Discussion

We have put forward the hypothesis that *Plasmodium* liver stage infection elicits a host apoptosis response that hampers successful terminus of hepatocyte infection leading to decreased parasite yield. This work revealed not only that Fas-mediated hepatocyte apoptosis is induced after *in vitro* infection, but also that an unpredicted hepatocyte-Kupffer cell crosstalk mediated by HGF sensitizes infected hepatocytes to undergo apoptosis.

Together, the results support the notion that after *in vitro* infection, hepatocytes respond by activating different apoptosis mechanisms. In particular, we show that hepatocyte apoptosis response to *Plasmodium* infection entails two distinct events: (1) a Fas-dependent Type II apoptosis response acting on both infected and non-infected hepatocytes and (2) a HGF-stimulated Type I apoptosis mechanism that preferably targets hepatocytes harboring early liver schizonts.

Fas pathway stimulation significantly increased apoptosis in hepatocyte cultures with a corresponding decrease in parasite yield, suggesting that susceptibility of infected hepatocytes to Fas-mediated apoptosis is an efficient way to control initial parasite liver stage transformation. Intriguingly, we found that FasL appear to be over produced by non-infected hepatocytes that surround individual infected hepatocytes, suggesting that FasL-expressing cells provide a paracrine signal to induce apoptosis of the infected cells. In fact, we confirmed previous reports [10,11] that the number of infected cells in hepatocyte cultures is considerable reduced across the time of liver stage development. These results strongly suggest that such spontaneous reduction of infected cells in culture is caused by hepatocyte apoptosis partially attributable to paracrine Fas pathway signaling (Figure IV-1). Interestingly, we noted that the ability to induce spontaneous apoptosis of individually infected cells in primary

hepatocyte cultures was restricted to underdeveloped parasite forms (Figure IV-2J) strongly suggesting that at later developmental stages the parasite confers resistance to apoptosis signals such as Fas pathway signaling.

Although susceptibility to Fas-mediated apoptosis plays a role in reducing parasite yield, this effect appeared to affect infected and non-infected hepatocytes equally, in the cultures. On the other hand, HGF signaling promotes a shift from Type II to Type I apoptosis [15] and preferentially targets infected cells in primary cultures. The results indicate that after exposure to HGF, hepatocytes become exquisitely sensitive to apoptosis, suggesting that apoptosis resistance conferred by the parasite can be overcome in hepatocytes carrying early schizonts.

The finding that Kupffer cells contain detectable levels of HGF *ex vivo* heightens the *in vivo* relevance of these results. Moreover, Kupffer cells were able to secret HGF *in vitro*, that presumably result in a reduction of parasite yield in hepatocyte primary cultures. It is plausible that during malaria liver stage, secretion of HGF by Kupffer cells provides a mechanism to target and destroy infected hepatocytes exhibiting mid-stage developed EEFs.

The decision of whether one infected hepatocyte undergoes apoptosis or support full parasite development may lay on time windows of host apoptosis mechanisms activation that are counteracted by effective parasite apoptosis-inhibiting activity. We have noted that the size of the EEFs found in TUNEL positive cells in untreated cultures is equivalent to an initial round parasite form, suggesting that hepatocyte apoptosis is able to deter parasite development at early stages. Furthermore, we observed that HGF treatment is capable of inducing apoptosis of hepatocytes containing

EEFs of larger sizes, indicating that this apoptosis pathway is effective at later stages of parasite development.

Together these observations indicate that the fate of infected hepatocytes is dictated by combinations of intracellular apoptotic signals and paracrine interactions between Kupffer cells and hepatocytes that are able to efficiently direct hepatocyte apoptosis. Manipulation of such apoptosis mechanisms may provide an effective tool to block or suppress liver stage progression and confer disease protection.

Material and Methods

Mice. All procedures involving laboratory mice were performed in accordance with national (Portaria 1005/92) and European regulations (European Directive 86/609/CEE) on animal experimentation and were approved by the Instituto Gulbenkian de Ciência's Ethics Committee and the Direcção-Geral de Veterinária. All experiments were conducted using male mice with 8 to 15 weeks of age from strains C57BL/6 and B6.lpr (B6.MRL-Fas^{lpr}/J). The mice were bred and maintained in conventional housing facilities at the Instituto Gulbenkian de Ciência.

Hepatocyte primary cultures. Mouse primary hepatocytes were prepared as previously described [20]. In short, mouse liver lobes were perfused with liver perfusion medium and liver digest medium (Gibco, Invitrogen) at 37°C. Dissociated cells from the tissue were separated using a 1.12 g/ml, 1.08 g/ml and 1.06 g/ml Percoll (GE Healthcare) gradient. Hepatocytes were collected from the gradient solution and cultured in William´s E complete medium (Gibco, Invitrogen) in plates or glass coverslips coated with 0.2% Gelatin. To our experience hepatocytes preparations can vary in yield and viability. We have been careful to take to experiments just preparations with high viability. On average, we find 30% of dead cells in freshly isolated preparations; most of these cells do not attach and are removed 12h post-plating (12h prior infection).

Fas blocking experiments used 10 µg/ml of anti-FasL antibody (CD178.1 Kay10, BD Pharmingen) added 1 hour prior infection. Fas pathway signaling was induced with 1 µg/ml of anti-Fas antibody (CD95, BD Pharmingen) 18h post- infection with *P. berghei*. HGF conditioning was performed by adding 100 ng/ml of recombinant mouse HGF

(eBioscience) 1 hour prior infection, with subsequent wash before infection.

Hepatocyte Infection. Green fluorescent protein (*gfp*)-expressing *P. berghei* ANKA [21] sporozoites were obtained from dissection of infected salivary glands from *Anopheles stephensi* mosquitoes bred in the insectarium of the Instituto de Medicina Molecular, Lisbon. Hepatocytes were infected with 1.5 to 3.5x10^4 *P. berghei* ANKA sporozoites, and non-infected controls were mock-infected with salivary glands from non-infected mosquitoes.

Hepatocyte apoptosis and microscopic parasite quantification. Hepatocytes cultured on coverslips were fixed 24h or 40h post-infection with 4% paraformaldehyde and incubated with 50 nM NH4Cl to extinguish PFA activity. Cells were permeabilized and blocked with blocking solution for 1 h (3% of Bovine Serum Albumin (Calbiochem), 100 mM of glycine, 0,1% of saponin and 10% of Foetal Calf Serum (Gibco, Invitrogen) in PBS 1X) and stained for apoptosis by TUNEL using "In Situ Cell Death Detection – TMR" (Roche); to stain EEFs, coverslips were then incubated 45 min. at room temperature with blocking solution containing anti-GFP IgG Alexa-488 conjugated antibody (Invitrogen); to stain FasL, cells were incubated 45 min. at room temperature with blocking solution containing anti-FasL (CD178.1 Kay10, BD Pharmingen) followed by DAPI staining (Invitrogen) and mounted with MOWIOL (Calbiochem). Cell culture images were acquired using an automated Nikon Eclipse TE2000-S inverted fluorescence microscope, with a 20x objective, covering the total coverslip area. ImageJ (NIH) software was used to differentially identify TUNEL positive or negative (live) cells and EEFs in each coverslip and to estimate single EEFs area. Results are expressed as the number of TUNEL positive cells per 100 microscopic fields.

Hepatocyte gene expression and *P. berghei* ANKA RNA quantification. Cells were collected at 24h or 48h post-infection lysed and reverse transcriptase reaction was performed using TaqMan Gene Expression Cell-to-CT kit (Ambion). *Cflar*, *Fadd*, *Casp8*, *Bid*, *Bax*, *Casp3*, *Pik3r1* and *Hgf* expression was quantified using TaqMan Gene Expression Assays from ABI (Mm01255576_m1, Mm00438861_m1, Mm00802247_m1, Mm00432073_m1, Mm01205549_m1, Mm01195084_m1, Mm01282780 and Mm01135185_m1, respectively). For *P. berghei* ANKA quantification specific primers for Taqman were, Forward 5'-CCG ATA ACG AAC GAG ATC TTA ACC T–3', Reverse 5'- CGT CAA AAC CAA TCT CCC AAT AAA GG-3' and Probe 5'– ACT CGC CGC TAA TTA G -3' (FAM/MGB). Endogenous controls (*Actb* (Mouse ACTB Endogenous Control, ABI) or *Gapdh* (Mouse GAPD Endogenous Control, ABI)) were used in multiplex PCR with target genes. PCR reactions were performed with ABI Prism 7900HT, and relative quantification was calculated using ΔΔCt method.

***Cflar* knock-down.** A mixture of three distinct siRNAs (s63907, s63908 and s63909 - Ambion), at a concentration of 20 μM was delivered to primary hepatocytes with Oligofectamine (Invitrogen). Control samples included untransfected cells and cells transfected with a negative control siRNA (Ambion) or a positive control targeting mouse GAPDH (Ambion).

Isolation of liver non-parenchymal cells. Non-parenchymal cells were obtained by adapting a method previously described [22]. Briefly, liver lobes were removed and perfused with liver perfusion medium (Gibco, Invitrogen) with 750 mg/l of Collagenase H (Roche) at 37°C. The resulting suspension was filtered through a 100 μm cell strainer (BD Falcon). The dissociated cells were suspended in liver perfusion medium and centrifuged for 10 min. at 1500 rpm, resuspended in RPMI complete medium (Gibco, Invitrogen), mixed in Percoll (GE Healthcare) solution

to give a final concentration of 30% Percoll and then centrifuged at 2000 rpm for 10 min. The cell pellet was resuspended in RPMI and carefully laid on 30% Percoll solution and centrifuged at 2000 rpm for 10 min. The cell pellet collected was washed and resuspended in ACK (NH4Cl 0.15 M, KHCO3 10 mM, Na2EDTA 2H2O 0.1 mM and pH 7.2) for 3 min. to lyse remaining erythrocytes. Cells were washed and centrifuged at 800 rpm for 20 sec. to discard the remaining hepatocytes; the supernatant was recovered, and non-parenchymal cells were collected at 1500 rpm for 5 min.

Kupffer cells FACS analysis and purification. Kupffer cells were stained, at 4°C, by incubating non-parenchymal cells with F4/80 APC (A3-1, Serotec) and subsequently performing a two-step HGF intracellular staining (H170, Santa Cruz and goat anti-rabbit IgG-FITC,) using the BD Cytofix/Cytoperm kit. FACScalibur cytofluorometer (Becton Dickinson) and FlowJo software were used for FACS analysis. Purified Kupffer cells were obtained through high-speed cell sorting (FACSAria, Becton Dickinson), after staining with F4/80 APC (Serotec).

Macrophage-hepatocyte transwell cultures. We used a 0.4 μm Transwell culture system (Millipore) to allow purified Kupffer cells (upper chamber) to exchange soluble factors during 12h with hepatocyte primary cultures (lower chamber). Hepatocytes were then infected with *P. berghei* ANKA sporozoites and fixed for parasite and apoptosis staining or collected for RNA analysis at 24h post infection.

HGF ELISA. HGF secretion in the supernatants from Transwell cultures was evaluated by sandwich ELISA. Briefly, Maxisorp plates were coated with a capture anti-HGF antibody (H-170, Santa Cruz) in 0.5 M Carbonate-Bicarbonate Solution. Detection used anti-HGF-biotinylated antibody (BAF2207, R&D). Samples were quantified against a standard curve with mouse recombinant HGF (eBioscience).

Statistical analysis. All experiments were performed with a minimum of triplicate samples and presented data are representative of at least three independent experiments. Results are presented as average values and error bars represent standard deviation. Statistical analysis used an unpaired Student t test and $p<0.05$ was considered statistically significant.

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CHAPTER 5

Conclusions

> "Each sick child represents our failure to prevent malaria, and each death represents our failure to treat soon enough."
>
> Louis H. Miller *et al.*, Nat Med **19** (2013).

General Conclusions

Liver is an obligatory site of infection in the natural development of malaria parasite within the vertebrate host. Although numerous publications in the last years evidenced host genetic predisposition to malaria infection and severe forms of the disease, the natural resistance of the host during the liver stage infection is largely unknown. The main objective of this thesis was to unveil host genetic factors that control *Plasmodium* exo-erythrocytic infection.

Malaria genetics research is conducted with the double aim of identifying host resistance factors that can be suggestive of novel therapeutic approaches, or resolving the complex pathogenesis mechanisms associated with *Plasmodium* infection. The vast majority of genetics research in human subjects is directed to the blood phase of infection. This brought light to various aspects of the RBCs infection mechanisms and pinpointed critical molecular players in iRBCs adhesion to endothelia and sequestration in different organs. Nevertheless, the role of host genetic factors intervening in the liver stage of infection is seldom addressed, due to practical difficulties in studying this phase of the infection in human subjects.

Mouse models have provided a valuable tool to understand the biology of liver stage infection and have helped in identifying host molecular pathways critical to this process. This has been achieved by

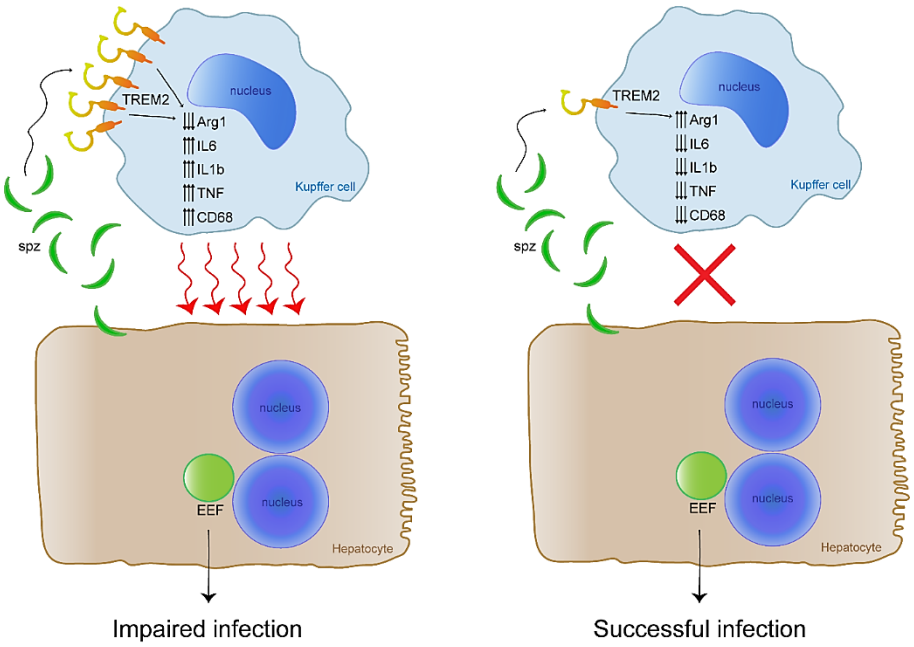
testing host factors hypothetically required for hepatocyte infection and parasite growth, through the use of defined mouse mutants to highlight the role of specific genes. By applying this approach, we studied molecules that are key to decide the fate of infected hepatocytes, which uncovered that the Fas pathway and HGF signaling are involved in inducing apoptosis of infected cells. Our findings indicate that, upon parasite infection, hepatocytes engage in a process of Fas-dependent programed cell death shortly after infection and that HGF produced by Kupffer cells is involved in inducing death of infected hepatocytes at later stages. These data unveil a strong host response directed to eliminate infected hepatocytes and suggests that success of *Plasmodium* liver stage infection represents a failure of host mechanisms to fully eliminate infection at the liver site. Therefore, the balance of the liver stage infection is dependent on the effective ability of the parasite to impede individual infected hepatocytes of committing suicide.

Forward genetics methodology is rarely used to pursue unbiased search for genetic factors related to natural resistance to *Plasmodium* liver stage. In this thesis, we provide an unprecedented example of using genetic mapping techniques, which ultimately led us to identify a gene underlying a quantitative trait locus that controls liver stage infection. Correlating TREM2 expression with the efficiency of liver stage infection is a novel finding that implicate genes expressed in macrophagic Kupffer cells controlling the course of liver stage infection. Our data introduces the view that Kupffer cells are activated by *Plasmodium* sporozoites through innate-like receptors and promote the elimination of infected hepatocytes. In contrast, we found that expression of MHC class II molecules in hepatocytes plays a role in decreasing parasite burden in liver stage infection, representing a hepatocyte autonomous response that operates through mechanisms yet to be elucidated.

Our work provides a view natural resistance to liver stage infection where multiple cellular and molecular components of the liver may intervene throughout the process of liver infection by the malaria parasite. The cellular architecture of the liver forces the parasite to engage in multiple contacts with liver cells, namely, Kupffer cells and hepatocytes. Such contacts elicit host responses that act in concert to fight the parasite and strive for infection control. Thus, it is expected that genes expressed in different cell types through molecular mechanisms that act in autocrine and paracrine fashions mediate genetic resistance to liver stage infection. Our work evidenced both hepatocyte intrinsic mechanisms and non-parenchymal cells paracrine effects that play a role at distinct time points of livers stage infection to induce elimination of infected hepatocytes and reduce the burden of infection in the liver.

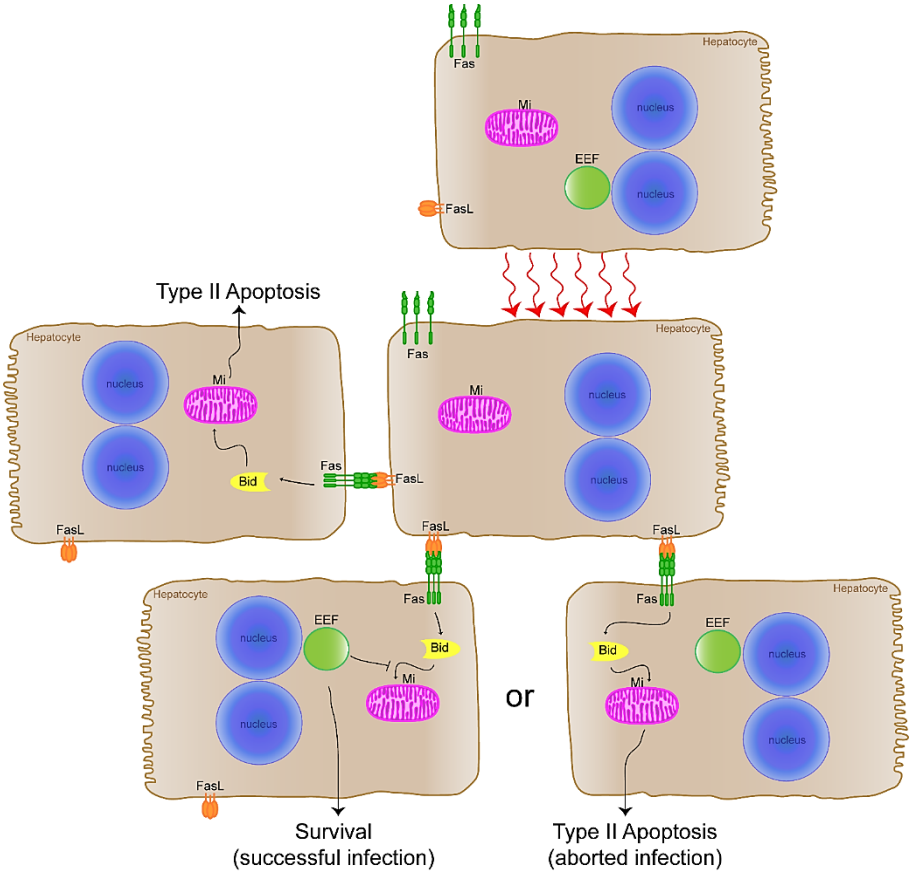
Genetic research on cell and molecular mechanisms involved in natural resistance to liver stage infection will offer insights on molecules specifically associated with liver infection, possibly providing a source of accessible infection biomarkers for this clinically silent phase of the disease. Furthermore, the identification of molecular pathways involved in disease pathogenesis may suggest possible therapeutic targets to abolish liver infection in a time window where the number of parasites represents a minimum load in the vertebrate host and therapy efficacy would be easier to achieve. Liver stage parasites are considered a valuable target of vaccine development but understanding the local liver responses to *Plasmodium* infection and host-parasite interactions at molecular level will be key to reveal parasite components that will serve as effective vaccine antigens.

Graphical Conclusions



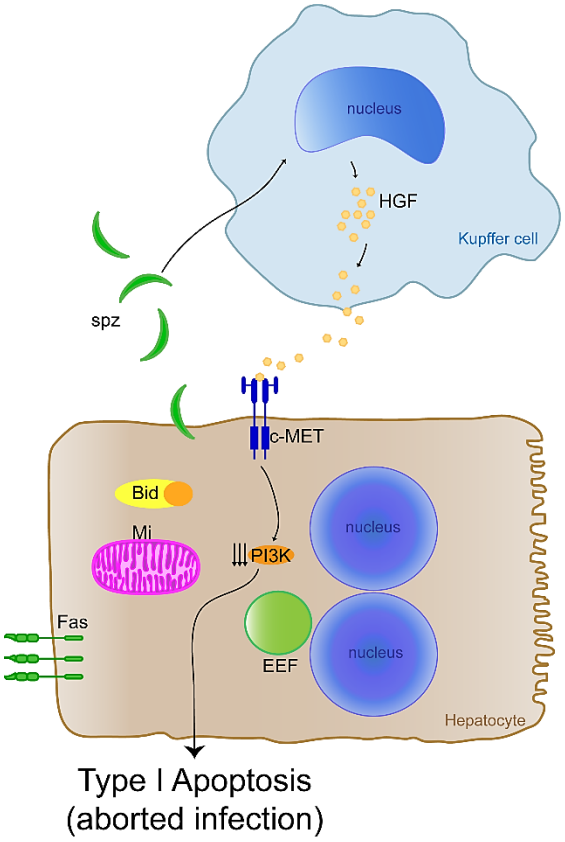
The role for TREM2 in controlling expansion of intra-hepatocytic malaria parasites

We hypothesize that sporozoites are recognized by TREM2 expressed in Kupffer cells surface in the course of endothelia traversing towards the liver parenchyma. TREM2 signaling directs Kupffer cell activation inducing a pro-inflammatory profile (M1 type). Activated Kupffer cells are able to reduce the yield of parasite infection upon direct contact with the hepatocyte. The effector functions mediating such infection protection mechanisms are still unknown.



Spontaneous paracrine induction of Fas cell death pathway in hepatocytes upon sporozoite infection

We suggest that the success of sporozoite infection in individual hepatocytes is significantly impaired by the induction of the Fas cell death pathway. In early stages of hepatocyte infection FasL is upregulated in non-infected hepatocytes and acts in a paracrine fashion to induce apoptosis of both infected and non-infected neighbor hepatocytes. This signal operates through a Type II apoptosis mechanism – mitochondrial dependent. Modulation of the Fas signaling in individual hepatocytes appears to be a critical factor in deciding infected hepatocyte survival and productive infection.



Plasmodium sporozoites induce HGF expression in Kupffer cells leading to apoptosis of infected hepatocytes

Kupffer cells secrete abundant amounts of HGF upon exposure to *Plasmodium* sporozoites. We suggest that HGF acts later stages of infection to sensitize infected hepatocytes to undergo apoptosis in a mitochondrial-independent fashion. We hypothesize that this mechanism is relevant for abortion of infection when the parasite has blocked mitochondrial-dependent apoptosis pathways.

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Twenty years from now, you will be more disappointed by the things that you didn't do than by the ones you did do, so throw off the bowlines, sail away from safe harbor, catch the trade winds in your sails. Explore, Dream, Discover.

Mark Twain

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