

Filarial nematodes protect against malaria in a murine co-infection model

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Index

ABBREVIATIONS	6
SUMMARY	7
1. INTRODUCTION	8
1.1. General concepts about malaria	8
1.1.1. Malaria epidemiology.....	8
1.1.2. Life cycle of <i>Plasmodium</i> sp.	10
1.1.3. Course of clinical disease.....	12
1.2. Immunology of malaria	13
1.2.1. <i>P. berghei</i> as a murine model for malaria	13
1.2.2. Immune responses against liver stage parasites.....	14
1.2.3. Immune responses against blood stage parasites	17
1.2.4. Cerebral malaria	18
1.3. General concepts about filariasis	23
1.3.1. Epidemiology of human filariasis	23
1.3.2. Life cycle of filarial nematodes.....	23
1.3.3. Onchocerciasis.....	24
1.3.4. Lymphatic filariasis	24
1.4. Immunology of filariasis	25
1.4.1. <i>L. sigmodontis</i> as a murine model for filariasis	25
1.4.2. Responses against adult <i>L. sigmodontis</i> worms	26
1.4.3. Responses against microfilariae.....	27
1.4.4. Immunomodulation by filarial worms.....	28
1.5. Co-infections of helminths and <i>Plasmodium</i>	31
1.5.1. Co-infections in humans.....	31
1.5.2. Murine models for filarial-plasmodial co-infection.....	33
1.6. Aims of the study	35
2. MATERIALS AND METHODS	36
2.1. Materials	36
2.1.1. Animals	36
2.1.2. Buffers and solutions.....	36
2.1.3. Consumables	41
2.1.4. Equipment	41

2.1.5. Computer software	41
2.2. Methods.....	42
2.2.1. Animal cycles and infections	42
2.2.1.1. Maintenance of <i>Anopheles stephensi</i> mosquitoes	42
2.2.1.2. <i>P. berghei</i> infection of mosquitoes	42
2.2.1.3. Preparation of <i>P. berghei</i> stock.....	44
2.2.1.4. <i>Litomosoides sigmodontis</i> lab cycle.....	44
2.2.1.5. <i>P.berghei</i> ANKA infections.....	44
2.2.2. Animal manipulations.....	45
2.2.2.1. Mouse perfusion	45
2.2.2.2. Macrophage depletion.....	45
2.2.2.3. <i>CD4⁺</i> and <i>CD8⁺</i> depletion	45
2.2.3. Laboratory methods.....	46
2.2.3.1. Assessment of body weight and splenomegaly	46
2.2.3.2. Haematocrit.....	46
2.2.3.3. Serum separation	46
2.2.3.4. Giemsa staining and assessment of parasitaemia.....	46
2.2.3.5. Microfilaraemia	46
2.2.3.6. Cell extraction.....	47
2.2.3.7. Flow cytometry.....	48
2.2.3.8. Enzyme linked immunosorbent assay	48
<i>TGF-β</i> ELISA.....	48
2.2.3.9. Assessment of RNA expression in brain.....	49
Extraction of brain RNA.....	49
Transcription to cDNA.....	49
Real-time PCR	50
2.2.4. Statistics	50
3. RESULTS.....	52
3.1. BALB/c mice.....	52
3.1.1. Malaria outcomes in co-infected BALB/c mice	52
3.1.1.1. 500 sporozoites render naive BALB/c mice malaria-positive	52
3.1.1.2. Prolonged survival in co-infected BALB/c mice	52
3.1.1.3. Characterization of malaria-positive and -negative co-infected BALB/c mice	56
3.1.2. Analysis of malaria-negative co-infected BALB/c mice.....	58
3.1.2.1. Malaria protection is not mediated by antibodies	58
3.1.2.2. Protective mechanisms are not directed against blood malaria parasites.....	60
3.1.2.3. Malaria protection is related to microfilaraemia.	60
3.1.2.4. <i>IL-10</i> is required for malaria protection in co-infected mice.....	62
3.1.2.5. Co-infection alters <i>CD8⁺</i> T cell distributions in spleen and liver.....	64
3.1.2.6. Subpatent parasitaemia is conditioned to parasite passage through the liver.....	66
3.1.3. Analysis of malaria-positive co-infected BALB/c mice.....	68
3.1.3.1. Prolonged survival and reduced parasitaemia in malaria-positive co-infected mice... 70	
3.1.3.2. Increased macrophage numbers in spleen in co-infected mice.....	70
3.1.3.3. Similar cytokine and immunoglobulin patterns in single and co-infected mice	72

3.2. C57BL/6 MICE	74
3.2.1. Protective effects of filarial infections on cerebral malaria in C57BL/6 mice	74
3.2.1.1. <i>Lower CM rates and parasitaemia in co-infected mice</i>	74
3.2.1.2. <i>CD8⁺ T cells accumulate in spleen but not in brain of co-infected mice</i>	74
3.2.1.3. <i>IL-10 is essential for CM protection</i>	78
3.2.1.4. <i>Improved control of parasitaemi is not mediated by CD4⁺ T cells</i>	80
4. DISCUSSION	81
4.1. Selection of mice and parasites	81
4.2. Co-infection in BALB/c mice	82
4.2.1. Protection against the liver stage of malaria	83
4.2.2. Improved outcome of blood stage malaria	87
4.3. Co-infection in C57BL/6 mice	89
4.4. Unique features of filarial worms protect against malaria	93
5. REFERENCES	97
6. ACKNOWLEDGEMENTS	111
7. DECLARATION/ERKLÄRUNG	112
8. CURRICULUM VITAE	113

Abbreviations

AAMq	alternatively activated macrophages	LT	lymphotoxin
Ab	antibody	Mal-	malaria negative
Ag	antigen	Mal+	malaria positive
APC	antigen presenting cell	Mf-	amicrofilaraemic
BBB	blood brain barrier	Mf	microfilaria
BSA	bovine serum albumin	Mf+	microfilaraemic
CAM	cell adhesion molecule	MHC	major histocompatibility complex
cDNA	complementary desoxyribonucleic acid	mRNA	messenger ribonucleic acid
CM	cerebral malaria	NK	natural killer cell
CNS	central nervous system	NKT	natural killer T cell
DC	dendritic cell	NO	nitric oxide
DNA	deoxyribonucleic acid	NP	neutrophil
dNTP	deoxyribonucleic triphosphate	OD	optical density
EDTA	ethylenediamine-tetraacetic acid	p.f.i.	post filarial infection
ELISA	enzyme linked immunosorbent assay	p.p.i.	post plasmodial infection
EP	eosinophil	PBMC	peripheral blood mononuclear cell
FACS	fluorescence activated cell sorting	PBS	phosphate-buffered saline
FCS	foetal calf serum	PCR	polymerase chain reaction
FITC	fluoresceine isothiocyanate	RNA	ribonucleic acid
GPI	glycosylphosphatidylinositol	RT	room temperature
i.p.	intraperitoneal	SD	standard deviation
i.v.	intravenous	SEA	<i>Schistosoma</i> egg antigen
ICAM	intercellular adhesion molecule	SMA	severe malarial anaemia
IFN- γ	interferon-gamma	sp	sporozoites
Ig	immunoglobulin	TGF- β	transforming growth factor-beta
IL	interleukin	Th1	T helper cell type 1
iRBC	infected red blood cell	Th2	T helper cell type 2
KO	knock out	TNFR	TNF receptor
LFA	leukocyte function-antigen	TNF- α	tumour necrosis factor-alpha
LPS	lipopolysaccharide	Tris	2-amino-2-hydroxymethyl-propan-1,3-diol
LSA	liver stage-antigen	uRBC	uninfected red blood cell
		w/v	weight per volume
		wt	wild type

Summary

Helminth infections such as filariasis can be life long due to immunomodulatory mechanisms elicited by the worms that prevent their elimination by the human host. Malaria prevalence is frequently high in areas where filarial nematode infections are endemic, originating co-infections. It is still an ongoing debate the extent to which worms can alter anti-plasmodial responses in a concomitantly infected host. In order to dissect the interactions occurring between both parasites, a murine model using the filarial nematode *Litomosoides sigmodontis* and the malaria parasite *Plasmodium berghei* ANKA in BALB/c and C57BL/6 mice has been established in the present work.

One third of helminth-infected BALB/c mice remained malaria negative and did not develop detectable parasitaemia after co-infection with plasmodial sporozoites. Such outcomes were not observed after co-infection with blood stage parasites, thereby circumventing the plasmodial liver stage, but were only originated after sporozoite inoculation. Protection depended on IL-10, since IL-10 deficient co-infected mice were fully susceptible to sporozoite challenge. However, systemic IL-10 levels were low, suggesting a local function of this cytokine. 44 h after *P. berghei* sporozoite inoculation increased CD8⁺ T cell numbers were found in the liver and spleen of co-infected mice. Interestingly, protection as well as numbers of CD8⁺ T cells correlated with the presence of microfilaraemia. Since microfilariae are potent stimulators of immune responses, their presence in the blood may enhance cross-protective responses against incoming sporozoites.

Co-infected C57BL/6 mice showed significantly reduced cerebral malaria (CM) rates. CD8⁺ T cell recruitment to brain, a critical condition for CM development, was reduced in co-infected mice. Furthermore, in contrast to *P. berghei* single-infected animals, groups of double infected mice presented high levels of circulating IL-10. The absolute requirement for IL-10 in CM protection was demonstrated by the total susceptibility to the pathology in IL-10 KO co-infected mice. Hence IL-10-dependent immunoregulatory mechanisms elicited by filarial nematodes might be able to suppress the overwhelming inflammatory reaction usually triggered against malaria parasites by C57BL/6 mice, preventing full progress to CM.

Taken together, this work shows that depending on the genetic background different mechanisms elicited by filarial worms improve control of both liver and blood stages of concomitant malaria parasites, and moreover ameliorate severe pathology in a murine model. The main beneficial effects driven by filariae rely on IL-10, which on the one hand prevents CM through immunomodulation but on the other enhances protective anti-malarial responses in the liver. Worm eradication programs are currently being carried out in endemic countries, but this study suggests that their implementation may cause unpredictable consequences on malaria control.

1. Introduction

The particular characteristics of plasmodial and filarial infections are the consequence of millions of years of evolution that have configured their life cycles, their strategies to survive as parasites and the pathologies they cause in the form we know today. Through the development of mechanisms to suppress host immune responses, filarial nematodes are the parasites with the longest life span in humans and cause a mild disease in most infected people. In contrast, despite being an old human parasite, a recent evolutionary bottleneck in *Plasmodium falciparum* populations is thought to have lead falciparum malaria to be one of the deadliest infectious diseases in the world, mainly due to the generation of an uncontrolled immune response by the host. Epidemiologically, malaria and filariasis share areas of distribution (Fig. 1.1), and thus co-infections are frequent. During co-infection antagonistic immune mechanisms driven by each parasite may alter the course of the single diseases. Theoretically, immunomodulation elicited by filariae could attenuate host immune responses against a concomitant plasmodial infection, preventing the host from developing severe malaria pathology. On the other hand, this suppression could lead to higher malaria morbidity and pathology due to an exacerbated malaria parasite multiplication. Opposing results from field studies supporting either a beneficial or a detrimental malaria outcome in nematode infected individuals currently divide the opinion on this matter in the scientific community. However, an urgent solution is needed since anti-filarial mass treatments are currently being carried out in endemic countries, potentially influencing malaria control.

The aim of the present study is to supply experimental data from a co-infection murine model for filariasis and malaria, using the parasites *Litomosoides sigmodontis* (Chandler, 1932) and *Plasmodium berghei* ANKA (Vincke, 1948), and thus contribute solving the present debate. The work focuses on the extent to which a prior filarial infection influences the pathological course of a concurrent plasmodial infection and the underlying immunological processes.

1.1. GENERAL CONCEPTS ABOUT MALARIA

1.1.1. Malaria epidemiology

Malaria is a mosquito-transmitted disease caused by a protozoan from the genus *Plasmodium* that accounts for one of the most devastating infectious diseases in the world. Four

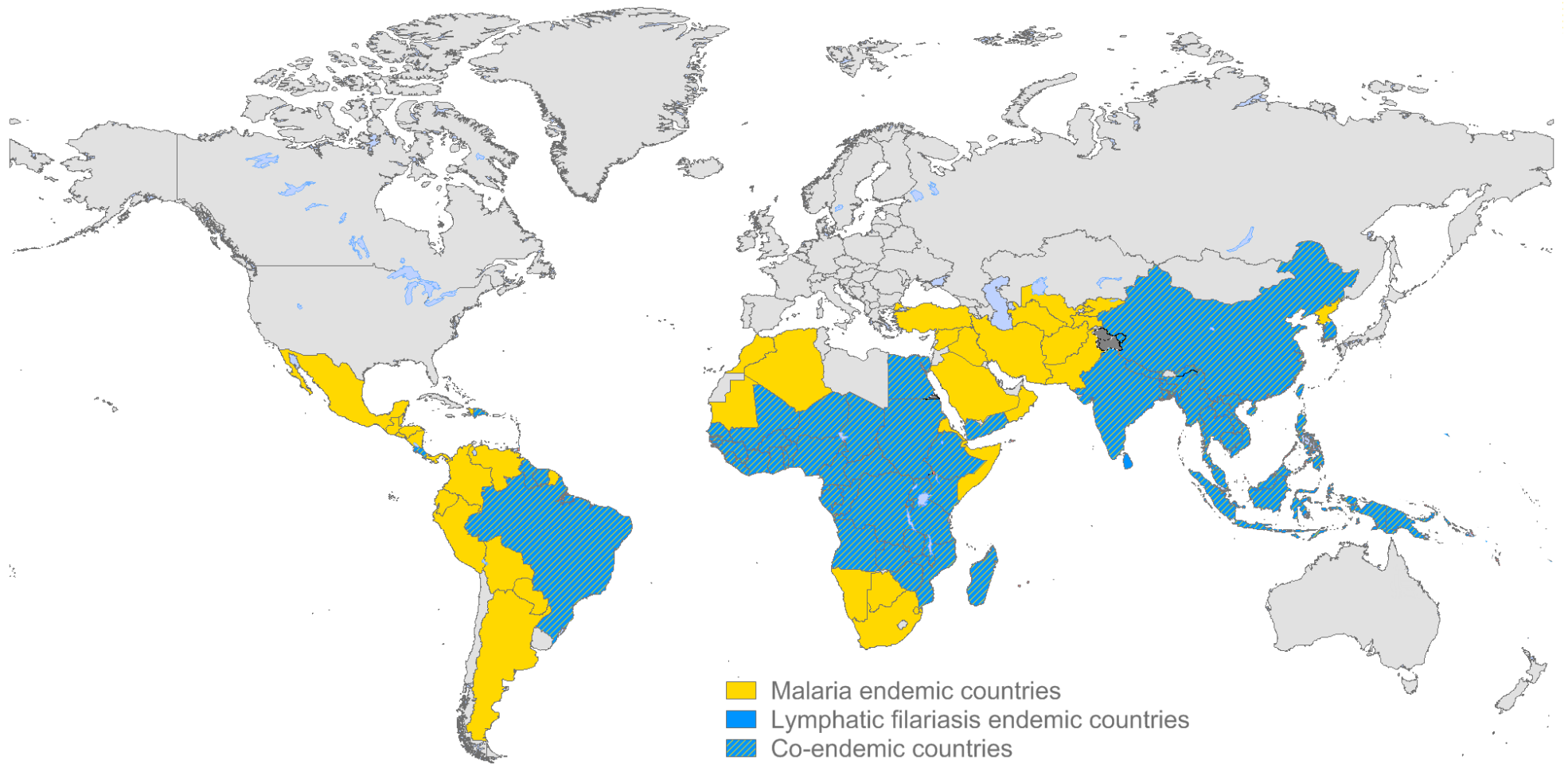


Figure 1.1: Global distribution of malaria and lymphatic filariasis. (Based on WHO, Public Health Mapping and GIS, <http://www.who.int/globalatlas/>)

plasmodial species can infect humans: *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium falciparum*.

Around 3.2 billion people live in malaria endemic areas encompassing 107 countries, and 350 to 500 million clinical malaria cases are recorded each year (277). Between 270 and 400 million of the reported cases are caused by *P. falciparum*, which is responsible for the majority of the deaths caused by malaria every year, estimated in 1 to 3 millions (128, 278). Children in Sub-Saharan Africa particularly contribute to this annual death toll. Indeed, 5 % of infected children die from malaria before reaching 5 years of age, and many others are born with seriously impaired survival chances due to the infection of their mothers during pregnancy (191). Unfortunately, despite malaria being curable, there are still high amounts of deaths in regions where poor infrastructures impede access to early treatment.

1.1.2. Life cycle of *Plasmodium* sp.

Plasmodial sporozoites are injected in the host during the blood meal of an infected female *Anopheles* mosquito. It has been estimated that only around 15 parasites are inoculated per bite (210). This number does not correlate with the total parasite load in the mosquito's salivary glands but with the number of sporozoites that glide into the salivary ducts before blood ingestion (80).

Once in the bloodstream sporozoites are transported to the liver, where they specifically attach (200). Trapped in the liver sinusoids sporozoites glide in search of Kupffer cells, interspersed with endothelial cells, and actively migrate across their cytoplasm accessing the liver parenchyma, where they infect hepatocytes (200). Surrounded by the parasitophorous vacuole derived from the cell membrane of the hepatocyte parasites undergo schizogony, a cell division process consisting of growth and subsequent division of nucleus and cytoplasm into multiple daughter cells within a parent cell, differentiating to liver schizonts of increasing size (79). Development in the liver is asymptomatic and lasts 6 to 15 days for human malaria parasites and around 50 hours for the murine infectious species *P. berghei* (156, 157, 191). After this time, blood parasites, called merozoites, are released from infected hepatocytes.

Merozoites invade erythrocytes, growing fast inside and multiplying through schizogony (39). Eventually the infected erythrocyte bursts and daughter merozoites are released to the bloodstream, where they immediately invade new erythrocytes. In parallel, some merozoites differentiate into

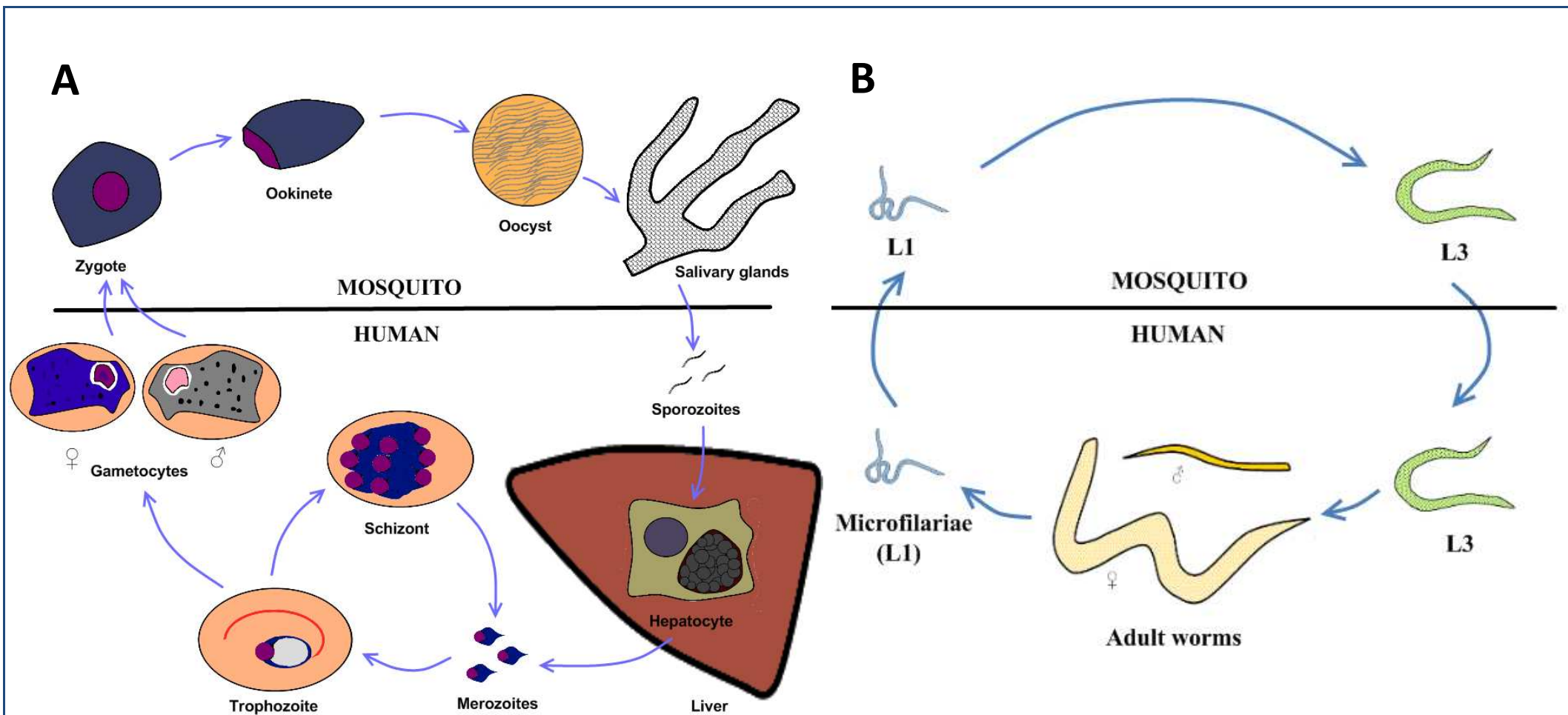


Figure 1.2: Life cycles of human plasmodial parasites and filarial worms. (A) *Life cycle of Plasmodium sp.* Sporozoites enter the host during the bloodmeal of an infected mosquito. After multiplication inside hepatocytes merozoites are released into the blood, where they invade erythrocytes and transform into trophozoites. These divide through schizogony and daughter merozoites are released when the erythrocyte breaks down. Some trophozoites differentiate into male and female gametocytes that merge to form the zygote inside the gut of a new mosquito. The zygote differentiates into an ookinete that settles in the wall of the mosquito's gut and develops to an oocyst, containing thousands of sporozoites. These are released in the mosquito haemolymph and transported to the salivary glands, where they mature, being able to infect a new host. (B) *Life cycle of filarial worms.* L3 larvae enter the bloodstream of the host after the feeding of an infected mosquito and perform two moulting episodes to become adults that produce L1 larvae (microfilariae). L1 larvae are ingested by a new vector and develop into L3 larvae in 2 moulting steps inside the mosquito's gut, migrating to the insect's proboscis to infect a new host after bloodmeal.

gametocytes, the sexual forms of the parasite, which complete the parasite's life cycle after ingestion by a new mosquito (Fig 1.2 A).

1.1.3. Course of clinical disease

Malaria pathology arises during the parasite's blood stage. The regular appearance of fever peaks in infected individuals is a consequence of the synchronic development of the parasites in blood: bursting of infected erythrocytes is accompanied by the release of parasite-derived pyrogenic factors causing acute febrile episodes (19). Intraerythrocytic growth rates of human plasmodial parasites differ regarding the species, generating fever episodes of characteristic periodicity (also known as malaria paroxysm). Namely, *P. vivax* and *P. ovale* (tertian malaria) cause fever episodes every 48 hours; *P. malariae* (quartan malaria) every 72 hours, and *P. falciparum* (designated as malignant tertian malaria) at 48 to 72-hour intervals.

Two features markedly distinguish *P. falciparum* from the other malaria species, and are the possible reasons why infections are of much higher severity. First, the capacity of infected red blood cells to adhere to the endothelial cells in the walls of capillaries of muscle and visceral organs; a phenomenon known as sequestration. In the brain, immune responses against sequestered parasites cause cerebral malaria (CM). Second, *P. falciparum* generates higher parasitaemia levels (around 10%) than other human malaria species (rarely exceeding 1%), giving rise to severe malarial anaemia (SMA). CM and SMA appearing separately or concomitantly define the term severe malaria pathology, which comprises all the processes that increase the risk of death from malaria (204, 233).

CM is a life-threatening immune-mediated pathology. As a consequence of the inflammatory environment created against malaria parasites, endothelial cells express high levels of adhesion molecules, favouring the accumulation of immune cells in the capillaries of the major organs of the body (225). *P. falciparum* infected red blood cells (iRBCs) are able to bind endothelial adhesion molecules too (15), which is interpreted as a strategy to avoid passage through the spleen, where parasites may be eliminated by immune cells. But as a consequence of the activity of effector immune cells against the parasites in the brain the blood-brain barrier is damaged, causing internal haemorrhages (225), and neuronal injury (154). At this stage the patient lapses into a coma and is frequently fatal.

Two different mechanisms have been proposed for onset of coma: first, neuronal hypoxia induced by the clogging of brain capillaries by sequestered immune cells and infected erythrocytes;

and second, deterioration of the brain endothelial cells due to the immune responses against sequestered parasites (147). The use of the model *P. berghei* ANKA in C57BL/6 mice, which reproduces most of the main features of human CM, like sequestration in the brain of immune cells and infected erythrocytes (147, 203), has facilitated understanding the mechanisms leading to this pernicious pathology. Data from this murine model suggest the second mechanism is the most important contributor to pathology, and this is explained in detail in section 1.2.4.

SMA results from massive loss of erythrocytes, but this pathology is not merely related to parasitaemia or to the direct destruction of erythrocytes by the parasite: indeed, severe anaemia may occur in individuals with chronic falciparum infection and low parasitaemia (279). At a given parasitaemia such as 1%, haemoglobin concentrations fall at a much more pronounced rate, to levels around 50%. In addition, an average of 8.5 uninfected red blood cells (uRBCs) are destroyed in addition to each iRBC (118). Intriguingly, an immune-mediated process has been shown to mediate loss of circulating uRBCs after *P. berghei* infection, involving CD4⁺ T cells and macrophages (73, 106). Hence an important component of SMA pathology may have an immunological aetiology.

1.2. IMMUNOLOGY OF MALARIA

1.2.1. *P. berghei* as a murine model for malaria

P. berghei is a model which is commonly used to study human falciparum malaria. The average protein identity between *P. falciparum* and *P. berghei* is 62.9%, and their average nucleotide identity is 70.3% (96).

Susceptibility to sporozoite infection differs between species. As mentioned before, a median of just 15 *P. falciparum* sporozoites were found to be injected into the human host from an infected mosquito (210). Regarding *P. berghei*, infections of BALB/c mice require at least 500 sporozoites (see section 3.1.1.1) or even more depending on the *P. berghei* ANKA strain, but C57BL/6 mice can be efficiently infected with a dose of 50 sporozoites (223). Once inside the hepatocytes *P. falciparum* development requires 5-6 days whereas *P. berghei* takes only 50 hours; this stage asymptomatic in both cases (223, 234).

P. berghei infections are lethal at the blood stage. BALB/c mice develop high parasitaemia and die after several weeks of progressive debilitation and loss of weight and hematocrit. Parasitaemia reaches levels of up to 80% (see section 3.1.1.1) in these mice, which markedly exceed the 10% achieved by *P. falciparum* (19). Unlike human malaria parasites *P. berghei* does not develop synchronously in blood. Nevertheless, parameters such as splenomegaly, extensive erythrocyte loss and decrease in haematocrit levels resemble the situation of *P. falciparum* infected individuals developing anaemia, and thereby *P. berghei* infected BALB/c mice have been used before for SMA studies (73).

As will be explained in section 1.2.4 in detail, strong Th1-type immune responses mainly involving IFN- γ and LT- α are elicited against *P. berghei* blood stages by C57BL/6 mice resulting in CM development (116). In this system murine CM resembles human pathology in multiple facets, such as the high levels of circulating Th1 cytokines, over-expression of adhesion molecules in brain endothelium causing immune cell and iRBC sequestration, or the generation of brain haemorrhages. A feature of *P. falciparum* markedly distinct from *P. berghei* is the accumulation of parasite-derived proteins in spots or “knobs” on the erythrocyte surface, which give the iRBC an irregular form (147). Knobs have been related to endothelium binding (14) and to aggregation of iRBCs to leukocytes and uRBCs (34), the latter being a known phenomenon termed rosetting that has been linked to full blown CM (34, 212). Nevertheless, though scarce, knobless parasites also exist in certain *P. falciparum* strains, presenting similar leukocyte-binding properties than knob-forming parasites *in vitro* (213). *P. berghei* iRBCs also sequester in brain capillaries (120). Absence of knobs in their surface may account for their comparatively lower sequestration capacity than *P. falciparum* iRBCs (147), but this fact does not impede CM generation.

1.2.2. Immune responses against liver stage parasites

Passage through the liver is a very delicate stage in the course of plasmodial infection. Since few parasites are injected into the host by the mosquito (210), only a low percentage of them will actually invade hepatocytes (7) and even fewer will succeed in completing this developmental phase (262). Malaria parasites have developed multiple strategies to ensure maximal success in this process.

Sporozoites progress within a few minutes from the site of injection to the liver (231). In such a short period the immune system of a naïve individual is unable to mount an efficient protective response against the parasite. A potentially feasible immune mechanism to prevent liver invasion is

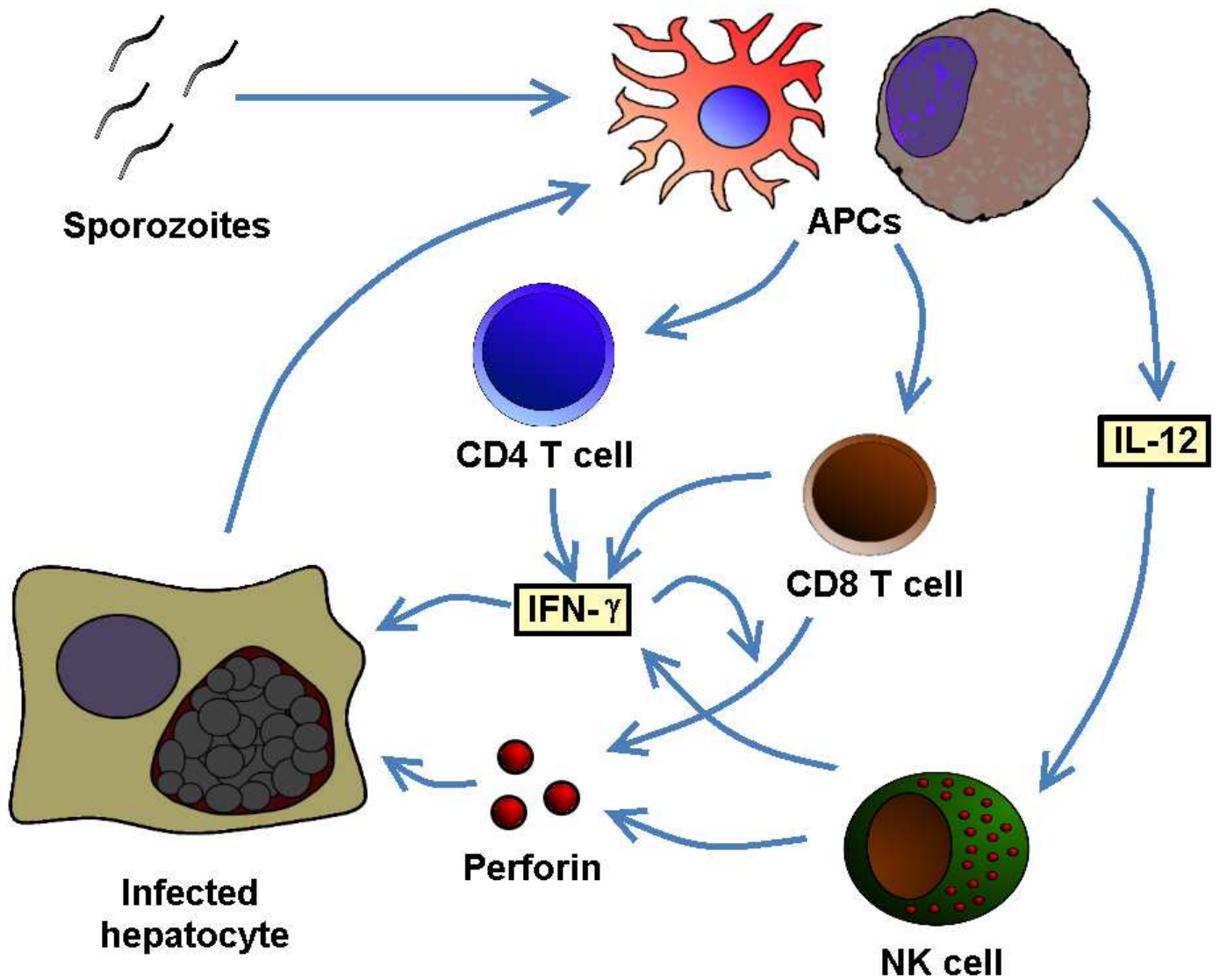


Figure 1.3: Immune response against liver stage malaria parasites. Plasmodial sporozoites as well as damaged intrahepatic parasites supply antigens to APCs (DCs and macrophages), which then prime parasite-specific T cells and produce IL-12, activating NK cells for IFN- γ production and cytotoxicity. Primed CD4⁺ and CD8⁺ T cells also produce IFN- γ , which directly kills intrahepatic parasites and induces cytotoxicity in CD8⁺ T cells. Adapted from (110).

through specific antibodies preformed after a previous infection, but even this is difficult to achieve (224). Monoclonal antibodies against the Pb44 epitope of the circumsporozoite protein (CSP), the most abundant in the sporozoite membrane, have been shown to provide resistance against infection in a murine model (197). However, repetitive sequences in CSP, which are strong B cell epitopes, seem to constitute a way of immunological evasion by a process of clonal dominance: numerous B cells specific for immunodominant repetitive sequences might hinder the binding of other B cells recognizing adjacent protective domains of CSP, avoiding the generation of efficient antibody responses (224). Moreover, though involved in motility, CSP is constantly released by gliding sporozoites, which leave a trail behind, possibly impairing sporozoite neutralization through anti- CSP antibodies (247).

Though some of the sporozoites inoculated by a mosquito are trapped in the lymphatic system and degraded in lymph nodes, supplying antigen for immune cell priming (7, 41), most of them have been found to reach the liver efficiently (210). Once inside hepatocytes malaria parasites are combated by specific cellular immune mechanisms elicited by the host (Fig. 1.3). As for any other intracellular pathogen, CD8⁺ T cells are the main mediators of anti-plasmodial cytotoxic responses at this stage. Hepatocytes, as any nucleated cell, constitutively present peptides derived from own proteins on their membrane. Those peptides are generated by the proteasome and loaded on major histocompatibility complex class I (MHC-I) proteins in the endoplasmic reticulum (127). Afterwards, complexes are transported in vesicles to the cell membrane (295). Upon infection by an intracellular microbe, pathogen-derived peptides are loaded on MHC-I molecules and carried to the cell surface through the same mechanism. CD8⁺ T cells scan MHC-I molecules on the membrane of nucleated cells in search for presented non-self antigens; indicating infection. Specific CD8⁺ T cells recognizing a particular peptide-MHC combination induce apoptosis (programmed death) of the infected cell (16, 18). Following this schema, hepatocytes infected with malaria parasites have been found to present parasite-derived peptides to specific CD8⁺ T cells (21), which in turn mediate protection against infection after immunization with attenuated sporozoites (60, 209, 227, 273).

CD8⁺ T cells are assisted by natural killer (NK) and natural killer T cells (NKT cells) in combating intrahepatic malaria parasites. The presence of NK cells is required for the induction of effective anti-plasmodial cytotoxic responses by CD8⁺ T cells (61). Additionally, NK and NKT cells have been proven able to kill intrahepatic parasites in an IFN- γ -dependent mechanism (193, 208). IFN- γ directly inhibits parasite growth through the induction of nitric oxide (NO) production by the hepatocytes (159). NKT cells artificially activated by α -galactosylceramide enhance IFN- γ production by CD8⁺ T cells in the liver, boosting elimination of malaria liver stages after suboptimal immunization with irradiated *P. yoelii* sporozoites (83). Thus, like CD8⁺ T cells, NK and NKT cells participate in the

clearance of liver stage parasites either directly, killing infected cells, or indirectly, inducing the production of IFN- γ by CD8⁺ T cells and increasing their cytotoxicity (Fig. 1.3). After the initial CD8⁺ T cell activation, specific IL-4-producing CD4⁺ T cells sustain the proliferation and maintenance of parasite-specific CD8⁺ T cells in an NK- and NKT-independent manner (35, 163, 274).

Since its intrahepatic development lasts merely 50 hours a fast immune response is required to block *P. berghei* infection in the liver. Dendritic cells (DCs) are able to generate specific and fully functional CD8⁺ T cells 6 to 12 hours after contact with an antigen (201). In the case of malaria, CD8⁺ T cell responses have been shown to start as soon as 8 hours after sporozoite infection (95). 24 hours after infection CD8⁺ T cell proliferation can be detected, as well as their secretion of IFN- γ and perforin. Cytotoxic elimination of liver parasites inside hepatocytes occurs between 24 and 48 hours (218). Mouse strains with varying susceptibility to *P. berghei* sporozoite infection differ in their ability to establish fast immune responses against liver stage malaria parasites. Thus, 44 hours after sporozoite inoculation BALB/c mice accumulate more infiltrating immune cells in the liver than C57BL/6 mice, killing liver parasites more efficiently and hence being more resistant to sporozoite challenge (223).

Nevertheless, malaria parasites have developed diverse mechanisms to impair the establishment of cytotoxic immune responses in the liver. For instance, sporozoites down-regulate MHC-I expression in Kupffer cells, impairing CD8⁺ activation (244). In addition, intrahepatic parasites can impede phosphatidylserine translocation to the outer membrane of infected cells that have been induced by CD8⁺ T cells to undergo apoptosis. Recognition of phosphatidylserine on the surface of an apoptotic cell is the signal that triggers its phagocytosis by macrophages. By blocking this process malaria parasites remain hidden during their development inside the hepatocytes (262). Indeed, it is parasite death (estimated to occur in 9 to 18% of the parasites that achieved hepatocyte invasion) that provides antigen for antigen presenting cells (APCs) for the development of specific protective CD8⁺ T cell responses after re-infection (141, 262). With such strategies plasmodial parasites maximize their chances of survival and are thus capable of effectively establishing blood infections with only a few sporozoites.

1.2.3. Immune responses against blood stage parasites

After completing their liver stage malaria parasites are released into the blood, where they infect erythrocytes. During this stage, the parasites grow and divide rapidly increasing their numbers

and making total elimination by the host immune system difficult. Malaria pathology exclusively develops during the blood stage. Indeed, important processes leading to the main manifestations of severe malaria pathology, CM and SMA, originate from inappropriate immune responses against blood parasites.

The capability of the immune system to combat blood stage malaria parasites is limited by the fact that erythrocytes lack expression of MHC-I molecules, which mediate the paradigmatic mechanism of defence against intracellular pathogens. Nevertheless, immune mechanisms are indeed elicited during blood infection. Effective control at the blood stage of the non-lethal murine parasite *P. chabaudi* is first mediated by CD4⁺ T cell-dependent activation of macrophages (286), and subsequently antibody production (269). IFN- γ secretion by CD4⁺ T cells has been shown to fully activate human macrophages for *P. falciparum* phagocytosis (179). Although Th1 cytokines like IFN- γ are required for the elimination of blood stage parasites, high levels may result in CM pathology (see next section and Fig. 1.4). Conversely, the absence of these cytokines may lead to exacerbated parasitaemia. Hence a precise level of inflammation is necessary for the successful clearance of a plasmodial infection in blood.

None of the classical mouse strains can spontaneously control *P. berghei* parasitaemia. A key factor involved in this susceptibility seems to be the way APCs respond to the parasites. DC maturation and capacity to cross-present antigens are impaired during *P. berghei* infection (282) due to high concentrations of systemic TNF- α (284). Moreover, *P. berghei* iRBCs also decrease IL-12 production by macrophages in an IL-10-dependent mechanism (287). A similar inhibition of human DC and macrophage function after phagocytosis of *P. falciparum* parasites has been described (228, 261), underlining the convenience of *P. berghei* to model blood stage human malaria in the laboratory.

1.2.4. Cerebral malaria

CM is the result of a strong inflammatory process against malaria parasites taking place in the brain. In a typical inflammatory reaction TNF- α released at the site of infection induces an increased expression of adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) by epithelial cells (196). Circulating immune cells are then recruited from circulation by binding ICAM-1 through leukocyte function-associated antigen-1 (LFA-1) (152), and extravasate to the tissue to combat the pathogen (24). A similar process occurs in the brain and other major organs during malaria: pro-inflammatory cytokines induce the up-regulation of adhesion molecules in the brain capillaries,

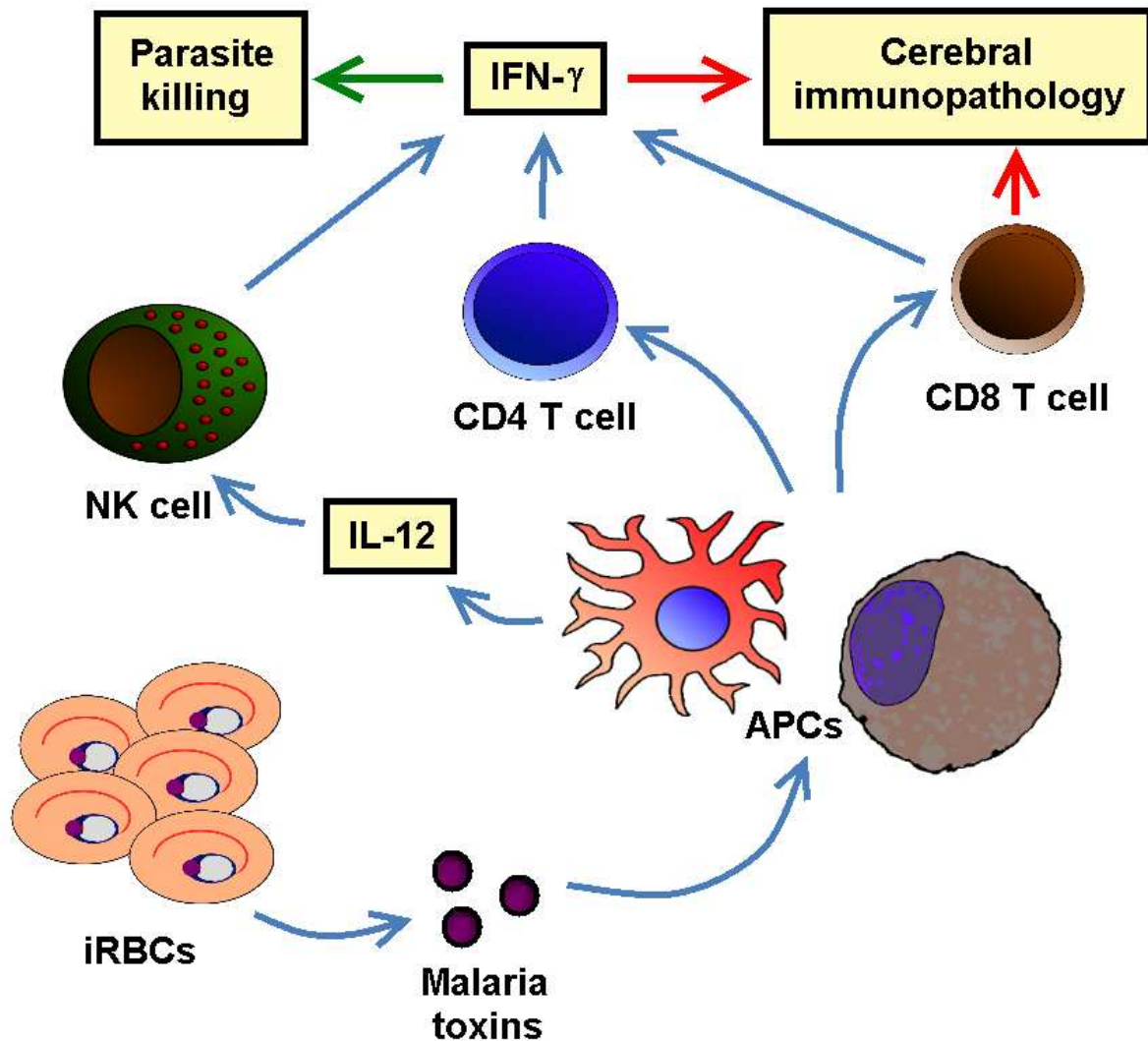


Figure 1.4: Immune response against blood stage malaria parasites. Parasite-derived catabolites activate APCs (DCs and macrophages) for T cell priming and IL-12 production, which in turn activates NK cells. NK cells, CD4⁺ and CD8⁺ T cells later produce IFN-γ, which on the one hand is essential for parasitaemia control but on the other can induce CD8⁺ T cell-mediated pathology at high concentrations. Adapted from (51)

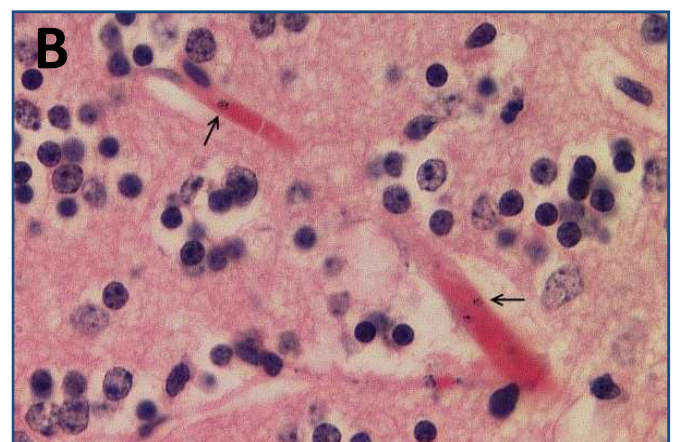
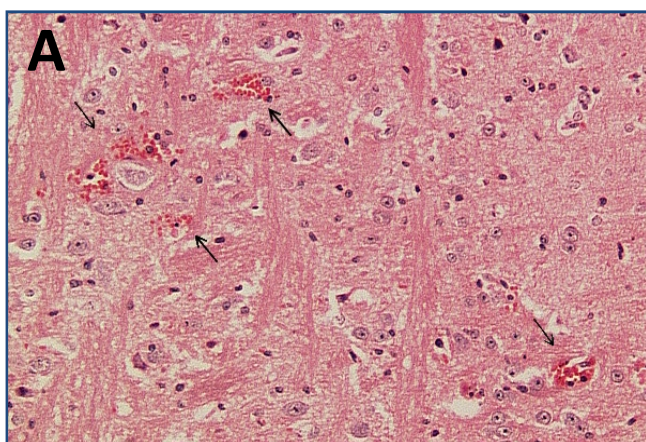


Figure 1.5: Brain histology during cerebral malaria. (A) Cerebral haemorrhages in brain parenchyma (arrows). (B) Sequestered erythrocytes in mouse brain capillaries during CM-development (arrows).

leading to the sequestration of immune cells and iRBCs and subsequently to endothelial damage (Fig. 1.5 A, B).

It is not TNF- α but a close relative, lymphotoxin α (LT- α), which has been found to be essential for CM development (71). Nevertheless TNF- α is produced by both murine and human macrophages when exposed to different plasmodial species (11, 192, 253), and also by microglia and astrocytes during CM (155). Microinjection of TNF- α in the brain has been demonstrated to reduce cerebral blood volume, and even to induce damage to the blood-brain barrier (BBB) (232), indicating that this molecule, though not essential, may worsen the course of the pathology.

On the other hand, ICAM-1 expression was found to be up-regulated in brain endothelial cells of CM-developing mice (89), resulting in the sequestration of immune cells (88). Enhancement of ICAM-1 expression is driven by TNF- α or LT- α signalling through the TNF receptor 2 (TNFR-2), which is up-regulated in CM-susceptible C57BL/6 mice during the onset of the pathology but not in CM-resistant BALB/c mice (148). Additionally, *Plasmodium*-derived glycosylphosphatidylinositol (GPI) directly induces ICAM-1 expression on endothelial cells (226).

Operating in an earlier step than TNF- α or ICAM-1, IFN- γ is critically involved in the cascade generating CM. Indeed, IFN- γ is produced by T cells to activate macrophages, enhancing their TNF- α production (37, 165). Furthermore, IFN- γ was shown to synergize with TNF- α and LT- α inducing increased expression of adhesion molecules by endothelial cells (272). In the context of *P. berghei* infection, deficiency in either IFN- γ or IFN- γ receptor prevents CM development, abolishing increases in TNF- α and ICAM-1 in the brain (5, 214, 289). When stimulated with malaria parasites *in vitro*, lymphocytes from CM susceptible mouse strains produce significantly higher IFN- γ levels than those from CM resistant mice (50).

Timing of cytokine peaks is a critical factor defining the eventual malaria outcome. An early IFN- γ peak produced by BALB/c mice 24 hours after non-lethal *P. chabaudi* or *P. yoelii* infections is thought to result in prevention of CM pathology (52). However, absence of the early IFN- γ peak and a later increase of this cytokine triggers CM in *P. berghei* ANKA infected mice. Interestingly, when mice are co-infected with two *P. berghei* strains from which one causes CM and the other does not, cerebral pathology is prevented and an early IFN- γ peak induced by the non-CM-inducing parasites can be measured (160).

CM is the result of a sequential cascade of events in which different cell types play defined roles at specific time points after the infection. Macrophages, neutrophils and platelets are involved in the initial steps for the induction of the pathology. Thus, in C57BL/6 mice CM development is

stopped when depletion of macrophages is done before day 5 p.p.i., but not later during the onset of CM (13, 40). Upon contact with the parasites macrophages release an initial wave of pro-inflammatory cytokines generating a Th1 environment required for progression to CM. Sensing of *P. falciparum* GPI by macrophages through TLR2 (133) activates them and induces IL-12, IL-6, TNF- α and NO production (294). Additionally, the complex formed by hemozoin, the residual product of the heme catabolism by the parasite, and plasmodial DNA engages TLR9-dependent responses in macrophages, enhancing IL-12 synthesis (186).

Neutrophils have also been shown to mediate CM (44, 229) by producing IFN- γ and TNF- α (43). However, like macrophages, their late depletion does not prevent pathology and hence their contribution to CM is done early after infection (13). Platelets, which express high levels of LFA-1 and sequester in the capillaries of brain and other major organs, also promote CM (90). Their early depletion hampers CM progression (90, 146), originating IFN- γ down-regulation and the increase of IL-10 levels in serum (264).

CD4⁺ T cells are a source of IFN- γ , and their depletion at day 4 after *P. berghei* infection, but not at day 6, prevents pathology (13). This places helper T cells in a more posterior stage of the immunological cascade which generates CM compared to macrophages or neutrophils. During *P. berghei* infection CD4⁺ T cells are induced to differentiate into Th1 cells. CD4⁺ T cells can belong to a Th1, Th2 or regulatory phenotype. Polarization of CD4⁺ T cells to each of these phenotypes can be driven between others by the presence of IFN- γ , IL-4 or IL-10 in their surroundings (59, 124). Whereas Th1 T cells are involved in IFN- γ production and full activation of cell-mediated immunity against intracellular pathogens, Th2 cells produce IL-4, IL-5 and IL-13, which mediate defence against extracellular parasites like helminths. Regulatory T cells are generated to suppress both Th1 and Th2 responses and prevent potential immune-derived damage to the host (124). The direction an adaptive immune response takes towards a Th1 or a Th2 type is thought to be determined by cells of the innate immune system; particularly NKT cells and DCs. These cells respond against the pathogen supplying an early wave of cytokines influencing CD4⁺ T cell polarization. NKT cells are able to produce IFN- γ or IL-4 upon contact with a pathogen, influencing the subsequent immune response (99). In accordance with this, shortly after *P. berghei* infection NKT cells from CM susceptible C57BL/6 mice induce the production of high amounts of IFN- γ by CD4⁺ T cells. Absence of NKT cells reduces CD4⁺-derived IFN- γ production in these mice and results in no pathology. In contrast, in the CM-resistant BALB/c background NKT cells induce IL-4 production in CD4⁺ T cells after contact with malaria parasites, and their absence leads to high IFN- γ production and CM development (100). Moreover, substitution of the NKC complex (a genetic region that regulates NK and NKT function) in CM resistant BALB/c mice for that of CM susceptible C57BL/6 mice turns transgenic BALB/c mice

susceptible to CM (98). Therefore, NKT cells regulate T cell commitment after *P. berghei* infection and also determine susceptibility to CM. On the other hand, priming of anti-plasmodial CD4⁺ T cells for IFN- γ production has been shown to be performed by CD11c^{high} DCs (55). Apart from DCs and NKT cells, neutrophils secreting IFN- γ shortly after infection may also contribute to Th1 differentiation and hence CM development.

CD8⁺ T cells are the ultimate effector cell in the cascade causing CM. Complete prevention of cerebral pathology can be achieved by depletion of these cells as late as day 6 after *P. berghei* infection, and even partial protection is seen after depletion during CM onset (13). After recognizing a peptide from the pathogen loaded in a MHC-I molecule, CD8⁺ T cells exert their cytotoxic activity inducing cell death through perforin (123). In the context of CM, perforin has been shown to induce apoptosis of brain endothelial cells, and mice deficient for this gene completely evade pathology development despite the presence of sequestered CD8⁺ T cells in the brain (172, 198). Thus, perforin-dependent apoptosis of brain endothelial cells is the last step in CM. It is important to remark at this point that it is not the mere sequestration of immune cells and infected erythrocytes in the capillaries of the brain that causes CM, but the immune responses elicited in the brain. However, there is still an open question about how activated CD8⁺ T cells destroy sequestered parasites and damage the brain endothelium if such parasites remain inside erythrocytes, which do not possess MHC-I molecules.

Apart from macrophages, CD4⁺ and CD8⁺ T cells, NK cells are also sequestered in the brain during CM in a CXCR3-dependent manner (97). CXCR3 is a chemokine receptor expressed mainly in activated Th1 lymphocytes and NK cells involved in the trafficking of activated T cells to inflammation sites in the CNS (47). NK cells enable T cells to migrate to the brain in a IFN- γ dependent mechanism by stimulating them for CXCR3 expression (97). This, however, might not be done directly, but through NK enhancement of DC function, which is also dependent on IFN- γ and TNF- α (266).

In summary, after *P. berghei* infection cells from the adaptive immune system orientate the subsequent immune response towards a Th1 type. Th1-polarized CD4⁺ T cells and NK cells produce IFN- γ , which contributes to full activation of macrophages and parasite phagocytosis. However, high levels of pro-inflammatory LT- α or macrophage-derived TNF- α also induce the brain endothelium to express adhesion molecules. As a consequence, macrophages, CD4⁺, CD8⁺ T cells and NK cells are all sequestered in the brain. Sequestered effector CD8⁺ T cells secreting perforin damage the brain endothelium and cause haemorrhages and eventually CM.

1.3. GENERAL CONCEPTS ABOUT FILARIASIS

1.3.1. Epidemiology of human filariasis

Filariasis is an infectious disease induced by parasitic nematodes. Several species can infect humans. *Wuchereria bancrofti* in Africa, and *Brugia malayi* and *Brugia timori* in Asia live in the lymphatic ducts and provoke lymphatic filariasis. *Onchocerca volvulus*, causing onchocerciasis or river blindness, *Loa loa* and *Mansonella streptocerca* settle in the subcutaneous tissue, and other filariae infect different organs (*Mansonella* spp. and *Dirofilaria* spp.) (113).

W. bancrofti, *B. malayi* and *O. volvulus* are responsible for the majority of the filarial infections in the world (184). Regarding lymphatic filariasis *W. bancrofti* causes 90% of the cases, being distributed in the tropics throughout the world. The remaining 10% corresponds to *Brugia malayi*, appearing in Southeast and Eastern Asia, and *Brugia timori*, in Timor and adjacent islands (158). *O. volvulus* is endemic in 28 Sub-Saharan countries in Africa, 6 in Latin America and Yemen. In these regions, endemic areas correspond to savannah and rain-forest close to watercourses where *Simulium* vectors breed (258). Distribution of the main filarial nematodes often coincides with that of *P. falciparum* (Fig. 1.1).

1.3.2. Life cycle of filarial nematodes

Filariae belong to the order Spirurida, in the phylum Nematoda. Adult filarial nematodes live in different lymphatic tissues and body cavities of the host, depending on the species. Mature female worms release microfilariae (Mf), which are first-stage larvae (L1). Mf migrate to blood or peripheral tissues, where they remain until ingested by an arthropod vector. In different regions Mf migration is performed selectively during the day or the night depending on the feeding behaviour of their endemic vector (19). Once inside the insect's gut, Mf penetrate the midgut wall and reach the hemocoel, being transported to the thoracic musculature. There they moult twice becoming infectious L3 larvae and migrate to the insect's proboscis. Such larvae gain access to the vertebrate host through the wound made by the insect during the blood feeding. Inside the new host they develop to adult worms through two moulting episodes (19) to complete the cycle (Fig. 1.2 B).

1.3.3. Onchocerciasis

O. volvulus worms reside in characteristic nodules of 1-5 cm surrounded by the host by fibrous tissue under the skin, known as onchocercomas. Their lifespan reaches 10-15 years (258). Millions of Mf are released by adult female worms and spread in the dermal tissue, where they are taken up by a new vector during a bloodmeal. Pathology is triggered in the skin by death of Mf, which become the target of immune attack. Eye inflammation after Mf death in the cornea causes blindness (108). The most common manifestation of the infection in endemic individuals is the generalized onchocerciasis, characterized by massive numbers of Mf in the skin but only mild signs of disease. In contrast, some individuals referred to as “sowda” patients present chronic dermatitis and lymphadenitis, which are derived from an excessive immune response against the worms. Numbers of Mf and adult worms are reduced in these patients as a consequence of such response (32), but at the cost of suffering disease (33). Finally, a small proportion of people living in the same areas and therefore under the same risk of infection remain free from helminth infection and are denoted “putatively immune” individuals (108). Generalized onchocerciasis is thought to reflect the ability of the worm to suppress immune effector mechanisms in the host to facilitate its own survival. In contrast, parasite clearance in sowda patients implies the failure of regulatory mechanisms, resulting in strong inflammation and severe disease (108).

1.3.4. Lymphatic filariasis

Lymphatic filariasis is considered the second leading cause of long-term disability in the world (276). In addition, severe social and psychological consequences follow the onset of certain pathological stages such as elephantiasis or hydrocoele (63).

The infection presents three stages: first, an incubation period involving the development of L3 larvae into adult worms. This stage is accompanied with mild symptoms and lasts one year or more. In some patients worms are eliminated without causing disease in the infected person (19). The second acute phase starts after the maturation of adult worms and Mf release. When adult worms die, their products trigger inflammation and swelling of arms and legs. In males the scrotal region is frequently affected, and results in hydrocoele. Finally, the chronic phase, affecting up to 10% of the infected population, involves the blockage of lymph vessels through the acute granulomatous response against dead and degenerating worms, provoking elephantiasis (19).

In correlation with onchocerciasis, areas endemic for lymphatic filariasis have three groups of coexisting subjects: asymptomatic microfilaraemia patients resemble those with generalized onchocerciasis and comprise the highest percentage of infected people, presenting Mf in blood but no evident signs of disease. In these patients, immunosuppression exerted by the worms is assumed to prolong parasite survival and prevent pathology development (184). Other individuals, like the sowda patients in onchocerciasis, are able to eliminate Mf, but develop pathology. Death of adult worms causes episodes of acute filarial lymphangitis (AFL), a local inflammatory response, which does not represent a strong clinical course (158). However, secondary bacterial infections elicit acute dermatolymphangioadenitis (ADLA), which consists of a local inflammatory reaction followed by oedema of the affected limb and eventually results in lymphoedema and elephantiasis (62, 64). Finally, individuals designated as “endemic normals” present no evidence of disease and are equivalent to putatively immune individuals in onchocerciasis areas, representing a third of the total population (less in onchocerciasis areas) (65, 271). Some of these individuals can be cryptically infected, appearing positive for infection due to the presence of circulating worm antigen in the blood (49).

1.4. IMMUNOLOGY OF FILARIASIS

1.4.1. *L. sigmodontis* as a murine model for filariasis

Brugia malayi, the human parasite, and *Brugia pahangi*, which naturally infects felines and canines, have all been used in experimental murine infections for immunological studies on filariasis. However, such systems are considered artificial since these worms cannot complete their life cycle in the mice (137). The establishment of the *L. sigmodontis* model in mice has become a valuable tool for immunological research on filariasis. BALB/c mice are fully susceptible to the infection, allowing worms to mature in their pleural cavity and produce Mf (188). *L. sigmodontis* belongs to the family Onchocercidae, which also comprises the human pathogens *O. volvulus* and *W. bancrofti* (285). The natural host of *L. sigmodontis* is the cotton rat (*Sigmodon hispidus*) (111).

After infection of the fully permissive BALB/c mice, L3 larvae migrate through the lymphatics to the lungs and then to the pleural cavity 4 days later, and moult twice there to become adults around 30 days post infection. Migration of immune cells to the pleural cavity starts as early as 4 days after infection (9). From day 55 onwards, Mf are released by adult female worms (111). A rate of roughly 50% of the *L. sigmodontis* infected mice become microfilaraemic, whereas the rest remain

negative (Mf-). Amicrofilaraemia in a susceptible mouse has been reported to be due to Mf clearance through a specific immune response mediated by antibodies (91) or to the decrease in fertility of female worms (8). The former situation is thought to occur more commonly, whereas the latter has been associated with high worm loads.

Adult worms elicit different immune-suppressive mechanisms, preparing a milder environment for the Mf at the moment of their release (109). Thus, Mf+ mice might be considered as those in which immunosuppression by the adult worms has been more efficient, whereas their amicrofilaraemic counterparts have been able to circumvent suppression and elicit efficient immune responses killing Mf. Both groups of mice resemble therefore different manifestations of filariasis in human patients. Unlike humans *L. sigmodontis* infected BALB/c mice do not develop any kind of pathology.

1.4.2. Responses against adult *L. sigmodontis* worms

Adult *L. sigmodontis* worms are eliminated in the pleural cavity through encapsulation in nodules formed by immune cells. These nodules are constituted by a first layer of neutrophils followed by a second layer of eosinophils. The same distribution of neutrophils and eosinophils can be found in nodules around *O. volvulus* worms in humans (281). Though eosinophils reach the pleural cavity shortly after *L. sigmodontis* infection they are unable to initiate worm encapsulation at this time. This process is started by neutrophils, arriving between days 30 to 60 p.f.i. in an IL-5-dependent mechanism (3). Macrophages also mediate anti-filarial immunity, being possibly activated by eosinophils for TNF- α -production (241) and then attracting neutrophils to the thoracic cavity (3). In *L. sigmodontis* infections of BALB/c mice the worm encapsulation processes start after the seventh week post infection, continuing over 4 to 5 weeks until all worms have been blocked. Finally, worms are progressively re-absorbed until week 20 p.f.i. (3).

Combined Th1 and Th2 responses are naturally developed by the vertebrate host against the different life stages of filarial worms. Absence of either IL-5 or IFN- γ impairs nodule formation and worm elimination (216). Indeed, both IL-5 and IFN- γ have been shown to work in a synergistic manner, and their double deficiency results in more increased worm numbers than the deficiency of IL-5 or IFN- γ alone (215). Filariae-induced Th1 and Th2 reactions are not antagonistic, since IL-4 depletion in BALB/c spleen cell cultures with worm antigen from *B. pahangi* does not lead to enhanced IFN- γ production (183). In contrast, it is the depletion of the regulatory cytokine IL-10 which results in the production of Th1 cytokines against the same filarial antigen (181). IL-4

deficiency allows prolonged worm survival and Mf release in the otherwise resistant C57BL/6 background (139). However, the additional deficiency of IL-10 restores resistance to worm infection (240). This underlines the concept of Th1 and Th2 responses acting simultaneously, but not exclusively, against the worms, and the role of IL-10 as a regulatory cytokine for both types of responses (252).

At the cellular level, in addition to eosinophils, neutrophils and macrophages, a variety of other cells contributes to the immune response against *L. sigmodontis* in BALB/c mice. CD4⁺ T cells are involved in the clearance of adult worms. Moreover, IL-4 and IL-5 responses, eosinophilia in the blood and pleural cavity and IgE levels are diminished in CD4⁺ T cell depleted mice (4). Production of IL-4, IL-5 and IL-10 is also driven by B1 cells, which are an ancient subtype of B cells found exclusively in pleural and peritoneal cavities producing T-cell independent responses (74), and their deficiency results in increased worm numbers (2). NK and NKT cells also migrate into the pleural cavity during *L. sigmodontis* infections, and their depletion leads to higher parasite loads (129). Depletion of CD8⁺ T cells has not been found to influence adult worm nor Mf elimination (129).

Susceptibility to *L. sigmodontis* infection varies among different mouse strains. Thus, BALB/c mice allow the complete development of its life cycle whereas C57BL/6 mice clear adult worms before they can produce Mf. Differences in immune responses against adult worms between susceptible and resistant mouse strains can be observed on days 10 and 30 after infection, when a higher migration of immune cells to the pleura, accompanied by higher levels of IL-4, IL-5, IL-10 and IFN- γ is seen in resistant C57BL/6 mice (9). However, worm loads are similar during this period and differences only occur in the ability of the helminth to sustain infection (9).

1.4.3. Responses against microfilariae

Mf are released by adult worms in the thoracic cavity and actively migrate to blood thereafter. When injected intravenously in naive C57BL/6 mice they are rapidly killed (within 6 days), whereas they are able to survive up to 45 days in susceptible BALB/c mice (190). Similar strain differences in elimination efficiency are observed after intrapleural injection of Mf. However, in this case Mf amounts in the pleural cavity at day 6 after injection are comparable in both mouse strains, indicating that their clearance only takes place in the blood (190). Adult worms in the pleural cavity are thought to downregulate immune responses against Mf to prolong their survival. Thus, transplantation of female worms to the pleural cavity of a resistant naïve mouse (e.g. C57BL/6) allows Mf injected thereafter to persist for several weeks, in an IL-10-dependent mechanism (112).

CD4⁺ T cells and B1 lymphocytes are involved in Mf clearance, since their depletion leads to higher Mf numbers in blood (2, 4). Macrophages, able to adhere to Mf, also contribute to their elimination (185). Absence of IL-4 or IL-5 in BALB/c mice causes higher microfilaraemia (3, 267). Patency in BALB/c mice is followed by high levels of circulating IFN- γ (182, 252) and the secretion of IgG2a, IgG2b and IgG3 (138), but IFN- γ deficiency does not lead to increased Mf loads in blood (215).

1.4.4. Immunomodulation by filarial worms

Parasitic filarial nematodes present a long lifespan inside the host due to the development of mechanisms modulating immune responses against them. This is reflected by the fact that, when cultured with filarial antigens, peripheral blood mononuclear cells (PBMCs) from subjects living in areas with elevated transmission rates, harbouring therefore high worm loads, proliferate less than those of people from low transmission areas (125). In addition, children living in endemic areas acquire fewer adult worms per year than older people, in whom immunomodulatory mechanisms have been established by the worms (67). Such mechanisms eventually result in a benefit for both host and parasite, since an excessive immune reaction against the worms reduces parasite loads but contributes to pathology (64). As an example, hyperreactive sowda onchocerciasis patients present increased numbers of mast cells and eosinophils in the skin, actively attacking live Mf. This results in Mf killing but also in the development of strong dermatitis (130). Skin lesions in such patients have been related to strong Th2 responses and absence of immunoregulation (259). In contrast, unresponsiveness allows high Mf loads in skin but prevents pathology in patients with generalized onchocerciasis (280). As a consequence of filaria-induced immune suppression, lower production of IL-5 and IFN- γ by Mf carriers compared to amicrofilaraemic individuals has been reported (27). A decrease in IL-5 production with increasing age in both microfilaraemic and amicrofilaraemic groups (220), as well as lower T cell proliferation (92) has been observed in individuals living in filaria endemic areas. This indicates that the establishment of immunoregulatory processes by filarial worms is a slow and long lasting process.

IL-10 is a major cytokine mediating immunosuppression by filarial nematodes. Its main function is modulating immune responses to avoid damage to the host, as indicated by the fact that IL-10 deficient mice develop chronic enterocolitis by reacting against normal bacterial flora in the intestine (134). IL-10 impairs APC function, production of Th1 cytokines and T cell co-stimulation by macrophages and DCs (53, 57, 72). Engagement of the IL-10 receptor by IL-10 triggers an intracellular cascade following the JAK-STAT pathway, involving the Jak family tyrosin kinases Jak1 and Tyk2 (77)



Figure 1.6: Immune responses against *L. sigmodontis* worms and their modulation. Mixed Th1 and Th2 responses are required to eliminate filarial worms. Adult worms are eliminated through encapsulation in nodules formed by macrophages (Mq), eosinophils (EP) and neutrophils (NP), in a process dependent on Th1 and Th2 cytokines. Microfilariae (Mf) are combated through adherence of activated macrophages and antibodies produced by B cells. However, adult worms induce IL-10 production by alternatively activated macrophages (AAMq) and regulatory T cells (Treg). IL-10 is capable of modulating both Th1 and Th2 responses. Therefore, adult worms protect their offspring and themselves through IL-10-mediated suppression of harmful host immune responses. Adapted from (109).

and the transcription factor STAT3, but also some other factors like STAT1 and 5 whose exact function remains unknown (161, 176). As a result, transcription of IFN-induced genes like ICAM-1, IP-10 or ISG54 is blocked (117). Also NF- κ B activation is suppressed by IL-10 (270). However, though its main function is immunosuppressive (177), IL-10 has been shown able to stimulate proliferation and cytotoxic activity of CD8⁺ T cells (45, 82, 93, 121, 219, 290) and NK cells (114, 293).

Filarial worms have taken advantage of the regulatory properties of IL-10, and stimulate its production in order to block immune onslaughts (151). Macrophages produce IL-10 in response to stimulation with filarial antigen, which in turn down-regulates their expression of co-stimulatory molecules CD80 and CD86 (28). Additionally, in vitro stimulated PBMCs from filarial-infected patients show an IL-10 mediated decrease in IFN- γ production (151), and IL-10 blockage restores proliferative capacity of lymphocytes (58, 126). IL-10 also skews Ig production by B cells to the IgG4 isotype, which does not bind complement and only binds weakly to Fc receptors, thus avoiding induction of inflammation (222).

Another important cytokine mediating immunosuppression is TGF- β . This cytokine engages a cellular cascade of Smad family proteins through ligand-induced activation of specific kinases. Smad proteins 2, 3 and 4 form a complex in the cytoplasm that translocates to the cell nucleus and induces expression of target genes (260). The main regulatory function of TGF- β is exerted on T cells, inhibiting proliferation of naive lymphocytes but not of activated cells (251). The latter are however impaired in their differentiation into Th1 or Th2 (85) by TGF- β , as well as the cytotoxic activity of CD8⁺ T cells is inhibited (235).

Filarial parasites induce the formation of regulatory T cells, which are capable of producing IL-10 and TGF- β (58), and suppressing proliferation of other T cells (221). At the molecular level, such suppression is mediated by CTLA-4, which is highly expressed in CD4⁺, CD8⁺ and CD4⁺CD25⁺ T cells of Mf⁺ individuals compared to Mf⁻ (243). Immunoregulation is also mediated by alternatively activated macrophages, characterized by the expression of arginase. In contraposition to classically activated macrophages, which perform respiratory burst and present enhanced TNF- α production upon IFN- γ stimulation (84), differentiation to an alternative phenotype is driven by IL-4 and IL-13 and these macrophages become potent suppressors of T cell proliferation (145). Further functions of alternatively activated macrophages are down-regulation of Th1 responses (103) and the production of arginase, a component necessary for wound repair (84). Classically and alternatively activated macrophages can be interconverted by their exposure to Th2 or Th1 cytokines respectively (87).

Similarly to human parasites, the rodent filaria *L. sigmodontis* develops multiple strategies to hinder the attack of the host immune system. Apart from IL-10 induction (112, 252), regulatory T

cells mediate hyporesponsiveness to *L. sigmodontis*, and their depletion leads to a faster clearance of the infection (256). Differentiation of CD4⁺ T cells to a regulatory phenotype is induced by alternatively activated macrophages (255). Moreover, conventional non-regulatory CD4⁺ T cells in the pleural cavity of *L. sigmodontis* infected mice present a strongly reduced proliferation capacity (254).

1.5. CO-INFECTIONS OF HELMINTHS AND *PLASMODIUM*

1.5.1. Co-infections in humans

Discrepancies exist in the scientific community as to whether the final effect of nematode worms on malaria is beneficial or detrimental for the host. In terms of both epidemiological and scientific data very little is known about the the particular case of filarial nematode worms and *Plasmodium* co-infections scarce background can be found in the literature. In contrast, some field studies have analysed the co-infection of other nematode species and malaria. Intestinal worms present similar properties to filarial nematodes. For instance, *Ascaris lumbricoides* is able to induce immunomodulation (38), and hookworms (*Ancylostoma duodenale* or *Necator americanus*) have been shown to evoke combined Th1/Th2 responses (202).

Several independent studies in different parts of the world have shown an association between intestinal nematode infections and higher malaria morbidity. More frequent malaria attacks (defined as body temperature higher than 38°C and a *P. falciparum* parasitaemia density higher than 5000 parasites/ μ l blood) were observed in subjects of 1-14 years of age co-infected with intestinal worms and *P. falciparum* in Senegal (242). Studies on adults in Thailand also showed similar results (42, 170). In concordance with this, higher percentages of mixed *P. falciparum* and *P. vivax* infections could be associated to *Ascaris* infection (167), suggesting that nematode immunomodulation might impair anti-parasitic immune responses and increase susceptibility to the plasmodial infection. Moreover, increased *P. falciparum* densities in the blood were found in Cameroonian children aged 0-14 carrying high loads of intestinal helminths (173). In addition to the co-infections with intestinal worms, *Schistosoma mansoni* infections have also been associated with increased malaria morbidity in Senegalese children (238). In this study, children presenting either high or low *Schistosoma* egg loads (defined as more than 400 or 1-100 eggs per gram stool respectively) presented increased malaria attacks compared to those which were *Schistosoma*-negative. However, no significant differences (indeed a trend for lower malaria incidence) were seen in children harbouring medium

worm loads (101 to 400 eggs per gram stool), and hence the interpretation of the results is complicated. Nevertheless, all these studies point to the same conclusion that a worm-mediated suppression impairs immune responses against malaria parasites increasing malaria morbidity. Druilhe et al. postulated that the production of the cytophilic antibodies IgG1 and IgG3, which are effective against blood stage malaria parasites (22), may have been shifted by the worms to the production of unprotective noncytophilic isotypes IgG2, IgG4 or IgM (23). Cytophilic antibodies are those whose constant regions (therefore unrelatedly to their antigen specificity) present affinity for receptors on certain cell types like macrophages or NPs, leading their engagement to the activation of cytotoxic and phagocytic effector functions in such cells. Unfortunately, no immunological parameters were measured in either of the co-infection studies to ascertain the immunological mechanism behind the observations.

In contrast to these results, a study on *S. haematobium*-*P. falciparum* co-infections showed lower malaria parasite loads in children with low amounts of *Schistosoma* eggs (1-9 eggs per ml urine), reflecting again the importance of worm loads in the outcome of *Schistosoma*-malaria co-infections (29). Moreover, in another study Madagascarean children over the age of 5 years were treated against *Ascaris lumbricoides* infection and presented an increase in *P. falciparum* densities in the blood. Surprisingly, this was not observed in younger children (30) or adults older than 15 years (31). Thus, age may also be an important factor in defining malaria outcome in co-infected patients.

On the other hand, Nacher et al. have shown in studies on adult patients that a prior *Ascaris* infection results in protection from CM (166). The same group reported a decrease in the intensity of fever peaks after *P. falciparum* infection in patients co-infected with *Necator americanus* (169). Moreover, jaundice and acute renal failure, both related to liver inflammation, were ameliorated in helminth co-infected patients (168). All three studies, performed on adult patients, are consistent with an immunosuppressive effect of parasitic nematode worms on malaria, reducing pathology. Thus, despite the possible increase of malaria incidence caused by worm infections, suppression of immune responses could account for the prevention of immune-derived malaria pathology. In contraposition to this, a study on Senegalese children by Le Hesran et al. showed a higher prevalence of severe malaria cases in *Ascaris* co-infected patients (140). The main difference between both studies seems to be again the age of the participants (being adults in the Thai study, with a median age of 24 years, and children in the Senegalese one, with a median age of 6.6 years).

Age has an important influence in the kind of response elicited against plasmodium or nematodes. Nematode infections are more prevalent in adults than children in endemic populations, which is interpreted as the long time required for worm infections to fully establish immune

suppression (68). Therefore, only responses to *Plasmodium* in adults, with a long history of worm exposure, are likely to be modulated by the latter.

Children living in endemic areas develop CM after several contacts with the parasite and the development of a strong pro-inflammatory response. Accumulation of inflammatory mediators after repeated infections triggers cerebral pathology. In a region of moderate transmission like Gambia, the median age of children developing CM is 3-4 years (105). CM in children is associated with strong IFN- γ production by T cells (205). In a context where suppressive mechanisms against the worms have still not been fully established, combined Th1/Th2 responses against the worms, and absence of regulation by IL-10 or TGF- β , may constitute an extra source of Th1 cytokines accelerating CM development. Supporting this explanation, a study by Diallo et al. on the co-infection of *Schistosoma haematobium* and *P. falciparum* showed co-infected children expressing more IFN- γ than *P. falciparum* single infected (56). Additionally, immunity to malaria pathology is gradually achieved in older children by the generation of a repertoire of antibodies against the parasites inhibiting cytoadherence (171, 206), erythrocyte invasion (94) or inducing phagocytosis by macrophages (180). An alteration in the antibody production inducing non-protective antimalarial antibody isotypes might delay the apparition of immunity in co-infected children. In adults, however, immunoregulatory mechanisms driven by the worms may modulate the production of Th1 cytokines resulting in lower CM rates. In the study by Diallo et al. co-infected adults presented higher levels of IL-10 and TGF- β , and higher ratios of IL-10/TNF- α than single infected patients. Interestingly, IFN- γ levels were not decreased in co-infected adults, but remained higher than those of *P. falciparum* single infected individuals. Moreover, parasitaemia in co-infected children and adult patients was not significantly higher than in single infected subjects (56), indicating that specific independent mechanisms are elicited during malaria, being not all of them equally susceptible to alteration by the worms.

Finally, another study in Uganda from a population living in an area with unstable malaria transmission worms were neither related to higher malaria susceptibility nor to stronger pathology (230), showing that undefined parameters in particular regions may add variability to the malaria outcome during co-infection.

1.5.2. Murine models for filarial-plasmodial co-infection

Several animal studies on the occurrence of concurrent malaria and filariasis can be found in the literature giving controversial results, which somehow reflect those from human studies. A

detrimental outcome was observed after non-lethal *P. chabaudi* co-infection of C57BL/6 mice previously infected with the intestinal nematode *Heligmosomoides polygyrus*. Co-infected mice developed higher parasitaemia and suffered higher mortality than their single infected counterparts. They presented lower IFN- γ and the cytophilic Th1-type immunoglobulin IgG2a, protective against malaria parasites, but higher IL-10 and TGF- β levels (248). Moreover, in the same co-infection setting immune-modulation by the worms decreased the efficiency of a blood-stage vaccine *P. chabaudi* antigen (249). Co-infection with another intestinal worm, *Echinostoma caproni*, and *P. yoelii* also resulted in higher parasitaemia and death rates in the co-infected group of mice (174). Moreover, impairment of anti-plasmodial immunity by the worm could be noticed at the liver stage of malaria in the same co-infection setting, as reflected by the higher susceptibility to sporozoite infection in co-infected mice (175). In a different model, co-infection with *Schistosoma mansoni* and *P. berghei* ANKA in C57BL/6 mice resulted in higher plasmodial parasitaemia. In this case circulating TNF- α , but not IFN- γ , was decreased in co-infected mice (102). Interestingly, this resembles the results obtained by Diallo et al. on *S. haematobium*-*P. falciparum* co-infection in Senegal, in which immune suppression in adults was not exerted on IFN- γ but on TNF- α levels (56). Furthermore, all these data resemble those from human studies in which higher risk of *P. falciparum* infection and malaria attacks were found in co-infected individuals (42, 167, 170, 242), and support the assumption that suppression of anti-plasmodial Th1 responses by the worms impair parasite control. On the other hand, consistently with the regulatory effect of worms on pathology development during subsequent plasmodial infections reported by Nacher et al. (166), CM was prevented in susceptible CBA/J mice by previous injection of *Brugia pahangi* L3 larvae. In this co-infection setting filarial infection induced high IL-4, IL-5 and IFN- γ , but downregulating cytokines like IL-10 or TGF- β were not measured (288).

Additionally, a study on co-infection of BALB/c mice with the non-lethal *P. chabaudi* and *L. sigmodontis* showed an important role of microfilaraemia defining malaria outcome. Consistent with the lack of detectable immunomodulation in amicrofilaraemic mice, these mice lost more weight and suffered more severe anaemia than their Mf+ counterparts (86). Interestingly, parasitaemia levels in Mf+ mice, (which suffered higher immunoregulation and consequently presented lower IFN- γ and IL-4 production by splenocytes during co-infection), were lower than those of Mf- mice, contradicting the assumption exposed above that immunoregulation is related to higher plasmodial parasitaemia. This is indeed in accordance with the observation that co-infected adult human patients do not present higher parasitaemia than *P. falciparum* single infected. Different unknown mechanisms may be elicited by filarial infections altering the natural means of control of blood parasites, giving a new perspective on the effects that the worm co-infection may have on malaria.

1.6. AIMS OF THE STUDY

Parasitological nematodes persist during an unusually long lifespan inside the infected host due to their capacity of inducing immunomodulation. Mechanisms involved in immunosuppression may however influence responses against concomitant plasmodial infections. Current literature does not provide a uniform answer on whether worm infections are beneficial or detrimental against malaria, leaving this question still under debate. Differences in several parameters such as the involved worm species, host age and environmental conditions between field studies may contribute to this heterogeneity, and specific analyses are therefore required to dissect the relationships between both kinds of parasites residing in the same host.

In order to contribute with experimental data to a better understanding of the mechanisms occurring during co-infection of filarial nematode worms and malaria, a murine model using the filarial worm *L. sigmodontis* and *P. berghei* ANKA has been established and analysed in the present work. The use of laboratory mice allows detailed studies of immune responses under well controlled conditions. Taking advantage of their different susceptibilities to the parasites, BALB/c and C57BL/6 mice were used to resemble the stages of the life cycles and a wide range of the manifestations that filarial and plasmodial infections present in humans.

The present study aimed at:

1. Assessing the effect of a *L. sigmodontis* infection to the susceptibility of a *P. berghei* sporozoite infection in BALB/c mice.
 - a. Does worm-derived immunoregulation alter susceptibility to sporozoite infection?
 - b. Does patency of filariae influence anti-plasmodial immunity?
2. Monitoring the effect of a *L. sigmodontis* infection on the susceptibility to blood stages of *P. berghei* in BALB/c mice.
 - a. Does worm-derived immunoregulation impair control of plasmodial blood stages?
 - b. Is the development of severe anaemia pathology altered by filarial infection?
3. Estimating the effect of a *L. sigmodontis* infection on the development of CM in C57BL/6 mice.
 - a. Does worm-derived immunoregulation impair control of plasmodial blood stages?
 - b. Given the immunological aetiology of CM, can worm-mediated immunomodulation reduce this pathology?

2. Materials and methods

2.1. MATERIALS

2.1.1. Animals

Sigmodon hispidus rats, *Ornithonyssus bacoti* mites, *Anopheles stephensi* mosquitoes and the different mice strains used, listed in the following table, were bred at the animal facilities of the Institute for Medical Microbiology, Immunology and Parasitology in Bonn.

BALB/c background	C57BL/6 background
Wild type	Wild type
IL-10 KO	IL-10 KO
μ MT	

2.1.2. Buffers and solutions

- Anesthetic

Components	Concentration	Company
Rompun		Bayer, Leverkusen, Germany
Kylazinhydrochloride	23.32 mg/ml	
Methyl-4-hydroxybenzoate	1.5 mg/ml	
Ketanest		Pfizer Pharma GmbH, Karlsruhe, Germany
Esketaminhydrochloride	25 mg/ml	

Preparation: 40 μ l Rompun + 10 μ l Ketanest per mouse (intramuscular injection).
Storage: 4°C

- Cell depletions

Components	Concentration	Company
Anti-Mouse CD4 monoclonal antibody	1 mg/ml	BIOZOL Diagnostica Vertrieb GmbH, Eching, Germany
Anti-Mouse CD8a monoclonal antibody	1 mg/ml	BIOZOL Diagnostica Vertrieb GmbH, Eching, Germany
Clodronate liposomes	250 mg/ml	See below

Preparation: see below

- ELISA

Components	Concentration	Company
<u>Coating buffer</u>		
NaH ₂ PO ₄	0.1 M	MERCK KGaA, Darmstadt, Germany
<u>Washing buffer</u>		
PBS	x1	MERCK KGaA, Darmstadt, Germany
Tween	0.05%	
<u>Blocking buffer</u>		
PBS	x1	SIGMA-ALDRICH CHEMIE GmbH, Munich, Germany
BSA	1%	
<u>Enzyme solution</u>		
PBS	x1	
Streptavidin		Roche, Mannheim, Germany
<u>Substrate buffer (pH=5.5)</u>		
NaH ₂ PO ₄	0.1 M	MERCK KGaA, Darmstadt, Germany
<u>Substrate (diluted in DMSO)</u>		
3,3',5,5'-Tetramethylenbenzidine (TMB)	6 mg/ml	Roth, Karlsruhe, Germany
<u>Developer</u>		
Substrate buffer	10 ml	
TMB	200 µl	
H ₂ O ₂	2 µl (30%)	
<u>Stopper</u>		
H ₂ SO ₄	2 N	MERCK KGaA, Darmstadt, Germany

- ELISA antibodies

Components	Concentration	Dilution	Company
<u>IL-10</u>			
Coating	1 mg/ml	1:500	BD Pharmingen, Heidelberg, Germany
Detection	0.5 mg/ml	1:500	
<u>IFN-γ</u>			
Coating	1 mg/ml	1:250	BD Pharmingen, Heidelberg, Germany
Detection	0.5 mg/ml	1:500	
<u>IgG1</u>			
Coating	0.5 mg/ml	1:500	BD Pharmingen, Heidelberg, Germany
Detection	0.5 mg/ml	1:500	
<u>IgG2a</u>			
Coating	0.5 mg/ml	1:500	BD Pharmingen, Heidelberg, Germany
Detection	0.5 mg/ml	1:500	
<u>TGF-β</u>			
Coating	0.5 mg/ml	1:250	BD Pharmingen, Heidelberg, Germany
Detection	0.5 mg/ml	1:500	

Materials and Methods

- Flow cytometry antibodies

Specificity	Fluorescence	Concentration	Dilution	Company
CD3	PerCP Cy5.5	0.2 mg/ml	1:100	BD Pharmingen
CD4	FITC	0.1 mg/ml	1:400	Caltag/Invitrogen
CD8	PE	0.1 mg/ml	1:400	Caltag/Invitrogen
CD25	APC	0.2 mg/ml	1:400	BD Pharmingen
CD19	FITC	0.1 mg/ml	1:400	Caltag/Invitrogen
F4/80	APC	0.2 mg/ml	1:400	Caltag/Invitrogen
Gr1	PE	0.1 mg/ml	1:400	Caltag/Invitrogen
DX5	PE	0.2 mg/ml	1:400	BD Pharmingen
NK1.1	PE	0.2 mg/ml	1:400	BD Pharmingen
r̢	PE	0.2 mg/ml	1:200	BD Pharmingen

- Freezing solution

Components	Concentration	Company
NaCl	0.72%	SIGMA-ALDRICH CHEMIE GmbH, Munich, Germany
Sorbitol	3.36%	SIGMA-ALDRICH CHEMIE GmbH, Munich, Germany
Glycerine	28%	MERCK KGaA, Darmstadt, Germany

Dilution: ddH₂O

Preparation: solve 0.9 g NaCl and 4.2 g sorbitol in 90 ml ddH₂O. Add 35 ml glycerine.

Storage: -20°C

- Giemsa

Components	Concentration	Company
Giemsa's azur eosin blue solution	-	MERCK KGaA, Darmstadt, Germany
<u>Buffer tablets pH 7.2</u>		MERCK KGaA, Darmstadt, Germany
Na ₂ HPO ₄ ·x2H ₂ O	0.63 g/l	
KH ₂ PO ₄	0.31 g/l	

Dilution (Buffer): ddH₂O

Preparation: One tablet was diluted per liter ddH₂O. 20ml buffer were mixed with 1ml Giemsa's azur eosin blue solution.

Storage: 4°C (buffer), RT (Giemsa's azur eosin blue solution). Giemsa solution was used fresh.

- Hinkelmann solution

Components	Concentration	Company
Eosin	0.5% (w/v)	MERCK KGaA, Darmstadt, Germany
Phenol	0.5% (w/v)	MERCK KGaA, Darmstadt, Germany
Formaldehyde	0.185% (w/v)	MERCK KGaA, Darmstadt, Germany

Dilution: ddH₂O

Storage: 4°C

- Paraformaldehyde solution

Components	Concentration	Company
Paraformaldehyde	4%	MERCK KGaA, Darmstadt, Germany

Dilution: 1xPBS

Preparation: diluted at 56°C.

Storage: RT (fixed worms). Paraformaldehyde solution for fixing cells was used fresh.

- Phenylhydrazine solution

Components	Concentration	Company
Phenylhydrazine	1.2 mg/ml	SIGMA-ALDRICH CHEMIE GmbH, Munich, Germany

Dilution: 1xPBS

Storage: -20°C

- (20x) Phosphate buffered saline (PBS)

Components	Concentration	Company
NaCl	160 g/l	SIGMA-ALDRICH CHEMIE GmbH, Munich, Germany
Na₂HPO₄·2H₂O	23.6 g/l	MERCK KGaA, Darmstadt, Germany
KH₂PO₄	4 g/l	MERCK KGaA, Darmstadt, Germany
KCl	4 g/l	MERCK KGaA, Darmstadt, Germany

Dilution: ddH₂O

Preparation: All components were diluted in H₂O and pH adjusted to 7.0; pH in 1xPBS was adjusted to 7.2.

Storage: RT

- Polymerase chain reaction reagents

Components	Company
Omniscript RT Kit	Qiagen, Hilden, Germany
Oligo-d(T) primer	Invitrogen, Karlsruhe, Germany
DNase I	Ambion, Hamburg, Germany
DNase inactivator	Ambion, Hamburg, Germany
RNAse inhibitor	Peqlab Biotechnologie GmbH, Erlangen, Germany
HotStar Taq polymerase	Qiagen, Hilden, Germany
SYBR Green	Roche, Mannheim, Germany

Materials and Methods

- RNA extraction

Components	Company
TRIzol reagent	Invitrogen, Karlsruhe, Germany
1-Bromo-3-chloropropan	SIGMA-ALDRICH CHEMIE GmbH, Munich, Germany
Isopropanol	MERCK KGaA, Darmstadt, Germany
Ethanol	MERCK KGaA, Darmstadt, Germany

- Supplemented RPMI Medium

Components	Concentration	Company
RPMI 1640		PAA laboratories, Pasching, Austria
Foetal calf serum (FCS)	10%	PAA laboratories, Pasching, Austria
L-Glutamine	2 mM	PAA laboratories, Pasching, Austria
Gentamicin sulphate	50 µl/ml	Cumbrex Bio Science, Walkerville, MD, USA

Preparation: 500ml RPMI medium were supplemented with 50ml FCS inactivated at 56°C for 30', 5 ml 200mM L-Glutamine and 500 µl 50 mg/ml gentamicin sulphate
Storage: -20°C (FCS, L-Glutamine), 4°C (RPMI 1640, gentamicin sulphate)

- Tris-Ammonium chloride

Components	Concentration	Company
NH ₄ Cl	0.1 M	MERCK KGaA, Darmstadt, Germany
C ₄ H ₁₁ NO ₃ (Tris)	0.17 M	Roth, Karlsruhe, Germany

Dilution: ddH₂O

Preparation: 100 ml Tris solution were prepared and pH adjusted to 6.65; and then 900 ml NH₄Cl. Both solutions were mixed and pH adjusted to 7.2.

Storage: 4°C

- Other solutions or products

Components	Company
Isofluran	Abbott GmbH, Wiesbaden, Germany
Methanol	MERCK KGaA, Darmstadt, Germany
Diethyl ether	MERCK KGaA, Darmstadt, Germany
Drabkins solution	SIGMA-ALDRICH CHEMIE GmbH, Munich, Germany
Lyophilized bovine haemoglobin	SIGMA-ALDRICH CHEMIE GmbH, Munich, Germany
Lymphocyte Separation Medium 1077	PAA Laboratories GmbH, Parsching, Austria
Percoll	SIGMA-ALDRICH CHEMIE GmbH, Munich, Germany

2.1.3. Consumables

Components	Company
1.5 ml-test tubes	Eppendorf, Hamburg, Germany
2 ml-test tubes	Eppendorf, Hamburg, Germany
EDTA-coated tubes	KABE Labortechnik GmbH, Nümbrecht-Elsenroth, Germany
15 ml-Falcon tubes, sterile	Greiner bio-one, Frickenhausen, Germany
50 ml-Falcon tubes, sterile	Greiner bio-one, Frickenhausen, Germany
Cryotube vials	Nunc, Roskilde, Denmark
Micro-haematocrit tubes	Brand GmbH, Wertheim, Germany
Neubauer cell-count chamber	Brand GmbH, Wertheim, Germany
Microscope slides	Engelbrecht, Germany
Glass Pasteur pipettes	Brand GmbH, Wertheim, Germany
50ml-syringes	B. Braun Melsungen, Melsungen, Germany
Butterflies	B. Braun Melsungen, Melsungen, Germany
S-Monovette blood collection system 10ml	Sarstedt, Nümbrecht, Germany
Steel beads (tissue homogenization)	PeqLab, Erlangen, Germany
3ml graduated Pasteur Pipettes unsterile	Copan, Brescia, Italy

2.1.4. Equipment

Device	Name
Spectra-photometer Plate reader	Spectra MAX 340PC, MTX lab systems, Virginia, USA
Flow Cytometer	FACS Canto, BD biosciences, Heidelberg, Germany
Cell counter	CASY model DT, Innovatis, Reutlingen, Germany
Centrifuge (cell extractions)	Heraeus Multifuge 4 KR, Kendro Laboratory Products GmbH, Hanau, Germany
Bead grinder	Precellys 24, PeqLab, Erlangen, Germany
RNA measurement	Eppendorf biophotometer, Eppendorf
Thermal cycler (PCR)	Primus, MWG biotech, Ebersberg, Germany
Thermal cycler (Real time-PCR)	RG-3000, Corbett Research, Sydney, Australia
Block Thermostat	HLC HBT-1 131, Progen Scientific Ltd, Mexborough, England
Centrifuge (RNA extraction)	Universal 32 R, Hettich Zentrifugen, Tuttlingen, Germany

2.1.5. Computer software

Action	Program
Data processing	Excel 2007
Statistical analyses	Graph Pad Prism 4.0
FACS	FACS Diva

2.2. METHODS

2.2.1. Animal cycles and infections

2.2.1.1. *Maintenance of Anopheles stephensi mosquitoes*

BALB/c mice were infected with sporozoites extracted from the salivary glands of mosquitoes. *Anopheles stephensi* mosquitoes were bred at the mosquito facility at IMMIP, Bonn, at 28°C and 80% air humidity. Solutions of 10% fructose in water were used for their daily feeding. 25 ml were placed in a 50-ml Erlenmeyer flask, and a scrolled paper was inserted in it to allow the mosquitoes to suck the solution. Sugar solutions were renewed every 2-3 days to avoid microbial contamination. Every mosquito cage was provided with a water glass to maintain air humidity.

Mosquito blood feeding for egg production was done by exposure to mice. 3 Naive NMRI mice were anesthetized (see anestheticum description above), and their ventral fur was shaved prior to exposure to hungered mosquitoes (sugar solution removed 24 hours before). Mice were laid on the mosquito cage for 20 minutes, allowing all the mosquitoes to feed. 2 days after the bloodmeal mosquitoes layed eggs on a water glass, which were then transferred to water cages. After another 2 days, larvae hatched out and were fed with pulverized commercial fish food. 10 to 14 days after the mosquito bloodmeal, emerging pupae were fished out and introduced in a new mosquito cage. Mosquitoes came out after 2 days. Blood feeding of new mosquitoes for a new cycle or for infection was done one week after the last addition of pupae to the cage.

2.2.1.2. *P. berghei infection of mosquitoes*

Frozen stock containing 8×10^7 iRBCs/ml was injected intraperitoneally (i.p.) into 2 naive NMRI mice. 4 days before the planned bloodmeal for the mosquitoes, 3 new naive NMRI mice were injected i.p. with 200 µg phenylhydrazine (10 µg per gram of body weight) to enhance their erythropoiesis. 2 days before mosquito bloodmeal, the mouse with the highest parasitaemia in the first group was bled from the retro-orbital vein, and blood was injected intraperitoneally to the phenylhydrazine-injected mice. 40-48 hours later, parasitaemia was measured and the presence of *P.berghei* gametocytes in blood assessed. Mice were anesthetized, shaved and exposed to hungered mosquitoes as described in the previous section. Immediately after feeding mosquitoes were transferred to an incubator at 21°C and 80% air humidity, optimal for plasmodial development. 5

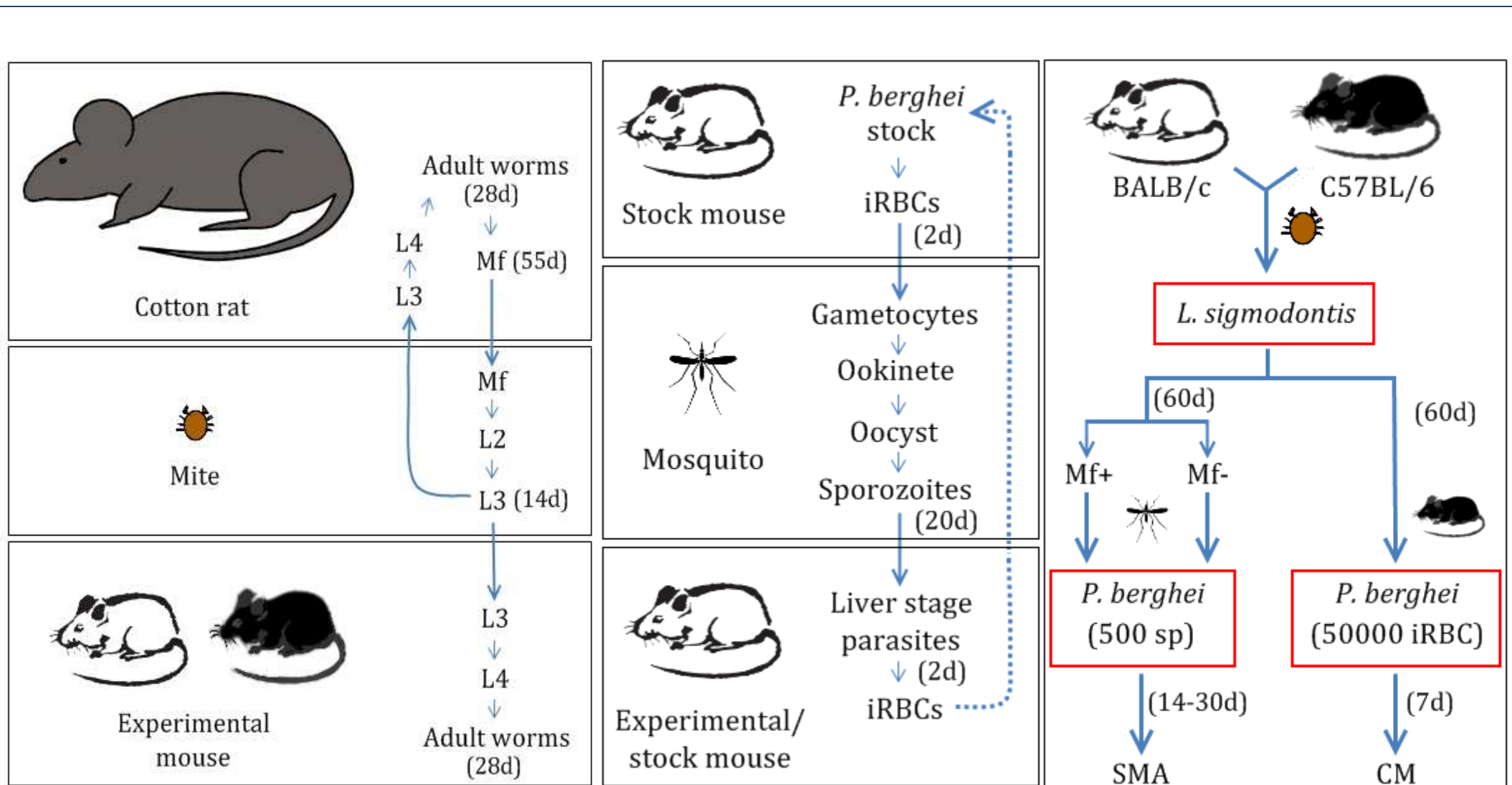


Figure 2.1: Parasite cycles and experimental design. (A) Laboratory cycle of *L. sigmodontis*. (B) Laboratory cycle of *P. berghei*. (C) Design of co-infection experiments. See text for detailed descriptions.

days after blood feeding mosquitoes were fed again with blood from naive mice following the standard protocol to boost parasite development. 10 days after infection 4-5 mosquitoes were anesthetized with diethyl ether and dissected to test the presence of oocysts in their gut, in order to evaluate the quality of the infection. From day 20 after infection mosquitoes were dissected for sporozoite recovery from salivary glands (Fig. 2.1, B).

2.2.1.3. Preparation of *P. berghei* stock

Mosquitoes were infected following the standard protocol (see above). Sporozoites were extracted from their salivary glands 20 days after infection. At least 20000 sporozoites per mouse were injected intravenously in NMRI mice. After 10 days, parasitaemia was assessed. Mice were bled from the retro-orbital vein and the blood diluted with PBS to a concentration of 1.6×10^8 parasites/ml. 250 μ l blood were quickly mixed with 250 μ l of freezing solution (see materials) in cryotube vials and frozen immediately in liquid nitrogen. Stock tubes were further stored in liquid nitrogen.

2.2.1.4. *Litomosoides sigmodontis* lab cycle

Ornithonyssus bacoti mites were maintained by allowing feeding on young mice replaced each 2-3 days. The natural host of *L. sigmodontis* is the cotton rat (*Sigmodon hispidus*), which can bear high Mf (L1 larvae) levels in blood. *Ornithonyssus bacoti* mites were infected through a blood meal on an infected rat, and placed in incubators at 25°C and 80% air humidity for 14 days. 10 days later some mites were dissected to assess infection quality. After 14 days mice were anesthetized and exposed to the infected mites, which transmit L3 larvae through the bloodmeal (Fig. 2.1, A).

2.2.1.5. *P. berghei* ANKA infections

P. berghei ANKA co-infections, either with sporozoites or iRBCs, were done 60 days after *L. sigmodontis* infection (Fig. 2.1, C).

Sporozoite infection

30 mosquitoes were anesthetized with diethyl ether and dissected. Salivary glands were collected in a 1.5 ml Eppendorf tube on ice containing 100 μ l PBS. Salivary glands were homogenized with a plastic mortar to release sporozoites. After assessing their concentration using a Neubauer chamber, sporozoites were diluted to a concentration of 500 per 200 μ l PBS per mouse, and injected intravenously in the lateral tail-vein.

iRBC infection

Frozen stock containing 8×10^7 iRBCs/ml in freezing solution was injected i.p. into 2 naive C57BL/6 mice 5 days before the beginning of the experiment. After assessing their parasitaemia and erythrocyte concentration in blood, a new blood sample was taken from the same mouse and diluted with PBS to the desired concentration of iRBCs for the infection. 200 μ l of the iRBC solution were injected per mouse via the tail vein. Mice were pre-warmed under red light in order to facilitate injections.

2.2.2. Animal manipulations

2.2.2.1. Mouse perfusion

A mouse was anesthetized by intramuscular injection (see above). The mouse's snout was placed within a 15ml-Falcon tube that contained a piece of paper soaked in isofluran until death. Immediately after respiratory arrest, the thoracic cavity was open and a butterfly connected to a 50ml-syringe filled with PBS was put in the left ventricle of the mouse. A cut was applied in the mouse's right auricle. Blood was collected from the pleural cavity with a monovette syringe. 10ml PBS were carefully flushed into the mouse's heart until proper complete perfusion of the liver, noted by colour fading from red to brown.

2.2.2.2. Macrophage depletion

Macrophages were depleted in *L. sigmodontis* co-infected mice by daily intravenous and intrapleural injections of 200 μ l chlodronate liposomes at 250 mg/ml for 4 consecutive days. Chlodronate liposomes were provided by Nico van Rooijen (Vrije Universiteit, Amsterdam, Netherlands).

2.2.2.3. CD4⁺ and CD8⁺ depletion

Depletions were done one day before *P. berghei* infection using 100 μ g anti-CD4⁺ or 50 μ g anti-CD8⁺ antibodies injected intravenously in 200 μ l PBS. Such amounts of antibodies had been shown by flow cytometry to completely eliminate the respective cell types for 7 days (data not shown).

2.2.3. Laboratory methods

2.2.3.1. Assessment of body weight and splenomegaly

Mice were daily weighed using a top-pan electronic balance. For splenomegaly assessment, the spleens were removed and placed in a tube with 10 ml RPMI medium, which was weighted before and after placing the spleen in it.

2.2.3.2. Haematocrit

10 µl tail blood was diluted in 200 µl Drabkins solution and pipetted in standard 96-well Flat-bottom plates. Lyophilized bovine haemoglobin was solved in Drabkins solution at 300 mg/ml and serially diluted to obtain a standard curve. Plates were incubated for 15 minutes at RT, and afterwards absorbance was measured at 570 nm.

2.2.3.3. Serum separation

For measurement of levels of circulating cytokines or immunoglobulins mice were bled every two days from tail vein. 50-60 µl blood were collected per mouse with micro-haematocrit tubes and stored in EDTA-coated tubes. After centrifugation at 8000 rpm for 5 min, supernatants were pipetted to new test tubes and stored at -20°C.

2.2.3.4. Giemsa staining and assessment of parasitaemia

Blood smears were dried and fixed with methanol for 5 minutes. Giemsa solution was prepared with 1 ml of Giemsa's azur eosin blue solution diluted in 20 ml of pH 7.2 buffer. Blood smears were soaked in giemsa solution for 12 to 15 minutes, washed with tap water and let dry before examination.

Samples were observed under the microscope using a 100x immersion objective. 3 fields were counted, containing at least 300 erythrocytes in total. In cases of low erythrocyte numbers due to high parasitaemia additional fields were counted until reaching 300 erythrocytes per slide.

2.2.3.5. Microfilaraemia

50 µl blood were extracted from the tail vein of *L. sigmodontis* infected mice one day before sporozoite co-infection and added to 300 µl of Hinkelman solution in a 0.5 ml test tube. After vortexing, tubes were kept at room temperature for 1 hour. Afterwards, they were centrifuged at

1800 rpm for 5 minutes. Supernatants were discarded with a glass Pasteur pipette, leaving 20-30 μ l on the pellet. Pellets were resuspended in that volume, pipetted on a glass slide and covered with a cover-glass. Mf through the whole preparation were counted under the microscope (20x objective).

2.2.3.6. Cell extraction

Spleen

Spleens were aseptically removed, weighed for splenomegaly assessment and homogenized through a metallic strainer in 10 ml of supplemented RPMI 1640. After centrifugation, pellets were resuspended in 10ml of cold Tris-ammonium chloride solution, incubated 10 min at RT and washed twice with PBS. Membrane debris was removed after the second wash by filtration through a nylon net. Finally, cells were resuspended in supplemented 10ml RPMI medium and kept at 4°C until further use.

Pleura: cell and worm extraction

Pleural cavity of the mice was flushed with 10 ml PBS with a plastic Pasteur pipette to collect cells and worms. The first ml pleural wash was pipetted to a 2 ml test tube, and further 9 ml were pipetted in a 15 ml tube. Cells in the 2 ml test tube were removed by centrifugation and supernatant stored for cytokine measurements. Pellet was resuspended in 1 ml PBS and pooled with the remaining 9 ml pleural wash. Worms were allowed to sediment, separated from the supernatant containing pleural cells and fixed in paraformaldehyde solution. Cells were then filtered through a nylon-screen, centrifuged, resuspended in 2 ml supplemented RPMI medium and counted.

Liver

After aseptic removal and homogenization in supplemented RPMI 1640 medium, cell suspensions were carefully pipetted over 5 ml of Lymphocyte Separation Medium 1077 and centrifuged for 20 minutes at 2000 rpm and 4°C without break. Cells in the interphase between LSM and RPMI medium were transferred to a new tube and washed twice with PBS. Cells were then resuspended in 2 ml supplemented RPMI medium and kept at 4°C until further use.

Brain

Brains were extracted to 15 ml tubes containing supplemented RPMI medium and homogenized to single-cell suspensions through a metallic strainer. After centrifugation supernatant was discarded and 5 ml 35% Percoll added. Tubes were centrifuged for 20 minutes at 1200 rpm and 4°C without break to allow the lymphocytes to sediment. Supernatant was then discarded and cells washed twice before further preparation for FACS analyses.

2.2.3.7. Flow cytometry

250 μ l cells were pipetted in a 96-well U-bottom cell culture plate and centrifuged. After discarding supernatants, Fc receptors were blocked by incubation at 4°C for 10 minutes with rat IgG2 antibodies diluted 1:100 in 50 μ l PBS. Plates were centrifuged, supernatants discarded and cells marked with the corresponding antibody dilution in a total volume of 50 μ l, by incubating at 4°C for 15 min. Non-binding antibodies were removed by two wash steps with PBS and cells were fixed in 4% paraformaldehyde and measured with a BD FACS Canto.

2.2.3.8. Enzyme linked immunosorbent assay

Concentrations of IL-10, IFN- γ were determined by specific two-site enzyme-linked immunosorbent assay (ELISA) using standard protocols: coating antibody was diluted in coating buffer and incubated on ELISA plates overnight at 4°C. After washing with wash buffer, plates were treated with blocking buffer for 1 hour at RT. Samples and standards were pipetted into corresponding wells and incubated overnight at 4°C. After 3 wash steps the biotinylated antibody was added and incubated for 1 h at RT. After addition of the streptavidin solution for 1 h and 5 wash steps, the developing solution containing TMB substrate was applied. Colouring reaction was stopped with sulphuric acid and absorbance measured with a spectra-photometer (λ_1 - λ_2 , being λ_1 =450nm, λ_2 =630nm).

Dilutions: pleura wash samples were used undiluted. Serum samples were diluted 1:10 in PBS/BSA for cytokine measurements. For IgG1 and IgG2a measurements samples were diluted 1:500000 and 1:100000 respectively.

TGF- β ELISA

At the blocking step of the standard ELISA protocol an additional ELISA plate was used to perform HCl-activation of TGF- β : Standards and samples were pipetted to the additional plate and HCl was added to reach a concentration of 0.02 N per well. After 15 min incubation at RT, NaOH was added to a final concentration of 0.02 N. Standards and samples were then transferred from this plate to the standard one, already coated and blocked, and processed normally as described above.

2.2.3.9. Assessment of RNA expression in brain

Extraction of brain RNA

Brain samples were frozen in liquid nitrogen and stored at -80 °C until RNA-isolation. RNA was homogenized in 1 ml TRIzol Reagent using a Precellys 24 grinder. After 5 min incubation at room temperature to permit the complete dissociation of nucleoprotein complexes, the homogenate was supplemented with 0.1 ml 1-bromo-3-chloropropane. Samples were then shaken vigorously for 1 min. The resulting mixture was incubated at RT for 10 min and centrifuged at 12000 g for 20 min at 4°C. Following centrifugation, the colourless upper aqueous phase containing RNA was transferred to a fresh tube. 500 µl Trizol-Reagent and 100 µl 1-Bromo-3-chloropropan were added, and samples shaken for 1 min again and centrifuged at 12000 g for 15 min at 4°C. RNA was precipitated by mixing supernatant with 0.8 ml isopropanol in a new tube. Samples were stored for 1 hour at -20°C and centrifuged at 12000 g for 15 min at 4°C. RNA pellet was washed twice with 1 ml of 75% ethanol, centrifuging at 12000 g for 10 min at 4°C. Ethanol was removed and the pellet dried at 37°C for 3-5 min. Then, the RNA was dissolved in 300 µl DEPC-water. To remove genomic DNA, 5 µl 10x buffer and 1 µl DNaseI were added and incubated for 30 min at 37°C. Quality of extracted RNA was assured by spectrophotometric determination at 260 nm.

Transcription to cDNA

1 µg of total RNA was reverse transcribed with an Omniscript RT Kit according to the manufacturer's instructions with oligo-d(T) primer, binding to the poly-A tail of mRNA. Reagents listed below were combined and vortexed for 5 sec. Template RNA was added and again vortexed for 5 sec. This was followed by 60 min incubation at 37°C to reverse transcribe the template RNA into cDNA. The cDNA was then stored at -20°C for further analysis.

Components	Volume	Final concentration
x10 buffer	2 µl	1x
dNTP mix	2 µl	0.5 mM (each dNTP)
Oligo-dT primer (10 µM)	2 µl	1 µM
RNAse inhibitor (10 units/µl)	1 µl	10 units
Omniscript reverse transcriptase	1 µl	4 units
RNAse free water	variable	
RNA	variable	

Total reaction volumen: 20 µl

Real-time PCR

Real Time PCR reactions were performed in a 20 μ l reaction volume containing the components listed below.

Components	β -actin	ICAM-1
Water	10.8	10.8
x10 buffer	2	2
MgCl ₂ (25 mM)	2.4	0.8
Forward primer (5 μ M)	1.2	1.2
Reverse primer (5 μ M)	1.2	1.2
dNTP mix (40 mM)	0.1	0.1
SYBR Green (1:1000)	0.2	0.2
Hotstar Taq polymerase (250 U/ μ l)	0.1	0.1
DNA	2	2

Total reaction volumen: 20 μ l (numbers denote μ l of the specific component per sample)

Reaction conditions in the light cycler were 15 min at 95°C followed by 45 cycles of 15 s at 94°C, 20 s at 58°C and 20 s at 72°C. Temperature change rates were 20°C/s. Copy numbers were normalized to expression of β -actin in each sample.

Primers

Gen	Forward	Reverse
β -actin	5'- AGA GGG AAA TCG TGC GTG AC- 3'	5' – CAA TAG TGA TGA CCT GGC CGT- 3'
ICAM-1	5'- CCG CAG GTC CAA TTC ACA CT - 3'	5' – TCC AGC CGA GGA CCA TAC AG - 3'

2.2.4. Statistics

A Kolmogorov-Smirnov test for normality was done in order to check data distribution and choose the appropriate test for the statistical analyses.

Parametrically distributed data was analyzed with unpaired T test (2 groups) or one-way ANOVA (more than 2 groups) and Tukey's multiple comparisons test to check differences between single groups. Parametric data were represented as mean \pm SD.

Non-parametrically distributed data were analyzed using Mann-Whitney test (2 groups) or Kruskal-Wallis test (more than 2 groups), plus Dunn's multiple comparisons test to check differences between single groups. Non parametric data were represented as median & range.

3. Results

3.1. BALB/c MICE

3.1.1. Malaria outcomes in co-infected BALB/c mice

3.1.1.1. 500 sporozoites render naive BALB/c mice malaria-positive

In order to resemble natural plasmodial infections, which are known to require very few sporozoites (210), co-infection of BALB/c mice was aimed to be performed using the lowest number of *P. berghei* sporozoites necessary to render naive mice malaria-positive. A preliminary experiment was performed to select the most suitable dose for co-infection. In that experiment different groups of naive mice were infected with a range of 250 to 15000 sporozoites. One out of 6 mice in the group infected with 250 sporozoites was found malaria-negative, but all other mice infected with 500, 5000 or 15000 sporozoites became malaria-positive (Fig. 3.1 A). Therefore, the dose of 500 sporozoites per mouse was considered the threshold for infection in BALB/c mice and further used for co-infection experiments. BALB/c mice cannot survive *P. berghei* infections and all malaria-positive animals succumbed within 27 days of plasmodial infection. No significant differences in survival could be found between groups ($p=0.1365$, logrank test, Fig. 3.1 A).

The course of parasitaemia in malaria-positive mice showed a typical pattern: a slow increase could be observed until day 11 post plasmodial infection (p.p.i.), followed by a steep raise until day 16 p.p.i. and the maintenance of constantly high levels thereafter, which progressively debilitated the mice until they eventually died. Despite of the use of markedly different doses a similar parasitaemia pattern was observed in all mice and no significant differences could be found throughout the experiment ($p>0.05$ for each day p.p.i., Kruskal-Wallis test, Fig. 3.1 B).

3.1.1.2. Prolonged survival in co-infected BALB/c mice

L. sigmodontis infected BALB/c mice were co-infected with 500 *P. berghei* sporozoites at day 60 p.f.i., during the period of Mf release in pleural cavity and hence the highest expected

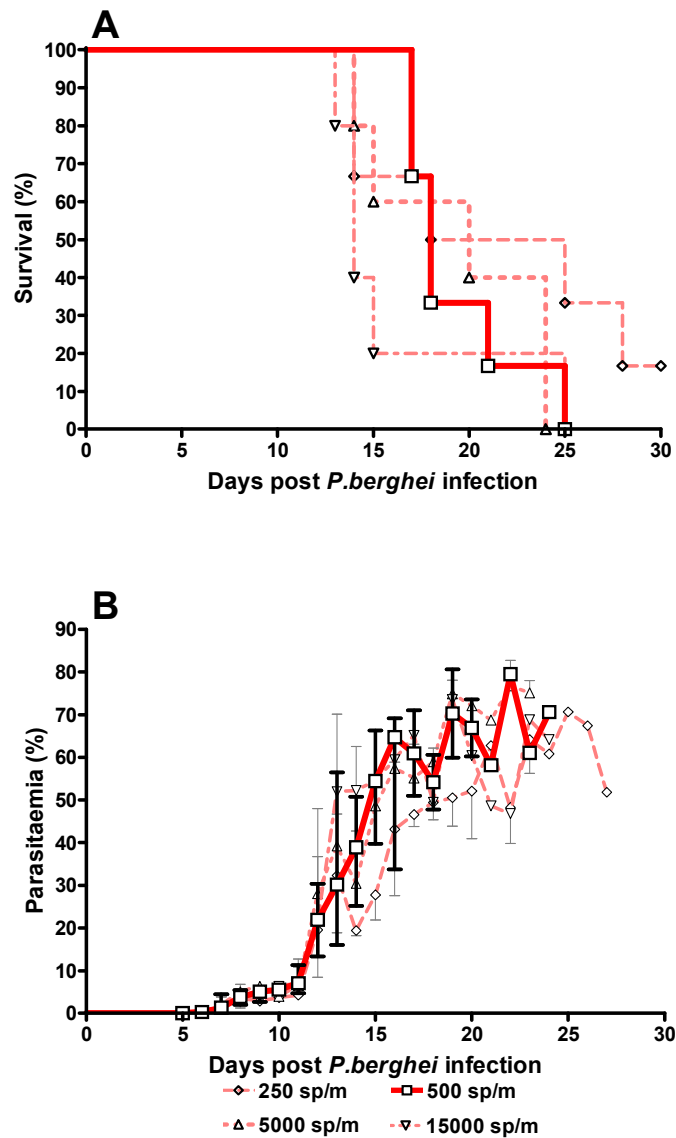


Figure 3.1: Range of *P. berghei* sporozoite infection of BALB/c mice. (A) *Survival*. Naive mice were infected with the indicated parasite doses and monitored for survival. The highlighted survival curve corresponds to mice infected with 500 sp, which was the infection dose elected for further co-infection experiments. No differences in survival were found ($p=0.1365$, logrank test). (B) *Development of *P. berghei* parasitaemia*. Lines represent median of parasitaemia and error bars denote interquartile range. Data corresponding to the group infected with 500 sp/mouse is highlighted. Only one side of the range is shown in the remaining groups for a better clarity of the graphic ($p>0.05$ for each day p.p.i., Kruskal-Wallis test). $n=5-6$ mice per group.

worm-mediated immunomodulation. Variations in survival, *P. berghei* parasitaemia and alterations of different physiological parameters related to malaria severity were monitored in single and co-infected mice after plasmodial infection.

Co-infected BALB/c mice survived *P. berghei* infection significantly longer than their single infected littermates ($p=0.0113$, logrank test, Fig. 3.2 A). Whereas the latter all died within the first 25 days p.p.i. 40% of the co-infected mice survived the whole observation period of the experiment, which was performed until day 40 p.p.i. *L. sigmodontis* infection is not lethal in BALB/c mice, and hence no animal infected only with filariae died.

In order to find out potential reasons for the prolonged survival of co-infected mice, *P. berghei* parasitaemia levels were assessed in the single and co-infected group. 100% of *P. berghei* single infected control mice became malaria-positive, presenting the characteristic parasitaemia pattern described before (Fig 3.1 B). In contrast, co-infected mice could be divided into 2 clear sub-groups: 64.6% of co-infected animals (7 out of 11) had malaria parasites in the blood, and were denoted as malaria-positive, and the remaining 36.4% (4 out of 11) did not show *P. berghei* parasitaemia and were referred to as malaria-negative mice (Fig. 3.2 B). Malaria-positive co-infected mice developed a course of parasitaemia similar to that of their *P. berghei* single infected littermates. At a particular time-point after plasmodial infection these mice showed significantly lower parasitaemia levels than their single infected counterparts (day 15 p.p.i., $p=0.0369$, Mann-Whitney test comparing malaria-positive single and co-infected mice, Fig. 3.2 B). Significant differences in parasitaemia between both groups were consistently found in further co-infection experiments, although the exact day p.p.i. they appeared was variable (see Fig. 3.8 B for cumulative analysis of parasitaemia from 13 independent experiments). Eventually all malaria-positive co-infected mice died. On the other hand, malaria-negative co-infected mice survived the whole experiment, contributing decisively to the prolonged survival observed in the co-infected group (Fig. 3.2 A).

Though consistently present in different independent experiments, the frequency of malaria-free co-infected mice was highly variable. A cumulative analysis of 13 independent co-infection experiments was performed to obtain a more precise estimation of the resistance to malaria in co-infected mice, resulting in a median of 31.7% malaria-negative mice per experiment (interquartile range 18.18-47.73%) compared to 0% in the single infected group (interquartile range 0-4.55%). Differences in percentages of malaria-positive mice between both groups were significant (Mann-Whitney test, $p<0.0001$, Fig. 3.2 C. See also Table 3.2).

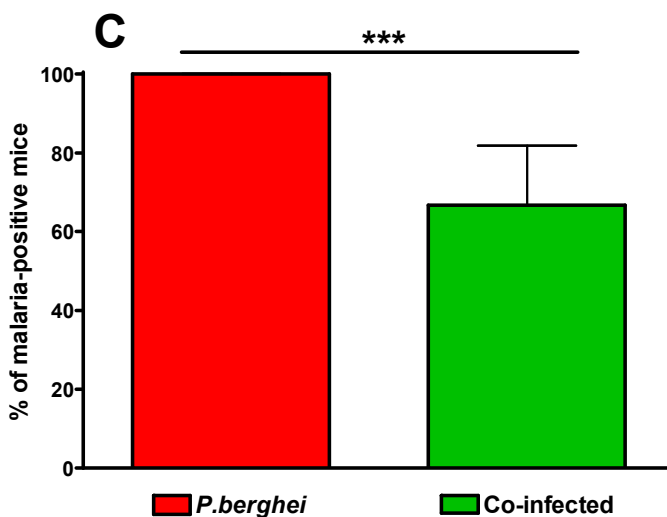
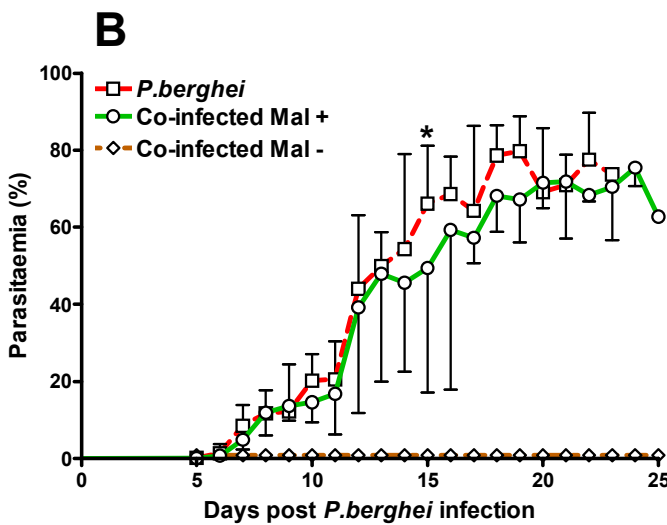
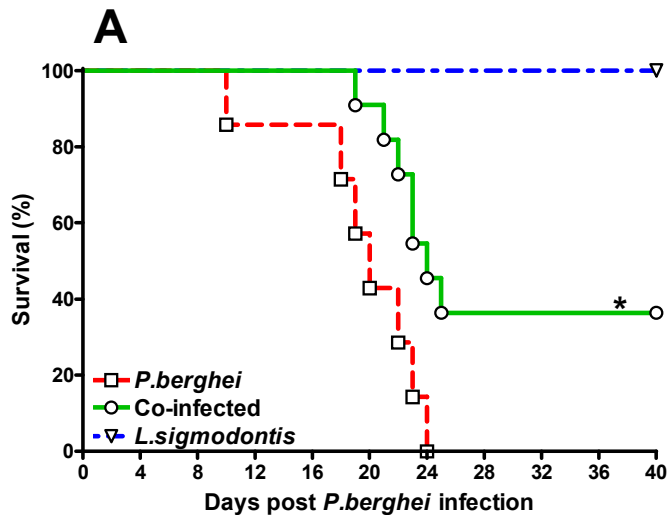


Figure 3.2: Differential malaria outcomes of the co-infection of BALB/c mice. Mice were co-infected with 500 *P. berghei* sporozoites at day 60 after *L. sigmodontis* infection. (A) *Survival*. Differences in survival to *P. berghei* infection in single and co-infected mice were calculated using logrank test (*, $p=0.0113$). Survival of *L. sigmodontis* single infected mice was excluded from the statistical analysis. (B) *Plasmodial parasitaemia*. Blood smears were done daily after *P. berghei* infection. The co-infected group was split into malaria-positive (co-infected Mal+) and malaria-negative mice (co-infected Mal-). Statistical analysis was done between *P. berghei* and co-infected Mal+ mice, using Mann-Whitney test (*, $p<0.05$). Lines and error bars denote median of parasitaemia and interquartile range respectively. Only one tail of the range was shown for a better clarity of the graphic. In A and B, data are representative for 2 independent experiments, illustrating consistently reproducible effects. 7 to 11 mice were used per experiment in each group. (C) *Percentages of malaria-positive single and co-infected mice*. Data were pooled from 13 independent experiments. A Mann-Whitney test was employed for the statistical analysis (***, $p<0.0001$). Columns represent median of percentage of malaria-positive mice per experiment. Error bars denote interquartile range.

Taken together, these data show that the filarial infection enhances the capacity of BALB/c mice to control malaria parasites and prolongs their survival to *P. berghei* malaria.

3.1.1.3. Characterization of malaria-positive and -negative co-infected BALB/c mice

Certain physiological parameters are altered during murine malaria. In order to further characterize the course of *P. berghei* infections in co-infected mice their body weight, hematocrit and spleen size were monitored and compared to single infected controls. Malaria-positive co-infected mice suffered strong reductions in body weight and haemoglobin concentrations as parasitaemia increased, showing no significant differences with their single infected counterparts (Fig. 3.3 A, B). Significant drops in body weight could be measured from day 10 p.p.i. in both groups compared to naive or *L. sigmodontis* single infected mice (*, $p < 0.05$, one-way ANOVA and Tukey's multiple comparison test, Fig. 3.3 A), whereas significant decreases in hematocrit became evident from day 12 p.p.i. (*, $p < 0.05$, one-way ANOVA and Tukey's multiple comparison test, Fig. 3.3 B). In contrast, malaria-negative co-infected mice presented unaltered values of both variables during the experiment, showing no significant differences with *L. sigmodontis* single infected and naive mice ($p > 0.05$, one-way ANOVA and Tukey's multiple comparison test, Fig. 3.3 A and B). As with the case of human patients infected with *P. falciparum* the spleen also increases in size during murine malaria (1, 153). At day 14 p.p.i. spleen size was comparable in *P. berghei* single infected and malaria-positive co-infected mice, and markedly higher than the rest of the groups (*, $p < 0.05$, one-way ANOVA and Tukey's multiple comparison test, Fig 3.3 C). As occurred for body weight and splenomegaly, malaria-negative co-infected mice presented similar values of spleen size than *L. sigmodontis* single infected and naive controls ($p > 0.05$, one-way ANOVA and Tukey's multiple comparison test, Fig 3.3 C). Thus, malaria-positive co-infected mice apparently followed a disease course similar to that of single infected mice, whereas malaria-negative co-infected mice resembled worm infected and naive mice, showing no evidence of being infected with malaria parasites.

Natural *L. sigmodontis* infections performed through the exposition of mice to infected mites entail a high variation in infection loads that each single mouse receives, potentially accounting for the different malaria outcomes observed in co-infected mice. Therefore the number of worms harboured by co-infected animals in each experiment (Fig. 3.3 C) was evaluated. Similar worm numbers ($p = 0.8513$, Mann-Whitney test) were found in malaria-positive and -negative mice (median of 12 with interquartile range 8-23 and 9 with interquartile range 5-21 respectively).

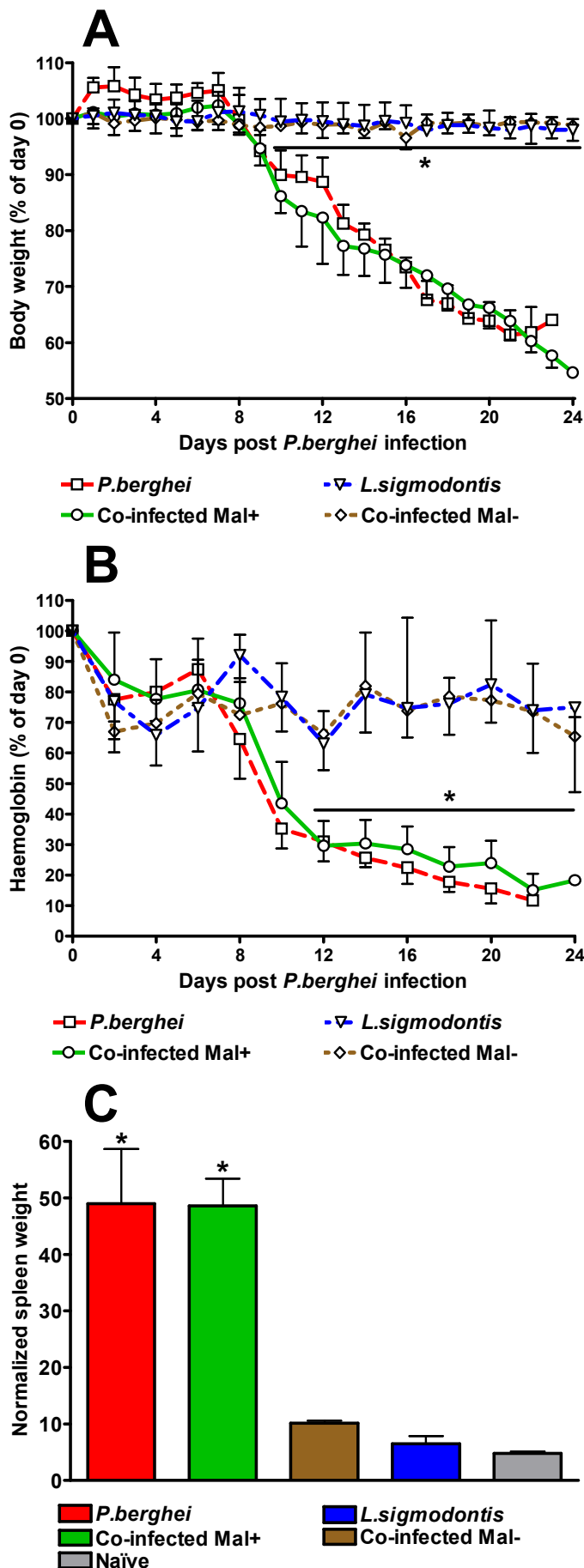


Figure 3.3: Characterization of vitality parameters after *P. berghei* co-infection of BALB/c mice. Mice were co-infected with 500 *P. berghei* sporozoites at day 60 after *L. sigmodontis* infection and indicated parameters evaluated thereafter. (A) *Body weight variations*. Lines and bars represent mean of % of initial body weight \pm SD of 7 mice. (B) *Hematocrit variations*. Lines and bars represent mean of % of initial haemoglobin concentration \pm SD of 7 mice. Naive control mice in A and B showed a similar behaviour than their *L. sigmodontis* single infected littermates and were not included in the present illustration. (C) *Splenomegaly*. Mice were sacrificed at day 14 p.p.i. for spleen recovery. Data were normalized to total mouse body weight at the same day following the formule (Spleen weight/total body weight)*1000. Columns and error bars indicate mean normalized spleen weight \pm SD of 7 mice. Comparisons between groups in A-C were done by one-way ANOVA and Tukey's multiple comparisons test (*, $p < 0.05$). Data are representative for 2 independent experiments.

3.1.2. Analysis of malaria-negative co-infected BALB/c mice: protective effects of filarial infections on the liver stage of malaria

Results concerning the analyses of those co-infected mice that remained malaria-negative after co-infection with 500 *P. berghei* sporozoites (Fig. 3.2 A-C) are shown in the following section.

Given the different stages of the malaria cycle in the mouse, several possibilities could be hypothesized to explain absence of plasmodial infection in some co-infected mice: a) blockage of sporozoite entrance in the liver; b) elimination of malaria parasites during their development inside hepatocytes, or c) once liver stage is completed, control of *P. berghei* parasitaemia. Different experimental approaches exposed below were undertaken to evaluate the suitability of these hypotheses to the co-infection model.

3.1.2.1. Malaria protection is not mediated by antibodies

Immune defense mechanisms against *L. sigmodontis* worms involve the generation of specific antibodies (2), and the presence of cross-reactive antibodies between helminth worms and plasmodial parasites has been reported before (194). Thus, antibodies synthesized against *L. sigmodontis* might cross-react with proteins on the surface of *P. berghei* sporozoites and block their entrance in the liver. To ascertain this possibility, μ MT mice, which are homozygous mutant mice with a targeted disruption of the membrane exon of the immunoglobulin μ chain gene and therefore deficient in mature B cells, were co-infected with *P. berghei* at day 60 after *L. sigmodontis* infection. B cell KO mice presented an equivalent degree of resistance to malaria sporozoites ($p=0.0078$, Fisher's exact test, Table 3.3) than their wild type counterparts ($p=0.001$, Fisher's exact test, Table 3.3). Furthermore, survival to *P. berghei* was significantly prolonged in wild type and B cell deficient co-infected mice compared to their respective single infected counterparts (*P. berghei* wt vs. co-infected wt; ***, $p<0.001$; *P. berghei* μ MT vs. co-infected μ MT; **, $p=0.0078$, logrank test, Fig. 3.4).

Malaria protection in co-infected mice was hence exerted at a later stage, either during intra-hepatic development or against the blood stage of the parasite.

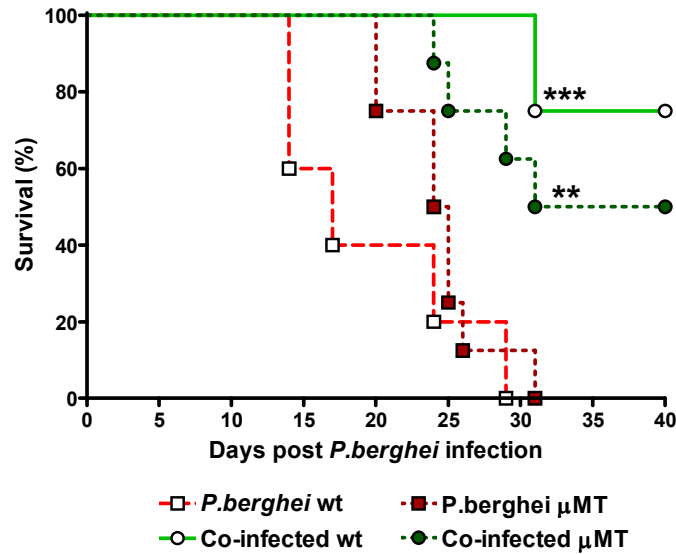


Figure 3.4. Survival of co-infected B-cell deficient μ MT mice. Naive or *L. sigmodontis* infected wild type BALB/c and μ MT mice (BALB/c background) were infected with 500 *P. berghei* sporozoites 60 days p.f.i. Statistical analyses of survival were performed using the logrank test (*P. berghei* wt vs. co-infected wt; ***, $p < 0.001$; *P. berghei* μ MT vs. co-infected μ MT; **, $p = 0.0082$). $n = 8$ mice per group. Data are representative for 2 independent experiments.

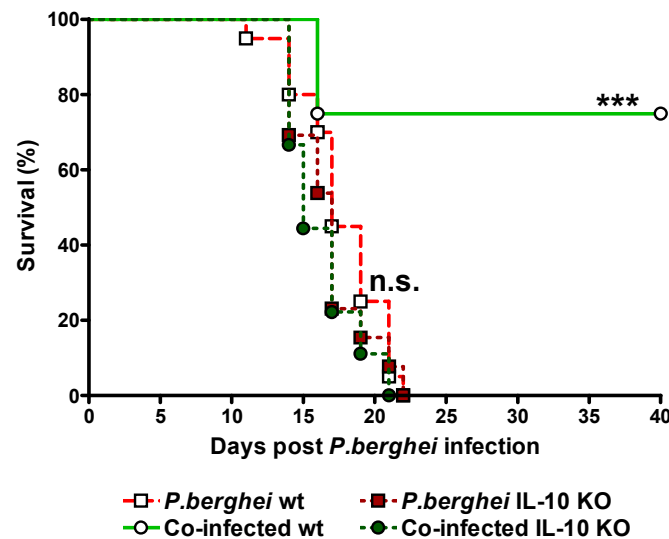


Figure 3.5. Survival of IL-10 KO co-infected mice. Naive and *L. sigmodontis* single infected wild type BALB/c and IL-10 deficient mice (BALB/c background) were infected with 500 *P. berghei* sporozoites at day 60 p.f.i. Significance was calculated using the logrank test (*P. berghei* wt vs. co-infected wt, ***, $p = 0.0008$; *P. berghei* IL-10 KO vs. co-infected IL-10 KO, n.s., $p = 0.4820$)

3.1.2.2. *Protective mechanisms are not principally directed against blood stage malaria parasites*

The presence of malaria-negative co-infected mice after blood smear examination could not be directly attributed to the development of complete sterile immunity against the parasites at this point, since these mice could have been able to control parasitaemia to levels below detection limit by microscopical observation, but still harbour blood stage malaria parasites. Such phenomenon has been described in human and murine malaria (257, 268). Therefore, to ascertain their real malaria status 500 μ l blood from each of 19 malaria-negative co-infected mice from 2 independent experiments were transferred i.p. to a respective naive mouse at day 9 p.p.i. Given that a naive mouse contains approximately 9×10^9 RBCs/ml blood, the amount of RBCs transferred was 4.5×10^9 . As a result, only 1/19 mice (5.26%) became positive for *P. berghei* blood stage parasites, indicating that the majority of malaria-negative co-infected mice are not cryptically infected (Table 3.1).

Experiment	Blood transfer (μ l)	Total mice	Malaria-positive recipient mice	% malaria-positive recipient mice
1	500	7	1	14.28
2	500	12	0	0
Total		19	1	5.26

Table 3.1: Malaria status of recipient BALB/c mice after blood transfer from malaria-negative co-infected donor mice. Mice were co-infected with 500 *P. berghei* sporozoites at day 60 after *L. sigmodontis* infection and malaria status assessed 9 days after *P. berghei* infection. Blood from each co-infected mouse proven malaria-negative was injected intraperitoneally the same day p.p.i. into a naive recipient. Malaria status of recipient mice was assessed at day 9 after blood transfer.

Thus, the mechanism responsible for generating malaria-protection in co-infected mice was very likely to occur during the liver stage of *P. berghei*.

3.1.2.3. *Malaria protection is related to microfilaraemia*

After maturation inside the thoracic cavity of BALB/c mice, female *L. sigmodontis* worms start releasing Mf from day 55 p.f.i. on. However, around 50% of the mice remain amicrofilaraemic during this period. Since Mf are known to stimulate potent immune responses (252), data pooled from 13 independent experiments were analysed to assess the relevance of microfilaraemia in the generation of malaria protection in the liver of co-infected mice (Table 3.2). As a result, malaria-

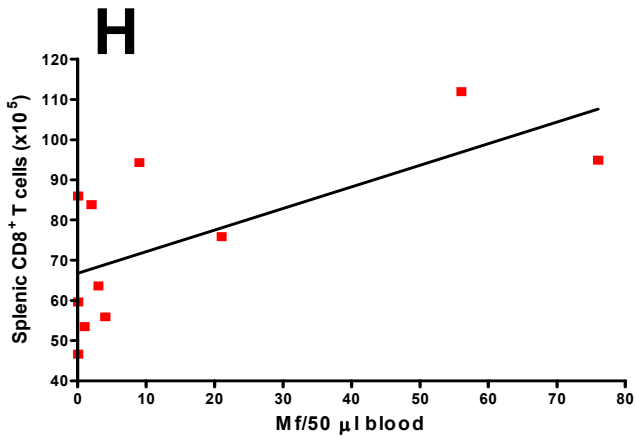
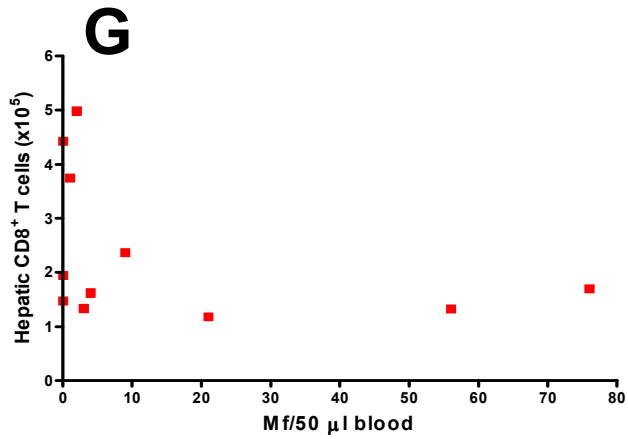
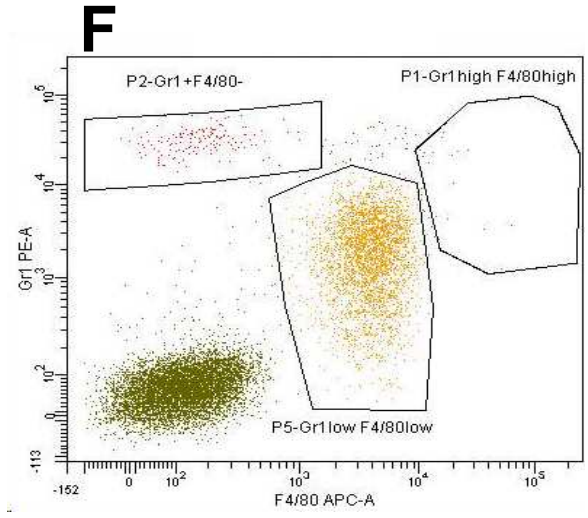
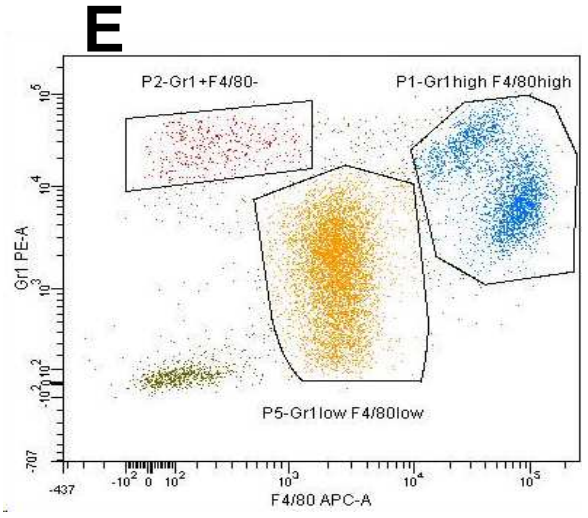
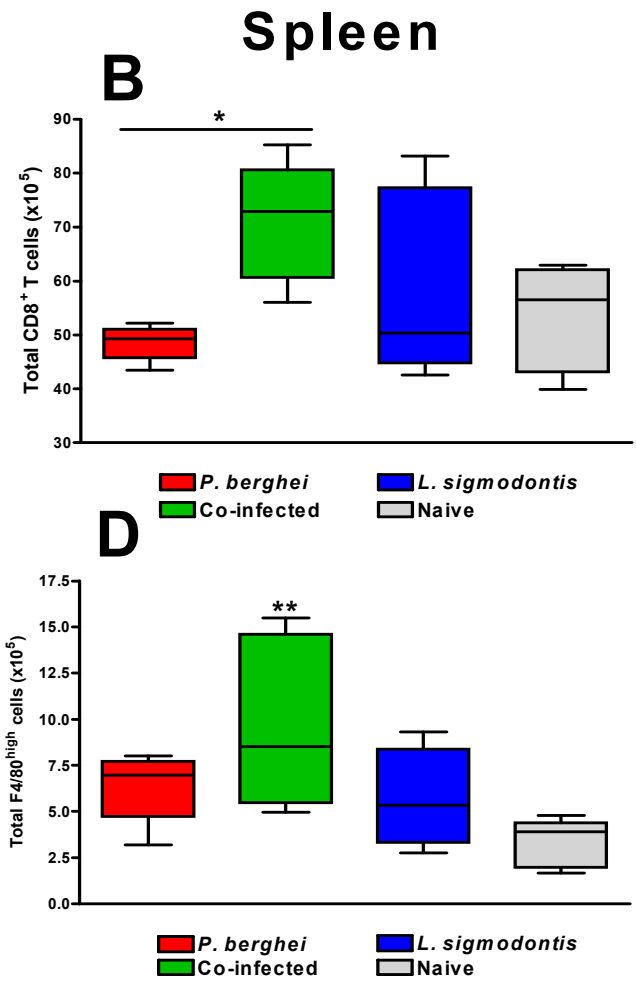
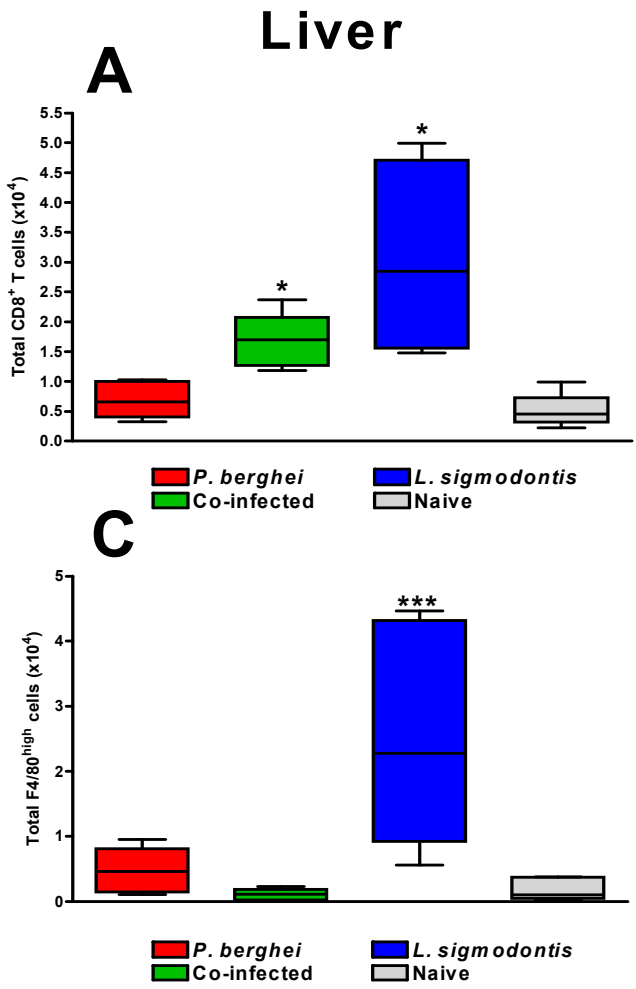


Figure 3.6 (see next page)

Figure 3.6 (see previous page): Cell distributions in the liver 40 hours after *P.berghei* co-infection of BALB/c mice. Mice were co-infected with 500 *P. berghei* sporozoites at day 60 after *L. sigmodontis* infection and sacrificed 40 hours later. Total amounts of CD8⁺ T cells and macrophages in liver (A and C respectively) or spleen (B and D respectively). Data were compared using one-way ANOVA test together with the Tukey's multiple comparison test (*, $p < 0.05$). Asterisks over the columns denote significance against naive mice. Data are representative for 2 independent experiments. Columns and error bars represent mean of cell numbers \pm SD. $n=7$ mice per group. Pleural cells from a non-depleted (E) and a macrophage-depleted mouse (F) infected with *L. sigmodontis*. Absence of these cells in the second figure demonstrates that Gr1^{high}F4/80^{high} cells are macrophages. Correlation of micro-filaraemia and CD8⁺ T cells in liver (G) and spleen (H). Significance was calculated using Spearman correlation ($r = -0.4679$, $p = 0.1467$ in liver; $r = 0.6422$, $p = 0.0331$ in spleen).

negative co-infected mice were clearly associated to the presence of microfilaraemia (26 out of 53 mice), whereas most of the amicrofilaraemic mice (51 out of 62 mice) were malaria-positive ($p = 0.0006$, Fisher's exact test, Table 3.2).

Group	<i>P. berghei</i> infection	Total mice	Malaria-negative	% malaria-negative	<i>p</i> -value
<i>P. berghei</i>	500 sp	108	5	4.62	
Co-infected	500 sp	115	37	32.17	<0.0001 ^a
Co-infected					
Mf+	500 sp	53	26	49.05	
Mf-	500 sp	62	11	17.74	0.0006 ^b

Table 3.2. Malaria status in co-infected BALB/c mice regarding microfilaraemia.

^a Comparisons were done by Fisher's exact test. Data were pooled from 13 independent experiments.

^b Co-infected group was sub-divided regarding microfilaraemia. Comparisons were done by Fisher's exact test.

3.1.2.4. IL-10 is required for malaria protection in co-infected mice

Since microfilaraemia was clearly related to protection in co-infected mice and a key cytokine involved in Mf survival in blood is IL-10 (151), IL-10 deficient BALB/c mice were used in order to assess the relevance of this cytokine in the present model. Unlike the wild type control group, in which usual rates of malaria-negative mice arose ($p = 0.0341$, Fisher's exact test, Table 3.3), co-infected IL-10 KO mice showed no malaria protection and became all positive,

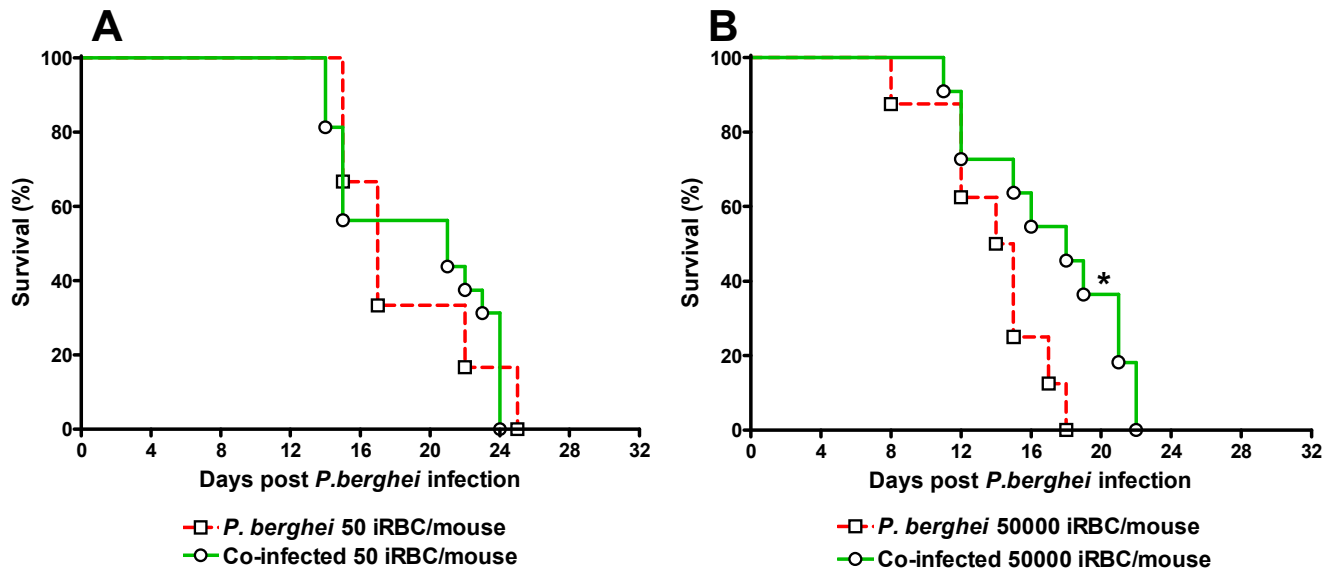


Figure 3.7. Survival to iRBC co-infection of BALB/c mice. Naive and *L. sigmodontis* single infected mice were co-infected with (A) 50 or (B) 50000 iRBC at day 60 p.f.i. Survival was analysed by logrank test (*P. berghei* 50 iRBC vs. Co-infected 50 iRBC, $p=0.9453$; *P. berghei* 50000 iRBC vs. Co-infected 50000 iRBC, *, $p=0.0355$). $n=8$ mice per group. Data representat 2 independent experiments.

Mice	Group	<i>P. berghei</i> Infection	Malaria-negative/ Total no. of mice	% Malaria-negative	Total no. of experiments	<i>p</i> -value
Wt	<i>P. berghei</i>	500 sp	5/108	4.62	13	
Wt	Co-infected	500 sp	37/115	32.17	13	<0.0001 ^a
	Mf+	500 sp	26/53	49.05		
	Mf-	500 sp	11/62	17.74		0.0006 ^b
Wt	Blood tr. [#]	-	18/19	94.73	2	
μ MT	<i>P. berghei</i>	500 sp	2/15	13.3	2	
μ MT	Co-infected	500 sp	10/15	66.6	2	0.0078 ^c
IL-10 KO	<i>P. berghei</i>	500 sp	0/15	0	2	
IL-10 KO	Co-infected	500 sp	0/15	0	2	n.s. ^d
Wt	<i>P. berghei</i>	50000 iRBC	0/14	0	2	
Wt	Co-infected	50000 iRBC	0/14	0	2	n.s. ^e
Wt	<i>P. berghei</i>	50 iRBC	0/14	0	2	
Wt	Co-infected	50 iRBC	0/28	0	2	n.s. ^f

Table 3.3 (see next page)

Table 3.3 (see previous page): Summary of experiments for the characterization of the mechanism of malaria protection in co-infected BALB/c mice. Mice were co-infected with indicated *P. berghei* stages at day 60 after *L. sigmodontis* infection.

Transfer of 500 µl blood from each malaria-negative co-infected mouse to a naïve animal.

^a One-tailed Mann-Whitney test of percentages of malaria-positive single and co-infected mice from 13 independent experiments.

^b Fisher's exact test contrasting numbers of a- or microfilaraemic co-infected mice against malaria status, positive or negative.

^c Fisher's exact test of B cell-deficient mice contrasting malaria status and type of infection (protection of co-infected Wt control mice in the corresponding experiments, $p=0.001$)

^d Fisher's exact test of IL-10 deficient mice contrasting malaria status and kind of infection (protection of co-infected Wt control mice in the corresponding experiments, $p=0.0341$)

^{e,f} Fisher's exact test contrasting malaria positive mice in the single or the co-infected group, for the indicated *P. berghei* iRBC dose.

like their single infected littermates ($p>0.999$, Fisher's exact test, Table 3.3). As a consequence, survival of wild type co-infected mice was significantly prolonged compared to their single infected littermates (***, $p=0.0008$, logrank test, Fig. 3.5) whereas single and co-infected IL-10 KO mice presented no differences in this parameter ($p=0.4820$, logrank test, Fig. 3.5). This demonstrated an implication of IL-10 in the generation of malaria-negative co-infected mice.

3.1.2.5. Co-infection alters CD8⁺ T cell distributions in spleen and liver

Data so far (Fig. 3.4 and Table 3.1) suggest that the major mechanisms mediating malaria protection in co-infected mice were elicited during the liver stage of *P. berghei*. To further investigate this hypothesis immune cell distributions in the liver were analysed in single and co-infected mice 40 hours after *P. berghei* infection with 500 sporozoites. At this time point malaria parasites were close to completing their intrahepatic development. Total CD8⁺ T cells were found to be increased in co-infected and *L. sigmodontis* single infected mice compared to controls, whereas no such increase could be observed in *P. berghei* single infected mice. (*, $p<0.05$, one-way ANOVA and Tukey's multiple comparison test, Fig. 3.6 A). In contrast, macrophages were increased only in *L. sigmodontis* single infected mice, but not in co-infected ones (*, $p<0.05$, one-way ANOVA and Tukey's multiple comparison test, Fig. 3.6 C). Macrophages were defined as Gr1^{high}F4/80^{high} cells, as assessed by depletion with chlodronate liposomes (Fig. 3.6 E, F).

Immune cell distributions were also assessed in spleen, a paradigmatic organ for generation of T cell responses. Compared to *P. berghei* single infected control mice, a significant increase in CD8⁺

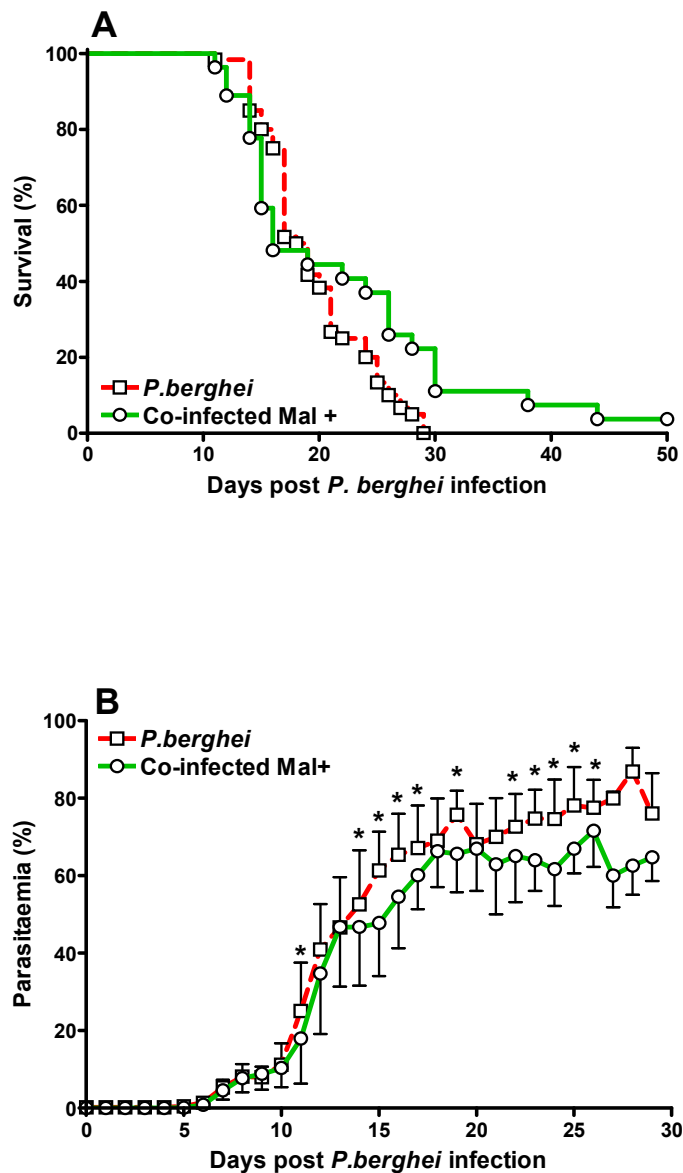


Figure 3.8: Cumulative analyses of survival and parasitaemia of single and co-infected BALB/c mice. Mice were co-infected with 500 *P. berghei* sporozoites at day 60 after *L. sigmodontis* infection. (A) *Survival*. Statistical analyses were done using logrank test ($p=0.1016$, analysis of the survival curve), or Fisher's exact test ($p=0.007$, analysis of contingency table in which mice were divided into those surviving further than day 30 p.p.i. or not. Only 4 co-infected mice (2 Mf+ and 2 Mf-) survived longer than day 30 p.p.i.). (B) *Plasmodial parasitaemia*. Statistical analysis was done using unpaired student t test (*, $p<0.05$). Data were pooled from 8 independent experiments.

T cells was found in the spleen of co-infected mice (*, $p < 0.05$, one-way ANOVA and Tukey's multiple comparison test, Fig. 3.6 B). Moreover, co-infected mice presented a significantly increased number of macrophages in the spleen compared to naive mice (**, $p < 0.01$, one-way ANOVA and Tukey's multiple comparison test, Fig. 3.6 D).

Since microfilaraemia has been linked to malaria resistance in co-infected mice and anti-malarial immune responses in the liver, elicited by CD8⁺ T cells (209), these cells were hypothesized to be relevant in this process, the relation between microfilarial loads in blood and CD8⁺ T cell numbers was analysed. Interestingly, microfilaraemia levels correlated with CD8⁺ T cell numbers in spleen ($p = 0.0331$, Spearman correlation, $r = 0.6422$, Fig. 3.6 H) but not in the liver of co-infected mice ($p = 0.1467$, Spearman correlation, $r = -0.4679$, Fig. 3.6 G). Thus, microfilaraemia seems to favour the increase in CD8⁺ T cells in the spleen.

3.1.2.6. Parasitaemia control at subpatent levels is conditioned to parasite passage through the liver

In light of the last results the major mechanisms implicated in malaria protection upon sporozoite co-infection seem to operate in the liver. Nevertheless, the blood transfer experiment from malaria-negative co-infected mice to naive counterparts described before (Table 3.2) showed that effective control of parasitaemia to levels undetectable by blood smear examination was achieved by some co-infected mice. Such capacity has never been reported before in *P. berghei* infected mice. In order to know the approximate amount of parasites within the infectious blood of donor mice naive BALB/c mice were intraperitoneally infected with varying doses of iRBCs (Table 3.4).

Infection dose (iRBC)	Total mice	Malaria-positive	% malaria-positive
50000	10	10	100
5000	10	10	100
500	10	10	100
50	10	10	100
5	10	6	60
1	10	3	30

Table 3.4: Range of *P. berghei* iRBC infection of BALB/c mice. Naive mice were injected intraperitoneally with the indicated amount of infected erythrocytes, and malaria status was assessed at day 12 p.p.i. in Giemsa stained blood smears.

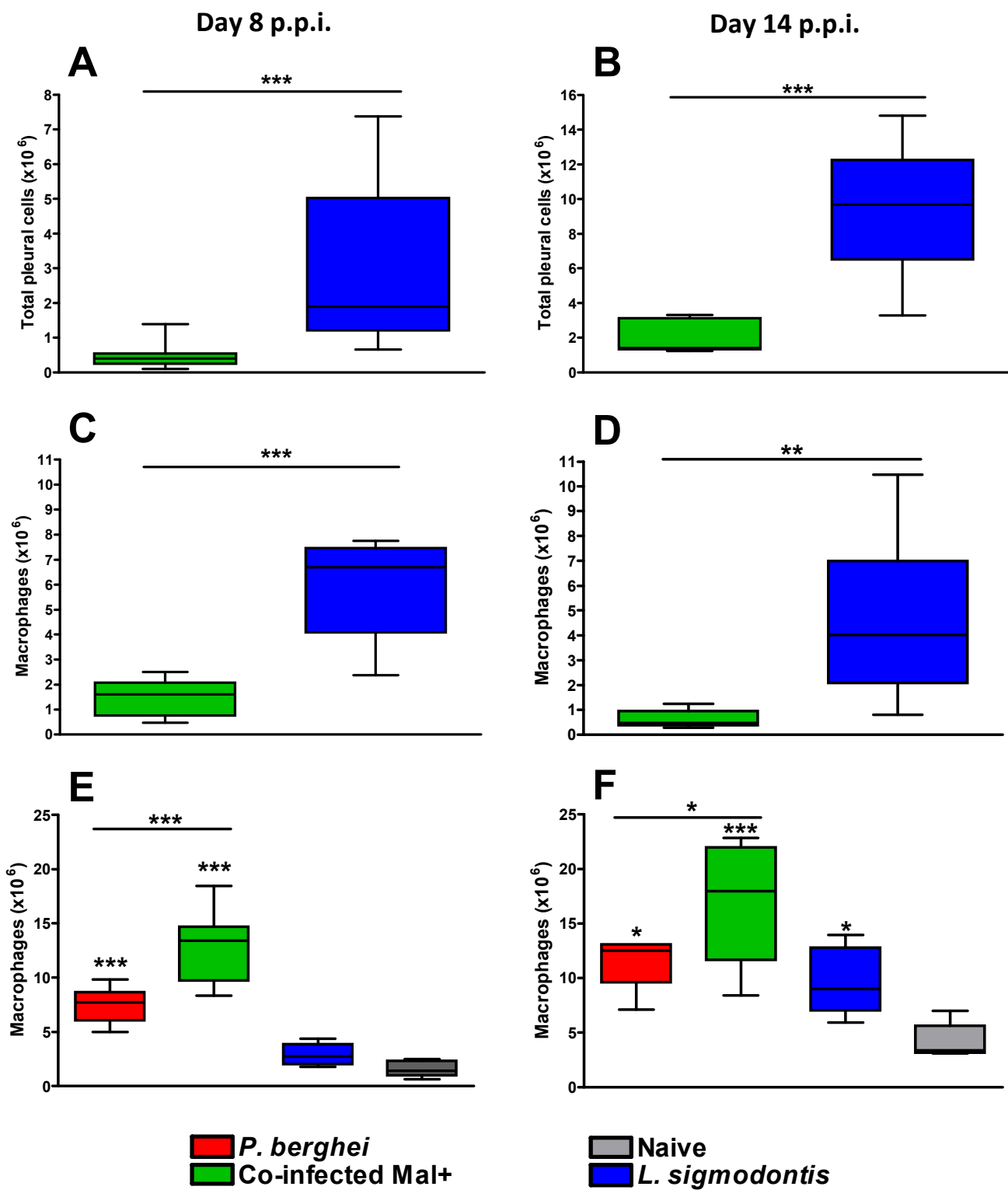


Figure 3.9: Cell distributions in pleural cavity and spleen after *P. berghei* co-infection of BALB/c mice. Mice were co-infected with 500 *P. berghei* sporozoites at day 60 after *L. sigmodontis* infection and sacrificed at days 8 or 14 after *P. berghei* infection. Total cells (A, B) and macrophage numbers in pleural cavity (C, D), and macrophage numbers in spleen (E, F) at the respective time points are depicted. In A-D scarce pleural cells in *P. berghei* single infected and naive mice are not shown. Significance was analysed using Mann-Whitney tests (A-D) or Kruskal-Wallis tests complemented with Dunn's multiple comparison test (E, F). 6 to 10 mice were used per group. Asterisks over columns denote significance with naive mice (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

50 iRBC/mouse was the lowest dose rendering all BALB/c mice positive, and was therefore the minimum amount of iRBCs that the mentioned donor mouse might have had. 50 iRBC contained in 500 μ l blood of the donor mouse corresponds to a parasitaemia of $1.11 \times 10^{-6}\%$, or 1 parasite per 100 million erythrocytes.

To further define the capacity of control of blood stage malaria parasites in double infected mice a new experiment was performed in which co-infections were done directly with iRBCs, hence skipping the malaria passage through the liver. A low dose of 50 iRBC per mouse, markedly lower than the theoretical number of merozoites released by one single sporozoite successfully completing the liver stage, estimated in 8000 to 10000 merozoites (81), and a standard dose of 50000 iRBCs, closer resembling the amount of merozoites arising from the liver in BALB/c mice, were used. All mice co-infected with either dose developed high parasitaemia and died ($p > 0.999$, Fisher's exact test, Table 3.3), being unable to hold blood infection under control. These results further underline the major importance of protective mechanisms actuating against liver stage than blood stage malaria parasites in negative co-infected mice. They also indicate that parasitaemia control at undetectable levels in malaria-negative co-infected mice might require parasite passage through the liver. Nevertheless, survival of BALB/c mice co-infected with 50000 iRBC but not with 50 iRBC was significantly longer than that of their respective single infected littermates (*P. berghei* 50 iRBC vs. Co-infected 50 iRBC, $p = 0.9453$, logrank test, Fig. 3.7 A; *P. berghei* 50000 iRBC vs. Co-infected 50000 iRBC, *, $p = 0.0355$, logrank test, Fig. 3.7 B), showing that additional beneficial mechanisms operate in co-infected mice during the patent blood stage of malaria.

3.1.3. Analysis of malaria-positive co-infected BALB/c mice: protective effect of filarial infections on the blood stage of malaria

As shown in Figs. 3.2 A-C a percentage of co-infected mice developed detectable plasmodial parasitaemia after challenge with 500 *P. berghei* ANKA sporozoites. The present section describes the analyses performed on such malaria-positive co-infected mice, and hence to the effects of worm infections on the blood stage of malaria in BALB/c mice.

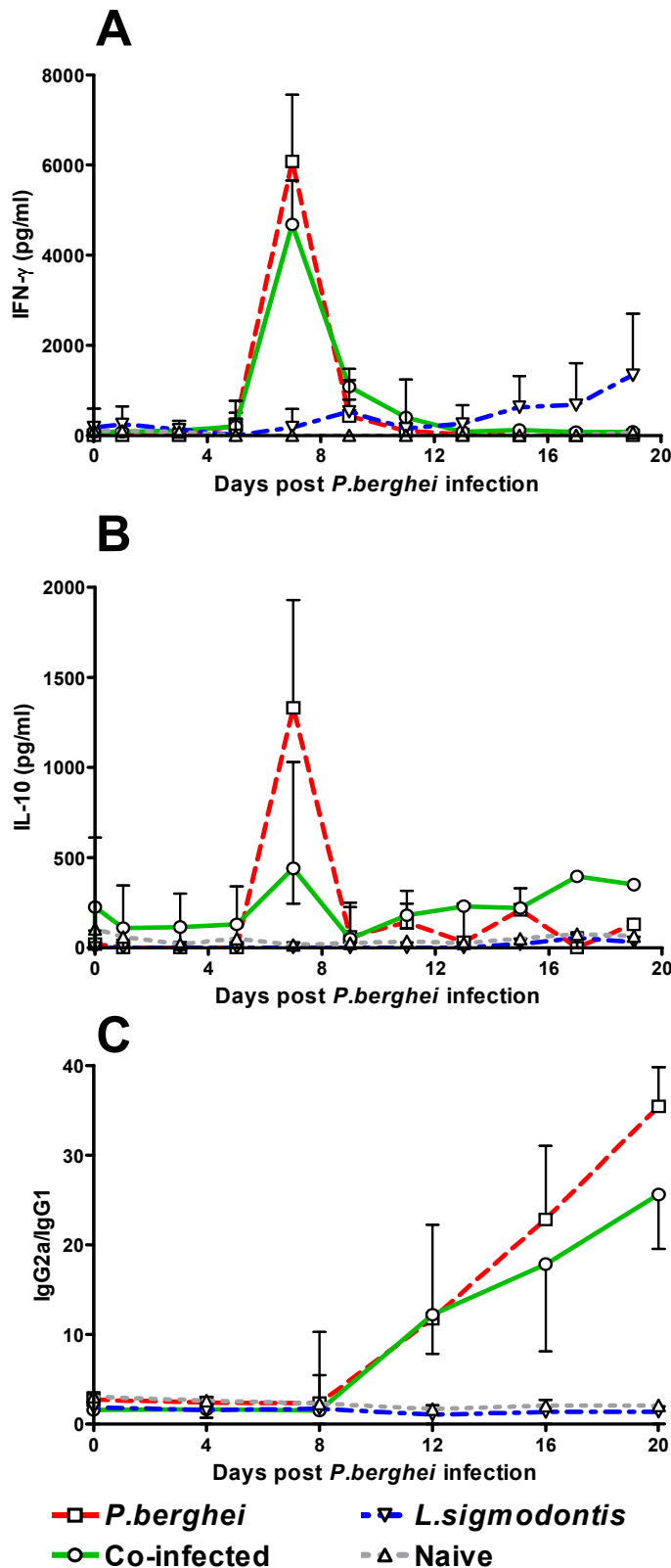


Figure 3.10: Courses of relevant circulating cytokine and immunoglobulin levels after *P. berghei* co-infection of BALB/c mice. Mice were co-infected with 500 *P. berghei* sporozoites at day 60 after *L. sigmodontis* infection and (A) IL-10, (B) IFN- γ and (C) ratio of IgG2a and IgG1 are represented. Cytokine and immunoglobulin concentrations were measured by ELISA in plasma samples taken daily. Data represent 2 independent experiments. Lines and error bars represent median of cytokine levels and interquartile range respectively of 5 to 7 mice. Only one side of the range was shown for a better clarity of the graphic. Statistical analyses were done using Kruskal-Wallis tests complemented with Dunn's multiple comparison test to specifically compare *P. berghei* single and co-infected mice. No significant differences were found ($p > 0.05$). Significance against *L. sigmodontis* and naive mice is not shown.

3.1.3.1. Prolonged survival and reduced parasitaemia in malaria-positive co-infected mice

After the single infection of 500 *P. berghei* sporozoites no naive BALB/c mouse survived longer than 30 days, a mark which was reached by some of their malaria-positive co-infected counterparts. For the statistical analysis of survival, animals in the single and co-infected group from different independent experiments were classified regarding their capacity to survive *P. berghei* infection below or beyond 30 days. As a result, a significantly increased number of long-surviving mice was found in the co-infected group compared to the single infected controls ($p=0.007$, Fisher's exact test, Fig. 3.8 A). Indeed numbers of co-infected mice presenting a prolonged survival were significantly higher compared to single infected from day 26 p.p.i. ($p=0.0203$, Fisher's exact test, Fig. 3.8 A).

Consistent differences in parasitaemia levels between single and co-infected mice had been regularly observed in each independent experiment (Fig. 3.2 B), but presenting high variability in intensity and frequency between experiments. Therefore a cumulative analysis of parasitaemia in single and double infected mice was done in data pooled from different independent experiments (Fig. 3.8 B). Parasitaemia levels were found lower in co-infected mice at late stages of the infection, namely day 11 and from day 14 p.p.i. onwards (*, $p<0.05$, unpaired T test, Fig. 3.8 B). However, the course of parasitaemia in both groups of mice during the early stage of the plasmodial infection, until day 10 p.p.i., presented no differences ($p>0.05$, unpaired T test, Fig. 3.8 B).

3.1.3.2. Increased macrophage numbers in spleen in co-infected mice

Malaria-positive single and co-infected mice were sacrificed at days 8 and 14 p.p.i., representative for an early and a late time point after infection respectively, to analyse the distribution of immune cells in the main sites where responses against the parasites take place: namely the spleen for *P. berghei* and the pleural cavity for *L. sigmodontis*. A pronounced decrease in the number of total immune cells in the thoracic cavity could be observed in co-infected mice compared to *L. sigmodontis* single infected controls at both day 8 (***, $p=0.0008$, Mann-Whitney test, Fig. 3.9 A) and 14 p.p.i. (*, $p=0.0317$, Mann-Whitney test, Fig. 3.9 B).

Macrophages are among the major cell types involved in the defence against *L. sigmodontis* infections in mice (3, 9). Pleural macrophage numbers were significantly decreased at day 8 (***, $p=0.0013$, Mann-Whitney test, Fig. 3.9 C) and 14 (**, $p=0.0027$, Mann-Whitney test, Fig. 3.9 D) after

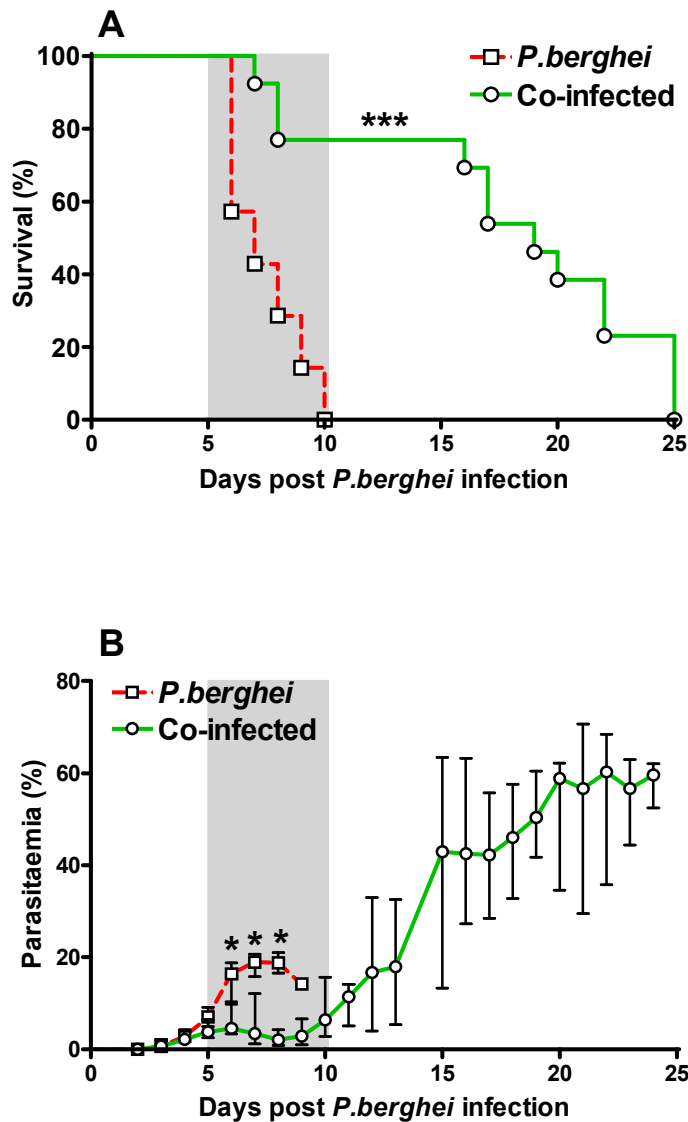


Figure 3.11: CM and parasitaemia development by *P. berghei* single- and co-infected C57BL/6 mice. Mice were co-infected with 50000 *P. berghei* iRBCs at day 60 after *L. sigmodontis* infection. (A) *Survival*. Significance was calculated using the logrank test (***, $p=0.0002$). (B) *Parasitaemia*. Lines represent median of parasitaemia and data range. Significance was calculated by Mann-Whitney test (*, $p<0.05$). Data in A and B represent 2 independent experiments. Shaded zones denote potential CM period. 10 to 12 mice were used in each group.

P. berghei infection. Macrophages are also involved in the defence against malaria parasites in the spleen (70). Higher numbers of macrophages were present in spleen of co-infected mice than *P. berghei* or *L. sigmodontis* single infected mice at day 8 (*, $p < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison test, Fig. 3.9 E) and 14 p.p.i. (***, $p < 0.001$, Kruskal-Wallis test with Dunn's multiple comparison test, Fig. 3.9 E, F).

3.1.3.3. Similar cytokine and immunoglobulin patterns in single and co-infected mice

In order to further characterize the response to *P. berghei* in single and co-infected mice circulating cytokines were measured in plasma. IFN- γ , which mediates macrophage activation and parasite phagocytosis (291), presented comparable levels in both single and co-infected mice, peaking at day 7 p.p.i. ($p > 0.05$, Kruskal-Wallis test and Dunn's multiple comparison test, Fig. 3.10 A). Similarly, the immunoregulatory cytokine IL-10 followed a similar pattern in both *P. berghei* infected groups, also peaking at day 7 p.p.i. ($p > 0.05$, Kruskal-Wallis test and Dunn's multiple comparison test, Fig. 3.10 B).

Different immunoglobulin G isotypes are secreted by B cells depending on the kind of polarization induced by the cytokine environment (236). In order to know the prevailing type of helper T cell response occurring in the mice, IgG1 (representative for Th2 responses) and IgG2a levels (representative for Th1 responses) were monitored after *P. berghei* infection. Both single and co-infected mice presented equivalent increases in IgG2a levels from day 8 p.p.i. on, whereas IgG1 remained at background levels throughout the infection ($p > 0.05$, Kruskal-Wallis test with Dunn's multiple comparison test, Fig. 3.10 C). In summary, the filarial infection did not substantially alter the cytokine patterns normally developed against malaria by BALB/c mice.

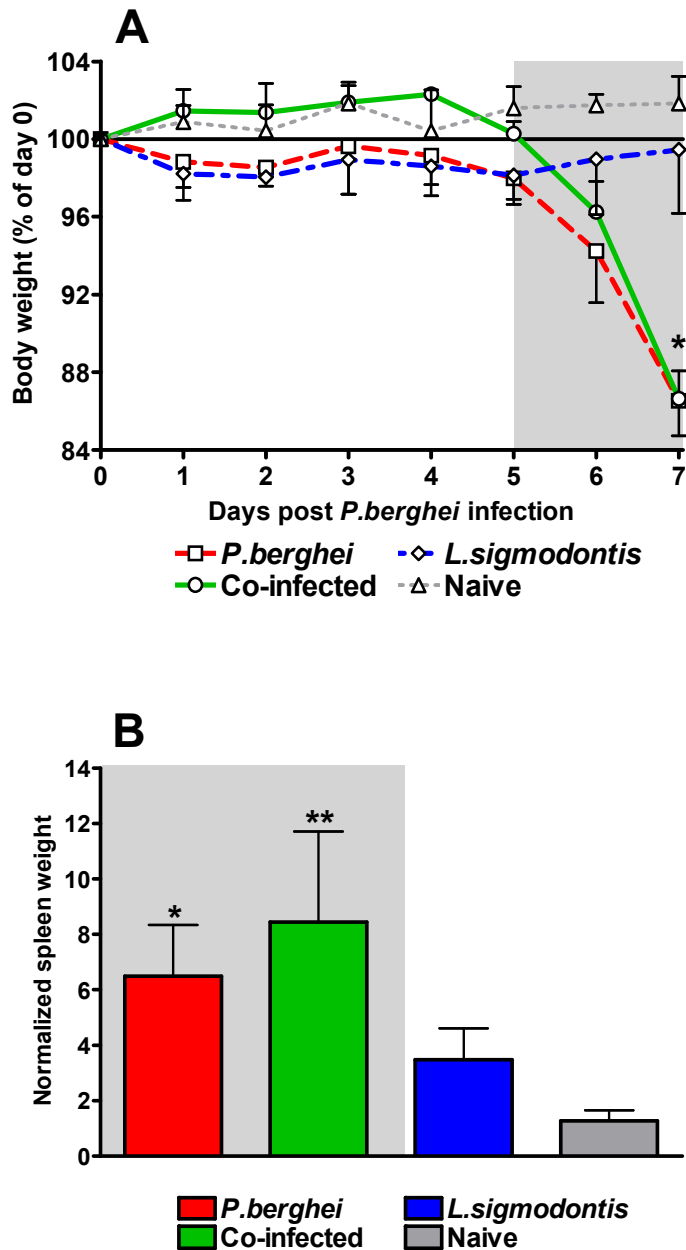


Figure 3.12: Physiological parameters after *P. berghei* co-infection of C57BL/6 mice. Mice were co-infected with 50000 *P. berghei* iRBCs at day 60 after *L. sigmodontis* infection. (A) *Body weight*. Variations in body weight were monitored after *P. berghei* infection until mice developed CM. Lines represent mean \pm SD of body weight. (B) *Splenomegaly*. Mice were sacrificed at day 7 p.p.i., during the CM period. Spleen weights were normalized with total body weights following the formula: (Spleen weight/total body weight)*1000. Columns represent mean of normalized spleen weight \pm SD of 7 mice. Data in A and B were compared by one-way ANOVA and Tukey's multiple comparison test for differences between groups (*, $p < 0.05$; **, $p < 0.01$), and represent 2 independent experiments. Shaded areas define potential CM period.

3.2. C57BL/6 MICE

3.2.1. Protective effects of filarial infections on cerebral malaria in C57BL/6 mice

P. berghei co-infection of *L. sigmodontis* infected C57BL/6 mice was performed on day 60 p.f.i. *P. berghei* infections were done with a dose of 50000 iRBCs, which generates CM pathology in 100% of the *P. berghei* single infected mice after 6 to 9 days.

3.2.1.1. Lower CM rates and parasitaemia in co-infected mice

Prior infection with *L. sigmodontis* resulted in a reduced number of mice developing CM after *P. berghei* infection. Whereas single infected mice developed typical pathological symptoms between days 6 and 10 p.p.i., 76.9% of co-infected mice survived this period and presented no evidence of severe pathology ($p=0.0002$, logrank test, Fig. 3.11 A). During the CM period, significantly lower parasitaemia was found in co-infected mice compared to their single infected littermates (*, $p<0.05$, Mann-Whitney test, Fig. 3.11 B).

Vitality parameters known to vary during malaria were monitored to compare the course of *P. berghei* infections. Co-infected mice suffered a comparable loss of body weight than the *P. berghei* single infected controls during the CM period (*, $p<0.05$, one-way ANOVA and Tukey's multiple comparison test, Fig. 3.12 A). Splenomegaly also occurred in a similar extent in single and co-infected mice (*, $p<0.05$, **, $p<0.01$, one-way ANOVA and Tukey's multiple comparison test, Fig. 3.12 B).

3.2.1.2. CD8⁺ T cells accumulate in the spleen but not in the brain of co-infected mice

Since many cell types are known to be involved in induction of malaria pathology, immune distributions were analyzed by flow cytometry in single and co-infected mice during the CM period at

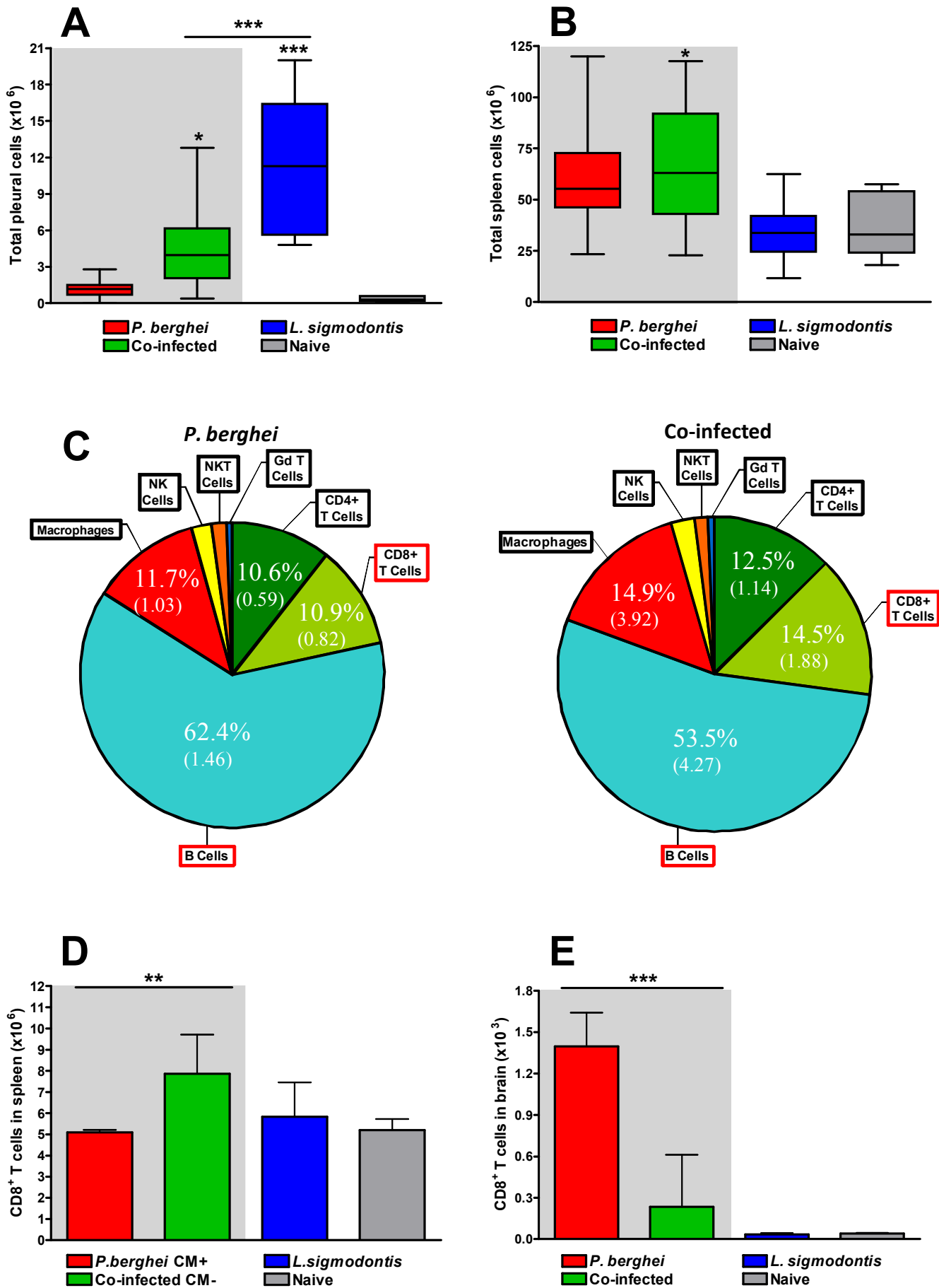


Figure 3.13 (see next page)

Figure 3.13 (see previous page): Cell distributions in spleen, pleural cavity and brain during CM in co-infected C57BL/6 mice. Mice were co-infected with 50000 *P. berghei* iRBCs at day 60 after *L. sigmodontis* infection and sacrificed 7 days later. Total cells in pleural cavity (A) and spleen (B) during the CM period. (C) Distribution of immune cells in the spleen of *P. berghei* single and co-infected mice during CM. The mean of cell population percentages are shown. Numbers in brackets denote SD. Cell names in red squares denote statistical differences between groups, and those in black squares correspond to non-significant differences. (D) Total CD8⁺ T cells in the spleen during CM. (E) Total CD8⁺ T cells in the brain during CM. In all figures mice were sacrificed at day 7 p.p.i. and cells analysed by flow cytometry. Columns in A, B, D and E represent mean of cell numbers \pm SD. Data were compared by one-way ANOVA and Tukey's multiple comparison test (**, $p < 0.01$; ***, $p < 0.001$). Asterisks over columns denote significance against naïve controls. Data in A and B were pooled from 2 independent experiments, whereas data in C, D and E are representative for at least 2 independent experiments. 5 to 7 mice per group were used in each experiment. Shadowed areas define potential CM period.

relevant sites involved in the development of immune responses against *P. berghei* and *L. sigmodontis*, namely pleura, spleen, and brain. In correlation to the findings in BALB/c mice, the total amounts of immune cells in the pleural cavity of co-infected C57BL/6 mice are significantly reduced compared to *L. sigmodontis* single infected controls at day 7 p.p.i. ($p < 0.001$, one-way ANOVA and Tukey's multiple comparison test, Fig. 3.13 A). At the same time in the spleen, only a modest increase in total cell numbers could be observed in co-infected mice compared to their naïve littermates ($p < 0.05$, one-way ANOVA and Tukey's multiple comparison test, Fig. 3.13 B).

The analysis of particular cell populations revealed that CD8⁺ T cells were present in significantly higher percentages in the spleen of co-infected mice compared to *P. berghei* single infected counterparts ($p < 0.05$, one-way ANOVA and Tukey's multiple comparison test, Fig. 3.13 C). Co-infected mice also showed a trend for increased percentages of macrophages and CD4⁺ T cells in the spleen. These increases were counterbalanced by a significant decrease in the percentages of B cells ($p < 0.05$, one-way ANOVA and Tukey's multiple comparison test, Fig. 3.13 C). No significant differences were found for NK cell, NKT cell or $\gamma\delta$ T cell numbers.

Absolute numbers of CD8⁺ T cells were significantly higher in the spleen of co-infected mice compared to their single infected littermates during the CM period ($p < 0.01$, Kruskal-Wallis test with Dunn's multiple comparison test, Fig. 3.13 D). No significant differences in total numbers could be found for other analysed cell types (data not shown). In turn, less total CD8⁺ T cells were found

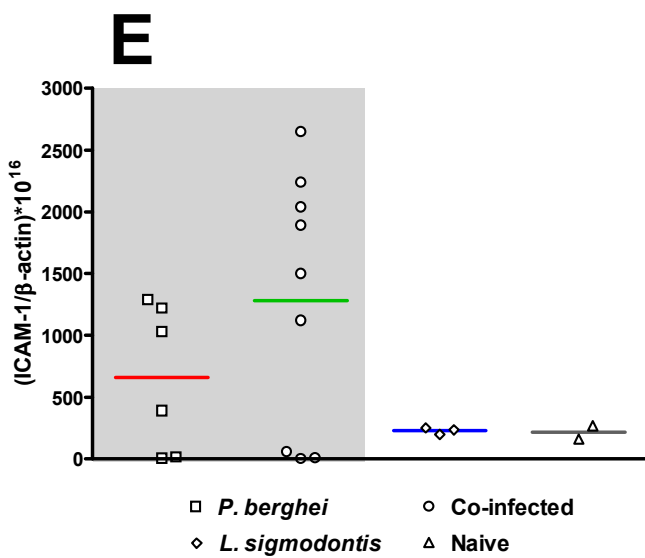
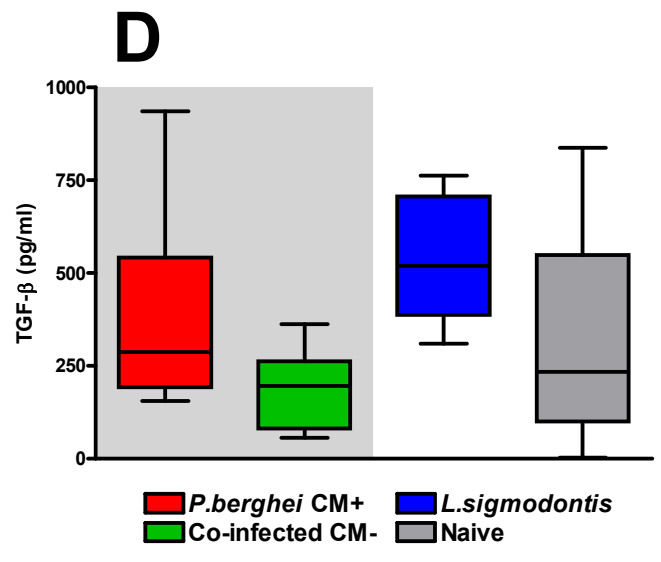
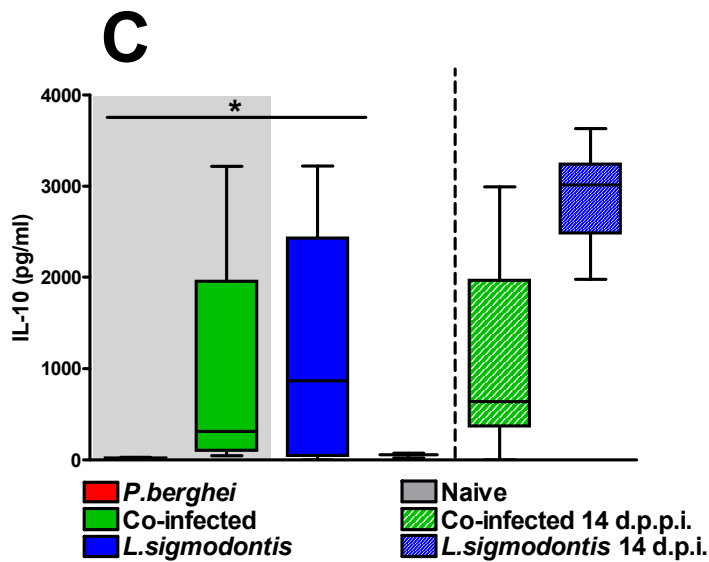
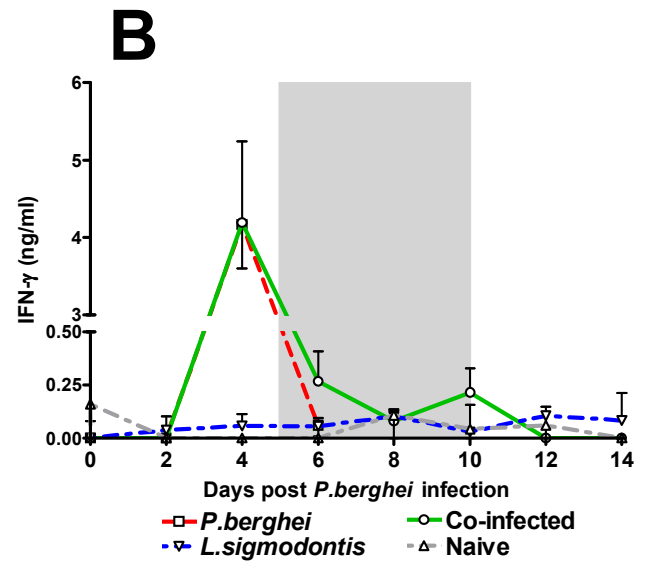
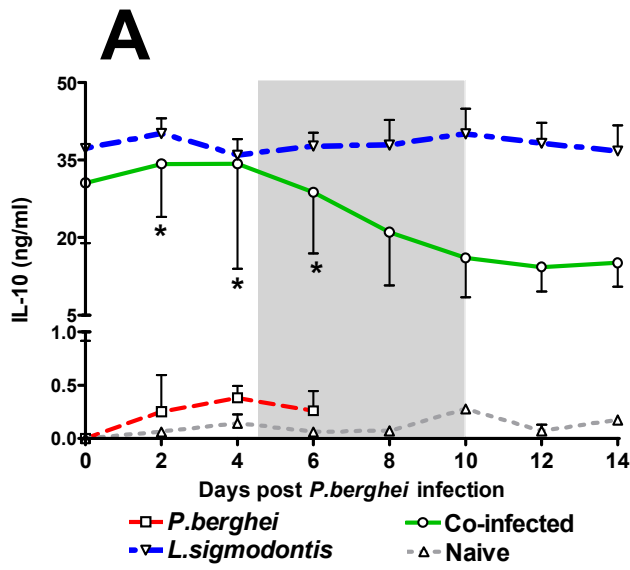


Figure 3.14 (see next page)

Figure 3.14 (see previous page): Cytokine levels after *P. berghei* co-infection of C57BL/6 mice. Mice were co-infected with 50000 *P. berghei* iRBCs at day 60 after *L. sigmodontis* infection. (A) *IL-10* and (B) *IFN- γ* course in plasma. Mice were bled every two days from the tail vein and cytokine concentrations measured by sandwich ELISA. Lines represent median and range of cytokine levels. Data were compared using Kruskal-Wallis test and Dunn's multiple comparison test for differences between groups (*, $p < 0.05$). Data are representative for 2 independent experiments. (C) *IL-10* and (D) *TGF- β* levels in the pleural cavity after *P. berghei* infection. Mice were sacrificed during the onset of CM (7 days p.p.i., left) or after the CM period (14 days p.p.i., right) and cytokine levels were measured in pleural wash by ELISA. Data were compared by Kruskal-Wallis test completed with Dunn's multiple comparison test for differences between groups (*, $p < 0.05$), and are representative for 2 independent experiments. Each group was composed of 5 to 7 mice. (E) *ICAM-1* expression in brain at day 7 p.p.i. Mice were sacrificed during onset of CM and RNA was extracted from brain for RT-PCR quantification. *ICAM-1* expression was normalized to β -actin copies. Shadowed zones represent potential CM period.

in the brain of co-infected mice at the same time-point p.p.i. (Fig. 3.13 E). The distribution of CD8⁺ T cells, critical mediators of CM (172), was markedly different in single and co-infected mice.

3.2.1.3. *IL-10* is essential for CM protection

Cytokine analyses in plasma were performed to evaluate the type of immune response against malaria parasites that developed in co-infected mice. Similarly to BALB/c mice, single and co-infected C57BL/6 mice showed a peak of circulating *IFN- γ* at day 4 post infection, which was comparable in both groups ($p > 0.05$, Kruskal-Wallis test with Dunn's multiple comparison test, Fig. 3.14 B, day 4 p.p.i.). However, strikingly high levels of the immune-regulatory cytokine *IL-10* could be measured in co-infected mice and *L. sigmodontis* single infected controls during the whole course of the experiment (*, $p < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison test, Fig. 3.14 B). Hence, worms seemed to induce an immune-regulation that was possibly altering the immune response against malaria parasites. Further pointing to a direct worm inducement of its production, *IL-10* levels were significantly high in all *L. sigmodontis* infected mice compared to *P. berghei* single infected or naïve mice at days 7 and 14 p.p.i. ($p < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison test, Fig. 3.14 C). *TGF- β* is another important immunomodulatory cytokine induced by *L. sigmodontis* (255). However, no significant differences between groups could be found in the thoracic cavity of the mice at day 7 p.p.i. ($p > 0.05$, Kruskal-Wallis test, Fig. 3.14 D).

In the search for the factors responsible for CM modulation in co-infected mice mRNA levels of the adhesion molecule *ICAM-1*, which mediates immune cell sequestration during CM (89), were

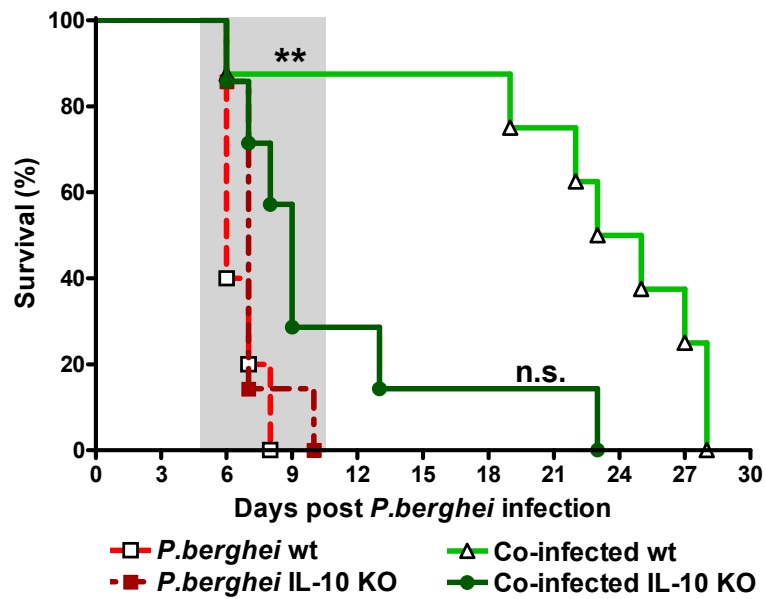


Figure 3.15: Survival of single- and co-infected IL-10-deficient C57BL/6 mice after *P. berghei* infection. Mice were co-infected with 50000 *P. berghei* iRBCs at day 60 after *L. sigmodontis* infection. Curves from wild type or KO mice were analysed separately with logrank test (wild type mice, **, $p=0.0018$; IL-10 KO mice, $p=0.1257$). Data represent 2 independent experiments. Groups contained 7 mice each. Shaded zone represents potential CM period.

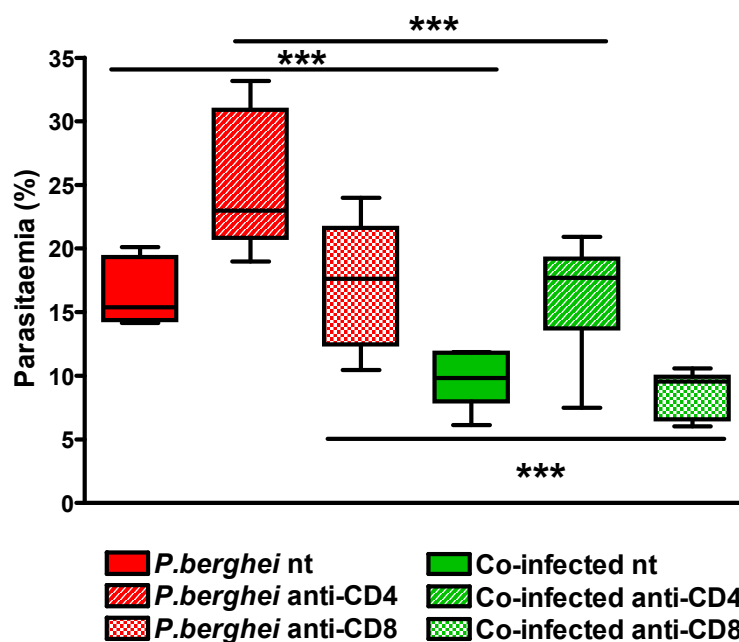


Figure 3.16: *P. berghei* parasitaemia during CM period in CD4⁺ or CD8⁺ depleted single and co-infected C57BL/6 mice. Mice were co-infected with 50000 *P. berghei* iRBCs at day 60 after *L. sigmodontis* infection. Depletion of CD4⁺ and CD8⁺ T cells with specific antibodies were performed 24 h before *P. berghei* infection, and blood smears were done 7 days p.i. Data were analyzed with Kruskal-Wallis test and Dunn's multiple comparison test (***, $p<0.001$). Each group contained 7 mice. nt=not treated.

measured in the brain. Surprisingly, both *P. berghei* single and co-infected mice showed increased ICAM-1 expression levels in brain ($p=0.0095$, Kruskal-Wallis test, Fig. 3.14 E).

Finally, in order to assess the relevance of high IL-10 levels on CM protection, co-infection of IL-10 deficient mice was performed, monitoring them for development of pathology. Both single and co-infected IL-10 KO mice developed high CM rates ($p=0.1257$, logrank test, Fig. 3.15), in contrast to their wild type co-infected counterparts, which showed the typical CM protection and increased survival ($p=0.0018$, logrank test, Fig. 3.15). Therefore, induction of IL-10 by the worms is an essential factor preventing CM development in co-infected mice.

3.2.1.4. Improved control of parasitaemia in co-infected mice is not mediated by CD4⁺ T cells

An important remaining question concerns the mechanism for improved parasite clearance exerted by co-infected mice during CM period. CD4⁺ T cells are a source of IFN- γ , which mediates elimination of malaria parasites (250, 292). Depletion of CD4⁺ and CD8⁺ T cells was done to assess the role of these cell types on parasitaemia. Parasitaemia in CD8⁺ T cell-depleted single and co-infected mice was comparable to that of non-depleted mice, excluding CD8⁺ T cells from playing any relevant role in control of blood stage *P. berghei* parasites ($p>0.05$, Kruskal-Wallis and Dunn's multiple comparison test, Fig. 3.16). However, depletion of CD4⁺ T cells led to higher parasitaemia in both single and co-infected mice, suggesting that CD4⁺ T cells strongly influence *P. berghei* parasitaemia control ($p<0.05$, Kruskal-Wallis and Dunn's multiple comparison test, Fig. 3.16). Nevertheless, such higher parasitaemia still showed the same ratio between the single and co-infected depleted group, with co-infected mice presenting lower levels than the single infected controls (***, $p<0.001$, Kruskal-Wallis and Dunn's multiple comparison tests, Fig. 3.16). Thus, a different cell type than CD4⁺ T cells must be responsible for the extra decrease in parasitaemia that all co-infected mice show compared to their respective single infected control groups.

4. Discussion

During infection immune cells receive through their multiple receptors danger signals from the pathogen or other cells that activate them, resulting in the development of specific mechanisms directed toward the clearance of the infective agents. Millions of years of evolution have trained the immune system to respond in specific ways to different infections. However, individuals are usually exposed to multiple perilous organisms, and the possibility of being concomitantly infected is high in particular environments. Co-infection implies the priming of the same immune cell types with different stimuli, and the result in terms of efficiency against the single pathogens may be profoundly altered. The final outcome of co-infection can be either detrimental or beneficial for the host, depending on the characteristics of the single infections (189).

Filarial nematodes are among the human parasites with the highest life span in the host. To achieve this, they have developed downregulatory mechanisms that prevent attack by the host immune system (109). Such mechanisms may however alter the course of concomitant infections such as malaria. In order to determine the extent in which susceptibility to plasmodial infections and malaria pathology is modified as a result of filarial-derived mechanisms a murine co-infection model has been established and analysed in the present work. Preceding filarial infections of *L. sigmodontis* improved malaria outcome in several ways; protecting BALB/c mice from sporozoite infection, modulating severe cerebral malaria pathology in C57BL/6 mice and even enhancing parasitaemia control in both mice strains.

4.1. SELECTION OF MICE AND PARASITES

In order to generate different manifestations of malaria and filariasis, a co-infection murine model was established using two mouse strains, BALB/c and C57BL/6, with contrasting susceptibilities to each of the parasites used (Table 4.1).

BALB/c mice are unique in their capacity to allow the complete development of adult *L. sigmodontis* worms (188). Some infected mice develop microfilaraemia whereas others do not, hence resembling filariasis in human patients. Thus, these mice provide the opportunity to study the effect of different stages of filarial parasites on the outcome of malaria in a co-infection model. Mf

appear in blood from day 55 p.f.i. and can be detected over a period of some weeks (8, 111). In order to analyse the influence of microfilariaemia on plasmodial infections, BALB/c mice were co-infected with *P. berghei* at day 60 after *L. sigmodontis* infection.

To simulate natural human plasmodial infections, which usually require the injection of very few sporozoites in the bloodstream (210), co-infections of BALB/c mice were performed with 500 *P. berghei* sporozoites; the lowest dose able to render 100% of naive mice malaria-positive (Fig. 3.1 A). This allowed analyses of relative variation in susceptibility to sporozoite challenge between single and co-infected mice. On the other hand, BALB/c mice develop severe malarial anaemia (SMA) after *P. berghei* infection in blood. Parasitaemia rises to high levels causing anaemia that progressively debilitates the mice, which die within 3-4 weeks after infection. Therefore *P. berghei* infections in BALB/c mice were also utilized to analyse SMA pathology in co-infection.

C57BL/6 mice are generally used to model cerebral malaria (CM), since they mount strong Th1 reactions against *P. berghei* and develop this pathology 5-10 days after infection, resembling most of the features of this pathology in humans. On the other hand, C57BL/6 mice are partially permissive to *L. sigmodontis*, allowing worm development to L4 stages (9). However, differently to the BALB/c strain, worms are cleared progressively from day 40 to 70 p.f.i. and all mice remain amicrofilaremic (240). Co-infections were done with 50000 *P. berghei* iRBCs injected intravenously, which is a dose already used in other studies (195) and can induce CM rates of 100% in naive mice.

4.2. CO-INFECTION IN BALB/C MICE

Co-infection of BALB/c mice generated two different malaria outcomes revealed by blood smear examination: approximately two thirds of the mice became malaria-positive and presented a parasitaemia pattern similar to that of their single infected counterparts, whereas the rest remained malaria-negative during the whole surveillance period (Figs. 3.2 B). Analysis of relevant physiological parameters further supported the division of co-infected mice into malaria-positive mice suffering from a characteristic malaria course, and malaria-negative without evident signs of infection or disease (Fig. 3.3 A-C). Hence, two separated lines of investigation, discussed in sections 4.2.1 and 4.2.2 respectively, were designed for the specific analysis of either subgroup.

Note that in this work the term “malaria-negative” is used for co-infected mice without patent parasitaemia, referring to the absence of pathology or disease (malaria) but not necessarily to the absence of the parasite (*Plasmodium*).

4.2.1. Protection against the liver stage of malaria in BALB/c mice

Three potential hypotheses could be formulated to explain the absence of patent plasmodial infection in malaria-negative co-infected BALB/c mice. Parasite elimination might have been achieved at the time of migration of the sporozoites to the liver, during their development inside the hepatocytes, or later during the blood stage.

Since sporozoites are able to enter hepatocytes within a few minutes after their injection into the host (231), the development of an effective sterilizing immune response is unlikely to occur in such a short time. Nonetheless, monoclonal antibodies against specific epitopes of the CSP have been shown to protect mice against sporozoite challenge (197). Analogously, in the present system antibodies generated against *L. sigmodontis* surface proteins might have cross-reacted with membrane proteins of the *P. berghei* sporozoites, triggering their fast clearance already in the bloodstream and rendering co-infected mice malaria-negative. The presence of antibodies against *Schistosoma mansoni* antigens that are cross-reactive with *P. falciparum* has been reported before (194). Therefore μ MT mice, deficient in mature B cells and therefore unable to generate antibodies, were used in the co-infection setting. The fact that such deficient mice were protected against sporozoite challenge to the same extent as their wild-type co-infected counterparts ruled out a role of antibodies in malaria protection upon co-infection (Fig. 3.4). This suggested that sporozoites possibly entered the liver and infected hepatocytes in co-infected mice, being parasites controlled either during their liver or blood stage.

The fact that malaria-negative co-infected mice did not present evidence of *P. berghei* parasitaemia did not necessarily mean that they were completely free of blood stage parasites. Persistent subpatent parasitaemia is a phenomenon observed in human (10, 78) and murine malaria (1, 268). To assess if protection in co-infected mice was sterile, blood was transferred from malaria-negative co-infected mice to naive counterparts, resulting in absence of infection in 94.74% (Table 3.1). This indicated that protective mechanisms allowed complete clearance of the parasites in these mice.

To further analyse the plasmodial stage at which protection was elicited, co-infection was performed with 50 or 50000 *P. berghei* iRBC, therefore circumventing the liver stage of malaria. As a result, all mice became malaria-positive (Fig. 3.7 A, B). If protection was directed against blood stages of *P. berghei*, iRBC co-infected mice should have been able to remain malaria-negative. Hence this result pointed to the liver as the main site where protective mechanisms against malaria parasites occurred in co-infected mice.

Further supporting the hypothesis of an enhanced hepatic anti-malarial response, distributions of immune cells in liver and spleen at the final period of the liver stage of *P. berghei* strongly differed in single and co-infected animals. More CD8⁺ T cells were also found in both organs in co-infected mice 40 hours after sporozoite infection (Fig. 3.6 A, B). Cytotoxic T cells are essential mediators of immunity against liver stage malaria parasites after immunization (164). Transfer of CD8⁺ T cell clones specific for epitopes of the circumsporozoite protein confers protection against parasite challenge (209). Since the liver stage of *P. berghei* lasts 50 hours, a fast response is required to achieve sterile immunity during this period. It has been observed that CD8⁺ T cell proliferation and development of effector functions (production of IFN- γ and perforin, and cytotoxic activity) occurs simultaneously 24 hours after contact with the parasites. The degree of protection against liver stage plasmodial forms has been shown to depend strictly on the total number of specific CD8⁺ T cells being formed during intra-hepatic development of the parasite (218). Increased cytotoxic cells observed in the liver in co-infected mice might therefore result in a higher clearance capacity of hepatic stages of *P. berghei*. The fact that *L. sigmodontis* single infected mice also presented similarly increased CD8⁺ T cell numbers in the spleen (Fig. 3.6 A) suggested that these cells may have been specific for *L. sigmodontis*-derived antigens rather than for *P. berghei* liver parasites. Nevertheless, a state of pre-activation of immune cells occurring in *L. sigmodontis* infected mice at patency may have boosted priming and proliferation of *P. berghei*-specific CD8⁺ T cells in the spleen of co-infected mice after sporozoite infection.

In accordance with this argument, microfilaraemia was related to protection in malaria-negative co-infected mice (Table 3.2), and microfilarial counts correlated with CD8⁺ T cell numbers in the spleen (Fig. 3.6 H). Once released by female worms in the pleural cavity, Mf must gain access to the lymphatic system and eventually to blood, in order to be taken up by a new vector. In this journey they must pass through capillary systems where they may be trapped because of their large size. 15 min after intravenous injection of *L. sigmodontis* Mf in cotton rats Wenk et al. could recover 32,9% in the liver and 6.4% from kidneys and spleen (275). Mf are known to stimulate strong immune responses (115, 182). Thus, trapped Mf may generate a local inflammatory environment favouring CD8⁺ T cell priming and proliferation.

Though comparatively more Mf may become trapped in the liver (275) no association between CD8⁺ T cell numbers and microfilaraemia could be found in this organ (Fig. 3.6 G). This suggested that the spleen was the main site involved in the generation of anti-plasmodial CD8⁺ T cells. Activated cytotoxic cells may later migrate to the liver to exert their anti-parasitical function. Indeed, an equivalent mechanism has been proposed before: CD8⁺ T cells specific for *P. berghei* liver stage parasites have been shown to be primed in draining lymph nodes after parasite inoculation through infected mosquitoes, and then exert their protective function in the liver (41). Since the spleen's main function is pathogen recognition and processing of blood (132), intravenous injection of sporozoites done in the present model might have resulted in CD8⁺ T cell priming in the spleen.

Potentially contributing to the establishment of more effective anti-sporozoite, macrophage numbers were found to be increased in the spleens of co-infected mice (Fig. 3.6 D). Macrophage migration from the pleural cavity to the blood after patency in *L. sigmodontis* infected mice has already been described (255), and in the work presented here, *L. sigmodontis* single infected mice showed significantly more macrophages in the liver than any other group (Fig. 3.6 C). Interestingly, macrophage numbers were lower in the pleural cavity of malaria-positive co-infected mice when compared to *L. sigmodontis* single infected controls (Figs. 3.9 C, D), suggesting that an additional number of these cells left the thoracic cavity after *P. berghei* infection. Increased macrophage numbers in the spleen of co-infected mice compared to *L. sigmodontis* single infected controls indicated that *P. berghei* infection may also drive their accumulation in the spleen. Induction of CD8⁺ T cell responses against *P. berghei* sporozoites would require antigen presentation by APCs, and macrophages are known to be able to exert this function (199). Therefore, an increased number of macrophages in the spleen of co-infected mice might augment the amount of specific cytotoxic T cells generated against plasmodial parasites and contribute to more efficient parasite elimination.

A factor potentially favouring T cell priming by macrophages in microfilaraemic mice is the presence of symbiotic *Wolbachia* bacteria in filarial worms. It has been observed that *Wolbachia*-derived antigens are sensed by APCs through TLR2 and 4 (25, 48). TLR engagement in macrophages induces their maturation and enhances APC functions (17, 207, 265). Trapping and eventual death of Mf in the spleen may result in the release of endosymbiotic *Wolbachia* bacteria and the induction of macrophage activation. Interestingly, malaria parasite-derived antigens also stimulate TLR2 and 4-dependent responses (133). Thus, bacteria may synergize with plasmodial antigens in co-infected mice in the induction of APC maturation and T cell priming.

As mentioned before, adult worms stimulate the production of downregulatory cytokines like IL-10 to suppress host immune responses against Mf, prolonging their survival until taken up by a

new vector (112). Since filarial patency correlated with malaria protection the effect of IL-10 in the model was assessed using IL-10 deficient mice. All IL-10 KO co-infected mice were rendered malaria-positive after sporozoite challenge, and thus malaria protection was proven to be dependent upon IL-10 (Fig. 3.6). Circulating IL-10 levels were not increased 60 days after *L. sigmodontis* infection (Fig. 3.10 B), indicating that the function of IL-10 is local rather than systemic. Interestingly, immunity to malaria in individuals living in endemic areas has been shown in several studies to be related to increased IL-10 levels. This cytokine is produced by peripheral blood mononuclear cells (PBMC) against *P. falciparum* liver stage antigen-1 (LSA-1) (162). High IL-10, but neither IFN- γ nor TNF- α responses to *P. falciparum* LSA-1 by PBMCs were associated to resistance to sporozoite infection in different studies in Kenya (135) and Gabon (150). In agreement with these studies, John et al. observed that individuals whose PBMCs generated IL-10 responses when exposed to LSA-1 during the dry season were more resistant to infection during the subsequent rainy season. In that work, an association between protection from *P. falciparum* re-infection and LSA-1-driven IL-10 but not TNF- α , IFN- γ , or IL-5 responses was shown (122). Moreover, IL-10 could not be related to down-regulation of immune responses against LSA-1 in another study on malaria immunity in children (149).

However, the mechanism by which IL-10 is related to protection from sporozoite challenge in these studies is unknown. Still, examples of an IL-10 mediated enhancement of CD8⁺ T cell function have been shown in several studies: IL-10 has been shown to increase proliferation (290) and cytotoxic activity (45, 82, 93, 121, 219) of effector CD8⁺ T cells. Therefore, in the present work worm induced IL-10 may favour the generation and function of cytotoxic T cells and favour elimination of malaria parasites.

Another possible explanation is an enhanced production of counterbalancing regulatory cytokines in IL-10 KO mice: deficiency in IL-10 has been shown to induce up-regulation of TGF- β (54, 144), which is another characteristic worm-induced immunoregulatory cytokine (58, 255) involved in wound healing (75). This cytokine might be up-regulated in co-infected IL-10-deficient mice to repair damage caused by excessive immune responses, for example against trapped Mf. TGF- β has been shown to mediate CD8⁺ T cell hyporesponsiveness against *P. yoelii* sporozoites caused by concomitant blood stage plasmodial infections (178). Compensation by TGF- β for the absence of IL-10 may account for the lack of protection in IL-10-deficient co-infected mice.

In summary, filarial infection has been shown to promote protection against plasmodial sporozoite challenge in a mechanism mediated by locally produced IL-10 and with the involvement of microfilaraemia and potentially CD8⁺ T cells and macrophages. Trapped Mf in the spleen of co-

infected mice might create a local inflammatory environment resulting in the accumulation of macrophages and the generation of CD8⁺ T cells specific for *P. berghei* intra-hepatic parasites. IL-10 might enhance proliferation and cytotoxic activity of these cells, increasing the efficiency of elimination of malaria parasites in the liver. Depletion of cytotoxic T cells should be performed in order to prove the relevance of these cells in resistance to sporozoite infection in co-infected mice.

4.2.2. Improved outcome of blood stage malaria in BALB/c mice

A trend could be seen in individual experiments suggesting a prolonged survival and lower parasitaemia in co-infected mice. Some animals in this group were found to survive *P. berghei* infection for more than 30 days, a phenomenon that single infected mice cannot achieve. Furthermore, mice co-infected with 50000 iRBC 60 days after *L. sigmodontis* infection survived significantly longer when compared with their single infected littermates ($p=0.0355$, logrank test, Fig 3.7 B). In order to obtain definitive proof of a beneficial effect of co-infection on the blood stage of malaria several independent experiments were pooled for the analysis of survival and *P. berghei* parasitaemia (Figs. 3.8 A, B).

No significant differences in survival could be found between groups using the logrank test ($p=0.1016$, Fig 3.8 A). However, the logrank test calculates significance throughout the whole survival curve, but in the present setting a specific analysis of late survival to malaria was of special interest. Hence, single and co-infected mice were classified regarding their capacity to survive malaria longer than a threshold defined at day 30 p.p.i. Using the Fisher's exact test then revealed that there was a significantly higher number of long-term survivors in the co-infected group when compared to *P. berghei* single infected mice ($p=0.007$, Fig. 3.8 A).

Compared to their single infected counterparts, co-infected mice presented a significantly lower parasitaemia during the late stage of infection: the first time point when differences could be found was on day 11 p.p.i, which coincided with a fast rising in parasitaemia. Later, during days 13-15 p.p.i. co-infected mice partially stabilized parasitaemia growth, which remained thereafter below the levels of their single infected counterparts (Fig.3.8 B).

Lower parasitaemia at a late stage of blood infection might explain the prolonged survival of co-infected mice by delaying anaemia. Immunological analyses were performed on day 8 p.p.i., an early time point in the responses of BALB/c mice to malaria (50), and day 14 p.p.i., a late time point

after co-infection consistently found to show differences in parasitaemia. Generally there was a reduced amount of immune cells in co-infected mice compared to their *L. sigmodontis* single infected littermates in the pleural cavity at both time points p.p.i. (Fig. 3.9 A, B). More specifically, macrophage numbers were strongly decreased in the pleural cavity of co-infected mice, but increased in the spleen (Fig. 3.9 C-F). The fact that a reduction in macrophage numbers could already be observed 8 days after *P. berghei* infection in the pleural cavity of co-infected mice compared to *L. sigmodontis* controls suggests that these cells actively leave this site. Since macrophages are considered long lived cells, with a life-span of several weeks (119), other possibilities such as cell death are unlikely to occur. Indeed, as commented before, macrophage migration from the pleural cavity to the periphery at day 60 post *L. sigmodontis* infection in single infected BALB/c mice has been previously reported (255), and the present results suggest that this process may be enhanced after *P. berghei* infection.

Macrophages can phagocyte and destroy blood stage malaria parasites (291). Therefore, higher macrophage numbers in the spleen, which is considered the main site of elimination of these pathogens (70), may allow a better control of parasitaemia by co-infected mice. Macrophage phagocytosis is enhanced through IFN- γ (179). Plasma levels of this cytokine in single and co-infected mice were comparable, peaking at day 7 p.p.i. with similar intensity (Fig. 3.10 A). This indicates that IFN- γ production driven by *P. berghei*, and therefore Th1 polarization, was not altered by the worms, and could not account for the observed differences in parasitaemia between both groups. Analysis of immunoglobulin patterns further supported this conclusion. IgG2a is an immunoglobulin produced in the context of a Th1 immune response, and isotype switching to IgG2a in B cells is driven by IFN- γ (237). In contrast, switching to IgG1 production is induced by IL-4 during Th2 responses (236). After day 8 p.p.i. a similar production of IgG2 was found in both groups. The similarities between single and co-infected mice were further emphasized in the development of anti-plasmodial immune responses; equivalent IL-10 levels in plasma were detected in both groups after infection, peaking at day 7 p.p.i. like IFN- γ (Fig. 3.10 B). Comparable cytokine and immunoglobulin patterns in single and co-infected mice excludes an important role for immune responses triggered by T or B cells and suggests that differences in parasitaemia and survival may be mainly due to the higher amount of macrophages present in spleens of co-infected mice.

In summary, co-infection of *L. sigmodontis* infected BALB/c mice with *P. berghei* at patency induces one third of mice remaining malaria-negative in an IL-10-dependent mechanism, and the rest malaria-positive. A more pronounced accumulation of CD8⁺ T cells in the spleen and liver takes place in co-infected mice, potentially improving sporozoite clearance. Additionally, macrophages

accumulate in the spleen and may account for improved CD8⁺ T cell priming against liver stage parasites and enhanced control of blood stage parasites in malaria-positive co-infected mice.

A beneficial effect of filarial patency against blood stage malaria parasites was also shown in a previous study of co-infection with non-lethal *P. chabaudi chabaudi* 60 days after *L. sigmodontis* infection (96). The present work could show in addition that microfilaraemia results advantageous against plasmodial liver stages.

4.3. CO-INFECTION IN C57BL/6 MICE

Co-infection of C57BL/6 mice with *P. berghei* at day 60 after *L. sigmodontis* infection resulted in a significant protection from CM. All single infected mice developed pathology within the first 10 days of infection, whereas more than 70% of their co-infected littermates survived the critical period when CM occurs (Fig. 3.11 A). Co-infected mice also presented a significantly lower parasitaemia than their single infected counterparts (Fig. 3.11 B). CM is an immune-mediated pathology triggered by the up-regulation of Th1 cytokines such as LT- α (71) or IFN- γ (214). Thus, CM prevention in co-infected mice might reflect a modulation of the inflammatory process exerted by the worms. Indeed, IL-10 levels in *L. sigmodontis* single and co-infected mice were significantly increased compared to *P. berghei* single infected or naïve controls in plasma and pleural cavity (Fig. 3.14 A, C). IL-10 is an immunomodulatory cytokine typically induced by filarial worms (58, 151, 181), suppressing not only Th1 but also Th2 responses (181, 240). The higher levels of this cytokine not only in plasma but also in the pleural cavity clearly indicated a worm-induced production in infected mice. In confirmation of this, co-infection of IL-10 deficient C57BL/6 mice resulted in absence of CM protection (Fig. 3.15).

Protective effects of IL-10 on malaria have been reported before. Injection of recombinant IL-10 in CM-susceptible mice abrogates pathology development after *P. berghei* infection (131). In contrast, infection of IL-10 deficient C57BL/6 mice with non-lethal *P. chabaudi* results in CM development and death of some mice (217), presenting increased levels of circulating IFN- γ and TNF- α (143). Depletion of TNF- α in IL-10 deficient mice significantly improves pathology development (144). Therefore, IL-10 naturally regulates excessive Th1 inflammation after *P. chabaudi* infection, and its deficiency leads to increased immune-mediated pathology. IL-10 regulatory function is complemented by TGF- β , another paradigmatic immunomodulatory cytokine. Increased levels of TGF- β are found in IL-10 KO mice after *P. chabaudi* infection, partially counterbalancing the defect in IL-10. Additional depletion of TGF- β in this model results in pathology development and death in all

mice (144). TGF- β has been found to mediate worm induced immunosuppression (58, 255). However, in the present co-infection model this cytokine was found at similar levels in all groups (Fig. 3.10 D), underlining the higher importance of IL-10 mediating immunoregulation.

The analysis of the course of IFN- γ after *P. berghei* infection showed no differences between single and co-infected mice (Fig. 3.14 B). Both groups presented a peak in a similar range at day 4 post-infection. It is of note to comment that a similar IFN- γ peak was observed in all single and co-infected C57BL/6 and BALB/c mice at days 4 and 7 post infection respectively. Taking into account that C57BL/6 mice were infected with blood parasites, and thus by passing the liver stage of *P. berghei*, this IFN- γ peak is likely to be triggered by a similar mechanism in both strains. As discussed before, CD4⁺ T cells are good candidates for the production of this peak (107). Since no differences could be found between single and co-infected mice, this cell population does not seem to be affected by worm-induced mechanisms in both mouse strains.

Differences between single and co-infected mice arose when examining the distribution of immune cells in the organs implicated in CM generation, namely spleen and brain. CD8⁺ T cells were found in lower numbers in the brain but higher in the spleen in co-infected mice compared to their single infected counterparts (3.13 D, E). CD8⁺ T cells are critical mediators of CM, accumulating in the brain and damaging the endothelium in a perforin-dependent manner (12, 13, 172, 198). Depletion of CD8⁺ T cells even during the onset of the pathology can prevent death in susceptible mice (13). Therefore the absence of these cells in the brain could explain the lack of pathology shown in co-infected mice.

Interestingly, ICAM-1 expression in the brain was similar in single and co-infected mice (Fig. 3.14 E). This result is therefore likely to reflect a particular alteration of CD8⁺ T cell migration, but not the complete abrogation of the inflammatory process in the brain, in co-infected mice. Supporting this, physiological parameters related to malaria like loss of body weight or splenomegaly were comparable in both *P. berghei* infected groups (Fig. 3.12 A, B), reflecting a similar development of pathology. The striking difference in susceptibility between single and co-infected mice was hence limited to a late step in the cascade of events triggering CM, namely CD8⁺ T cell migration and effector function. Indeed, a very similar mechanism of impairment of CD8⁺ T cell migration to the brain resulting in CM prevention has been described before: depletion of CD4⁺CD25⁺Foxp3⁺ T cells (natural regulatory T cells) in *P. berghei* infected C57BL/6 mice surprisingly arrested progression to CM (6). Protection was found to be linked to a decreased specific recruitment of CD8⁺ T cells in the brain, and to their accumulation in the spleen, in a process mediated by IL-10. Interestingly, in that study ICAM-1 levels were up-regulated in brain in both depleted and non-depleted mice (6). The

chemokine receptor CCR5 has been related to CD8⁺ T cell migration to the brain during CM, and CCR5 KO mice do not develop pathology (12). CCR5 expression has been shown to be down-regulated in CD4⁺ T cells in the presence of IL-10 (187). Analogously, high levels of circulating IL-10 might prevent CCR5 expression and migration of CD8⁺ T cells to the brain, protecting co-infected mice from CM.

Despite the proven regulatory properties of IL-10 in macrophages, neutrophils and eosinophils (53, 211, 283), which are the main mediators of immune responses against worms, high IL-10 levels in the pleural cavity and plasma did not hinder parasitaemia control by co-infected animals, which indeed developed a significantly lower parasitaemia than the single infected controls during the whole period of infection (Fig. 3.11 B). Recent advances in understanding the peculiarities of TLR stimulation in alveolar macrophages under immunomodulatory conditions may however explain this phenomenon. Alveolar macrophages are found in the lung, where constitutively high IL-10 expression by bronchial epithelial cells keeps a state of permanent immunosuppression (20). Despite this, they are able to efficiently respond to pathogens upon cross-stimulation of their TLR2, 4 or 9 by specific agonists (76). In the present co-infection model immune cells were found in lower numbers in pleural cavity of co-infected mice compared to *L. sigmodontis* single infected controls at day 7 p.p.i., during the CM period (Fig. 3.13 A). In contrast, more cells were found in the spleen of co-infected mice (Fig. 3.13 B). Though not significant, a trend of increased macrophage numbers could be observed (Fig. 3.13 C). Similarly to co-infected BALB/c mice, macrophages might leave the pleural cavity to home to the spleen upon *P. berghei* co-infection in C57BL/6 mice. Malaria parasites are recognized by macrophages through TLR2, 4 and 9 (133, 186), and therefore it is possible that the cross-activation mechanism described for alveolar macrophages may also apply in co-infected C57BL/6 mice. An extra number of macrophages leaving the thoracic cavity and homing to the spleen might be cross-activated by malaria parasites and allow an improved control of parasitaemia. Indeed, macrophage cross-activation might already occur in the pleural cavity since *Wolbachia* engagement of TLRs can also activate macrophages for TNF- α production through TLR2 and 4 (25). Release of *Wolbachia* from damaged worms is known to elicit neutrophil accumulation for granuloma formation and worm elimination (26). Therefore macrophages may be pre-activated in the pleural cavity through *Wolbachia* and migrate to blood upon co-infection, resulting in an improved control of the malaria parasites.

CD4⁺ T cells are known to contribute to parasitaemia clearance through IFN- γ production and macrophage activation (136, 179, 246). In order to further clarify the mechanism of reduction of parasitaemia in co-infected mice, CD4⁺ and CD8⁺ T cells were depleted and the parasitaemia measured at day 7 p.p.i., during the CM period. All depleted mice were protected from CM, as expected (data not shown). Depletion of CD4⁺ T cells lead to an increase in parasitaemia in both

single and co-infected mice, but surprisingly, CD4⁺ depleted single infected mice still had significantly higher parasitaemias than their co-infected counterparts (Fig. 3.16), suggesting that an additional mechanism in the co-infected mice is responsible for the further reduction of parasitaemia. This result supports the hypothesis that pre-activated macrophages leaving the pleural cavity after *P. berghei* infection might be involved in the enhanced parasite elimination.

A remaining question refers to the source of IL-10. Many cell types are able to produce IL-10: CD4⁺ T cells, CD8⁺ T cells, B cells, mast cells, eosinophils, macrophages and DCs (reviewed in (161, 177)). In the co-infection model used here, *P. berghei* infection caused extensive loss of B and T cells in CM-surviving mice after the CM period (data not shown). Selective apoptosis of B and T cells in the *P. chabaudi* model (101), as well as specific anti-*P. berghei* CD4⁺ T cells have been already reported (107). However, since elevated IL-10 levels do not significantly decrease after the CM period when compared to those of *L. sigmodontis* single infected mice (at day 12 p.p.i., $p=0.0927$, Mann-Whitney test, Fig. 3.10 A), a cell type other than B and T cells is more likely to produce IL-10. From the immune cells mentioned before macrophages and eosinophils could be good candidates, since they are the main mediators of the response against *L. sigmodontis* in the thoracic cavity (3), and therefore susceptible to be altered by the worms. Macrophages have been shown to produce IL-10 upon contact with *S. mansoni* eggs (46) and *L. sigmodontis* (239).

In summary, a delicate equilibrium must be achieved for a successful outcome after a plasmodial infection: a Th1 reaction is necessary to kill the parasites, but an exaggerated response will derive in pathology. In this model, co-infection seems to provide a mechanism to establish such equilibrium. First, co-infected mice presented markedly enhanced levels of circulating IL-10 that prevent pathology, potentially impeding CD8⁺ T cell migration to the brain. But second, high IL-10 levels do not interfere with the production of IFN- γ , necessary to kill the parasites (5, 104, 245), and additionally an extra number of macrophages coming from the pleural cavity may contribute in holding the parasitaemia at lower levels in co-infected mice.

In agreement with the results presented here, development of cerebral pathology was abrogated in CM-susceptible CBA/J mice previously injected with *Brugia pahangi* L3 larvae (288). Splenic cells were found to produce increased levels of IL-4, IL-5 and IFN- γ at the moment of their co-infection, thus developing combined Th1 and Th2 responses typically generated against filarial nematodes (215), but no IL-10 measurement was performed and the data shown does not allow a comparison to the mechanisms of CM protection presented in the model here. Interestingly, in contrast with the results of this work, *P. berghei* parasitaemia was markedly increased in *B. pahangi*-*P. berghei* co-infected mice when compared to their single infected littermates, reflecting a profound

immunosuppression in these mice. In the present *L. sigmodontis*-*P. berghei* model, improved parasitaemia elimination might have been achieved by an extra number of macrophages that escaped pleural cavity to combat malaria parasites after long exposure to *L. sigmodontis*, as discussed before. However, in *B. pahangi*-*P. berghei* co-infected mice a significant macrophage accumulation in the spleen might not have taken place since co-infection was performed only 10 days after the first L3 injection.

In summary, a prior filarial infection significantly protected C57BL/6 mice from CM development after *P. berghei* infection in an IL-10-dependent manner, resulting in a lower recruitment of CD8⁺ T cells in the brain. Moreover, *P. berghei* parasitaemia was reduced in co-infected mice notwithstanding high circulating IL-10 levels.

4.4. UNIQUE FEATURES OF FILARIAL WORMS PROTECT AGAINST MALARIA

Filarial infections have been shown to play important beneficial roles against malaria in the co-infection model studied here, increasing resistance to sporozoite infection, modulating CM development and prolonging the survival of the mice. Important protective mechanisms against malaria in co-infected mice were found to rely on IL-10 in both mouse strains. The genetic background of the mice critically determined the intensity of IL-10 responses against the parasites: whereas BALB/c mice showed low systemic levels of this cytokine, which probably acted locally, C57BL/6 mice presented a clearly enhanced production resulting in high plasma concentrations at day 60 after *L. sigmodontis* infection. Such differences produced completely different results: IL-10 strikingly increased resistance of BALB/c mice against sporozoite infection, but also mediated a reduction of CM rates in C57BL/6 mice, which may be more “expected” outcome, given its major downregulatory function (161). Both mechanisms involved CD8⁺ T cells. Though high systemic IL-10 concentrations may prevent migration of these cells to the brain in C57BL/6 mice, low systemic levels in BALB/c mice may not be sufficient to suppress CD8⁺ T cell accumulation in the liver. Moreover, IL-10-mediated enhancement of CD8⁺ T cell function may be only possible at low concentrations.

A different relevance of IL-10 on altering specific immunological mechanisms was further supported by similar findings in both strains showing that despite the differences in IL-10 levels, *P. berghei* parasitaemia was combated more efficiently, and that blood stage plasmodial-induced IFN- γ peaks were unaltered in co-infected animals. Particularly intriguing is the observation that C57BL/6 mice were able to control parasitaemia more efficiently than their single infected counterparts despite their high systemic IL-10 levels and clearly shows that independent mechanisms mediate the elimination of blood stage malaria parasites and development of immune pathology, even though all rely on Th1 responses, and all are differently affected by IL-10.

Macrophage numbers were decreased in pleural cavity and elevated in the spleen of co-infected mice from both strains. This fact may be advantageous since more phagocytic cells could exert a better control of plasmodial parasitaemia. In the particular case of C57BL/6 mice, macrophages that have been constantly exposed to high IL-10 levels in the pleural cavity may overcome IL-10-mediated downregulation, being cross-activated by malaria parasites in blood through TLR engagement.

How do these results from a laboratory model fit with data obtained from other models and from human studies of helminth-plasmodial co-infection in endemic areas?

As explained in section 1.3, co-infections of intestinal nematodes or *S. mansoni* and *P. falciparum* were shown in multiple studies to result in stronger malaria attacks defined by increased parasitaemia linked to fever, or to multiplicity of plasmodial infections (42, 167, 170, 173, 238, 242). A link between young age and worsened malaria could be found in some studies; older co-infected patients are less affected by a detrimental effect of the worms (30, 31). Shift to a Th2-like response or suppression of anti-plasmodial Th1 responses has been hypothesized to explain this effect (66). Though the immune status of human patients in endemic areas strongly differs from that of naive laboratory mice, such results are underlined by some experimental data from mice co-infected with *S. mansoni* and *P. chabaudi* (102), *H. polygyrus* and *P. chabaudi* (248) or *E. caproni* and *P. yoelii* (174). Moreover, a previous infection with the intestinal worm *E. caproni* resulted in enhanced susceptibility to *P. yoelii* sporozoites in BALB/c mice (175). Other co-infection systems with *S. mansoni* and *P. berghei yoelii* did not find alterations in parasitaemia (142), but this might be due to the different capacity of *S. mansoni*-derived antigens to skew Th1 responses (36, 69). Since Th1 responses are necessary for blood or liver stage malaria parasite control, any alteration consisting in its down-regulation or shift to Th2 may impair parasite elimination. Results from the present work seem to disagree with these studies.

On the other hand, worm infections have been linked to protection from malaria pathology in adult patients (166, 169), in whom worms are more prevalent than in children (68). Results were mirrored by studies in mice showing that *P. berghei* co-infection after injection of *B. pahangi* L3 larvae were also protected from CM (263). It is expectable that if worms have immunoregulatory properties impairing Th1-dependent parasitaemia control, they might also modify the outcome of the Th1-dependent CM pathology. Results showed here agree with previous literature in the latter case.

There are unique features of *L. sigmodontis* and by extension filarial infections that are not shared with other helminths and make them unique in a co-infection setting with malaria, resulting beneficial even in enhancing anti-malarial mechanisms which are usually hampered by immune responses mediated by non-filarial worms: a) the presence of worms in the pleural cavity and Mf in blood (in BALB/c mice) allows a close contact between worm stages and immune cells; b) different stages of *L. sigmodontis* induce different kinds of responses; thus, though adult worms induce immunoregulation, Mf stimulate inflammation; and c) a Th1 component of anti-*L. sigmodontis*

immunity is linked to responses against *Wolbachia* bacteria emerging from damaged worms (25), possibly allowing cross-activation of immune cells by engaging similar TLRs than malaria parasites.

Most of the relevant variables associated with protective malaria outcomes in the present co-infection model (microfilaraemia, IL-10-derived immunosuppression, similar combined Th1/Th2 responses against the worms), are present in filaria patients living in endemic areas, making it theoretically possible to directly compare between the results presented here and the human situation. Filariae may constitute a natural counterbalance of the high virulence of *P. falciparum*, protecting co-infected individuals from pathology and high parasitaemia and favouring their survival to malaria. By doing so, filariae would not only protect the host, but they would ultimately ensure their own survival as parasites, and indeed they would allow malaria transmission from co-infected subjects for prolonged periods of time.

In order to solve the current debate on the effect of worms and plasmodial co-infections in human populations more precise investigations linking immunological variables (such as APC function, T cell or antibody isotype polarization, cytokine patterns) with the characteristics of the life cycles of particular nematodes and with diverse transmission intensities and patient's age should be undertaken. Given the heterogeneity of body niches occupied by different nematode groups and the variable capabilities of immune stimulation that they exert on the host it seems unlikely, however, that a unifying conclusion on a protective or detrimental effect of any worm infection on malaria can be raised. Thus, whereas a clear beneficial outcome has been described here involving different mechanisms derived from intrinsic characteristics of filarial worms, co-infection studies using other nematode species caused impaired malaria control.

Given the similarities between the murine parasites used here and their human counterparts, it is likely that immune mechanisms occurring in individuals co-infected with filariae and *Plasmodium* reflect those describe here, and that these subjects may enjoy a natural protection against malaria parasites. In consequence, since anti-filarial mass-treatments are currently being carried out in endemic countries, there is a clear risk of impairing such mechanisms, increasing malaria morbidity and mortality. Urgent analyses should be performed in co-infected patients to determine the extent in which data from the present murine model is representative for human beings.

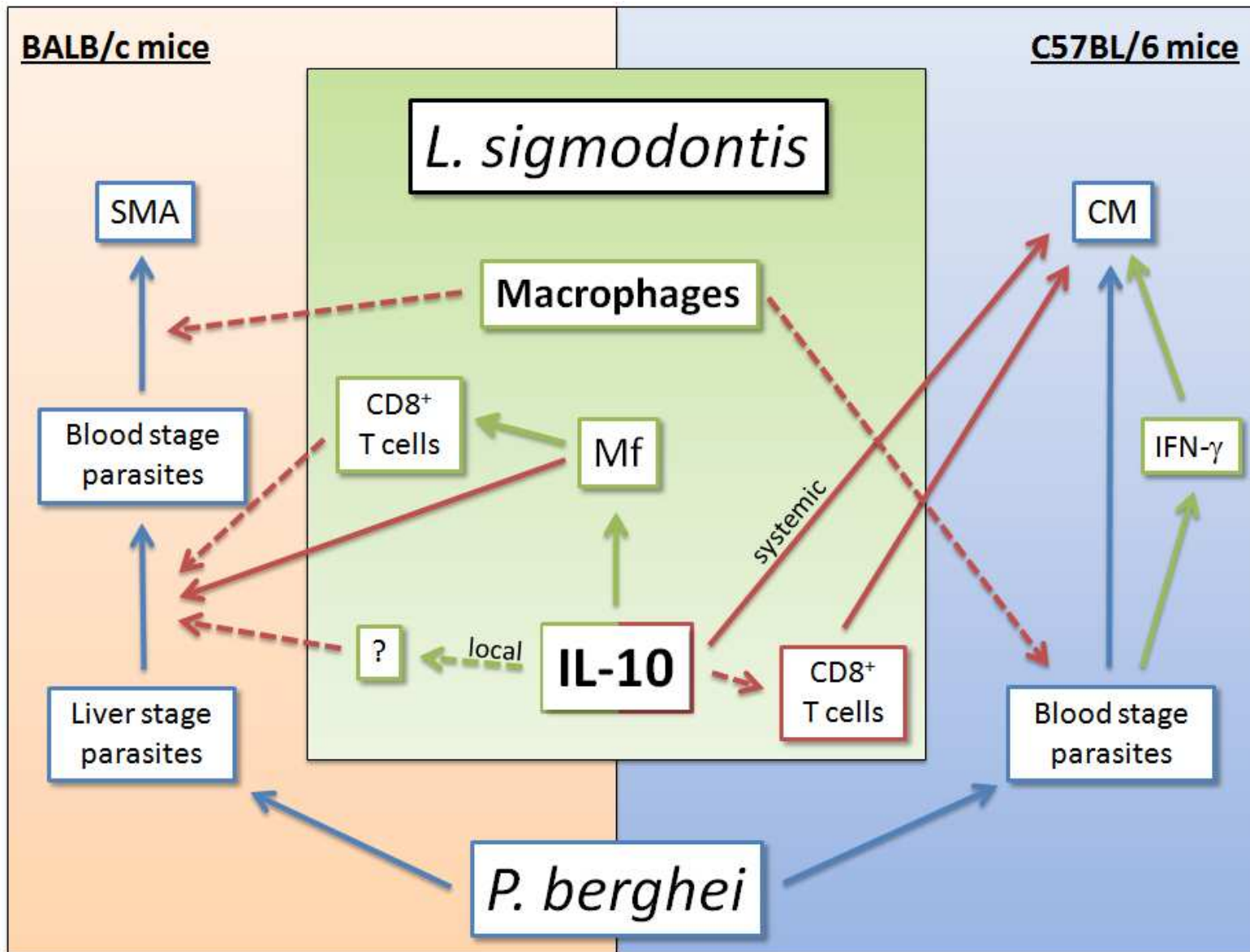


Figure 4.1: Summary of the effects of *L. sigmodontis* on *P. berghei* after co-infection. *L. sigmodontis* has been shown to influence the course of *P. berghei* infections through different mechanisms that were dependent on IL-10. This cytokine induced protection against *P. berghei* sporozoite challenge in BALB/c mice. Such protection was related to the presence of Mf in blood, favoured by IL-10, which in turn correlated with higher CD8⁺ T cell numbers in spleen. Additionally, CD8⁺ T cell numbers were increased in the liver of co-infected mice, potentially impairing intrahepatic parasite development. In co-infected C57BL/6 mice CM was prevented in an IL-10-dependent manner, presenting these mice lower CD8⁺ T cells in the brain. IFN- γ levels remained unaltered in co-infected mice. Macrophages were increased in the spleen in co-infected mice from both strains, potentially accounting for improved parasitaemia control. Red arrows denote impairment, whereas green arrows represent enhancement. Dotted lines denote not fully demonstrated hypotheses.

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7. Declaration/Erklärung

An Eides statt versichere ich, dass ich die Arbeit mit dem Titel "Filarial nematodes protect against malaria in a murine co-infection model" selbst und ohne jede Hilfe angefertigt habe, dass diese oder eine ähnliche Arbeit noch keine anderer Stelle als Dissertation eingereicht würde. Ich habe früher noch keinen Promotionsversuch unternommen.

This thesis has been written independently and with no other sources and aids than stated

Bonn, April 2008

Daniel Fernández Ruiz

Communications

Oral presentations

Fernández Ruiz D, Dubben B, Hoerauf A, and Saeftel M “Filarial infection leads to prolonged survival in *Plasmodium berghei* (ANKA) co-infected mice” 9th symposium "Infection and Immune Defence", Rothenfels, Germany, 11th-13th March 2005

Fernández Ruiz D, Dubben B, Killian T, Stephan C, Hoerauf A, and Saeftel M. “Previous filarial infection protects *Plasmodium berghei* (ANKA) co-infected C57BL/6 mice against death through cerebral malaria% 36. Jahrestagung der Deutschen Gesellschaft für Immunologie (36th Annual Meeting of the German Society of Immunology), Kiel, Germany, 21st-24th September 2005

Fernández Ruiz D, Dubben B, Killian T, Stephan C, Hoerauf A, and Saeftel M. “Previous filarial infection protects *Plasmodium berghei* (ANKA) co-infected C57BL/6 mice against death from cerebral malaria” 22.Jahrestagung der Deutsche Gesellschaft für Parasitologie (22nd Annual Meeting of the German Society of Parasitology), Vienna, Austria, 22nd-25th February 2006

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Posters

Saeftel M, Fernández Ruiz D, Dubben B, and Hoerauf A “Co-infection with filariae leads to prolonged survival in *Plasmodium berghei* (ANKA) infected mice” First Annual BioMalPar Conference on Biology and Pathology of the Malaria Parasite, Heidelberg, Germany, 2nd-4th March 2005

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Publications

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