Analysis of parasite-specific T cells and cellular interactions in the spleen during *Plasmodium berghei* induced experimental cerebral malaria

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Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig und ohne fremde Hilfe verfasst habe. Andere als die angegebenen Quellen und Hilfsmittel wurden nicht benutzt und wörtlich oder inhaltlich entnommenen Stellen der angegebenen Werke sind als solche kenntlich gemacht. Ferner versichere ich, dass ich diese Dissertation an keiner anderen Universität eingereicht habe, um ein Promotionsverfahren eröffnen zu lassen.

Abstract

Vector-transmitted parasitic infections are a global health problem. Diseases such as malaria are a major health threat and economic burden for developing countries. In Sub-Saharan Africa malaria causing *Plasmodium* parasites may evoke life threatening complications, which mainly affect children under the age of five years. Pathogenesis of cerebral malaria is a multifactorial complex process that involves inflammatory mediators such as effector T cells and IFN-γ. Time, origin and cellular and molecular factors involved in early immune responses priming effector T cells responsible for the pathogenesis of cerebral malaria have been less investigated. In our study we were able to determine the presence of parasite specific cytotoxic T cells in spleen and brain during *Plasmodium berghei* infection. This enabled us to analyse cellular interactions involved in the priming of T cells directed against the parasite. The place of essential T cell priming during *Plasmodium* infections was determined in splenectomised mice, in which effector responses were reduced and experimental cerebral malaria (ECM) pathology was absent. Depletion of antigen-presenting cells, involved in maintaining the organized splenic structure, abrogated T cell priming and resulted in the loss of effector responses. Without either macrophages, DCs or B cells lytic activity and IFN-γ production by parasite-specific T cells was diminished and in the absence of effector responses ECM progression was suppressed. In detail, we provide evidence that macrophages, B cells and dendritic cells, as well as $CD4^{+}$ T cells together with TLR-9 and IL-12 signalling comprise complex interactions affecting T cell generation during blood stage malaria leading to neuropathology.

Taken together our study suggests, that early immune responses during *P. berghei* infection are generated in the spleen and that distinct cells and cytokines drive generation of parasite-specific T cells leading to subsequent pathology.

Abstrakt

Durch Vektoren übertragene Parasiteninfektionen stellen weltweit eine große Herausforderung dar. Krankheiten, wie z.B. Malaria bergen große gesundheitliche, aber auch ökonomische Risiken. Malaria, ausgelöst durch eine *Plasmodium*-Infektion, ist eine schwerwiegende Krankheit mit zum Teil lebensgefährlichen Komplikationen, die insbesondere Kinder unter fünf Jahren betreffen. Die Pathogenese von zerebraler Malaria ist ein multifaktorieller und komplexer Entzündungsprozess, bei dem Effektor T-Zellen und IFN-γ eine wichtige Rolle spielen. Obwohl T-Zellen stark in die Entwicklung zerebraler Malaria involviert sind, ist über Ursprung, zeitliche Abfolge und zelluläre und molekulare Prozesse der frühen Immunantwort, die für die Aktivierung von Effektor T-Zellen verantwortlich sind, bisher wenig bekannt. Wir konnten die Existenz von Parasiten-spezifischen zytotoxischen T-zellen in der Milz und im Gehirn während einer *Plasmodium berghei* ANKA Infektion in einem Mausmodell nachweisen. Das Entfernen der Milz ging mit einem reduziertem Risiko der zerebrale Malaria und einer verminderten Entzündungsreaktion einher. Dies zeigte, dass Effektor T-Zellen in der Milz generiert werden. Durch die Depletion Antigen-präsentierender Zellen, welche strukturgebend für die Milzarchitektur sind, wurde die Generierung und Aktivierung von Effektor T-Zellen verhindert und inflammatorische Reaktionen unterbunden. In Abwesenheit von Makrophagen, dendritischen Zellen, oder B-Zellen war die zytotoxische Aktivität und die INF-γ Antwort reduziert und neuropathologische Symptome blieben aus. Dies deutet darauf hin, dass komplexe Interaktionen von Makrophagen, B-Zellen, und dendritische Zellen, sowie CD4^{+} T Helfer Zellen zusammen mit TLR-9 und IL-12 Signalwegen, die T-Zell-Generierung in einer *Plasmodium*-Infektion und somit die Pathogenese von zerebraler Malaria stark beeinflussen. Zusammengefasst liefert dieses Projekt Hinweise dafür, dass die frühe Immunantwort gegen den Parasiten in der Milz hervorgerufen wird, wobei spezifisch Immunzellen und Zytokine an der Bildung von Effektor T-Zellen beteiligt sind, welche wiederum zu der Entstehung von neurologischen Schäden führen.

List of Abbreviations

α	anti-
AdOVA	recombinant *Adenovirus* type 5 expressing ovalbumin
Ag	antigen
APC	antigen-presenting cell
AT	autotransplantation
BBB	blood-brain barrier
BMDC	bone marrow derived dendritic cell
BSA	bovine serum albumine
CD	cluster of differentiation
CloLip	clodronate liposome
CM	cerebral malaria
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DOG	CD11c DTR Ovalbumin green fluorescent protein
dpi	days post infection
DTR	diphtheria Toxin Receptor
DTX	diphtheria Toxin
ECM	experimental cerebral malaria
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme linked immuno sorbent assay
FACS	fluorescence activated cell sorter
FITC	fluorescein
GPI	glycosyl-phosphatidyl-inositol
i.p.	intraperitoneal
i.v.	intravenous
ICAM	intercellular adhesion molecule; CD54

IDO	indoleamine 2,3-dioxygenase
IFN	interferon
Ig	immune globuline
IL	interleukin
iRBC	infected red blood cell
KO	knock-out
LPS	lipopolysaccharide
LT	lymphotoxin
mAb	monoclonal antibody
MHC	major histocompatibility complex
mM	millimolar
MMM	metallophillic marginal zone macrophage
MSP	merozoite surface protein
MZ	marginal zone
MZM	marginal zone macrophage
NfκB	nuclear factor kappa B
μg	microgram
ng	nanogram
NK	natural killer
NKT	natural killer T cell
NO	nitric oxide
ODN	oligodeoxynucleotide
OVA	ovalbumin
p.i.	post infection
PALS	periarteriolar lymphoid sheath
PAMP	pathogen associated molecular pattern
PbA	*Plasmodium berghei* ANKA
PBS	phosphate buffered saline
PbTG	recombinant *Plasmodium berghei*expressing ovalbumin
PRR	pattern recognition receptor
RAG	recombinant activation gene deficient mice
RBC	red blood cell
rpm	rotations per minute
RT	room temperature

S8L	SIINFEKL, OVA-derived MHC class I peptide
SCID mice	nude severe combined immunodeficient mice
SNP	single nucleotide polymorphism
SplX	splenectomy
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF	tumour necrosis factor

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1 Introduction

Developing countries, especially in Sub-Saharan Africa, have to cope with diseases like HIV, tuberculosis and malaria. In 2008 around 250 million cases and almost a million deaths (WHO, 2010) were caused by malaria, a disease induced by infection with the vector-transmitted parasite *Plasmodium* spp.. Investigations of mechanisms involved in protective and detrimental immune responses against the protozoan parasite remain far from being unveiled. This study aimed to elucidate early parasite-specific immune responses in the spleen involved in the pathogenesis of cerebral malaria.

1.1 General concepts about malaria

1.1.1 Epidemiology

Five species of the protozoan parasite from the genus *Plasmodium* are relevant for humans. Infection with *Plasmodium vivax, P. ovale, P. malariae*, *P. falciparum* and *P. knowlesi* can cause disease, with complications ranging from benign to very severe. Usually, these parasites are well adapted to their host species. The primate parasite *P. knowlesi*, however, is also infectious for humans and pathology is comparable to the other infective strains. Widely distributed and adopted to various conditions *Plasmodium* transmitting *Anopheles* mosquitoes render half the world population in 108 countries and territories at risk of infection. In tropical regions, especially in sub-Saharan Africa, *Plasmodium falciparum* is the major cause of severe debilitating illness that accounts for an estimated 1 million deaths worldwide and is responsible

for 20% of infant mortality in Africa. Besides health issues, malaria has a high socio-economic burden for developing countries and despite being curable, in areas of poor infrastructure access to early treatment is difficult (WHO, 2010).

1.1.2 Life cycle

Infected female *Anopheles* mosquitoes transmit *Plasmodium*-sporozoites to the host during the blood meal. Sporozoites migrate out of the salivary gland and are injected into the host. Sporozoites are either directly injected into the blood stream or into the skin from where they migrate into the blood vessels to enter blood circulation (Yamauchi et al., 2007). With the blood circulation some sporozoites reach the liver where they pass through several tissue cells inducing the first immune responses to the parasite before they invade hepatocytes. Within the hepatocytes sporozoites reside in a parasitophorous vacuole, in which they reproduce by multiple fission generating thousands of daughter parasites. This developmental stage is asymptomatic and lasts 6-15 days. When the division is completed, schizonts release single merozoites into the blood stream. New erythrocytes are quickly invaded, where daughter parasites multiply through schizogonie very fast. After 48 to 72 hours parasitized erythrocytes burst and released merozoites that immediately infect new erythrocytes. In parallel, some merozoites differentiate into male and female gametocytes, which complete the life cycle when taken up by another mosquito (Bogitsh et al., 2005).

1.1.3 Clinical disease

Clinical pathology of malaria arises from the blood stage of infection. Infected individuals may experience regularly reoccurring fever peaks. This is caused by the synchronized parasite discharge from infected erythrocytes, which is accompanied by the release of pyrogenic mediators. Differing growth rates of human plasmodial parasites lead to a species-specific characteristic periodicity. Tertian malaria originating from *P. vivax* and *P. ovale* causes febrile episodes every 48 hours. After infection with *P. malariae* (quartan malaria) patients experience fever bursts every 72 hours. Erythrocyte release from *P. falciparum* is less synchronized and does not

Figure 1.1: Life cycle of *Plasmodium* spp.
Female *Anopheles* mosquitoes transfer sporozoites during a blood meal to the vertebrate host. After inoculation parasites travel to the liver where they invade hepatocytes. Intracellular division takes place within hepatocytes and after 2-7 days parasites are released into the blood stream from ruptured cells. Free merozoites readily infect erythrocytes in which asexual reproduction takes place followed by the release of daughter cells and invasion of fresh cells. In infected erythrocytes some parasites develop into male and female gametocytes, which then can be taken up by a blood feeding vector and the cycle starts over (adapted from Miller et al. (2002)).

show a stringent pattern in fever episodes, which may occur in 48 - 72 hour intervals (Bogitsh et al., 2005). 80% of the worlds malaria cases are caused by *Plasmodium falciparum*, which is also responsible for severest complications increasing the risk of death. Two characteristic traits of this species are associated with pathology. For one *P. falciparum* has the ability to infect any erythrocyte without regard to age or infectious status, leading to higher parasite burdens. Increased destruction of infected and uninfected erythrocytes can cause severe malaria anaemia (SMA). Involvement of the immune system causing anemia has been proposed but detailed mechanisms remain to be described (Schofield and Grau, 2005). Secondly, expression of parasite

cell-surface proteins on host erythrocytes alter adhesion properties and infected erythrocytes thereby escape immune-recognition in the spleen through tissue sequestration. Site-specific adhesion of parasitized red blood cells, concomitantly with host inflammatory reactions contribute to drastic disease progression. Cerebral malaria is defined by the presence of unarousable coma, asexual forms of *P. falciparum* in blood and exclusion of other causes of encephalopathy (Newton and Warrell, 1998). Exact mechanisms leading to neuropathogenesis are still not fully understood. In recent years perception of cerebral malaria pathogenesis has shifted from tissue sequestration being the sole cause towards a more global notion, in which sequestration, inflammation and dysregulated hemostasis are considered to be critically involved in disease progression (van der Heyde et al., 2006; Clark and Alleva, 2009; Combes et al., 2010). Despite immense research efforts and increasing knowledge, the multifactorial cerebral malaria syndrome is far from being understood let alone from being eradicated. Challenges in treating CM-patients arise i) early diagnosis of CM is difficult as reliable predictive markers are scarce (Martins et al., 2009), symptoms are rarely distinct and are sometimes misdiagnosed (Milner, 2010), ii) access to health care and medical treatment in countries with high CM incidences is limited (WHO, 2010).

1.2 Immunology of malaria

Despite having strictly defined clinical criteria, cerebral malaria is a heterogeneous condition with several factors contributing to pathogenesis. Although critically debated, excessive host immune responses are now considered to be at least partially responsible for disease progression. Clinical studies examining parasite-host interactions before and during neuropathogenesis are strongly restricted by ethical constraints and remain descriptive.

1.2.1 Rodent model of cerebral malaria

Human studies focusing on local immune responses in specific organs after *P. falciparum* infection are, for obvious ethical reasons, limited to post-mortem examina-

tions. However, important information regarding host-pathogen interactions and immunological responses in time and placed tissue tropism can be examined in an experimental murine model of cerebral malaria. *Plasmodium berghei* infection in susceptible strains (Rest, 1982; Curfs et al., 1993b) induces pathology in mice, which is comparable to pathology seen in humans. Although the similarities of the murine model of experimental cerebral malaria (ECM) continue to be debated (de Souza et al., 2010; White et al., 2010). The absence of erythrocyte sequestration but presence of leukocytes and platelets in ECM positive mice as well as differences in inflammatory responses against the parasite are some of the criticized differences to human cerebral malaria. Nevertheless, sequestration in brain capillaries being the major factor causing neuropathology seems to be overrated, as some tissue samples from deceased cerebral malaria patients were devoid of parasite and erythrocyte sequestration (Clark et al., 2003). Several reviews elucidated and discussed the factors involved and sequence of events leading to *Plasmodium* induced neuropathogenesis (Milner, 2010; Clark and Alleva, 2009; van der Heyde et al., 2006) coming to the conclusion that sequestration, inflammation or dysregulated homeostasis can all be associated with pathology. In patients, analysis of inflammatory responses during *Plasmodium* infection are limited to peripheral blood, and in rare cases liquor and post-mortem tissue samples. Thus experimental models, despite incompletely resembling human disease, give the possibility to match age, genetic background and experimental readouts regarding immune responses at specific time points in specific organs. Hence, giving valuable insights into the complex interactions of host immunity and parasite infection.

1.2.2 The spleen

The spleen as secondary lymphoid organ is involved in clearing blood borne infections and generation of adaptive immune responses (Engwerda et al., 2005). Highly specialized compartments constitute the splenic architecture, in which very efficient innate and adaptive immune responses can be mounted (Mebius and Kraal, 2005). Circulating blood flows through arterioles into the marginal sinus and red pulp. The marginal sinus, a part of the marginal zone (MZ) divides the red and the white pulp regions. Specialized macrophage populations, the

marginal metallophillic macrophages (MMMs) and marginal zone macrophages (MZMs) together with B cells and trafficking dendritic cells (DCs) and T cells make up the MZ. Marginal zone macrophages are highly efficient in removing blood pathogens and pathogen particles. Metallophillic macrophages, in addition to phagocytic properties, have recently been shown to transfer processed antigen to dendritic cells which prime effector T cells through cross-presentation (Backer et al., 2010). The marginal zone directs antigen-presenting cells (APCs) to the white pulp, which consists of the B cell follicles and the periarteriolar lymphoid sheath (PALS). In the PALS trafficking dendritic cells come into contact with resident T cells, which are readily primed by antigen recognition.
During *Plasmodium* infection the spleen clears infected erythrocytes, and parasite particles. This property has been linked to increased incidence of splenomegaly and reduced parasite burden, but also to severe malaria anaemia (Buffet et al., 2009). Splenectomised malaria patients have been described to have increased parasite burden (Chotivanich et al., 2002; Looareesuwan et al., 1993). In a murine ECM model, splenectomy reduced the generation of cerebral pathology (Hermsen et al., 1998; Eling, 1980). Moreover, structural changes of the spleen have been observed in lethal cases of *P. falciparum* infection. The white pulp especially shows increased disorganisation with B cells absent from the marginal zone, unsegregated T cell areas and T cells diffusely scattered in the white pulp (Urban et al., 2005). Immune responses in the spleen are on the one hand required to control parasitemia and generate anti-parasitic responses. On the other hand, early interactions between parasite and immune cells can lead to effector cell generation potentially harmful for the host.

1.2.3 Antigen-presentign cells (APCs)

Blood circulation transports pathogens and pathogen particles into the spleen, where cells of the innate immune system are the first to encounter micro-organisms. After phagocytosis, antigens are processed and peptides presented either in a major histocompatibility complex (MHC) class I or MHC class II context. T cells recognize peptide-MHC complexes via the T cell receptor and this interaction, together with co-stimulatory signals, leads to priming of T cells.

Macrophages

Macrophages, situated in the spleen at the interface where blood passes through, are the first cells to come across pathogens. Receptor recognition of pathogen-surface molecules induces phagocytosis and intracellular destruction in macrophage lysosomes. Elimination of micro-organisms is followed by processing antigenic peptides, although not very efficient, these can be presented to activated T cells to induce effector function. Activated macrophages secrete a wide range of inflammatory mediators, including chemokines and cytokines, which participate in initiation of adaptive immune responses (Janeway et al., 2001). Macrophages have been implicated in the pathogenesis of cerebral malaria in humans (Baratin et al., 2005) and murine experimental models of CM (Pais and Chatterjee, 2005; Couper et al., 2010). Parasites are cleared mainly by monocytes/macrophages either directly by phagocytosis and intracellular destruction or by the release of toxic mediators (Artavanis-Tsakonas et al., 2003; Malaguarnera and Musumeci, 2002). Early recognition of parasites leads to increased production of pro-inflammatory cytokines (IL-12, IL-1, and IL-6) necessary for activation and differentiation of other immune competent cells, e.g. NK cells and CD4^{+} T cells. Although important for *Plasmodium* clearance, macrophages contribute to a pro-inflammatory cytokine environment reinforcing adaptive immune reactions which might have deleterious consequences for the host.

B cells

B cells have several different functions during infection. Their membrane bound immune-globulins serve as receptors for antigen, which when bound to the antibody will be taken up by B cells, processed and presented to T cells. Additionally, activation of B cells is induced after antibody-antigen recognition and interaction with T cells and macrophages. Activated B cells differentiate and clonaly expand generating plasma and memory cells. Plasma cells are able to produce secreted antibodies with specificity to one epitope on an antigen. During infection, antibodies bind to specific antigens on the pathogen, thus facilitating destruction by phagocytes. Vaccination studies describe B cells to influence blood stage *Plasmodium* in-

fection in an antibody dependent manner (Good et al., 2005). Acute malaria is associated with disruption of germinal centres in murine spleens (Carvalho et al., 2007) and depletion of B cells in the marginal zone in lethal *P. falciparum* infections (Urban et al., 2005). These data implicate a strong involvement of B cells in maintaining the organized structure of the spleen, although a direct involvement of B cells in pathogenesis of cerebral malaria has not been found (Yañez et al., 1996). However, the role of B cells in antigen presentation has not been addressed and their role during cerebral malaria has to be further elucidated.

Dendritic cells

Another set of antigen-presenting cells are dendritic cells. Linking innate and adaptive immune responses, mature dendritic cells presenting antigen potently stimulate naïve T cells. On the one hand, DCs can recognize and take up pathogens, peptides are generated in intracellular vesicles, loaded into MHC class II molecules and transported to the cell surface. On the other hand, dendritic cells can cross-present protein-derived antigens, which are taken up from the extracellular environment, processed and presented in an MHC class I context. Any infected nucleated cell may present antigens from intracellular pathogens bound to MHC class I molecules and thereby together with co-stimulatory signals induce effector function of $CD8^+$ cytotoxic T cells. However, cross-presentation by dendritic cells greatly enhances activation and expansion of effector T cells, which are critically involved in antiviral, anti-tumour immunity and in maintaining T cell tolerance to self antigens (Kurts et al., 2010). Depending on the strain of *Plasmodium*, dendritic cells have different functions during infection (Lundie, 2010). After *Plasmodium chabaudi* infection, $CD8^-$ DCs present parasite antigens to $CD4^+$ T cells and induce a shift towards Th2 responses characterized by IL-10 and IL-4 cytokines in mice, thus providing effective help for blood-stage parasite specific antibody responses (Sponaas et al., 2006). Nonetheless, $CD8^+$ DCs cross-present *P. berghei* specific antigens to $CD8^+$ T cells (Lundie et al., 2008), but presentation is short-lived (Lundie et al., 2010) and pro-inflammatory cytokines and T cell activity influence disease progression. deWalick et al. (2007) showed that conventional DCs are critically involved in cerebral malaria after *P. berghei* infection. Information on human dendritic cells

and their role in cerebral malaria is scarce and are mainly confined to *ex vivo* or *in vitro* studies. Nonetheless, haemozoin, a parasite-detoxification product released into the blood stream when infected erythrocytes rupture is known to inhibit monocyte function and further differentiation into dendritic cells (Stevenson and Urban, 2006).

1.2.4 Toll-like receptors

Invading pathogens are sensed by the innate immune system of the host through Toll-like receptors (TLRs). Pathogen associated molecular patterns (PAMPs) stimulate these pathogen recognition receptors (PRRs) inducing a variety of host defence mechanisms (Takeda et al., 2003). Cellular distribution of TLRs depends on the molecules recognized. Some are located on the cell surface to sense extracellular pathogens, for example LPS from gram-negative bacteria binds to TLR-4. Intracellular pathogens like viruses or intracellularly degraded micro-organisms are recognized in late endosomal compartments, e.g. TLR-7 senses the presence of single stranded RNA motifs and TLR-9 binds to short unmethylated CpG motifs (Takeda et al., 2003). Binding of PRRs to TLRs leads to the recruitment and phosporylation of adapter proteins and subsequent signalling cascades induce transcription factors culminating in the production of inflammatory mediators. Most TLRs share the adapter protein MyD88 and signalling leads to downstream activation of NF-κB, inducing the expression of pro-inflammatory cytokines like IFN-γ and IL-12 (Wagner, 2004). *Plasmodium* derived glycosylphosphatidylinositol (GPI) is known to stimulate TLR-2 and to a lesser extend, TLR-4; plasmodial DNA bound to haemozoin is recognized by TLR-9 (Krishnegowda et al., 2005; Parroche et al., 2007). TLR-9 is expressed mainly by plasmacytoid and conventional DCs, but are also found on B cells and macrophages. An involvement of TLR-9 signalling in CM pathogenesis is debated, with some studies having shown that neuropathology is TLR-9 dependent, while evidence from others argues against an influence of TLR-9 in disease progression (Coban et al., 2007; Togbe et al., 2007; Lepenies et al., 2007). However, human studies analysing host genetic factors found that single nucleotide polymorphisms (SNPs) in TLR-4 and TLR-9 predispose infected persons to severe malaria (Leoratti et al., 2008; Mockenhaupt et al., 2006).

1.2.5 Cytokines

Circulating cytokines such as tumour necrosis factor (TNF), interleukin-1 (IL-1), interleukin-6 (IL-6), lymphotoxin (LT) and interferon-γ (IFN-γ) have been associated with the development of severe malaria (Artavanis-Tsakonas et al., 2003; Hunt and Grau, 2003). Since inflammatory responses are recognized to be involved in pathology, lymphocytes and inflammatory mediators have increasingly become the focus of current research.

Interferon-γ

Interferon-γ, mainly produced by T and NK cells, induces activation of macrophages and subsequent microbicidal functions, increases MHC class I and II expression and enhances antigen presentation. Moreover, IFN-γ produced by Th1 cells is involved in antibody-class switching to protective IgG isotypes. This cytokine is also associated with CD8$^+$ T cell differentiation and involved in proinflammatory responses. Presence of IFN-γ during *Plasmodium* infection has versatile effects on the host. On the one hand IFN-γ is known to have anti-plasmodial properties in *P. chabaudi* infection, stimulating macrophages to produce nitric oxide (NO) and other toxic reactive oxygen radicals (Meding et al., 1990; Su and Stevenson, 2000). On the other hand, IFN-γ has been linked to cerebral malaria development, stimulating macrophages to exert anti-pathogenic activity and initiating antigen-presentation in endothelial cells (Geppert and Lipsky, 1985; Galea et al., 2007). In the absence of IFN-γ (Yañez et al., 1996) or IFN-γ- receptor (Amani et al., 2000; Rudin et al., 1997) mice are resistant to CM development. Knock-out mice showed reduced downstream effector functions like reduced intracellular adhesion molecule (ICAM-1) expression, reduced indolamin-2,3-dioxygenase (IDO) activity and overall reduced inflammatory reactions. Genetic studies in humans have revealed that people with a certain polymorphism in the IFN-γ gene had a lower incidence of CM. Some studies associate serum IFN-γ with development of severe malaria and neuropathology, whereas others negatively correlated this cytokine to acute malaria (Hunt and Grau, 2003; Malaguarnera and Musumeci, 2002; McCall

and Sauerwein, 2010). Thus the exact role of IFN-γ in malaria disease is still to be described.

Interleukin-12

Production of IFN-γ is induced by IL-12, a pro-inflammatory cytokine produced by dendritic cells and phagocytes upon TLR stimulation. IL-12p40 and IL-12p35 subunits comprise the heterodimeric IL-12p70 protein, signalling through the IL-12 receptor, which itself is composed of two chains (IL-12R-β1 and IL-12R-β2). Several cytokines belong to the IL-12 super-family sharing different subunits and receptors (Trinchieri, 2003). The IL-12p40 subunit together with IL-23p19 builds IL-23, whereas IL-12p35 and Ebi3 make up IL-35 (figure 4.1). IL-12p40 can also be found as a homodimer to form IL-12p80, which is described to have regulatory functions (Holscher, 2004). However, IL-12p70 is critically required for induction of IFN-γ production by NK cells and both cytokines are needed for Th1 cell differentiation and generation of cytotoxic CD8$^+$ T cells. Although IL-12 plays a central role in cell-mediated immune responses to induce parasite clearance, parasites and increased TLR-9 stimulation is reported to paralyse DC maturation dampening pro-inflammatory immune responses facilitating parasite survival and reducing immune-mediated damage (Reis e Sousa et al., 1999; Wingender et al., 2006; Urban et al., 2001). While IL-12 is known to be involved in strong pro-inflammatory reactions, in *P. falciparum* infection severity of disease is actually correlated to lower levels of IL-12 (Malaguarnera et al., 2002), and a heterozygous polymorphism in the promotor region of the IL-12B gene (encoding IL-12p40) has been associated with reduced IL-12 protein in serum samples that conferred an associated increased risk of CM development (Marquet et al., 2008).

Lymphotoxin

The tumour necrosis factor (TNF)/tumour necrosis factor receptor (TNF-R) superfamily comprises several cytokines with a variety of functions in immune responses. Two members of this family lymphotoxin-α (LT-α) and lymphotoxin-β (LT-β), despite their similar structures, have distinct roles in innate and adaptive immunity.

A common signalling network involves five distinct receptors which are shared between TNF-family cytokines and exert overlapping biological functions (Ware, 2005). Secreted mainly by activated lymphocytes like T cells, but also by macrophages and dendritic cells, LT activates and induces NO production, TNF activates vascular endothelium and increases permeability leading to local and systemic inflammation. Besides their involvement in immune responses, these cytokines and their receptors are required for lymphoid organ development. In the absence of lymphotoxin-β or LT-βR, development of the splenic architecture is disrupted and the migratory properties of DCs are impaired. Similar effects are seen in the absence of other TNF family members (Alimzhanov et al., 1997; Ware, 2005; Fütterer et al., 1998). Cerebral malaria pathology has been associated with increased serum levels of TNF and a single nucleotide polymorphism within the TNF promoter region correlated with greater cytokine transcription, conferring a higher risk of neuropathology (Hunt and Grau, 2003; Randall and Engwerda, 2010). Experimental data from murine cerebral malaria models, however, reinforce the importance of lymphotoxin rather than TNF in disease progression since the development of ECM required functional TNF and LT receptors (Togbe et al., 2008; Randall et al., 2008). Nonetheless, as LT-βR deficient mice have a disrupted splenic micro-architecture a failure to generate effector T cells may be hold responsible for the absence of neuropathology.

1.2.6 T cells

T cell responses of the adaptive immune system require processed antigens of invading pathogens for activation and effector functions. T cells recognize corresponding peptides bound to MHC molecules through their T cell receptor (TCR). T cells can be divided into two groups distinguished by surface expression of co-receptor molecules CD4 and CD8. Together with T cell receptors, CD4 and CD8 determine effector function of the cell. T cells expressing CD4, recognize antigens presented in an MHC class II context and differentiate into T helper (Th) cells that can be categorized into two main subsets, Th1 and Th2 cells. Cytokine environment influences differentiation into either subset, IL-12 induces Th1 differentiation, while e.g. IL-4 is responsible for e.g. Th2 development. Th1 and Th2 subsets have differ-

ent functions during immune responses. Th1 pro-inflammatory cytokines like IFN-γ, IL-12 and TNF assist in cell mediated immunity by activating DCs, macrophages and cytotoxic T cells. Excessive inflammation and tissue damage can also be associated with this subset. Th2 cells secrete IL-4 and IL-5 thereby promoting antibody dependent responses and activation of eosinophils.

Plasmodium induced cytokines like TNF and IFN-γ skew the differentiation of CD4$^+$ cells into Th1 cells. These cells are discussed to reduce parasite burden, but also promote disease in a murine *Plasmodium* infection (Hirunpetcharat et al., 1999). Moreover, early interactions between NK cells and *P. falciparum* parasites induce activation and function of CD4$^+$ T cells *in vitro*, possibly assisting in parasite clearance (Horowitz et al., 2010; Horowitz and Riley, 2010). Association of CD4$^+$ T cells with cerebral malaria is confined to the mouse model, in which these cells have been shown to contribute to neuropathology (Belnoue et al., 2002; Yañez et al., 1996; Amante et al., 2010).
Secreted cytokines like IFN-γ and IL-12 from Th1 cells participate in activation of cytotoxic CD8$^+$ T cells, thereby supporting clearance of pathogens through cytokine secretion, phagocyte activation and cytotoxic activity. However, if not tightly controlled, CD8$^+$ T cells can be involved in tissue destruction. Naive CD8$^+$ T cells encounter antigen bound to MHC class I molecules on the surface of antigen-presenting cells like dendritic cells and macrophages. Recognition and co-stimulation leads to maturation and clonal expansion before antigen-specific effector functions take place. Although, efficiently clearing virus infections, during ECM pathogenic T cells mediate blood brain barrier disruption by sequestration to endothelial cells with the resulting perforin-mediated destruction of the same (Nitcheu et al., 2003). The predominant role of CD8$^+$ T cells in ECM pathology was shown in depletion experiments performed by Belnoue et al. (2002) and Renia et al. (2006), which showed that depletion six days post infection and shortly before onset of CM development rescued WT mice from neurological disease and subsequent death. Moreover, RAG2$^{-/-}$ mice, which lack mature lymphocytes, do not develop neurological complications as seen in BL/6 WT mice (Finley et al., 1982; Grau et al., 1986; Yañez et al., 1996). However, reconstitution with primed effector T cells reversed protection from ECM (Nitcheu et al., 2003). The role for effector T cells during human cerebral malaria has not been evaluated systematically, as sample

retrieval is constrained by obvious ethical reasons. However, studies performed in the mid 1980s reported an increased frequency of circulating CD8$^+$ T cells in *P. falciparum* infections (Troye-Blomberg et al., 1984; Stach et al., 1986). Early interactions of APCs with effector T cells in the spleen, as well as antigen-specific immune responses during *Plasmodium* infection and neuropathology are insufficiently investigated, requiring further investigations.

1.3 Aims and Objectives

Cerebral malaria has been proposed to be the result of excessive immune mediated reactions induced by *Plasmodium* infection. Some factors and cells are known to be involved in blood brain barrier (BBB) disruption and neuropathology. Time, origin and cellular and molecular factors involved in early immune responses priming effector T cells, responsible for the pathogenesis of cerebral malaria, have not been well investigated. Immunopathology has more than one cause and depends on the interaction of several factors. In this work the spleen as an immunologic compartment involved in early priming of pathogenic responses during experimental cerebral malaria was elucidated.
Moreover, macrophages, B cells and dendritic cells that compose the highly organised architecture of the spleen and their involvement in effector cell generation were analysed in this study.

1. It is well known that effector T cells are critically involved in disease progression of cerebral malaria. Our study aimed to elucidate if detrimental responses were a result of unspecific hyper-reactive T cell responses or if effector T cells were directed specifically towards parasite antigens, and were thus restrictively involved in neuropathology?
2. T cell priming towards blood-bourne antigen takes place in the spleen. It is known that the spleen is involved in detrimental immune responses and splenectomy is described to reduce the incidence of cerebral malaria in susceptible mice. Is the cellular organisation and an intact micro-architecture a necessity for early priming events during *Plasmodium* infection and are cells

maintaining the splenic structure of importance in generation of antigen-specific T cell responses and subsequent pathogenesis?

3. Antigen-presentation and specific priming of T cells is a requirement for effective T cell responses. Which cells in the spleen participate in antigen presentation and parasite-specific cytotoxic T cell generation? Are interactions between macrophages, dendritic cells and B cells, all of which reside within distinct compartments in the spleen, responsible for priming of $CD8^+$ responses leading to cerebral malaria?

4. The activation of antigen-presenting cells is dependent on parasite recognition through TLRs. This in turn leads to production of cytokines like IL-12 required for the activation of $CD4^+$ T cells. Therefore the question arose, is parasite recognition through TLR-9 and subsequent IL-12 production important during *P. berghei* infection and is TLR-9 stimulation and IL-12 signalling involved in T cell priming? Another prerequisite for APC and T cell activation is the help of $CD4^+$ T cells, which are activated by IL-12. During *Plasmodium* infection, is CD4 T cell help involved in effector T cell generation and blood-brain barrier disruption and neuropathology?

2 Material and Methods

2.1 Material

2.1.1 Animal maintenance

C57 BL/6 mice were obtained from Janvier (Le Genest Saint Isle, France), Charles River Laboratories (Sulzfeld, Germany), our own animal facility or the House of Experimental Therapy (HET, Bonn). All mice were kept in animal facilities of the institute and were provided with food and water *ad libitum*.

2.1.2 Buffers and solutions in alphabetical order

Anaesthesia: longterm

Rompun 23,32 mg/ml(Bayer, Leverkusen, Germany)	10 μl
Ketanest 50 mg/ml (Medistar, Ascheberg, Germany)	10 μl
PBS 1x (PAA Laboratories, Austria)	30μl

50 μl injected intra muscular (i.m.)

Anaesthesia: short-term

Inhalation of Isofluran (Abbott, Wiesbaden, Germany)for 30-90 seconds.

Antibodies

ELISA

Antibodies and corresponding recombinant protein for cytokine ELISA were purchased from BD Pharmingen (Heidelberg, Germany).

IFN-γ ELISA

Capture antibody	R4-6A2, 2μg/ml
Detection antibody	MG1.2, 1μg/ml
Recombinant protein	19301T

FACS

For FACS-staining of murine surface-molecules or intracellular cytokines, the following antibodies were purchased from BD Pharmingen or eBiosciences. All antibodies were either biotinylated or labelled with a fluorochrome (FITC, PE or APC) and used in amounts previously determined by titration. The antibodies were directed against the following murine epitopes (clone names are given in parentheses): anti-CD8α (53.-6.7), anti-IFN-γ (R4-6A2) Fc receptors were always blocked by adding 2.4G2 (rat) antibody. H-2kb/SIINFEKL tetramers labeled with fluorochromes were purchased from ProImmune.

Depletion

GK1.5 antibody to deplete CD4$^+$ T cells was kindly provided by the Institute of Molecular Medicine, Bonn.

Cell culture media

All reagents, unless otherwise stated, were purchased from PAA Laboratories. BMDCs were cultured in IMDM supplemented with 10% FCS, 2 mM Glutamine, 100 IU/ml Penicillin and 100 μg/ml Streptomycin. Splenocytes and brain lymphocytes were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM Glutamine, 100 IU/ml Penicillin and 100 μg/ml Streptomycin. Work was carried out under sterile conditions and solutions were kept sterile at 4°C.

Collagenase A buffer

50 mg/ml collagenase type I A (*Clostridium histolyticum*) from Sigma Aldrich GmbH, Munich, was diluted 1:125 in 1x PBS.

CFSE stock

5,6-Carboxy-Succinimidyl-Fluoresceine-Ester (CFSE, Invitrogen, Darmstadt, Germany) was dissolved in DMSO (10 mM) and stored in 10 μl aliquots at –20°C.

EDTA 200 mM

18.61 g Ethylendiamintetraacetat (EDTA) ($C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$) was dissolved in 250 ml ultra-pure water and adjusted to pH 8.0 with NaOH. The solution was autoclaved and stored at room temperature.

ELISA buffer

Coating buffer	0.1 M Na_2PO_4 in aqua bidest, pH 9.0
Washing buffer	1 M PBS, 0.05% Tween (Sigma Aldrich GmbH)
Blocking solution	1 M PBS with 1% BSA (Roth, Karlsruhe, Germany)
Substrate buffer	0.1 M $NaH_2PO_4 \cdot H_2O$, pH 5.5
Substrate	60 mg 3,3',5,5'Tetramethylenbenzidine (Roth) dissolved in 10 ml DMSO
Substrate solution	10 ml substrate buffer 200 μl substrate and 2 μl H_2O_2
Stop solution	2 M H_2SO_4

FACS buffer

1x PBS containing 1% FCS, prepared under sterile conditions and stored at 4°C.

FarRed stock

FarRed (Invitrogen) was dissolved in DMSO (1 mM) and stored in 10 μl aliquots at -20°C.

MACS buffer

1x PBS containing 1% FCS and 2mM EDTA was stored under sterile conditions at 4°C.

NaCl

Diaco, Serag Wiessner KG, Naila, Germany.

PBS - Phosphate buffered saline

PAA Laboratories, Austria

Peptides

Peptides were obtained from Pineda (Berlin, Germany), dissolved in DMSO for 20 mM stock solutions and stored at -20°C.

Origin	Name	Sequence	MHC Haplotype
OVA	S8L	SIINFEKL	H-2kb
MSP	F8L	FITKFEEL	H-2kb
	L8L	LGITYKSL	H-2kb
	D10L	DGLQNIYAEL	H-2db
	K10L	KPIENIQDDI	H-2db
	T10L	TELVNLIKTL	H-2db

2.1.3 Equipment

Spectra-photometer Platereader	Spectra MAX 340PC, MTX lab systems, Virginia, USA
Flow Cytometer	FACS Canto, BD biosciences, Heidelberg, Germany
Cell counter	CASY TT® , Schärfe System, Reutlingen, Germany
Centrifuge	Heraeus Multifuge 4 KR - LH-4000 Rotor, Kendro Laboratory Products GmbH, Hanau, Germany

2.1.4 Computer software

Data processing	Open Office Calc (Sun Microsystem Inc.)
Statistical analyses	Graph Pad Prism 5.0 (Molecular Devices, Sunnyvale, USA)
	PASW 18 (IBM, New York, USA)
FACS	FACSDiva Software (BD Pharmingen, Heidelberg, Germany)
	FlowJo Software (Treestar Inc., Ashland, USA)

2.2 Methods

2.2.1 Identification of H-2kb and H-2db specific peptides from *Plasmodium berghei* MSP-1

MSP-1 specific peptides were searched with SYFPEITHI, a database of MHC ligand- and peptide motifs. The NCBI amino acid sequence (NCBI Reference: XP_678505) of *P. berghei* merozoite surface protein-1 (MSP-1) was placed into the database of SYFPEITHI. Our model of interest was the C57 BL/6 mouse, in which MHC class I molecules have the H-2kb or H-2db haplotype. Length of the peptides was set to 8-10 amino acids, the optimal size for the MHC class I peptide binding groove. Peptides with the best binding properties were selected and synthesized from Pineda (Berlin, Germany).

2.2.2 Experimental animal handling and knock-out mice

Starting day four post infection, mice were punctured in the tail vein for daily blood-smears. Before day 4, parasite levels were almost undetectable (1 dpi and 2 dpi) or very low (3 dpi). For evaluation of disease onset, mice were monitored twice daily for ECM symptoms. ECM development was scored according to the following symptoms: 0= without symptoms, 1= ruffled fur, 2= hunching, 3= wobbly gait, 4= limb paralysis, 5= convulsions, 6= coma (Amante et al., 2007).

Table 2.1: Mouse strains

Strain	**Phenotype**	**Reference**
CD11c-DTR-OVA-GFP tg	Express under CD11c promoter human Diphtheria toxin receptor and express OVA and GFP	N. Garbi, Heidelberg
CD19-DTR- tg	Express under CD19 promoter human Diphtheria toxin receptor and express OVA and GFP	Buch et al. (2005)
IL-12p35$^{-/-}$	IL-12 deficient	Cousens et al. (1999)
IL-12p35/p40$^{-/-}$	IL-12 and IL-23 deficient	Provided by G. Alber, Leipzig
IL-12p40$^{-/-}$	IL-12 and IL-23 deficient	Provided by G. Alber, Leipzig
IL-23p19$^{-/-}$	IL-23 deficient	Provided by G. Alber, Leipzig
IL-12Rβ1$^{-/-}$	Deficient in IL-12 signalling	Provided by M. Freudenberg, Freiburg
RAG1$^{-/-}$	Lack B and T cells	Provided from IMMEI
TLR-9$^{-/-}$	TLR-9 deficient	Hemmi et al. (2000)

2.2.3 *Plasmodium berghei* infection

In all experiments *P. berghei* infected red blood cells (pRBCs) were used to inoculate mice. Mice were infected intravenously (i.v.) with $5x10^4$ pRBCs obtained from mice that had been previously infected intraperitoneally (i.p.) with stock solution. Stock solution contained $1x10^7$ iRBCs in glycerine and was stored in liquid nitrogen. Stock mice were of the same background as experimental animals. Experiments were conducted with either *P. berghei* ANKA (PbA) or *P. berghei* expressing OVA (PbTG). The latter was kindly provided by Rachel Lundie (Lundie et al., 2008). Experimental setup and *Plasmodium* strains used are denoted in the figure caption or results section.

2.2.4 Splenectomy and autotransplantation

Splenectomy was performed seven to five days prior to infection (SplX). Splenic pedicles were ligated and subsequently severed. Then the spleen was removed and the body wall and skin sutured. For autotransplantation (AT), the spleen was placed in a dish with sterile PBS and cut into pieces of equal size. The pieces were then re-implanted into the same animal by placing them in the renal pelvis. During the whole procedure mice were anaesthetised with Isofluran (Abbott).

2.2.5 *Adenovirus* infection

$1x10^8$ pfu of *Adenovirus* type 5 expressing ovalbumin (AdOVA) was administered i.v. 6 days prior to analysis.

2.2.6 Depletion of cell types

Macrophages
Macrophages were depleted at one day prior to infection (d-1) and 4 days after parasite inoculation (4 dpi) by intravenous injection of 0.2 ml of PBS containing approximately 1 mg of dichloro-methylenediphosphonate (Cl_2-MDP) encapsulated in

liposomes (kindly provided by Nico van Rooijen, Faculty of Medicine, Amsterdam University, Amsterdam, The Netherlands).

Dendritic cells
CD11c DTR OVA GFP mice were injected i.p. with 40 ng/g diphtheria toxin (DTX) (Sigma-Aldrich GmbH) dissolved in 100 μl NaCl. Treatment regimen was on a daily basis, starting d0 and ending d4 p.i. with *P. berghei*.

B cells
CD19 DTR OVA GFP mice were injected i.p. with 40 ng/g diphtheria toxin (DTX) (Sigma-Aldrich GmbH) dissolved in 100 μl NaCl. Treatment regimen was on a daily basis, starting d0 and ending d4 p.i. for early depletion, and d4 till d7 p.i. for late depletion.

$CD4^+$
100 μl GK1.5 antibody was given i.p. one day post infection or 5 days post infection.

2.2.7 Perfusion and lymphocyte isolation

In order to analyse lymphocytes that were sequestered to vascular endothelium or extravasated into the brain tissue, long-term anaesthetised mice were intracardially perfused for 5 minutes with 1x PBS. Spleens and brains were removed, placed in MACS buffer or collagenase A buffer, respectively, and placed on ice. Spleens were gently pressed through a sieve, washed with 1x PBS containing 1% FCS and 2 mM EDTA and centrifuged (10 min., 1500 rpm with the Heraeus Multifuge 4 KR), cell numbers were determined with the CASY® TT cell counter (Schärfe Systems). Cells were adjusted to $2x10^7$ per ml medium, and 50 μl were plated into 96 well plates. Brain tissue was cut into small pieces and incubated in 2 ml collagenase A buffer for 30 min. at 37°C in a water bath. MACS-buffer was added to the tissue, which was then homogenized to a single cell suspension using a metal sieve. Cells were then centrifuged at 1500 rpm for 8 min. at 4°C. The cell pellet was suspended in 5 ml 30% Percoll that was then layerd onto a two step Percoll gradient made of 3 ml 37% Percoll and 3 ml 70% Percoll. Samples were centrifuged at room temperature for 20 minutes without brakes at 2000 rpm (Heraeus Multifuge 4 KR). After

percoll separation, two white interphases were isolated and transferred into a fresh tube and washed with MACS buffer. After a second centrifugation step, cells were resuspended in 1 ml RPMI. Cells were counted with the CASY® TT cell counter. (10 μl in 10 ml). Peptides (OVA or MSP-1) were added in a final concentration of 2 μM.

2.2.8 Generation of bone marrow derived dendritic cells (BMDCs)

Hind legs from naive C57 BL/6 donor mice were cut at the pelvis and disinfected with ethanol. After removal of skin and muscle tissue, bones were washed in sterile PBS. The ends of the bones were cut off and bone marrow was flushed with a PBS-filled syringe into 50 ml Falcon tubes. Cells were then washed with PBS, centrifuged for 10 minutes, 1500 rpm at 4°C (Heraeus Multifuge 4 KR) and resuspended in GM-CSF (PAN^{TM} Biotech GmbH, Aidenbach, Germany) complemented IMDM medium. The suspension was adjusted to 5x10^5/ml and 10 ml were plated on to sterile petri dishes. After four days cells were incubated for 2 minutes with 2 mM EDTA at 37°C, all cells were transferred into a new 50 ml tube and washed with sterile PBS and centrifuged for 10 minutes at 4°C . After resuspension in IMDM supplemented with GM-CSF cells were re-plated. Seven days after culture BMDCs were harvested and co-cultured with splenocytes or brain lymphocytes for 24 hours in RPMI.

2.2.9 Cell culture

1x10^6 splenocytes or 1x 10^5 brain lymphocytes were co-cultured with 1x10^6 BMDCs in 96-well culture plates (Greiner) at 37°C and 5 % CO_2 for 24 hours. Supernatants were taken for cytokine analysis and stored at -20°C until needed.

2.2.10 Evans Blue Assay to detect BBB disruption

Six days p.i. with *P. berghei* mice were injected intravenously with 200 μl 2% Evans Blue (Sigma Aldrich, St. Louis, USA). Mice were sacrificed one hour later and

brains were isolated to assess vascular leakage of the dye into the brain parenchyma. After photo documentation, brains were weighed and incubated in 2ml formamide for 48h at 37°C. 100 μl of the solution were analysed in triplicate on a microtiter plate. Absorbance was measured at 620nm in an ELISA reader. Concentration of Evans Blue was calculated with a standard curve (200- final point μg/ml) and is expressed as μg Evans Blue per gram brain tissue.

2.2.11 *In vivo* cytotoxicity assay

Five days after infection, cytotoxicity of antigen-specific $CD8^+$ T cells can be measured. Intravenously administered splenocytes (target cells) from syngenic donors, were labeled with antigen-specific class I peptides and lysed by antigen-specific cytotoxic T lymphocytes (CTLs) of infected animals. The target cells were labelled with CFSE to allow visualization via flow cytometry. Target cells were pulsed with OVA class I peptide SIINFEKL (1 μM) or a MSP-1 peptide mix containing F8L, K10L, T10L, D10L peptides (1 μM), samples were incubated for 15 minutes at 37°C. Subsequently cells were labelled with 1 μM CFSE or FarRed (high). These target cells should be recognized by CTLs. Reference cells without peptide were labelled with 0.1 μM CFSE. The amount of reference cells should remain stable as they should not be recognized by immune cells. Staining took place at 37°C for 30 minutes. After two washing steps cells were counted and mixed 1:1:1 from each population. Intensity of CFSE labelling, was analysed via flow cytometry by mixing 50 μl of each sample and 50 μl of unlabelled cells together. Peptide loaded cells (1 μM dye) and reference cells (0.1 μM dye) should differ by one log-step in the mean fluorescence intensity (MFI), whereas the difference between unlabelled cells and reference cells should be 1.5 to 2 log steps. Mice were injected i.v. with $5x10^6$ target cells from each colour and the same amount of reference cells. After 18 hours spleens were removed and homogenised. The ratio of lysed target cells to reference cells was determined by flow cytometry. To calculated specific cytotoxicty following formula was used:
specific cytotoxicity % = 100 – ($CFSE_{high} \div CFSE_{low}$) of immunized animal ÷ ($CFSE_{high} \div CFSE_{low}$) of naïve control x 100
and for FarRed labelled cells:

specific cytotoxicity % = 100 – ($FarRed_{high}$ ÷ $CFSE_{low}$) of immunized animal ÷ ($FarRed_{high}$ ÷ $CFSE_{low}$) of naïve control x 100

2.2.12 Enzyme Linked Immunosorbent Assay (ELISA)

Cytokines in the supernatant were measured by specific sandwich ELISA using microlon high bonding microtiter plates (Greiner Bio One, Frickenhausen, Germany). Antibodies were used according to standard protocol provided by the company. Briefly, plates were incubated over night at 4°C with 50 μl coating antibody diluted in coating buffer. Following two washes with wash buffer, plates were incubated with 150 μl/well blocking solution for one hour at room temperature and washed again. 50 μl/well of samples and a series of standards, diluted in 1% BSA-PBS were added and incubated over night a 4°C. After five washes, 50 μl biotinylated detection antibodies diluted in 1% BSA-PBS were added to each well. After 60 minutes incubation, plates were washed five times, and incubated with 50 μl streptavidin-peroxidase complex (1:5000, Boehringer, Mannheim, Germany) for 45 minutes at room temperature. After five washes 50 μl substrate solution was added to each well. The reaction was stopped after colour development by addition of 50 μl stop solution. Plates were read at 450nm using a Spectra Max 340 Microwellreader (Molecular Devices, Sunnyvale, California, USA). Concentration was determined from the standard curve.

2.3 Statistical analysis

Graphpad Prism 5.0 software was used for data organisation and statistical analysis, additionally PASW 18.0 software was used for statistical calculations. Differences in survival between groups were calculated with the Mantel-Cox log-rank test. ECM scores over time are presented as median and range. Statistical differences for three or more groups of IFN-γ levels in the supernatant (displayed as μg/ml), Evans Blue leakage (displayed as μg/g brain tissue), and cytotoxicity (displayed as % cytotoxicity) were determined with Analysis of Variance (ANOVA)

with the Bonferroni post-hoc test. For the comparison of two groups the Student-t Test was chosen. P-values < 0.05 were considered significant. Unless otherwise stated data is displayed as mean.

3 Results

3.1 Antigen-specific immune responses during PbA induced ECM

Immune mediated damage has been described to be critically involved in the development of neuropathology in cerebral malaria. The relevance of adaptive immunity contributing to pathology, especially the involvement of T cells has been known for a while. Especially CD8$^+$ T cells are responsible for fatal neurologic complications and immune mediated damage in the brain. However, the precise mechanisms of CD8$^+$ T cell participation in the pathogenesis of cerebral malaria are still unclear. The place and time of *Plasmodium*-induced CD8$^+$ T cell generation and subsequent responses involved in cerebral malaria were of particular interest in this study.

3.1.1 Identification of H-2kb specific peptides of PbA MSP-1

In order to study antigen-specific T cell responses, parasite specific peptides needed to be identified. *P. berghei* merozoite surface protein-1 (MSP-1) was selected to generate immunogenic peptides. Peptides were searched with SYFPEITHI, a database of MHC ligands and peptide motifs. Our model of interest was the C57 BL/6 mouse, in which MHC class I molecules have the H-2kb or H-2db haplotype. Five peptides (table 3.1) predicted to be presented by the respective MHC haplotypes were selected and synthesized. Splenocytes from PbA infected mice co-cultured with BDMCs were restimulated with these five peptides and IFN-γ levels in the supernatant were examined with ELISA. All peptides stimulated parasite-specific T cells, as naive mice did not show increased IFN-γ levels (data not shown).

Table 3.1: Selected MSP-1 specific peptides

Name	Sequence	MHC Haplotype
F8L	FITKFEEL	H-2kb
L8L	LGITYKSL	H-2kb
D10L	DGLQNIYAEL	H-2db
K10L	KPIENIQDDI	H-2db
T10L	TELVNLIKTL	H-2db

3.1.2 Antigen specific cytotoxic T cells in ECM

To investigate the presence of parasite specific T cells in spleen and brain during *P. berghei* infection, isolated lymphocytes from naïve or infected C57 BL/6 mice were analysed for IFN-γ production after restimulation with MSP-1 peptides presented in a MHC I dependent manner. *Plasmodium* infection induced the generation of IFN-γ producing CD8^{+} T cells in the spleen (figure 3.1a) and increased CD8^{+}T cell numbers in the brain (figure 3.1c) 6 days post infection (dpi), the same time when first symptoms of cerebral malaria occurred. To analyse antigen-specificity, splenic or brain lymphocytes from naïve or PbA infected mice (WT) were incubated with peptide loaded BMDCs and analysed for IFNγ production after 24 hours. After restimulation with the MSP-1 derived peptide D10L, IFN-γ levels in the supernatant of splenocytes (figure 3.1c) derived from PbA infected mice were increased ($p<0.0001$). This strongly suggests the presence of antigen specific CD8^{+} T cells. Brain lymphocytes from diseased mice produced ten times higher amounts of IFN-γ after restimulation with D10L than brain lymphocytes from healthy naïve animals (figure 3.1d). In order to analyse antigen-specific cytotoxicity, the transgenic *Plasmodium* strain expressing ovalbumin PbTG (Lundie et al., 2008) was used to infect C57 BL/6 mice. This strain was of great advantage for our experiments as it expresses the model antigen ovalbumin in addition to parasite specific proteins. MHC class I peptides SIINFEKL (S8L) from OVA, or MSP-1 specific peptides K10L, D10L and F8L are presented by the C57 BL/6 MHC class I haplotypes H-2kb and H-2db. Syngenic target cells were labelled with either S8L or a mixture of MSP-1 specific peptides and transferred to recipient mice, which had been infected for five

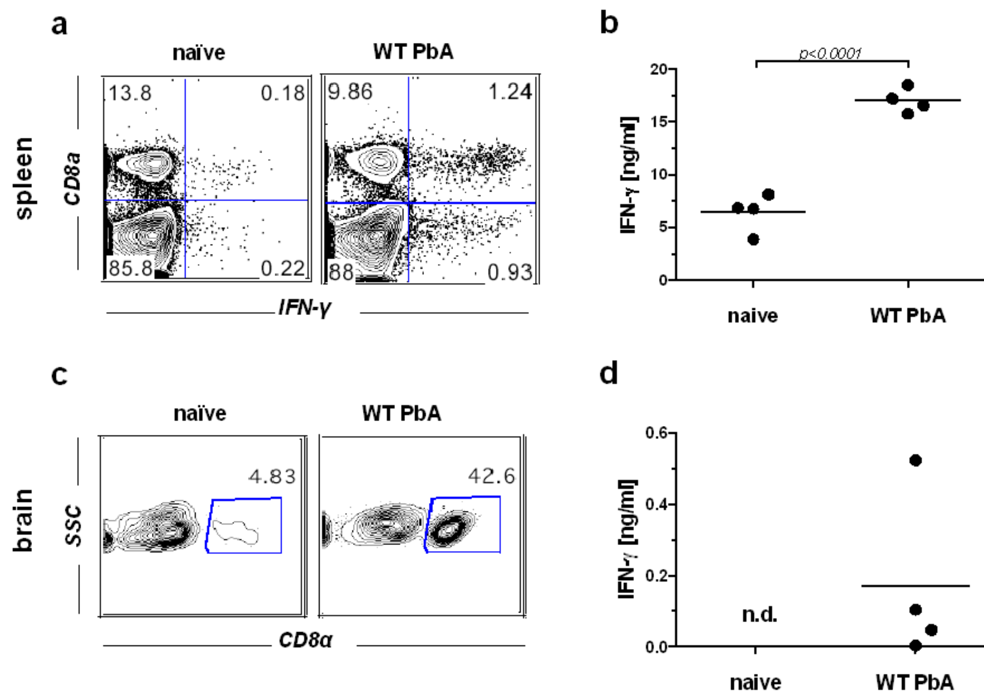

Figure 3.1: Antigen specific $CD8^+$ T cells can be detected in spleen and brain.
6 days post PbA infection splenocytes and brain lymphocytes were isolated from naive or PbA infected WT mice (n=4). Spleen $CD8^+$ cells from naive and PbA infected WT mice analysed by FACS (a). IFN-γ released from spleen $CD8^+$ cells incubated with BMDCs from naive C57 BL/6 donors restimulated with D10L (b). Brain $CD8^+$ cells from naive and PbA infected mice analysed by FACS (c). Same as b but for brain lymphocytes (d). IFN-γ levels are displayed as mean $p<0.0001$ (Student-t Test). One representative data set shown in each panel.

days with PbTG. 18 hours after cell transfer lytic activity of T cells was analysed with an antigen-specific *in vivo* cytoxicity assay. In the presence of antigen-specific $CD8^+$ T cells the target cells were lysed, giving insights on effector T cell generation and their activity during infection.

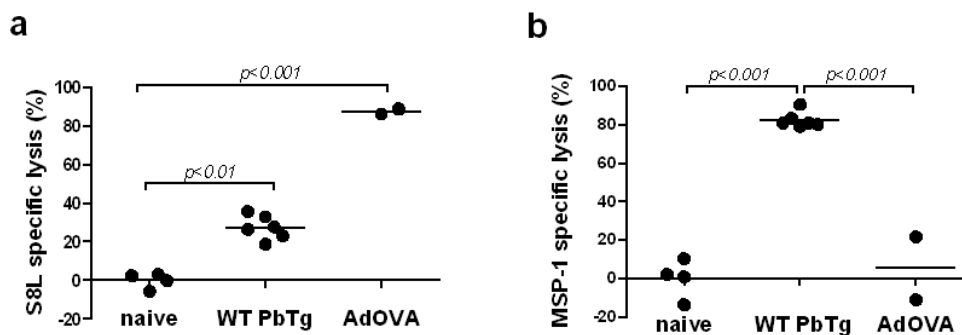

Figure 3.2: antigen-specific cytotoxic $CD8^+$ T cells are present in the spleen of *Plasmodium berghei* infected mice
In vivo cytotoxicity assay was performed in C57 BL/6 naïve (n=4), PbA (WT) (n=6) or AdOVA (n=2) infected mice 6 dpi . MSP-1 or S8L labelled syngenic target cells were injected 18h prior to analysis. H-2kb specific lysis of S8L labelled (a) or MSP-1 labelled (b) target cells was measured with flow cytometry. Specific lysis depicted as mean $p<0.01$ tested with ANOVA and adjusted with Bonferroni post-hoc test.

Mice infected with OVA expressing *Adenovirus* (AdOVA) at d0 were included as positive controls. In AdOVA infected control mice lysis of S8L labelled cells was around 90%, whereas T cells from naive mice did not show any lytic activity ($p<0.001$). In WT (PbTG infected) animals cytotoxic activity was lower (30%) than in AdOVA control, lytic capacity was considerably increased compared to naive animals ($p<0.01$) (figure 3.2a). Moreover, for the first time parasite-specific cytoxocity was detectable in *Plasmodium berghei* OVA (PbTG) infected, cerebral malaria positive mice. 80% of the syngenic target cells (H-2kb) loaded with MSP-1 derived peptides (K10L, D10L, F8L) were eliminated (figure 3.2b), whereas T cells from naïve mice did not show any specific lytic activity ($p<0.001$). The detection of parasite-specific cytotoxic $CD8^+$ effector T cells propose an involvement of antigen-specific, cytotoxic $CD8^+$ T cells in the pathogenesis of cerebral malaria.

3.2 Immune responses against *Plasmodium berghei* are generated in the spleen

During blood stage infection with *Plasmodium* spp., the spleen functions as an important lymphoid organ, where infected erythrocytes are removed from the blood stream and T and B cell responses are generated. Clearance of parasitized erythrocytes leads to possible antigen uptake by antigen-presenting cells (APCs), e.g. macrophages, but also dendritic cells and B cells. These cells in turn are capable of inducing the maturation and activation of effector T cells. The involvement of the architecture and cells associated with the structure of the spleen in the priming of $CD8^+$ T cells and disease progression, the splenic compartment as a whole and single cell types (macrophages, B cells and dendritic cells) were removed before or during *Plasmodium* infection.

3.2.1 The intact structure of the spleen is critically involved in disease progression and T cell activation

Splenectomy was one tool to prove the hypothesis that the spleen as a lymphoid organ is of importance in the pathogenesis of cerebral malaria. One week after surgical removal of the spleen, mice were infected with $5x10^4$ PbA infected erythrocytes (iRBCs) and ECM development and survival was monitored. The removal of the spleen resulted in ameliorated or absent neuropathology (data not shown) as well as in significantly increased ($p<0.0001$) survival (figure 3.3a). Mice died between day 20 and day 30 post infection from hyperparasitaemia, anaemia or metabolic dysfunction. In a different infection model, with OVA expressing recombinant *Adenovirus*, Beatrix Schumak (PhD Thesis) has shown that without the spleen cytotoxic T cell lysis was absent. PbA infected splenectomised mice did not show any signs of neuropathology, indicating that immunological events in the spleen are critically involved in ECM pathogenesis. Brain lymphocytes of splenectomised mice showed reduced IFN-γ production after co-culture with BMDCs and restimulation with S8L compared to IFN-γ levels in infected WT mice and IFN-γ levels were similar to the background levels of naive mice (figure 3.3c).

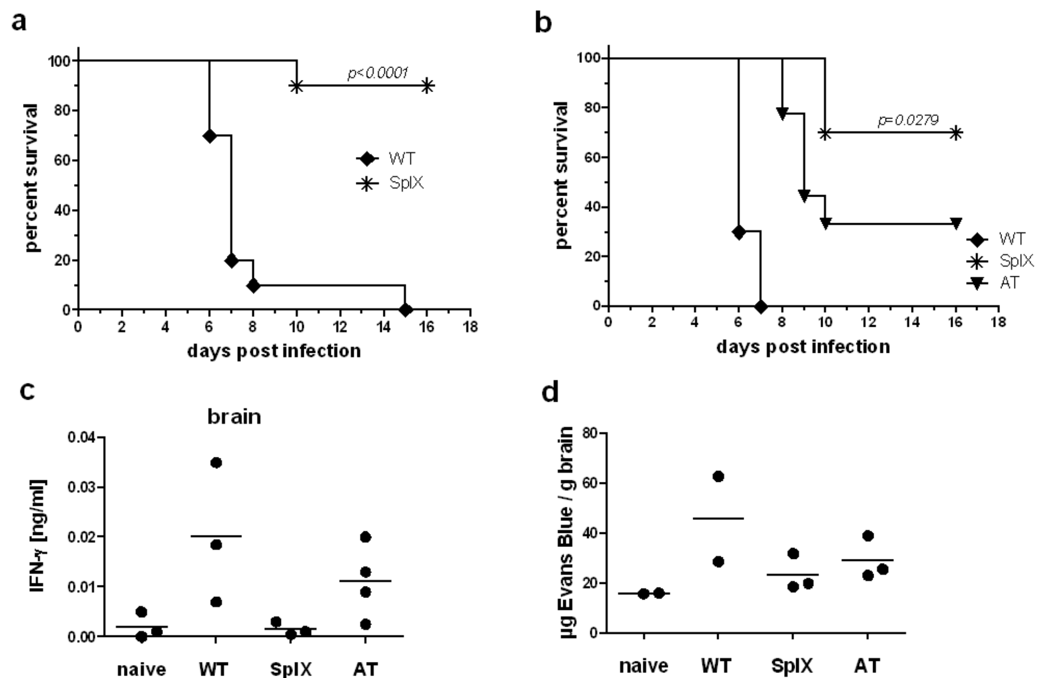

Figure 3.3: Presence of an intact spleen is required for ECM and activity of antigen-specific T cell responses
C57 BL/6 WT were infected with *Plasmodium berghei* ANKA, indicated groups were splenectomised or autotransplanted one week prior to infection. Survival was monitored in WT, splenectomised (SplX) (a and b) and in autotransplanted (AT) mice (b). Differences in survival were calculated with Log-rank Test. IFN-γ production after S8L-specific restimulation of brain lymphocytes on BMDCs (c) and Evans Blue leakage into brain parenchyma (d) was analysed six days post PbTG infection. Data is displayed as mean, statistical analysis with ANOVA and Bonferroni post-hoc test did not reach $p<0.05$.

Blood brain barrier disruption and intra-cerebral infiltration, measured by Evans Blue leakage into brain parenchyma was decreased in mice lacking the spleen (figure 3.3d), supporting the hypothesis that the spleen as a lymphoid compartment is critically involved in the generation of detrimental immune responses.

3.2.2 Autotransplantation of the spleen reverses protection

In the absence of the spleen, mice were unable to mount an immune response against the parasite. In parallel, antigen-specific T cells were absent in the blood

and brain, and blood brain barrier (BBB) disruption as well as neuropathology were undetectable. To further analyse the unique functions of the spleen regarding its complex architecture and its pivotal role as a lymphoid organ in ECM, splenic pieces were implanted into splenectomized mice prior to PbA infection and parasitological and immunological parameters were assessed. A post-mortem study of splenectomised mice showed that the implanted spleen pieces were revascularised 6 days after surgery. Autotransplanted *Plasmodium* infected mice showed decreased survival comparable to WT mice (figure 3.3b).
Furthermore, brain lymphocytes from autotransplanted mice produced increased amounts of IFN-γ after restimulation with S8L than the restimulated lymphocytes of splenectomised *Plasmodium* infected mice. Cytokine levels in restimulated brain cells after retransplantation were half the levels found in WT mice (figure 3.3c). Although T cell responses were present, Evans Blue leakage into brain parenchyma was reduced in autotransplanted mice. ECM positive WT mice had tissue concentrations of the dye twice as high as those in autotransplanted mice (figure 3.3d). Revascularised pieces of the spleen were sufficient for generating detrimental parasite specific immune responses, adding evidence to the hypothesis that the spleen is an important source of inflammatory cells and substantially involved in ECM pathogenesis.

3.2.3 The splenic architecture is involved in T cell priming and disease progression

Further evidence for the importance of the spleen could be elucidated in mice with perturbed cellular structure in this organ. Mice lacking mature lymphocytes (RAG2$^{-/-}$) as well as mice genetically deficient in lymphotoxin-β receptor (LT-βR$^{-/-}$) have defects in lymphoid organ development and show disturbed architecture of the spleen (Fütterer et al., 1998; Mueller et al., 2002). Previous experiments have shown that CTL responses against AdOVA were inhibited in RAG2$^{-/-}$ mice despite reconstitution with splenic T cells prior to *Adenovirus* infection. Moreover, *Plasmodium berghei* infected RAG2 deficient mice did not develop ECM specific symptoms, but died around day 25 p.i. from hyperparasitemia (PhD Thesis Beatrix Schumak). Are RAG2 deficient mice protected against ECM development

due to devoid T and B cells or is the disturbed splenic architecture involved in induction of neuropathology? To clarify this question RAG2$^{-/-}$ mice were reconstituted with CD8^{+} T cells isolated 6 dpi from WT mice. Transfer of effector T cells reversed protection against cerebral malaria. Between day six and day eight after PbA infection and T cell substitution RAG2$^{-/-}$ mice developed neurologic pathology with 50% dying shortly after transfer of the T cells. However, RAG2$^{-/-}$ mice which received T cells from naive donors did not develop cerebral malaria similar to unreconsituted PbA infected littermates (data not shown). These experiments demonstrate that reconstitution with naive T cells is insufficient for inducing host immune responses and additionally suggest that priming of T cells in an intact spleen is essential for malaria pathogenesis.
As lymphotoxin-β receptor deficient mice are known to have disturbed lymphoid organ development (Fütterer et al., 1998) and are resistant to ECM development (Togbe et al., 2008) we hypothesized that disrupted lymphotoxin-β signalling is not solely responsible for ameliorated disease progression after PbA infection, but rather disorganisation of the spleen inhibits early immune responses and thus results in changes in disease outcome. Transferring primed effector cells from infected WT mice into LT-βR deficient mice and subsequent infection with PbTG led to increased neurological deficits (figure 3.4b), 1-3 days later mice recovered but died later of hyperparasitemia (figure 3.4a). LT-β receptor deficient mice had decreased parasite-specific IFN-γ responses in the spleen (figure 3.4c) and brain (figure 3.4d). Moreover, specific cytotoxicity of effector T cells was strongly reduced ($p<0.05$ compared to WT mice) in knock-out mice (figure 3.4e). The absence of Evans Blue leakage, and thus BBB disruption in LT-βR$^{-/-}$ mice was reduced to the same background levels seen in naive mice (figure 3.4f) and are in line with the observed prolonged survival and absence of neuropathology.

3.3 Antigen-presenting cells are required for the generation ECM

Different antigen-presenting cells like dendritic cells, monocytes, macrophages and B cells reside in the spleen and are able to take up, process and present parasite

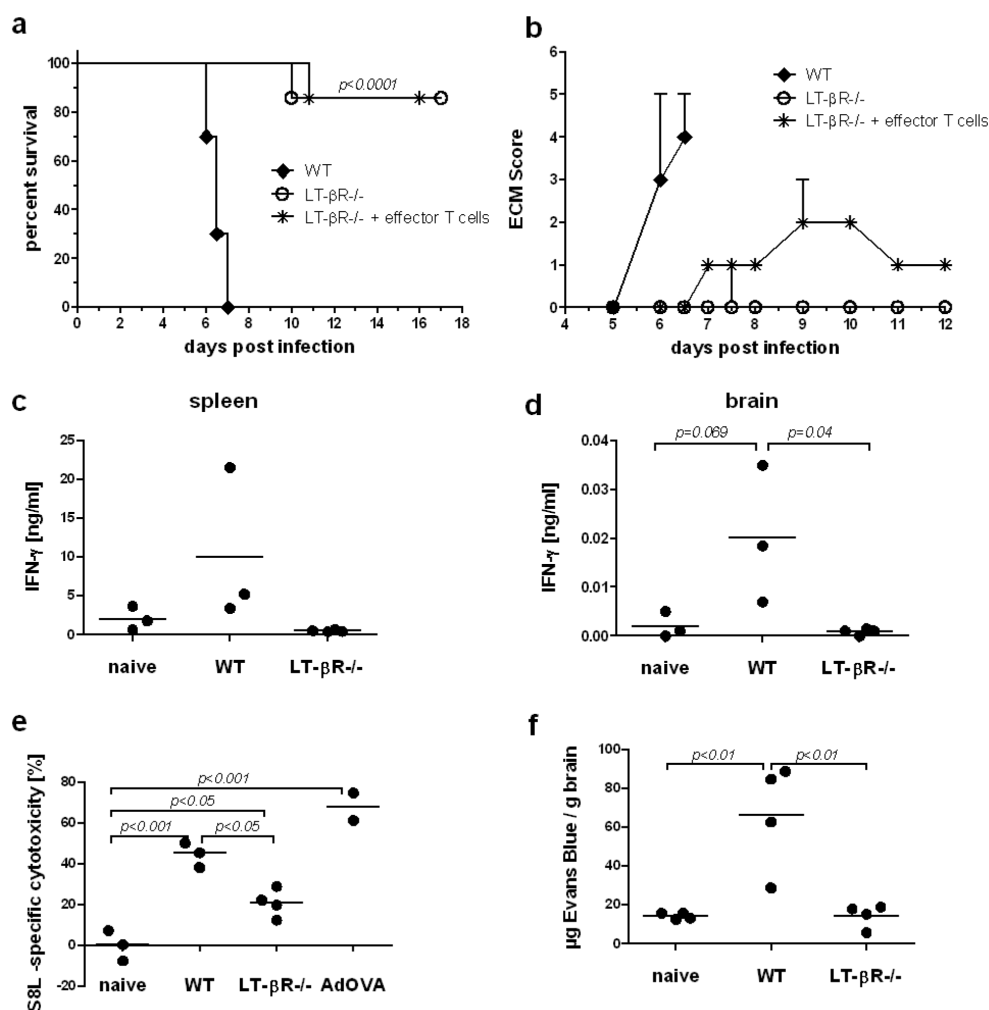

Figure 3.4: LT-βR$^{-/-}$ mice are protected against ECM and lack antigen-specific CD8^{+} T cells. PbTG infected WT (n=10), LT-βR$^{-/-}$ (n=7) or LT-βR$^{-/-}$mice reconstituted at day 0 with WT effector cells isolated from PbTG infected WT mice six dpi (n=7). Cumulative survival (a) and time course of ECM development (b) was monitored. Differences in survival were calculated with Log-rank Test ($p<0.0001$), ECM score is displayed as median and range. IFN-γ concentration in supernatant of BMDC *ex vivo* co-culture from naive (n=3), PbTG infected WT (n=3) and LT-βR$^{-/-}$ (n=4) splenocytes restimulated with F8L (c) and brain lymphocytes restimulated with S8L (d), all isolated 6 dpi when first symptoms were observed. Percent S8L specific lysis (e), *Adenovirus* infected mice were included as positive control (n=2). Extravasation of Evans Blue into brain tissue (f) was colorimetrically measured in naive (n=2), WT (n=2) and LT-βR$^{-/-}$ (n=4) mice. Data are the mean and p-values below 0.05 were considered significant, calculated with ANOVA with Bonferroni post-hoc test.

derived particles. Each cell type has distinct mechanisms, and is more or less efficient in antigen-presentation and T cell stimulation. Our study aimed to elucidate the role of different splenic APCs during priming (early), or effector (late) phase of *Plasmodium berghei* infection.

3.3.1 Macrophages are involved in the generation of parasite-specific T cell responses and ECM pathogenesis

Macrophages are the first immune-cells to encounter invading pathogens and are very efficient in taking up whole parasites and parasite particles, but antigen presentation is dependent on prior activation and is less efficient compared to DCs. To analyse macrophage involvement in ECM development, C57 BL/6 mice were depleted of this cell type by injection of clodronate liposomes (CloLip), which induce apoptosis after being phagocytosed. Following depletion of macrophages one day (d-1) prior to PbTG infection, 80% of the mice survived ($p<0.0002$) without ECM development, but subsequently died from hyperparasitemia. In this experiment only 10% of WT mice survived *Plasmodium berghei* infection (figure 3.5a). Depletion later in the infection (d4) protected the mice to the same extent as early macrophage depletion (data not shown). Further, lack of macrophages resulted in drastically ($p<0.01$) decreased *in vivo* lysis of syngenic S8L labelled target cells when measured 6 dpi (figure 3.5d). Clodronate liposome administration d-1 did not only decrease cytotoxicity of effector cells. Splenocytes and brain lymphocytes from these mice were unable to produce IFN-γ (figure 3.5b and c, respectively). In the absence of macrophages, MSP-1 restimulated splenocytes produced four time less IFN-γ than WT splenocytes ($p<0.001$). In contrast to cytokine production in infected WT brain lymphocytes, no IFN-γ was detected in those of naive or macrophage depleted groups after antigen-specific restimulation. Macrophage depletion minimised BBB disruption, as seen by notably reduced Evans Blue leakage ($p<0.01$ compared to WT mice) into brain tissue (figure 3.5e).
These results support the hypothesis that macrophages are involved in detrimental immune responses during *Plasmodium berghei* infection. We conclude from these data that generation of parasite-specific responses depend on the presence of these phagocytic antigen-presenting cells.

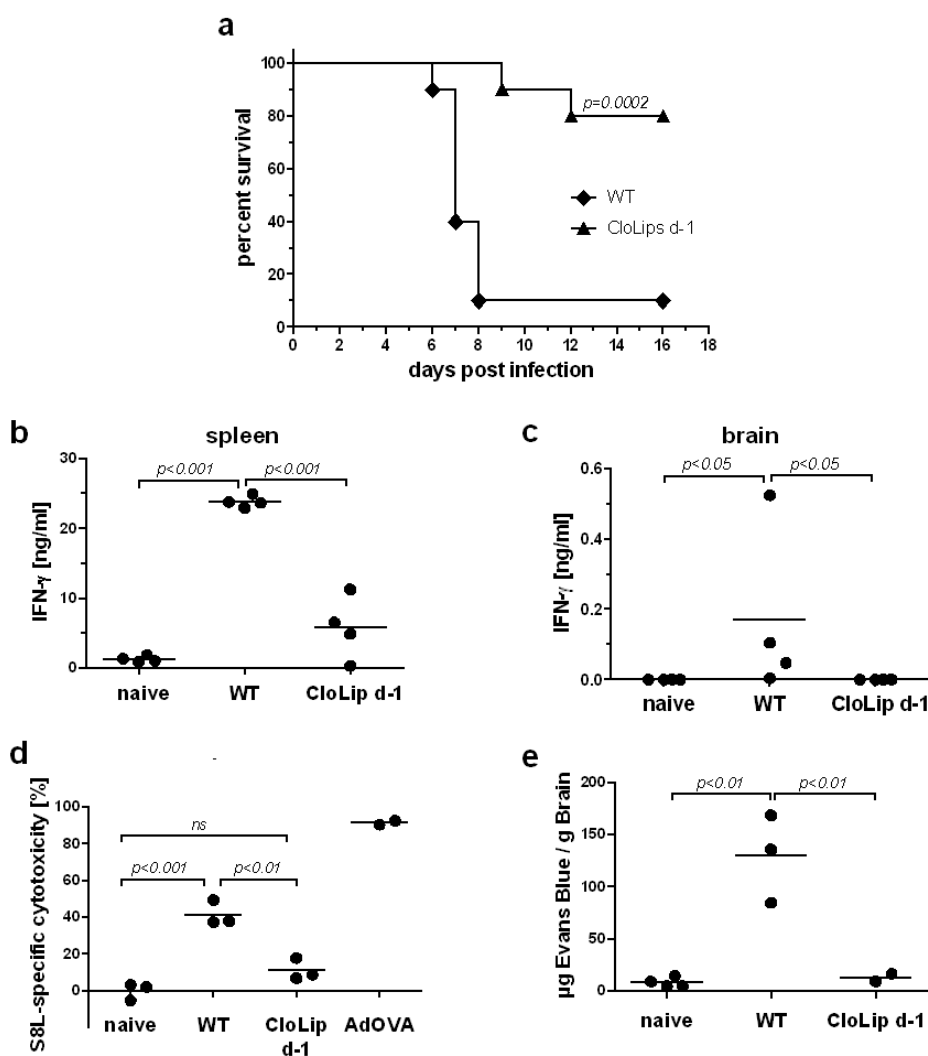

Figure 3.5: Macrophages are involved in antigen-specific responses and ECM development. Survival (a) of PbTG infected C57 BL/6 WT (n=10), d-1 clodronate liposome (CloLips) administered C57 BL/6 mice (n=10). Differences in survival were calculated with Log-rank Test ($p<0.0002$). IFN-γ concentration in supernatant of *ex vivo* BMDC co-culture with splenocytes restimulated with D10L (b) and brain lymphocytes restimulated with K10L (c) of naive, PbTG infected WT and d-1 CloLip administered mice (for all groups n=4). Percent S8L specific lysis (e), a group *Adenovirus* infected mice was included as positive control (n=2). Amount Evans Blue leakage into brain tissue (f) was colorimetrically measured in naive (n=4), WT (n=3) and d-1 CloLip (n=2) mice. Cells were isolated 6 dpi. Data are the mean and $p<0.05$ was considered significant, calculated with ANOVA and with the Bonferroni post-hoc test.

3.3.2 B cells are involved in antigen-specific effector responses and neuropathology

B cells are also antigen-presenting cells. They posses the unique ability to bind soluble antigen with surface immune-globulin receptors. After antigens have bound to Ig-receptors, this complex is internalized and processed. To investigate the role of B cells during *Plasmodium berghei* infection, inducible B cell knock-out mice were inoculated with parasites and survival as well as cellular responses were analysed. Mice expressing the diphtheria toxin receptor (DTR) under the control of the CD19 promoter can be depleted of $CD19^+$ B cells by daily administration of 40ng/g diphtheria toxin (DTX). Survival of mice lacking B cells during early (d0-d4) or late (d4-d7) infection with *P. berghei* ANKA was monitored. Early absence of B cells clearly increased survival of PbA infected mice ($p<0.0014$), 80% of d0-d4 depleted mice survived without symptoms of cerebral malaria (figure 3.6a). In contrast, late depletion of B cells only delayed ECM pathology (figure 3.6b) and mice died due to neurological complications 14 dpi. Further investigations confirmed that the presence of B cells early in infection were required for efficient effector cell priming. Depletion of these cells shortly after infection strongly reduced antigen-specific IFN-γ responses in splenocytes ($p<0.01$) and brain lymphocytes (figure 3.6c and d). Due to low cell numbers after brain lymphocyte isolation, measured cytokine levels after restimulation are widely scattered. These results imply that in the absence of B cells the generation of antigen-specific immune responses during early infection are inhibited and the ECM pathogenesis suppressed.

3.3.3 Dendritic cells are promote the generation of antigen-specific T cells and ECM development

Dendritic cells are professional APCs, like macrophages or B cells. They stand out, however, in their enhanced ability to prime and stimulate naïve T cells or B cells during primary immune responses. Conditional knock-out mice, which express the Diphtheria-Toxin-Receptor (DTR) under the control of the CD11c promoter (Hochweller et al., 2008) were used to analyse the role of $CD11c^+$ DCs during *Plasmodium berghei* infection. Daily administration of 40ng/g Diphtheria Toxin i.p.

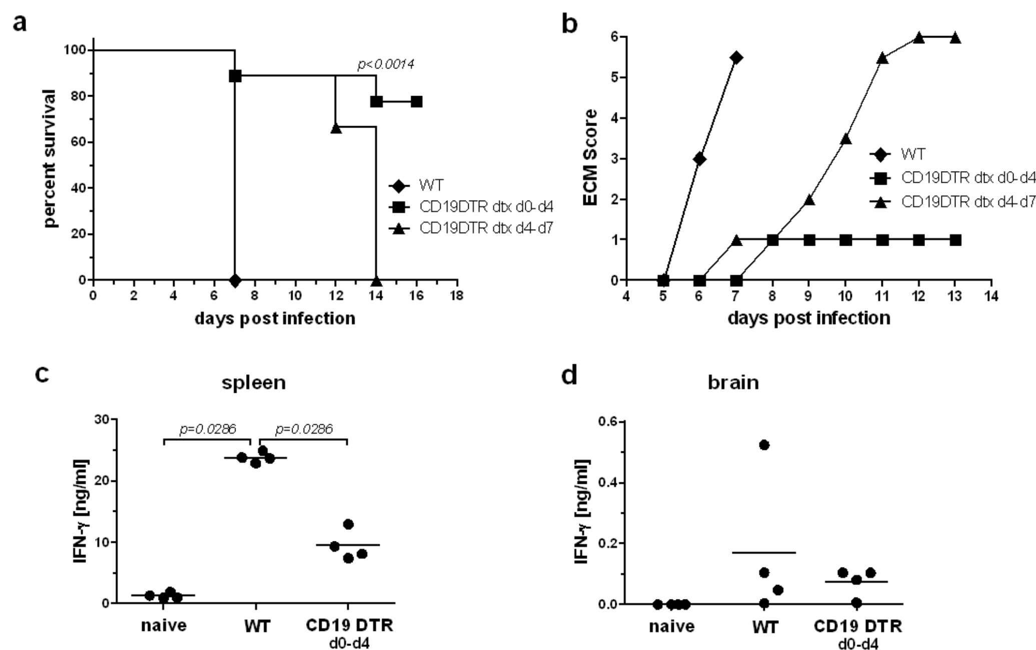

Figure 3.6: Priming of parasite-specific T cell responses and ECM development are dependent on CD19^{+} B cells. PbA infected C57 BL/6 WT mice (n=10) and transgenic CD19 DTR mice, administered daily with 40ng diphtheria toxin (DTX) either from d0-d4 (n=9) or from d4-d7 (n=9) post infection were monitored for survival (a) and ECM development (b). The Log-rank Test ($p<0.0014$) was used to calculate differences in survival, ECM score is displayed as median. 6dpi naive, PbA infected WT and d0-d4 post infection DTX administered CD19 DTR mice (for all group n=4) were sacrificed and supernatant of BMDC co-cultured splenocytes (c) and brain lymphocytes (d) restimulated with D10L and K10L respectively, was analysed for IFN-γ. Results are displayed as mean and p-values below 0.05, calculated with ANOVA and with the Bonferroni post-hoc test, were considered significant.

depleted all CD11c^{+} cells. After depletion of dendritic cells in the first 4 days after PbA infection none of the mice developed cerebral malaria ($p<0.0002$) whereas 100% of the WT mice were moribund (figure 3.7a). Lack of DCs resulted in diminished antigen-specific IFN-γ responses in splenocytes (figure 3.7b) and brain lymphocytes (figure 3.7c). In conjunction with reduced IFN-γ production, absence of CD11c^{+} cells reduced lytic activity of S8L antigen-specific T cells (figure 3.7d). Compared to S8L specific lysis in PbTG infected WT mice, cytotoxicity in CD11c depleted animals was low, similar to levels in naïve mice (WT vs. DTX d0-d4 $p<0.05$). CD11c^{+} dendritic cells are critically involved in pathogenesis of cerebral malaria, as seen in prolonged survival and reduced antigen specific effector responses. Re-

duced Evans Blue leakage into the brain parenchyma of CD11c depleted mice reinforces previous results (figure 3.7e).

Previous results, showing that DCs are involved in malaria (deWalick et al., 2007), are supplemented by our data, demonstrating a role of dendritic cells in generating effector cells and promoting disease. All these experiments provide evidence that dendritic cells, macrophages and B cells are critically involved in priming of antigen-specific T cells and ECM pathogenesis.

3.4 Toll-like receptor 9 is involved in ECM pathogenesis and but not in effector cell generation

In recent years, evidence increased that cerebral damage in *Plasmodium* infection is caused by excessive pro-inflammatory responses. Effector cells, pro-inflammatory cytokines and chemokines are involved in neuropathology. Especially, $CD8^+$ T cells and IFN-γ mediate fatal destruction in affected tissue. This work provides strong evidence that indeed cytotoxic, IFN-γ producing effector cells are engaged in pathological processes. Macrophages, B cells and dendritic cells have been shown to be involved in detrimental responses during *Plasmodium* (see section 3.3.1, 3.3.2 and 3.3.3) infection. These cells are the first cells to encounter pathogens and pathogen derived particles, thus early recognition and signal-transduction appears to be essential for distinct immune responses. Toll-like receptor signalling has been discussed to be involved in the pathogenesis of ECM. In particular the role of TLR-9 has been debated and arguments for and against TLR-9 engagement during malaria have been presented. Signal-cascades induced by TLR-9 ligation result in pro-inflammatory responses. We elucidated inflammatory mechanisms in ECM development, especially antigen-specific effector responses in dependency of TLR-9. *Plasmodium berghei* infected mice with a genetic TLR-9 deficiency were monitored for ECM development and survival. The lack of TLR-9 signalling reduced the incidence of fatal neuropathology in PbA infected mice to 50% ($p<0.0001$), whereas all WT mice died with obvious ECM symptoms (figure 3.8a). Although 50% of TLR-$9^{-/-}$ mice did not develop cerebral malaria, splenic $CD8^+$ T cells showed the ability after restimulation with MSP-1 peptides to produce IFN-γ (figure 3.8b).

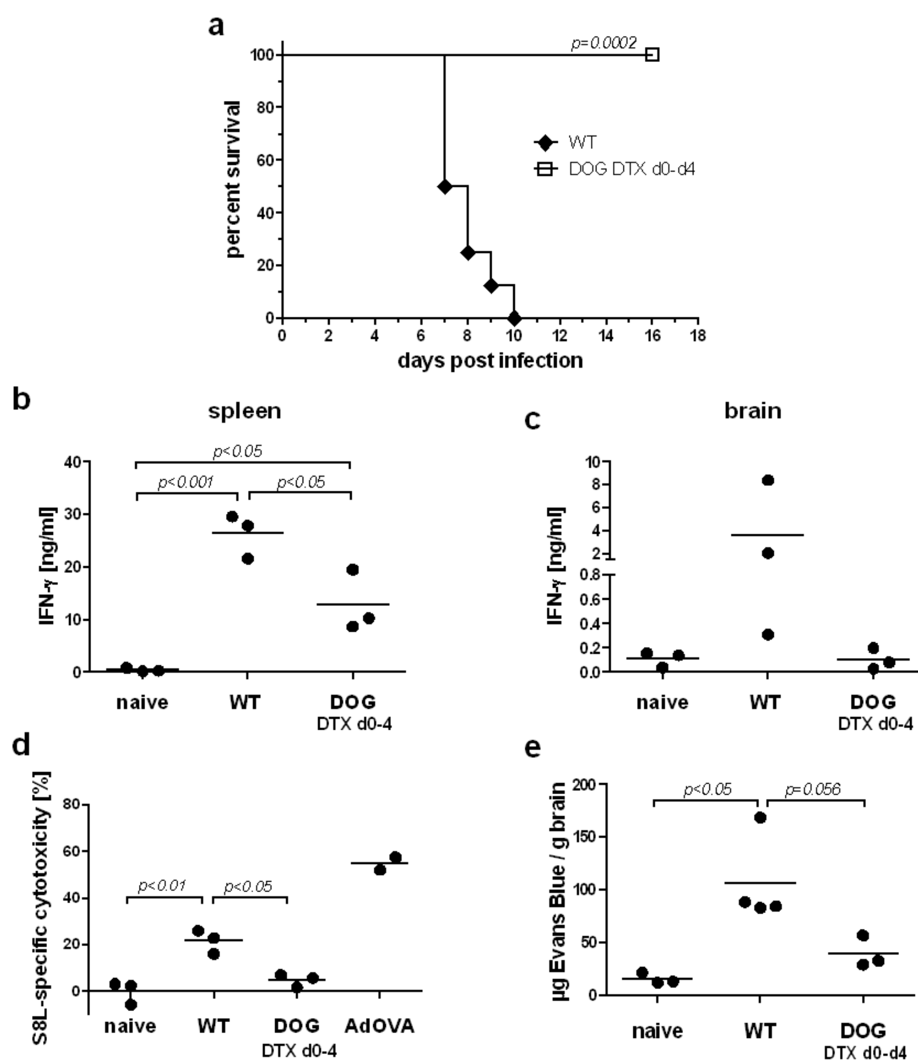

Figure 3.7: Dendritic cells are involved in ECM and required for antigen-specific responses. Cumulative survival (a) of PbA infected C57 BL/6 WT mice (n=8) and transgenic C11c DTR mice, administered daily with 40ng diphtheria toxin (DTX) d0-d4 (n=6). The Log-rank Test (p<0.0002) was used to calculate differences in survival. Six days post infection naïve, PbA infected WT and d0-d4 post infection DTX administered CD11c DTR mice (for all group n=3) were sacrificed and supernatant of BMDC co-cultured splenocytes (b) and brain lymphocytes (c) restimulated with F8L, was analysed for IFN-γ. Percent S8L specific lysis (d), a group *Adenovirus*-OVA d0 infected mice was included as positive control (n=2). Evans Blue leakage into brain tissue (e) was colorimetrically quantified in naïve (n=3), WT (n=4) and CD11c DTR dtx d0-d4 (n=3) mice. Data are the mean and p-values below 0.05 were considered significant, tested with ANOVA and adjusted with the Bonferroni post-hoc test.

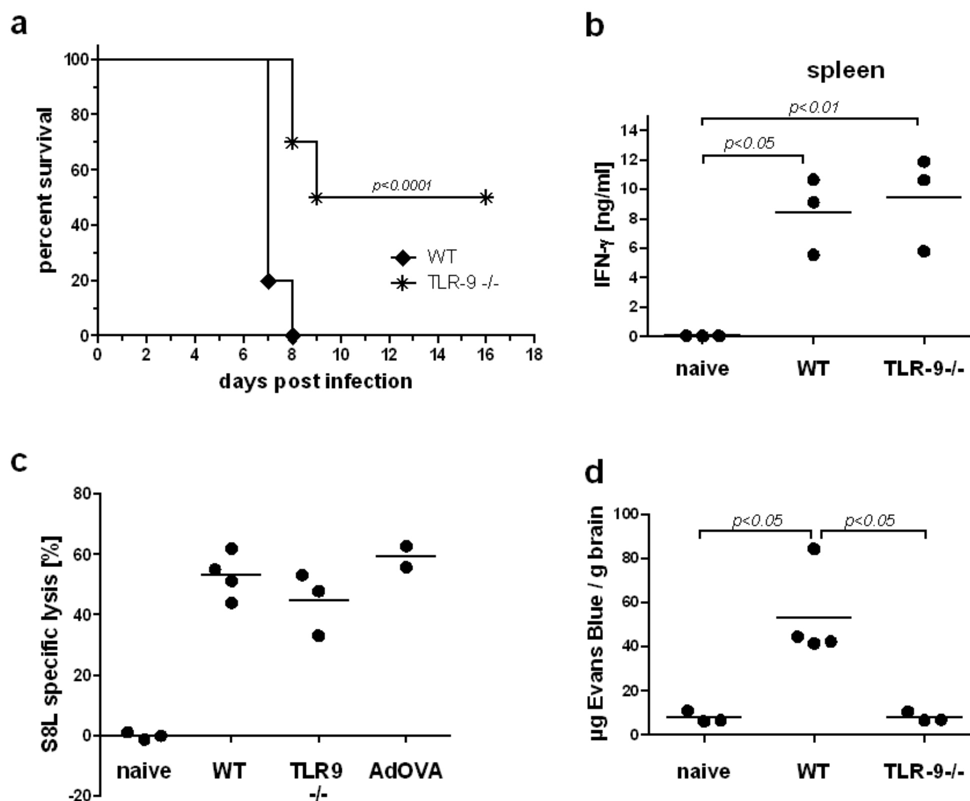

Figure 3.8: TLR-9 signalling is needed for ECM induction but not for antigen-specific immune responses. Survival of PbA infected C57 BL/6 WT (n=10) and TLR-9$^{-/-}$ (n=10) mice was observed. Differences in survival were calculated with the Log-rank Test (p<0.0001). Six days post infection supernatant of a BMDC co-culture with splenocytes (b) restimulated with F8L from naïve, PbA infected WT and TLR-9$^{-/-}$ mice (n=3 for all groups) was assessed for presence of IFN-γ. Percent cytotoxic activity against S8L labelled target cells (c) was assessed 6 dpi in naïve (n=3), PbTG infected WT (n=4), TLR-9$^{-/-}$ (n=3) and d0 *Adenovirus*-OVA infected WT (n=2) mice. Blood-brain barrier disruption determined by Evans Blue leakage into brain parenchyma (d) was quantified in naïve (n=3), PbA infected WT (n=4) and TLR-9$^{-/-}$ (n=3) mice. Data is displayed as mean and statistically tested with ANOVA and Bonferroni correction, p-values below 0.05 were considered significant.

Secreted IFN-γ levels were slightly higher than in infected WT mice but strongly increased compared to cytokine concentrations in restimulated naïve splenocytes (p<0.01). Moreover, no reduction in antigen-specific cyotoxicity of splenic T cells could be observed in the absence of TLR-9 (figure 3.8c). Although, generation and stimulation of antigen-specific effector T cells was not inhibited in TLR-9$^{-/-}$ mice, blood brain barrier damage was reduced to observed background levels seen in

naïve mice. In the absence of TLR-9 Evans Blue leakage (figure 3.8d), indicating BBB disruption was reduced (compared to WT mice $p<0.05$). Accumulation of the dye in brain tissue of naïve and TLR-9$^{-/-}$ mice was five times lower compared to concentrations of Evans Blue in WT mice. Altogether, these results demonstrate an involvement of TLR-9 signalling in ECM development. Moreover, our data suggest that parasite-specific T cell generation in the spleen is independent of TLR-9 signalling. Nonetheless, reduced Evans Blue leakage and an intact BBB support survival data showing increased survival and reduced neuropathology in TLR-9 deficient mice.

3.5 *Plasmodium* induced cerebral malaria is dependent on Interleukin-12 and Interleukin-12 signalling

The presence of DCs as well as functional TLR-9 responses are critically involved in the process of ECM development. Ligation of TLR-9 with its ligand results in the production of IL-12, which in turn has the ability to stimulate CD4^{+} T helper cells as well as NK cells and is known to be a potent inducer of IFN-γ . Therefore the downstream mechanism of TLR-9 signalling, especially the role of IL-12 in generation of parasite specific responses were analysed. IL-12 deficient mice were infected with PbA and survival was monitored. Around 40% of IL-12p35$^{-/-}$, 60% of IL-12p40$^{-/-}$ and 100% IL-12Rβ1$^{-/-}$ PbA infected mice were protected against ECM development ($p<0.0001$), while none of the WT mice survived PbA infection (figure 3.9a). An involvement of IL-23p19, a cytokine belonging to the IL-12 superfamily and sharing the IL-12p40 subunit, could be excluded as IL-23p19 knock-out mice died of cerebral malaria around the same time as infected WT mice. Parasite-specific responses 6 dpi were measured in an *ex vivo* co-culture, and splenocytes were restimulated for 24h with F8L. Then IFN-γ concentration in supernatant was quantified by ELISA. Antigen-specific IFNγ response in IL-12p35 deficient mice was notably reduced ($p<0.001$ compared to WT mice) reaching concentrations almost as low as in restimulated splenocytes from naïve donors (figure 3.9b). Furthermore, lysis of target cells labelled with parasite-specific antigens was decreased in the absence of IL-12p35 (figure 3.9c). On average, MSP-1 specific cytotoxicity in

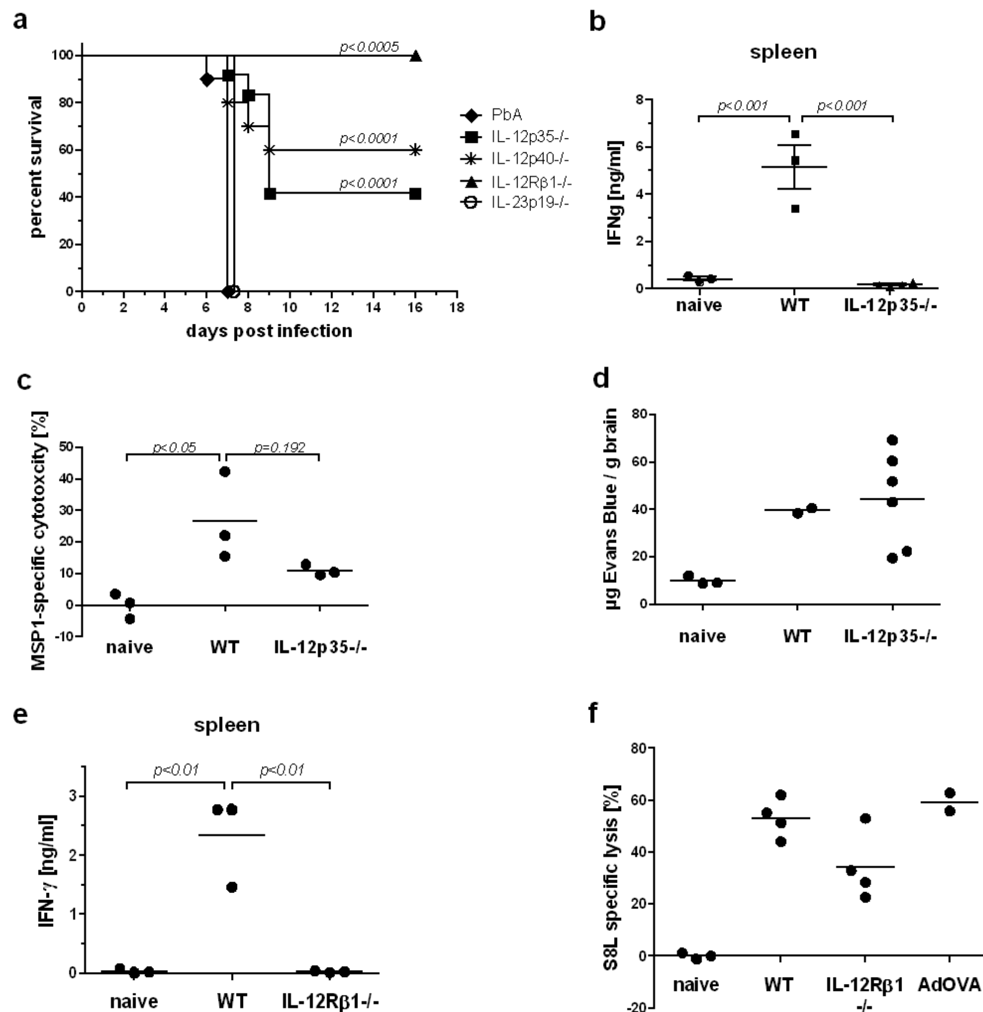

Figure 3.9: Development of parasite-specific T cell responses and ECM are dependent on functional IL-12 and IL-12 signalling. C57 BL/6 WT (n=10), IL-p35$^{-/-}$ (n=12), IL-12p40$^{-/-}$ (n=10), IL-12p19$^{-/-}$ (n=12) all on BL/6 background and IL-12Rβ1$^{-/-}$ (n=5) on SV129 background were infected with PbTG and monitored for survival (a). Differences in survival were calculated with Log-rank Test (WT vs. IL-12p35$^{-/-}$ p<0.0001, WT vs. IL-12p40$^{-/-}$p<0.0001, WT vs. IL-12Rβ1$^{-/-}$ p<0.0005) IFN-γ concentration in supernatant of *ex vivo* BMDC co-culture with splenocytes from naïve, PbTG WT and IL-12p35$^{-/-}$ mice (n=3) for all groups) restimulated with F8L (b). Percent MSP-1 specific lysis (c). Extravasation of Evans Blue into the brain (d) was colorimetrically analysed in naïve (n=3), WT (n=2) and IL-12p35$^{-/-}$ (n=6) mice. Splenocytes were isolated 6 dpi from naïve, PbA infected WT and IL-12Rβ1$^{-/-}$ mice (n=3 for all groups) and restimulated on BMDCs with F8L (e). S8L specific target cell lysis (f) was analysed in naïve (n=3), PbTG infected WT (n=4) and IL-12Rβ1$^{-/-}$ mice. *Adenovirus*-OVA infected mice (n=2) were used as positive control. Data are the mean and p-values below 0.05 were considered significant, calculated with ANOVA and with the Bonferroni post-hoc test.

IL-12p35$^{-/-}$ mice was 9% , whereas cyotoxic effector cells in WT mice lysed 26% of the target cells (p=0.192). Despite reduced antigen-specific IFN-γ production and cytotoxicity, leakage of Evans Blue into brain parenchyma was not changed in IL-12p35$^{-/-}$ compared to WT mice (figure 3.9d). All analysed *Plasmodium berghei* infected mice showed signs of cerebral malaria (data not shown). Intact IL-12 signalling during *P. berghei* infection is of great relevance during ECM development. In the absence of a functional IL-12-receptor (formed by IL-12Rβ1 and IL-12-Rβ2), mice did not die of specific neuropathology. Moreover, almost no antigen-specific IFN-γ production after restimulation could be detected in IL-12R$\beta 1^{-/-}$ ($p<0.05$), and IFN-γ concentrations were comparable to those of naïve mice (figure 3.9e). Antigen-specific lysis of target cells was decreased compared to WT mice (figure 3.9f), however, this difference was not significant. Altogether these results suggest that IL-12 and IL-12 signalling influences adaptive immune responses directed against the *Plasmodium* parasite leading to fatal complications.

3.6 CD4$^+$ T cells have a vital role in generating parasite-specific responses and subsequent ECM induction

As reviewed by Renia et al. (2006), CD8$^+$ T cells have been shown to be the predominant cell type involved in the pathogenesis of cerebral malaria . The principal role of antigen-specific effector T cells and their dependency on APCs could be shown in our study, extending current knowledge. Nonetheless, CD8$^+$ T cell responses and APC activity are also dependant on CD4$^+$ T helper cells. Inhibition of ECM pathogenesis after early CD4$^+$ T cell depletion has been described previously (Belnoue et al., 2002 and Yañez et al., 1999). To further analyse the role of T helper cells in parasite-specific inflammatory responses, we depleted CD4$^+$ T cells with neutralising antibodies, either early (1 dpi) or late (4 dpi) in infection.

40% of the mice depleted of CD4$^+$ T cells 5 dpi survived without neuropathological symptoms compared to 10% survival in *Plasmodium berghei* infected WT mice (figure 3.10a). Differences in survival were subtle (p=0.0607), but a trend could

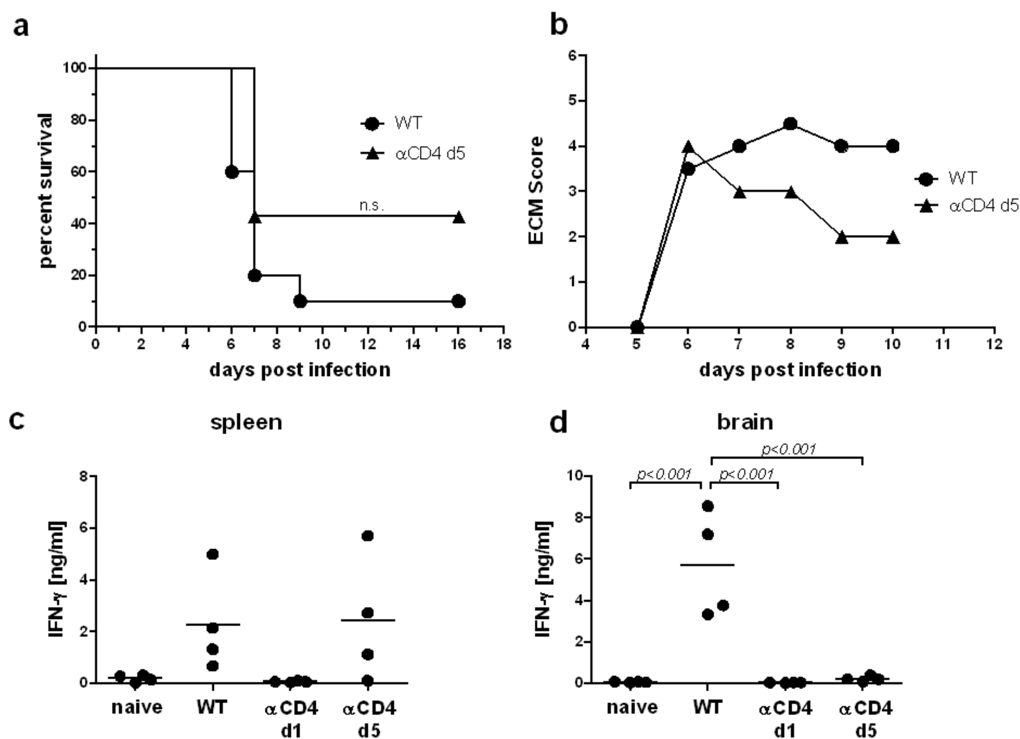

Figure 3.10: CD4$^+$ T cell responses are required for antigen-specific effector cell generation and ECM development
PbA infected C57 BL/6 WT mice (n=10) and WT mice administered 5 dpi with CD4 neutralizing antibodies (n=7) were monitored for survival (a) and ECM development (b). The Log-rank Test was used to calculate differences in survival, ECM score is displayed as median. Six days post infection naïve, PbA infected WT and WT administered with CD4 antibodies either 1 dpi or 5 dpi (for all group n=4) were sacrificed and BMDC co-culture with splenocytes (c) and brain lymphocytes (d) restimulated with F8L and K10L respectively, was analysed for IFN-γ in the supernatant. Results are displayed as mean and $p<0.05$, calculated with ANOVA and with the Bonferroni post-hoc test, was considered significant.

be seen. Specific neurological symptoms were observed in depleted animals to a similar extent as in WT mice (figure 3.10b). Further, the relevance of CD4$^+$ T cells for antigen-specific T cell priming was examined. Splenocytes and brain lymphocytes were isolated from naïve, PbA infected WT, αCD4 +d1 and αCD4 d5 administered animals and restimulated with MSP-1- or OVA-specific peptides. Depletion of CD4$^+$ T cells resulted in drastically decreased IFN-γ production after restimulation with F8L (figure 3.10c). Cytokine levels after early depletion were comparable to those of naïve splenocytes. Late neutralization, however, did not decrease antigen-

specific responses and IFN-γ concentrations in splenic lymphocytes and were similar to concentrations of infected WT mice. A similar cytokine pattern could be seen in K10L restimulated brain lymphocytes (figure 3.10d). After early neutralisation of $CD4^+$ T cells, INF-γ responses were absent. Despite disease progression in late α-CD4 administered mice effector responses in the brain remained at background levels of naïve mice. Taken together, these results provide strong evidence that early CD4 help is critically required for priming and activation of antigen-specific T cell responses during malaria. Later in infection, $CD4^+$ T helper cells had little involvement, ECM pathogenesis and already primed effector cells cannot be inhibited by α-CD4 treatment.

4 Discussion

Parasitic infections are a major health threat in developing countries. Malaria, caused by *Plasmodium* spp., is one of the leading infectious disease with high lethality. Despite extensive research, mechanisms of disease development and protective or detrimental immune responses during *Plasmodium* infection are not fully understood. In the last years, however, evidence has increased that malaria is an immune-mediated disease, in particular excessive pro-inflammatory host immune reactions might be responsible for severe disease (van der Heyde et al., 2006; Clark and Alleva, 2009; Milner, 2010). Using a mouse model of *P. berghei*-induced cerebral malaria parasite-specific responses in spleen and brain were analysed. Immunological processes, e.g. *in vivo* cytotoxicity, antigen-specific IFN-γ production and blood brain barrier (BBB) disruption in *P. berghei* infected mice, were examined 6 dpi. It is known that immune-mediated damage in the brain linked to ECM pathology can be detected five to six days after inoculation, whereas it is unknown where priming and generation of immune responses take place. Although many cellular and molecular factors involved in cerebral malaria pathogenesis have been identified, the organ where cellular immune responses against the parasite are generated remains to be elucidated.

The aim of this thesis was to investigate the engagement of the spleen, and cellular interactions within this organ in the development of *Plasmodium berghei*-induced cerebral malaria. Another focus of this study was the detection and analysis of parasite-specific T cell responses involved in pathogenesis. Results acquired in this study point out the pivotal role of an intact splenic architecture and cell-cell interaction in the spleen in generating parasite-specific immune responses. The unique internal structure of the spleen allows the interaction of antigen-presenting cells with effector T cells leading to potent immune responses. Presentation of para-

site antigen by APCs as well as TLR-9 signalling and cytokine environment have the potential to efficiently generate effector T cells, with pathogenic capacity. This study demonstrates that experimental depletion of DCs, macrophages, B cells, cytokines or the removal of the whole splenic compartment prevents the generation of antigen-specific effector T cells resulting in reduced pathogenicity and ameliorated disease development.

4.1 Mouse Model of cerebral malaria

Mechanisms involved in the detrimental disease progression of cerebral malaria are far from being completely understood. Insight into the complexity of immune reactions in human cerebral malaria is limited to post-mortem and *in vitro* studies. *Plasmodium berghei* ANKA infected C57 BL/6 mice represent a widely used and well established animal model displaying similar features of human cerebral malaria. Albeit similarity, plausibility of this murine model of ECM is debated (White et al., 2010; Riley et al., 2010). Some hallmarks of human cerebral malaria, e.g. sequestration of iRBCs to endothelium, low parasite burden or vascular obstruction, are less pronounced or absent in the murine model (White et al., 2010). Nevertheless, translation of results from murine to human studies provide the only option for detailed observation of *Plasmodium* infection and pathology. Cerebral malaria is a complex sequence of distinct interactions between host and parasite, and human studies are limited to descriptive or correlative assertions. In the murine model of cerebral malaria, information on immunological and parasitological events during pathogenesis can be dissected. Although, mouse models do not correlate completely with human malaria, yet they reveal valuable directions in malaria research, some with great importance to mankind (de Souza et al., 2010).

4.2 Detection of antigen-specific pathogenic T cells and the correlation with cerebral malaria pathogenesis

Pathogenesis of cerebral malaria has been strongly linked to pro-inflammatory reactions and pathogenic T cells leading to accidental immune-mediated damage of the host, over the course of infection (Belnoue et al., 2002; Nitcheu et al., 2003; Hunt and Grau, 2003; Renia et al., 2006).
We hypothesized that priming of parasite-specific T cells is one of the essential steps in immunopathogenesis of cerebral malaria. The importance of T cells has been investigated extensively, however, only a few reports on parasite-specific immune responses and their involvement in ECM have been published. Genetically altered parasites expressing the model antigen ovalbumin (OVA) can be used to detect antigen-specific immunity (Miyakoda et al., 2008 and Lundie et al., 2008). For the first time we were able to detect cytotoxic T cells that specifically lysed target cells labelled with the OVA peptide S8L after PbTG infection. More important, however, with this experimental set-up we could determine that *Plasmodium berghei* MSP-1 specific cytotoxic T cells were active after PbA infection. Additionally, effector function of CD8$^+$ T cells in the spleen and brain could be detected after restimulation with MSP-1 peptides. Therefore, our study is the first demonstrating the presence and the activity of parasite-specific (MSP-1) effector T cells in splenic and brain tissues during *Plasmodium* infection. Activity and effector function of T cells was evaluated in an indirect assay in which isolated cells were restimulated with distinct parasite peptides and IFN-γ production was quantified with ELISA since the numbers of isolated brain lymphocytes was too low for intracellular FACS analysis. The transgenic OVA-expressing strain PbTG, kindly provided by Rachel Lundie (Lundie et al., 2008), as well as *Plasmodium berghei* ANKA were used to analyse antigen-specific immune responses in the spleen and brain. Apart from the OVA class I peptide S8L, MHC class I peptides derived from MSP-1 were used to examine antigen-specific responses during malaria. These peptides were chosen on the basis of their peptide sequence in order to be presented by MHC-class I and are thus, hypothetically, only able to stimulate CD8$^+$ T cells.
Six days post infection with *Plasmodium berghei*, S8L-specific CTLs were present and active in the spleen (figure 3.2a). Moreover, we were able to detect increased MSP-

1-specific cytotoxicity in infected mice (figure 3.2b). In later experiments MSP-1 specific lysis was less pronounced in infected animals, alterations might have been caused by differences in peptide binding to target cells. Moreover minor variations between infections and subsequent effector cell generation cannot be ruled out. Apart from cytotoxicity, cells from infected animals produced IFN-γ in an antigen-specific manner (figure 3.1). BBB disruption and strong increases of IFN-γ producing cytotoxic cells in affected tissue in ECM positive mice strongly suggest a relationship between neuropathology and effector T cells. This hypothesis is supported by results provided by Nitcheu et al. (2003), who showed that tissue damage in the brain is dependent on the presence of activated cytotoxic T cells, as lymphocyte deficient ECM resistant RAG2$^{-/-}$ mice reconstituted with primed effector T cells showed neuropathology. These results are in line with previous studies demonstrating that brain infiltrating perforin^{+} cytotoxic T lymphocytes (Nitcheu et al., 2003) and activated antigen-specific T cells (Miyakoda et al., 2008; Lundie et al., 2008) are responsible for *P. berghei* induced neuropathology. It was shown that the onset of cerebral malaria is dependent on T cell migration from the spleen to the brain by chemokine promoted recruitment (Nie et al., 2009; Miu et al., 2008) and T cell adhesion via ICAM-1 (Favre et al., 1999; Bauer et al., 2002). Taking these results into account it is well conceivable that cerebral malaria is a directed process rather than an uncontrolled overreaction.

Interferon-γ is a key cytokine mediating disease progression during cerebral malaria in mice (de Kossodo and Grau, 1993; Rudin et al., 1997). However, exact mechanisms involved in pathology are still being investigated and the relevance of IFN-γ in human cerebral malaria is debated (reviewed in McCall and Sauerwein (2010)). Although, not the classical type of antigen-presenting cells, IFN-γ activated endothelial cells possess the ability to take up antigens (Geppert and Lipsky, 1985) and present peptides via MHC class I to effector T cells leading to BBB damage (Galea et al., 2007). The role of IFN-γ during *Plasmodium* infection, however, could be double-edged. During *P. chabaudi chabaudi* and *P. yoelii* infection IFN-γ seems to have protective properties by activating macrophages, which in turn increase parasite clearance (Meding et al., 1990). Interferon-γ secreted mainly by T cells, NK cells, APCs and NKT cells has the strong ability to suppress growth of intra- and extracellular protozoan parasites like *Toxoplasma* (Suzuki et al., 1988), *Leishmania*

(Heinzel et al., 1991) and *Trypansoma cruzi* (Reed, 1988) and possesses protective properties in animal models of the named infections.

This is the first report about endogenous *Plasmodium*-specific effector responses of T cells and results add evidence that IFN-γ producing cytotoxic T cells are directly involved in cerebral malaria pathogenesis (figure 3.1 and 3.2).

4.3 ECM development is dependent on an intact splenic architecture and a functional interaction between APCs and effector T cells

Plasmodium infected erythrocytes, free parasites and parasite particles are transported to the spleen with the bloodstream (reviewed in Engwerda et al. (2005)). In the spleen, different professional antigen-presenting cells may take up, process and present malaria antigens. Several studies suggest that T cell priming after *Plasmodium* infection takes place in the spleen (Hermsen et al., 1998; Eling, 1980), but details on cellular events have been less investigated. In order to analyse the location where immune responses against the parasite are generated, we removed or autotransplanted the spleen prior to *Plasmodium berghei* infection.
Splenectomy reduced the incidence of ECM (figure 3.3a) and also impaired the generation of antigen-specific T cells and their subsequent responses in the brain (figure 3.3c). Autotransplantation of splenic pieces restored parasite specific IFN-γ responses in the brain and reversed protection to neuropathology (figure 3.3b). BBB disruption and vascular leakage were decreased in splenectomised and in autotransplanted mice (figure 3.3d). It has to be taken into account that the impact of the surgical procedure and later wound healing might influence disease progression. In a model of *Streptococcus pneumoniae* infection, autotransplantation restored immune responses controlling infection (Fernandes et al., 2009), whereas splenectomy rendered the mice susceptible to disease progression. Although this study shows protective effects after autotransplantation, it is in line with our data showing that the spleen is indispensable for the generation of effector responses, in the case of PbA infection with detrimental outcome. In the absence of the spleen, *in*

vivo cytotoxicity and *ex vivo* restimulation experiments were performed with isolated blood lymphocytes, but due to minimal cell numbers analysis and evaluation of results were impossible.
The spleen is an organ with a highly organised cellular architecture. Therefore, the question arose whether the splenic structure and ordered cellular interaction may be indispensable for effector cell generation and disease progression. To test this assumption, RAG2 deficient mice and LT-βR deficient mice, both resistant to *Plasmodium berghei* induced ECM development (Randall et al., 2008; Nitcheu et al., 2003), were used in our experimental set-up. Lack of functional T and B cells or lymphotoxin (LT) signalling causes disturbed lymphoid organ development, leading to disintegrated cellular compartments (Mebius and Kraal, 2005; Ware, 2005). Therefore, these mice were suitable to examine the involvement of the spleen architecture during ECM. Reconstitution of RAG2 deficient mice with naïve T cells did not decrease survival, but reconstitution with primed T cells from *P. berghei* infected mice showed effector activity and neurologic symptoms were obvious (data not shown and PhD Thesis Beatrix Schumak). Similar results were obtained when LT-βR$^{-/-}$ mice were reconstituted with primed T cells, but in this case only weak symptoms of CM were seen. However, the mice recovered from disease and died later from hyperparasitemia (figure 3.4a and b). Infected LT-βR$^{-/-}$ mice, in correlation to resistance of ECM development, have markedly decreased cytotoxic and antigen-specific T cells (figure 3.4). Nevertheless, disrupted splenic architecture (Fütterer et al., 1998) is not solely responsible for the absence of neuropathology in lymphotoxin-βR$^{-/-}$ mice. Togbe et al. (2008) ascribed a critical role in neuroinflammation and lymphocyte recruitment to the brain to LT-βR expressed on endothelial cells and the lack of this pro-inflammatory cytokine may have yet to be discovered downstream effects. Thus, permeability of the blood-brain barrier may be influenced by LT-β signalling. However, our results from RAG2 and LT-βR deficient mice suggest that strict organisation of splenic compartments has a major role during antigen-specific T cell induction. Previous investigations in human and mice revealed that *Plasmodium* spp. infection changes splenic micro-architecture and cellular composition (Stevenson and Kraal, 1989; Urban et al., 2005; Carvalho et al., 2007; Beattie et al., 2006). Additionally, our experiments imply that changes in the structured compartmentalisation of the spleen prior to or early during infection may influence immune reactions.

Effector cell generation is inhibited in the absence of an intact spleen, but priming of T cells in the spleen requires a complex sequence of events involving activation of antigen-presenting cells, promotion of a pro-inflammatory cytokine environment, cell-cell and receptor interactions and signalling between cells (figure 4.2).

4.3.1 Generation of parasite specific T cells and ECM development is macrophage dependent

Phagocytic cells such as macrophages and monocytes home to the spleen. These cells are especially important during blood borne infections. While they remove pathogens and damaged or infected erythrocytes, they are also able to alter the cytokine environment and contribute to inflammatory processes during infection. Macrophages have a distinct location in the spleen supporting the organisation and function of the spleen as a lymphoid organ. To test our assumption that macrophages are relevant in immune mediated pathology of *Plasmodium berghei* infection, we depleted macrophages. Antigen-specific IFN-γ production and cytotoxicity were drastically reduced when macrophages were lacking during the priming phase of *Plasmodium* infection. (figure 3.5b-d). The absence of antigen-specific effector T cells after macrophage depletion minimised fatal neurological conditions, seen as prolonged survival and significantly reduced Evans Blue leakage (figure 3.5a and e). Several studies address the role of macrophages during *Plasmodium* spp. infection, however, the results contradict one another. Depletion of macrophages shortly before the onset of CM did not protect mice against fatal neuropathology (Belnoue et al., 2002). This is contrary to our preliminary observations, where depletion of macrophages four days post infection increased survival chances (data not shown). This outcome would argue for the importance of the presence of macrophages during the priming phase. The influence of macrophages later during infection has recently been investigated. Whole parasites and parasite micro-particles like haemozoin or GPI (glycosylphosphatidyl-inositol) activate macrophages inducing high levels of circulating inflammatory mediators (Couper et al., 2010), which could possibly further contribute to immune-mediated damage in course of *Plasmodium* infection.

Macrophages are able to generate a pro-inflammatory environment in which DC function and T cell priming is optimized, removal of this cell type terminates the development of cerebral malaria induced by *Plasmodium berghei* K173 (Curfs et al., 1993a). However Pais and Chatterjee (2005), described a close relationship between activation of brain macrophages, so called microglia cells, and the development of cerebral malaria. Other cells from the macrophage lineage may also have a role in disease progression. However, we would argue that macrophages have a decisive role in the priming phase in the spleen. This hypothesis is supported by a recent study from Backer et al. (2010) demonstrating that marginal metallophilic macrophages, residing at the interface of the marginal zone, collaborate with dendritic cells to generate cytotoxic T cells. Together with our findings, splenic resident macrophages, included in the network of well structured compartments in the spleen, have a considerable role during disease development (figure 4.2). At the present further experiments are being conducted to investigate the role of macrophages during cerebral malaria in detail.

4.3.2 B cells are involved in priming effector T cells and *Plasmodium berghei* induced neuropathogenesis

The spleen harbours not only T cells, macrophages and dendritic cells, but also B cells. This cell type is of great importance in mounting antibody-mediated immunity. B cells, as well as the other cells have a specific localisation in the spleen, adding to a structured micro-architecture. To analyse the function of $CD19^+$ B cells in priming of effector responses during malaria, we used mice expressing Diphtheria-Toxin-Receptor under the control of CD19. By injection of diphtheria toxin (DTX) B cells expressing CD19 could be depleted. Depletion of $CD19^+$ cells early in PbA infection increased survival chances and reduced IFN-γ responses after antigen-specific restimulation (figure 3.6). Interestingly, the absence of $CD19^+$ cells in a later phase of infection delayed but did not inhibit ECM development. Neuropathology, as measured by the ECM score, increased till day 13 post infection in mice depleted of $CD19^+$ cells from d4 till d7 post infection but not in early depleted mice. One day later ECM positive mice succumbed to severe brain damage (figure 3.6b). This study did not elucidate the role of B cells as antibody pro-

ducer, therefore, assumptions concerning functions other than their importance in antigen-specific T cell priming during cerebral malaria cannot be made. The involvement of B cells during cerebral malaria has been excluded (Yañez et al., 1996). However, antibody-secreting B cells have been correlated with protective immunity to secondary *Plasmodium* infection (Nunes et al., 2009; Stephens et al., 2009). Nevertheless, we could show that B cell deficiency resulted in abrogated T cell responses and led to prolonged survival without neurological complications. Our findings indicate an involvement of the spleen and its functional micro-architecture in parasite-specific effector responses causing detrimental outcome of malaria. We ascribe similar functions as dendritic cells and macrophages to B cells, i) in sustaining the splenic micro-architecture and ii) in antigen presentation and generation of antigen-specific responses. Our hypothesis is further supported by recent findings describing CD19^{+} cells (You et al., 2009) and B cells in general (Nolte et al., 2004) to be critical to marginal zone development and maintenance. The absence or gradual depletion of B cells led to a loss of metallophilic and marginal zone macrophages. The relationship of B cells, DCs and macrophages determining their localisation in splenic compartments and the generation of antigen-specific T cells and their role during ECM pathogenesis must be further investigated. Histological examination of the spleen from ECM positive WT or APC deficient mice would have to be analysed, providing more information on how the micro-architecture and cellular interactions affect T cell priming.

4.3.3 Dendritic cells drive antigen specific T cell differentiation and induce cerebral malaria

Dendritic cells provide a critical link between innate and adaptive immunity and have the unique ability to take up antigen without prior activation, rapidly process antigens and activate and prime naïve T cells. As we have previously shown antigen-specific T cells are readily present in cerebral malaria positive mice, we predicted that priming of those cells might be linked to the presence of dendritic cells. We demonstrated in this study that the early absence of DCs increased survival, reduced antigen-specific cytotoxicity and ameliorated extravasation of Evans Blue into brain tissue (figure 3.7). These data supplement previous results from deWal-

ick et al. (2007) showing that conventional dendritic cells are critically required for antigen specific responses by T helper cells (deWalick et al., 2007). Depletion of conventional CD11c$^+$ DCs, especially in the early phase of *Plasmodium berghei* infection reduced T cell responses and reduced cerebral inflammation. On the one hand, DC function is inhibited during *Plasmodium* infection (Millington et al., 2007) leading to reduced T cell priming or a switch in T helper responses towards Th2 and potentially limiting damaging processes (Sponaas et al., 2006). On the other hand, DCs have been described to be critically involved in shaping protective CD4$^+$ Th1 responses during a parasitemia model with blood stage *P. chabaudi* (Ing et al., 2006). Contrasting reports on dendritic cell biology during *Plasmodium* infection originate most likely from the use of different mouse and/or parasite strains. However, even the same strain of *Plasmodium* (PbA) might induce slightly divergent immune responses (Amani et al., 1998).Wilson et al. (2006) discuss *P. berghei* induced inhibition of cross-presentation by DCs and subsequent reduction of CD8$^+$ cell priming. On the contrary, activation of dendritic cells by iRBC internalisation (Seixas et al., 2009) and proliferation and activation of CD8$^+$ effector cells driven by CD8α DCs in an ECM model has been demonstrated (Lundie et al., 2008). Results of our study are in line with the latter two articles, demonstrating that early presence of DCs and potent presentation of parasite antigen to effector cells is crucially involved in cerebral malaria development (figure 4.2).

Our experiments provide evidence that antigen-presenting cells are critically involved in priming of antigen-specific T cells. In the absence of either B cells, macrophages or dendritic cells generation of effector T cells is inhibited or reduced and disease progression to cerebral malaria is ameliorated. The importance of the early priming phase in the spleen, involved cells and implications for disease development were demonstrated in our study. Involvement of APCs as effector cells in the late phase of cerebral malaria is currently investigated in our laboratory. Taken together, these findings indicate the spleen and its complex architecture to be of great importance for the induction of antigen-specific responses during cerebral malaria.

4.4 Toll-like-receptor-9 signalling and IL-12 promote neuropathology in *Plasmodium berghei* infection

Pathogen derived molecules such as unmethylated oligonucleotides, lipopolysaccharides (LPS), GPI (glycosylphosphatidylinositol) or RNA are recognised by the innate immune system through pattern recognition receptors (PRR). A special subset of PRRs are the Toll-like receptors (TLRs), mainly expressed on cells of the innate immune system. TLR-9, expressed by DCs, macrophages and B cells has the ability to sense short DNA sequences and to signal via the adaptor molecule MyD88 to induce strong pro-inflammatory responses. Based on our previous results, we hypothesised that production of IFN-γ and IL-12 are at least partially a consequence of TLR-9 signalling. Our results demonstrate that TLR-9 deficient mice survived *Plasmodium berghei* infection better than WT littermates. Fewer animals developed neurological symptoms (figure 3.8a) compared to C57 BL/6 WT mice, but ECM negative mice died later suffering from hyperparasitemia. Interestingly, the absence of TLR-9 did not reduce antigen-specific IFN-γ production by T cells *ex vivo* (figure 3.8b). Nevertheless, a drastic decrease of cytokine production in restimulated brain cells was observed (figure 3.8c). The role of TLR-9 signalling in ECM development has been controversially debated (Togbe et al., 2007; Lepenies et al., 2007; Coban et al., 2007). Therefore, it is reasonable that our results reflect mechanisms of TLR-9 involvement in immune cell priming which, up to now have, not been considered. Signalling through the TLR-9 pathway results in pro-inflammatory reactions including strong upregulation of type I interferons and IL-12 followed by induction of adaptive immune responses (Wagner, 2004). *Plasmodium* parasites have been shown to induce upregulation of TLR expression, essential to initiate IL-12 and IFN-γ responses (Franklin et al., 2009). Reduced IL-12 responses in TLR-9 deficient mice might lead to reduced IFN-γ production, which in turn is involved in ECM development. The absence of TLR-9 signalling alters adaptive immune responses and lymphocytes, especially antigen-specific T cell migration to lungs during *Cryptococcus neoformans* infection (Zhang et al., 2010). Our results show that antigen-specific cytotoxic T cells are present in TLR-9 deficient mice despite the absence of ECM, together with the study from Zhang et al. (2010), it is conceivable that the lack of TLR-9 inhibits migration of splenic antigen-specific effector cells to brain

tissue, possibly leading to a reduction in IFN-γ production in the brain. Several human genetic studies have addressed the role of single nucleotide polymorphisms in toll-like receptor genes or associated promoter regions. A SNP in the TLR-4 gene predisposes African children to severe malaria and disease manifestation (Mockenhaupt et al., 2006), and SNPs in TLR-9 could be correlated with increased incidence of CM and increased serum IFN-γ (Sam-Agudu et al., 2010). Complex interaction between APCs, effector cells and cytokines during cerebral malaria are sensitive to changes and compensation through other mechanisms is only partially possible.

TLR-9 activation induces the production of interleukin-12 important for differentiation of CD4^{+} T helper cells and activation of T cells and NK cells. IL-12 is a Th1 promoting cytokine, mainly produced by DCs but also by macrophages and monocytes, that potently stimulates NK and T cells to produce IFN-γ. As we have shown that macrophages as well as DCs were involved in antigen-specific T cell priming and induction of neuro-inflammation, we wanted to analyse whether IL-12 was also a part of this complex network. Moreover, we hypothesised that antigen-specific IFN-γ responses, known to be crucial for ECM development, were inhibited in the absence of IL-12. We analysed IL-12p35$^{-/-}$ mice and mice deficient in IL-12Rβ1 for ECM development, survival, BBB disruption and their ability to produce IFN-γ after antigen-specific restimulation. Mice survived passed the ECM development window when they lacked IL-12p35, IL-12p40 or IL-12Rβ1 (figure 3.9a). As the IL-23p19 forms a heterodimer with the IL-12p40 subunit the possibility that this IL-12 related cytokine was involved in cerebral malaria needed to be excluded and found not to be the case (figure 4.1). The regulatory cytokine IL-35, consisting of the IL-12p35 subunit and Ebi3 protein was ruled out to be of major importance in T cell generation and ECM development, as mice lacking the proteins IL-12p40, IL-12p35 or both had ameliorated disease progression and decreased T cell responses. In the absence of IL-12 or IL-12 signalling, splenic T cells were unable to produce IFN-γ after stimulation with parasite peptides (figure 3.9b, e). However, protection against *Plasmodium berghei* induced pathology was not complete and effector responses incompletely abrogated in IL-12p35 deficient, suggesting that IL-12 independent mechanisms contribute to immune damage. IL-12Rβ1 deficiency completely protected mice against ECM development and splenocytes produced no IFN-γ upon restimulation, although specific lysis was not greatly altered. IL-12Rβ1$^{-/-}$ mice

were on the 129 Sv background have the H-2kb and H-2db haplotype, also found in C57 BL/6 mice. 129 Sv mice are described to be susceptible to *P. berghei* infection and develop cerebral malaria with haemorrhage and leukocyte sequestration (Amani et al., 2000). We did not analyse *Plasmodium berghei* infected WT 129 Sv mice for survival but *ex vivo* experiments showed increased vascular leakage of Evans Blue into the brain parenchyma, whereas Evans Blue remained on background levels in ECM-resistant IL-12Rβ1$^{-/-}$ mice (data not shown). Nevertheless, increased

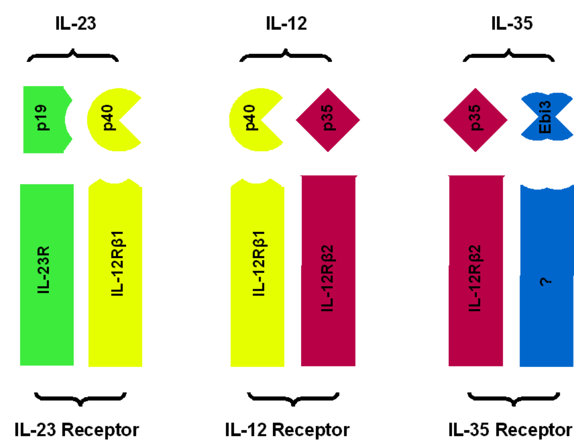

Figure 4.1: Family of IL-12 and IL-12 receptors

survival, reduced pro-inflammatory and cytotoxic potential of splenocytes argue for a strong involvement of IL-12 and IL-12 signalling in ECM development. Previous reports only examined the role of IL-12 during blood-stage infection with *Plasmodium chabaudi* AS, a model for severe malaria anaemia. Splenic macrophage derived IL-12p70 was increased in C57 BL/6 mice clearing *P. chabaudi* AS infection and showing no fatal anaemia (Sam and Stevenson, 1999b). Moreover, expression analysis of resistant BL/6 mice infected with *P. chabaudi* AS has shown that IL-12Rβ1 and β2 mRNA was upregulated in the spleen (Sam and Stevenson, 1999a). It has been discussed that induction of protective IFN-γ responses are critically required for parasite clearance and required for antibody-mediated immunity (Su and Stevenson, 2002). Besides protective properties, IL-12 has been assigned to have pathogenic potential. Blood stage *Plasmodium berghei* NK65 infection is ameliorated after IL-12 depletion (Yoshimoto et al., 1998). In ECM resistant IFN-γR deficient mice, reduced mRNA levels of IL-12 were observed (Rudin et al., 1997). During human malaria, reduced levels of IL-12 have been associated with increased

development of severe symptoms. Moreover, a polymorphism in the promoter region of the gene encoding IL-12p40 was linked to reduced IL-12p70 serum concentrations and correlated with a higher risk for CM development. Diverging functions of IL-12 during malaria may be attributed to differing models and different study designs. IFN-γ clearly has pathogenic functions during *Plasmodium berghei* ANKA infection, hence it is conceivable that the IFN-γ inducing cytokine IL-12 might also have immunopathogenic properties. However, results from human studies are contradictory to those of murine models. In *Plasmodium berghei* infection, IL-12 and IFN-γ expression is limited to a specific time frame in which progression to ECM is determined (Mitchell et al., 2005; McCall et al., 2010). Cytokine kinetics and organ specific expression patterns from human patients are lacking making it difficult to determine the relevance and implication of the human results. Our study demonstrates the importance of IL-12 for mediating effector cell generation and pathogenesis during parasite infection. In order to draw further conclusions, the source of IL-12 and cell types that are directly activated still need to be elucidated.

In conclusion, a pro-inflammatory cytokine environment, especially IL-12 signalling is a prerequisite in antigen-specific T cell generation. The skew to a more pro-inflammatory response during *Plasmodium* infection depends on the functional TLR-9 signalling and following changes in cytokine milieu involved in T cell priming and activation.

4.5 CD4$^+$T helper cells affect generation of parasite-specific effector responses and ECM development

CD4$^+$ T cells, also called T helper cells, are required for shaping adaptive immunity, especially CD8$^+$ dependent responses. DCs and macrophages which have detected pathogenic material via toll-like receptors produce high levels of IL-12. This cytokine in turn activates CD4$^+$ T cells which are then able to recognize antigen presented in an MHC class II context. Antigen recognition and co-stimulation through surface molecules and cytokines culminate in distinct patterns of signal

cascades leading to initiation of most adaptive immune responses. Activation of $CD4^+$ T cells by IL-12 shapes differentiation towards Th1 phenotype. Activated Th1 cells are able to produce high amounts of IFN-γ , critically required for MHC class I upregulation and macrophage activation. Studies from Belnoue et al. (2002) and (Yañez et al., 1996) have shown that early depletion of $CD4^+$ decreases susceptibility to cerebral malaria, depletion at a later time point at the onset of symptoms did not inhibit disease progression. However, mice deficient in $CD4^+$ T cells due to genetic knock-out were protected against fatal complications from *P. berghei* infection. We could show that the absence of $CD4^+$ T cells later in infection did not substantially alter ECM development and survival compared to WT mice (figure 3.10a and b). Additionally our results suggest that the presence of $CD4^+$ T cell help early in infection is highly relevant for the generation of antigen-specific effector T cells (figure 3.10c). Parasite specific IFN-γ responses in the spleen were diminished after early depletion, whereas late depletion did not alter cytokine responses. The presence of antigen-specific responses in the spleen in mice lacking $CD4^+$ T cells late in infection could partially explain ECM susceptibility. Interestingly, these mice had reduced parasite-restricted IFN-γ responses in brain cells, comparable to those seen in early $CD4^+$ depleted mice (figure 3.10d). Our early depletion experiments suggest that effector T cell origin is dependent on the presence of T helper cells. Nonetheless, when priming of effector cells is accomplished, $CD4^+$ T cells have no influence on effector activity. Recently **?** described the interdependency of $CD4^+$ and $CD8^+$ T cells and parasite tissue sequestration. Together with our results this would suggest that besides effector T cells, further mechanisms like tissue sequestration are responsible for ECM pathology. Although, Th1 cells are discussed to influence parasite growth by strong induction of IFN-γ production and by activating other immune cells (Hirunpetcharat et al., 1999; Hirunpetcharat and Good, 1998), they also have a relevance in inducing pathology. Thus $CD4^+$ T cell activity is not solely restricted to effector T cell induction but may also influence cytokine environment which may be independent of T cells, harmful for the BBB integrity (Togbe et al., 2008; Randall et al., 2008). The above section clearly demonstrates that *Plasmodium* induced cerebral malaria is dependent on early $CD4^+$ T cell help, which is a prerequisite for generation of parasite-specific responses. The absence of IFN-γ responses in the brain of late CD4 depleted ECM-susceptible mice, however, is not elucidated and further research is needed.

4.6 The effector phase of cerebral malaria

According to our hypothesized model, priming of parasite-specific responses in the spleen early in *Plasmodium berghei* infection is a critical step in the development of neuropathology. We propose that ECM development can be divided into a priming and an effector phase. As analysed in this study, priming of antigen-restricted T cell responses requires an intact spleen architecture and is dependent on antigen-presentation by macrophages, B cells and DCs using TLR-9 and IL-12 signalling and $CD4^+$ T cell help. However, this study did not, and was not intended to address the topic of the effector phase and possible mechanisms in depth. Depletion of dendritic cells early in infection (d0-d4 post infection) led to increased survival and ameliorated pathology and reduced antigen-specific responses in spleen and brain. When DCs were absent later during infection (d4-d7 post infection), mice developed ECM symptoms and died shortly thereafter. Although disease progression was not altered in comparison to ECM-susceptible WT mice, no reduction in cytokine production after restimulation with parasite peptides could be seen. However, antigen-specific cytotoxicity could be observed when DCs were absent late in the infection (data not shown).
Mice administered with clodronate liposomes one day prior to infection (-d1) survived and displayed no symptoms (figure 3.5a). Although treatment with clodronate liposomes 4 days post infection did increase survival chances of *P. berghei* infected mice, the lack of macrophages late in infection did not inhibit generation of antigen-specific cytotoxic T cells and IFN-γ responses after restimulation of splenocytes (data not shown, further experiments are currently being conducted). In contrast, the early absence of macrophages reduced parasite-specific effector functions. We hypothesize that the absence of macrophages during the priming phase inhibits T cell generation, but that macrophages may have another role during ECM development as seen in ameliorated pathology after clodronate liposome administration later in the infection. Clodronate liposomes do not cross the blood-brain barrier (Getts et al., 2008) and therefore do not eliminate brain macrophages, which have been discussed to contribute to local $CD8^+$ T cell responses during cerebral pathology (Pais and Chatterjee, 2005). However, recent findings indicate that micro-particles derived from infected erythrocytes drive macrophage activa-

tion and contribute to systemic inflammation (Couper et al., 2010). The absence of macrophages in the late phase of infection when micro-particle shedding and concentration is the highest could possibly lead to reduced overall inflammation and decreased ECM development.
Complementary outcomes were seen in mice administered with neutralizing CD4 antibodies, where we found that survival chances and IFN-γ levels after restimulation were similar to WT mice (figure 3.10). Together our results suggest that detrimental T cell responses are generated early in infection in the spleen and are dependent on macrophages, DCs and CD4$^+$ T cells. Despite involvement early in priming of effector responses, absence later in infection does not influence disease outcome. We hypothesise that T cell priming is completed around 5 dpi and that these cells are not, or are only partially, involved during effector phase when first symptoms become obvious. This hypothesis is supported by several studies that have shown that depletion of macrophages, neutrophils and CD4$^+$ T cells in the effector phase does not ameliorate ECM development (Belnoue et al., 2002; Chen et al., 2000). However, their involvement was not further elucidated. Gene expression analysis of ECM-susceptible mice also adds evidence that the clinical course of *Plasmodium berghei* infection can be divided into two phases. Early in disease, changes in expression levels in the brain are only subtle, whereas drastic upregulation of genes involved in apoptotic mechanisms and inflammatory responses, especially those regulated by IFN, were seen shortly before the onset of characteristic symptoms (Lovegrove et al., 2007; Delahaye et al., 2007).

Our study, for the first time, provides evidence that early cellular interactions in the spleen are responsible for detrimental disease development. Most cells involved in the priming phase are dispensable for the effector phase when T cell priming is completed and interventions by cell depletion are insufficient for the inhibition of ECM. These results extend the global rationale of early mechanisms involved in *P. berghei* induced cerebral pathogenesis by identifying the spleen as the lymphatic tissue where immune responses are primed. The spleen is the organ in which immune responses against the parasite are generated and outcome of disease is determined. Interpolation from the rodent model to human infection must be analysed carefully and with a complete understanding of what the model can and cannot tell us. The

better the understanding of immune responses leading to fatal complications, the greater are the possibilities to find interventions against cerebral malaria.

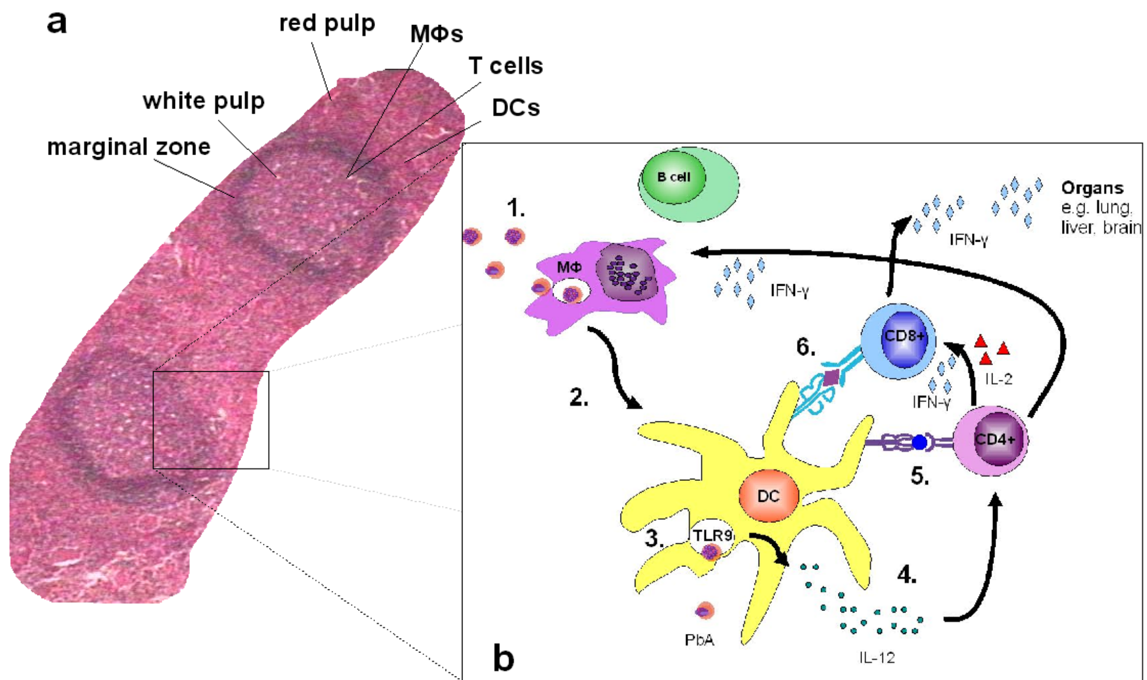

Figure 4.2: Proposed model of cellular interactions in the spleen during priming phase of *Plasmodium berghei* infection
a) Schematic view of the splenic structure. b) model of interactions in the priming phase of *Plasmodium berghei* infection 1. Phagocytic uptake of iRBCs, whole parasites or parasite particles by macrophages and possibly by B cells. 2. Macrophages are assumed to transfer processed antigen DCs 3. Parasite recognition through TLRs and induction of IL-12. 4. Secreted IL-12 drives Th1 T cell differentiation and activation. 5. Activated $CD4^+$ Th1 cells recognize antigen-MHC class II complexes on DCs via TCR, thus licensing DCs for cross-presentation and produced cytokines drive cytotoxic T cell differentiation and macrophage activation. 6. $CD8^+$ T cells recognize parasite antigen presented in a MHC class I context, this leads to priming of effector cell activity. Cytotoxic $CD8^+$ T cells migrate to affected tissue to exert their effector function and secrete high levels of IFN-γ, both steps critically required in ECM development.

5 Conclusion

The spleen is an important lymphoid organ involved in early immune responses against invading pathogens. Our results demonstrate an involvement of the spleen and its intact micro-architecture in the pathogenesis of cerebral malaria. Early interactions between antigen-presenting cells and effector cells during *Plasmodium berghei* infection is critically required for the generation and priming of pathogenic T cells. These interactions include dendritic cells, macrophages and B cells as APCs to present parasite antigen to T cells. Specific cell-type depletion can lead to a disrupted architecture in the spleen resulting in abrogation of the cellular interactions necessary for effector responses. In the case of *Plasmodium berghei* infection, priming of antigen-specific T cells in the context of an intact spleen is an essential step in neuropathogenesis. Besides named antigen-presenting cells, recognition of parasite particles through TLR-9 and subsequent production of the pro-inflammatory IL-12 as well as CD4 T cell help is indispensable for the generation of cytotoxicity and antigen-specific responses that will lead to pathology. Our study emphasizes the role of pathogenic T cells during cerebral malaria as determined by the generation of effector responses, which are dependent on structural, cellular and molecular factors. For the first time we could associate parasite-specific T cells and their close interrelationship with spleen-dependent immune responses with pathology. Detailed insights into mechanisms of disease progression are valuable for further investigations and should be considered in future drug research and treatment strategies.

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