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Departamento de Biologia Animal

Role of macrophages during the malaria liver stage

Dissertação

Francisco Miguel de Oliveira Barbosa Vidal

Mestrado em Biologia Humana e Ambiente

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Dissertation supervised by

Prof. Doutor Peter Liehl, PhD (IMM)

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Acknowledgments

This last year was anything but perfect. The initial project I had been accepted in was cancelled due to bureaucracy and financial issues which I had nothing to do with, so I ended up developing my thesis on an unplanned scientific area, which defeated the purpose of doing the master course in the first place. A few months after being accepted in an excellent group I realized again the whole thing wasn't going to work as well as I expected despite the circumstances, because I still was frustrated and because I refused to follow the 'rules'.

Sadly, now more than ever I realize education in this country is just a business like pretty much everything else, and that a scientific career is often built on too much dishonesty, greed and ineptitude to change what is wrong. I don't even see the point of having a degree anymore, master or otherwise, but I'm hoping to be proven wrong eventually. It seems that knowledge, the skills and everything else is useless unless you are really lucky, you know someone at the right place, or you don't mind being trampled by the so called 'professionalism' of this unprofessional career. I guess it's safe to say I probably won't do any more scientific research in this country unless things change significantly.

Nevertheless this is the acknowledgements section, and I obviously have to thank a few people. I would like to thank my group leader Maria Mota for the opportunity and the pep talks and to both my lab supervisor Peter Liehl and Vanessa Luis for teaching me a lot of technical stuff despite the unpleasantness here and there. Thanks to everyone else from the group that helped me in any way. Thanks to prof. Teresa Rebelo for the support and suggestions. Thanks to my classmates, especially Francisco Mouro and Filipa Brito, for their friendship throughout the last two years. A special thank you goes out to Gonçalo Ramos, my partner in crime, for giving me the faith to go on when I just wanted to quit. I also wish to thank my brothers and sisters Joana Sousa, João Jorge, Marco Granada, Sílvia Santos and Vera Silva for keeping the insanity at bay, as always. Last but not least I have to thank my father for the invaluable financial support.

Education is an admirable thing, but it is well to remember from time to time that nothing that is worth knowing can be taught.

Oscar Wilde

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Sumário

A infecção por malaria começa com uma picada de um mosquito *Anopheles* infectado, através da transmissão de esporozoítos do protozoário pertencente ao género *Plasmodium*. Uma vez na circulação sanguínea, os esporozóies chegam rapidamente ao fígado onde atravessam a camada de células constituída por células endoteliais e células de Kuppfer (macrófagos residentes do fígado), acabando por alcançar e infectar os hepatócitos do fígado. Uma vez dentro destas células, cada um dos merozoítos inicia uma série de ciclos replicativos até se diferenciar numa nova forma parasitária chamada merozoíto. São estes merozoítos que uma vez libertados para a corrente sanguínea, vão infectar eritrócitos e desencadear a fase simptomática da doença.

O principal objectivo deste trabalho é estudar o papel dos macrófagos durante a fase hepática da infecção por malária, adoptando a hipótese de que as células de Kupffer são suprimidas após infecção pelos esporozoítos. Para isso usou-se *Plasmodium berghei* ANKA obtido por dissecação de glândulas salivares de mosquitos *Anopheles* infectados, bem como o modelo murino C57BL/6 (espécie *Mus musculus*). A análise foi efectuada com recurso a microscopia confocal e ao nível do RNA através do uso de extractos de fígado de murganhos infectados e não infectados.

Os nossos resultados sugerem que murganhos desprovidos de macrófagos induzem após infecção por esporozoítos, um recrutamento massivo de macrófagos para o fígado, bem como uma expressão genética substancial de genes pró-inflamatórios. Também se verificou que esse recrutamento de macrófagos para o fígado poderá ser desencadeado por substâncias presentes nas glândulas salivares de mosquitos e não por esporozoítos.

Palavras-chave: *Plasmodium*, malária, esporozoítos, fígado, células de Kupffer

Abstract

Malaria infection starts with the bite of an infected *Anopheles* mosquito. *Plasmodium* sporozoites, the parasite form transmitted by mosquitos, are first deposited in the skin of the vertebrate host. After entering circulation sporozoites rapidly reach the liver, cross the sinusoidal cell layer composed of endothelial cells and Kupffer cells (the resident macrophages of the liver) and infect hepatocytes. Inside these cells the parasites replicate and develop into thousands of new parasites, called merozoites. When released into the blood stream, merozoites infect red blood cells causing the symptomatic stage of the disease.

The main goal of this project is to study the role of macrophages during the liver stage of malaria infection, under the hypothesis that Kupffer cells are suppressed upon sporozoite infection. To this end we used the rodent model parasite *Plasmodium berghei* ANKA, which was obtained from the dissection of salivary glands of infected *Anopheles* mosquitos, and the rodent host C57BL/6 mice (species *Mus musculus)*. The analysis was performed at the confocal microscopy level as well as at the RNA level using liver extracts of infected and non-infected animals. Genes of interest were screened and analyzed by qRT-PCR.

Our results suggest that macrophage depleted mice induce upon sporozoite infection a massive recruitment of macrophages to the liver blood vessels and a substantial expression of pro-inflammatory genes. In addition, the recruitment of macrophages to the liver seems to be triggered by components present in salivary gland material of mosquitos rather than by sporozoites.

Keywords: *Plasmodium*, malaria, sporozoites, liver, Kupffer cells

Abbreviations

cDNA: complementary DNA

Clo-lip: clodronate liposomes

DAPI: 4',6-diamidino-2-phenylindole

$_{d}$H20: diethylpyrocarbonate-treated water

DMEM: Dulbecco's modified Eagle's medium

DNA: desoxyribonucleic acid

Hprt: hypoxanthine-guanine phosphoribosyltransferase

LPS: lipopolysaccharide

mRNA: messenger RNA

PBS: phosphate Buffer Saline

PCR: polymerase chain reaction

PBS-lip: PBS liposomes

qRT-PCR: quantitative RT-PCR

RNA: ribonucleic acid

RT-PCR: real time PCR

TRL: toll-like receptor

µg: microgram

µl: microliter

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Introduction

Historic background

Malaria is a disease that despite all the scientific and humanitarian efforts towards its eradication remains the 21st century world's most deadly vector-borne infectious disease.

References to conditions that bear several of the hallmarks of malaria such as fevers and splenomegaly have been quoted from Chinese records (5.000 years ago), Egyptian texts (3,500 to 4,000 years ago) and Indian scriptures (3,500 to 1,900 years ago). Around 2,000 years later, in 500 B.C. malaria had already appeared in Greek texts, and later in the works attributed to Hippocrates (460 to 377 B.C.) there is reference to malaria's benign tertian and malignant subtertian forms. In 323 B.C., beyond Mesopotamia on the route to India, Alexander the Great is said to have died of malaria. By the beginning of the Christian era, malaria was widespread around the Mediterranean, in southern Europe and southern Asia and it probably began to spread into northern Europe in the dark and middle ages. Malaria seems to have coincided with human pioneering and land clearing for agriculture. In fact, during the time of the voyages of Columbus until the mid-19th century, European trade and colonization in the tropics were marked by massive losses of life from infectious disease. It was around this time malaria reached its global limits and exacted its highest toll of sickness to the point of killing 1 in 10 affected people. Then, from toward the end of the 19th century, throughout North America and Northern and Western Europe, malaria started to decline toward its present extinction in these regions (Carter and Mendis, 2002).

The first malaria parasites were discovered in 1880 in the blood of malaria patients by Alphonse Laveran. Seventeen years later, William MacCallum discovers the blood stages in birds infected with a related haematozoan, *Haemoproteus columbae*. In the same year, Ronald Ross elucidates the whole transmission cycle in culicine mosquitoes and birds infected with *Plasmodium relictum*. In 1898 a group of Italian malariologists demonstrated that human malaria was also transmitted by mosquitoes, in this case anophelines. Henry Shortt and Cyril Garnham discovered that malaria parasites developed in the liver before entering the blood in 1948, and the final stage in the life cycle, the presence of dormant stages in the liver, was demonstrated in 1982 by Wojciech Krotoski (reviewed in Cox, 2010).

Epidemiology and global distribution

The disease is typically transmitted to people by mosquitoes belonging to the genus *Anopheles*, although in rare cases it can be contracted through contaminated blood or through pregnancy to the fetus when the mother is infected. Since these mosquitoes belong to one of the most abundant arthropod populations in the world, their role as vectors for the parasite constitutes one of the main reasons for malaria's prevalence. *Anopheles* mosquitoes breed in water and while some species feed on both humans and other mammals, the primary malaria vectors feed almost exclusively on humans. With more than 20 different *Anopheles* species in nature acting as malaria vectors, the transmission essentially depends on climatic conditions such as humidity and temperature that may not only affect the survival of mosquitoes but the parasites as well. Human immunity is also an important factor since it can provide a moderate protection from severe disease if it's developed throughout the years. For this reason, the specific risk groups include young children, semi or non-immune pregnant women, people with HIV/AIDS and travelers from non-endemic areas (WHO, 2010. NIAID Science Education, 2007. Matuschewski, 2006).

At the moment malaria is a life threatening risk for 3.3 billion people over 100 countries in the Asian, South American and African continents (**Fig.1**). Surprisingly malaria mortality rates have fallen by more than 25% since 2000, with the largest percentage reductions seen in the European (99%), American (55%), Western Pacific (42%) and African Regions (33%). Out of 99 countries with ongoing malaria transmission, 43 recorded decreases of more than 50% in the number of malaria cases between 2000 and 2010. Another 8 countries recorded decreases of more than 25%. The European Region could actually be the first to eliminate malaria in the next few years, since almost all remaining malaria cases in 2010 were reported from just two countries, Azerbaijan and Tajikistan and case numbers are continuing to fall in both countries (WHO, 2011). Moreover, malaria has a huge impact on the affected countries' economy, being annually responsible for the reduction of the gross domestic product of as much as 1.3% in countries with high levels of transmission, and has consequently lead to socioeconomic discrepancies between affected and non-affected countries (WHO, 2010).

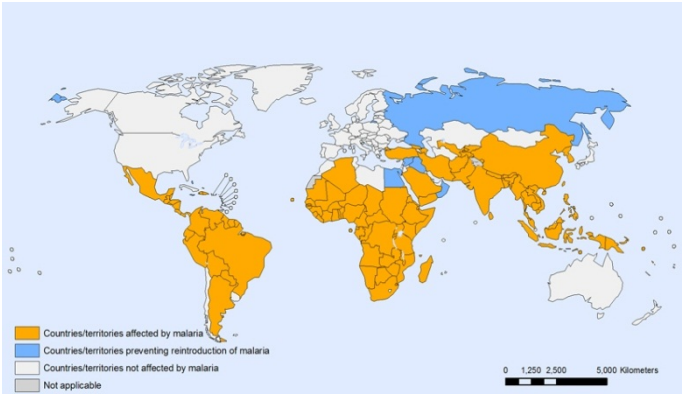

Figure 1: Countries and territories affected by malaria in 2010 (WHO, 2012).

Control strategies

There are different approaches regarding the control of malaria, including the development of affordable and efficient malaria vaccines, chemotherapy treatment with anti-malarial drugs, and development of insecticides to prevent the vector from spreading. Chloroquine and dichlorodiphenyltrichloroethane were used for several years as control methods for the parasite and the vectors respectively, but resistance eventually emerged, rendering those drugs ineffective. Nowadays, significant advances have been made in vector control strategies and especially in parasite control with artemisinin based combination therapy. However, a licensed malaria vaccine is still not available (Crompton *et al*, 2010).

Clinical pathology

Clinical manifestations of malaria infection are induced by the asexual stages of the parasite, when it is developing inside the red-blood cells and during sequestration. This phenomenon occurs when the parasites bind to the capillary and venous endothelium in various organs. The main symptoms often encountered in malaria are periodic fever, abdominal discomfort, headache, chills, joint muscle aches, vomiting and fatigue. The periodic fever is commonly named tertian and quartian malaria, which refer to the typical feature of an acute febrile episode that returns every three or four days. The major complications of severe malaria include splenomegaly, acute renal failure, bleeding, severe anemia, pulmonary edema, metabolic disorders (acidosis and hypoglycemia), cerebral malaria and placental malaria (reviewed in Trampuz *et al*, 2003).

The parasite

Malaria parasites have probably been so almost since there were potential hosts, at least half a billion years ago. At an early stage their evolution they acquired an asexual and usually intracellular form of reproduction called schizogony thus increasing their proliferative potential. Their ancestor is believed to have been a chloroplast-containing, free-living protozoan. This ancestor would become adapted to live in the gut of certain aquatic invertebrates, more specifically aquatic insect larvae including those of early Dipterans. As it is shown in **Fig.2**, these insects first appeared around 150 million to 200 million years ago, and since then many different lines of malaria and malaria-like parasites evolved and radiated (Carter and Mendis, 2002).

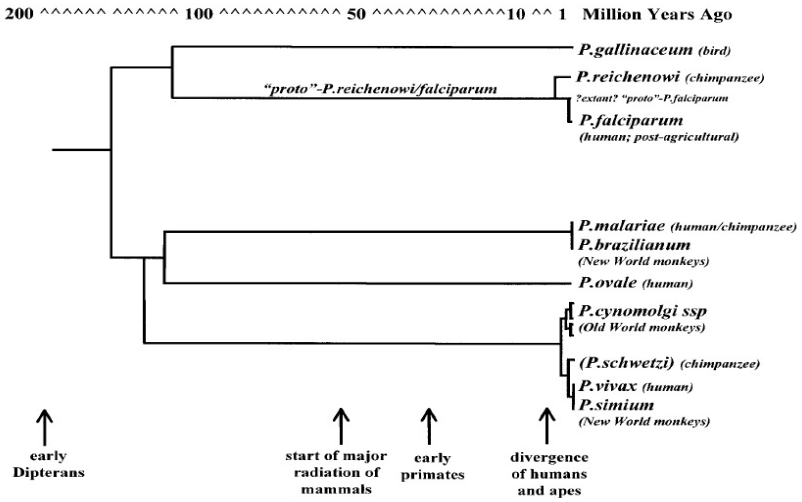

Figure 2: Phylogeny of the malaria parasites of humans and other species (Carter and Mendis, 2002)

Malaria parasites are protozoans that belong to the *Plasmodium* genus and to the Apicomplexa phylum in which we can find other species such *Cryptosporidium parvum* and *Toxoplasma gondii*. (Kim and Weiss, 2004) This phylum is characterized by having a plastid organelle; the apicoplast (**Fig.3**).The apicoplast is prokaryotic in origin and derived from endosymbiosis of a plastid bearing red algae. During the course of evolution, it lost its photosynthetic function and in *Plasmodium spp* it is important for both intraerythrocytic and intrahepatic development in the human host (Yeh and DeRisi, 2011).

There are more than 100 species of Plasmodium which can infect several animal species such as birds, reptiles and many mammals. Nevertheless, they have an amazingly restricted host range infecting only related vertebrate species. Only four species of *Plasmodium* infect humans; *P. falciparum, P. ovale, P. malariae* and *P. vivax* (Cowman and Kappe, 2006). However, it has been reported that humans have also been infected by a fifth species, *P. knowlesi*, which is a natural parasite of long-tailed and pig-tailed monkeys from south-east Asia. It has also been discovered that *P. falciparum* can parasite a few species of primates such as gorillas and chimpanzees (reviewed in Antinori *et al*, 2012).

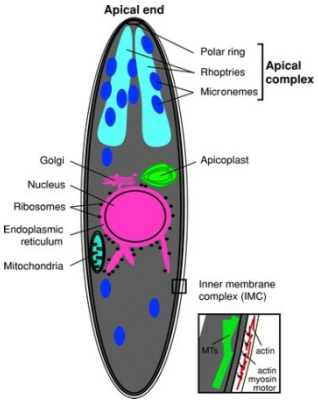

Figure 3: Schematic of a *Plasmodium spp* sporozoite with an apicoplast (green) (Kappe *et al*, 2004).

Regarding human infection, *P. malariae* is frequently clinically silent, although it can develop a chronic infection. Although rarely fatal, *P. vivax* and *P. ovale* are usually just responsible for febrile illness that can result in anemia. *P. falciparum* is responsible for the majority of the cases of severe disease and deaths. Every species show evidence of recurrence after treatment with the exception of *P. ovale* (reviewed in Schofield and Grau, 2005).

Relatively to the two most important human *Plasmodium* species' distribution, the global area at risk of *P. falciparum* malaria is distributed between America (20.30%), Africa (61.10%) and Asia (18.60%) while the people at risk of *P. vivax* transmission worldwide are distributed with different percentages with the vast majority (91%) inhabiting in Asia, 5.5% living in America and 3.4% living in Africa (Guerra *et al*. 2010. Hay *et al*. 2009).

The life cycle

Plasmodium's life cycle is very complex, cycling between an invertebrate and a vertebrate host, and it is transmitted to humans when infected female *Anopheles* mosquitoes inject elongated parasite forms called sporozoites during blood meals in order to carry out their egg production (**Fig.4**) (Ponnudurai *et al.* 1990). The survival of the malaria parasite in the mosquito depends on several factors, two of them being the atmospheric conditions such as temperature and humidity, and whether the *Anopheles* survives long enough to allow the parasite to complete its cycle in its gut. The cycle is comprised of both sexual and asexual stages.

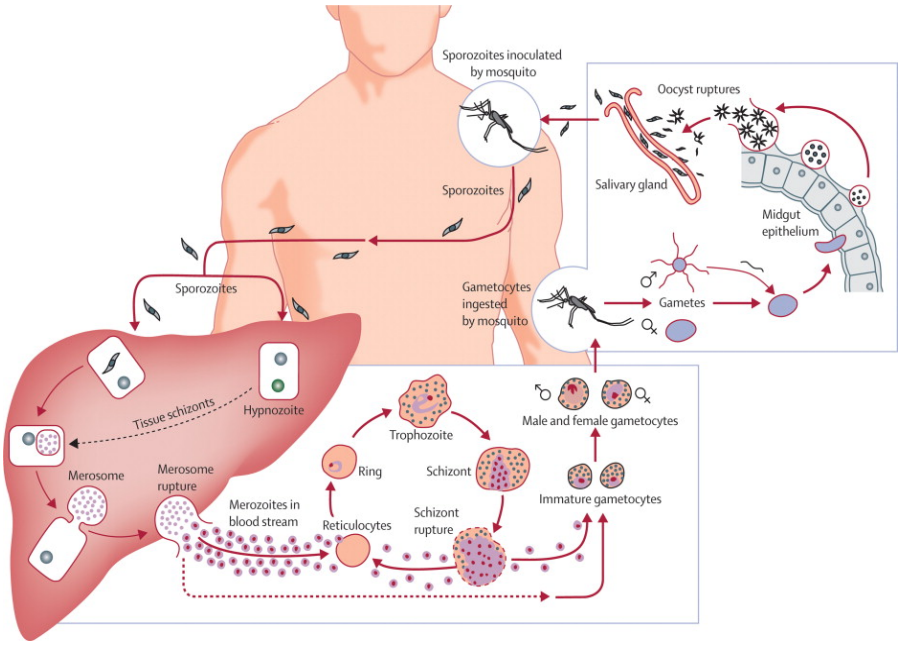

Figure 4: Plasmodium spp life cycle (Mueller *et al.* 2009)

The sexual stage

This stage occurs inside the mosquito and starts with the uptake of the male and female gametocytes during a blood meal. After the gametocytes fusion, the motile diploid zygotes are formed. They then endure meiosis and genetic recombination in order to originate the motile ookinetes which migrate through the mosquito midgut epithelium and start differentiating into sessile oocysts, the only extracellular stage of the parasite. When fully matured, each oocyst undergoes several mitotic divisions to form sporoblasts from which thousands of sporozoites start budding and enter the hemocoel. Afterwards, the sporozoites migrate into the mosquito's salivary glands and

gain access to the salivary duct where they accumulate and finish their maturation (Aly *et al*. 2009 and reviewed in Frevert *et al.* 2006). Sporozoite migration to the salivary glands is mediated by the circumsporozoite protein, a major surface protein of the parasite. This protein can be found on the sporozoite's surface and not only presents homology with other apicomplexan parasite proteins but is also highly conserved between the different species (Myung, 2004). The mature sporozoites are then injected into the mammalian host when the infected mosquito takes it next blood meal, starting the asexual stage of the parasite's life cycle. Although the salivary glands of an infected mosquito can contain thousands of sporozoites, less than 100 of these are transmitted in any one bite (Rosenberg *et al*. 1990).

The asexual stage

This stage includes a pre-erythrocytic stage, which is the first stage of infection in humans where sporozoites are inoculated to infect the hepatocytes, and an erythrocytic stage, which is the asexual reproduction of the parasite in the blood that causes the clinical symptoms of the disease.

The pre-erythrocytic or liver stage begins when sporozoites are deposited in the avascular dermal tissue of a vertebrate mammalian host where they can stay between 1 to 3 hours. The injected sporozoites start to move by gliding motility until they reach a dermal blood capillary from where they are simply transported by circulation since they are not competent to infect erythrocytes in a direct way. However, some sporozoites may enter the lymphatic nodes via the lymphatic vessels, where they are eliminated by dendritic cells. Others are simply destroyed by phagocytes while in the dermis (Amino *et al.* 2006). They rapidly reach the liver and they cross the liver sinusoidal cell layer traversing several hepatocytes, before invading a final hepatocyte. There, they form a parasitophorous vacuole, where they undergo a cycle of asexual replication for a period of a minimum of 5.5 days for the human malaria species. The migration through several hepatocytes leaves the membranes disrupted and although the cells can repair the damage and survive, death occurs in some cases. This migration also seems to be important for infection since it activates sporozoites for the infection itself and increases the susceptibility of host hepatocytes (Mota *et al.* 2001, Doolan and Good, 1999). Inside de parasitophorous vacuole each sporozoite differentiates into an exoerythrocitic form that grows and multiplies by schizogony into thousands of erythrocyte-infective merozoites,

without killing the host cell. After maturation, the merozoites are released in the liver sinusoid where they enter bloodstream, marking the end of the asymptomatic pre-erythrocytic stage (reviewed in Vaughan *et al*. 2008 and Sturm *et al*. 2006). In *P. vivax* and *P. ovale* a dormant form that does not undergo asexual replication, called hypnozoite, can persist in the liver and can cause disease relapses by invading erythrocytes weeks or years later (reviewed in Markus, 2011. Durante *et al.* 2003).

The erythrocytic or blood stage starts when the merozoites invade the erythrocytes with the formation of a parasitophorous vacuole. After several rounds of invasion, growth and division the merozoites develop into three distinct morphological stages: the ring stage forms, the trophozoites and the erythrocyte schizonts, leading to the formation of new merozoites. When fully matured, these merozoites rupture the host cell and start invading other blood cells in order to perpetuate the cycle (**Fig.5**). This process is gradually amplified by repeated cycles of invasion, intracellular growth, multiplication and re-invasion, which differs from 24 to 72 hours depending on the *Plasmodium spp* (reviewed in Silvie *et al*. 2008 and reviewed in Bannister and Mitchell, 2003).

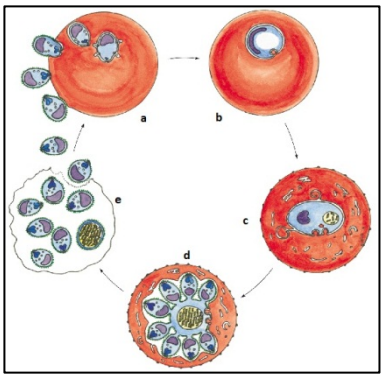

Figure 5: Detailed schematic of *Plasmodium spp* blood stage with blood cell invasion (**a**), ring form (**b**), trophozoite (**c**), schizont (**d**), and merozoites release (**e**) (adapted from Bannister and Mitchell, 2003).

Innate immunity and macrophages

Human immunity comprises innate and adaptive responses that collaborate together in order to destroy pathogens. The innate immune system employs cells that include monocytes/macrophages, dendritic cells, mast cells, natural killer cells and neutrophils to provide immediate defense against infection. These cells are then responsible for initiating adaptive immune responses, for example via antigen presentation or the release of cytokines.

These innate immune cells are able to recognize pathogen associated molecular patterns. These patterns can be components of microorganisms such as lipopolysaccharide (LPS), lipoproteins, bacterial desoxyribonucleic acid (DNA) and viral ribonucleic acid (RNA). They also recognize endogenous ligands released by damaged or necrotic host cells via their pattern-recognition receptors, which include receptors for bacterial carbohydrates and toll-like receptors (TLRs). It is the interaction between these elements that results in targeted destruction of foreign organisms, infected or tumor cells, by the release of cytotoxic agents or phagocytosis (reviewed in Liaskou *et al*, 2012).

Even though all macrophages are ultimately derived from the bone marrow, they may propagate at the site of their final destination. They are generally grouped in subpopulations with different functions depending on the type of activation. The classically activated macrophages develop in response to *Ifn-γ* and microbial products. They can be identified through their ability to present antigens, produce pro-inflammatory cytokines and by their increased endocytic functions. The alternatively activated macrophages are responsible for secreting chemokines, recruit eosinophils, basophils and T cells to the site of infection and promote anti-inflammatory immune responses (Martinez, 2011).

Macrophages are important in both cell mediated and humoral mechanisms against malaria infection. In fact, as some studies have shown, they can secrete factors such as pro-inflammatory cytokines and release nitric oxide or reactive oxygen species. On the other hand, data from further studies have shown that their functions appear to decrease during malaria infection, more specifically by the production of hemozoin generated after hemoglobin degradation by the parasite, which impairs both antigen presentation and immunomodulatory functions of the macrophages (Schwarzer *et al*, 1998).

The Kupffer cells

These liver macrophages are named after the pathologist C. von Kupffer who was the first to identify this non-parenchymal cell type. They are recruited from the stem cells of the bone marrow and mature through differentiation under the influence of specific signals such as interleukin-3 and macrophage colony stimulating factor, until they reach the liver sinusoids (**Fig.6**) (reviewed in Decker, 1990).

Kupffer cells are the largest and functionally most important population of fixed tissue macrophages of the body (Phillips *et al*, 1987). However, there is evidence that they can migrate along sinusoidal walls with a mean speed of 4.6 microns/min. (McPhee *et al*, 1992). They can be found in the periportal area of the lobule (43%) as well as in the midzonal areal (28%) and in the central area (29%). They account for 15% of the total liver cell population, being constantly in contact with a perfusion of oxygenated arterial blood (20%) and venous blood rich in nutrients and bacterial endotoxins (80%) (reviewed in Liaskou *et al*, 2012).

They are involved in liver metabolism, homeostasis, innate immune defense, liver injury and portal vein tolerance (continuous inflammatory response with anti-inflammatory cytokines, as well as pro-inflammatory responses) (reviewed in Frevert *et al*, 2006). In fact, aided by their strategic position in the liver sinusoid (**Fig.7**), these resident phagocytes efficiently clear the bloodstream from foreign substances such as microorganisms and toxic agents since they are the first cells to be exposed to materials absorbed from the gastrointestinal tract (Klotz and Frevert, 2008. Kolios *et al*, 2006).

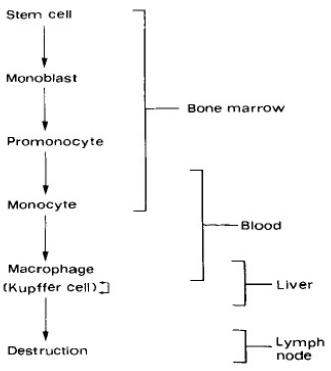

Figure 6: Maturation of Kupffer cells (Decker, 1990).

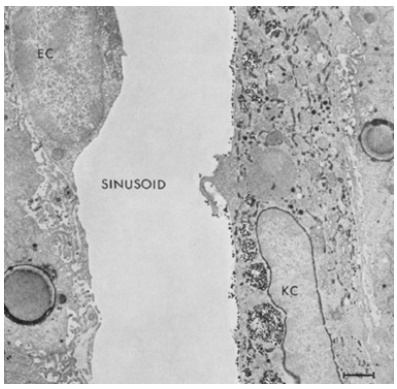

Figure 7: Electron micrograph (x9800) of a hepatic sinusoid, a Kupffer cell (KC) and an endothelial cell (EC) (adapted from Widmann *et al*, 1972).

During local liver injury or infection, resident Kupffer cells and monocyte/macrophages initiate an immune response. Upon phagocytosis of the pathogenic material, phagocytes release a variety of chemical messengers such as tumor necrosis factor alpha (*Tnf-α*), interleukin-1 (*Il-1*), and interleukin-6 (*Il-6*) that initiate the acute-phase response and inflammation (reviewed in Liaskou *et al*, 2012).

Kupffer cells can also phagocyte damaged, senescent or even parasitized red blood cells during malaria infection. It is believed that this extensive erythrophagocytosis leads to an increased physical load of red blood cells in the cytoplasm of Kupffer cells, to macrophage toxicity caused by the parasite or to an excessive iron and free radicals release after hemoglobin is broken down. Ultimately, Kupffer cell's organelle motion is decreased, compromising the host defenses (Bellows *et al*, 2010).

As it is shown in **Fig.8**, to infect hepatocytes sporozoites must cross the sinusoidal cell layer through Kupffer cells and the space of Disse. Moreover, they start to accumulate in the hepatocytes through the interaction of circumsporozoite protein with heparin sulfate proteoglycans, which are present in the basolateral pole of the liver cells (reviewed in Frevert *et al,* 2006. Silvie *et al* 2008). It was proposed that the parasites could pass through the fenestrations on the liver endothelial cells, but it was shown the gaps were not wide enough (Mota, 2002).

In addition, osteopetrosis mutant mice, which are deficient for macrophages including Kupffer cells, are significantly more resistant to sporozoite liver infection (Klotz and Frevert, 2008). These data further support the hypothesis that Kupffer cells are used by sporozoites to enter hepatocytes.

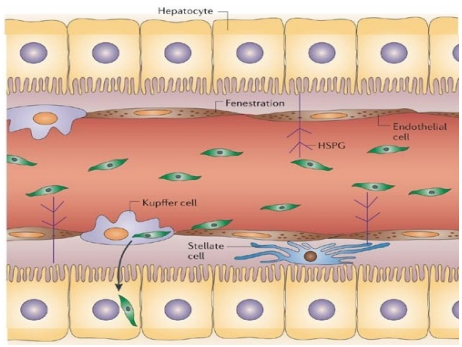

Figure 8: Sporozoites (green) traversing through Kupffer cells as they reach the liver sinusoids (adapted from Prudêncio *et al.* 2006).

Another *ex vivo* study, showed that Kupffer cells from mice challenged with irradiated sporozoites are able to induce the expression of pro-inflammatory cytokines, Major Histocompability Complex class I and co-stimulatory molecules. In contrast, their expression was suppressed in mice infected with infectious live sporozoites compared to naïve mice (Steers *et al,* 2005). Thus, by suppressing the pro-inflammatory response and Major Histocompability Complex class I expression, the parasites down-modulate the antigen presenting cell function of these macrophages and avoids recognition by the immune system. Strikingly, live sporozoites are not eliminated inside Kupffer cells (reviewed in Frevert *et al,* 2006). In fact, sporozoites seem to have mechanisms to suppress Kupffer cell activation. An *in vitro* study by Usynin *et al* showed that the circumsporozoite protein, which is a constitutively secreted protein by sporozoites, blocks the production of reactive oxygen species – a powerful macrophage defense mechanism (Usynin *et al*, 2007).

Altogether, sporozoites seem to be able to selectively recognize and traverse Kupffer cells, without being killed due their capacity to modulate basic anti-microbial macrophage function thus supporting the enormous growth of its liver stages, culminating with the release of thousands of merozoites into the blood (Baer *et al*, 2006. Klotz and Frevert, 2008).

Theoretical aspects of Quantitative Real-Time PCR (qRT-PCR)

The polymerase chain reaction (PCR) is a genetic technique that allows the specific enzymatic amplification of DNA or complementary deoxyribonucleic acid (cDNA) sequences (amplicons). It is most used for quantitative analysis of DNA copy number or gene expression. Quantitative Real-Time PCR is an extension of the PCR methodology. The basic principle of real-time PCR (RT-PCR) is the monitoring of the PCR product accumulation at each cycle during the reaction, instead of at the end point detection which happens with conventional quantitative PCR. Some of its advantages include the absence of post-PCR manipulation which reduces the contamination risk and the fact it can be graphically monitored. It just requires minimal amounts of DNA and it is faster, since there's no need to examine the fluorescence of products of the PCR amplification reaction via the staining of the sample separated by gel

electrophoresis. The reaction can be measured by a great number of fluorescent detection methods such as the generic double-stranded DNA binding dye SYBR®Green, sequence-specific probes such as TaqMan®, or adjacent hybridization probes to name a few. While SYBR®Green labeling can be applicable to any PCR product, the lack of specificity means that fluorescence from an unintended product is indistinguishable from that generated from the intended target sequence thus potentially resulting in inaccurate quantitation (Walker, 2000. Arya *et al*, 2005). Real-time PCR assays have the potential to detect low levels of parasitemia, identify mixed infections, and allow for precise differentiation of species via melting curve analysis. Thus, it becomes easier to identify a given infection of a person without the need for multiple blood specimens or even microscopic analysis (Mangold *et al*, 2005).

Theoretical aspects of Immunofluorescence

Immunofluorescence is an immunohistochemistry technique used to search for cell or tissue antigens ranging from amino-acids and proteins to infectious agents such as parasites. It relies on fluorescent dyes that are conjugated with antibodies which in turn bind to the antigen of interest allowing its detection. The fluorescence can then be quantified and visualized using several imaging techniques such as confocal microscopy. There are two labeling methods: direct, in which the antibody against the molecule of interest is conjugated to a fluorescent dye, and indirect. In the indirect method, the antibody specific for the molecule of interest (called the primary antibody) is unlabeled, thus requiring a second anti-immunoglobin antibody (called secondary antibody) tagged with the fluorescent dye to be directed to the first antibody (**Fig.9**). The indirect method is more sensitive and allows amplification of the signal because more than one secondary antibody can attach to each primary antibody. However, it can lead to cross-reactivity and requires more stages of antibody incubation as well as the need to find primary antibodies from different species when performing multiple-labeling experiments (reviewed in Matos *et al,* 2010. Robinson *et al*, 2010).

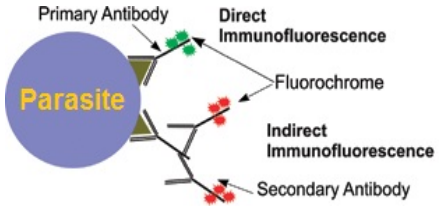

Figure 9: Schematic of direct and indirect immunofluorescence (adapted from Robinson *et al*, 2010)

Theoretical aspects of macrophage depletion with clodronate

Macrophages can be depleted using dichloromethylene bisphosphonate, or clodronate liposomes, which are artificially made spheres of concentric phospholipid bilayers divided by aqueous solution (**Fig.10**). This method is based on the natural process of phagocytosis of liposomes by macrophages: once ingested by a macrophage, it instantly recognizes the clodronate liposomes as foreign particles and proceeds with their destruction. Once inside, the liposomes are engulfed into a phagosome which later fuses with a lysosome forming a phagolysosome. The lysosome contains many enzymes such as phospholipases and its membrane has proton pumps that will lower the internal pH of the phagolysosome.

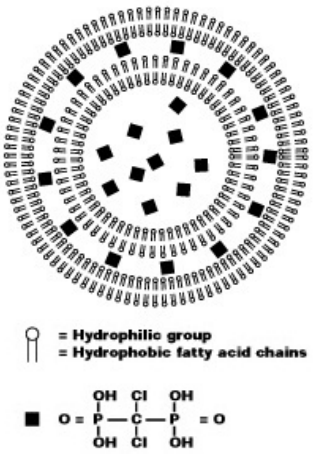

Figure 10: Schematic of a liposome with encapsulated clodronate (squares) (Rooijen and Sanders, 1994).

The phospholipid bilayers of the liposomes are then disrupted under the influence of the lysosomal phospholipases and the low internal pH, releasing the clodronate. As a result, clodronate accumulates in the cell because it's unable to cross the cell membranes (**Fig.11**) (Rooijen and Sanders, 1994).

Once in the cytosol and as it starts to accumulate, clodronate is mistakenly metabolized into to a β-ɣ-methylene (AppCp-type) analog of ATP. The β-ɣ-methylene inhibits mitochondrial oxygen consumption by a mechanism that involves competitive inhibition of the ADP/ATP translocase. This causes the collapse of the mitochondrial membrane potential, loss of the inner membrane integrity and the release of a series of molecular signals that initiate cell death via apoptosis. Clodronate released in the circulation from dead macrophages or by leakage from liposomes cannot cross cell membranes neither penetrate vascular barriers and has an extremely short half-life in circulation and body

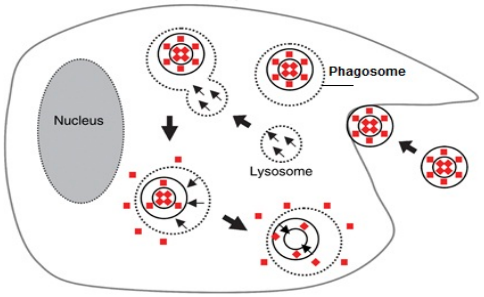

Figure 11: Mechanism of macrophage depletion with clodronate (red squares) (adapted from ClodronateLiposomes.org).

fluids (Lehenkari *et al*, 2002 and Frith *et al*, 1997). Therefore it represents an advantageous alternative to genetic approaches for macrophage depletion that theoretically not only deplete macrophages at the site of interest but also on any tissue that contains macrophages.

Clodronate liposomes are usually dosed intravenously to mice about 1% of their body weight regardless of the injection site. This large volume dosage along the fact that the suspension is slightly viscous increases the risk of adverse reactions during experimental procedures. That risk is increased in cases when the animals become immunosuppressed after treatment. Although free clodronate is hydrophilic and therefore rapidly cleared by the renal system, encapsulated clodronate cannot leave the bloodstream through veins and arteries and some of it is actually destroyed upon entering the bloodstream. The sinusoidal capillaries of the liver, bone marrow and spleen are the only areas where the liposomes can interact with cells outside the vascular system since most of the liposomes will be filtered out by these organs (Patrakka and Tryggvason, 2010. Rooijen and Sanders, 1994).

Aims

The main goal of this project was to study the role of macrophages during the liver stage of malaria infection, under the hypothesis that Kupffer cells are suppressed upon sporozoite infection. To this end, the main aims were to further investigate if macrophage function is suppressed by the parasite, explore if the host responds to infection when the liver macrophages are depleted, and decipher what could be specifically inducing the immune response when liver macrophages are depleted.

Materials and Methods

Mice models

Many strains of mice can be used to study the basic biology of the malaria parasite or the immune mechanisms it elicits. Despite the numerous mouse strain-parasite combinations that can be used in malaria research, previous studies have shown that infection of C57BL/6 strain mice with *Plasmodium berghei* is the optimal model for investigating the malaria liver stage. In fact, this model is the most susceptible to sporozoite invasion and development into microscopically detectable hepatic schizonts in the liver since it shows a delayed onset of cellular responses against these exoerythrocitic stages, compared to other strains (Scheller *et al*, 1994).

For every experiment, pathogen-free C57BL/6 mice were purchased from Charles River Laboratories, and maintained in the animal facilities of Instituto de Medicina Molecular on 12h light/12h dark cycle with access to water and food *ad libitum*, and at the appropriate biosafety level. Experiments were generally performed with mice with ages ranging from 6 to 10 weeks. Mice were anesthetized with Isoflurane (IsoFlo®, Esteve) and injected via the orbital venous sinus. Infected animals were injected with $5x10^4$ sporozoites in 200µl of Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/Invitrogen). For the salivary gland controls, animals were injected with salivary gland material from uninfected mosquitoes in 200µl DMEM. Uninfected animals were injected with 200µl of DMEM. All the work was conducted at Instituto de Medicina Molecular and was approved by the Animal Care Committee.

Sporozoites extraction and purification

Although several species of rodent malaria parasites have been described, only *P. berghei*, *P. yoelii*, *P vinckei* and *P. chabaudi* have been used as valuable models in malaria research even though none of them is a natural pathogen of the laboratory mouse. Despite the phylogenetic distance, these murine parasites are analogous to human parasites in many biochemical and genetic aspects with the advantage of also being good models for genetic manipulation (Déchamps *et al*, 2010).

Plasmodium berghei (ANKA strain) sporozoites were obtained from 4-8 day old laboratory-reared female *Anopheles stephensi* mosquitoes previously kept in mesh nylon cages at 27^oC with constant access to a sucrose solution on filter paper. Mosquitoes were dissected under a workbench magnifier lamp after they had taken an

infectious blood meal. The salivary glands were separated from the rest of the body, collected in DMEM, and then transferred to an Eppendorf tube and lysed mechanically in order to release the sporozoites. Afterwards, the solution was filtered through a 70µm pore diameter cell strainer into a Falcon tube and centrifuged for 3 minutes at 700 rpm. After diluting (1:5) the solution, the sporozoites were counted in a Neubauer improved counting chamber that was previously kept in a moist chamber for 10 minutes. The final concentration was estimated by the average of sporozoites per quadrant x 10^4 x dilution factor. Irradiated sporozoites were treated in an Irradiator Gammacell® 3000 ELAN (Best Theratronics).

Liver extraction and homogenization

To obtain the livers for RNA extraction, the mice were anesthetized with Isoflurane and sacrificed by cervical dislocation 42 hours post-infection. After being secured on their backsides with pins, their belly fur sprayed with 70% ethanol, the animals were opened and their livers were removed into phosphate buffer saline (PBS). The livers were mechanically homogenized with a manual homogenizer in 3 ml of denaturating solution (4 M guanidine thiocyanate; 25 mM sodium citrate pH 7; 0,5 % N-Lauroyllsarcosine; and 0,7% of freshly added β-mercaptoethanol in diethylpyrocarbonate-treated water, dH_20). Two plastic beakers containing dH_20 were used to wash the manual homogenizer and finally at least one aliquot of 1000µl was made for each homogenate.

RNA isolation and quantification

RNA extraction was made using the RNeasy MiniKit (250) from Qiagen. 100µl of liver lysates were resuspended in 900µl RLT solution and mixed with 900µl of 70% ethanol. 600µl of each sample was loaded in an RNeasy mini kit column and washed with Buffer RW1 and RPE, always discarding the flow-through after each wash. The RNA was resuspended in 50µl of DNase/RNAse free water. The RNA concentration was estimated spectrophotometrically by 260/280 absorbance, measured in a NanoDrop-1000 Spectrophotometer (Thermo Scientific).

Quantitative Real-Time PCR

For every experiment, the first strand cDNA was synthesized from the total RNA templates using random hexamers and the Transcriptor First Strand cDNA Synthesis kit (Roche) according to the manufacturer's protocols and the amplification programme: 25ºC/10 min, 55ºC/30 min and 85ºC/5 min. Relative quantification of messenger ribonucleic acid (mRNA) by real-time PCR was done on an ABI Prism 7500 Fast Real-Time System (Applied Biosystems) using the DyNAmo^{TM}HS SYBR$^{®}$Green qPCR kit (Finnzymes), according to manufacturer's instructions. Thermal cycling conditions were 50ºC/2 min and 95ºC/10 min, followed by 50 cycles of 95ºC/15 sec and 60ºC/1 min, and one cycle of 95ºC/15 sec, 60ºC/1 min, 95ºC/30 sec and 60ºC/15 sec. Data was normalized using the expression of hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) housekeeping gene as an endogenous reference and analyzed by the comparative C_T method ($\Delta\Delta C_T$) to produce relative gene expression levels. Parasite load was calculated as the relative amount of *P. berghei*'s 18S cDNA copies against *Hprt* cDNA copies. Primer sequences for every gene used in RT-PCR assays using SYBR$^{®}$Green are displayed next:

PbA 18S	F	**AAGCATTAAATAAAGCGAATACATCCTTAC**	*Hprt*	F	**TTTGCTGACCTGCTGGATTAC**
	R	**GGAGATTGGTTTTGACGTTTATGTG**		R	**CAAGACATTCTTTCCAGTTAAAGTTG**
Saa3	F	**AGAGACATGTGGCGAGCCTAC**	*Mcp-1*	F	**CTTCTGGGCCTGCTGTTCA**
	R	**CAGCACATTGGGATGTTTAGG**		R	**CCAGCCTACTCATTGGGATCA**
Cd68	F	**ACTCATAACCCTGCCACCAC**	*F4/80*	F	**CCCCAGTGTCCTTACAGAGTG**
	R	**GATTTGAATTTGGGCTTGGA**		R	**GTGCCCAGAGTGGATGTCT**
Clec4f	F	**TGAGTGGAATAAAGAGCCTCCC**			
	R	**TCATAGTCCCTAAGCCTCTGGA**			

Immunofluorescence

Livers were fixed with 4% paraformaldehyde for 3 hours and washed with PBS. Each liver was sectioned with a 50µm thickness in a Vibratome 1000S (Leica Microsystems). The sections were labeled indirectly. The slices were blocked at room temperature with a solution of 0.5% Triton® X-100 (Sigma-Aldrich) and 1% albumin from bovine serum (Sigma-Aldrich) in PBS for 1 hour to avoid unspecific reactions. Each slice was incubated with primary antibodies Pan Tissue Fixed Macrophage and Monocytes/Macrophages (F4/80) during an entire day at 4ºC and washed with blocking

solution, followed by an overnight incubation with secondary antibodies 4',6-diamidino-2-phenylindole (DAPI) and anti-GFP 488 at 4ºC in the dark. Every stained section was finally washed with PBS and mounted between 2 glass slides with Fluoromount-G (Southern Biotech). DAPI (1:1000) was obtained from Sigma, anti-GFP AF488 (1:100) from Invitrogen (Molecular Probes), Pan Tissue Fixed Macrophage (1:400) from Fitzgerald and Monocytes/ Macrophages F4/80 (1:200) from RPI.

Imaging

The fixed immunofluorescent slices were examined under a Zeiss LSM 510 META laser point-scanning confocal microscope. Images were taken with LSM 5 software and were acquired using a 40x oil objective. Fluorescence was excited by a 405-nm diode laser, a 488-nm argon laser and a 594-nm diode laser. Every section of each slice was analyzed for parasite and macrophage presence. Image processing such as image merging was performed with Image J software and in a few cases the contrast and brightness levels were optimized using Adobe Photoshop CS3. Images were only enhanced as a whole and no other alterations were done.

Results

Little is known about the role of liver macrophages, such as Kupffer cells, during the malaria liver stage. Preliminary results in the host laboratory, indicated that macrophage depleted mice induce, after infection with *Plasmodium* sporozoites, a strong expression of pro-inflammatory genes in the liver.

I started my master thesis project first by confirming the efficiency of macrophage depletion in uninfected mice. I injected Clodronate liposomes (Clo-lip) into mice and PBS liposomes (PBS-lip) into the control animals. 42h after the injection I measured the expression of two macrophage cell marker genes (*Cd68, F4/80*) and one Kupffer cell receptor marker gene (*Clec4f*), in liver extracts by qRT-PCR. Expression of all three genes was strongly reduced in Clo-lip injected mice (**Fig. 12**).

Next, I sought to measure the efficiency of macrophage depletion in *P. berghei* sporozoite infected mice. For this purpose I included another two groups of mice which were injected with Clo-lip or PBS-lip two days prior to infection with $5x10^4$ *P. berghei* sporozoites. 42h after infection I determined the expression of all three macrophage markers in liver extracts by qRT-PCR. In agreement with the first experiment, expression of all three mRNAs was strongly reduced in uninfected mice.

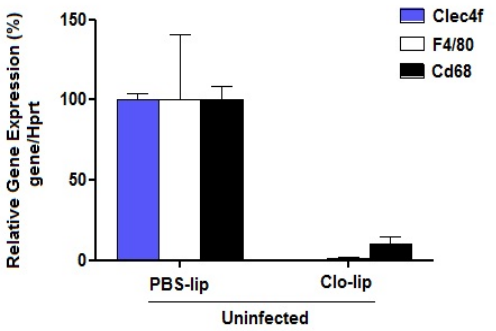

Figure 12: Relative expression of macrophage gene markers was assessed by real time PCR in uninfected animals treated with PBS-lip or Clo-lip.

In sporozoite infected mice depletion of macrophages also led to a dramatically reduced expression of *Clec4f*. However, surprisingly, we found that *Cd68* and *F4/80* mRNA expression in sporozoite infected animals depleted of macrophages was not impaired (**Fig.13**).

Interestingly, qRT-PCR analysis of parasite liver load in mice treated with clo-lip was not significantly different compared to controls treated with PBS-lip (**Fig. 14**)

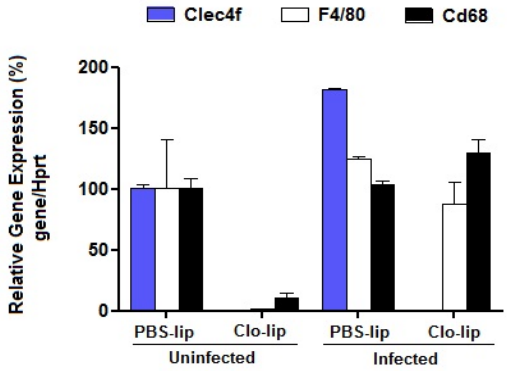

Figure 13: Relative gene expression of macrophages markers assessed by real time PCR in uninfected and infected animals treated with PBS-lip and Clo-lip.

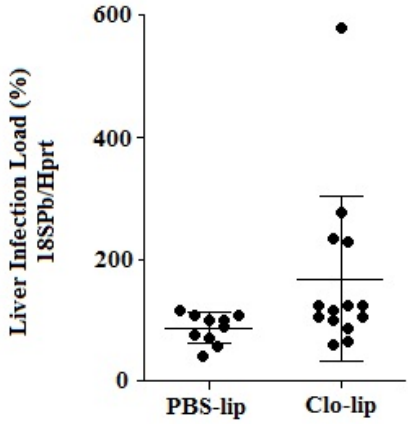

Figure 14: Parasite load in the liver accessed by real-time PCR in infected animals treated with PBS-lip and Clo-lip.

To further explore the possibility that macrophages are recruited to the liver during the infection, we decided to visualize these innate immune cells in liver sections of uninfected and infected mice using confocal microscopy. The macrophages were stained with antibodies directed against a macrophage-specific marker (anti-F4/80 antibody). We found a significant reduction in macrophages in mice treated with Clo-lip compared to PBS-lip treated mice. Strikingly, in infected animals treated with Clo-lip, we observed massive macrophage accumulation in the liver blood vessels (**Fig.15**).

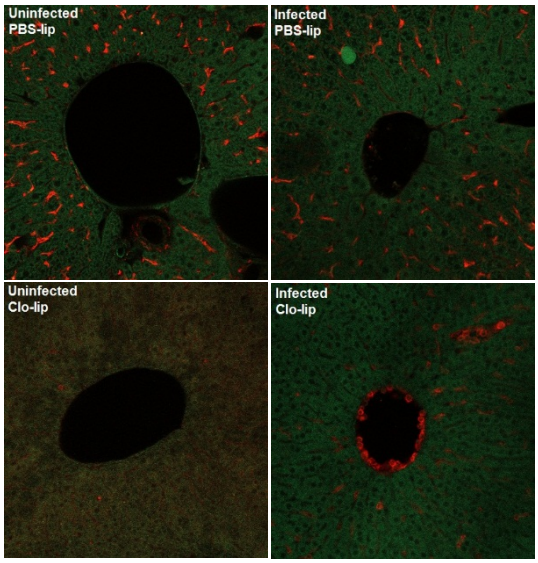

Figure 15: Immunofluorescence images of macrophages (anti-F4/80 antibody, red) in liver sections of uninfected and infected mice. Note the difference in macrophage numbers between Clo-lip and PBS-lip treated mice (40X objective).

Next I decided to monitor whether transcript expression of one chemokine (*Mcp-1*) and one acute phase protein (*Saa3*) is altered in infected mice treated with clodronate. Expression of *Saa3* and *Mcp-1*, in uninfected animals was similar in both PBS-lip and Clo-lip treated groups (**Fig. 16**). However, in the infected mice, the expression of both genes was considerable higher in Clo-lip than in PBS-lip treated animals. Their expression was also significantly higher when compared to uninfected animals treated with Clo-lip.

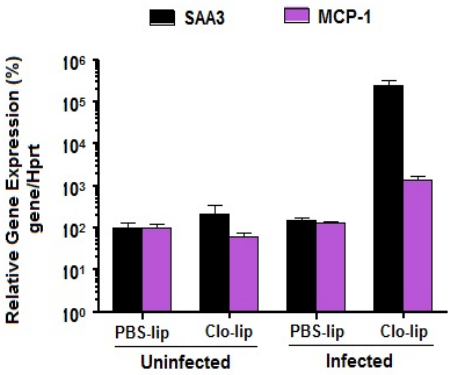

Figure 16: Relative gene expression of two pro-inflammatory markers assessed by real time PCR in uninfected and infected animals, treated with PBS-lip or Clo-lip.

Is the response specifically activated by sporozoites?

In the second phase of the project we decided to monitor whether the induction of this innate response is specifically induced by sporozoites in clodronate treated mice. For this, I decided to measure the response in PBS-lip and Clo-lip treated mice in three additional experimental conditions. The first group of mice was injected with salivary gland material from uninfected mosquitoes, a second group of mice was injected with heat-killed sporozoites, which do not reach the liver and do not infect hepatocytes, and a third group of mice was challenged with irradiated sporozoites, which are unable to replicate in hepatocytes. By injecting uninfected salivary gland material, I wanted to see if the innate response could be caused by microbes, such as bacteria or viruses present on and inside the mosquitos. By injection of heat inactivated sporozoites, I wanted to determine if the sporozoites need to be alive to trigger the inflammatory response, and finally by injecting irradiated sporozoites I wanted to determine whether parasite replication and development inside hepatocytes is required to induce the response.

Depletion of macrophages led to a dramatically reduced expression of *Clec4f* mRNA in all conditions. Expression of *Cd68* and *F4/80* in animals depleted of macrophages was strongly impaired in mice injected with medium. However, their

expression was not impaired in mice injected with salivary glands, heat-killed sporozoites, irradiated sporozoites nor live sporozoites (**Fig.17**).

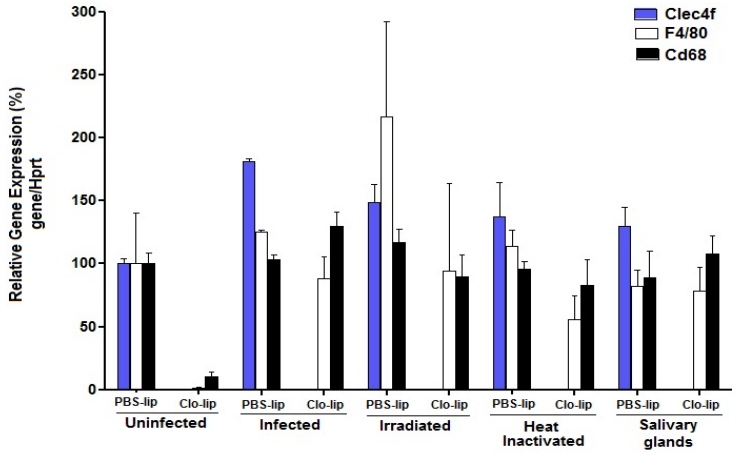

Figure 17: Relative gene expression of three macrophage markers assessed by real time PCR in animals injected with medium, salivary gland material from uninfected mosquitoes, heat-killed and live sporozoites, and treated with PBS-lip and Clo-lip.

I also found that injection of uninfected salivary gland material in clodronate treated animals led to substantial macrophage recruitment to liver blood vessels (**Fig.18**).

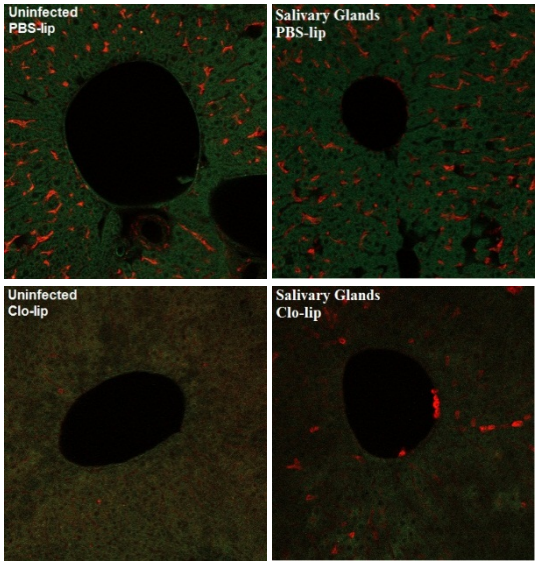

Figure 18: Immunofluorescence images of macrophages (anti-F4/80 antibody, red) in liver sections of uninfected mice and mice injected with salivary gland material from uninfected mosquitoes. Note the difference in macrophage numbers between Clo-lip and PBS-lip treated mice (40X objective).

Finally, expression of *Mcp1* and *Saa3* was strongly up-regulated in clodronate treated mice that were injected with salivary gland material from uninfected mosquitoes (**Fig.19**)

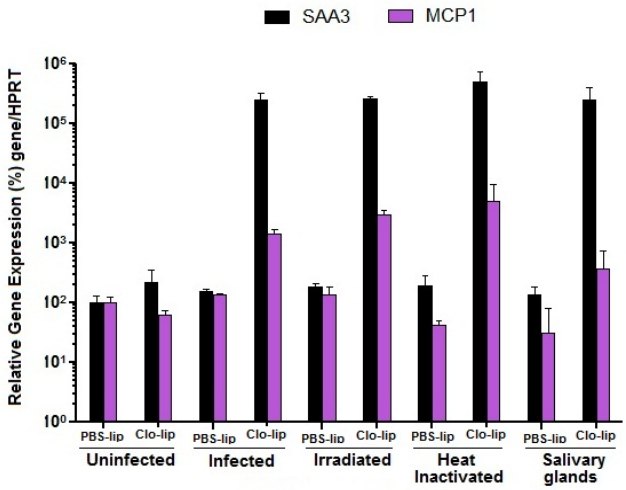

Figure 19: Relative gene expression of two pro-inflammatory markers assessed by real time PCR in animals injected with medium, salivary gland material from uninfected mosquitoes, heat-killed and live sporozoites, treated with PBS-lip or Clo-lip.

Is the response triggered by LPS?

In the last part of this project I tried to determine what could be the ligand that triggers this response. LPS is a major component of the bacterial cell wall and is able trigger innate immune responses by binding to TLR4. Since mosquitoes do not live under sterile condition in the insectary, it is very likely that they are contaminated with bacteria and LPS. Therefore, we tested next whether we could reproduce this innate immune response simply by injecting LPS into mice depleted of macrophages. Two groups of mice each treated with PBS-lip or Clo-lip were injected either with medium (control) or different concentrations of LPS.

Fig.20 shows the expression levels of *Cd68*, *F4/80* and *Clec4f* in Clo-lip treated mice challenged with either LPS or medium. I found that expression of all three macrophage marker genes was impaired in Clo-lip treated mice injected with medium but also after injection of different concentrations of LPS.

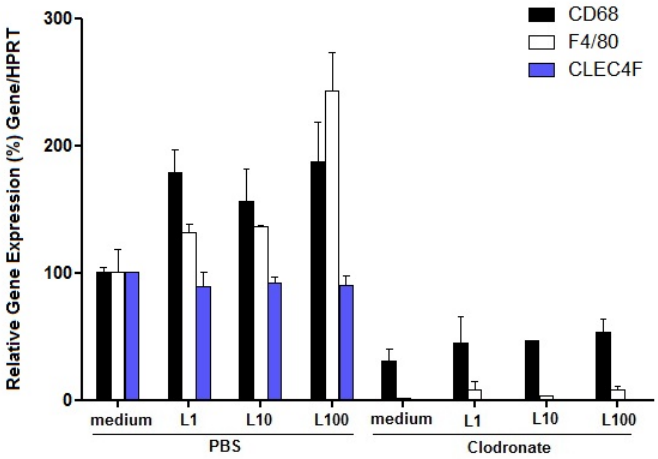

Figure 20: Relative gene expression of three macrophage markers assessed by real time PCR in non-infected animals treated with PBS-lip or Clo-lip. Both groups had animals injected with medium as a control, and animals injected either with 1µg/Kg (L1), 10µg/Kg (L10) or 100µg/Kg (L100) of LPS.

Inflammatory genes *Mcp-1* and *Saa3* were also analyzed for this experiment (**Fig.21**) and the expression of these two genes was not induced in LPS Clo-lip treated animals compared to LPS PBS-lip treated animals.

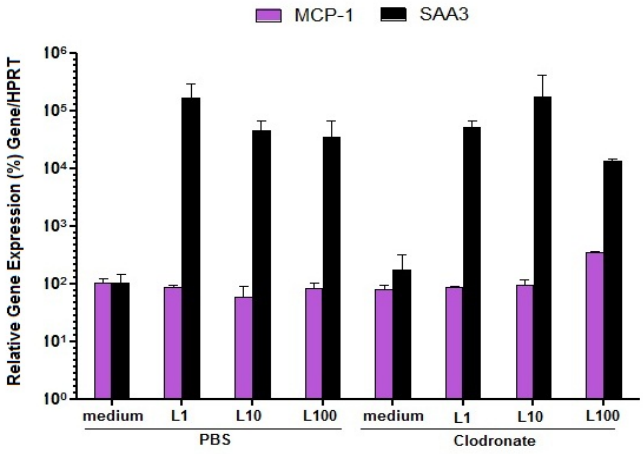

Figure 21: Relative gene expression of two pro-inflammatory markers assessed by real time PCR in non-infected animals treated with PBS-lip or Clo-lip. Both groups had animals injected with medium as a control, and animals injected either with 1µg/Kg (L1), 10µg/Kg (L10) or 100µg/Kg (L100) of LPS.

Discussion and Conclusion

In this project our main goal was to study the role of macrophages during the liver stage of malaria, since their role is not well known in this stage. To reach this goal we used a simple approach to explore macrophage cell function by depleting them using dichloromethylene bisphosphonate, or clodronate liposomes (Clo-lip), based on the natural process of phagocytosis of liposomes by macrophages.

After injection of Clo-lip, phagocytes are specifically killed via apoptosis mainly in the bone marrow, spleen and the liver (Rooijen and Sanders, 1994). Also, neutrophils and lymphocytes do not seem to be affected by it (Feng *et al*, 2011) and previous data from Jordan *et al*, 2003 suggests that clodronate liposomes are non-selective regarding the different macrophage subtypes. This selectivity was confirmed in our results by the low expression levels of two macrophage cell marker genes (*Cd68, F4/80*) and one Kupffer cell receptor marker gene (*Clec4f*) in clodronate treated uninfected mice (**Fig.12**). Interestingly, the expression levels of *Cd68* and *F4/80* did not show the same decrease in infected animals treated with macrophages (**Fig.13**). This indicates that during a malaria liver infection Kupffer cells are efficiently depleted while other motile macrophages might be recruited to the liver to respond to the infection. However, measurement of the parasite load in the liver was not significantly different in animals treated with clodronate compared to control animals treated with PBS (**Fig.14**). This result (i) contrasts to another study where it was shown that in clodronate treated animals, *Plasmodium* infection was enhanced possibly due to the gaps left by removal of the Kupffer cells, which would explain why sporozoite microneme protein essential for cell traversal (SPECT)-deficient sporozoites are still able to infect (reviewed in Frevert *et al*, 2006. Ishino, 2004), and (ii) also suggests that macrophages do not have an obvious impact on the *Plasmodium* liver.

Concerning the macrophage recruitment described above, our *F4/80* immunohistochemistry and subsequent confocal microscopy results showed a great accumulation of macrophages in the liver blood vessels of infected animals treated with clodronate (**Fig.15**) This imaging approach not only validated once again the efficiency of clodronate to deplete liver resident macrophages, but also further indicated that macrophages are indeed recruited to the liver after infection with *Plasmodium* sporozoites.

The immune response was also analyzed by measurement of the expression of one chemokine (*Mcp-1*) and one acute phase protein (*Saa3*). Results showed a higher expression level of these pro-inflammatory markers in infected animals treated with clodronate (**Fig.16**). This supports previous studies where it was shown that sporozoites induce a selective suppression in the production of pro-inflammatory markers such as *Tnf-α, Il-6* and *Mcp-1*, and enhancement of the anti-inflammatory cytokine, which suggests that sporozoites render Kupffer cells insensitive to pro-inflammatory stimuli and eventually eliminate these macrophages by forcing them into programmed death (Klotz and Frevert, 2008).

Altogether, these results indicate that macrophage depleted mice induce upon sporozoite infection a massive recruitment of macrophages to the liver blood vessels and a substantial expression of pro-inflammatory genes.

Results regarding the specific cause for the innate response in clodronate treated mice showed that despite the different experiments designed to isolate each possible cause, the expression level of *Cd68* and *F4/80* was only impaired in uninfected animals treated with clodronate. Expression of *Clec4f* was impaired in every group, confirming that Kupffer cells were removed (**Fig.17**). Moreover, the fact the expression level of *Cd68* and *F4/80* was not reduced in clodronate treated animals injected with salivary gland material from uninfected mosquitoes, suggests that the recruitment of macrophages to the liver is not triggered by sporozoites but by other components present in salivary gland material of mosquitos. In agreement with these results, our *F4/80* immunohistochemistry and following confocal microscopy results showed an accumulation of macrophages in the liver blood vessels of mice injected with salivary gland material from uninfected mosquitoes (**Fig.18**). Expression levels of pro-inflammatory markers *Mcp-1*and *Saa3* were also higher in clodronate treated animals injected with salivary gland material from uninfected mosquitoes, which confirmed the presence of the acute phase response (**Fig.19**).

Concerning LPS experiments, expression levels of *Cd68* and *F4/80* were impaired in animals treated with clodronate (**Fig.20**), which contrasts with the higher expression levels in clodronate treated animals injected with salivary gland material from uninfected mosquitoes (**Fig. 17**). This shows that there is no macrophage recruitment after injection of LPS.

Mcp-1 and *Saa3* expression was also analyzed, and again, the results contrasted with those observed in clodronate treated animals injected with salivary gland material from uninfected mosquitoes (**Fig.18**), because there was no induction of these pro-inflammatory markers in LPS clodronate treated animals (**Fig.21**), thus excluding LPS as a trigger for the immune response.

In conclusion, understanding the mechanisms of macrophage function during the malaria liver stage is important, since is a direct relation with the host's immune response. Our study assessed this, using a simple macrophage depletion technique. One interesting result was the recruitment of these phagocytic cells into the liver, as well as a higher immune response, after the Kupffer cells had been removed. Also, in our study, salivary gland material from uninfected mosquitoes was responsible for a stronger immune response and phagocytic cell recruitment when macrophages were depleted, pointing out that agents other than parasites and LPS might be acting as triggers.

In future experiments, it might be interesting (i) to decipher which ligand triggers the induction this response and (ii) to determine from which source of the body (e.g. the bone marrow) the macrophages are recruited and whether macrophage depletion mobilizes and activates hematopoietic stem cells (HSC).

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