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Mechanisms of inhibition of *Plasmodium* liver infection by amino acid supplementation

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Mechanisms of inhibition of *Plasmodium* liver infection by amino acid supplementation

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Resumo

A malária é uma das doenças infeciosas mais prevalentes no mundo[1]. É causada por parasitas protozoários do género *Plasmodium*, os quais são transmitidos aos seus hospedeiros mamíferos através da picada de um mosquito *Anopheles* fêmea infetado[1,2]. A primeira fase da infeção, obrigatória e assintomática, ocorre no fígado e é iniciada quando os esporozoítos injetados invadem os hepatócitos do hospedeiro[2,3]. Aí, os parasitas diferenciam-se e desenvolvem-se até se formarem merozoitos, que são libertados na corrente sanguínea, infetando ciclicamente os eritrócitos e causando os sintomas da malária[2,3].

A multiplicação dos parasitas durante a fase hepática da infeção é sustentada pela obtenção de nutrientes a partir do seu hospedeiro. Um desses nutrientes é a arginina (Arg), cujo metabolismo é crucial para o desenvolvimento intra-hepático do parasita[4].

A Arg tem-se tornado cada vez mais popular na suplementação nutricional dada a sua capacidade de estimular o sistema imunitário[5], sendo a única suplementação baseada em aminoácidos avaliada no contexto da malária. No entanto, embora a suplementação com Arg possa aumentar a produção de óxido nítrico (NO), diminuir a parasitemia, e melhorar a sobrevivência em modelos animais de infeção por *Plasmodium*, os resultados obtidos na clínica têm sido inconclusivos [6,7,8].

Resultados preliminares do laboratório de acolhimento demonstraram que a suplementação de murganhos C57Bl/6 com RKV, em que a administração de Arg (R) é combinada com a de Lisina (K) e Valina (V), dois aminoácidos descritos como inibidores da arginase, leva a uma diminuição acentuada da infeção hepática, sobretudo através da redução do número de hepatócitos infetados, sugerindo uma eliminação dos parasitas. No entanto, permanece por esclarecer se este fenótipo é recapitulado noutras estirpes de murganhos. Assim, o primeiro objetivo desta tese foi caracterizar a infeção hepática por *Plasmodium* em murganhos BALB/c após suplementação com Arg e RKV. Demonstrámos que a suplementação com Arg é suficiente para inibir a infeção hepática por *Plasmodium* em murganhos BALB/c, enquanto a suplementação com RKV pode levar tanto ao aumento como à diminuição da infeção hepática, em ambos os casos afetando principalmente o número de hepatócitos infetados. A razão desses resultados contraditórios permanece desconhecida.

O segundo objetivo desta tese foi elucidar o mecanismo de eliminação hepática do parasita em murganhos C57Bl/6 suplementados com RKV. Demonstrámos que a eliminação do parasita pela suplementação com RKV não depende nem da produção de NO, nem da estimulação da resposta de interferão tipo-I (IFN), anteriormente relatadas como cruciais para o controlo da infeção hepática[9,10]. Utilizando murganhos *knockout* e eliminando populações de células imunes específicas, identificámos as Células Linfóides Inatas (ILCs) como potenciais células imunes efetoras envolvidas na eliminação do parasita dependente de RKV. Adicionalmente, a sinalização através de MyD88 parece ser essencial para a eliminação hepática do parasita após suplementação com RKV, embora as células envolvidas nessa sinalização permaneçam desconhecidas.

Este projeto irá melhorar o nosso conhecimento quanto aos aspetos fundamentais da biologia de *Plasmodium* e quanto à resposta do hospedeiro face à infeção, abrindo

caminho para o desenvolvimento de potenciais novas estratégias que possam vir a ser usadas para controlar a infeção por *Plasmodium*.

Palavras-chave: *Plasmodium*, infeção hepática, Arginina, suplementação, resposta imune

Abstract

Malaria is an acute febrile illness and one of the most prevalent infectious diseases in the world[1]. It is caused by protozoan parasites of the genus *Plasmodium* that enter their mammalian host in the form of sporozoites, via the bite of an infected female *Anopheles* mosquito[1,2]. The first, obligatory and asymptomatic phase of infection occurs in the liver and is initiated when injected sporozoites invade their host's hepatocytes[2,3]. There, parasites differentiate and develop until merozoites are formed and released into the bloodstream, cyclically infecting red blood cells and causing the malaria symptoms[2,3].

During the liver-stage of infection, *Plasmodium* parasites scavenge host nutrients to support their multiplication. One of these nutrients is arginine (Arg), whose metabolism is crucial for the parasite's intrahepatic development[4].

Arg is becoming increasingly popular in nutritional supplementation for its ability to boost the immune system[5]. Arg is so far the only amino acid-based supplementation evaluated in the context of malaria. However, although Arg supplementation can enhance nitric oxide (NO) production, decrease parasitaemia and improve survival in animal models of *Plasmodium* infection, unclear results have been obtained in the clinic[6,7,8].

Preliminary results from the host laboratory have shown that supplementation of C57Bl/6 mice with RKV, which combines Arg (R) with Lysine (K) and Valine (V), two amino acids described as arginase inhibitors, but not Arg alone, leads to a striking decrease of hepatic infection, mostly by reducing the number of infected hepatocytes, suggesting that parasites are being eliminated. However, whether this phenotype was also recapitulated in other mouse strains remained unclarified. Thus, the first aim of this thesis was to characterize *Plasmodium* liver infection in BALB/c mice upon Arg and RKV supplementation. We found that Arg supplementation is sufficient to impair *Plasmodium* liver infection in BALB/c mice and, on the contrary, RKV supplementation can lead to either an increase or a decrease in *Plasmodium* hepatic infection, in both cases mostly by affecting the number of infected hepatocytes. The reason for these contradictory results remains unknown.

The second aim of this thesis was to elucidate the mechanism of hepatic parasite elimination in C57Bl/6 mice upon RKV supplementation. We found that parasite elimination by RKV supplementation does not rely on NO production nor on a boost of the type-I interferon (IFN) response, previously reported as crucial to control liver-stage infection[9,10]. Employing knockout mice and depleting specific immune cell populations, we identified Innate Lymphoid Cells (ILCs) as potential effector immune cells involved in RKV-dependent parasite elimination. Additionally, signalling via MyD88, seems to be essential for hepatic parasite's elimination upon RKV supplementation, although the cells in which this signalling occurs remain unidentified.

This project will enhance our knowledge of fundamental aspects of *Plasmodium* biology and of the host's response to infection, paving the way to the development of potential new strategies that may ultimately be employed to control *Plasmodium* infection.

Keywords: *Plasmodium*, liver-stage infection, Arginine, RKV supplementation, immune response

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Abbreviations

AIM2	Absent in melanoma 2
ACK	Ammonium-Chloride-Potassium
ACTs	Artemisinin-based combination therapies
ADMA	Asymmetrical dimethylarginine
ALI	Acute lung injury
ALRs	Absent in melanoma 2-like receptors
Anti-Gr-1	Anti-Granulocyte receptor-1
APCs	Antigen presenting cells
AQ	Amodiaquine
AQ+SP	Amodiaquine + sulfadoxine-pyrimethamine
Arg	Arginine
ASC	Apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain
BSA	Bovine Serum Albumin
CAT2A/B	Cationic amino acid transporters 2A and B
CCR2	C-C Motif chemokine receptor type 2
CD68	Cluster of Differentiation 68
CD115	Cluster of Differentiation 115
cDNA	Complementary DNA
Clec4f	C-Type Lectin Domain Family 4 Member F
CQ	Chloroquine
CSF-1	Colony-stimulating factor 1
DAMPs	Danger-associated molecular patterns
DCs	Dendritic cells
DEPC	Diethyl pyrocarbonate
ECM	Experimental cerebral malaria
EDTA	Ethylenediaminetetraacetic acid
EEF	Exoerythrocytic form
Eomes	Eomesodermin
FACS	Fluorescence-activated cell sorting

FBS	Fetal Bovine Serum
FcRs	Receptors for the fragment crystallizable portion of antibodies
FcεRI	High-affinity IgE receptor
FELASA	Federation of European Laboratory Animal Science Associations
Flt3	Fms-like tyrosine kinase 3
GATA3	GATA-binding protein 3
G-CSF	Granulocyte colony-stimulating factor
Gfi1	Growth factor independent 1
GFP	Green fluorescent protein
GLUT1	Glucose transporter class I
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPCRs	G-protein-coupled receptors
GPI	Glycosylphosphatidylinositol
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigens
hpi	Hours post-infection
Hprt	Hypoxanthine-guanine phosphoribosyltransferase
i.p.	Intraperitoneal
i.v.	Intravenous
Id2	DNA-binding protein
IFNAR	Interferon α receptor
IFN	Interferon
Ig	Immunoglobulin
IGC	Instituto Gulbenkian de Ciência
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-2Rβ	Interleukin-2 receptor β
IL-3Rα	Interleukin-3 receptor α
IL-7Rα	Interleukin-7 receptor α
IL-18R	Interleukin-18 receptor
ILCs	Innate lymphoid cells
ILC1s	Group 1 Innate lymphoid cells

ILC2s	Group 2 Innate lymphoid cells
ILC3s	Group 3 Innate lymphoid cells
ILC4s	Group 4 Innate lymphoid cells
iMM	Instituto de Medicina Molecular João Lobo Antunes
iNOS	Specific isoform of nitric oxide synthase
IPTi	Intermittent preventive treatment for infants
IPTp	Intermittent preventive treatment in pregnancy
IRS	Indoor residual spraying
ISGs	Interferon stimulated-genes
ITNs	Insecticide-treated mosquito nets
IVC	Inferior vena cava
LDM	Liver digestion medium
L-FABP	Liver-fatty acid binding protein
LLNs	Long-lasting nets
LPM	Liver perfusion medium
LTis	Lymphoid tissue inducer
Ly6C	Lymphocyte antigen 6 complex, locus C
Ly6G	Lymphocyte antigen 6 complex locus G6D
Lys	Lysine
M1	Classically activated macrophages
M2	Alternatively activated macrophages
MAVS	Mitochondrial antiviral signalling protein
MDA5	Melanoma differentiation-associated gene 5
MHC	Major histocompatibility complex
Mrgprb2	Mas-related G-protein coupled receptor member B2
MRGX2	Mas-related G-protein-coupled receptor X2
MyD88	Myeloid differentiation primary response 88
NAC	N-Acetyl Cysteine
NETs	Neutrophil extracellular traps
Nfil3	Nuclear factor, IL-3
NF-κB	Nuclear factor-κappa B
NK	Natural killer cells

NK T	Natural killer T cells
NLRs	Nucleotide-binding oligomerization domain
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
PbHT	*P. berghei* hexose transporter
PbNPT1	*P. berghei* Novel Putative Transporter 1
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PFA	Paraformaldehyde
PLP1	Perforin-like protein 1
PQ	Primaquine
PRRs	Pattern recognition receptors
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
qPCR	Quantitative real-time PCR
RBCs	Red blood cells
RDT	Rapid diagnostic tests
RIG	Retinoic acid-inducible gene
RORα	Retinoic acid receptor-related orphan receptor-α
RORγτ	Retinoic acid receptor-related orphan receptor-γτ
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SLC7	Solute carrier family 7
SMC	Seasonal malaria chemoprevention
SP	Sulfadoxine-pyrimethamine
T-bet	T-box transcription factor-Tbx21
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th	T helper
Th1	Type 1 T helper
Th2	Type 2 T helper

Abbreviations

Th9	Type 9 T helper
Th17	Type 17 T helper
TIR	Toll/interleukin-1 receptor
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor α
Treg	Regulatory T cells
UIS3	Up-regulated in infectious sporozoites 3
UIS4	Up-regulated in infectious sporozoites 4
Val	Valine
WHO	World Health Organization
WT	Wild-type
αLP	α-lymphoid precursor

Chapter I – Introduction

General introduction to Malaria

Parasitic diseases infect over half a billion people worldwide and are a tremendous public health burden. Malaria is an acute febrile illness and one of the most prevalent infectious diseases in the world[1]. It is a leading cause of death and disease across many tropical and subtropical countries, pregnant women and children being the most affected groups[1].

i) *Plasmodium* parasite species

Malaria is caused by protozoan parasites of the genus *Plasmodium* that are transmitted to the host through the bite of an infected female *Anopheles* mosquito[1]. There are five *Plasmodium* species known to cause disease in humans, four of which, *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, have long been recognized to infect humans[11,12,13], and one, *P. knowlesi*, which naturally infects monkeys and has been recently reported to cause human cases of malaria in certain forested areas of South-East Asia[14]. Among these, *P. falciparum* and *P. vivax* are the most prevalent and account for the majority of the global malaria cases[1,11,15]. *P. falciparum* is responsible for most deaths attributed to malaria and is most prevalent in Africa[1,11]. *P. vivax* is much less predominant in this region due the absence of Duffy antigen receptor for chemokines on the surface of red blood cells (RBCs), essential for *P. vivax* invasion [11], and occurs mostly in the Americas and South-East Asia[1]. In 2016, *P. vivax* malaria was responsible for only 4% of the estimated malaria cases globally. *P. vivax* can also generate dormant liver-stage forms termed hypnozoites, which lead to malaria relapses[2].

ii) Cases and Deaths

People who suffer from malaria are typically very sick with periodic fever, shaking chills, shivering, headache, nausea, and vomiting[11]. Left untreated, they may develop severe complications, including cerebral malaria, placental malaria, malaria associated acute lung injury, acute renal failure, severe anaemia and liver injury[16], which often leads to death. The 2017 World Malaria Report estimated that a total of 216 million cases of malaria occurred worldwide in 2016, an increase of 5 million cases in comparison

with the previous year[1]. The total tally of malaria deaths reached 445.000 globally, 80% of which in sub-Saharan Africa, and mostly in children aged under 5 years old[1]. Although malaria case incidence and mortality rate have fallen 18% globally between 2010 and 2016, the rate of decline has stalled and even reversed in some regions since 2014[1].

iii) Funding

In 2016, US$ 2.7 billion were invested to accelerate progress towards malaria control and elimination[1]. However, this value represents less than half of the minimum investment of US$ 6.5 billion estimated to be required annually in order to achieve the 2030 targets of the World Health Organization (WHO) global malaria strategy[1]. Additionally, US$ 686 million were also estimated to be required annually for malaria research and development between 2016 and 2030[1].

iv) Strategies for malaria control and elimination

WHO recommends the use of vector control measures to reduce malaria transmission; chemoprevention, providing drugs that suppress blood-stage infections in specific population subgroups (pregnant women, children and other high-risk groups); and case management, which includes diagnosis and treatment of infections[1].

Vector control

Vector control can be achieved using insecticide-treated mosquito nets (ITNs), long-lasting nets (LLNs) or indoor residual spraying (IRS)[1]. In 2016, an estimated 54% of people at risk of malaria in sub-Saharan Africa were sleeping under an ITN, which represents a considerable increase of level coverage since 2010[1]. However, the proportion of the population at risk protected by IRS declined globally from a peak of 5.8% in 2010 to 2.9% in 2016[1].

Chemoprevention

Chemoprevention is recommended by WHO for pregnant women and young children[1]. In sub-Saharan Africa, intermittent preventive treatment in pregnancy (IPTp) with 3 or 4 doses of sulfadoxine-pyrimethamine (SP), starting as early as possible in the second trimester, has been shown to reduce maternal anaemia, low birth weight and

perinatal mortality[1]. In 2016, an estimated 19% of eligible pregnant women received 3 or more doses of IPTp, an increase of 6% since 2014[1].

Intermittent preventive treatment for infants (IPTi) with SP, which provides protection against clinical malaria and anaemia, was not implemented in any country, similarly to what was reported in 2015[1]. For children aged 5-59 months living in areas of highly seasonal malaria transmission, WHO recommends seasonal malaria chemoprevention (SMC) with amodiaquine (AQ) plus SP (AQ+SP), which reduced the incidence of clinical attacks and severe malaria by about 75%[1]. In 2016, about 15 million children were included in SMC programmes in 12 countries and 91% of those received SMC at least once[1].

Chemoprophylaxis is also recommended for travellers from non-endemic countries to countries with ongoing local malaria transmission, preferably in addition to other personal protective measures, such as the use of insect repellents, long sleeves, long pants, and ITNs[17,18]. Currently, the most common prophylactic drugs for travellers are cloroquine (CQ), Atovaquone/Proguanil (known as Malarone), Doxycycline, Mefloquine and primaquine (PQ)[17,18]. The preventive drug recommended varies with the country of origin and the availability of antimalarial drugs in that country, the species of *Plasmodium* and the presence or not of drug resistance in the country of destination, as well as, the time the traveller will spend there[17,18].

v) Diagnostic and Treatment

Early diagnosis and treatment of malaria is the most effective intervention to reduce disease and prevent death[1]. Effective case management may also help to reduce the transmission, by improving malaria case management in remote communities[1].

WHO recommends that every suspected malaria case be confirmed by either microscopy or a rapid diagnostic test (RDT) before treatment[1]. Accurate diagnosis ensures that antimalarial medication is used only when necessary[1]. In 2016, 87% of suspected malaria cases received a parasitological test, compared to 36% in 2010[1]. Artemisinin-based combination therapies (ACTs), combining artemisinin, which confers rapid and potent effectiveness, with a longer-lived antimalarial (such as lumefantrine,

mefloquine, piperaquine, AQ or SP) as a partner drug[19], were adopted for first-line treatment of *P. falciparum* uncomplicated malaria[1]. Quinine remains the official first-line treatment of severe malaria [1]. CQ is still used as an effective first-line treatment for *P. vivax* in many countries[1]. CQ or ACTs can also be combined with PQ to eliminate hypnozoites and prevent relapses[1,2].

Life cycle of *Plasmodium* parasites

Plasmodium spp. have a complex life cycle, alternating between female *Anopheles* mosquitoes and vertebrate hosts[2] (Fig. 1.1). *Plasmodium* infection begins with the bite of an infected female *Anopheles* mosquito that inoculates under the skin of the mammalian host *Plasmodium* parasites, termed sporozoites at this stage[2] (Fig. 1.1).

Within minutes to a few hours after inoculation[20], a proportion of the sporozoites use a form of locomotion, known as gliding motility, to reach the host's vasculature. Sporozoites that successfully enter blood vessels rapidly access the liver by a process known as cell traversal, that involves crossing the sinusoidal barrier comprising fenestrated endothelial cells and macrophage-like Kupffer cells[21,22,23,24,25]. Once within the liver parenchyma, sporozoites continue traversing several hepatocytes before invading a final one. Sporozoites initially invade cells inside transient nonreplicative vacuoles by an active moving junction-independent process and then, using pH sensing and perforin-like protein 1 (PLP1), they exit these vacuoles and, only then, enter the parasitophorous vacuole (PV)[26]. Once hepatocyte infection is established, each sporozoite inside a PV develops into an exoerythrocytic form (EEF), through a process called schizogony. This process of transformation and intense replication defines the obligatory and asymptomatic pre-erythrocytic phase of infection and lasts 5-17 days in humans[27,28,29] and 2 days in rodents[12]. Sporozoites development culminates in release of newly formed haploid parasites, termed merozoites at this stage, into the bloodstream by budding of parasite filled vesicles called merosomes[30]. Once in the circulation, merosomes can reach the lung capillaries where they rupture releasing the erythrocyte-infectious merozoites that initiate the erythrocytic stage of malaria infection[3]. *P. vivax* and *P. ovale* are capable of forming dormant pre-erythrocytic forms, called hypnozoites, that can be activated at later time points to cause relapsing blood-stage infections[31].

During the symptomatic erythrocytic stage of infection, the cycles of parasite asexual replication inside erythrocytes are shorter than during exoerythrocytic schizogony (24, 48 or 72h, depending on parasite species) [28,32,]. *Plasmodium*'s asexual development in erythrocytes is complex, with 3 successive morphological stages: the ring stage, which is established following invasion of the red blood cell by a merozoite; the trophozoite stage, characterized by rapid parasite growth and the appearance of haemozoin (remnant of digested haemoglobin) in the food vacuole; and finally, the schizont stage, during which it generates daughter merozoites that, after rupture of the host cell, invade new erythrocytes[33]. The erythrocytic stage constitutes a continuous cycle of infection that allows parasite numbers to reach billions within weeks and is responsible for causing the classical symptoms of the disease [31]. This is not only due to the massive destruction of RBCs but also to the sequestration of infected RBCs in the microvasculature, leading, for example, to cerebral or placental malaria pathologies through the sequestration in the brain or placenta, respectively[31]. During the blood-stage of infection, a proportion of asexually reproducing merozoites are reprogrammed to undergo a process of gametocytogenesis[2]. Within 15 days, the sexual parasite forms, termed gametocytes at this stage, sequester and develop within the bone marrow. Once mature, gametocytes enter the peripheral circulation and are taken up by an *Anopheles* mosquito upon a blood meal, where they experience a drop in temperature, a change in pH and exposure to xanthurenic acid, which together trigger their maturation into extracellular male (microgamete) and female (macrogamete) gametes in the mosquito midgut[2,34,35]. Mating occurs by fusion of a microgamete with a macrogamete to form a diploid zygote that develops into a motile diploid ookinete[2]. This ookinete migrates through the mosquito midgut epithelium and develops into oocysts that burst after 1 or 2 weeks of incubation in the midgut, releasing motile sporozoites into the hemolymph[2,34]. Finally, these sporozoites travel to the salivary glands where they can be injected into the next host during the mosquito blood meal, which ensures the transmission of the parasite[12,2].

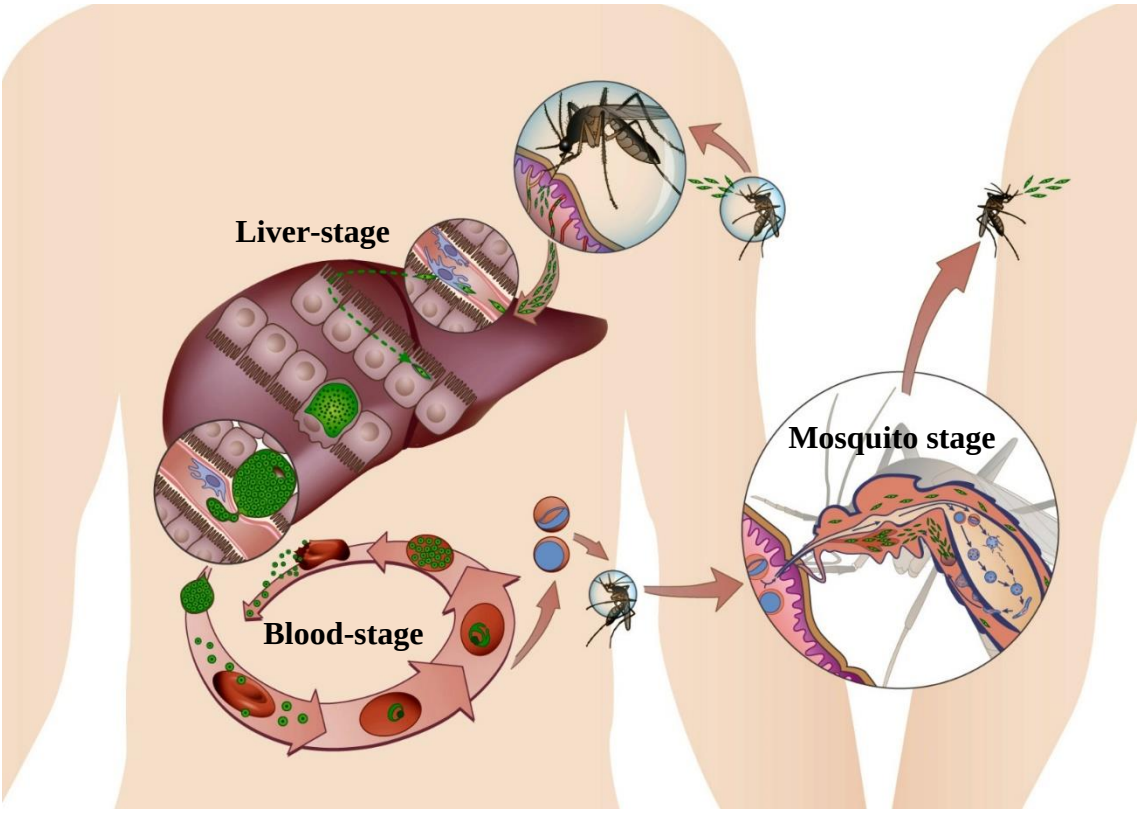

Figure 1.1 – Life cycle of *Plasmodium* parasites.

Nutrient Acquisition by *Plasmodium* in the liver

The pre-erythrocytic stage of *Plasmodium* infection starts with the deposition of *Plasmodium* sporozoites under the skin of the mammalian host and culminates in the release of newly formed merozoites into the bloodstream. During this stage, *Plasmodium* parasites undergo an obligatory phase of extensive replication inside their mammalian host´s hepatocytes[4], with 10^4- to 10^5-fold replication of its genome, which constitutes one of the fastest known growth rates among eukaryotic cells[3,36]. However, *Plasmodium* can neither synthesize all nutrients that they need nor store host molecules, requiring therefore a constant supply of nutrients[37]. In order to meet their anabolic requirements and proficiently multiply within hepatocytes, these parasites must scavenge host cell nutrients[37]. They achieve this by transforming its PV into a highly permeable compartment [38], with pores across its membrane (PVM), leading to an interconnection of the host cytoplasm with the vacuolar space. This allows the passive diffusion of small molecules from host cytosolic pools[39], such as lipids [fatty acids[40]; cholesterol[37]; and

phosphatidylcholine (PC)[41]]; glucose[42,43,44]; iron[45], lipoic acid[46]; and amino acids [asparagine[47]; and arginine (Arg)[4]], as described below.

i) Lipids

Lipids play key roles in many biological processes, ranging from a structural role in membranes to signalling, in addition to being sources of metabolic energy[41].

Intrahepatic parasites actively regulate the expression of several host genes involved in lipid metabolism [48,49], highlighting the aptitude of the parasite in modifying the host's basic metabolism to increase the available pool of nutrients[37].

Fatty acids

The malaria parasite is able to synthesize fatty acids through an apicoplast-located synthesis pathway[50]. However, the enormous growth of hepatic parasites implies that fatty acids are scavenged from the host cell. Up-regulated in infectious sporozoites 3 (UIS3) and UIS4 were identified as being essential for liver-stage growth and development[39,51]. UIS3 was also identified as being one of the liver-stage PVM-resident proteins [39], which interacts directly with the host liver-fatty acid binding protein (L-FABP)[37]. L-FABP, the main cytoplasmic carrier of fatty acids in host hepatocytes, acts as a shuttle of fatty acids for import, storage and export as well as delivery to intracellular destinations[37], and has been reported to be involved in parasite uptake of fatty acids[40]. Liver-stage parasite development is inhibited upon down-modulation of L-FABP expression, supporting a critical role of L-FABP for successful malaria liver-stage infection [37].

Phosphatidylcholine

The rapid replication of *Plasmodium* parasites in hepatocytes requires important lipid resources to support organelle and membrane neogenesis and the growth of the PVM[3,41]. PC, the major membrane phospholipid, is taken up from the host cell by both *P. falciparum* and *P. berghei*, and supports both the enlargement of the PVM and membrane neogenesis for newly-formed merozoites[41]. PC is therefore essential for parasite survival[41].

Cholesterol

Since malaria parasites cannot synthesize sterols, they must scavenge these lipids from the host[37]. *Plasmodium* continuously diverts cholesterol from hepatocytes until merozoites are released[37]. *Plasmodium* parasites have moderate need of sterols for optimal development in hepatocytes and can adapt to survive in cholesterol-restrictive conditions by exploiting accessible sterols derived from alternative sources in hepatocytes[37].

ii) Glucose

Because *Plasmodium* parasites do not have intracellular energy stores during most of their life cycle, they require a constant supply of glucose from their hosts [52]. Glucose is the primary source of energy and a key substrate for most cells, being essential during *Plasmodium* intrahepatic development[42,43,44]. Glucose has been shown to be transported into infected hepatocytes mainly by glucose transporter class I (GLUT1), and then taken up by the parasite via a parasite-encoded facilitative hexose transporter [*P. falciparum* hexose transporter (PfHT) and its orthologue, the *P. berghei* hexose transporter (PbHT)][42,43,44]. Inside hepatocytes, glucose plays an essential role during the liver-stage of infection by the malaria parasite, modulating the liver-stage development both *in vitro* and *in vivo*[44].

iii) Iron

Iron availability also plays a role on *Plasmodium* liver development[45]. It was shown that blood-stage parasite density above a certain threshold can inhibit the next wave of sporozoite invasion and development, by decreasing both the number and size of EEFs, through modulation of hepcidin[45]. Hepcidin regulates iron homeostasis and distribution by targeting the iron exporter ferroportin for degradation[45]. In this context, hepcidin redistributes iron away from the liver, which impairs *Plasmodium* liver infection[45].

iv) Lipoic acid

Lipoic acid is an essential cofactor for enzymes that participate in key metabolic pathways in most organisms[46]. Protein lipoylation in the plasmodial apicoplast relies on

de novo lipoic acid synthesis while lipoylation of proteins in the mitochondrion depends on scavenging of lipoic acid from the host cell[46]. Mitochondrial lipoic acid scavenging is essential for *P. berghei* liver-stage development, specifically to the process of schizogony[46].

v) Amino acids

Amino acids are the molecular building blocks of proteins, the precursors of various biologically important molecules and are essential sources of carbon, nitrogen and energy metabolism[53,54]. During the liver-stage of infection, it was reported that asparagine and Arg are essential for *Plasmodium* hepatic infection.

Asparagine

Asparagine is one of the most abundant amino acids in *Plasmodium* proteins[55]. *Plasmodium* parasites retained asparagine synthetase that catalyses the formation of asparagine from aspartate[47]. Upon deletion of asparagine synthetase, *Plasmodium* liver-stage development is impaired, and survival may be maintained through the extracellular addition of asparagine[47]. These findings indicate that liver-stage parasites are capable of taking up asparagine from the host hepatocytes and that this amino acid is essential for *Plasmodium* hepatic infection[47].

Arginine

Arg is a semi-essential cationic amino acid, transported into hepatocytes mostly via the cationic amino acid transporters 2A and B (CAT2A/B), a member of the solute carrier family 7 (SLC7)[4]. Arg can subsequently enter the parasite via the recently described *P. berghei* Novel Putative Transporter 1 (PbNPT1)[56].

Once inside the cell, Arg can be metabolized into nitric oxide (NO), ornithine, urea, polyamines, proline, glutamate, creatine, and/or agmatine[54]. In mammals, the pathways involving the enzyme arginase and a specific isoform of nitric oxide synthase (iNOS) are quantitatively the most important for Arg catabolism[54] (Fig. 1.2). Arginase catalyses the hydrolysis of Arg, being its activity important for maintaining ornithine levels for polyamine synthesis[4] (Fig. 1.2). There are 2 isoforms of this enzyme: a cytosolic arginase I, abundantly expressed in the liver[54]; and a more widely distributed mitochondrial arginase II[54]. On the other hand, Arg is also a substrate of iNOS for the

production of NO[53,57,58] (Fig. 1.2). Therefore, arginase and iNOS compete for Arg[53]. A marked elevation in arginase activity, which reduces NO synthesis, provides a mechanism responsible for the survival of immunologically challenged parasites[59]. Contrarily, increasing extracellular concentrations of Arg, increases NO production by endothelial cells and activated macrophages[60], which can lead to parasite killing.

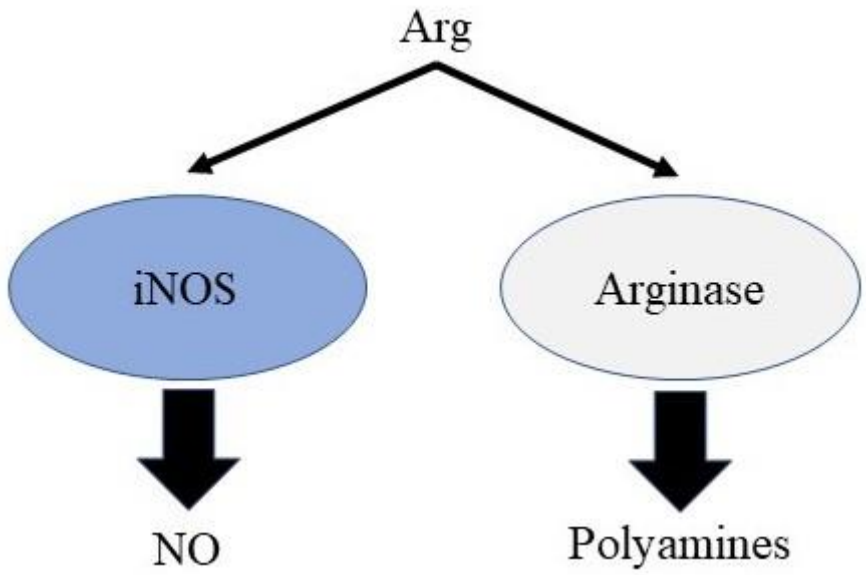

Figure 1.2 - Two main pathways of Arg metabolism.

Arg:Arginine; iNOS: inducible nitric oxide synthase; NO: nitric oxide.

The host lab defined an essential role of Arg-dependent polyamine biosynthesis in the normal developmental process of liver-stage *P. berghei* parasites[4]. *Plasmodium* preferentially uses its own biosynthesis pathway to obtain the polyamines it requires to proliferate inside hepatocytes but this requirement of its own arginase pathway may be bypassed through the action of the host's arginase and ornithine decarboxylase enzymes[4]. On the other hand, iNOS-dependent NO production does not seem to play a role during liver infection by malaria parasites[4].

Arginine supplementation and disease

Arg is known to play an important role in cell division, growth and immune regulation[61,62]. Arg is becoming increasingly popular in nutritional supplementation for its ability to boost the immune system, as it has been linked to proliferation of T

lymphocytes, activation of macrophages and increased antibody production[5]. Several clinical studies showed that Arg supplementation can lead to an improved outcome in patients with major traumas, cancer and Human immunodeficiency virus (HIV) infections[8,63].

Arginine supplementation and malaria

In the context of malaria infection, Arg is the only amino acid-based supplementation that has been evaluated so far. Nonetheless, its impact on malaria infection and pathology remains difficult to define.

Studies in African children with severe malaria demonstrated impaired production of NO[64]; low plasma concentrations of Arg[65] (hypoargininemia); and impaired endothelial function[66]. Endothelial dysfunction is a measure of endothelial activation and may play a role in the pathogenesis of severe malaria by increasing the adhesion of parasitized erythrocytes to the endothelium and thereby worsening microcirculatory obstruction and oxygen delivery[67]. Arg infusion was described to be able to improve NO bioavailability and endothelial function in patients with severe malaria[66]. Studies in healthy volunteers, in patients with cardiovascular disease and in patients hospitalized with severe malaria considered that Arg infusion was safe and with minimal side effects[68,69,70,71]. However, one study of intravenous Arg infusion in Indonesian adults with severe malaria, did not find any improvement in endothelial NO production[72]. In the context of animal models, Arg pre-treatment drastically improved animal survival and led to better resolution of parasitemia in *P. yoelii* 17XL-infected BALB/c mice during the early blood-stage of infection, by improving a protective Th1 response[6]. On the other hand, it was associated with an increase of experimental cerebral malaria (ECM) in *P. berghei* ANKA-infected C57Bl/6 mice[73]. Arg was also reported to further inhibit the development of sexual stages in mosquitoes, through increased production of NO, resulting in decreased formation of zygotes and ookinetes *in vitro* and oocysts *in vivo*[74].

In summary, although Arg supplementation has been shown to be beneficial in some aspects of the malaria pathology, a clear role for this amino acid in the context of the disease has not yet been defined.

Immune system overview

The immune system refers to a collection of cells and proteins that function to protect the organism from foreign antigens, such as microbes (bacteria, fungi and parasites), viruses, cancer cells and toxins. The immune system can be simplistically viewed as having 2 lines of defense: innate immunity and adaptive immunity[75].

i) Innate Immunity

The innate immune system is an evolutionarily conserved first line defense to an intruding pathogen[75,76]. It is an antigen-independent defense mechanism that is used by the host immediately or within hours of encountering an antigen[75]. The innate immune response encompasses anatomic and physiologic barriers, which include intact skin, vigorous mucociliary clearance mechanisms, low stomach pH, and bacteriolytic lysozyme in tears, saliva, and other secretions[77]. After overcoming this first line of defense, the innate immune cells play a crucial part in the initiation and subsequent development of adaptive immune responses[78].

Numerous cells are involved in the innate immune response such as: myeloid cells [eosinophils, basophils, mast cells, dendritic cells (DCs), neutrophils, and monocytes/macrophages] and lymphoid cells [innate lymphoid cells (ILCs)][77]. Moreover, recent research has documented the capacity of innate immune cells such as natural killer (NK) cells, monocytes and macrophages to recall a first encounter with a pathogen demonstrating that a non-specific innate immune compartment is capable of memory[78].

In addition to these cellular defenses, innate immunity also has a humoral component that includes well-characterized components such as complement proteins, lipopolysaccharide binding protein, C-reactive protein and other pentraxins, collectins, and anti-microbial peptides including defensins[77], which will not be covered in detail in this section.

The present section addresses the intervening cells in the innate immune response.

Myeloid cells

Eosinophils

Eosinophils are major effector cells in the immune system that are produced by the bone marrow from haematopoietic stem cells in a process called "granulopoiesis"[79]. Together with basophils and neutrophils, they constitute the granulocytes, a type of immune cell that has granules (small particles) with enzymes that are released during infections, allergic reactions, and asthma[79]. Besides their role as destructive end-staged effector cells, eosinophils are also important regulators of local immunity and remodelling/repair[79]. Therefore, they have different features: (i) are nonspecific destructive and cytotoxic cells; (ii) are an omnipresent cellular infiltrate of the asthmatic lung; and (iii) are a necessary and ubiquitous host defense against parasitic infections[79]. These cells express a large panel of cytokines: both Type 1 T helper (Th1) and Type 2 T helper (Th2) cytokines [such as: interleukin (IL)-12 and interferon (IFN)-γ; IL-13 and IL-25; respectively]; acute proinflammatory cytokines [such as: Tumor necrosis factor-α (TFN-α), IL-1β and IL-6] and immune inhibitory cytokines [such as: Transforming growth factor-β (TGF-β) and IL-10][79]. Eosinophils also express pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) 1-5 and 9, which allows them to recognize pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs)[79]. These cells interact with B cells and T cells, contributing for plasma B-cell survival through cytokines production or playing an important role in T cell selection in the thymus[79].

Basophils

Basophils are the least common granulocytes, representing less than 1% of peripheral blood leukocytes in both mice and humans[80,81]. Basophils differentiation from myeloid progenitors is driven by IL-3 and their expression of IL-3 receptor α (IL-3Rα) chain enables them to be distinguished from mast cells[80,81]. These cells circulate in the peripheral blood and migrate to sites of inflammation, where they become activated by antigen crosslinking of high-affinity immunoglobulin E receptor (FcεRI) and therefore, undergo rapid degranulation and release their cellular contents, which includes histamine[80,81]. In addition, basophils can also be activated without immunoglobulin (Ig) E crosslinking by IL-3, IL-18, IL-33 or TLR ligands, which induce the production of IL-

4[80,81]. They also can interact with Th2 cells, group 2 ILCs (ILC2s), eosinophils and inflammatory macrophages during the inflammation process[80,81].

Mast cells

Mast cells are long-lived tissue-resident cells derived from the bone marrow, functionally related to basophils[82]. They are located in virtually all tissues and are noticeably abundant in the skin and mucosa[82]. They are filled with secretory granules, where a huge panel of mediators is stored (such as: histamine, heparin, tryptase, chymase)[82]. The process of these granule secretion (degranulation) is triggered by two main classes of receptors: (i) receptors for the fragment crystallizable portion of antibodies (FcRs; such as FcεRI, which is common to basophils) that allows mast cells to specifically respond to antibody-target antigens; and (ii) G-protein-coupled receptors [GPCRs; such as: Mas-related G-protein-coupled receptor X2 (MRGX2) in humans and Mas-related G-protein coupled receptor member B2 (Mrgprb2) in rodents] that allow mast cells to degranulate in response to soluble mediators[82]. Although mast cells function and secretory granules release are involved in physiologic tissue homeostasis, excessive activation of these cells plays a crucial role in the pathogenesis of allergy and of organ-specific inflammation disorders (such as: psoriasis and inflammatory bowel disease)[82]. These cells also can interact with eosinophils, basophils and T cells[82].

Dendritic cells

DCs are bone marrow-derived leukocytes that constitute 1% or less of total haematopoietic cells of any lymphoid organ or blood, being however the most potent type of antigen-presenting cells[83]. DCs are specialised to capture and process antigens, converting proteins to peptides that are presented on Major histocompatibility complex (MHC) molecules recognised by T cells, which play an extremely important role in initiating tolerance, memory, and polarised Th1, Th2 and Type 17 T helper (Th17) differentiation[83]. These cells also play an essential role in maintaining tissue homeostasis and immune tolerance[83]. Human and mouse DCs can be classified in 4 categories, which are defined in the Table 1.1 together with the respective functions[83].

Table 1.1 – Categories and respective functions of DCs in human and mouse.

pDC – plasmacytoid DC; MoDC – monocyte-derived DC

Category Human	Category Mouse	Functions
CD141$^+$ DC	CD8$^+$/CD103$^+$	Major producers of type-III IFN, defined as important mediators against viruses.
CD1c$^+$ DC	CD11b$^+$ DC	Main producers of IL-12, being implicated in immune responses to mycobacterial and fungal infections and in Th2-mediated allergic responses.
pDC	pDC	Major IFN-I producers, playing a critical role in anti-viral immunity.
MoDC	MoDC	Rapidly differentiated from monocytes, infiltrate tissues in response to inflammation. These cells are specialized in driving local responses (such as: activation of tissue resident effector memory T cells).

Neutrophils

Neutrophils are the predominant immune cell population in the human blood, produced daily by the bone marrow from haematopoietic stem cells in a process called "granulopoiesis" and reside mainly in the peripheral vasculature[84]. Granulocyte colony-stimulating factor (G-CSF) is the predominant factor regulating the neutrophil's life cycle by increasing cell proliferation, survival, differentiation, and trafficking/mobilization[84]. Although neutrophils have been considered as short-lived effector cells of the immune system, *in vivo*-labelling studies demonstrated a life span of 5.4 days for human neutrophils[85], suggesting that neutrophils shape immune responses beyond rapid host-pathogen interactions.

Neutrophils have different roles in the innate immunity: (i) they are rapid and potent phagocytes; (ii) they were found to extrude their own nuclear or mitochondrial DNA as neutrophil extracellular traps (NETs), a phenomenon that almost always results in NETosis, a novel form of cell death[86]; (iii) they interact with a variety of immune and non-immune cells, such as DCs, monocytes/macrophages (recruitment of monocytes

involves production of cytokines, such as IFN-γ), NK cells and T lymphocytes[84]; (iv) and play a key role in the acute inflammation, being the first cells recruited to sites of infection and inflammation[84].

Monocytes/Macrophages

Monocytes and macrophages are included in the family of mononuclear phagocytes, alongside with DCs and osteoclasts[87]. This family shares distinctive properties: (i) distribution through multiple tissue compartments during development and adult life via blood and lymph; (ii) a common origin from haemopoietic stem cells and progenitors in specialised niches; (iii) serving as sentinels of change and stress, being versatile and adapting to widely differing environments[87].

Monocytes are produced by the bone marrow and circulate for a few days before they migrate into tissue where they develop into different types of macrophages[88]. These cells can be divided in 3 subpopulations, depending on the presence of CD14/CD16 and Ly6C/CD43 markers, in humans and mice, respectively (Table 1.2). They are multifunctional, with established roles in homeostasis, immune defense and tissue repair[88].

Table 1.2 – Nomenclature of monocyte populations in human and mouse.

The + and ++ denotes an expression level of 10-fold and 100-fold above the isotype control, respectively.

Monocyte Subpopulation	Human	Mouse
Classical	$CD14^{++}CD16^{-}$	$Ly6C^{++}CD43^{+}$
Intermediate	$CD14^{++}CD16^{+}$	$Ly6C^{++}CD43^{++}$
Nonclassical	$CD14^{++}CD16^{++}$	$Ly6C^{+}CD43^{++}$

Macrophages are cells specialised in the detection, phagocytosis and destruction of bacteria and other harmful organisms. These cells can derive either from embryonic yolk sac and foetal liver precursors or blood monocytes that leave the circulation to differentiate in different tissues[87]. Therefore, tissue macrophages consist of a mixed population of resident macrophages of embryonic origin and bone marrow-derived blood monocytes[87].

Macrophages are involved in both the inflammatory response and the subsequent tissue repair and regeneration. They are capable of detecting endogenous danger signals (PAMPs through TLRs)[77].

Macrophages can also interact with the adaptive response, being activated by cytokines that are secreted by lymphocytes, which modulate macrophage differentiation in 2 sub-phenotypes: classically activated macrophages (M1) and alternatively activated macrophages (M2)[76,87]. Th1 lymphocytes produce cytokines, such as IFN-γ, that induces the M1 phenotype, whereas the cytokines produced mainly by Th2 lymphocytes, such as IL-4 and IL-13, promote the M2 phenotype[76,87]. Th1 and Th2 lymphocytes will be covered in detail in the Adaptive Immunity section.

Lymphoid cells

Innate lymphoid cells

ILCs are a family of immune cells that play a crucial role as primary line of defense against different types of pathogens, by modelling the immune response[89].

ILCs are similar to lymphoid cells in their development but their α-lymphoid precursor (αLP) expresses the inhibitor of DNA-binding protein (Id2), which inhibits the expansion of B and T cells[89]. These cells have been classified into 4 groups, separating the cytotoxic ILCs and helper ILCs mirroring $CD8^+$ T cytotoxic cells [group 1 ILCs (ILC1s)] and $CD4^+$ T helper (Th) cells [ILC2s, group 3 ILCs (ILC3s) and group 4 ILCs (ILC4s)], respectively[90,91].

The unifying characteristic of ILC1s is their ability to produce IFN-γ. Apart from this common attribute, significant differences exist between the known ILC1 subsets. ILC1s comprise NK cells and cells that produce type 1 inflammatory cytokines, particularly IFN-γ and tumor necrosis factor (TNF-α). The Th1 cell associated transcription factor T-box transcription factor-Tbx21 (T-bet) cooperates with eomesodermin (Eomes) in order to control the development and function of NK cells[89]. NK cells are lymphocytes developed in the bone marrow and also in the thymus, that circulate in the peripheral blood, and rapidly migrate into sites of immune reactions in peripheral tissues[89]. It is well recognized that NK cells have cytotoxic activity and are also capable to produce inflammatory molecules and various lytic enzymes upon

activation[89], such as perforin and granzyme B, which induce cell death in tumor cells and virally infected cells[92]. Perforin facilitates the entry of the granzymes into the target cell cytoplasm, by forming pores, which leads to activation of apoptosis[89]. Granzyme B cleaves target cell proteins[89]. Granulysin is also expressed by NK cells, being responsible for apoptosis in a perforin dependent manner, similar to granzymes[93]. Other IFN-γ-secreting ILCs have been described and are referred to as ILC1s that are distinct from NK cells[89]. In humans, this subset of ILC1s expresses high levels of T-bet, and to a moderate level retinoic acid receptor-related orphan receptor-γt (RORγt), but lacks the expression of CD117 and Eomes transcription factor[94]. These ILC1s are found in both mouse and human intestine and lung tissues, where they participate in type 1 inflammation[95,96]. They are essentially implicated in immune response against intracellular pathogens and have been shown to be associated with human inflammatory bowel, Crohn's disease[89].

ILC2s comprise cells that produce type 2 cytokines such as: Granulocyte macrophage colony-stimulating factor (GM-CSF), IL-3, IL-4, IL-8, IL-9, IL13, IL-21[89]. ILC2s development requires IL-7, IL-25 and IL-33 to produce Th2 cell-associated cytokines[97,98,99]. Moreover, ILC2s require the transcription factors GATA-binding protein 3 (GATA3) and RORα[100,101,102]. These cells are involved in: (i) protection against parasites mainly through the production of IL-4, IL-5, IL-9 and IL-13; (ii) allergy, when coordinating with DCs and $CD4^+$ T cells; (iii) metabolic homeostasis; (iv) and tissue repair[89].

ILC3s comprise cells that produce IL-17A with or without IL-22[89]. ILC3s development and function depends on the transcription factor RORγt as well as IL-7 receptor α (IL-7Rα)[89]. This group comprises: (i) lymphoid tissue inducer (Ltis) cells, which are the first cells to populate the lymph nodes and were recognized as vital regulators of lymphoid tissue architecture after birth; (ii) IL-22-producing ILC3s, which play a critical role in tissue repair, regeneration of inflamed intestine, and antibacterial immunity[89].

ILC4s comprise cells that produce IL-17 and IFN-γ[89]. ILC4s require, for development and function, the transcriptional factors T-bet and RORγt, and also IL-2 receptor β (IL-2Rβ)[89]. Their role is not yet quite clear, being until now associated with diseases such as multiple sclerosis and psoriasis.

Innate Immunity and Inflammasome activation

Innate immunity provides a first line of defense against invading pathogens. The host cell is equipped with a set of germ-line-encoded PRRs that recognizes the basic structural units that make up microorganisms[103]. These receptors are located on the cell surface, endosomal membrane and in the cytoplasm[103]. The membrane-bound TLRs serve to detect ligands from the extracellular and endosomal milieu, whereas nucleotide-binding oligomerization domain and leucine-rich repeat-containing receptors (NLRs), the absent in melanoma 2 (AIM2)-like receptors (ALRs) and retinoic acid-inducible gene (RIG) I-like receptors provide immune surveillance[103]. Of these, NLRs and ALRs have the ability to form an inflammasome[103]. Among NLR inflammasome complexes, the NLRP3 inflammasome has been the most widely characterized, being a multi-protein entity with the capacity to activate the cysteine protease caspase-1, and ultimately induce proteolytic cleavage of the proinflammatory pro-IL-1β and pro-IL-18[104,105,106] (Fig. 1.3). Activation of this NLRP3 inflammasome requires at least two signals: signal 1, also known as the priming signal, is mediated by microbial ligands recognized by TLRs or cytokines such as TNF-α[107] (Fig. 1.3). Engagement of TLRs triggers intracellular signalling cascades through a set of toll/interleukin-1 receptor (TIR)-domain-containing adaptors, which includes Myeloid differentiation primary response protein 88 (MyD88)[108]. Therefore, during signal 1, the TLRs signal via the adapter molecule MyD88 consequently activates the Nuclear factor-κappa B (NF-κB) pathway, which represents a family of inducible transcription factors that regulates a large array of genes involved in different processes of the immune and inflammatory responses[109] (Fig. 1.3).

NF-κB pathway, in this context, leads to upregulation of pro-IL-1β and NLRP3 protein levels[110,111] (Fig. 1.3). The signal 2 is mediated by numerous PAMP or DAMP stimulation which activate the functional NLRP3 inflammasome by initiating assembly of a multi-protein complex consisting of NLRP3, the adapter apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) and pro-caspase 1, ultimately leading to the activation of pro-caspase-1 into its cleaved form[107] (Fig. 1.3). Several molecular mechanisms have been suggested for NLRP3 activation to induce caspase-1 activation, such as pore formation and potassium (K^+) efflux[112,113], lysosomal destabilization and rupture[114,115], and mitochondrial reactive oxygen species (ROS) generation[107,115,116] (Fig. 1.3). Caspase-1 is known as an inflammatory caspase that then

plays a role in the maturation of IL-1β and IL-18 into active cytokines and, under certain conditions, in the induction of pyroptosis, a form of programmed inflammatory cell death[107] (Fig. 1.3). IL-1β is a key pro-inflammatory cytokine that affects nearly every cell type and mediates inflammation in a variety of tissues, being involved in various systemic inflammatory diseases[117]. IL-18 is another pro-inflammatory cytokine that is involved in polarized Th1 cell and NK cell immune responses[118].

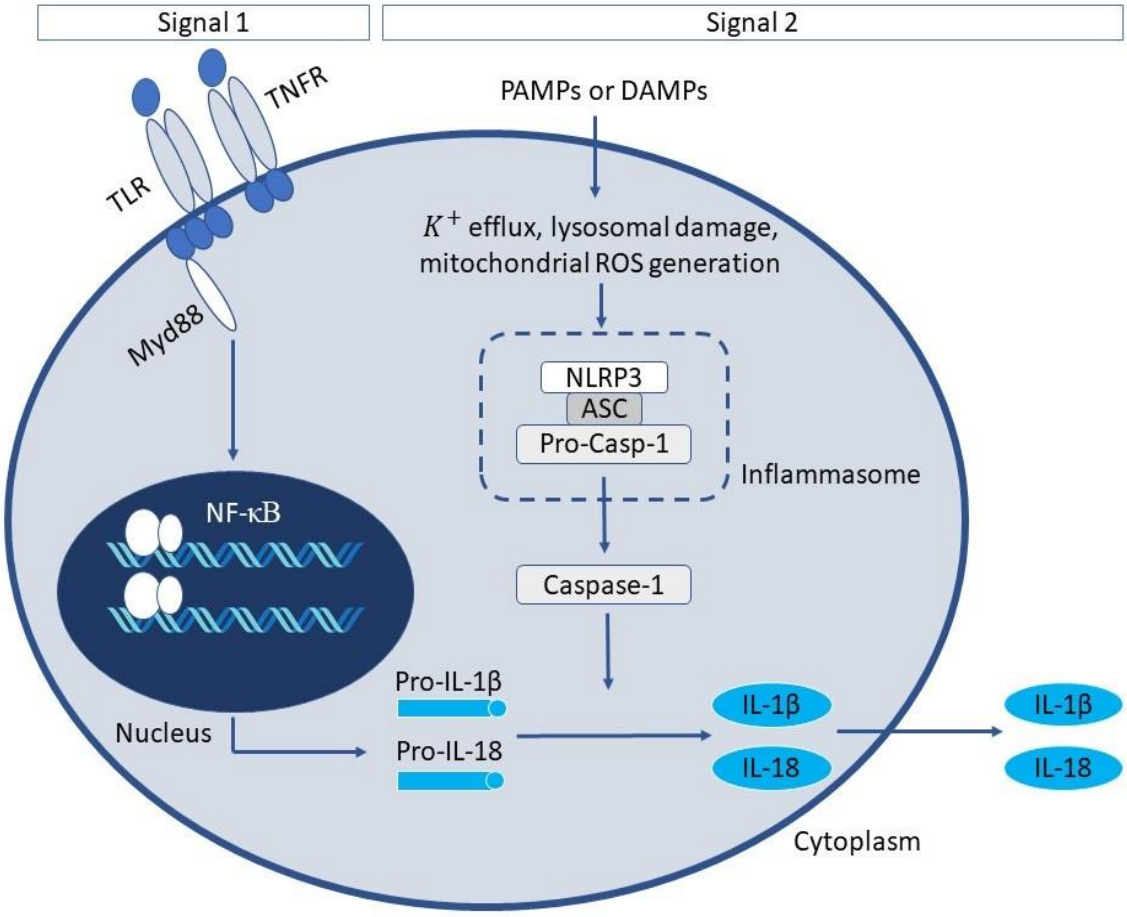

Figure 1.3 - A Two-Signal Model for NLRP3 Inflammasome Activation (Adapted from [107])

ASC: apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain; DAMPs: damage-associated molecular patterns; MyD88: myeloid differentiation primary response 88; NF-κB: nuclear factor κappa B; PAMPs; pathogen-associated molecular patterns; Pro-Casp-1: pro-caspase-1; TLR: toll-like receptors; TNFR: tumor necrosis factor receptor.

ii) Adaptive immunity

The innate immune system provides critical mechanisms for the rapid sensing and elimination of pathogens, recognizing those with germline-encoded receptors (such as TLRs)[76]. Conversely, adaptive immune responses depend on receptors that are custom tailored and selected through a process of somatic recombination of a large array of gene segments[119]. This response is developed only when the innate immunity is ineffective in eliminating infectious agents and the infection is established[75]. During this response, an immunologic memory is developed, which provides the capacity for rapid response in the event of re-exposure[119].

The cells of the adaptive immune system include the lymphoid cells: (i) T cells, which mature in the thymus and are activated through the action of antigen presenting cells (APCs); (ii) and B cells, which arise in the bone marrow and secrete antibodies[119].

T cells

T cells derive from haematopoietic stem cells in bone marrow or foetal liver and, following migration, mature in the thymus[75,119]. These cells express a unique antigen-binding receptor on their membrane, known as the T cell receptor (TCR)[119].

A major group of T cells express αβ TCR, which are involved in the activation of T cells through an interaction with the surface of APCs (usually DCs, macrophages, B cells, fibroblasts and epithelial cells) that recognize a specific antigen[119]. The surfaces of APCs express cell-surface proteins, known as MHC[119]. MHC are classified as either class I [human leukocyte antigens (HLA)-A, HLA-B and HLA-C], which present endogenous (intracellular) peptides; or class II (HLA-DR, HLA-DQ and HLA-DP), which present exogenous (extracellular) peptides and are inducible by innate immune stimuli, including ligands for TLRs[119]. This antigen presentation process stimulates T cells to differentiate into either cytotoxic T cells ($CD8^+$ single-positive) or Th cells ($CD4^+$ single-positive) through interaction of MHC class I or class II, respectively[119].

Th cells play a role in establishing and maximizing the immune response, through release of cytokines that influence the activity of many cell types. Several categories have been defined, according to the cytokines produced. Th1 response is characterized by the production of IFN-γ, which is involved either in the activation of mononuclear

phagocytes, NK cells and cytotoxic T cells, or in the induction of B cells to make opsonizing and neutralizing antibodies. Th2 response is characterized by the production of IL-4, IL-5, IL-10 and IL-13, which are involved in the activation and/or recruitment of B cells, mast cells and eosinophils[119]. Th17 cells are another type of Th cells that are involved in the production of IL-17, which is a potent pro-inflammatory cytokine capable of inducing IL-6 and TNF-α production, as well as driving granulocyte recruitment and tissue damage[120]. Type 9 T helper (Th9) was also reported as another type of Th cells, involved in the production of IL-9, a potent mast cell growth factor and mediator of helminthic immunity[121122]. Finally, regulatory T cells (Treg) were described as Th cells involved in the control of aberrant immune responses to self-antigens and in the development of auto-immune disease[123].

Cytotoxic T cells ($CD8^+$ T cells) are involved in the destruction of cells infected by foreign agents, by killing host cells in a contact-dependent mechanism, which ultimately leads to apoptotic cell death through the release of perforin, granzyme and granulysin[119], similarly to NK cells.

Natural Killer T (NK T) cells represents another subset of T cells that also express αβ TCR, together with NK cell antigens (CD56 in humans and NK1.1 in mice)[119]. These cells recognize nonpeptide antigens, such as lipids, presented by non-classic MHC molecules of the CD1 family[119]. NK T cells, when activated, are capable of rapid and substantial production of cytokines, including IL-4, which has been implicated in allergic pathogenesis[119,124].

A small subset of T cells, found in high numbers in the gastrointestinal epithelium, express γδ TCR and are $CD4^-$ $CD8^-$ double-negative cells: γδ T cells. These cells recognize antigens not in the context of MHC but rather as presented by nonclassical MHC molecules of the CD1 family, like NK T cells[119]. The γδ T cells are an important subset of T cells that can attack cells directly through their cytotoxic activity or indirectly through the production of cytokines (such as IFN-γ, TNF-α, IL-17)[119].

B cells

B cells derive from haematopoietic stem cells in the bone marrow and, following maturation, leave the bone marrow expressing a unique antigen-binding receptor on their membrane. B cells can recognize antigens directly, without need for APCs[119].

B cell's antigen-binding receptor recognizes and binds to the antigen in its native form, which activates them[119]. Activated B cells undergo proliferation and differentiation into 2 pathways: either they immediately become short-lived plasma cells secreting low-affinity antibody, or they enter a follicle to establish a germinal center, where B cells can change between 5 types of antibodies (IgA, IgD, IgE, IgG and IgM)[125]. The immune response to the first exposure to an antigen is called primary response. It is relatively slow response and leads to production of predominantly IgM antibody with relatively low-affinity. During this response, long-lived memory T and B cells are generated. In a subsequent exposure to the same antigen, these cells are activated more quickly in the comparison with the primary response, producing high-affinity IgG[119].

Immune response to liver-stage parasites

The pre-erythrocytic stage is an obligatory and asymptomatic stage of malaria infection. For a long time, it was thought that this stage of *Plasmodium* infection was immunologically silent. Nonetheless, the presence of inflammatory cells in the liver of infected mice constitutes evidence that the host senses *Plasmodium* hepatocyte infection and responds to it[126].

It has been shown that viable sporozoites can induce a rapid and generalized inflammation within the liver of the host, which becomes evident at around 4 hours post-infection (hpi) and progresses to the formation of well-defined granulomas by 24 hpi[126]. Additionally, immune cell infiltrates around developing hepatic schizonts were also observed at about 40 hpi, being eosinophils their main component [126].

In 2014, 2 independent studies with *P. berghei* ANKA and *P. yoelii* 17XNL have elucidated the role of type-I IFN signalling in controlling liver-stage *Plasmodium* infection[10,9].

One of these studies has shown that infection of hepatocytes by *Plasmodium* parasites induces a type-I IFN response (Fig. 1.4; step 1)[10]. This pathway requires signalling through the mitochondrial antiviral signalling protein (MAVS), that is independent of TLR signalling, and which was shown to be triggered by the Melanoma differentiation-associated gene 5 (MDA5)[10] (Fig. 1.4; step 1). The activation of this cytosolic pathway leads to the release of type-I IFNs into the extracellular environment, where it binds to the single interferon α receptor (IFNAR) on the surface of hepatocytes (Fig. 1.4; step 2), propagating the response in an autocrine and paracrine manner via the production of interferon stimulated-genes (ISGs) (Fig. 1.4; step 3). Despite being known to mediate the functional antiviral responses, ISGs are not able to eliminate *Plasmodium* parasites[10]. Therefore, it was hypothesized that activated hepatocytes, which produced type-I IFN, recruit leukocytes and other innate immune cells to the liver that will specifically target infected hepatocytes (Fig. 1.4; step 4 and 5, respectively)[10], which play a key role in parasite elimination (Fig. 1.4; step 6).

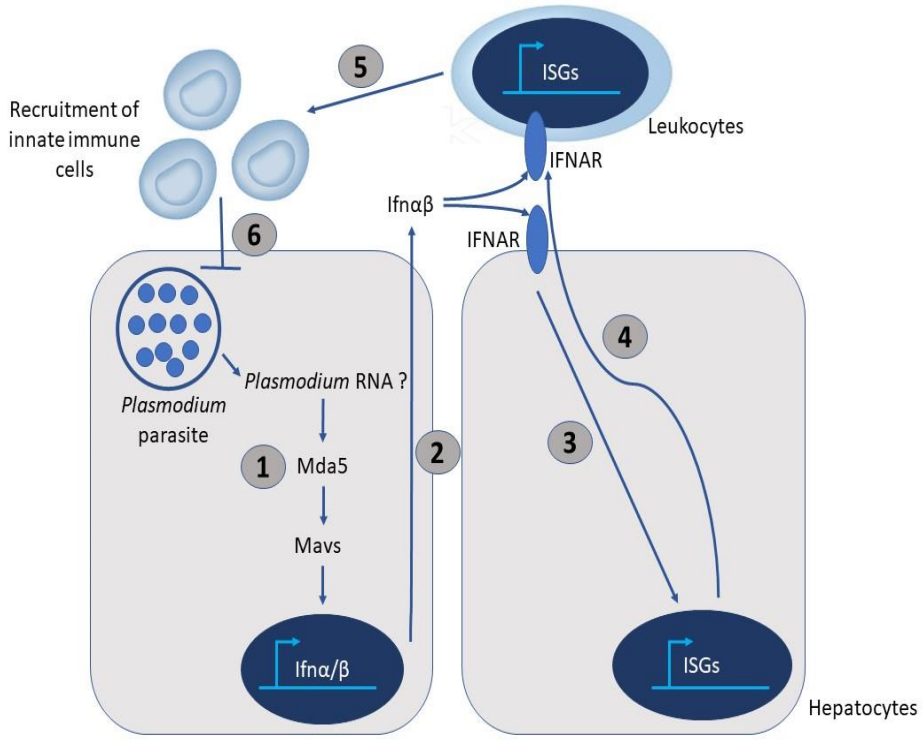

Figure 1.4 - Schematic representation of the type-I IFN-signalling occurring in the liver after *Plasmodium* hepatic infection. (Adapted from[10])

IFNAR: interferon α receptor; Ifnα/β: interferon-α/β; ISGs: interferon stimulated-genes; Mavs: mitochondrial antiviral signalling protein; Mda5: melanoma differentiation-associated gene 5

The other study has shown that NK T cells increase in the liver following infection and are able to inhibit liver-stage infection through the production of IFN-γ[9]. This was also supported by a previous study demonstrating that addition of the glycolipid α-galactosylceramidem, which activates NK T cells, inhibits *Plasmodium* liver infection[9].

Together, these studies revealed that type-I IFN signalling is protective against liver-stage *Plasmodium* infection, by facilitating sensing of parasite within hepatocytes via MDA5 and MAVS, as well as recruitment of NK T cells[10,127]. Importantly, this type-I IFN response has been shown to constitute a major defense mechanism against sporozoite reinfection[128,127].

RKV supplementation

During the asymptomatic liver-stage of malaria infection, *Plasmodium* parasites scavenge host nutrients to support their rapid growth and replication[3,36]. Arg was described as one of these nutrients, whose metabolism was defined as crucial for the parasite's intrahepatic development[4].

Arg metabolism has been shown to be regulated by dietary amino acids, such as Lysine (Lys), Valine (Val) and Ornithine, which have been described as arginase inhibitors[129,130]. Preliminary results from the host laboratory have shown that supplementation of C57Bl/6 mice with RKV, which combines Arg with Lys (K) and Val (V), leads to a striking decrease of hepatic infection, resulting in impaired parasite growth but, mostly, in a reduction in the number of infected hepatocytes, supporting a role of the host's immune system on parasite elimination. It was also demonstrated that this parasite elimination does not rely on NO production nor on a boost of the type-I IFN response, previously reported as crucial to control liver-stage infection[10,128]. Also, the reduction in liver parasite load typically observed in RKV-supplemented mice is abolished by lethal mouse irradiation and in MyD88$^{-/-}$ mice, supporting a role for the immune system in the mechanism of hepatic parasite elimination.

Thesis aims

The aims of the present thesis are: (i) to characterize *Plasmodium* liver infection in BALB/c mice upon Arg and RKV supplementations; (ii) to elucidate the mechanism of hepatic parasite elimination in C57Bl/6 mice upon RKV supplementation.

Arg supplementation has been previously shown to improve survival and decrease parasitemia in BALB/c mice infected with blood-stage *P. yoelii* parasites. However, the effect of Arg supplementation during liver-stage infection remains unclarified. Thus, the first aim of this thesis is to assess whether Arg and/or RKV supplementation, which is efficacious in the case of C57Bl/6 mice, would have an impact on *Plasmodium* liver-stage infection in BALB/c mice. To this end, liver parasite load was assessed by qPCR following infection with *P. berghei* sporozoites, and immunofluorescence microscopy was employed to compare the number and size of hepatic parasites in control and supplemented mice. Additionally, the effect of both supplementations on *ex vivo* hepatic infection was also assessed by measuring the luminescence intensity of lysates of infected mouse primary hepatocytes collected from the livers of BALB/c mice.

It was described in the previous section that RKV supplementation leads to a striking decrease of *Plasmodium* hepatic infection in C57Bl/6 mice, mostly by reducing the number of infected hepatocytes. Although, the parasites seem to be eliminated by the host's immune system, the mechanism of hepatic parasite elimination remains unknown. Thus, the second aim of this thesis is to elucidate the mechanism of parasite elimination upon RKV supplementation in C57Bl/6 mice. In order to identify the cells involved in the RKV-dependent parasite elimination and to clarify the involvement of some cytokines in the mechanism of action of RKV supplementation, knockout mice and depleting antibodies were employed to assess the involvement of specific immune cell populations.

Chapter II - Materials and Methods

Mice

Wild-type (WT) BALB/c and C57Bl/6 mice were purchased from Charles River. iNOS$^{-/-}$ mice were purchased from Jackson Laboratories. IFNAR$^{-/-}$, MyD88$^{-/-}$ and Rag2$^{-/-}$ mice were purchased from Instituto Gulbenkian de Ciência (IGC). IL1α$^{-/-}$, IL17$^{-/-}$, Genista[131], Ragγc$^{-/-}$, TCRδ$^{-/-}$ mice were bred at Instituto de Medicina Molecular João Lobo Antunes (iMM, Lisbon, Portugal). Experiments were performed in male and/or female animals, as indicated in the results section, depending on the availability of each mouse strain. The animals were housed in the facilities of iMM. All animal experiments were performed in strict compliance to the guidelines of iMM´s animal ethics committee and the Federation of European Laboratory Animal Science Associations (FELASA).

Isolation of *Plasmodium berghei* sporozoites

Green fluorescent protein- (GFP-) or luciferase-expressing *P. berghei* ANKA sporozoites were isolated from the salivary glands of infected female *Anopheles stephensi* mosquitoes, bred at iMM. The salivary glands are located at the anterior portion of the thorax and can be isolated in non-supplemented Roswell Park Memorial Institute (RPMI) medium, first by using forceps to remove the head of the mosquito, and after by holding and gently pushing down the thorax with the probes. Salivary glands isolated in non-supplemented RPMI were then smashed to release the sporozoites, filtered through a 40 µm cell strainer and spun. Sporozoites suspension were diluted with non- supplemented RPMI (1:5 or 1:10 dilution if less or more than 20 mosquitoes, respectively) and counted on a Neubauer chamber.

Isolation and infection of mouse primary hepatocytes

Mouse primary hepatocytes were isolated using a modified two-step perfusion protocol [132],[133] followed by a Percoll purification step. Briefly, mice were euthanized by CO_2 inhalation and immediately processed for cannulation of the portal vein using a 26-gauge needle. Upon successful cannulation, the inferior vena cava (IVC) was cut to allow fluid to drain. The liver was perfused with 30 mL of liver perfusion medium (LPM, Gibco) at 8–9 mL/min followed by digestion with 40 mL of liver digestion medium (LDM, Gibco) at the same rate. Intermittent clamping of the IVC (3 s clamp every 30 s)

was performed during LDM perfusion to improve tissue digestion. After digestion, the liver was excised and the cells were liberated by tearing and shaking of the liver with forceps. The cell suspension was then sequentially filtered through a 100 μm and a 70 μm cell strainer, and centrifuged at 50xg for 4 min at 4ºC. The cell pellet was resuspended in Williams' E Medium containing 10% (v/v) Fetal Bovine Serum (FBS) and carefully overlaid on a 45% (v/v) or 60% (v/v) Percoll solution [5 mL Percoll 100% (v/v) [9 mL Percoll stock and 1 mL Phosphate-buffered saline (PBS) 10x] and 7 mL non-supplemented RPMI; or 7 mL Percoll 100% (v/v) and 5 mL non-supplemented RPMI, respectively] in case of primary hepatocytes isolated from BALB/c mice or C57Bl/6 mice, respectively. The cell suspension was fractionated by centrifugation at 750xg for 20 min, without break or acceleration, at 20 °C. Viable hepatocytes deposited in the pellet were washed with Williams' Medium E with 10% (v/v) FBS, centrifuged at 50xg for 4 min at 4ºC and resuspended in complete Williams' Medium E [supplemented with 4% (v/v) FBS and 1% (v/v) penicillin/streptomycin]. Hepatocytes were then plated at a density of $2.0x10^4$ per well in 96-well plates well plates and infected on the following day with $1.0x10^4$ luciferase-expressing *P. berghei* ANKA sporozoites, followed by centrifugation at 1800xg for 5 min at room temperature (RT). The medium was replaced approximately 2 hpi by medium supplemented with a physiological concentration of Arg (100 μM), with a supraphysiological concentration of Arg (1 mM), and with 1 mM Arg, 20 mM Lys and 20 mM Val, which correspond to the Control, Arginine and RKV experimental conditions, respectively. Cell viability and purification yield were assessed by trypan blue staining.

Ex vivo hepatic infection measurement by luminescence

Overall hepatic infection was determined by measuring the luminescence intensity of lysates of mouse primary hepatocytes infected with firefly luciferase-expressing *P. berghei* ANKA sporozoites. Mouse primary hepatocyte infection was measured 48 hpi by a bioluminescence assay (Biotium), according to the manufacturer's instructions. The effect of the different treatments on the viability of the cells was assessed by adding 100 μL of 1:20 dilution of Alamar Blue (Promega) in culture medium per well, followed by incubation at 37ºC for 1 h. Luminescence and fluorescence measurements were performed on a multiplate reader Infinite M200 (Tecan).

In vivo amino acid supplementation

The drinking water of mice was replaced by sterilized water containing 2.5% (w/v) L-arginine hydrochloride (FisherScientific) alone or in combination with 2.5% (w/v) L-lysine (FisherScientific) and 2.5% (w/v) L-valine (FisherScientific), which is designated RKV. The mice were allowed to drink *ad libitum* for 4 weeks. Unsupplemented sterilized water was provided to control mice.

In vivo P. berghei infection

Mice were infected i.v. through retro-orbital injection of $1.0x10^4$ GFP- or luciferase-expressing *P. berghei* ANKA sporozoites, obtained as previously described.

Mouse irradiation

C57Bl/6 mice were placed in an Irradiator Gammacell ELAN 3000 and exposed to 900 rads/mouse, one day before infection with *P. berghei* ANKA sporozoites.

Depletion of immune cells *in vivo*

Specific immune cell depletion was achieved by the intraperitoneal (i.p.) injection of the following antibodies: anti-Ly6G, anti-CCR2, anti-CD115, anti-Gr-1 and anti-NK1.1, as described in Table 2.1. Control animals in these experiments were injected with 200 µL of PBS i.p. Clodronate liposomes and the corresponding PBS liposomes were administered intravenously (i.v.), in the amounts and schedules listed in Table 2.1.

Table 2.1 - List of reagents used to deplete specific target cell populations.

hpi: hours post-infection; d0: day of the infection; d-4, d-3, d-2 and d-1: four, tree, two or one day before infection; d+1: one day after infection.

Treatment	Clone	Supplier	Dose	Time of Injection	Target Cell Population
Clodronate	-	Clodronate Liposomes	200 µL/mouse i.v.	d-2	Macrophages, Kupffer cells and Dendritic cells
anti-Ly6G	1A8	BioXCell	250 µg/mouse i.p.	2 hpi	Neutrophils
anti-CCR2	MC-21	Kindly provided by Mathias Mack[134]	20 µg/mouse i.p.	d-2, d-1, d0, d+1	Monocytes
anti-CD115	AFS98	BioXCell	300 µg/mouse i.p.	d-3, d-1, d+1	Monocytes

anti-Gr-1	RB6-8C5	BioXCell	250 µg/mouse i.p.	2 hpi	Neutrophils and monocytes
anti.NK1.1	PK136	BioXCell	150 µg/mouse i.p.	d-1	NK and NK T cells

N-Acetyl Cysteine treatment

N-Acetyl Cysteine (NAC), an inhibitor of ROS production, was resuspended in PBS and 150 mg of NAC/kg of mouse was administered i.p. on C57Bl/6 mice every 12 h for 5 days (from d-2 until d+2).

Organ Collection

Mice were euthanized at 45 hpi by overdose with Isoflurane (Abbot Laboratories) and the livers were collected and divided in two parts. The largest lobe of each liver was fixed in 4% (v/v) Paraformaldehyde (PFA) for immunohistochemical staining and the rest was homogenized in 3 mL of denaturing solution [4 M guanidine thiocyanate; 25 mM sodium citrate pH 7; 0.5% (v/v) N-lauroylsarcosine sodium salt solution and 0.7% (v/v) β-mercaptoethanol in Diethyl pyrocarbonate (DEPC)-treated water] for RNA extraction. In the experiments in which a specific immune cell population was depleted, part of the livers was used for isolation of leukocytes and the spleens were also collected for lymphocyte isolation, to confirm depletion by flow cytometry.

Immunohistochemical staining of liver sections

For microscopy, PFA-fixed liver lobes were cut in 50 µm sections and were incubated in permeabilization/blocking solution [2% (w/v) Bovine Serum Albumin (BSA), 0,5% (v/v) Triton-X100 in PBS 1x] at RT for 1 h. Parasites were stained with 1:500 dilution of anti-UIS4 antibody (SicGen) overnight at 4ºC, followed by three washes with permeabilization/blocking solution. Liver slices were further incubated in a 1:200 dilution of anti-goat Alexa 568 (Life Technologies) in the presence of a 1:250 dilution of anti-GFP Alexa 488 (Roche) and a 1:1000 dilution of Hoechst (Invitrogen). Additional washes were carried out with permeabilization/blocking solution and, finally, with PBS 1x. Coverslips were mounted on microscope slides with Fluoromount (SouthernBiotech). Confocal images were acquired using a Zeiss LSM 710 confocal microscope. Widefield

images for size and number determination of parasites were acquired in a Leica DM5000B microscope. Images were processed with ImageJ software (version 1.49).

Isolation of splenocytes

Collected spleens were homogenized and filtered using a 70 µm cell strainer and the piston of a plastic syringe, followed by centrifugation at 400xg for 8 min at 6ºC. The supernatant was discarded and the cell pellet was then resuspended in 3 mL Ammonium-Chloride-Potassium (ACK) buffer [155 mM Ammonium chloride; 10 mM Potassium bicarbonate; and 0,1 mM Ethylenediaminetetraacetic acid (EDTA) in DEPC-treated water] for 4 min at RT to lyse RBCs. Fluorescence-activated cell sorting (FACS) buffer [2% (v/v) FBS in PBS] was added to stop the reaction. After centrifugation at 400xg for 8 min at 6ºC, the resulting pellet was resuspended in FACS buffer and filtered through a 40 µm cell strainer. After a final centrifugation at 400xg for 8 min at 6ºC, cells were resuspended in 1 mL of FACS buffer and kept on ice for extracellular FACS staining.

Isolation of liver leukocytes

Mouse liver lobes were homogenized and filtered using a 100 µm cell strainer and a piston of a plastic syringe, followed by centrifugation at 400xg for 8 min. To separate leukocytes from the other hepatic cells, 10 mL of a 35% (v/v) Percoll solution (4 mL Percoll 100% and 8 mL non-supplemented RPMI) was added to the cell pellet and centrifuged at 1360xg for 20 min, without break or acceleration, at 20 °C. The supernatant and the layer containing the other cells was then removed, the cell pellet was washed with PBS 1x, and then centrifuged at 400xg for 8 min. Subsequently, the pellet was resuspended in 3 mL ACK buffer for 3 min at RT, after which FACS buffer was added to stop the lysis of RBCs. After a final centrifugation at 400xg for 8 min, the cells were resuspended in 200 µL of FACS buffer for extracellular FACS staining.

Extracellular FACS staining

One hundred µL of isolated splenocytes and liver leukocytes were pipetted into a 96-well plate with round bottom and centrifuged at 800xg for 3 min at 4ºC. The supernatant was removed and 15 µL of a 1:50 dilution of Fc block (anti-CD16/CD32) was added to block unspecific binding. Cells were incubated on ice for 20 min and washed

twice with PBS 1x. Fifteen µL of an extracellular antibody mix diluted in PBS 1x, with the specific antibodies to analyse the cell depletion (see Table 2.2) and a dye to allow exclusion of dead cells [dil 1:200 LIVE/DEAD Fixable near-IR Dead Cell Stain (Life Technologies)], were added to each well and incubated 20 min on ice, protected from light. Cells were washed twice with FACS buffer and finally were resuspended in 250 µL of FACS buffer, before being transferred into acquisition tubes (Corning Incorporated). Cells were analysed on a BD LSR Fortessa X-20 flow cytometer.

Table 2.2 - List of the conjugated antibodies used for extracellular FACS staining to assess depletion of specific immune cell populations.

Treatment	Immune cell population (s) depleted	Clone	Antibody staining	Supplier
anti-Ly6G	Neutrophils	1A8	dil 1:400 PE.Cy7 anti-mouse Ly-6G Antibody	Biolegend
		HK1.4	dil 1:400 Brilliant Violet 605™ anti-mouse Ly-6C Antibody	Biolegend
		M1/70	dil 1:400 FITC anti-mouse CD11b Antibody	Biolegend
		N418	dil 1:300 PE anti-mouse CD11c Antibody	eBiosciences
anti-CCR2	Monocytes	1A8	dil 1:400 PE.Cy7 anti-mouse Ly-6G Antibody	Biolegend
		HK1.4	dil 1:400 Brilliant Violet 605™ anti-mouse Ly-6C Antibody	Biolegend
		M1/70	dil 1:400 FITC anti-mouse CD11b Antibody	Biolegend
		N418	dil 1:300 PE anti-mouse CD11c Antibody	eBiosciences
anti-CD115	Monocytes	1A8	dil 1:400 PE.Cy7 anti-mouse Ly-6G Antibody	Biolegend
		HK1.4	dil 1:400 Brilliant Violet 605™ anti-mouse Ly-6C Antibody	Biolegend
		M1/70	dil 1:400 FITC anti-mouse CD11b Antibody	Biolegend
		N418	dil 1:300 PE anti-mouse CD11c Antibody	eBiosciences
		SA011F11	dil 1:300 PerCP/Cy5.5 anti-mouse CX3CR1 Antibody	Biolegend
anti-Gr-1	Neutrophils and monocytes	1A8	dil 1:400 PE.Cy7 anti-mouse Ly-6G Antibody	Biolegend
		HK1.4	dil 1:400 Brilliant Violet 605™ anti-mouse Ly-6C Antibody	Biolegend
		M1/70	dil 1:400 FITC anti-mouse CD11b Antibody	Biolegend
		N418	dil 1:300 PE anti-mouse CD11c Antibody	eBiosciences
anti-NK1.1	NK and NK T cells	H57-597	dil 1:300 APC/Cy7 anti-mouse TCR β chain Antibody	Biolegend
		PK136	dil 1:100 FITC anti-mouse NK-1.1 Antibody	Biolegend

RNA extraction and quantification

Total RNA was extracted from the livers with the TripleXtractor direct RNA kit (Grisp), according to the manufacturer's protocol. The amount of RNA in each sample was assessed by absorbance measurement on a NanoDropND-2000 spectrophotometer.

cDNA synthesis and qPCR

Complementary DNA (cDNA) was synthesized from 1 µg of RNA using the NZYTech cDNA synthesis kit, according to the manufacturer´s instructions. The cDNA was synthesized employing the following thermocycling parameters: 25°C for 10 min 55°C for 30 min, and 85°C for 5 min. Quantitative real-time PCR (qPCR) reaction was performed in a total volume of 8 µL in a Viia7 Real-Time PCR System (Thermo Fisher) using the iTaq Universal SYBR Green Supermix 5000 (BioRad) as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min; the melting stage consisted of 95°C for 15 s, 60°C for 1 min, and 95°C for 30 s. Parasite load was quantified using primers specific to *P. berghei* 18S RNA, and the normalization was done with primers for the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) (Table 2.3). The efficiency of macrophage depletion by clodronate liposomes was assessed by quantifying the expression of three specific markers: CD68 (Cluster of Differentiation 68), which is a marker of the macrophage lineage; Clec4f (C-Type Lectin Domain Family 4 Member F), which is specifically expressed by Kupffer cells; and F4/80, which is expressed by mature macrophages (Table 2.3). The delta-delta CT relative quantification method was used for analysis of qPCR results.

Table 2.3 - List of the primer sequences (forward and reverse) used.

Gene	Forward primer (5'-3')	Reverse primer (3'-5')
Pb18S	AAGCATTAAATAAAGCGAATACATCCTTAC	GGAGATTGGTTTTGACGTTTATGTG
Hprt	TTTGCTGACCTGCTGGATTAC	CAAGACATTCTTTCCAGTTAAAGTTG
CD68	AGCTGCCTGACAAGGGACACT	AGGAGGACCAGGCCAATGAT
Clec4f	TGAGTGGAATAAAGAGCCTCCC	TCATAGTCCCTAAGCCTCTGGA
F4/80	CCCCAGTGTCCTTACAGAGTG	GTGCCCAGAGTGGATGTCT

Statistical Analysis

Statistical analysis were performed using the GraphPad Prism 5 software. One-way ANOVA with post-test Dunnett, Two-tail Mann-Whitney test or Unpaired t-test were used for significance of the differences observed, as indicated in each figure. ns – not significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Chapter III – Project Background and State-of-the-Art

Impact of amino acid supplementation in C57Bl/6 mice

RKV supplementation impacts *Plasmodium* hepatic infection *in vivo*

Having shown that Arg-dependent polyamine synthesis is crucial for parasite hepatic development[4], the host laboratory wondered whether *Plasmodium* liver-stage infection could be impaired by appropriate amino acid supplementation that would interfere with Arg metabolism. Lys (K) and Val (V) have been described as arginase inhibitors[129,130], suggesting they could be employed to inhibit the polyamine synthesis pathway. The combination of Arg with these 2 amino acids was designated RKV. The effect of Arg supplementation alone on liver infection was also assessed because it has been described that it can enhance NO production, decrease parasitaemia and improve survival in animal models of *Plasmodium* blood-stage infection[8,74,7]. Specifically, in BALB/c mice, Arg pre-treatment drastically improved survival and led to better resolution of parasitemia during early blood-stage of infection[6], contrarily to what is reported in C57Bl/6 mice, where Arg supplementation does not affect parasitemia nor the survival of the mice[7], being also associated with aggravation of ECM[73].

The drinking water of WT C57Bl/6 mice was supplemented with 2.5% (w/v) Arg alone or with the RKV supplementation (2.5% w/v of each amino acid). After 4 weeks of supplementation, mice were infected, through i.v. injection, with $1.0x10^4$ *P. berghei* ANKA sporozoites and liver parasite load was assessed 45 hpi by qPCR. The results show that Arg supplementation is not sufficient to impair *Plasmodium* liver infection in C57Bl/6 mice (Fig. 3.1). These results are in agreement with a previous report that shows that Arg supplementation of this mouse strain with blood-stage infection does not affect parasitemia nor survival of the mice[7]. Interestingly, RKV supplementation, which combines Arg with Lys and Val, leads to a significant decrease in *Plasmodium* liver load (Fig. 3.1).

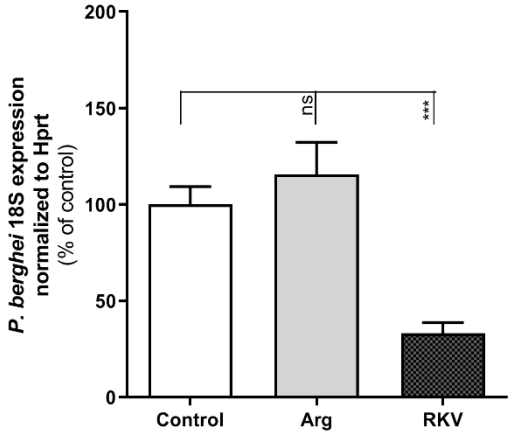

Figure 3.1 - Amino acid supplementation impacts *Plasmodium* liver infection in C57Bl/6 mice. The drinking water of the mice was replaced by sterilized water supplemented with 2.5% (w/v) of Arg alone or in combination with 2.5% (w/v) Lys and 2.5% (w/v) Val, which was designed by RKV. Mice were allowed to drink *ad libitum* for 4 weeks after which they were infected through i.v. injection of $1.0x10^4$ *P. berghei* ANKA sporozoites. Liver parasite load was assessed by qPCR at 45 hpi and was plotted as percentage of control. Pool of 7 independent experiments. Control: n = 34; Arg: n = 15; RKV: n = 29 (all males). Error bars represent SD. One-way ANOVA with post-test Dunnett. ns - not significant and *** $p < 0.001$.

To further clarify the effect of the RKV supplementation on *Plasmodium* liver infection, 50 µm liver sections were analysed by immunofluorescence microscopy in order to quantify the number and size of hepatic parasites in control and RKV-supplemented mice. The results obtained show a small but statistically significant reduction on parasite size in the livers of supplemented mice, showing that the RKV supplementation partially impairs parasite development (Fig. 3.2A and C). Importantly, the observed reduction in the number of parasites per liver area in supplemented mice is much more pronounced, suggesting that parasites are being eliminated from the liver of these mice (Fig. 3.2B).

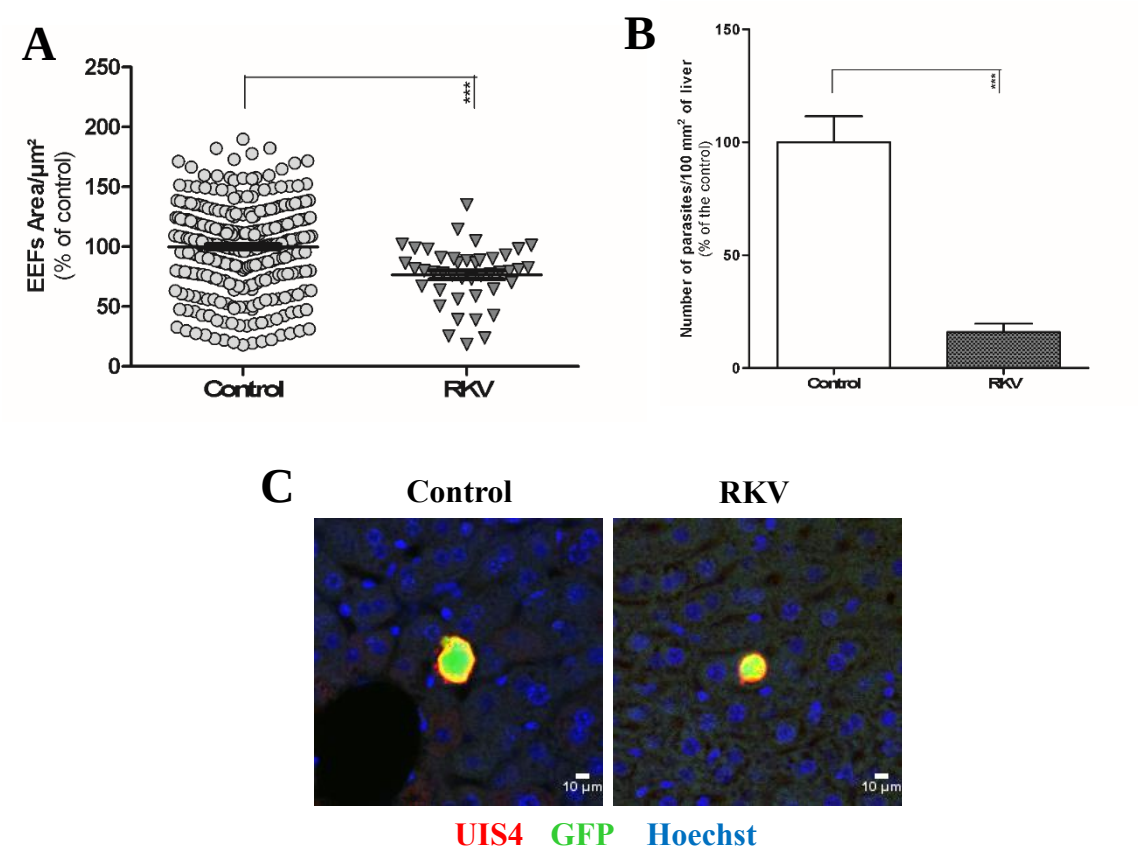

Figure 3.2 - *In vivo* RKV supplementation impairs parasite growth and decreases the number of *P. berghei* parasites in the liver of C57Bl/6 mice. Immunofluorescence microscopy quantification of **(A)** EEF areas, and **(B)** number of parasites, in the liver sections of control or RKV-supplemented mice. Both panels: plotted as percentage of control; pool of 2 mouse per group (males) from 2 independent experiments. Error bars represent SD. Two-tailed Mann-Whitney test. *** $p < 0.001$. **(C)** Representative confocal images of *P. berghei* parasite size in both groups of mice. Scale bar, 10 µm.

RKV supplementation impacts *Plasmodium* hepatic infection *ex vivo*

The impact of Arg and RKV supplementation was also assessed in the context of an *ex vivo P. berghei* infection. Mouse primary hepatocytes were extracted from the liver of C57Bl/6 mice, cultured on a 96-well plate and infected with $1.0x10^4$/well of firefly luciferase-expressing *P. berghei* ANKA sporozoites. Infection was quantified by measuring the luminescence intensity of the infected cell lysates at 48 hpi. These results show that supplementation of the culture medium of infected hepatocytes with supraphysiological concentrations of Arg, which corresponds to the experimental condition of Arg supplementation *in vivo*, leads to a small but significant reduction of hepatic infection (Fig. 3.3). Importantly, a much stronger reduction of hepatic infection was observed when mouse primary hepatocytes were supplemented with Lys and Val, under supraphysiological concentrations of Arg, a condition that partially mimics the *in vivo* RKV experimental condition (Fig. 3.3).

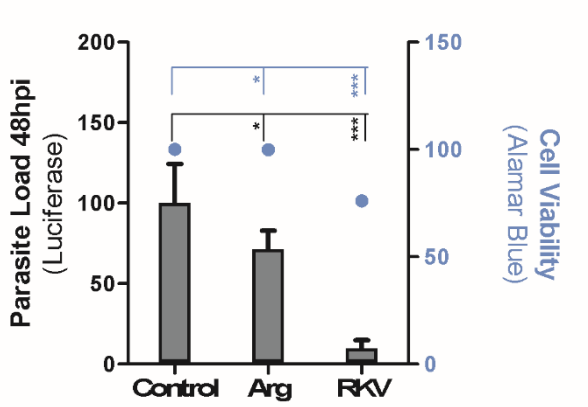

Figure 3.3 - Amino acid supplementation impacts hepatic *Plasmodium* infection *ex vivo*. Mouse primary hepatocytes were infected with luciferase-expressing *P. berghei* ANKA sporozoites and 2 hours later the culture medium was replaced by medium supplemented with a physiological concentration of Arg (100 µM), with a supraphysiological concentration of Arg (1 mM), and with 1 mM Arg, 20 mM Lys and 20 mM Val, which correspond to the Control, Arg and RKV experimental conditions, respectively. *Ex vivo* hepatic infection (luminescence) and cell viability (Alamar Blue) were assessed at 48 hpi and plotted as percentage of control. Pool of 2 independent experiments. Error bars represent SD. One-way ANOVA with post-test Dunnett. ns - not significant, * $p < 0.05$ and *** $p < 0.001$.

In order to further dissect how these amino acid supplementations influence hepatic infection *ex vivo*, an established flow cytometry-based approach that makes use of GFP-expressing *P. berghei* ANKA parasites to determine the number of infected cells and to measure parasite development[135] was employed. It was observed that, *ex vivo* supplementation with supraphysiological concentrations of Arg, condition that corresponds to the experimental condition of Arg supplementation *in vivo*, leads to significant increase of parasite development (Fig. 3.4A). Conversely, supplementation with Lys and Val at supraphysiological concentrations of Arg, which corresponds to the experimental condition of RKV supplementation *in vivo*, were effective at reducing parasite development (Fig. 3.4A). The results also show that a supraphysiological concentration of Arg is not sufficient to impair the number of infected cells at 48 hpi (Fig. 3.4B). On the contrary, the number of infected cells at 48 hpi in mouse primary hepatocytes supplemented with Lys and Val at supraphysiological concentrations of Arg were reduced compared to control, but to a much lower extent than the reduction observed *in vivo* (Fig. 3.4B).

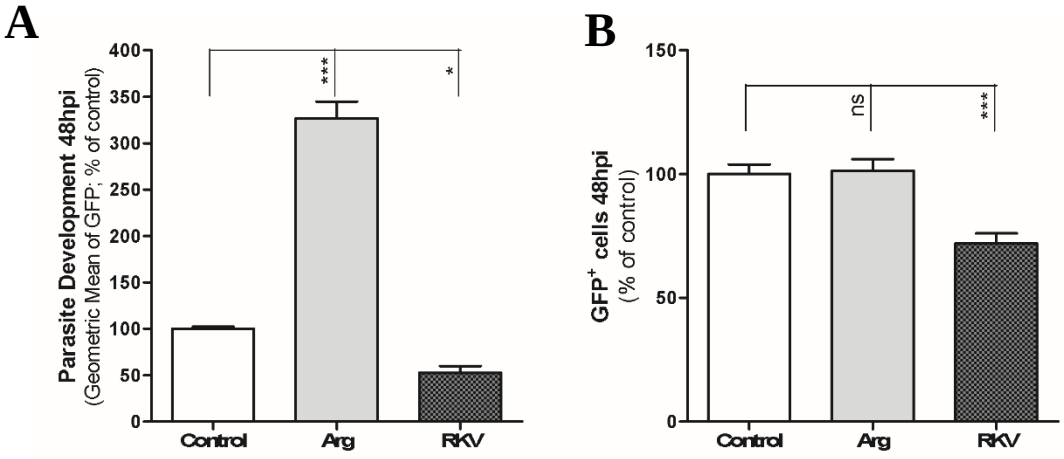

Figure 3.4 – Amino acid supplementation impacts parasite development *ex vivo*. Mouse primary hepatocytes were infected with GFP-expressing *P. berghei* ANKA sporozoites and 2 hours later the culture medium was replaced by medium supplemented with a physiological concentration of Arg (100 μM), with a supraphysiological concentration of Arg (1 mM), and with 1 mM Arg, 20 mM Lys and 20 mM Val, which correspond to the Control, Arg and RKV experimental conditions, respectively. **(A)** Parasite development in mouse primary hepatocytes was assessed by determining the fluorescence intensity of GFP^+ cells by flow cytometry at 48 hpi. Pool of 3 independent experiments. **(B)** Percentage of GFP^+ cells was quantified by flow cytometry at 48 hpi.

Pool of 3 independent experiments. For all panels: Error bars represent SD; One-way ANOVA with post-test Dunnett. ns - not significant, * $p < 0.05$ and *** $p < 0.001$.

Overall, these data show that *Plasmodium* hepatic infection is impaired both *in vivo* and *ex vivo* by RKV supplementation. It was demonstrated that *in vivo* this impairment is essentially due to a decrease in the number of parasites per liver area, while *ex vivo* both the number of infected cells in mouse primary hepatocytes and, mainly, the parasite development were affected. This supports the idea that the parasites were eliminated from the mouse livers, suggesting a role for the immune system in this process.

RKV-dependent parasite elimination does not rely on NO production

RKV supplementation *in vivo* leads to a striking decrease in the number of infected hepatocytes, suggesting that parasites are being eliminated. As one of its components is Arg, which can be metabolized into NO or polyamines by iNOS or arginase, respectively, the host laboratory hypothesized that Lys and Val could be inhibiting arginase's activity, consequently channelling Arg for NO production, which would ultimately lead to parasite elimination. To test this hypothesis, 1 group of C57Bl/6 WT and 1 group of iNOS$^{-/-}$ mice were allowed to drink *ad libitum* control water while another group of C57Bl/6 WT and of iNOS$^{-/-}$ mice were allowed to drink *ad libitum* RKV-supplemented water. The mice were infected by i.v. injection of $1.0x10^4$ *P. berghei* ANKA sporozoites and liver parasite load was then assessed by qPCR at 45 hpi. The results obtained show that the animals that cannot produce NO via iNOS display a reduction in liver parasite load similar to that which is typically observed in RKV-supplemented WT mice, suggesting that parasite elimination does not rely on NO production (Fig. 3.5).

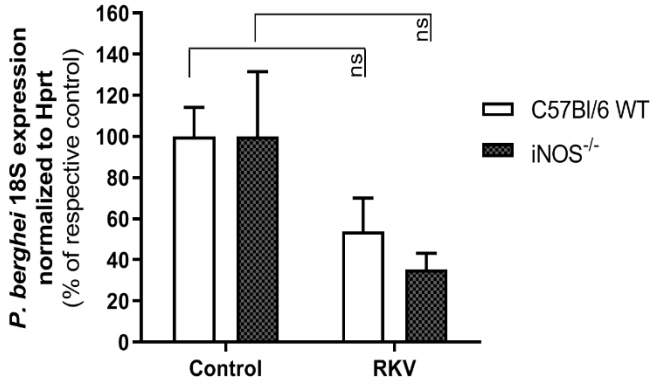

Figure 3.5 – NO production is not involved in RKV-dependent parasite elimination. C57Bl/6 WT and iNOS$^{-/-}$ mice were infected through i.v. injection of 1.0x10^4 *P. berghei* ANKA sporozoites. Liver parasite load was assessed by qPCR at 45 hpi and plotted as percentage of control. One experiment with 5 mice per group (males). Error bars represent SD. Unpaired t-test. ns - not significant.

RKV supplementation does not boost the Type-I IFN response

It has been described that type-I IFN signalling is involved in the elimination of liver-stage *Plasmodium* parasites [9,10]. To assess if RKV supplementation leads to parasite elimination through the stimulation of the type-I IFN signalling, mice lacking the type-I interferon receptor (IFNAR$^{-/-}$ mice) were employed. Thus, 2 groups of C57Bl/6 WT were allowed to drink *ad libitum* control or RKV-supplemented water and 2 groups of IFNAR$^{-/-}$ mice were also allowed to drink *ad libitum* control or RKV-supplemented water. Liver parasite load was then assessed by qPCR 45 h after infection with 1.0x10^4 *P. berghei* ANKA sporozoites. These data showed that lacking type-I IFN signalling does not abolish the reduction in liver parasite load typically observed in RKV-supplemented mice (Fig. 3.6A), excluding a boost of this response as the mechanism of parasite killing induced by RKV supplementation.

This conclusion is also supported by the data obtained by measuring the expression of several ISGs, namely Ifit1, Ifi44, Usp18, Ifit3 and Irf7, in the livers of control and RKV-supplemented mice, at different time points of infection. RKV supplementation induces a similar expression of these genes as non-supplemented mice, and therefore, type-I IFN response, even though being active, it is not responsible for the parasite elimination observed in RKV-supplemented mice (Fig. 3.6B).

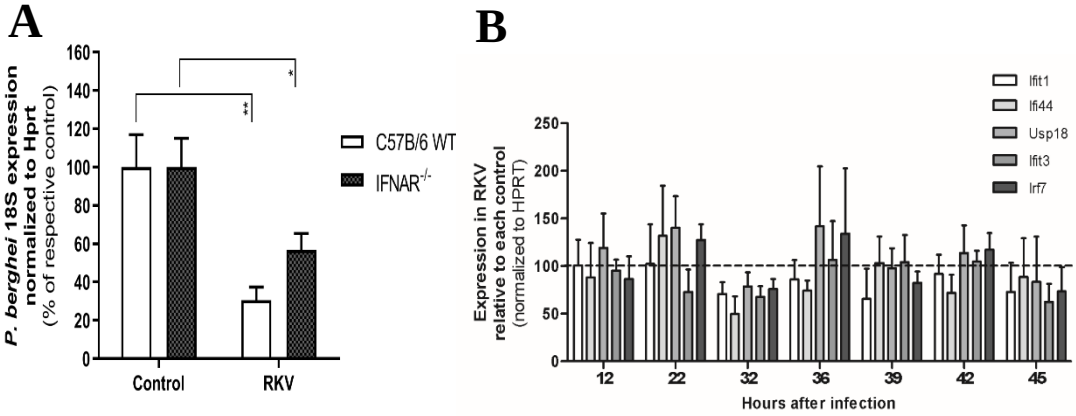

Figure 3.6 – Type-I IFN response is not involved in RKV-dependent parasite elimination. (A) Control and RKV-supplemented C57Bl/6 WT and IFNAR$^{-/-}$ mice were infected through i.v. injection of 1.0x10^4 *P. berghei* ANKA sporozoites. Liver parasite load was assessed by qPCR at 45 hpi and plotted as percentage of control. Pool of 2 independent experiments with 3 to 6 mice per group (males and females). Error bars represent SD. Unpaired t-test. * $p < 0.1$ and ** $p < 0.01$. **(B)** C57Bl/6 WT mice were allowed to drink *ad libitum* control or supplemented water for 4 weeks. Mice were infected through i.v. injection of 1.0x10^4 *P. berghei* ANKA sporozoites and the livers were collected at different time points of infection: 12 hpi, 22 hpi, 32 hpi, 36 hpi, 39 hpi, 42 hpi and 45 hpi. Expression of ISGs (Ifit1, Ifi44, Usp18, Ifit3 and Irf7) was assessed by qPCR for all the timepoints. The expression of these genes in RKV supplemented mice was plotted relatively to non-supplemented mice, which are represented as a dashed line in the graph.

Overall, these results show that the RKV supplementation does not elicit a stronger type-I IFN response in the supplemented mice than the one observed in control mice, indicating that RKV-dependent parasite elimination is not related to a boost of the type-I IFN response.

RKV-dependent parasite elimination is mediated by the immune system

Having excluded the role of the two most obvious mechanisms that could explain RKV-dependent parasite elimination, NO production and type-I IFN response, the host laboratory wondered whether the immune system could be involved. To assess this, 2 groups of C57Bl/6 WT mice were allowed to drink *ad libitum* control water while other 2 groups drank RKV-supplemented water for 4 weeks, after which 1 group from each treatment was lethally irradiated with 900 rads one day before infection with 1.0x10^4 *P. berghei* ANKA sporozoites. Lethal irradiation kills dividing cells, including bone marrow and other haematopoietic progenitors, suppressing the host's immune system. Liver parasite load was then assessed by qPCR 45 h after infection with 1.0x10^4 *P. berghei* ANKA sporozoites. The results show that irradiation completely abolished the reduction in liver parasite load typically observed in RKV-supplemented mice, suggesting that the immune system is implicated in parasite elimination (Fig. 3.7).

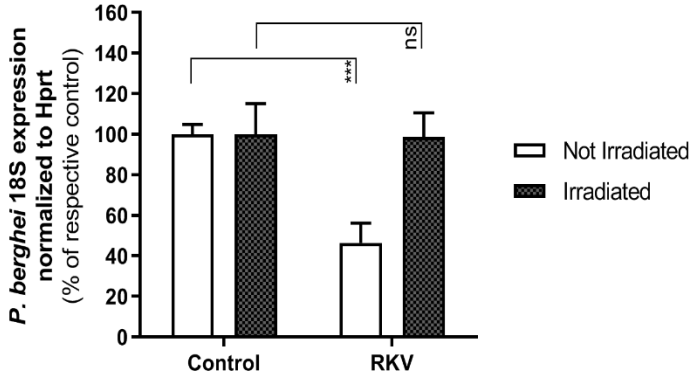

Figure 3.7 – RKV-dependent parasite elimination is dependent on a functional immune system. Control and RKV-supplemented C57Bl/6 mice were irradiated with 900 rads one day before infection with $1.0x10^4$ *P. berghei* ANKA sporozoites. Non-irradiated control and RKV-supplemented mice were used as controls. Liver parasite load was assessed by qPCR at 45 hpi and was plotted as percentage of the corresponding control. Pool of 2 independent experiments with 36 mice in total (males). Error bars represent SD. Unpaired t-test. ns - not significant and *** $p < 0.1$.

MyD88 signalling is involved in RKV-dependent parasite elimination

The reduction in liver parasite load typically observed in RKV-supplemented mice is abolished by lethal mouse irradiation, suggesting a role for the immune system in this mechanism. As the liver-stage of infection takes only 2 days in mice, it is a very short time to mount an adaptive immune response. Therefore, the innate immune system would be the obvious candidate to be involved in the mechanism of RKV-mediated parasite elimination. Most of the cells that compose the innate immune system are from the myeloid lineage. To assess the possible involvement of myeloid cells in the mechanism of parasite elimination by RKV supplementation, $MyD88^{-/-}$ mice were employed. MyD88 encodes a cytosolic adapter protein that plays a central role in innate immune responses, being an essential signal transducer in the IL-1 and TLRs signalling pathways[108].

Two groups of C57Bl/6 WT and 2 groups of $MyD88^{-/-}$ mice were allowed to drink *ad libitum* either control or RKV-supplemented water for 4 weeks. The mice were infected through i.v. injection with $1.0x10^4$ *P. berghei* sporozoites and liver parasite load was then assessed by qPCR at 45 hpi. The results obtained show that lacking MyD88 signalling abolished the reduction in liver parasite load that is typically observed in RKV-

supplemented mice, suggesting that MyD88 signalling is essential for parasite elimination (Fig. 3.8).

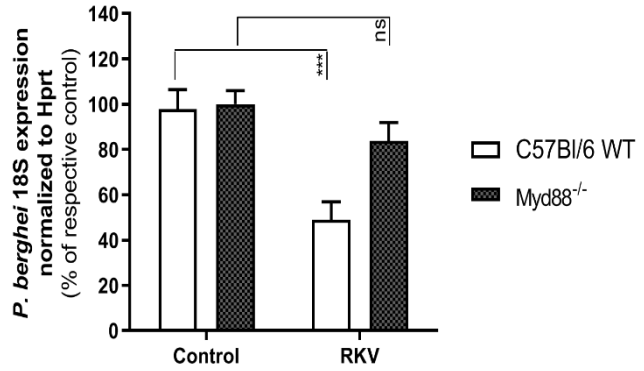

Figure 3.8 –MyD88 signalling is essential for hepatic parasite's elimination upon RKV supplementation. Control and RKV-supplemented C57Bl/6 and MyD88$^{-/-}$ mice were infected through i.v. injection of 1.0x10^4 *P. berghei* ANKA sporozoites. Liver parasite load was assessed by qPCR at 45 hpi and plotted as percentage of control. Pool of 5 experiments with 4 to 5 mice per group (males and females). Error bars represent SD. Two-tailed Mann-Whitney test. ns - not significant and *** $p < 0.001$.

Overall, these results point to a possible involvement of myeloid cells in the mechanism of *Plasmodium* hepatic elimination mediated by RKV supplementation. Thus, the role of individual myeloid cell population had to be assessed. Also, the role of the lymphoid lineage still remained unclarified.

Chapter IV - Results

The results presented in chapter III showed that RKV supplementation leads to a decrease of liver parasite load in C57Bl/6 mice. However, whether this phenotype was also recapitulated in other mouse strains remained unclarified. Therefore, this chapter is divided in two parts: the first part focuses on the effect of Arg and RKV supplementation on the liver parasite load of BALB/c mice, while the second part focuses on the elucidation of the mechanism behind the phenotype observed in C57Bl/6 mice.

BALB/c mice

Arg supplementation has been previously shown to decrease parasitemia in BALB/c mice infected with blood-stage *P. yoelii* parasites[6]. However, no reports exist on the effect of Arg on the liver-stage of *Plasmodium* infection. Thus, we decided to assess whether Arg and/or RKV supplementation, which leads to a decrease of hepatic *P. berghei* infection in C57Bl/6 mice, would also have an impact on *Plasmodium* liver-stage infection in BALB/c mice. Similarly to what has been done for C57Bl/6 mice, BALB/c mice were supplemented for 4 weeks with Arg or RKV, after which they were infected through i.v. injection of $1.0x10^4$ *P. berghei* ANKA sporozoites. Liver parasite load was then assessed by qPCR at 45 hpi. In accordance to its reported effects on blood-stage infection[6], Arg supplementation alone is sufficient to impair *Plasmodium* liver infection in BALB/c mice. Surprisingly, contradictory results were obtained upon RKV supplementation, resulting in either an increase or a decrease in liver parasite load from one experiment to the next. For simplicity, the two results obtained with RKV supplementation of BALB/c mice were designated RKV phenotype 1 and RKV phenotype 2. (Fig. 4.1).

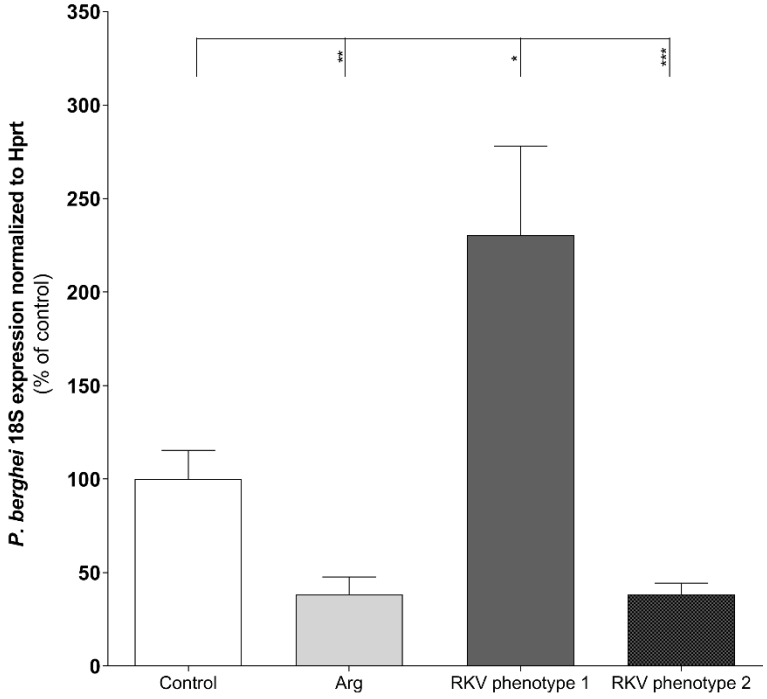

Figure 4.1- *Plasmodium* liver infection in BALB/c mice is modulated by Arg and RKV supplementation. The drinking water of the mice was replaced by sterile water supplemented with Arg or RKV and mice were allowed to drink *ad libitum* for 4 weeks. Liver parasite load was assessed by qPCR at 45 hpi and plotted as percentage of control. Pool of 7 independent experiments. Control: n = 31; Arg: n = 11; RKV phenotype 1: n = 13; RKV phenotype 2: n = 17. Error bars represent SD. One-way ANOVA with post-test Dunnett. ns - not significant, * $p < 0.1$, ** $p < 0.01$ and *** $p < 0.001$.

To further clarify the effect of Arg and RKV supplementation on *Plasmodium* liver infection in BALB/c mice, 50 µm liver sections were analysed by immunofluorescence microscopy in order to compare the number and size of hepatic parasites in control and supplemented mice. The results obtained show a significant reduction in both the number of parasites per liver area and on the size of the parasites in Arg-supplemented mice, suggesting that Arg supplementation impairs both parasite intrahepatic survival and parasite development (Fig. 4.2). In RKV-supplemented mice, an increase in parasite size was observed in both RKV phenotype 1 and RKV phenotype 2 (Fig. 4.2A and C). The number of parasites per liver area is tendentially higher in RKV phenotype 1 and lower in RKV phenotype 2, but these differences were not statistically significant (Fig. 4.2B). Even so, these results suggest that parasite numbers account for the overall liver parasite load observed for each phenotype upon RKV supplementation.

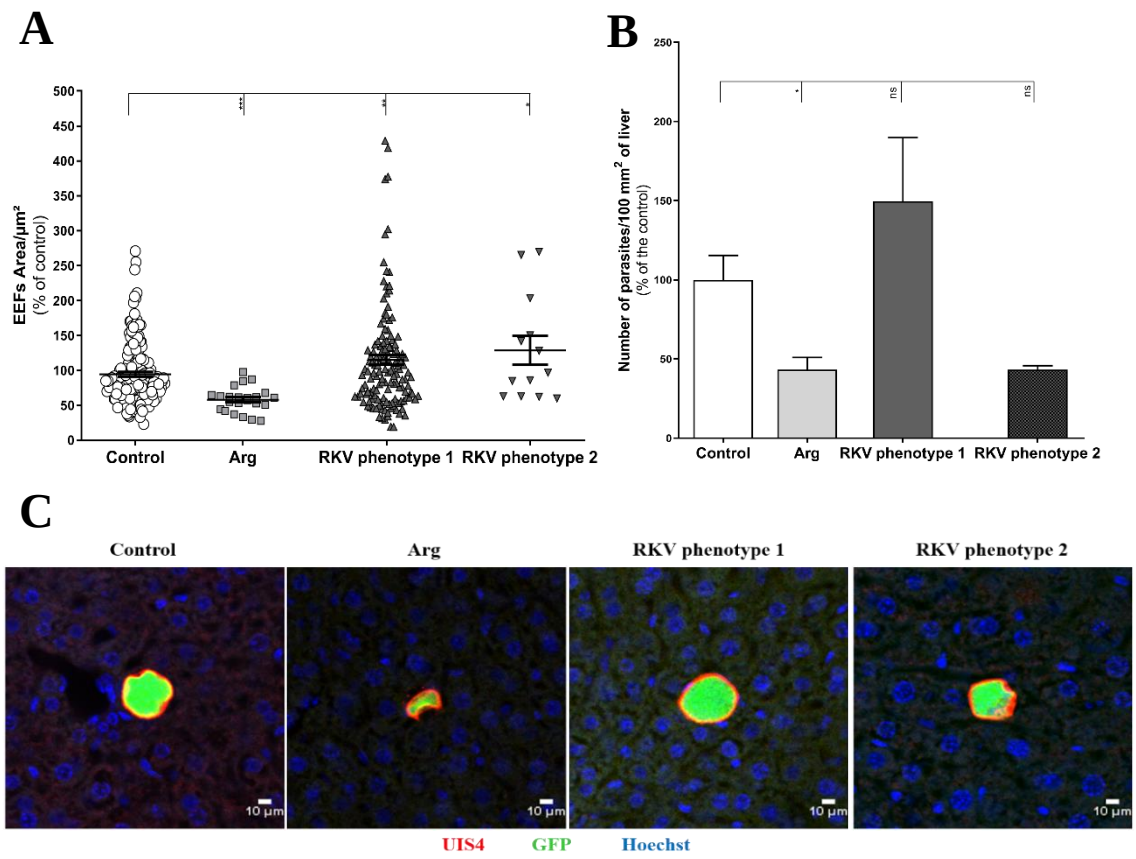

Figure 4.2 - *In vivo* supplementation with Arg or RKV impacts the number of *P. berghei* parasites in the liver of BALB/c mice. (A) Immunofluorescence microscopy quantification of **(A)** EEF areas, and **(B)** the number of parasites, in the liver sections of control, Arg- or RKV-supplemented mice. Both panels: plotted as percentage of control; pool of 2 to 3 mice per group from 2 to 3 independent experiment. Error bars represent SD. One-way ANOVA with post-test Dunnett. ns - not significant, * $p < 0.1$, ** $p < 0.01$ and *** $p < 0.001$. **(C)** Representative confocal images of *P. berghei* parasites in the livers of mice from several experimental groups. Scale bar, 10 µm.

A similar experiment was performed under *ex vivo* conditions, employing mouse primary hepatocytes collected from the liver of BALB/c mice and infected with $1.0x10^4$ luciferase-expressing *P. berghei* ANKA sporozoites. *Ex vivo* hepatic infection was determined by measuring the luminescence intensity of lysates of the infected cells at 48 hpi. Similarly to what was observed with primary hepatocytes from C57Bl/6 mice, a supraphysiological concentration of Arg, which corresponds to Arg supplementation *in vivo*, leads to a small but significant reduction on hepatic parasite load. The supplementation of the culture medium with Lys and Val under a supraphysiological

concentration of Arg, which corresponds to RKV, leads to an even higher impact on hepatic parasite infection, displaying a reduction of approximately 90% (Fig. 4.3).

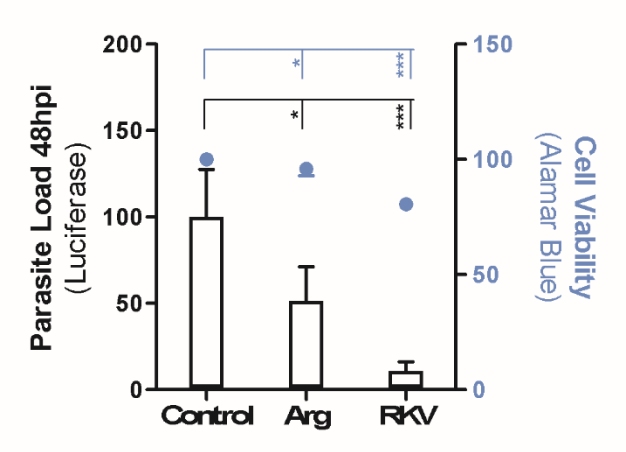

Figure 4.3 - Hepatic *Plasmodium* infection is modulated by amino acid supplementation *ex vivo*. Mouse primary hepatocytes were infected with $1.0x10^4$ luciferase-expressing *P. berghei* ANKA sporozoites and 2 hours later the culture medium was replaced by medium supplemented with a physiological concentration of Arg (100 μM), with a supraphysiological concentration of Arg (1 mM), and with 1 mM Arg, 20 mM Lys and 20 mM Val, which correspond to the Control, Arg and RKV experimental conditions, respectively. *Ex vivo* hepatic infection (luminescence) and cell viability (Alamar Blue) were assessed at 48 hpi and plotted as percentage of control. Pool of 2 independent experiments. Error bars represent SD. One-way ANOVA with post-test Dunnett. ns - not significant, * $p < 0.05$ and *** $p < 0.001$.

Overall, these data show that *Plasmodium* hepatic infection in BALB/c mice can be modulated *in vivo* and *ex vivo* by Arg supplementation, similarly to what has been reported for blood-stage infection[6]. On the other hand, RKV supplementation, which has a strong inhibitory effect on liver infection in C57Bl/6 mice and was our main object of study, gave contradictory results. These inconsistent results led us to abandon the first aim of the thesis and proceed only with the objective of clarifying the mechanism of RKV-dependent immune system stimulation and parasite elimination in C57Bl/6 mice.

C57Bl/6 mice

As described in the previous results section, RKV supplementation leads to a striking decrease of hepatic *Plasmodium* infection in C57Bl/6 mice, mostly by reducing the number of infected hepatocytes. This parasite elimination does not rely on NO production nor on a boost of the type-I IFN response, previously reported as crucial to control liver-stage infection[10,128]. The reduction in liver parasite load typically observed in RKV-supplemented mice is abolished by lethal mouse irradiation and in MyD88$^{-/-}$ mice, suggesting that parasites are being eliminated by the host's immune system and that MyD88 signalling is involved in this elimination.

To further assess whether the mechanism of RKV-dependent parasite elimination relies on the immune system, we employed knockout mice and depleting antibodies to eliminate specific immune cell populations, and therefore evaluate their role in this process. In all experiments, C57Bl/6 WT and knockout or antibody-depleted mice were allowed to drink *ad libitum* control or RKV-supplemented water for 4 weeks. Mice were then infected through i.v. injection of 1.0x10^4 *P. berghei* ANKA sporozoites and liver parasite load was assessed by qPCR at 45 hpi.

Myeloid cells

Previous results from the host laboratory suggest that MyD88 signalling is involved in the mechanism of RKV-mediated parasite elimination. As this molecule plays a crucial role in the signalling and function of myeloid cells[136], we decided to assess whether the observed RKV-dependent parasite elimination is dependent on one or more of the cell populations from this branch of the immune system.

We started by depleting *in vivo* phagocytic cells, namely Kupffer cells, macrophages and DCs, through the administration of clodronate-filled liposomes. Once delivered into phagocytic cells, clodronate is released from the liposomes and, because it cannot escape from the cell, it accumulates intracellularly leading to cell death[137]. A control and a RKV-supplemented group of C57Bl/6 mice were injected i.v. with 200 µL of PBS-filled liposomes, which were used as controls, while another control and RKV-supplemented groups were injected with 200 µL of clodronate-filled liposome formulation 48 hours before infection with 1.0x10^4 *P. berghei* ANKA sporozoites. Liver

parasite load was then assessed by qPCR at 45 hpi. As described previously, the efficiency of the depletion was assessed by quantifying the expression of three specific markers by qPCR: Clec4f, which is specifically expressed by Kupffer cells; CD68, which is a marker of the macrophage lineage; and F4/80, which is expressed by mature macrophages (Fig. 4.4A). Although DCs can be theoretically depleted *in vivo* through administration of clodronate-filled liposomes, DC depletion upon clodronate administration was not specifically confirmed in these experiments.

While Kupffer cells were completely eliminated by the administration of the clodronate-filled liposome formulation, as shown by the absence of expression of Clec4f, some expression of the CD68 and F4/80 markers could be detected, which likely results from monocytes and macrophages that were recruited to the liver as a consequence of *Plasmodium* infection (Fig. 4.4A). Importantly, the liver infection results obtained show that depleting Kupffer cells, macrophages and DCs does not abolish the reduction in liver parasite load typically observed upon RKV supplementation, suggesting that these cells are not involved in hepatic parasite elimination upon RKV supplementation (Fig. 4.4B).

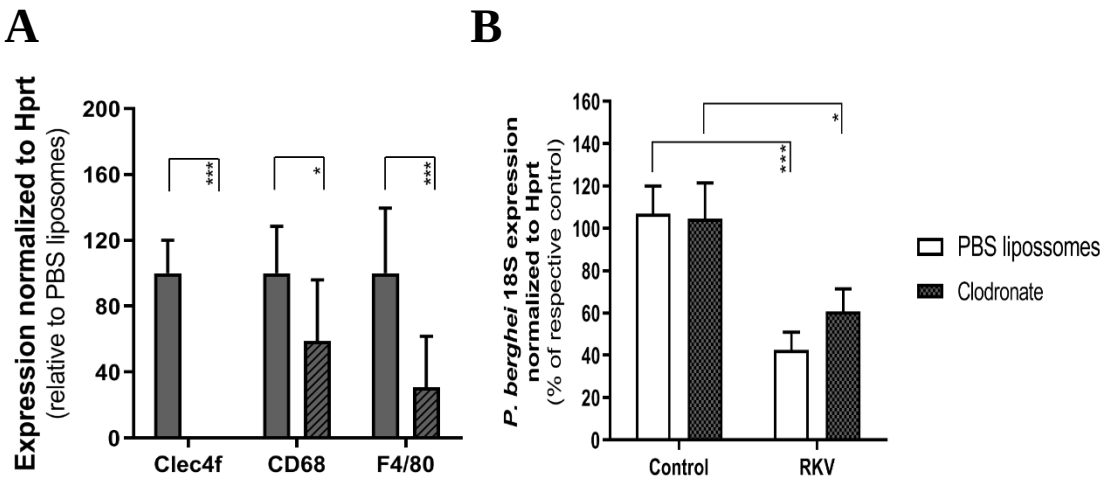

Figure 4.4 – Kupffer cells and macrophages are not involved in the mechanism of RKV-mediated parasite elimination. C57Bl/6 mice received 200 μL of the PBS- or clodronate-filled liposome formulation by i.v. injection 48 hours before infection with $1.0x10^4$ *P. berghei* ANKA sporozoites. **(A)** Assessment of hepatic phagocyte depletion by qPCR at 45 hpi. Clec4f is a specific marker for Kupffer cells, CD68 is a marker of the macrophage lineage, and F4/80 is a marker of mature macrophages. Expression of Clec4f, CD68 and F4/80 was plotted as percentage relative to the control. **(B)** Liver parasite load

was assessed by qPCR at 45 hpi and plotted as percentage of control. Pool of 3 experiments with 4 to 5 mice per group (males and females). For all panels: Error bars represent SD. Two-tailed Mann-Whitney test and Unpaired t-test for panels A and B, respectively. ns - not significant, * $p < 0.1$ and *** $p < 0.001$.

We then wondered whether neutrophils would be involved in the mechanism of hepatic elimination by RKV supplementation. To assess this, neutrophils were depleted *in vivo* by the administration of anti-Lymphocyte antigen 6 complex locus G (Ly6G) antibody. Ly6G is a 21-25 kDa member of the Ly-6 superfamily of Glycosylphosphatidylinositol (GPI)-anchored cell surface proteins, which is expressed differentially during development by cells in the myeloid lineage including monocytes, macrophages and granulocytes, which include neutrophils[138]. However, while neutrophils retain Ly6G expression, making this a good cell surface marker for these populations, monocytes only express Ly6G transiently during development, its expression being subsequently lost[88,139].

A control or a RKV-supplemented group of C57Bl/6 mice were injected i.p. with 200 µL of PBS, which were used as controls, while another control and RKV-supplemented group were injected i.p. with 250 µL of anti-Ly6G antibody 2 hours after infection with *P. berghei* sporozoites. Liver parasite load was assessed at 45 hpi by qPCR. The depletion of neutrophils was assessed by flow cytometry analysis (Fig. 4.5A). The results obtained show that depleting neutrophils seems to not abolish the reduction in liver parasite load typically observed with the RKV supplementation, suggesting that these cells are also not involved in parasite elimination (Fig. 4.5B).

As RKV supplementation in these experiments led only to a decrease of approximately 35% of the liver parasite load, we decided to further confirm our conclusions employing Genista mice, a mouse model that lacks mature neutrophils. Genista mice have a point mutation in the transcription factor Growth factor independent 1 (Gfi1), resulting in the generation of small numbers of atypical $CD11b^{+}$ $Ly\text{-}6G^{int}$ neutrophils capable of mediating a split pattern of inflammatory and anti-infectious responses[131]. Additionally, an increase of monopoiesis has been observed in these mice, possibly as a consequence of the blockade of terminal granulopoiesis.

Therefore, 1 group of C57Bl/6 WT and 1 group of Genista mice were allowed to drink *ad libitum* control water while another group of C57Bl/6 WT and of Genista mice were allowed to drink *ad libitum* RKV-supplemented water. Liver parasite load was assessed following infection with *P. berghei* sporozoites by qPCR. The results show that RKV-supplemented Genista mice displayed the typical reduction observed in RKV-supplemented WT mice, thus confirming that neutrophils are not involved in the mechanism of RKV-mediated parasite elimination (Fig. 4.5C).

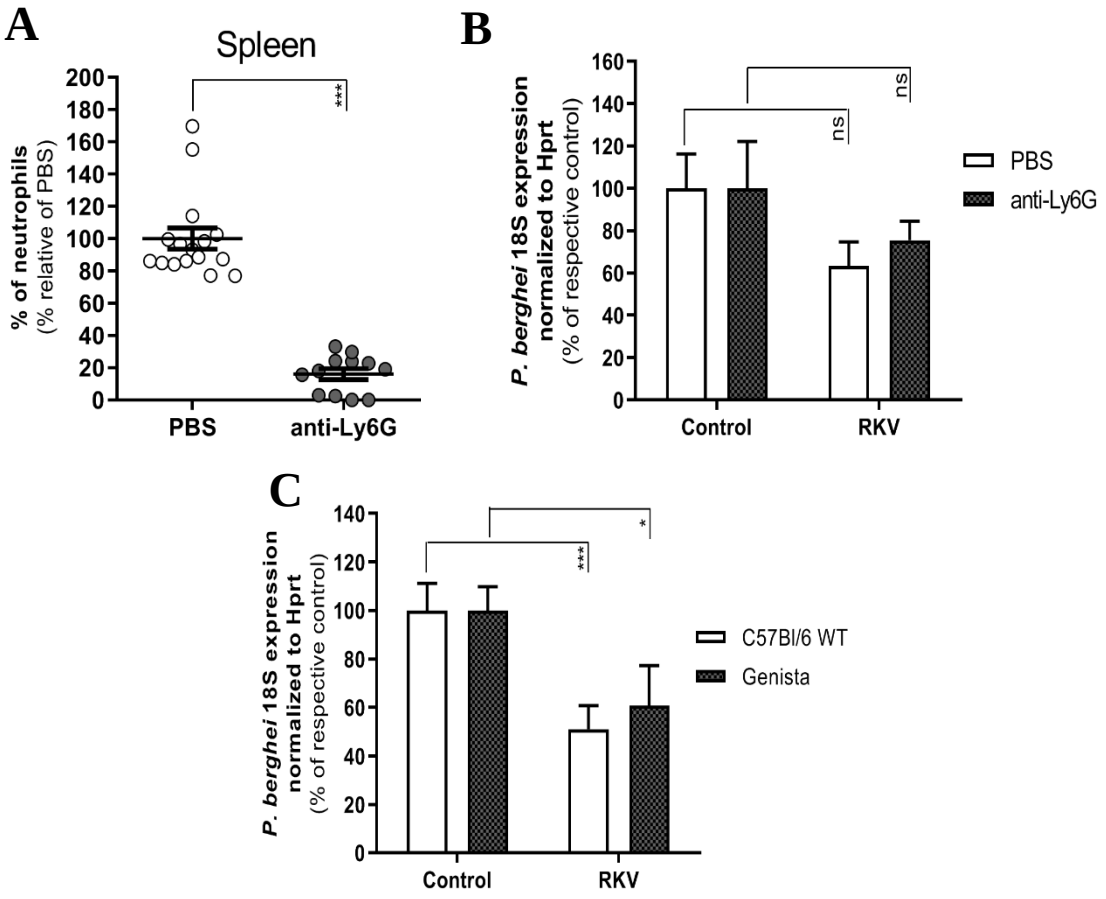

Figure 4.5 – Neutrophils do not appear to be involved in RKV-dependent parasite elimination. (A) C57Bl/6 mice received PBS or 250 µg of anti-Ly6G antibody by i.p. injection 2 hours after infection with $1.0x10^4$ *P. berghei* ANKA sporozoites. Neutrophils (represented as a % of $Ly6G^+$ cells, gated in $CD11b^+CD11c^-$ population) depletion was assessed in isolated splenocytes of mice that received PBS (non-depleted; control) or anti-Ly6G antibody (depleted) by flow cytometry analysis. Plotted as percentage of control. **(B)** Liver parasite load was assessed by qPCR at 45 hpi and plotted as percentage of control. Pool of 2 independent experiments with 3 to 5 mice per group (males).

(C) C57Bl/6 and Genista mice were infected through i.v. injection of $1.0x10^4$ *P. berghei* ANKA sporozoites. Liver parasite load was assessed by qPCR at 45 hpi and plotted as percentage of the corresponding control. Pool of 3 independent experiments with 3 to 8 mice per group (males and females). For all panels: Error bars represent SD. Two-tailed Mann-Whitney for panels A and C; Unpaired t-test for panel B. ns - not significant, * $p < 0.1$ and *** $p < 0.001$.

Having excluded neutrophils, we further ascertained whether monocytes could be involved in parasite elimination by RKV supplementation. To this end, monocytes were depleted *in vivo* by administration of anti-CCR2 and anti-CD115 antibodies. C-C Motif chemokine receptor type 2 (CCR2) is homogeneously expressed on monocytes and on T cells, playing an important role in the recruitment of monocytes/macrophages and T cells[134]. In turn, Cluster of Differentiation 115 (CD115) is the sole cell-surface receptor for colony-stimulating factor 1 (CSF-1) identified to date, which regulates the proliferation and differentiation of cells in the monocytic lineage[140]. Monocytes were depleted in both control or RKV-supplemented groups of C57Bl/6 mice through daily i.p. injections of 20 µg of anti-CCR2 for 4 days. Monocytes were also depleted in another control and RKV-supplemented groups of C57Bl/6 mice by administration of 300 µg of anti-CD115 through i.p. injection. In both approaches, the control group were injected i.p. with 200 µL of PBS. The depletion of monocytes by administration of anti-CCR2 and anti-CD115 was assessed by flow cytometry analysis (Fig. 4.6A and B). Since the anti-CD115 antibody depletes preferentially patrolling monocytes from circulation, the efficacy of depletion by this antibody has to be verified in the blood (Fig. 4.6B), not in the liver and spleen, as is the case for anti-CCR2 (Fig. 4.6A). Unfortunately, the depletion with anti-CD115 was not very successful, and thus, these results are inconclusive. Consequently, only the results obtained with the anti-CCR2 antibody allowed to infer about the role of monocytes in the mechanism of RKV-mediated parasite elimination. Liver infection, which was assessed at 45 hpi by qPCR, was approximately the same between all groups of RKV-supplemented mice, suggesting that monocytes do not also play a role in parasite elimination by RKV supplementation (Fig. 4.6C).

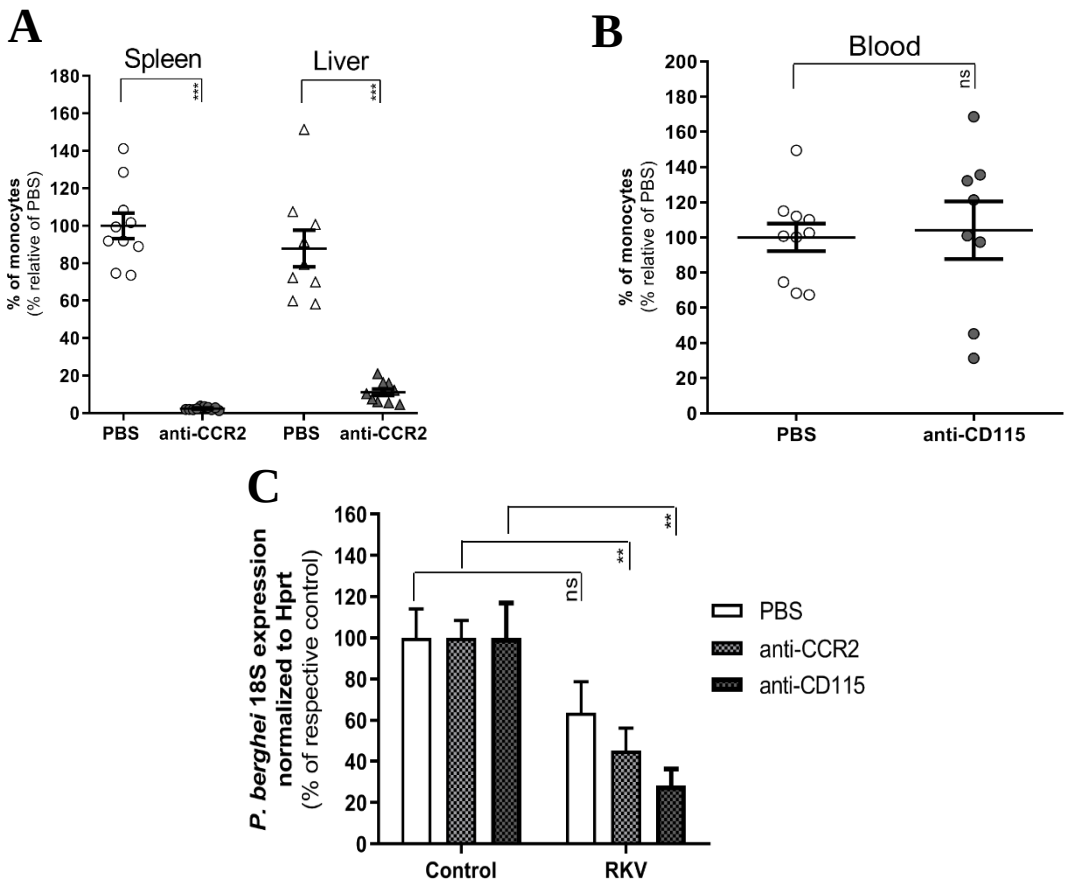

Figure 4.6 – Monocytes are not involved in hepatic parasite elimination by RKV supplementation. (A) C57Bl/6 mice received 200 µL of PBS, 20 µg of anti-CCR2 or 300 µg of anti-CD115 antibodies by i.p. injection at d-2, d-1, d0, d+1 or d-3, d-1, d+1, respectively. Mice were infected with $1.0x10^4$ *P. berghei* ANKA sporozoites. Monocytes (represented as a % of $Ly6C^+$ cells, gated in $CD11b^+CD11c^-$ population) depletion was assessed in blood of mice that received PBS (non-depleted; control) or anti-CD115 (depleted) by flow cytometry analysis. Plotted as percentage of control. **(B)** . Monocytes (represented as a % of $Ly6C^+$ cells, gated in $CD11b^+CD11c^-$ population) depletion was assessed in isolated splenocytes and liver leukocytes of mice that received PBS (non-depleted; control) or anti-CCR2 (depleted) by flow cytometry analysis. Plotted as percentage of control. **(C)** Liver parasite load following infection was assessed by qPCR at 45 hpi and plotted as percentage of the corresponding control. One experiment with 5 mice per group (males). For all panels: Error bars represent SD; Unpaired t-test. ns - not significant, ** $p < 0.01$ and *** $p < 0.001$.

Neutrophils and monocytes were individually excluded to be involved in the mechanism of RKV-mediated parasite elimination. However, since these cell populations have similar functions, they can compensate for the absence of each other, thus justifying the fact that we did not abolish the reduction of the liver parasite load typically observed in RKV-supplemented mice. Then, to assess if neutrophils and monocytes were definitely not involved in the mechanism of hepatic parasite elimination, we depleted these 2 cell populations through the administration of anti-Gr-1 in C57Bl/6 and in Genista mice.

Anti-Granulocyte receptor 1 (anti-Gr-1) antibody reacts strongly with mouse Ly6G and weakly with mouse Ly6C[141]. Lymphocyte antigen 6 complex, locus C (Ly6C) is a 14-17 kDa member of the Ly-6 superfamily of GPI-anchored cell surface proteins, which is expressed by neutrophils, DCs, and subsets of monocytes, macrophages, and lymphocytes whereas Ly6G is a marker for neutrophils[141]. Thus, anti-Gr-1 administration leads to deplete *in vivo* not only neutrophils but also other Gr-l$^+$ (Ly6C$^+$) cells, which include Gr-1$^+$ (Ly6C$^+$) blood monocytes that are precursors of inflammatory macrophages[141].

Control and RKV-supplemented groups of C57Bl/6 mice were injected i.p. with 200 µL of PBS, while another control or a RKV-supplemented group of C57Bl/6 mice were injected i.p. with 250 µg of anti-Gr-1. Liver parasite load was assessed at 45 hpi infection with *P. berghei* sporozoites. Neutrophils and monocytes depletion was assessed by flow cytometry analysis (Fig. 4.7A and B, respectively). The results show that depleting neutrophils and monocytes in C57Bl/6 mice does not abolish the reduction in liver parasite load typically observed with the RKV supplementation (Fig. 4.7C).

In a second approach, we administered the anti-Gr-1 antibody in Genista mice. The rational of this experiment was to use anti-Gr-1 to deplete monocytes in a mouse model that was described as having no neutrophils.

In this experiment, control and RKV-supplement groups of C57Bl/6 and Genista mice were injected with 200 µL of PBS, which were both used as controls, while another control and RKV-supplemented groups of Genista mice were injected i.p. with 250 µg of anti-Gr-1 antibody. Neutrophils and monocytes depletion was assessed by flow cytometry analysis (not shown; depletion similar to what was observed in Fig. 4.7A). Liver parasite load, which was assessed by qPCR following infection with *P. berghei* sporozoites, show

that Genista mice that were injected with anti-Gr-1 antibody, display the typical reduction observed in RKV-supplemented WT and Genista mice, confirming that neither neutrophils or monocytes are involved in parasite elimination by RKV supplementation (Fig. 4.7D).

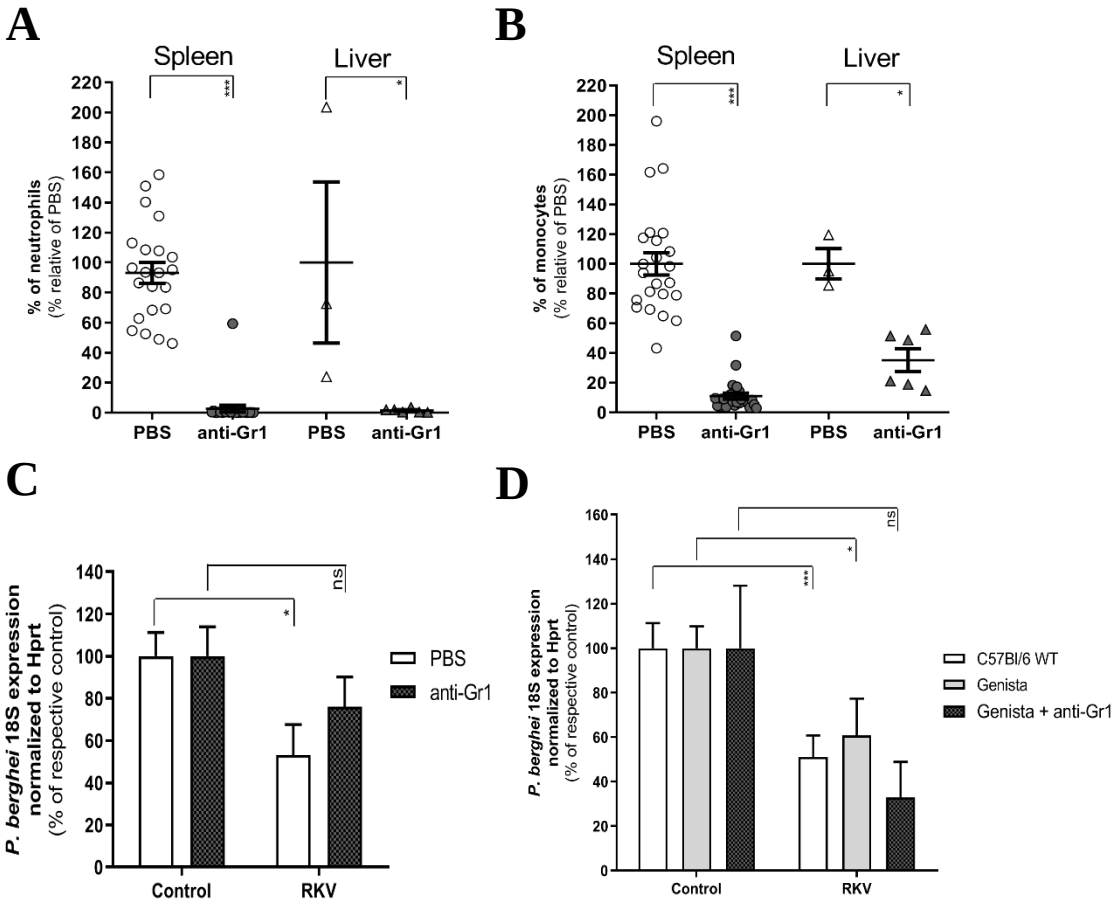

Figure 4.7 – Neutrophils and monocytes are not involved in RKV-dependent parasite elimination. (A) C57Bl/6 mice received 200 µL of PBS or 250 µg of anti-Gr-1 antibody by i.p. injection 3 hpi with $1.0x10^4$ *P. berghei* ANKA sporozoites. Neutrophils (represented as a % of $Ly6G^+$ cells in total live cells) depletion was assessed in isolated splenocytes and liver leukocytes of mice that received PBS (non-depleted; control) or anti-Gr-1 antibody (depleted) by flow cytometry analysis. Plotted as percentage of control. **(B)** Monocytes (represented as a % of $Ly6C^+$ cells in total live cells) depletion was assessed in isolated splenocytes and liver leukocytes of mice that received PBS (non-depleted; control) or anti-Gr-1 antibody (depleted) by flow cytometry analysis. Plotted as percentage of control. **(C)** Liver parasite load following infection was assessed by qPCR at 45 hpi and plotted as percentage of control. Pool of 3 independent

experiments with 3 or 5 mice per group (males). **(D)** C57Bl/6 mice received 200 µL of PBS by i.p. injection. Genista mice received 200 µL of PBS or 250 µg of anti-Gr-1 by i.p. injection 3 hpi with $1.0x10^4$ *P. berghei* ANKA sporozoites. Liver parasite load following infection was assessed by qPCR at 45 hpi and plotted as percentage of control. Pool of 3 independent experiments with 3 to 8 mice per group (males and females). For all panels: Error bars represent SD. Two-tailed Mann-Whitney for panels A, B and D; Unpaired t-test for panel C. ns – not significant, * $p < 0.1$ and *** $p < 0.001$.

Having excluded most myeloid cells from being involved in the mechanism of parasite elimination by RKV supplementation, except eosinophils, basophils and mast cells, which are present in very low abundance in the blood or organs[142,80,81,82,83], we decided to proceed to investigate the involvement of lymphoid cells in the RKV-mediated parasite elimination.

Lymphoid cells

Lymphoid cells include B cells, T cells, γδ T cells, NK cells, NK T cells and ILCs. To investigate the role of these cells in the mechanism of RKV-mediated parasite elimination we started by employing $Rag2^{-/-}$, $TCR\delta^{-/-}$ and $Rag\gamma c^{-/-}$ mice, which lack specific cell populations or groups of populations.

Control and RKV-supplemented groups of C57Bl/6 mice and $Rag2^{-/-}$ mice, which lack B cells, T cells, γδ T cells and NK T cells, were firstly employed. All groups of mice were infected with $1.0x10^4$ *P. berghei* sporozoites and, after 45 hpi, liver parasite load was assessed by qPCR. Liver infection results show that RKV-supplemented $Rag2^{-/-}$ mice display the typical reduction observed in WT mice, suggesting that B cells, T cells, γδ T cells and NK T cells are not involved in parasite elimination by RKV supplementation (Fig. 4.8A).

Control and RKV-supplemented $TCR\delta^{-/-}$ mice were also employed to confirm that γδ T cells would not, in fact, be involved in the mechanism of RKV-mediated parasite elimination. The results show lacking γδ T cells does not abolish the typical reduction obtained with RKV supplementation, thus confirming that these cells are not involved in the mechanism of parasite elimination by RKV supplementation (Fig. 4.8B).

Having excluded most of the lymphoid cells with the Rag2$^{-/-}$ mouse strain, only the roles of NK cells and ILCs remained to be clarified. With this in mind, control and RKV-supplemented groups of C57Bl/6 and Ragγc$^{-/-}$ mice were employed. Ragγc$^{-/-}$ mice, similarly to Rag2$^{-/-}$ mice, lack B cells, T cells, γδ T cells, NK T cells, however, this mouse strain additionally lacks NK cells and ILCs. Liver parasite load of these mice was then assessed by qPCR 45 h after infection with *P. berghei* sporozoites. The results obtained employing Ragγc$^{-/-}$ mice, whose only difference relative to Rag2$^{-/-}$ mice is additionally lacking NK cells and ILCs, abolishes the reduction in liver parasite load observed upon RKV supplementation, suggesting that at least one of these cells might be involved in the mechanism of RKV-mediated impairment of liver parasite load (Fig. 4.8C).

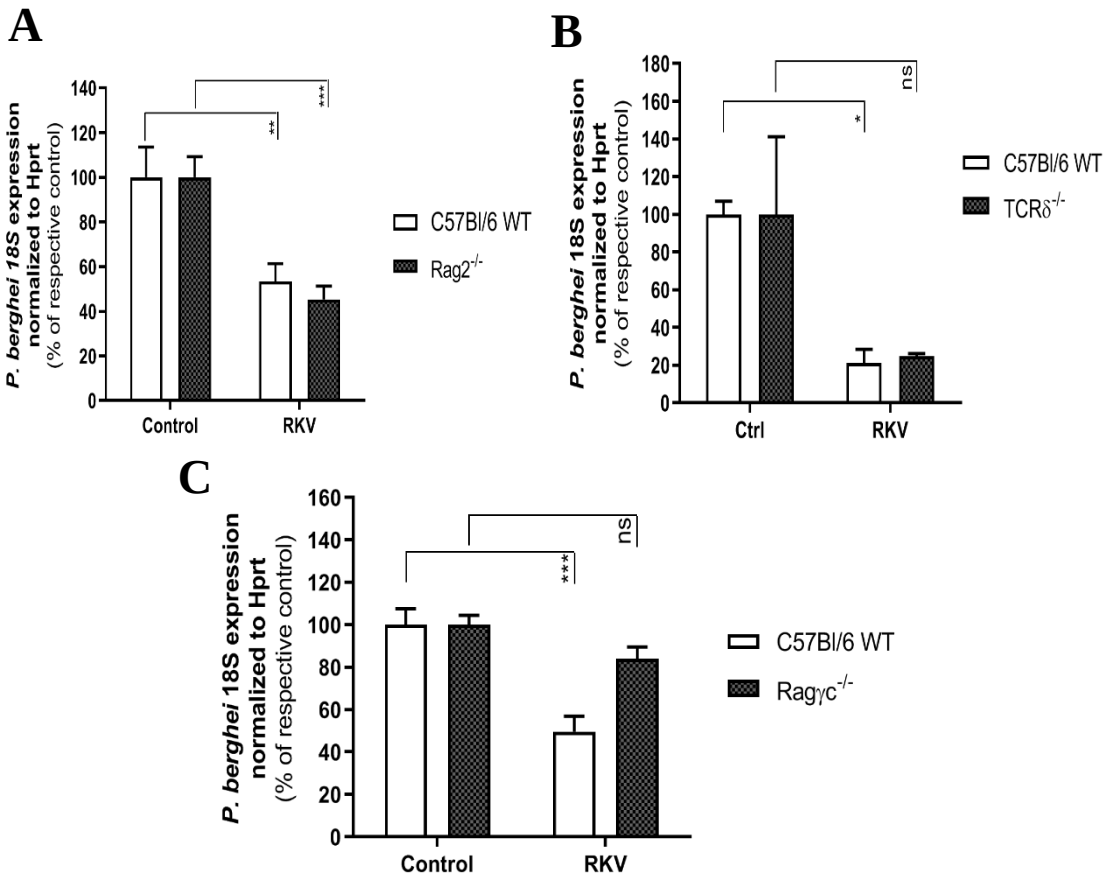

Figure 4.8 – ILCs and NK cells are potential candidates to be involved in RKV-dependent parasite elimination. C57Bl/6 and knockout mice were infected through i.v. injection of 1.0x10^4 *P. berghei* ANKA sporozoites. Liver parasite load was assessed by qPCR at 45 hpi and plotted as percentage of control. **(A)** Liver parasite load following infection of C57Bl/6 and Rag2$^{-/-}$ mice. Pool of 4 independent experiments with 4 to 5

mice per group (males and females). **(B)** Liver parasite load following infection of C57Bl/6 and TCR$\delta^{-/-}$ mice. One experiment with 3 to 4 mice per group (females). **(C)** Liver parasite load following infection of C57Bl/6 and Rag$\gamma c^{-/-}$ mice. Pool of 6 independent experiments with 4 to 5 mice per group (males and females). For all panels: Error bars represent SD. Two-tailed Mann-Whitney test for panels A and B; Unpaired t-test for panel C. ns - not significant, * $p < 0.1$, ** $p < 0.01$ and *** $p < 0.001$.

Next, we decided to investigate the role of NK cells by depleting these cells *in vivo* through the administration of anti-NK1.1 antibody. NK1.1 is a type-II integral membrane glycoprotein with a C-type lectin domain expressed by NK and NK T cells[143,144] that plays a role in NK cell activation and differentiation, IFN-γ production, cytotoxic granule release, and is thought to be involved in the generation of Th2 cells[145]. Therefore, injecting anti-NK1.1 will deplete both NK and NK T cells. However, as we already excluded the possibility of NK T cells being involved in the mechanism of parasite elimination upon RKV supplementation, and there is no other way to deplete NK cells without depleting also NK T cells, the results obtained with anti-NK1.1 will reflect whether NK cells play a role in this process. Control and RKV-supplemented groups of C57Bl/6 mice were injected i.p. with 200 µL of PBS, which were used as controls, while another control and RKV-supplemented groups of C57Bl/6 were injected with 150 µg of anti-NK1.1. NK cells depletion was assessed by flow cytometry analysis (Fig. 4.9A), while liver parasite load was assessed by qPCR following infection with *P. berghei* sporozoites. Liver infection results show that depleting NK cells does not abolish the reduction in the liver parasite load typically observed upon RKV supplementation, which also excludes their involvement in the RKV-related hepatic parasite impairment (Fig. 4.9B).

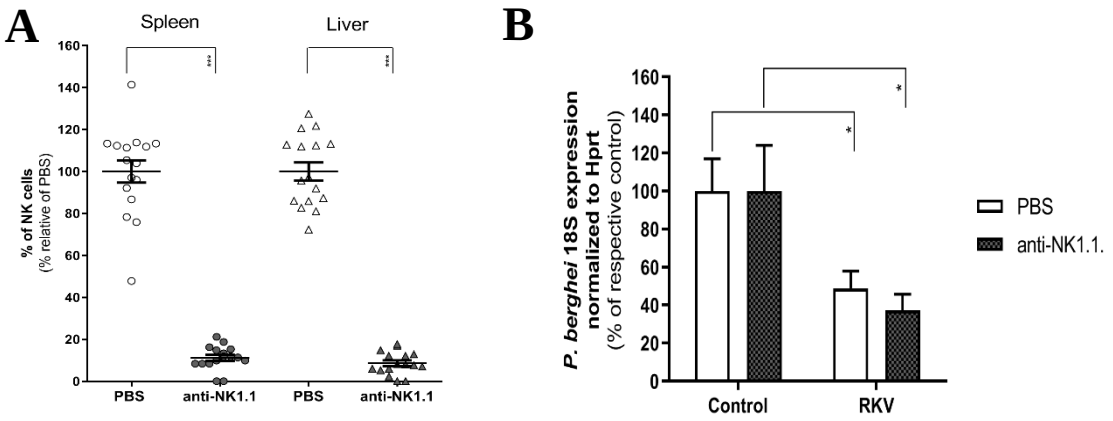

Figure 4.9 – NK cells are also not involved in the mechanism of hepatic RKV-mediated parasite elimination. C57Bl/6 mice received PBS or 150 µg of anti-NK1.1. antibody by i.p. injection one day before infection with $1.0x10^4$ *P. berghei* ANKA sporozoites. **(A)** NK cells (represented as a % of $NK1.1^+$ cells in total live cells) depletion was in isolated splenocytes and liver leukocytes of mice that received PBS (non-depleted; control) or anti-NK1.1 antibody (depleted) by flow cytometry analysis. Plotted as percentage of control. **(B)** Liver parasite load following infection was assessed by qPCR at 45 hpi and plotted as percentage of control. Pool of 2 independent experiments with 4 mice per group (males and females). For all panels: Error bars represent SD. Unpaired t-test and Two-tailed Mann-Whitney test for panels A and B, respectively. ns - not significant, * $p < 0.1$ and *** $p < 0.0001$.

Having excluded B cells, T cells, γδ T cells, NK cells and NK T cells, only the role of ILCs as candidates to be involved in the RKV-dependent hepatic parasite elimination remains to be elucidated. Therefore, their role in this mechanism of parasite elimination will be clarified in future experiments.

Cytokines

Cytokines are small secreted proteins released by cells that mediate and regulate immunity, inflammation and hematopoiesis[146]. Key pro-inflammatory cytokines include IL-1, IL-6, TNF-α, IL-17 and IFNs[146]. We investigated the role of 2 of these cytokines: IL-1α and IL-17A.

The IL-1 family of cytokines includes 11 members, one of them being IL-1α, a potent inflammatory cytokine that is synthesised by multiple cell types including

monocytes, macrophages, neutrophils, hepatocytes, and tissue macrophages throughout the body, and was expressed constitutively in many cell types[146,147]. IL-1α functions both as a secreted and as a membrane-bound cytokine, having an important role in the inflammatory process[146,147] and, consequently in the innate immunity. Specifically, we ascertain the involvement of IL-1α in the mechanism of action of RKV supplementation since it has been demonstrated that an increase of this cytokine correlated with early neutrophil recruitment[148,149,150].

To assess the role of IL-1α in the process of parasite elimination by RKV supplementation, control and RKV-supplemented groups of C57Bl/6 and IL-1α$^{-/-}$ mice were employed. These mice were then infected with *P. berghei* sporozoites and liver parasite load was assessed by qPCR at 45 hpi. The results show that RKV-supplemented IL-1α$^{-/-}$ mice display a reduction in the liver parasite load similar to that observed in the WT mice, suggesting that this cytokine is not involved in the inhibition of *P. berghei* hepatic infection upon RKV supplementation (Fig. 4.10A). This is in agreement with our previous results that suggested that neutrophils are not involved in the mechanism of RKV-mediated parasite elimination.

We also investigated the role of IL-17A since it was previously identified by the host laboratory as being upregulated in liver leukocytes isolated from RKV-supplemented C57Bl/6 mice when compared with liver leukocytes from their control counterparts.

IL-17 is a family of cytokines that includes 6 members, one of which is IL-17A, a cytokine that is expressed by several lymphocyte populations such as Th17, γδ T cells, NK T cells, NK cells and ILC3s, and that plays key protective roles in host defense against certain pathogens at epithelial and mucosal barriers and also in the inflammatory process[151]. Thus, IL-17A is considered to be placed at the interface between the innate and adaptive immunity[152]. The involvement of IL-17A in the mechanism of parasite elimination upon RKV supplementation was assessed by employing control and RKV-supplemented groups of C57Bl/6 and IL-17A$^{-/-}$ mice. Liver parasite load was assessed by qPCR following infection with *P. berghei* sporozoites. Liver infection results show that the absence of IL-17A does not abolish the reduction in the liver parasite load typically observed with the RKV supplementation, suggesting that this cytokine is also not

involved in the inhibition of *P. berghei* hepatic infection upon RKV supplementation (Fig. 4.10B).

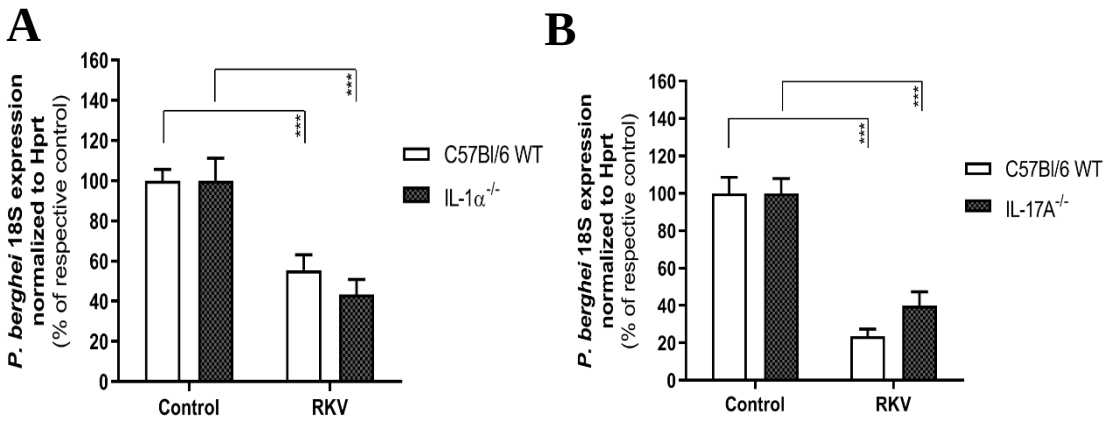

Figure 4.10 – IL-1α and IL-17 are not involved in the mechanism of action of the RKV supplementation. C57Bl/6 WT and knockout mice were infected through i.v. injection of $1.0x10^4$ *P. berghei* ANKA sporozoites. Liver parasite load was assessed by qPCR at 45 hpi and plotted as percentage of control. **(A)** Liver parasite load following infection of C57Bl/6 WT and IL-$1\alpha^{-/-}$ mice. Pool of 2 experiments with 3 to 8 mice per group (females). **(B)** Liver parasite load following infection of C57Bl/6 WT and IL-$17^{-/-}$ mice. Pool of 3 experiments with 3 to 5 mice per group (females). For all panels: Error bars represent SD. Unpaired t-test and Two-tailed Mann-Whitney test for panels A and B, respectively. ns - not significant and *** $p < 0.001$.

Production of Reactive oxygen species

ROS, including NO, superoxide, and peroxynitrite, have been shown to kill intraerythrocytic malarial parasites[153]. The most cited mechanism for parasite killing is that acute *Plasmodium* infection induces IFN-γ-producing Th1 cells, which in turn activate macrophages to secrete parasiticidal NO and ROS[153]. However, although ROS have not been described as being involved in controlling liver-stage malaria infection, an enhancement of NO production was observed in the context of Arg supplementation during blood-stage malaria infection. As previously mentioned, RKV-dependent parasite elimination does not rely on NO production. Nevertheless, we wondered whether other free radicals derived by oxygen would be involved in parasite elimination upon RKV supplementation. To assess this, ROS production was inhibited by treating the mice with

NAC. NAC is a precursor of L-cysteine that leads to an elevation of glutathione biosynthesis[154]. It acts directly as a scavenger of free radicals, especially oxygen radicals[154].

A control and an RKV-supplemented group of C57Bl/6 mice were injected i.p. with 200 µL of PBS, while another control and RKV-supplemented groups of C57Bl/6 mice were treated with NAC every 12 h for 5 days. Mice were then infected through i.v. injection with $1.0x10^4$ *P. berghei* sporozoites and liver parasite load was then assessed by qPCR at 45 hpi. The results show that the inhibition of ROS production does not abolish the reduction in the liver parasite load typically observed upon RKV supplementation. However, as we were unable to confirm that the production of ROS was indeed reduced by this treatment, this result must be considered with caution (Fig. 4.11).

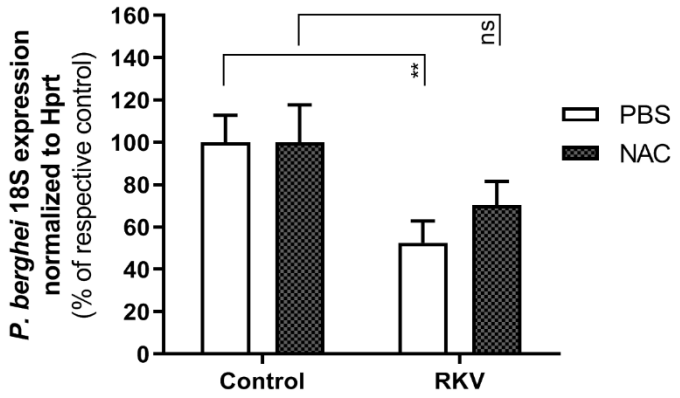

Figure 4.11 – Hepatic parasite elimination by RKV supplementation does not rely on ROS production. C57Bl/6 mice received 200 µL of PBS or 150 mg NAC/kg of mouse by i.p. injection every 12 h for 5 days (from d-2 until d+2). C57Bl/6 mice were infected through i.v. injection of $1.0x10^4$ *P. berghei* ANKA sporozoites. Liver parasite load following infection was assessed by qPCR at 45 hpi and plotted as percentage of control. Pool of 2 independent experiments with 5 mice per group (males). Error bars represent SD. Unpaired t-test. ns - not significant and * $p < 0.1$.

Modulation of the microbiota

Important advances in the fields of immunology and gut microbiology have emerged in recent years. It has been clearly demonstrated that diet has a considerable

effect on the composition of the gut microbiota, which in turn, influences the innate and adaptive immune responses[155,156]. Dietary amino acid supplementations have been associated with a modification of gut microbiota[155,156,157,158,159]. Of note, dietary Arg supplementation was reported as capable of inducing changes in the gut microbiota, which contributes to the activation of the innate immune response[159].

Therefore, we hypothesized that possible microbiota changes caused by RKV supplementation could be related to the phenotype typically observed in RKV-supplemented mice (reduction in liver parasite load), since a strong connection between the gut microbiota and the immune system has been defined[155,157]. To assess this, 2 groups of C57Bl/6 mice were allowed to drink control water while another group drank RKV-supplemented water. At the end of each week of supplementation, faecal pellets were collected from the cage receiving supplemented water (designated RKV donor) and transferred to one of the cages with control water (designated Control receptor)[160,161]. During cohousing, animals may feed on faecal pellets (also known as coprophagy) or more likely ingest faecal material by self-grooming[160,161]. The other group receiving control water were used as controls. All these mice were infected with $1.0x10^4$ *P. berghei* ANKA sporozoites and liver parasite load was assessed at 45 hpi by qPCR. The results show that transferring the faecal pellet from RKV-supplemented mice to the non-supplemented mice cage did not lead to an impairment of liver parasite infection, showing that any RKV-dependent modelling of the microbiota is not related or responsible for the reduction in liver parasite load typically observed upon RKV supplementation (Fig. 4.12).

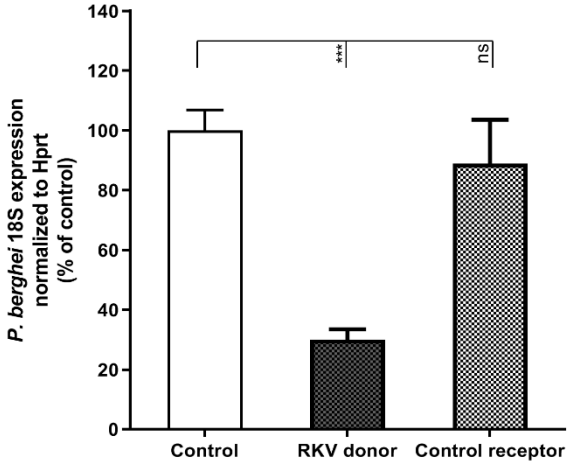

Figure 4.12 – Modulation of microbiota is not responsible for the reduction in liver parasite load observed upon RKV supplementation. C57Bl/6 WT mice were allowed

to drink control or supplemented water *ad libitum* for 5 weeks. Faecal pellets were collected from a cage in which the mice drank supplemented water (RKV donor) and transferred to a cage in which the mice drank control water (Control receptor), so that these mice contacted with the pellets from the RKV-supplemented mice for 4 weeks. C57Bl/6 mice were infected through i.v. injection of $1.0x10^4$ *P. berghei* ANKA sporozoites and liver parasite load was assessed by qPCR at 45 hpi and plotted as percentage of control. One experiment with 5 mice per group (females). Error bars represent SD. One-way ANOVA with post-test Dunnett. ns - not significant and *** $p < 0.001$.

Discussion

Plasmodium parasites are obligate intracellular pathogens within their mammalian hosts, infecting and replicating inside hepatocytes during the asymptomatic liver-stage of infection and, later, inside RBCs, during the blood-stage of infection, responsible for the symptoms of malaria. Despite its asymptomatic nature, the intrahepatic stage of infection is an obligatory step of *Plasmodium* life cycle and involves a massive multiplication of each parasite, with a 10^4- to 10^5-fold replication of its genome. This remarkable cellular and genomic expansion inside hepatocytes is possible because the parasite takes advantage of the extremely metabolite- and nutrient rich environment of these cells, scavenging nutrients from them[4,47]. One of these nutrients is Arg, whose metabolism into polyamines was defined as crucial for the parasite's intrahepatic development[4].

Arg is becoming increasingly popular in nutritional supplementation for its ability to boost the immune system being, in the context of malaria infection, the only amino acid-based supplementation evaluated. However, its impact on malaria infection and pathology remains difficult to define. In this project, we evaluated the impact of Arg and RKV supplementation, which combines Arg with Lys and Val, defined as arginase inhibitors[129], in *Plasmodium* liver infection of BALB/c and C57Bl/6 mice.

Our results show that Arg supplementation is sufficient to impair liver parasite load in BALB/c mice. This data is in agreement with previous reports showing that Arg supplementation decreases parasitemia and improves survival in BALB/c mice infected with blood-stage *P. yoelli* parasites[6]. Conversely, Arg supplementation is not sufficient to impair *Plasmodium* liver infection in C57Bl/6 mice, which is also in agreement with the previous report that shows that Arg supplementation of C57Bl/6 mice with blood-stage infection does not affect parasitemia nor survival of the mice[73].

In turn, RKV supplementation, which constituted the main object of our study, leads to a striking decrease of *Plasmodium* liver infection of C57Bl/6 mice. However, surprisingly, either an increase or a decrease in liver parasite load was observed upon RKV supplementation of BALB/c mice. Once inside hepatocytes, Arg is mainly catabolized by the arginase pathway rather than the iNOS pathway[53,162,163]. As arginase

and iNOS compete for the same substrate, if most Arg is being metabolized by the arginase pathway into polyamines, it becomes unavailable for iNOS, thereby limiting NO production[164,165,166]. Polyamines (putrescine, spermidine and spermine) are low molecular weight organic cations that are synthesized in all cells but occur in particularly high concentrations in rapidly proliferating cells, such as cancer cells and protozoan parasites[167,168,169]. These molecules and their derivatives have the ability to interact electrostatically with most polyanionic macromolecules in the cells and thereby mediate several important cellular processes, including cellular differentiation and proliferation[170,171,172,173]. Polyamines have been shown to be some of the major metabolites present within malaria parasites, highlighting the importance of these molecules to those rapidly dividing parasites[172,173,174]. In fact, an essential role of polyamines for survival and rapid proliferation of the intraerythrocytic *Plasmodium* parasites has been reported[170,172,173]. Therefore, in this case, the polyamines produced can contribute to parasite growth, which could explain the increase in liver parasite load observed in 50% of the experiments performed, termed RKV phenotype 1. However, in the other 50% of the experiments performed, a decrease in liver parasite load was observed, which we designated RKV phenotype 2. In this case, RKV supplementation might have been effective at blocking polyamine synthesis through the inhibition of arginase activity by Lys and Val, consequently channelling Arg for NO production by iNOS, and contributing to parasite elimination. The reason that different phenotypes observed upon RKV supplementation in BALB/c mice from one experiment to the next remains unknown. We can speculate that the outcome of *Plasmodium* infection in BALB/c mice upon RKV supplementation could reflect the balance between the 2 metabolic pathways, which was, in fact, illustrated in the outcome of many parasitic infections, including *Leishmania*, *Trypanosoma* and also *Plasmodium*[172]. Reciprocal regulatory interactions between these 2 enzymatic systems have been defined[175]. On one hand, arginase inhibits NO production via several potential mechanisms, including: (i) competition with iNOS for the substrate Arg[175]; (ii) uncoupling of iNOS resulting in the generation of NO scavenger, superoxide and peroxynitrite[175]; (iii) repression of the translation and stability of inducible iNOS protein[175]; (iv) inhibition of iNOS-mediated NO production through the generation of urea[175]; (v) by asymmetrical dimethylarginine (ADMA), which is an endogenous inhibitor of iNOS[175]. On the other hand, iNOS can also

influence arginase activity, since N^G-hydroxy-L-arginine, an intermediate formed during the catalysis of Arg by iNOS, was defined as a potent inhibitor of the arginase enzyme[53].

Arg and RKV supplementations also displayed distinct effects on two different mouse strains, BALB/c and C57Bl/6. The Th1/Th2 balance differs in mice with different genetic background, and this balance is extremely important for the outcome of infection[176]. T cells from BALB/c mice favour Th2 cytokine production with low IFN-γ and high IL-4, which facilitates humoral immune responses, whereas those from C57Bl/6 mice preferentially produce Th1 cytokines with high IFN-γ and low IL-4, which provides help for cell-mediated immune responses[177]. Thus, the discrepancies observed in the outcome of infection upon either Arg or RKV supplementation may be due to the difference in the immune response of these mouse strains, since BALB/c and C57Bl/6 mice are respectively regarded as Th2- and Th1-dominant mouse strains[178]. Of significant interest, it was reported that these differences in their immune response were associated with different susceptibility to a variety of pathogens including *Leishmania major*[179], *Trypanosoma cruzi*[180], *Yersinia enterocolitica*[181], *Mycobacterium avium*[182], *Listeria monocytogenes*[183] and also, *Plasmodium*[184,185]. In the context of *Plasmodium* infection, despite overall similarities, BALB/c mice are relatively resistant and C57Bl/6 mice are highly susceptible to liver-stage infection by *P. berghei* sporozoites[186]. Additionally, it was also suggested that *Plasmodium* parasites behave differently within hepatocytes of BALB/c and C57Bl/6 mice[187]. This is supported by the fact that different immune schedules were required to induce protective immunity by gamma-irradiated sporozoites in these mouse strains[188]. BALB/c mice are relatively easy to protect, requiring fewer numbers and doses of irradiated sporozoites to induce a long lived protection against experimental sporozoite challenge[188]. In contrast, C57Bl/6 mice require multiple doses and larger numbers of irradiated sporozoites to induce complete protection, which is short lived[187]. Furthermore, also a different susceptibility to the development of ECM was identified in these mouse strains, having been described that C57Bl/6 mice infected with *P. berghei* ANKA develop ECM symptoms and die, while BALB/c mice infected with *P. berghei* ANKA are ECM-resistant[189,190]. Interestingly, another mouse strain, DBA/2 mice, when infected with *P. berghei* ANKA constitutes a model for malaria-associated acute lung injury (ALI), where the cause of death is respiratory failure and not ECM[191].

In summary, the immune responses to *Plasmodium* infection are mouse-strain dependent, resulting in different outcomes of *Plasmodium* infection.

Overall, these results suggest that the impact of Arg and RKV supplementations is mouse-strain dependent, possibly because they alter the metabolism and/or the immune response differently in each mouse strain. The inconsistent results obtained regarding the effect of RKV supplementation in liver parasite load in BALB/c mice, led us to abandon the first aim of the thesis and proceed only with the objective of clarifying the mechanism of RKV-mediated parasite elimination in C57Bl/6 mice.

The second aim of this project was to elucidate the mechanism of hepatic parasite elimination in C57Bl/6 mice upon RKV supplementation. We started by assessing the role of the two most obvious mechanisms, NO production and type-I IFN response, both described as involved in controlling malaria infection[64,128,133,192]. However, both mechanisms were excluded, suggesting that an unknown mechanism would be involved. Thus, since parasites are being eliminated, we wondered whether the immune system could be involved in the process of inhibition of *Plasmodium* hepatic infection by RKV supplementation. In fact, it was demonstrated that the typical reduction in the liver parasite load observed in RKV-supplemented mice is abolished by lethal mouse irradiation, a process that almost completely destroys the immune system of the mice, suggesting that the immune system is implicated in the mechanism of parasite elimination by RKV supplementation.

As the liver-stage of infection takes only about 50 h in mice, there is no time to mount an adaptive immune response. Therefore, we started by assessing the involvement of myeloid cells in the process of parasite elimination by RKV supplementation, the main cells of the innate immune compartment. Employing MyD88$^{-/-}$ mice, we found that the absence of MyD88 signalling completely abolished the reduction in liver parasite load, supporting that parasites are being eliminated by the host's immune system and that MyD88 signalling is involved in this parasite elimination.

MyD88 is a molecule that is very important for the signalling and function of the myeloid cells[136]. However, the most abundant myeloid cells in the blood or organs, which include macrophages, neutrophils and monocytes, were excluded from being involved in

the mechanism of hepatic parasite elimination by RKV supplementation. Additionally, the involvement of IL-1α, a potent cytokine involved in neutrophils recruitment, was also excluded, in agreement with the exclusion of neutrophils from an involvement in the mechanism of RKV-mediated hepatic parasite elimination. Nevertheless, the role of eosinophils, basophils, mast cells and DCs was not elucidated. Although DCs can be theoretically depleted *in vivo* through administration of clodronate-filled liposomes, we did not confirm DC depletion upon clodronate administration, which implies that the involvement of these cells in the mechanism of RKV-mediated parasite elimination cannot be definitively excluded. However, although an involvement of eosinophils and basophils in the control of *Plasmodium* infection has not reported so far, such a role has been defined for mast cells and DCs[193,194,195,196]. Mast cells contribute to parasite clearance and TNF production in rodent malaria, which appears to have a role in promoting innate immune activation since they produce fms-like tyrosine kinase 3 (Flt3) ligand during blood-stage malaria infection in mice, an essential growth factor for DCs[193,194], which induces DCs proliferation[194]. These cells were shown to be efficient in the phagocytosis and phagosomal maturation of infected erythrocytes[195]. Also, plasmacytoid DCs (pDCs) are activated through TLR9-MyD88 signalling pathway by infected erythrocytes, which promotes γδ T cell proliferation and IFN-α production[196]. Together, these highlights the key role of mast cells and DCs in immunoregulation and immunopathogenesis of blood-stage malaria infection[196]. In the future, the role of these myeloid cells, and specially mast cells and DCs, in parasite elimination by RKV supplementation should be further investigated.

The fact that our results excluded the most abundant myeloid cells from playing a role in the RKV-mediated inhibition of *Plasmodium* liver infection suggested that RKV-dependent parasite elimination is not dependent on one or more of the cell populations from this branch of the immune system. However, MyD88 signalling seems to be essential for hepatic parasite elimination by RKV supplementation. MyD88 is the canonical adaptor for inflammatory signalling pathways downstream of members of the TLR and IL-1 receptor families, which includes the interleukin-1 receptor (IL-1R) and interleukin-18 receptor (IL-18R)[108]. MyD88 plays an important role in the activation of AIM2 and NLPR3 inflammasome[107,197]. Once activated, these inflammasomes are responsible for activating caspase-1[107]. In its turn, caspase-1 will cleave pro-IL-1β and

pro-IL-18 into the active cytokines, IL-1β and IL-18, which propagate the inflammatory response and, under certain conditions, can induce pyroptosis[107]. Of significant interest, AIM2 inflammasome was reported to be inducible by type-I IFN signalling, a mechanism which was excluded to be involved in RKV-mediated hepatic parasite elimination. Therefore, both inflammasomes, but particularly the NLRP3 inflammasome, may be activated through MyD88-dependent signalling which consequently leads to hepatic parasite elimination, through an unknown mechanism. Recent reports highlighted the important role of the NLRP3 inflammasome in host response to the pathogenesis of infections caused by intracellular protozoan parasites, such as *Leishmania*, *Trypanosoma cruzi*, *Toxoplasma gondii* and also, *Plasmodium*[198]. It was described that NLRP3 inflammasome was, in fact, activated during blood-stage malaria infection[198]. However, the specific activities of this inflammasome and the consequences of its activation during malaria infection remain unclarified. Of interest, it has been suggested that the phagocytosis of infected erythrocytes effectively participates in protective immunity against malaria while generating the inflammasome-mediated response, which strongly supports a physiologic role for the inflammasome during blood-stage malaria infection[199].

Although most lymphoid cells belong to the adaptive branch of the immune system, some of them, specifically NK cells and ILCs, belong to the innate immune system. Thus, these cells could be involved in the mechanism of RKV-dependent parasite elimination. Employing a mouse strain that lacks only the lymphoid cells belonging to the adaptive immune system ($Rag2^{-/-}$ mice, which lack B cells, T cells, γδ T cells and NK T cells) and another mouse strain that lacks all the lymphoid compartment ($Rag\gamma c^{-/-}$, which also lack NK and ILCs), we were able to reduce our candidate cell populations to NK and ILCs. However, the involvement of NK cells in the mechanism of hepatic parasite elimination upon RKV supplementation was also excluded through the administration of anti-NK1.1 antibody, which depletes these cells *in vivo*. Thus, only the role of ILCs remains to be clarified.

ILCs are a family of immune cells that have been classified into 4 groups: (i) ILC1s, which comprise NK cells and cells that produce type 1 inflammatory cytokines, particularly IFN-γ and TNF-α; (ii) ILC2s, which comprise cells that produce type 2 cytokines, such as GM-CSF, IL-3, IL-4, IL-8, IL-9, IL-13, IL-21; (iii) ILC3s, which

comprise cells that produce IL-17A with or without IL-22; (iv) and, ILC4s, which comprise cells that produce IL-17 and IFN-γ. ILCs play crucial roles in protection against pathogens. Of note, the involvement of IL-17 in the mechanism of RKV-mediated parasite elimination was also experimentally excluded, restricting the possible candidates to the ILC1 and ILC2 subsets. In fact, the roles of ILC1s and ILC2s in protective immunity to *Toxoplasma gondii* and *Schistosoma*, respectively, have already been described [204,201,206]. Importantly, a recent report has showed that IL-33 protects the mice against ECM by inducing a population of ILC2s, that produce type 2 cytokines (IL-4, IL-5 and IL-13)[203]. This leads to the polarization of the anti-inflammatory M2 macrophages, which in turn, expand Tregs, and all these cells combined decrease the inflammation levels and, therefore, prevent ECM[203]. In this way, the role of ILCs as immune effector cells in the mechanism of action of RKV supplementation has to be clarified. For this, we will employ mice lacking transcription factor nuclear factor, IL-3, known as Nfil3 or E4BP4 (Nfil3$^{-/-}$ mice), which is a key factor for the development of all ILC subsets[204].

Taken together, our results suggest a role of the immune system in the mechanism of RKV-dependent parasite hepatic elimination, possibly through the action of liver ILCs. MyD88 signalling is essential for parasite elimination, however, the cell in which this signalling is occurring remains unidentified, as well as, which are the consequences of this signalling. Alb.Cre-MyD88$^{flox/flox}$ and LysM.Cre-MyD88$^{flox/flox}$ mice, which lack MyD88 in hepatocytes and myeloid cells, respectively, will be employed to identify whether MyD88 is being activated in the hepatocytes and to definitely exclude a role for myeloid cells in the mechanism of RKV-dependent parasite elimination.

We propose 2 different models that can explain the mechanism in which RKV supplementation leads to parasite elimination in C57Bl/6 mice:

(i) In the first model, stimulation of TLRs, possibly the endosomal TLRs (TLR3, TLR7/8 or TLR9[205]), leads to activation of MyD88 signalling, which occurs in the infected hepatocytes (Fig. 5.1, Model 1, steps 1 and 2), and leads to inflammasome activation (Fig. 5.1, Model 1, steps 3 and 4), possibly the NLRP3 inflammasome. Additionally, ILCs, whose activation may or may not be MyD88-dependent (Fig. 5.1, Model 1, step 5), can directly or indirectly lead to the elimination of infected cells, through the production of cytokines or chemokines (Fig. 5.1, Model 1, step 6).

(ii) In the second model, stimulation of TLRs, IL-1R or IL-18R leads to activation of MyD88 signalling only in the ILCs (Fig. 5.1, Model 2, steps 1 - 4) that were recruited by the infected hepatocytes through an unknown mechanism (Fig. 5.1, Model 2, step 5), leading to their activation. These consequently results in parasite elimination by ILCs, directly or indirectly through the production of cytokines or chemokines (Fig. 5.1, Model 2, step 6).

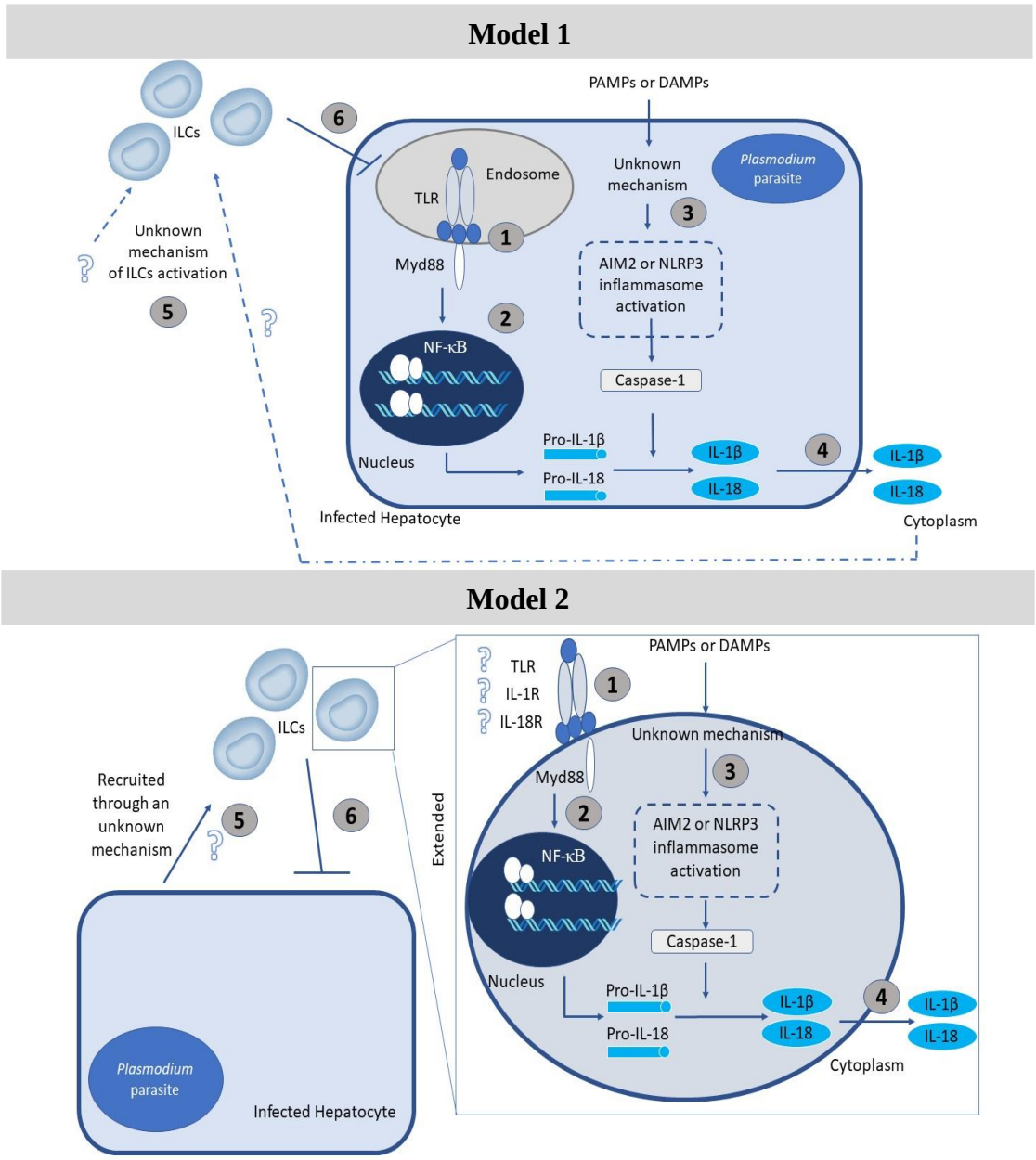

Figure 5.1 – Two models proposed to explain the mechanism of action of RKV supplementation.

Ultimately, we expect that this project will provide new insights into the biology of the host-parasite interactions at play during liver-stage infection by malaria parasites, new knowledge in how these interactions can be manipulated and pave the way for the development of novel intervention strategies against this disease.

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