Uncovering the role of IFNAR1 in Experimental Cerebral malaria

Elizabeth Ann Ball

Dissertation presented to obtain the Ph.D degree in Biology.

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Research work coordinated by:

FUNDAÇÃO CALOUSTE GULBENKIAN
Instituto Gulbenkian de Ciência

Oeiras,
April, 2013

INSTITUTO
DE TECNOLOGIA
QUÍMICA E BIOLÓGICA
/UNL

Knowledge Creation

This dissertation was completed with the financial support by Fundação para a Ciência e Tecnologia. Apoio financeiro da FCT e do FSE no âmbito do Quado Comunitário de Apoio, Grant No. SFRH / BD / 33564 / 2008

Preface

This thesis is the result of my work carried out at the Instituto Gulbenkian de Ciência during the time from September 2008 to September 2012, where I was enrolled in the internal Doctoral Program PGD2008, Instituto Gulbenkian de Ciência, under the supervision and guidance of Dr. Carlos Penha-Gonçalves.
All work presented here was carried out at the Instituto Gulbenkian de Ciência.
Financial support was provided by Fundação para a Ciência e Tecnologia, Portugal, through the Ph.D. fellowship grant:
SFRH / BD / 33564 / 2008.

This thesis is constructed of four chapters:

Chapter one comprises a general introduction to malaria, with emphasis on the host immune responses during an infection, and the usage of mouse models in Experimental Cerebral malaria. This chapter also includes the thesis objectives.

Chapter two presents the development and results of a novel cell transfer protocol designed during this thesis work.

Chapter three includes the manuscript comprising my Ph.D. thesis work, accepted for publication.

Chapter two and three are comprised of author contributions, summary and introduction, detailed material and methods, with results and discussion.

Chapter four comprises the general discussion for this thesis work, including conclusions and future prospectives.

Acknowledgments

Firstly I would like to thank Dr. Rosario Sambo for the work she conducted in Angola, and to the children and staff who participated in the original study at Hospital Pediátrico David Bernardino. These results led us to this thesis work, for which I will always be grateful. Secondly, to my supervisor Carlos Penha-Gonçalves, for his endless patience in teaching me, his careful guidance, and especially his support during the final year of this Ph.D. work.

I would like to thank Monica Dias and especially Miguel Soares for being a part of my thesis committee. Thank you for your valuable words of advice and wisdom, even though they were often stern, they helped me finally reach this point. Thank you for your care and commitment.

Thank you especially to Rui Garder, Telma Lopes and Claudia, for the many hours spent sorting and the dedication you gave to my work and the results, without you I would have very few Ph.D. results! And also to Manuel Rebelo, thank you for your support and maintenance of the wonderful mice I worked with.

I would like to thank all my friends at the IGC and colleagues who welcomed me into this very special community. Thank you for introducing me to 'lanche', and to the importance of lunch away from the desk, where we talked away the worries and set the world to right. These times were an education in life as well as science, especially to Andreia Lino, Nadia and Luciana, I will always remember the many life lessons you've taught me!

Thank you to my group, the wonderful women who supported and taught me during these years, and became friends

along the way, Joana Corte-Real, Nadia Duarte, Lurdes Duarte and Joana Rodo, Luciana Moraes and Lígia Gonçalves. Thank you for every moment together, you all taught me different things that I will keep with me always. Especially thank you to Joana CR and Nadia – sitting next to you both, definitely pushed me to work harder as you were the power pair and showed how much you can achieve when your time is put to good use! I would especially like to thank Lígia, you taught me from the word go, sharing your experience and wealth of knowledge of malaria, research techniques and everything in-between. I am very lucky to have been taught by you. And also to Luciana, you helped me during the final few years of my work and we became close friends. I saw you as an 'Aunty' and we shared many moments of fun, food and facs together! Thank you Lu for being so wonderful and always there to help and talk.

I would like to thank also Susana, Bahti and Zelia. My adopted group, who went from 'borrowing' reagents to real friends. Your words of support and scientific discussion, and many IGC canteen feasts together (Zelia) made the daily hours of work in our student's office feel like friends together learning, thank you.

I would like especially to thank the group of 2008 students, from the internal Ph.D. program, Computational program and Ph.D. Champaulimaud Neuroscience program, whom I was so lucky to have had classes with at the beginning of my Ph.D. These were the best times for me and I felt 'a part' of science and research from this moment onwards. It was such a wonderful experience to be taught in a classroom with physicists and engineers and to have sat amoungst you as a young, naïve medical student – you showed me the level of talent and friendship that can come from a passion and interest. Thank you all dearly. Especially thank you to Daniel Damineli, a firm friend from the beginning, who shared tea with me

and made me feel at home, and who I count as such a great friend. I would also like to thank Ines Bento, we became friends from the classes, you are a wonderful person, and a special friend. Thank you for all your help and support through the years, your passion for learning, giving and helping people will always be inspiring to me.

Thank you to Manuel Pita, I am so happy to have met you here, and whom I now consider a very dear friend. As well as Maria Serrano, I am so happy to have worked next to you and to build our friendship. Thank you for always supporting me with a friendly face, for the all the hours talking and a shoulder to cry on.

I would like to thank also my wonderful teacher and friend Isa Guitana and my dear friend Stef and Ana, and the students at Casa Vinyasa, you all have been instrumental in the years of my Ph.D., letting me follow my dreams and always supporting. Casa Vinyasa is such a special place in my heart.

Especially I would like to thank my family. My loving boyfriend Nuno, who first brought me to Lisbon, thank you for always pushing me to my best and for loving me so much. Thank you also to Albino, Dona São, Rita and Alex, my Portuguese family! Thank you for supporting me all these years, and for the many family dinners, relaxing together, they mean so much to me. Thank you to my Aunty Frieda, Shirley and Di, for your continued belief in me and support in everything I do, and for the encouragement to keep going during the final few months.

Thank you to my two beautiful sisters, Alice and Lucy. You both inspire me so much everyday to be a better person, and to follow my dreams. I love you both very much. And lastly thank you to my Mum and Dad for everything. For all the years you have supported and believed in me. Thank you for the many hours over the years listening and giving words of advice and wisdom, and for

your enthusiasm whilst I explained how to stain a brain blue. I share this achievement with you, I love you very much.

Abbreviations

Ab – Antibody

Ag – Antigen

C57BL/6 – C57black/6 (wildtype), mouse strain

CM – Cerebral malaria

CTL – Cytolytic T cell

CXCR3 – Chemokine (C-X-C motif) receptor 3

BBB – Blood brain barrier

DC – Dendritic cells

ECM – Experimental Cerebral malaria

EC – Endothelial Cell

GFP – Green fluorescent protein

GrB – Granzyme B

IFNAR1 – Interferon Type I, alpha, beta receptor

IFN I – Type I Interferon

IP-10 – Interferon gamma-induced protein 10

i.p – intraperitoneal

iRBC – infected red blood cells

IRF – Interferon regulatory factor

ISG – Interferon stimulated genes

ISREs – Interferon stimulated response elements

JAK – Janus activated kinase

PAMPs – Pathogen associated molecular patterns

PbA – Plasmodium berghei ANKA

pDC – plasmocytoid DC

PI – post-infection

pRBC – parasitized red blood cells

PRR – Pattern recognition receptors

SNPs – Single nucleotide polymorphisms

STAT – Signal transducer and activator of transcription

TLR – Toll like receptors

Tnf-α – Tumor necrosis factor alpha

Summary

Cerebral malaria is a severe and fatal form of clinical *Plasmodium falciparum* infection, resulting in brain injury from a damaging cascade of vascular, inflammatory and immunological host responses. However progression to cerebral malaria can be modified by host genetic factors. This thesis work extensively reveals the role of Interferon type I receptor (IFNAR1) in the development of Experimental cerebral malaria, through the use of the mouse model *Ifnar1*$^{-/-}$. We found *Ifnar1*$^{-/-}$ mice protected from Experimental cerebral malaria upon infection with *Plasmodium berghei* ANKA-GFP, compared with susceptible wild-type C57BL/6 mice. *Ifnar1*$^{-/-}$ mice showed diminished blood brain barrier breakage, despite parasite accumulation in the periphery and accumulation of immune cells within the brain tissue during infection. To elucidate the cellular basis of *Ifnar1*$^{-/-}$ resistance we developed a novel cell transfer protocol that enables spleen cell priming in absence of disease. This further led us to discover that IFNAR1 expression in CD8^{+} T cells is crucial, and can abrogate resistance to Experimental cerebral malaria in *Ifnar1*$^{-/-}$ mice. We found splenic CD8^{+} T cells from *Ifnar1*$^{-/-}$ mice are functionally activated upon infection, yet unable to mediate Experimental cerebral malaria development within the brain tissue.

Our findings prove that IFNAR1 signaling unleashes CD8^{+} T cell effector capacity, vital for cerebral malaria and concludes the essential role of IFNAR1 in CD8^{+} T cell triggering. Moreover this suggests a possible role of CD8^{+} T cells in human cerebral malaria development, and highlights IFNAR1 as a potential therapeutic target in future preventative measures against cerebral malaria.

Sumário

A malária cerebral é uma forma clínica severa e fatal da infecção por *Plasmodium falciparum*, que causada por uma cadeia de respostas vasculares, inflamatórias e imunológicas que determinam lesões cerebrais. O risco de ocorrência de malária cerebral em indivíduos infectados está dependente de factores genéticos. Esta tese revela o papel do gene IFNAR1 (receptor 1 do interferão alpha) num modelo experimental de malária cerebral, pela análise de ratinhos *Ifnar1*$^{-/-}$. Os ratinhos *Ifnar1*$^{-/-}$ demonstraram resistência à indução de malária cerebral por *Plasmodium berghei* ANKA-GFP. Os ratinhos *Ifnar1*$^{-/-}$ infectados mostraram reduzida incidência de quebra da barreira hemato-encefálica apesar de mostrarem acumulação de eritrócitos parasitados e de células do sistema imunitário no tecido cerebral. Para determinar a base celular da resistência à malária cerebral nos ratinhos *Ifnar1* $^{-/-}$ desenvolvido um protocolo de transferência celular que impede a transferência simultânea do parasita quando são transferidas células esplénicas que foram desfiadas pelo parasita. Este protocolo permitiu evidenciar que a expressão de IFNAR1 nas células CD8+ é crucial e é suficiente para induzir malária cerebral nos ratinhos *Ifnar1* $^{-/-}$. As células T CD8^{+} dos ratinhos *Ifnar1*$^{-/-}$ infectados revelaram estar funcionalmente activadas mas incapazes de provocarem cerebral malária.

Este trabalho mostra que a sinalização pelo IFNAR1 acciona a capacidade efectora das células T CD8^{+} é critico para o desenvolvimento da malária cerebral. Este trabalho propõe um papel relevante para o gene *IFNAR1* no desenvolvimento da malária cerebral em seres humanos e sugere o IFNAR1 como um potencial alvo terapêutico na prevenção da malária cerebral.

Table of Contents

Chapter 1

General Introduction

1.1 Epidemiology and Malaria infection.

1.1.1 Epidemiology

Research into malaria first began through the work of Ettore Marchiafava and Amico Bignami in 1890's (Autino et al., 2012). They were amongst the first to recognize that the cause of malaria could be transported through the mosquito, and Marchiafava named this parasite, *Plasmodium*. *Plasmodium* is a protozoan parasite (Sinnis and Zavala, 2012), of which there are four species that infect humans, *P. ovale*; *P. vivax*; *P. malariae*; and *P. falciparum* (Haldar et al., 2007), and most recently a fifth species now found to infect humans, *P.knowlesi* (Servonnet et al., 2012).The parasite *Plasmodium falciparum* has been classed as one of the most successful human pathogens (Urban et al., 1999). Malaria was first recorded in around 2700 BC in ancient Chinese texts, and yet *Plasmodium* still fathoms research by its evading capacities and no effective cure has been found to date. *Plasmodium also* continues to challenge strategies of elimination and eradication and procure successfully both the human and mosquito host.

More than 40% of the world's population is at risk of infection by *Plasmodium* parasites, with roughly 216 million cases of malaria being recorded in 2010 (WHO report, 2012). *P.falciparum* infections are mainly responsible for cases leading to severe malaria (Rosenthal, 2008), a complicated and severe form, which presents with clinical manifestations including, anemia; hypoglycemia; metabolic acidosis; repeated seizures; coma and multiple organ failure (Snow et al., 2005). One % of severe cases of infection can further develop into Cerebral malaria (CM) (de Souza et al., 2010), a clinical form of severe malaria dominated by a

neurological syndrome, and occurs within the incidence of 1-1.2 million cases/year, in endemic regions of Africa (Idro et al., 2010), with up to 20% of cases being fatal (Idro et al., 2005). Its peak incidence is in children under 5 years of age, and approximately 500,000-800,000 children develop CM each year in sub Saharan Africa alone (Reis et al., 2012). Economically, the 213 million annual 'attacks' of malaria, has led to over 800 million sick days being taken per year in Africa (Breman et al., 2004), thus showing what a catastrophic effect malaria infections cause in endemic countries.

1.1.2 Lifecycle of Malaria infection

The *Plasmodium* parasite is transmitted through the bite of an infected female Anopheles mosquito, as well as a yet unclassified species of mosquito recently found (Stevenson et al., 2012). Female mosquitoes seek a human blood meal for fertilization of their eggs. During this piercing of the skin, the parasite passes through the saliva of the mosquito and is deposited in the skin of the human, now classified as a host. The initial deposit within the dermis, is then followed by gliding of the sporozoites to the bloodstream in which it is transported to the liver, an obligatory stage of development (Prudencio et al., 2006). Infection in the human host occurs in two phases, the first asymptomatic liver stage and the second blood stage of infection that is often symptomatic. Following passage in the liver sinusoids, individual sporozoites traverse a number of hepatocytes before residing in the hosting hepatocyte where they expand and develop into merozoites (Sturm et al., 2006). Merozoites are released from infected hepatocytes inside vesicles termed merosomes, into the

blood stream, where they invade red blood cells and follow a second stage of asexual development (Liehl and Mota, 2012). Infected red blood cells (iRBC), eventually rupture and release daughter merozoites that then can invade fresh erythrocytes (Haldar et al., 2007). This process of iRBC bursting and invasion occurs cyclically every 48 hours, triggering host immune responses and causes the onset of fever, sweating and chills (Sinnis and Zavala, 2012), the first apparent symptoms of a malaria infection. *P. falciparum* is unique in its cycle, whereby every 24-32 hours, upon maturation into its trophozoite (ring) stage, iRBC can then adhere to endothelial cells in the circulation of particular organs, such as liver, placenta and brain (Haldar et al., 2007). A select number of parasites within iRBC can differentiate into male and female gametocytes, which are taken up by a feeding mosquito (Michalakis and Renaud, 2009), and undergo sexual development within the mosquito to form sporozoites within the mid-gut, ready to be deposited in the next blood meal and thereby completing the parasite lifecycle. The passage between the human host and mosquito vector leads to loss of parasite numbers but the asexual and sexual replication within the human and mosquito, ensures expansion of these numbers (Sinnis and Zavala, 2012), and a successful transmission. Figure 1.1 represents an example of the malaria lifecycle.

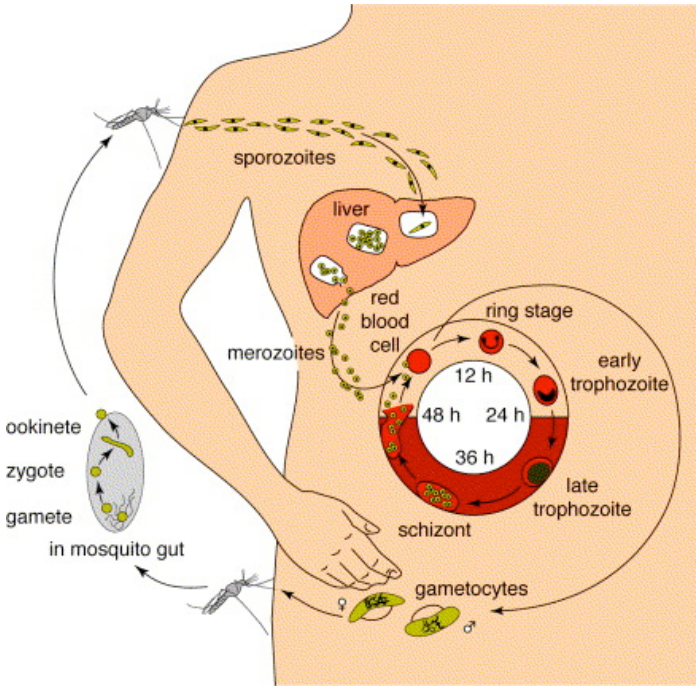


Figure 1.1 Malaria lifecycle of P.falciparum (van Agtmael et al., 1999).. From the bite of an infected female Anopheles mosquito, sporozoites are transmitted through the salivary glands into the blood stream of the human host. They migrate to the liver where they traverse and then reside in hepatocytes, within one hour of infection. Sporozoites develop asexually into merozoites. Within 5-7 days after initial infection, infected hepatocytes release thousands of merozoites, which then invade red blood cells. The parasite further develops within red blood cells, and every 48 hours mature schizonts are released by rupture of infected red blood cells into the bloodstream, where they are available to invade fresh red blood cells. This cyclical rupture leads to the host immune responses and development of malaria symptoms, such as high fever and chills. (Adapted and re-printed with author permission).

1.1.3 Clinical disease

Malaria cases present with fever and chills along with common symptoms of dizziness; diarrhea; nausea; vomiting and dry cough (Trampuz et al., 2003), however there is great difficulty in

clinical diagnosis and treatment for malaria as often these symptoms appear flu-like (Murphy and Oldfield, 1996). Severe and complicated malaria is characterized by anaemia; hypoglycemia; respiratory distress; altered consciousness and general malaise and can progress to major complications, including pulmonary edema; acute renal failure; multi-organ failure and cerebral malaria (CM) (de Souza et al., 2010; Haldar et al., 2007).

Severe anemia from malaria infection accounts for a large proportion of deaths in children living in endemic regions of Africa (Murphy and Breman, 2001). Anemia has been primarily linked to the bursting of iRBC, and the clearance of deformed RBC from the circulation (Haldar and Mohandas, 2009). The role that the spleen plays in malaria infection is also extensive, and enlargement of the spleen (splenomegaly) is often seen during infection (Ahmed et al., 2011). It has been claimed that the spleen can work as a 'double-edged sword' in malaria, harbouring both non-infected and iRBC, which can lead to a decrease in total body parasite burden, therefore decreasing the risk of progression to CM, but at the same time increasing the risk of severe malaria anemia (Buffet et al., 2009).

Measurement of peripheral parasitemia is the most common method in diagnosing a malaria infection. It might be thought that the higher the levels of parasite in the body, the increased risk of developing a severe form of the disease. This is often the argument for human CM cases, however a clinical study did find 7 out of 31 cases of *P.falciparum* CM without high levels of pRBS in the brain tissue (Taylor et al., 2004), and that further cases of hyperparasitemia (high levels of parasitemia) have been recorded in non-CM patients also (Craig et al., 2012).

In the case of the mouse model, recent work by (Haque et al., 2011a) stated that early immune responses, primarily led by $CD4^+$ T cells, aimed at decreasing parasite burden and predicts disease severity and risk of death. However work by (McQuillan et al., 2011) found that treatment with anti-malaria quinine protected mice from ECM development, despite increase in peripheral parasitemia. Therefore it is still not confirmed whether levels of parasitemia can be used in predicting disease outcome.

1.2 Cerebral Malaria

In *P.falciparum* cases of malaria, roughly 1% of these develop into the severe form of CM, with 10-20% of these cases having a fatal outcome in the absence of treatment, translating into roughly 300,000-500,000 deaths per year (de Souza et al., 2010). CM develops very rapidly, in the case of a few hours, following the initial days of fever from the blood stage of infection.

The only recognized form of clinical diagnosis for CM is unarousable coma, not attributed to other causes, defined by the World Health Organization (WHO) (Molyneux et al., 1989; Teasdale and Jennett, 1974). Further symptoms of CM can include general malaise; headaches; fits and vomiting and diarrhea (de Souza et al., 2010; Newton and Krishna, 1998) and these early symptoms of CM can quickly progress to increases in intracranial pressure; paralysis; ataxia and coma (de Souza et al., 2010). The clinical presentation of CM can vary widely amongst patients, making it very hard to distinguish from other neurological diseases. This difficulty in diagnosis, combined with the poor treatment options, begs for early resolutions at catching the onset of CM in malaria

cases. Even CM cases that received treatment with anti-malaria drugs, Artemisinin and quinine, resulted in death in 15% and 22% of the cases (Dondorp et al., 2005). Therefore it is imperative that research continues to devour the triggers and preventative measures of CM development.

1.2.1 Host genotype and malaria infection

Treatment options for malaria are limited to anti-parasite drugs selected at targeting and diminishing the blood stage of infection and its symptoms. Therefore in endemic areas of malaria, due to the lack of knowledge and protection against the initial (silent), liver stage of disease, a person can be re-exposed to infections throughout life (Aidoo and Udhayakumar, 2000). Even after effective anti-malaria treatment for cerebral malaria (CM), patients can still suffer long term neurological sequellae (Shikani et al., 2012). Therefore after apparent elimination of the parasite this does not resolve the clinical consequences of an infection and furthermore the process of parasite re-exposure can lead to long-term influences on human genetics.

Genetic risk factors for severe malaria have shown that roughly 25% of the risk in progressing to a severe form of malaria is determined by human genetic factors (Mackinnon et al., 2005; Rihet et al., 1998). For our interests, further evidence indicates from both human and mouse model studies of CM, that host-genetic components influence the outcome of CM, and that this outcome may be polygenic (Driss et al., 2011; Hunt et al., 2006; Sambo et al., 2010).

Further genetic variants associated with CM risk include polymorphisms in the N-terminal region of I-CAM1, found to

increase risk of CM in African populations (Craig et al., 2000); single-nucleotide-polymorphisms (SNPs) encoding IL-13 were found in a Tanzanian population to increase the risk of CM (Manjurano et al., 2012); SNPs in Interferon-alpha receptor-1 (*IFNAR1*) gene show protection against CM (Aucan et al., 2003); polymorphisms in the promoter region of the Interferon-gamma receptor-1 (IFGR1) gene are associated with CM susceptibility (Koch et al., 2002) and polymorphisms in the promoter region of the TNF gene, have been found to increase risk of neurological symptoms in Gambian children (McGuire et al., 1994; McGuire et al., 1999). The polymorphism in the TNF2 allele, although disadvantageous for severe malaria anaemia risk, is still carried within the population suggesting it is biologically advantageous, which may represent a balancing selection phenomenon also associated with the sickle cell anemia gene (Ferreira et al., 2011).

A recent report also suggests that host variation interplays with infection and that genetic variation in children pre-disposes them in mounting an effective immune response towards a malaria infection (Idaghdour et al., 2012). Collectively, these studies provide genetic links to CM susceptibility, and have begun to pinpoint inflammatory mediators that influence the development of CM. Therefore it is crucial in CM research to decipher the role of these mediators within common pathogenic mechanisms leading to CM development.

1.2.2 Mouse model *vs* Human cases

There is a large debate in the field of malaria research to the advantages behind the use of mouse models in CM research. I believe this will always be a hot topic, and so it is imperative that

researchers focus on how the mouse model can help us understand human cases of CM development.

One published article on human *vs* mouse models caused debate when it stated that the model of *Plasmodium berghei* ANKA (*PbA)* was limited in its use for understanding the pathogenesis of human CM (White et al., 2010). It was met with much dispute by leading researchers who use the mouse model, termed Experimental cerebral malaria (ECM). Together they support the essential role the mouse model contributes in CM understanding (Carvalho, 2010; de Souza et al., 2010; Renia et al., 2010; Riley et al., 2010). In particular Carvalho and colleagues and Hunt and colleagues, (Carvalho, 2010; Hunt et al., 2010) raise the issue of decreased publications of human CM cases, and express concerns that researchers should focus on the similarities between the mouse and human models and how essential discoveries were made by the mouse model for human studies. The example of our recently published article in Chapter three, is one such paper that led from the discovery of genetic polymorphisms amongst human patients, to the understanding and discovering of IFNAR1 roles in CM through the use of ECM mouse models.

The apparent controversial difference lies between human CM development being caused by sequestration of pRBC *vs* mouse ECM being a consequence of leukocyte sequestration (Cabrales et al., 2010; Craig et al., 2012). However autopsies from a small number of human cases that died from severe malaria, with no CM, have shown signs of sequestration of pRBC within the brain (Pongponratn et al., 2003). Furthermore, as *P.falciparum* is the only human parasite species that sequesters within the brain and the main species that causes CM development (Sarkar and Bhattacharya, 2008), these cases suggest pRBC sequestration

may not be the determining factor for human CM development (Pino et al., 2005). Given that human and mouse CM both occur through obstruction of micro-vessels within the brain tissue, by pRBC or leucocytes or both, this could represent a common mechanism in CM development (Craig et al., 2012). In support iRBC have been imaged within post-capillary venules in *P.falciparum* infections, as seen in ECM (Dondorp et al., 2004; Nacer et al., 2012)

Two main hypotheses exist for the cause of CM in human cases (Hunt et al., 2006). The mechanical obstruction hypothesis whereby CM is a consequence of parasitized (p)RBC adhering to endothelial cells, leading to vascular obstruction and cerebral oxygen deprivation (hypoxia) (Berendt et al., 1994), opposed to the cytokine response hypothesis that supports a central role for cytokines in CM immuno-pathology (Clark and Rockett, 1994). Therefore the ECM model is also currently used to investigate the role of cytokines in ECM development.

The largest advantage of the mouse model is the ability of researchers to closely examine the brain tissue during CM occurrence, a limiting factor in human case studies. Knockout strains of mice as well as genetically altered mouse strains have been instrumental in exploring in-depth potential inflammatory and immune regulatory mechanisms that lead to ECM development (Grau et al., 1986; Pamplona et al., 2007; Parekh et al., 2006; Serghides et al., 2011; Zhu et al., 2012). In conclusion it is of the role of the researcher to provide an appropriate experimental system that aims at deepening our understanding of human CM cases, rather than distancing the gap between the human and mouse models.

1.2.3 Experimental Cerebral Malaria pathogenesis

Consistent with human studies of CM, genetic background, age, degree of infection and clonal variations in parasite strains, all play a role in the pathogenesis of Experimental Cerebral Malaria (ECM) (de Souza et al., 2010; Lackner et al., 2006).

Infections with the *PbA* strain of parasite, together with the C57BL/6 mouse strain, has set up an established susceptible mouse model for ECM (Rest, 1982), and this model is accepted as the most available experimental model, replicating many events seen during human CM (de Souza et al., 2010).

Mice develop symptoms of ECM between days 5-7 post-infection, expressing relatively low parasitemia levels and die within 2-3 days from disease onset. Symptoms of ECM include ataxia; convulsions; roll over; paralysis and coma (de Souza et al., 2010; Favre et al., 1999; Lackner et al., 2006). However not all of these symptoms are consistently found in the disease state, and as like human cases, there is variation also (Linares et al., 2012).

A well-documented account of ECM development, and its neurological events has been reported by Lackner and colleagues (Lackner et al., 2006). This has helped record the pathogenic events leading up to ECM, and more importantly provide a framework to evaluate how these events correlate to a human infection. After the onset of neurological symptoms, a decrease in body temperature is seen in the mice – a reliable indicator of terminal illness - 36 hours prior to death, motor functions and co-ordination are lost and respiratory rate becomes altered, which is consistent with brainstem lesions. General muscle tone and strength is lost and this can be used as a good marker to distinguish ECM from non-ECM mice also.

It can be difficult to assess symptoms of ECM, alike clinical diagnosis of human CM. The SHIRPA score was developed to mimic the general, neurological and psychological assessments used in human cases (Rogers et al., 1997). The score produces quantitative data for each mouse at three different stages of cerebral disease, collectively giving a specific profile of the mouse related to loss of function. It is not a widespread or mandatory form of ECM assessment, but it has been suggested as useful to document the beginning stages of cerebral disease (Lackner et al., 2006), and does allow for abnormalities between mouse strains to be detected (Rogers et al., 1997). A similar score for ECM assessment has also been documented, a quantitative, rapid murine coma and behavioural score (RMCBS) (Carroll et al., 2010). This score was developed to assess ECM in the *PbA* mouse model, aimed at identifying symptoms preceding ECM onset, for use in potential treatment trials. Together these assessments and the experience of the researcher are the standard diagnostic procedures used for ECM models.

1.2.4 Brain tissue damage

The general assessment for brain tissue damage in mice is through analysis of brain sections and by evaluating the level of permeability and leakage of the blood brain barrier (BBB). Brain tissue damage by cerebral malaria development has been found to include disruption of vessel walls; endothelial degeneration and cerebral edema, as well as adherence of pRBC and blood leucocytes in brain vessels (Lackner et al., 2006).

Many authors questioned whether there is a specific area of the brain that is affected most during malaria. The work of (Lackner

et al., 2006) found that lesions were most significant in the forebrain, brain stem, with most damage seen in the rhinencephalon area. In a study with Malawian children areas of vascular damage in the brain also showed myelin loss and axonal damage (Dorovini-Zis et al., 2011), further verified in adult Vietnamese populations (Medana et al., 2002). However unless the research study is focused upon identifying lesions or damage to the brain, this is not a specific objective in CM assessment, and a general concensus is that there is no one specific area of damage within the brain cortex of mice or human cases of CM.

1.2.4.1 Blood brain barrier

Blood brain barrier (BBB) breakage is a hallmark of ECM, however it is not generally seen in human cases of CM (Clark et al., 1992). Breakage of the BBB is confirmed only after death, through experimental *in vivo* injection of dye into the circulation and followed by anatomical analysis (Favre et al., 1999). The exact point at which breakage of the barrier occurs is much a question of whether ECM development leads to BBB breakage, or if BBB breakage is the cause of ECM progression.

The BBB is described as a selective barrier formed by endothelial cells (EC) that line the brain microvasculature (Abbott et al., 2006). There are in fact three types of barriers, the physical barrier, made up of tight junctions between the EC; a transport barrier between the luminal and subluminal membranes of the EC, controlling the entry of essential and harmful molecules into the brain and a metabolic barrier, governed by intra and extracellular enzymes (Abbott et al., 2006). Together these barriers regulate the homeostasis of the brain and keep it guarded and selectively

separate from the body. In this way brain interstitial fluid (ISF), which bathes the brain cells is maintained at optimal conditions for neuronal function.

The BBB is found predominantly in the brain capillaries, but there are also more modulatory barriers within the arterioles and venules of the brain (Abbott et al., 2006). Figure 1.2 shows the makeup of the BBB. Within this figure there is also note of astrocyte cells, the brain perivascular cells that help to regulate the BBB function (Abbott et al., 2006), enhancing protection and avoidance of un-necessary compound entry into the brain parenchyma.

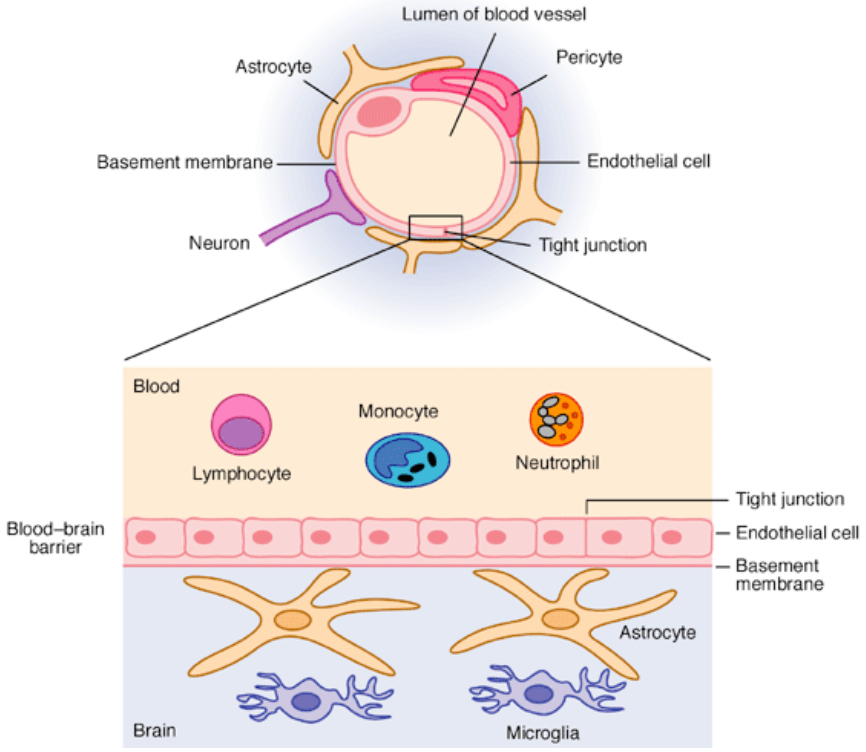


Figure 1.2. The blood brain barrier (BBB). (Francis et al., 2003). The BBB is created by tight junctions of EC that line the blood vessels within the brain, forming a barrier between the circulation and brain parenchyma. Lymphocytes, monocytes and neutrophils (blood-borne cells) cannot penetrate the barrier. The thin basement membrane also adds mechanical support, aiding the barrier function, it is made up of lamin, fibronectin and other proteins and surrounds the EC and pericytes. The BBB is critical in preventing pathogen and antibody entry into the CNS, protecting the brain tissue from inflammatory damage due to immune responses. (Adapted and re-printed with authors permission).

Endothelial cells and astrocytes themselves form a close barrier, supporting each other in correcting cellular associations between EC and pericytes, and the EC support growth and differentiation of the astrocytes (Abbott et al., 2006). EC are supported further by pericytes, cells that wrap themselves around the EC and occupy a key position (Armulik et al., 2005; Bordon, 2013), where they help regulate the BBB and brain vasculature permeability (Armulik et al., 2010). It is the collective work of these cell types that together closely regulates the BBB functions.

The BBB may appear to sound as a barrier that is either fixed or broken, however it is a permeable support that can be altered by modulators that lead to the barrier becoming leaky or permeable (Abbott, 2005), and weakening of the junctions. In cases when important molecules need to access the brain, this leaky property is vital, but if altered can lead to the permanent damage and huge infiltration of blood mononuclear cells, pathogens or viruses into the brain parenchyma, altering the highly protected homeostatic conditions within the brain.

Entry of immune cells into the brain parenchyma, across the BBB occurs through two different steps, the physiological BBB and neuro-inflammatory BBB (Owens et al., 2008), and early reports

suggest that cytokine release can lead to activation of brain neuroglial and microglial cells, due to the opening of the BBB (Toro and Roman, 1978). Therefore in malaria infection, during the time window of CM induction the parasite is residing in iRBC at different maturity stages, from merozoite to schizont. The parasite is sitting in perfect position to be carried in the brain microvasculature and potentially exposed to unleash damage onto the brain parenchyma. Furthermore it has been shown that soluble factors from *P.falciparum* iRBC induce apoptosis in human brain vascular endothelial cells *in vitro*, suggesting that longtime exposure to such factors, could be associated to vascular; neuronal and tissue injury in malaria patients (Wilson et al., 2008). It is still a debatable topic as to whether the presence of the parasite causes damage directly, or whether it is the inflammatory responses acting on a normally quiescent brain parenchyma, that leads to the damaging cascade of events towards barrier breakage, coma and eventual death.

The breakage of the barrier during CM holds a general consensus at occurring during the final stages of CM induction, and is seen as the final unleashing of the inflammatory immune response upon the brain tissue. In some cases, if the gradual opening and increased permeability of the BBB is caught early enough in a malaria infection, symptoms of CM can be reversed and the patient saved from the induction of neurological downfall and death. One such study suggests that presence of the iRBC leads to constriction of the micro-vessels, leading to opening of the EC tight junctions, and that administration of anti-malaria drugs at the onset of human CM can abrogate this opening and cause survival of patients (Brewster et al., 1990; Nacer et al., 2012).

1.3 Innate immune response to Malaria

During malaria infection a host can develop partial immunity due to repeated exposure over a number of years, leading to a "pre-munition" state. Nevertheless this tends to wane in absence of exposure and upon moving away from endemic regions, clinical malaria immunity decays and is eventually lost (Autino et al., 2012). This 'pre-munition' is due to parasite strategies that evade sterile immunity, and build up of the host's immune response, preventing clinical disease. This build up of immunity can be triggered by the shedding of protein parts from parasite polymorphic antigens during prolonged exposure in endemic regions (Autino et al., 2012). Therefore vital information is held within individual's immune responses, and comparisons made within human populations living in malaria endemic areas against those in elimination areas, can provide clues in regard to the immune response properties of the malaria infection.

1.3.1 Cytokines

It has been reported numerous times, that it is the imbalance of pro and anti-inflammatory responses that causes the plethora of symptoms associated with severe malaria infections, and recent work has begun to show associations of cytokines and their receptors to malaria pathogenesis (Khor et al., 2007).

Extensive work has shown the role of IFN-γ related to CM development (de Kossodo and Grau, 1993; Villegas-Mendez et al., 2012), where it was found that *IFN-γ*$^{-/-}$ mice are protected from ECM induction upon *PbA* infection (Amani et al., 2000), with no leukocytes or parasites being found within the brain of these mice and absence of brain hemorrhages seen also (Villegas-Mendez et

al., 2012). In fact deletion of the IFN-γR, led also to protection from ECM in *PbA* infection in 129P2Sv/ev mice, (Belnoue et al., 2008), who showed further that leukocyte accumulation in the brain was hindered. This has led the way for showing the vital roles cytokines and Interferons play in ECM induction. This is further supported by (Parekh et al., 2006), who suggested that ECM development may be due to concentration of pro-inflammatory cytokines, such as IFN-γ, due to sequestered iRBC at the brain tissue site. Moreover increased levels of inflammatory cytokines involved in trafficking of T cells, such as MIP-1α and MIP-1β (Ochiel et al., 2005) and IP-10 (Armah et al., 2007) have been shown in cases of CM patients. Finally IFN-γ has been supported as being a key cytokine in providing a strong T helper 1 biased immune response, critical in the clearance of intra-cellular pathogens (Khor et al., 2007) and ECM has been referenced to as a classical IFN-γ mediated disease (Villegas-Mendez et al., 2012).

1.3.2 Toll like receptors

The response of type I IFN is first stimulated through the recognition of a pathogen by the germ-line encoded pattern recognition receptors (PRR), Toll like Receptors (TLR). PRRs recognize on each pathogen, pathogen associated molecular patterns (PAMPs), which initiates the first line of immune defense by the innate immune response (Janeway, 1989).

TLR were the first type of PRR to be identified (Medzhitov et al., 1997) and so far ten TLRs have been indentified in humans (Takeda and Akira, 2004), and 12 identified in mice (Leulier and Lemaitre, 2008). TLRs are located either on the cell surface or intra-cellular vesicles (Kawai and Akira, 2011), with each TLR being

distinct in its recognition of PAMPs, for example TLR 4 recognizes Lipopolysaccharide (LPS); TLR 3, double-stranded RNA, and TLR 9 binds DNA motifs (Akira and Takeda, 2004; Kawai and Akira, 2011). Figure 1.3 illustrates the pathway of activation after a TLR has encountered a foreign microbe, such as a virus; bacteria or parasite. Upon recognition of their PAMPs each TLR recruits specific adaptor molecules that lead to an innate immune response, releasing inflammatory cytokines; chemokines and type I IFN. These responses further recruit macrophages; activate neutrophils and set in motion the stimulation of IFN genes (Dunne and O'Neill, 2003; Kawai and Akira, 2010), together leading to eradication of the invading pathogen.

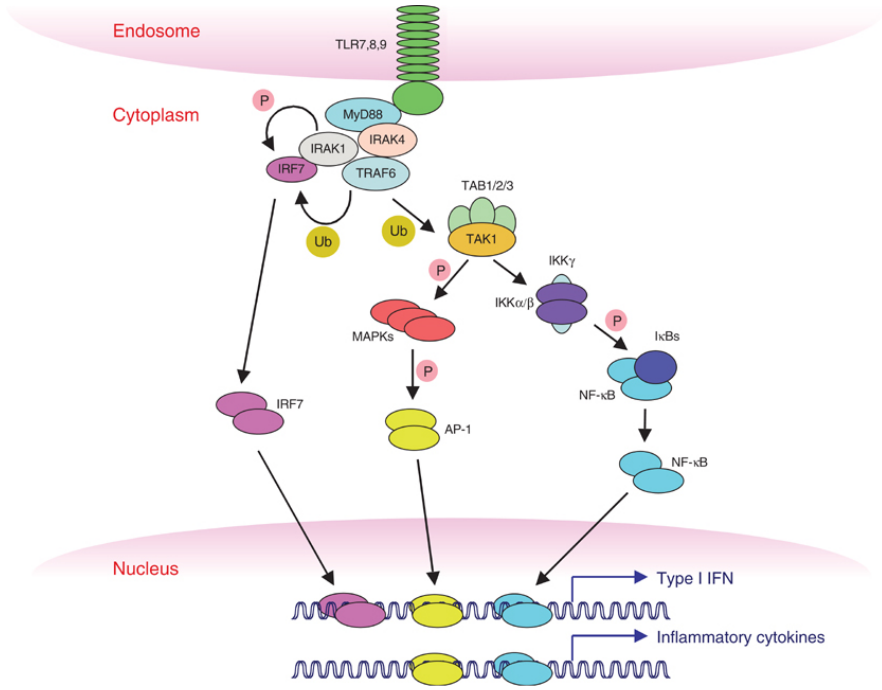


Figure 1.3 TLR9 pathway (Kawai and Akira, 2006). TLR7, 8, 9 are located in the endosome of specific cells. After ligand attachment to the TLRs, this signals the MyD88-dependent pathway. In plasmacytoid DCs, Interferon regulatory factor (IRF) 7 is constitutively expressed and forms a signaling complex with MyD88, Interleukin-1 receptor-associated kinase 1 (IRAK1) and IRAK4. In response to ligand stimulation, IRF7 is phosphorylated by IRAK1, dimerized and translocated to the nucleus. TNF-receptor associated factor (TRAF) 6-dependent ubiquitination is also needed for IRF7 activation, and together with NF-κB and Activator protein-1 (AP-1), this regulates expression of Type I IFN.

1.4 Type I IFN and IFNAR1

Analysis of Interferons has always been at the forefront of cytokine research (Taniguchi and Takaoka, 2002), with Interferons being first discovered by Alick Isaacs and Jean Lindemann in 1956, as cytokines released by virus infected cells (Isaacs and Lindenmann, 1957). Type I IFN was the first cytokine to be discovered, and was named so after its potential to “interfere” with viral effects (Isaacs and Lindenmann, 1987). Activation of type I IFN has been found to be anti-viral; anti-proliferative (Basler and Garcia-Sastre, 2002; Delhaye et al., 2006; Huys et al., 2009); arise anti-tumoral effects (Kawamoto et al., 2004), as well as govern overall immunomodulatory mechanisms as reviewed by (Gonzalez-Navajas et al., 2012; Tompkins, 1999).

All nucleated mammalian cell types have the ability to produce type I IFN in response to viral infections (Ito et al., 2006), however it is a particular subset of plasmocytoid dendritic cells (pDC), that have been found to produce the most type I IFN (Cella et al., 1999), and with which have the ability to produce up to a hundred to a thousand times more type I IFN following viral infection (Siegal et al., 1999).

There are 15 different subtypes of type I IFN, comprising 14 IFN-α subtypes, and one IFN-β subtype, both bind and act through the type I IFN receptor – IFNAR (Stockinger and Decker, 2008; Uze et al., 2007). The IFNAR is a heterodimeric receptor composed by two membrane glycoproteins, IFNAR1 (α) and IFNAR2 (β) (Takaoka et al., 2000; Wang and Fish, 2012). IFNAR1 exists as a single form, whilst IFNAR2 can exist as either a soluble (IFNAR2a), short (IFNAR2b), or long form (IFNAR2c) (Mogensen et al., 1999; Prejean and Colamonici, 2000). The *IFNAR1* and *IFNAR2* genes are located on chromosome 21q22.1, located in a cluster with genes that encode critical proteins for the immune response (Diop et al., 2006). Although the type I IFN receptors are expressed at low levels on the cell surface, from 10^2-10^3, they can efficiently transmit signals to the cell interior (Takaoka et al., 2000).

Figure 1.4 depicts the pathway leading to type I IFN stimulation. The pathway begins upon binding of either IFN α/B subunits to the IFNAR2 region of the heterodimer. However, IFNAR1 is considered the 'signaling' receptor, even though it does not bind directly to a IFN molecule, it is essential for the type I IFN signal transduction (Domanski and Colamonici, 1996). Each receptor subunit interacts specifically with a Janus activated kinase (JAK); IFNAR1 constitutively associates with tyrosine kinase 2 (TYK2), and IFNAR2 with JAK1 (Darnell, 1998; Ihle, 1995). The initiating step in the type I IFN pathway is the activation of these JAKS by auto-phosphorylation following ligand (IFNα/β) attachment and dimerization of the receptor (Platanias, 2005; Silvennoinen et al., 1993). Activation of the JAKs then leads to activation of the classic JAK- signal transducer and activator of transcription (STAT) pathway, including STAT1, 2, 3 and 5 (Darnell, 1998), which form either homo or hetero-dimers, such as STAT1-STAT3, that then

translocate to the cell nucleus where they bind to specific sites in the promoter region of the IFN stimulated genes (ISGs) (Darnell, 1997; Stark et al., 1998).

A unique element in the type I IFN pathway remains the formation of the complex ISG factor 3 (ISGF3), formed by STAT1, STAT2 and IFN regulatory factor (IRF) 9. This collective complex, ISGF3, binds to specific IFN-stimulated response elements (ISREs) in the promoter regions of the ISGs leading to the transcription of the type I IFN genes (Platanias, 2005). It is this original model of the JAK-STAT pathway discovered in the 1990s that remains principle in the type I IFN immune response. There are a number of varying pathways that can be stimulated alongside the JAK-STAT pathway, which could explain the pleiotrophic roles that Type I IFN plays in a host immune response (Platanias, 2005).

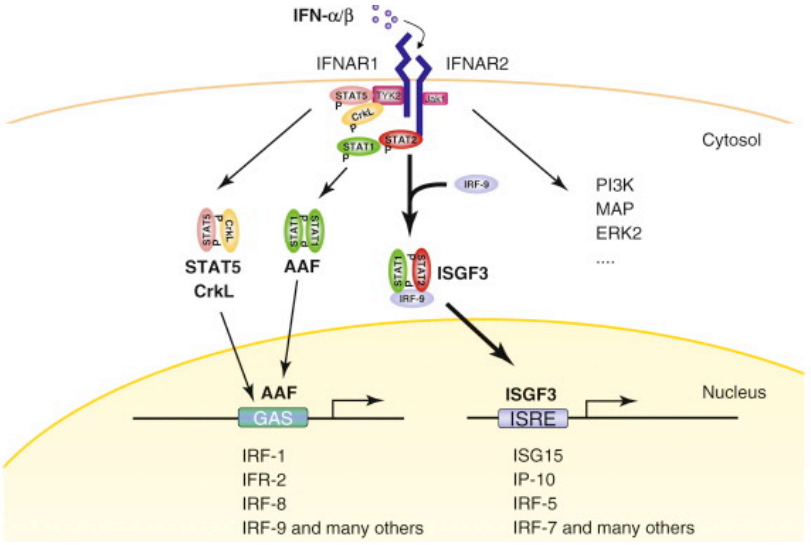


Figure 1.4. Interferon Type I pathway. (Prinz and Kalinke, 2010), All types of Type I IFN act through the single cell surface receptor composed of the α-chain IFNAR1 and β-chain IFNAR2. The two chains are linked to tyrosine kinase TyK2 and Jak1. Upon binding of either IFN-α/β, to the IFNAR1+2 chains, this induces the transcription factor, signal ransducers and activators of transcription (STAT) 2 and IFNAR2 binding. STAT1 is then recruited to STAT2 and upon tyrosine phosphorylation is released and associated with Interferon regulatory factor (IRF) 9. Together with p48, this forms a heterodimeric IFN stimulated gene factor (ISGF) 3 complex that translocates to the nucleus and binds to IFN-stimulated regulatory elements (ISRE). At the same time STAT1 homodimers (AAF) and other STAT complexes, STAT3, STAT5, STAT1/STAT3, STAT5/CrKL heterodimers also form. AAF and STAT5/CrKL translocate to the nucleus and bind to IFN-γ activated sequences (GAS). (Adapted and re-printed with permission).

Interferons are also found to be highly species specific (Erdmann et al., 2011), which potentially explains the vast array of responses induced by Interferons during immune responses in the body, and with currently over 300 ISGs identified (de Veer et al., 2001), this supports the monumental role type I IFNs play within the innate immune response.

Innate immune response to malaria infection is evidenced by the first TLR associated with malaria parasite recognition being TLR9 (Pichyangkul et al., 2004). It was discovered that extracts from schizont stage parasites during *P.falciparum* infection may contain the ligand detected by TLR9 which stimulates the innate immune response. Further, the recent work of (Sharma et al., 2011) has extensively explored the mechanism by which parasites are detected within the innate immune system. During the blood stage of infection, parasites digest haemoglobin, producing hemozoin, an inert crystal. It is this crystal hemozoin, that the authors report carries a *Plasmodium* DNA motif (identified as ATTTTTAC), that is the trigger of TLR9 and innate immune recognition. The ingestion of

the hemozoin into the endosomal compartment, then allows CpG motifs on the crystalline surface to be recognized. Further destabilization of the phagosome leads to the release of material into the cell cytosol, containing the AT-rich stem loop DNA motif, and triggering innate immune responses. The response through TLR9 signaling leads to up-regulation of IFN-I stimulatory genes, (Sharma et al., 2011), and supports the involvement of type I IFN in malaria innate immune responses.

Recent publications have also arisen studying the role of type I IFNs during CM. Strong evidence supports their multi-functional role in malaria due to the large increase of IFN-stimulated genes (ISGs) and essential gene components of the IFN signaling pathway, such as signal transducers and the IRF family, being up-regulated during *PbA* infection (Miu et al., 2008). Further, both type I IFN and TLR have been hypothesized as bridging the adaptive and innate immune response (Biron, 2001; Khor et al., 2007; Le Bon and Tough, 2002).

1.5 Adaptive immune response to malaria

1.5.1 T cells

The role of T cells has been paramount in CM pathogenesis research. It has been corroborated that $CD4^+$ T cells intervene during the induction stage of ECM, whilst $CD8^+$ T cells play their prominent role in the effector phase of the immune response in the brain tissue. This has been shown extensively in ECM research, begun by (Grau et al., 1986), who gave evidence of the necessity of T cells in mounting a CM response, and supported the early findings of the role of T cells in CM by (Finley et al., 1982).

T cell responses that help drive susceptibility versus resistance to parasite infection have been linked with $CD4^+$ T cell involvement (Mosmann and Coffman, 1989). However, early research findings contrast in their results, naming $CD4^+$ T cells responsible for ECM induction (Grau et al., 1986); opposed to solely $CD8^+$ T cells (Waki et al., 1992); opposed to both $CD4^+$ and $CD8^+$ T cells (Hermsen et al., 1997). Discrepancies they conclude, could be due to differing mouse strains or *P.berghei* strains of infection, however together these early studies clearly showcased the essential involvement of T cells within CM development.

In support, complete lymphocyte ablation in the mouse model of *Rag2*$^{-/-}$ (Nitcheu et al., 2003) and *Rag1*$^{-/-}$ (Amante et al., 2010) results in 100% protection from ECM development, as well as individual depletion of $CD4^+$ T cells, via MHC-II knockout (*A 0/0*) and $CD8^+$ T cells, via MHC-I knockout mice (*β2m 0/0*) also result in protection from ECM, (Yanez et al., 1996). However this suggests a need for deciphering between the exact role of T cells as opposed to the major histocompatibility complex (MHC) involvement in ECM induction.

Abundant literature is focused on the role of $CD8^+$ T cell in ECM, including, CD8 knockout mice; antibodies against $CD8^+$ T cells and depletion of $CD8^+$ T cells (Belnoue et al., 2002; Hermsen et al., 1997; Yanez et al., 1996). Together this work showed that the cytotoxic function of $CD8^+$ T cells (CTL) via perforin and Granzyme B (Haque et al., 2011b; Nitcheu et al., 2003), is instrumental to their pathogenic role as effector T cells in CM development. However these works have lacked in exploring the ultimate trigger for the cytotoxic role of the activated $CD8^+$ T cell in CM.

1.5.2 Effector $CD8^+$ T cells

Cytotoxic T cells (CTL) are a subset of $CD8^+$ T lymphocytes, whose role once activated, is to kill infected, damaged or dysfunctional cells, and are critical in maintaining proper functioning of the immune system (Jerome et al., 2003). A review by (Aidoo and Udhayakumar, 2000) presented how CTL T cells are seen as a potential target for drug vaccine development and suggest a natural CTL induction might be influenced by parasite variants, host genes and transmission patterns (Aidoo and Udhayakumar, 2000).

CTLs have a specialized mechanism to induce apoptosis of infected host cell, triggered by activation of the T cell receptor (TCR), upon recognition of a specific antigen (Ag), that is presented in the context of MHC class I, on the surface of a target cell (Jerome et al., 2003). Apoptosis is induced in this target cell, which requires direct effector-target cell contact (Trapani and Smyth, 2002) and induces release of Granzyme B (GrB) and Perforin into the target cell, triggering apoptosis via the caspase cascade (Smyth and Trapani, 1995). GrB is a serine protease that requires perforin to form channels for its entry into the target cell and released from the endosome into the target cell cytoplasm where it cleaves caspases, activating the caspase casade of apoptosis (Shi et al., 1997). GrB can induce rapid apoptosis within four hours in most cell types (Heusel et al., 1994), and has also been shown to work independent of the caspase cascade (Sarin et al., 1997).

GrB and perforin have been shown to be instrumental to ECM development (Haque et al., 2011b; Nitcheu et al., 2003), and in the context of malaria infection it appears both knockout mice are protected from ECM development, independent of the other molecule. Moreover GrB mRNA has also been shown to be

increased in response to murine malaria infections (Miu et al., 2008), most strongly during *PbA* infections.

A secondary apoptosis pathway is also used by CTL T cells in cell target directed killing, the Fas/FasL pathway (Van Parijs and Abbas, 1996), however mice deficient for Fas (*gld*$^{-/-}$), or FasL (*Lpr*$^{-/-}$), are still susceptible to ECM development (Potter et al., 2006), therefore concluding that apoptosis via cytoplasmic granules is the pathway used in malaria infections.

1.5.3 Chemokines

Chemokines have also gradually showed their vital roles in mediating both the type I IFN and CD8^{+} T cell roles within CM development. Both ECM resistant and susceptible mouse strains have shown up regulation of chemokine genes in response to *PbA* infection, in the brain; liver and spleen, representing the large part chemokines play in the innate immune response (Hanum et al., 2003).

Elegant work by (Campanella et al., 2008), showed the essential need of CXCR3 receptor expression for migration of CD8^{+} T cells to the brain, through the use of *CXCR3*$^{-/-}$ mice, and the ability to induce ECM by adoptive wildtype CD8^{+} T cell transfers to *CXCR3*$^{-/-}$ mice. In keeping with this response, depletion of natural killer (NK) cells from C57BL/6 mice, therefore down-regulation of CXCR3, led to protection from ECM also (Hansen et al., 2007). Furthermore down regulation of inflammatory response via neutralization of IP-10, the ligand of CXCR3, and *IP-10*$^{-/-}$ mice, protected development of ECM in *PbA* lethal infections (Nie et al., 2009). This led to increased retention of parasites within the spleen, controlling parasite burden throughout and decreased T cell

mediated trafficking to the brain, resulting in protection from ECM.

Evidence thereby classifies chemokines as essential supporters in the vital effects T cells incur within the brain tissue leading to development of ECM. However, it still remains to be seen whether susceptibility to CM is correlated with a particular pattern of cytokine production (de Kossodo and Grau, 1993).

In conclusion Figure 1.5 depicts the current understanding of the ECM mouse model and the key players mediating the development and pathogenesis of this disease state. This thesis aims now forth to discuss in detail the role of type I Interferon, mediated through the IFNAR1, during CM development.

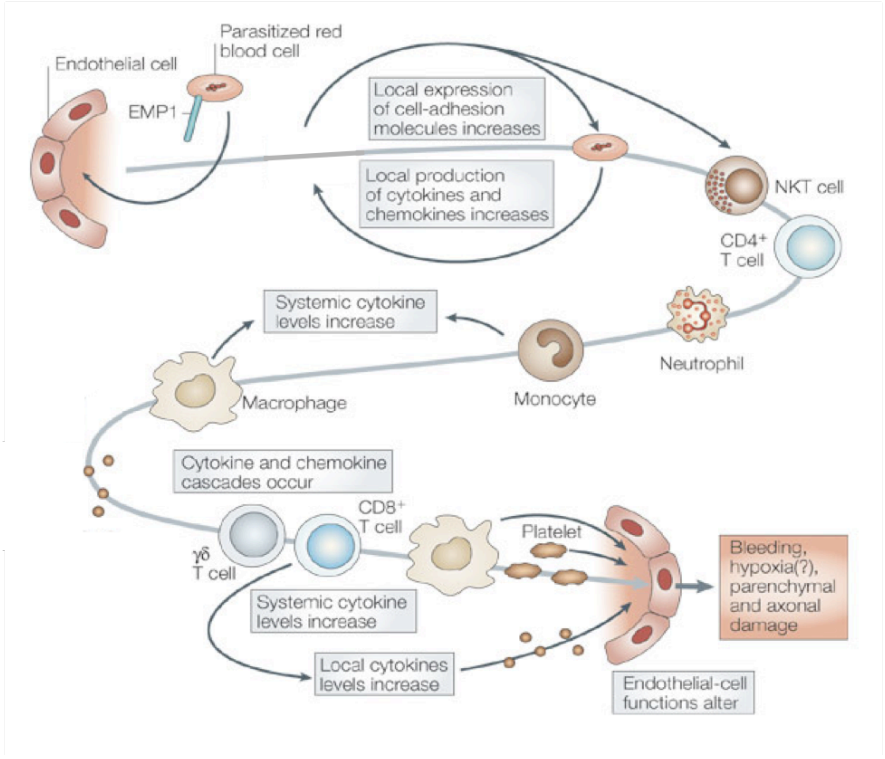


Figure 1.5 Experimental Cerebral malaria pathogenesis. (Schofield and Grau, 2005). pRBC adhere to receptors expressed by brain microvascular EC. When merozoites are released from pRBC ~4 hr later, parasite glycosylphosphatidylinositol (GPI), functions as a pattern-associated molecular pattern and toxin, stimulating the host inflammatory response. A local response is induced, involving activation of endothelium and production of cytokines and chemokines. Following the next 24hr this cycle is exacerbated. Natural killer T cells (NKT) cells, recruit $CD4^+$ T cells. Chemokines further recruit monocyte and activate neutrophils. Monocytes develop into macrophages, enhanced by IFN-γ, releasing further chemokines, amplifying this cycle. Platelets can be further arrested in microvessels, along with macrophages leading to hightened pro-inflammatory effects. Finally damage to endothelium is caused, leading to possible perivascular haemorrhage, axonal injury and alterations of the brain parenchyma. (Adapted and re-printed with authors permission).

1.6 Aims of this thesis

Cerebral Malaria is the result of an overwhelming immune response to infection by *Plasmodium falciparum* parasite. Current research is trying to understand the exact mechanisms that lead to CM development, where there might be potential aims for therapeutic intervention.

The clinical diagnosis of CM is difficult in both the human and mouse models (de Souza et al., 2010), thus making it particularly hard to target onset of CM disease. Research from human cases in endemic areas of malaria infection provides clues as to how host immunity is built and how the immune system fights against infection from the *Plasmodium* parasite. Type I interferon has been shown to govern the innate immune response (Delhaye et al., 2006; Gonzalez-Navajas et al., 2012), and evidence is mounting for its role as a potential candidate for CM prevention strategies (Aucan et al., 2003; Driss et al., 2011).

In the case of ECM development, $CD8^+$ T cells are instrumental in this fight (Belnoue et al., 2008; Hermsen et al., 1997), yet their intrinsic triggering mechanism to unleash their pathological role still remains unsolved.

The overall goal of this thesis work was to evaluate the resistance of *Ifnar1*$^{-/-}$ mice to ECM induction, and uncover the mechanism by which IFNAR1 is important during a malaria infection. We aimed specifically to assess whether a particular immune cell type governs the type I IFN response during ECM development, and successfully showed this through the design of a novel cell transfer protocol. We aimed further to characterize the activation status and activity of immune cells that abrogated the $CD8^+$ T cell profile, and to understand the involvement of type I IFN upon CD8+ T cell pathogenic function, during *PbA* infection.

1.7 References

Abbott, N.J. (2005). Dynamics of CNS barriers: evolution, differentiation, and modulation. Cellular and molecular neurobiology *25*, 5-23.

Abbott, N.J., Ronnback, L., and Hansson, E. (2006). Astrocyte-endothelial interactions at the blood-brain barrier. Nature reviews Neuroscience *7*, 41-53.

Ahmed, S., Adil, F., Shahzad, T., and Yahiya, Y. (2011). Severe malaria in children: factors predictive of outcome and response to Quinine. JPMA The Journal of the Pakistan Medical Association *61*, 54-58.

Aidoo, M., and Udhayakumar, V. (2000). Field studies of cytotoxic T lymphocytes in malaria infections: implications for malaria vaccine development. Parasitol Today *16*, 50-56.

Akira, S., and Takeda, K. (2004). Toll-like receptor signalling. Nature reviews Immunology *4*, 499-511.

Amani, V., Vigario, A.M., Belnoue, E., Marussig, M., Fonseca, L., Mazier, D., and Renia, L. (2000). Involvement of IFN-gamma receptor-medicated signaling in pathology and anti-malarial immunity induced by Plasmodium berghei infection. European journal of immunology *30*, 1646-1655.

Amante, F.H., Haque, A., Stanley, A.C., Rivera Fde, L., Randall, L.M., Wilson, Y.A., Yeo, G., Pieper, C., Crabb, B.S., de Koning-Ward, T.F.*, et al.* (2010). Immune-mediated mechanisms of parasite tissue sequestration during experimental cerebral malaria. J Immunol *185*, 3632-3642.

Armah, H.B., Wilson, N.O., Sarfo, B.Y., Powell, M.D., Bond, V.C., Anderson, W., Adjei, A.A., Gyasi, R.K., Tettey, Y., Wiredu, E.K.*, et al.* (2007). Cerebrospinal fluid and serum biomarkers of cerebral malaria mortality in Ghanaian children. Malaria journal *6*, 147.

Armulik, A., Abramsson, A., and Betsholtz, C. (2005). Endothelial/pericyte interactions. Circulation research *97*, 512-523.

Armulik, A., Genove, G., Mae, M., Nisancioglu, M.H., Wallgard, E., Niaudet, C., He, L., Norlin, J., Lindblom, P., Strittmatter, K.*, et al.* (2010). Pericytes regulate the blood-brain barrier. Nature *468*, 557-561.

Aucan, C., Walley, A.J., Hennig, B.J., Fitness, J., Frodsham, A., Zhang, L., Kwiatkowski, D., and Hill, A.V. (2003). Interferon-alpha receptor-1 (IFNAR1) variants are associated with protection against cerebral malaria in the Gambia. Genes and immunity *4*, 275-282.
Autino, B., Corbett, Y., Castelli, F., and Taramelli, D. (2012). Pathogenesis of malaria in tissues and blood. Mediterranean journal of hematology and infectious diseases *4*, e2012061.
Basler, C.F., and Garcia-Sastre, A. (2002). Viruses and the type I interferon antiviral system: induction and evasion. International reviews of immunology *21*, 305-337.
Belnoue, E., Kayibanda, M., Vigario, A.M., Deschemin, J.C., van Rooijen, N., Viguier, M., Snounou, G., and Renia, L. (2002). On the pathogenic role of brain-sequestered alphabeta CD8+ T cells in experimental cerebral malaria. J Immunol *169*, 6369-6375.
Belnoue, E., Potter, S.M., Rosa, D.S., Mauduit, M., Gruner, A.C., Kayibanda, M., Mitchell, A.J., Hunt, N.H., and Renia, L. (2008). Control of pathogenic CD8+ T cell migration to the brain by IFN-gamma during experimental cerebral malaria. Parasite immunology *30*, 544-553.
Berendt, A.R., Tumer, G.D., and Newbold, C.I. (1994). Cerebral malaria: the sequestration hypothesis. Parasitol Today *10*, 412-414.
Biron, C.A. (2001). Interferons alpha and beta as immune regulators--a new look. Immunity *14*, 661-664.
Bordon, Y. (2013). Cell migration: Pericytes--route planners. Nature reviews Immunology *13*, 5.
Breman, J.G., Alilio, M.S., and Mills, A. (2004). Conquering the intolerable burden of malaria: what's new, what's needed: a summary. The American journal of tropical medicine and hygiene *71*, 1-15.
Brewster, D.R., Kwiatkowski, D., and White, N.J. (1990). Neurological sequelae of cerebral malaria in children. Lancet *336*, 1039-1043.
Buffet, P.A., Safeukui, I., Milon, G., Mercereau-Puijalon, O., and David, P.H. (2009). Retention of erythrocytes in the spleen: a double-edged process in human malaria. Current opinion in hematology *16*, 157-164.
Cabrales, P., Zanini, G.M., Meays, D., Frangos, J.A., and Carvalho, L.J. (2010). Murine cerebral malaria is associated with a

vasospasm-like microcirculatory dysfunction, and survival upon rescue treatment is markedly increased by nimodipine. The American journal of pathology *176*, 1306-1315.
Campanella, G.S., Tager, A.M., El Khoury, J.K., Thomas, S.Y., Abrazinski, T.A., Manice, L.A., Colvin, R.A., and Luster, A.D. (2008). Chemokine receptor CXCR3 and its ligands CXCL9 and CXCL10 are required for the development of murine cerebral malaria. Proceedings of the National Academy of Sciences of the United States of America *105*, 4814-4819.
Carroll, R.W., Wainwright, M.S., Kim, K.Y., Kidambi, T., Gomez, N.D., Taylor, T., and Haldar, K. (2010). A rapid murine coma and behavior scale for quantitative assessment of murine cerebral malaria. PloS one *5*.
Carvalho, L.J. (2010). Murine cerebral malaria: how far from human cerebral malaria? Trends in parasitology *26*, 271-272.
Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A., and Colonna, M. (1999). Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nature medicine *5*, 919-923.
Clark, I.A., MacMicking, J.D., Gray, K.M., Rockett, K.A., and Cowden, W.B. (1992). Malaria mimicry with tumor necrosis factor. Contrasts between species of murine malaria and Plasmodium falciparum. The American journal of pathology *140*, 325-336.
Clark, I.A., and Rockett, K.A. (1994). The cytokine theory of human cerebral malaria. Parasitol Today *10*, 410-412.
Craig, A., Fernandez-Reyes, D., Mesri, M., McDowall, A., Altieri, D.C., Hogg, N., and Newbold, C. (2000). A functional analysis of a natural variant of intercellular adhesion molecule-1 (ICAM-1Kilifi). Human molecular genetics *9*, 525-530.
Craig, A.G., Grau, G.E., Janse, C., Kazura, J.W., Milner, D., Barnwell, J.W., Turner, G., and Langhorne, J. (2012). The role of animal models for research on severe malaria. PLoS pathogens *8*, e1002401.
Darnell, J.E., Jr. (1997). STATs and gene regulation. Science *277*, 1630-1635.
Darnell, J.E., Jr. (1998). Studies of IFN-induced transcriptional activation uncover the Jak-Stat pathway. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research *18*, 549-554.

de Kossodo, S., and Grau, G.E. (1993). Profiles of cytokine production in relation with susceptibility to cerebral malaria. J Immunol *151*, 4811-4820.
de Souza, J.B., Hafalla, J.C., Riley, E.M., and Couper, K.N. (2010). Cerebral malaria: why experimental murine models are required to understand the pathogenesis of disease. Parasitology *137*, 755-772.
de Veer, M.J., Holko, M., Frevel, M., Walker, E., Der, S., Paranjape, J.M., Silverman, R.H., and Williams, B.R. (2001). Functional classification of interferon-stimulated genes identified using microarrays. Journal of leukocyte biology *69*, 912-920.
Delhaye, S., Paul, S., Blakqori, G., Minet, M., Weber, F., Staeheli, P., and Michiels, T. (2006). Neurons produce type I interferon during viral encephalitis. Proceedings of the National Academy of Sciences of the United States of America *103*, 7835-7840.
Diop, G., Hirtzig, T., Do, H., Coulonges, C., Vasilescu, A., Labib, T., Spadoni, J.L., Therwath, A., Lathrop, M., Matsuda, F.*, et al.* (2006). Exhaustive genotyping of the interferon alpha receptor 1 (IFNAR1) gene and association of an IFNAR1 protein variant with AIDS progression or susceptibility to HIV-1 infection in a French AIDS cohort. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie *60*, 569-577.
Domanski, P., and Colamonici, O.R. (1996). The type-I interferon receptor. The long and short of it. Cytokine & growth factor reviews *7*, 143-151.
Dondorp, A., Nosten, F., Stepniewska, K., Day, N., and White, N. (2005). Artesunate versus quinine for treatment of severe falciparum malaria: a randomised trial. Lancet *366*, 717-725.
Dondorp, A.M., Pongponratn, E., and White, N.J. (2004). Reduced microcirculatory flow in severe falciparum malaria: pathophysiology and electron-microscopic pathology. Acta tropica *89*, 309-317.
Dorovini-Zis, K., Schmidt, K., Huynh, H., Fu, W., Whitten, R.O., Milner, D., Kamiza, S., Molyneux, M., and Taylor, T.E. (2011). The neuropathology of fatal cerebral malaria in malawian children. The American journal of pathology *178*, 2146-2158.
Driss, A., Hibbert, J.M., Wilson, N.O., Iqbal, S.A., Adamkiewicz, T.V., and Stiles, J.K. (2011). Genetic polymorphisms linked to susceptibility to malaria. Malaria journal *10*, 271.

Dunne, A., and O'Neill, L.A. (2003). The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. Science's STKE : signal transduction knowledge environment *2003*, re3.
Erdmann, J., Vitale, G., van Koetsveld, P.M., Croze, E., Sprij-Mooij, D.M., Hofland, L.J., and van Eijck, C.H. (2011). Effects of interferons alpha/beta on the proliferation of human micro- and macrovascular endothelial cells. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research *31*, 451-458.
Favre, N., Da Laperousaz, C., Ryffel, B., Weiss, N.A., Imhof, B.A., Rudin, W., Lucas, R., and Piguet, P.F. (1999). Role of ICAM-1 (CD54) in the development of murine cerebral malaria. Microbes and infection / Institut Pasteur *1*, 961-968.
Ferreira, A., Marguti, I., Bechmann, I., Jeney, V., Chora, A., Palha, N.R., Rebelo, S., Henri, A., Beuzard, Y., and Soares, M.P. (2011). Sickle hemoglobin confers tolerance to Plasmodium infection. Cell *145*, 398-409.
Finley, R.W., Mackey, L.J., and Lambert, P.H. (1982). Virulent P. berghei malaria: prolonged survival and decreased cerebral pathology in cell-dependent nude mice. J Immunol *129*, 2213-2218.
Francis, K., van Beek, J., Canova, C., Neal, J.W., and Gasque, P. (2003). Innate immunity and brain inflammation: the key role of complement. Expert reviews in molecular medicine *5*, 1-19.
Gonzalez-Navajas, J.M., Lee, J., David, M., and Raz, E. (2012). Immunomodulatory functions of type I interferons. Nature reviews Immunology *12*, 125-135.
Grau, G.E., Piguet, P.F., Engers, H.D., Louis, J.A., Vassalli, P., and Lambert, P.H. (1986). L3T4+ T lymphocytes play a major role in the pathogenesis of murine cerebral malaria. J Immunol *137*, 2348-2354.
Haldar, K., and Mohandas, N. (2009). Malaria, erythrocytic infection, and anemia. Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program, 87-93.
Haldar, K., Murphy, S.C., Milner, D.A., and Taylor, T.E. (2007). Malaria: mechanisms of erythrocytic infection and pathological correlates of severe disease. Annual review of pathology *2*, 217-249.

Hansen, D.S., Bernard, N.J., Nie, C.Q., and Schofield, L. (2007). NK cells stimulate recruitment of CXCR3+ T cells to the brain during Plasmodium berghei-mediated cerebral malaria. J Immunol *178*, 5779-5788.
Hanum, P.S., Hayano, M., and Kojima, S. (2003). Cytokine and chemokine responses in a cerebral malaria-susceptible or -resistant strain of mice to Plasmodium berghei ANKA infection: early chemokine expression in the brain. International immunology *15*, 633-640.
Haque, A., Best, S.E., Ammerdorffer, A., Desbarrieres, L., de Oca, M.M., Amante, F.H., de Labastida Rivera, F., Hertzog, P., Boyle, G.M., Hill, G.R.*, et al.* (2011a). Type I interferons suppress CD4(+) T-cell-dependent parasite control during blood-stage Plasmodium infection. European journal of immunology *41*, 2688-2698.
Haque, A., Best, S.E., Unosson, K., Amante, F.H., de Labastida, F., Anstey, N.M., Karupiah, G., Smyth, M.J., Heath, W.R., and Engwerda, C.R. (2011b). Granzyme B expression by CD8+ T cells is required for the development of experimental cerebral malaria. J Immunol *186*, 6148-6156.
Hermsen, C., van de Wiel, T., Mommers, E., Sauerwein, R., and Eling, W. (1997). Depletion of CD4+ or CD8+ T-cells prevents Plasmodium berghei induced cerebral malaria in end-stage disease. Parasitology *114 (Pt 1)*, 7-12.
Heusel, J.W., Wesselschmidt, R.L., Shresta, S., Russell, J.H., and Ley, T.J. (1994). Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell *76*, 977-987.
Hunt, N.H., Golenser, J., Chan-Ling, T., Parekh, S., Rae, C., Potter, S., Medana, I.M., Miu, J., and Ball, H.J. (2006). Immunopathogenesis of cerebral malaria. International journal for parasitology *36*, 569-582.
Hunt, N.H., Grau, G.E., Engwerda, C., Barnum, S.R., van der Heyde, H., Hansen, D.S., Schofield, L., and Golenser, J. (2010). Murine cerebral malaria: the whole story. Trends in parasitology *26*, 272-274.
Huys, L., Van Hauwermeiren, F., Dejager, L., Dejonckheere, E., Lienenklaus, S., Weiss, S., Leclercq, G., and Libert, C. (2009). Type I interferon drives tumor necrosis factor-induced lethal shock. The Journal of experimental medicine *206*, 1873-1882.

Idaghdour, Y., Quinlan, J., Goulet, J.P., Berghout, J., Gbeha, E., Bruat, V., de Malliard, T., Grenier, J.C., Gomez, S., Gros, P.*, et al.* (2012). Feature Article: Evidence for additive and interaction effects of host genotype and infection in malaria. Proceedings of the National Academy of Sciences of the United States of America *109*, 16786-16793.
Idro, R., Jenkins, N.E., and Newton, C.R. (2005). Pathogenesis, clinical features, and neurological outcome of cerebral malaria. Lancet neurology *4*, 827-840.
Idro, R., Marsh, K., John, C.C., and Newton, C.R. (2010). Cerebral malaria: mechanisms of brain injury and strategies for improved neurocognitive outcome. Pediatric research *68*, 267-274.
Ihle, J.N. (1995). The Janus protein tyrosine kinase family and its role in cytokine signaling. Advances in immunology *60*, 1-35.
Isaacs, A., and Lindenmann, J. (1957). Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci *147*, 258-267.
Isaacs, A., and Lindenmann, J. (1987). Virus interference. I. The interferon. By A. Isaacs and J. Lindenmann, 1957. Journal of interferon research *7*, 429-438.
Ito, T., Kanzler, H., Duramad, O., Cao, W., and Liu, Y.J. (2006). Specialization, kinetics, and repertoire of type 1 interferon responses by human plasmacytoid predendritic cells. Blood *107*, 2423-2431.
Janeway, C.A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harbor symposia on quantitative biology *54 Pt 1*, 1-13.
Jerome, K.R., Sloan, D.D., and Aubert, M. (2003). Measurement of CTL-induced cytotoxicity: the caspase 3 assay. Apoptosis : an international journal on programmed cell death *8*, 563-571.
Kawai, T., and Akira, S. (2006). TLR signaling. Cell death and differentiation *13*, 816-825.
Kawai, T., and Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature immunology *11*, 373-384.
Kawai, T., and Akira, S. (2011). Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity *34*, 637-650.
Kawamoto, S., Oritani, K., Asakura, E., Ishikawa, J., Koyama, M., Miyano, K., Iwamoto, M., Yasuda, S., Nakakubo, H., Hirayama, F.*, et al.* (2004). A new interferon, limitin, displays equivalent

immunomodulatory and antitumor activities without myelosuppressive properties as compared with interferon-alpha. Experimental hematology *32*, 797-805.
Khor, C.C., Vannberg, F.O., Chapman, S.J., Walley, A., Aucan, C., Loke, H., White, N.J., Peto, T., Khor, L.K., Kwiatkowski, D.*, et al.* (2007). Positive replication and linkage disequilibrium mapping of the chromosome 21q22.1 malaria susceptibility locus. Genes and immunity *8*, 570-576.
Koch, O., Awomoyi, A., Usen, S., Jallow, M., Richardson, A., Hull, J., Pinder, M., Newport, M., and Kwiatkowski, D. (2002). IFNGR1 gene promoter polymorphisms and susceptibility to cerebral malaria. The Journal of infectious diseases *185*, 1684-1687.
Lackner, P., Beer, R., Heussler, V., Goebel, G., Rudzki, D., Helbok, R., Tannich, E., and Schmutzhard, E. (2006). Behavioural and histopathological alterations in mice with cerebral malaria. Neuropathology and applied neurobiology *32*, 177-188.
Le Bon, A., and Tough, D.F. (2002). Links between innate and adaptive immunity via type I interferon. Current opinion in immunology *14*, 432-436.
Leulier, F., and Lemaitre, B. (2008). Toll-like receptors--taking an evolutionary approach. Nature reviews Genetics *9*, 165-178.
Liehl, P., and Mota, M.M. (2012). Innate recognition of malarial parasites by mammalian hosts. International journal for parasitology *42*, 557-566.
Linares, M., Marin-Garcia, P., Perez-Benavente, S., Sanchez-Nogueiro, J., Puyet, A., Bautista, J.M., and Diez, A. (2012). Brain-derived neurotrophic factor and the course of experimental cerebral malaria. Brain research.
Mackinnon, M.J., Mwangi, T.W., Snow, R.W., Marsh, K., and Williams, T.N. (2005). Heritability of malaria in Africa. PLoS medicine *2*, e340.
Manjurano, A., Clark, T.G., Nadjm, B., Mtove, G., Wangai, H., Sepulveda, N., Campino, S.G., Maxwell, C., Olomi, R., Rockett, K.R.*, et al.* (2012). Candidate human genetic polymorphisms and severe malaria in a tanzanian population. PloS one *7*, e47463.
McGuire, W., Hill, A.V., Allsopp, C.E., Greenwood, B.M., and Kwiatkowski, D. (1994). Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. Nature *371*, 508-510.

McGuire, W., Knight, J.C., Hill, A.V., Allsopp, C.E., Greenwood, B.M., and Kwiatkowski, D. (1999). Severe malarial anemia and cerebral malaria are associated with different tumor necrosis factor promoter alleles. The Journal of infectious diseases *179*, 287-290.
McQuillan, J.A., Mitchell, A.J., Ho, Y.F., Combes, V., Ball, H.J., Golenser, J., Grau, G.E., and Hunt, N.H. (2011). Coincident parasite and CD8 T cell sequestration is required for development of experimental cerebral malaria. International journal for parasitology *41*, 155-163.
Medana, I.M., Day, N.P., Hien, T.T., Mai, N.T., Bethell, D., Phu, N.H., Farrar, J., Esiri, M.M., White, N.J., and Turner, G.D. (2002). Axonal injury in cerebral malaria. The American journal of pathology *160*, 655-666.
Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A., Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature *388*, 394-397.
Michalakis, Y., and Renaud, F. (2009). Malaria: Evolution in vector control. Nature *462*, 298-300.
Miu, J., Hunt, N.H., and Ball, H.J. (2008). Predominance of interferon-related responses in the brain during murine malaria, as identified by microarray analysis. Infection and immunity *76*, 1812-1824.
Mogensen, K.E., Lewerenz, M., Reboul, J., Lutfalla, G., and Uze, G. (1999). The type I interferon receptor: structure, function, and evolution of a family business. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research *19*, 1069-1098.
Molyneux, M.E., Taylor, T.E., Wirima, J.J., and Borgstein, A. (1989). Clinical features and prognostic indicators in paediatric cerebral malaria: a study of 131 comatose Malawian children. The Quarterly journal of medicine *71*, 441-459.
Mosmann, T.R., and Coffman, R.L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual review of immunology *7*, 145-173.
Murphy, G.S., and Oldfield, E.C., 3rd (1996). Falciparum malaria. Infectious disease clinics of North America *10*, 747-775.
Murphy, S.C., and Breman, J.G. (2001). Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and

complications of pregnancy. The American journal of tropical medicine and hygiene *64*, 57-67.
Nacer, A., Movila, A., Baer, K., Mikolajczak, S.A., Kappe, S.H., and Frevert, U. (2012). Neuroimmunological blood brain barrier opening in experimental cerebral malaria. PLoS pathogens *8*, e1002982.
Newton, C.R., and Krishna, S. (1998). Severe falciparum malaria in children: current understanding of pathophysiology and supportive treatment. Pharmacology & therapeutics *79*, 1-53.
Nie, C.Q., Bernard, N.J., Norman, M.U., Amante, F.H., Lundie, R.J., Crabb, B.S., Heath, W.R., Engwerda, C.R., Hickey, M.J., Schofield, L.*, et al.* (2009). IP-10-mediated T cell homing promotes cerebral inflammation over splenic immunity to malaria infection. PLoS pathogens *5*, e1000369.
Nitcheu, J., Bonduelle, O., Combadiere, C., Tefit, M., Seilhean, D., Mazier, D., and Combadiere, B. (2003). Perforin-dependent brain-infiltrating cytotoxic CD8+ T lymphocytes mediate experimental cerebral malaria pathogenesis. J Immunol *170*, 2221-2228.
Ochiel, D.O., Awandare, G.A., Keller, C.C., Hittner, J.B., Kremsner, P.G., Weinberg, J.B., and Perkins, D.J. (2005). Differential regulation of beta-chemokines in children with Plasmodium falciparum malaria. Infection and immunity *73*, 4190-4197.
Owens, T., Bechmann, I., and Engelhardt, B. (2008). Perivascular spaces and the two steps to neuroinflammation. Journal of neuropathology and experimental neurology *67*, 1113-1121.
Pamplona, A., Ferreira, A., Balla, J., Jeney, V., Balla, G., Epiphanio, S., Chora, A., Rodrigues, C.D., Gregoire, I.P., Cunha-Rodrigues, M.*, et al.* (2007). Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. Nature medicine *13*, 703-710.
Parekh, S.B., Bubb, W.A., Hunt, N.H., and Rae, C. (2006). Brain metabolic markers reflect susceptibility status in cytokine gene knockout mice with murine cerebral malaria. International journal for parasitology *36*, 1409-1418.
Pichyangkul, S., Yongvanitchit, K., Kum-arb, U., Hemmi, H., Akira, S., Krieg, A.M., Heppner, D.G., Stewart, V.A., Hasegawa, H., Looareesuwan, S.*, et al.* (2004). Malaria blood stage parasites activate human plasmacytoid dendritic cells and murine

dendritic cells through a Toll-like receptor 9-dependent pathway. J Immunol *172*, 4926-4933.
Pino, P., Taoufiq, Z., Nitcheu, J., Vouldoukis, I., and Mazier, D. (2005). Blood-brain barrier breakdown during cerebral malaria: suicide or murder? Thrombosis and haemostasis *94*, 336-340.
Platanias, L.C. (2005). Mechanisms of type-I- and type-II-interferon-mediated signalling. Nature reviews Immunology *5*, 375-386.
Pongponratn, E., Turner, G.D., Day, N.P., Phu, N.H., Simpson, J.A., Stepniewska, K., Mai, N.T., Viriyavejakul, P., Looareesuwan, S., Hien, T.T.*, et al.* (2003). An ultrastructural study of the brain in fatal Plasmodium falciparum malaria. The American journal of tropical medicine and hygiene *69*, 345-359.
Potter, S.M., Chan-Ling, T., Rosinova, E., Ball, H.J., Mitchell, A.J., and Hunt, N.H. (2006). A role for Fas-Fas ligand interactions during the late-stage neuropathological processes of experimental cerebral malaria. Journal of neuroimmunology *173*, 96-107.
Prejean, C., and Colamonici, O.R. (2000). Role of the cytoplasmic domains of the type I interferon receptor subunits in signaling. Seminars in cancer biology *10*, 83-92.
Prinz, M., and Kalinke, U. (2010). New lessons about old molecules: how type I interferons shape Th1/Th17-mediated autoimmunity in the CNS. Trends in molecular medicine *16*, 379-386.
Prudencio, M., Rodriguez, A., and Mota, M.M. (2006). The silent path to thousands of merozoites: the Plasmodium liver stage. Nature reviews Microbiology *4*, 849-856.
Reis, P.A., Estato, V., da Silva, T.I., d'Avila, J.C., Siqueira, L.D., Assis, E.F., Bozza, P.T., Bozza, F.A., Tibirica, E.V., Zimmerman, G.A.*, et al.* (2012). Statins decrease neuroinflammation and prevent cognitive impairment after cerebral malaria. PLoS pathogens *8*, e1003099.
Renia, L., Gruner, A.C., and Snounou, G. (2010). Cerebral malaria: in praise of epistemes. Trends in parasitology *26*, 275-277.
Rest, J.R. (1982). Cerebral malaria in inbred mice. I. A new model and its pathology. Transactions of the Royal Society of Tropical Medicine and Hygiene *76*, 410-415.
Rihet, P., Traore, Y., Abel, L., Aucan, C., Traore-Leroux, T., and Fumoux, F. (1998). Malaria in humans: Plasmodium falciparum

blood infection levels are linked to chromosome 5q31-q33. American journal of human genetics *63*, 498-505.
Riley, E.M., Couper, K.N., Helmby, H., Hafalla, J.C., de Souza, J.B., Langhorne, J., Jarra, W.B., and Zavala, F. (2010). Neuropathogenesis of human and murine malaria. Trends in parasitology *26*, 277-278.
Rogers, D.C., Fisher, E.M., Brown, S.D., Peters, J., Hunter, A.J., and Martin, J.E. (1997). Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. Mammalian genome : official journal of the International Mammalian Genome Society *8*, 711-713.
Rosenthal, P.J. (2008). Artesunate for the treatment of severe falciparum malaria. The New England journal of medicine *358*, 1829-1836.
Sambo, M.R., Trovoada, M.J., Benchimol, C., Quinhentos, V., Goncalves, L., Velosa, R., Marques, M.I., Sepulveda, N., Clark, T.G., Mustafa, S.*, et al.* (2010). Transforming growth factor beta 2 and heme oxygenase 1 genes are risk factors for the cerebral malaria syndrome in Angolan children. PloS one *5*, e11141.
Sarin, A., Williams, M.S., Alexander-Miller, M.A., Berzofsky, J.A., Zacharchuk, C.M., and Henkart, P.A. (1997). Target cell lysis by CTL granule exocytosis is independent of ICE/Ced-3 family proteases. Immunity *6*, 209-215.
Sarkar, S., and Bhattacharya, P. (2008). Cerebral malaria caused by Plasmodium vivax in adult subjects. Indian journal of critical care medicine : peer-reviewed, official publication of Indian Society of Critical Care Medicine *12*, 204-205.
Schofield, L., and Grau, G.E. (2005). Immunological processes in malaria pathogenesis. Nature reviews Immunology *5*, 722-735.
Serghides, L., Kim, H., Lu, Z., Kain, D.C., Miller, C., Francis, R.C., Liles, W.C., Zapol, W.M., and Kain, K.C. (2011). Inhaled nitric oxide reduces endothelial activation and parasite accumulation in the brain, and enhances survival in experimental cerebral malaria. PloS one *6*, e27714.
Servonnet, A., Rapp, C., Delacour, H., Bigaillon, C., Pilo, J.E., and Merens, A. (2012). Plasmodium knowlesi: an emerging species in humans? Medecine et sante tropicales *22*, 417-421.
Sharma, S., DeOliveira, R.B., Kalantari, P., Parroche, P., Goutagny, N., Jiang, Z., Chan, J., Bartholomeu, D.C., Lauw, F., Hall, J.P.*, et al.* (2011). Innate immune recognition of an AT-rich stem-loop DNA

motif in the Plasmodium falciparum genome. Immunity *35*, 194-207.
Shi, L., Mai, S., Israels, S., Browne, K., Trapani, J.A., and Greenberg, A.H. (1997). Granzyme B (GraB) autonomously crosses the cell membrane and perforin initiates apoptosis and GraB nuclear localization. The Journal of experimental medicine *185*, 855-866.
Shikani, H.J., Freeman, B., Lisanti, M.P., Weiss, L.M., Tanowitz, H.B., and Desruisseaux, M.S. (2012). Cerebral Malaria: We Have Come a Long Way. The American journal of pathology.
Siegal, F.P., Kadowaki, N., Shodell, M., Fitzgerald-Bocarsly, P.A., Shah, K., Ho, S., Antonenko, S., and Liu, Y.J. (1999). The nature of the principal type 1 interferon-producing cells in human blood. Science *284*, 1835-1837.
Silvennoinen, O., Ihle, J.N., Schlessinger, J., and Levy, D.E. (1993). Interferon-induced nuclear signalling by Jak protein tyrosine kinases. Nature *366*, 583-585.
Sinnis, P., and Zavala, F. (2012). The skin: where malaria infection and the host immune response begin. Seminars in immunopathology *34*, 787-792.
Smyth, M.J., and Trapani, J.A. (1995). Granzymes: exogenous proteinases that induce target cell apoptosis. Immunology today *16*, 202-206.
Snow, R.W., Guerra, C.A., Noor, A.M., Myint, H.Y., and Hay, S.I. (2005). The global distribution of clinical episodes of Plasmodium falciparum malaria. Nature *434*, 214-217.
Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., and Schreiber, R.D. (1998). How cells respond to interferons. Annual review of biochemistry *67*, 227-264.
Stevenson, J., St Laurent, B., Lobo, N.F., Cooke, M.K., Kahindi, S.C., Oriango, R.M., Harbach, R.E., Cox, J., and Drakeley, C. (2012). Novel vectors of malaria parasites in the western highlands of Kenya. Emerging infectious diseases *18*, 1547-1549.
Stockinger, S., and Decker, T. (2008). Novel functions of type I interferons revealed by infection studies with Listeria monocytogenes. Immunobiology *213*, 889-897.
Sturm, A., Amino, R., van de Sand, C., Regen, T., Retzlaff, S., Rennenberg, A., Krueger, A., Pollok, J.M., Menard, R., and Heussler, V.T. (2006). Manipulation of host hepatocytes by the

malaria parasite for delivery into liver sinusoids. Science *313*, 1287-1290.
Takaoka, A., Mitani, Y., Suemori, H., Sato, M., Yokochi, T., Noguchi, S., Tanaka, N., and Taniguchi, T. (2000). Cross talk between interferon-gamma and -alpha/beta signaling components in caveolar membrane domains. Science *288*, 2357-2360.
Takeda, K., and Akira, S. (2004). TLR signaling pathways. Seminars in immunology *16*, 3-9.
Taniguchi, T., and Takaoka, A. (2002). The interferon-alpha/beta system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. Current opinion in immunology *14*, 111-116.
Taylor, T.E., Fu, W.J., Carr, R.A., Whitten, R.O., Mueller, J.S., Fosiko, N.G., Lewallen, S., Liomba, N.G., and Molyneux, M.E. (2004). Differentiating the pathologies of cerebral malaria by postmortem parasite counts. Nature medicine *10*, 143-145.
Teasdale, G., and Jennett, B. (1974). Assessment of coma and impaired consciousness. A practical scale. Lancet *2*, 81-84.
Tompkins, W.A. (1999). Immunomodulation and therapeutic effects of the oral use of interferon-alpha: mechanism of action. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research *19*, 817-828.
Toro, G., and Roman, G. (1978). Cerebral malaria. A disseminated vasculomyelinopathy. Archives of neurology *35*, 271-275.
Trampuz, A., Jereb, M., Muzlovic, I., and Prabhu, R.M. (2003). Clinical review: Severe malaria. Crit Care *7*, 315-323.
Trapani, J.A., and Smyth, M.J. (2002). Functional significance of the perforin/granzyme cell death pathway. Nature reviews Immunology *2*, 735-747.
Urban, B.C., Ferguson, D.J., Pain, A., Willcox, N., Plebanski, M., Austyn, J.M., and Roberts, D.J. (1999). Plasmodium falciparum-infected erythrocytes modulate the maturation of dendritic cells. Nature *400*, 73-77.
Uze, G., Schreiber, G., Piehler, J., and Pellegrini, S. (2007). The receptor of the type I interferon family. Current topics in microbiology and immunology *316*, 71-95.

van Agtmael, M.A., Eggelte, T.A., and van Boxtel, C.J. (1999). Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication. Trends in pharmacological sciences *20*, 199-205.
Van Parijs, L., and Abbas, A.K. (1996). Role of Fas-mediated cell death in the regulation of immune responses. Current opinion in immunology *8*, 355-361.
Villegas-Mendez, A., Greig, R., Shaw, T.N., de Souza, J.B., Gwyer Findlay, E., Stumhofer, J.S., Hafalla, J.C., Blount, D.G., Hunter, C.A., Riley, E.M.*, et al.* (2012). IFN-gamma-producing CD4+ T cells promote experimental cerebral malaria by modulating CD8+ T cell accumulation within the brain. J Immunol *189*, 968-979.
Waki, S., Uehara, S., Kanbe, K., Ono, K., Suzuki, M., and Nariuchi, H. (1992). The role of T cells in pathogenesis and protective immunity to murine malaria. Immunology *75*, 646-651.
Wang, B.X., and Fish, E.N. (2012). The yin and yang of viruses and interferons. Trends in immunology *33*, 190-197.
White, N.J., Turner, G.D., Medana, I.M., Dondorp, A.M., and Day, N.P. (2010). The murine cerebral malaria phenomenon. Trends in parasitology *26*, 11-15.
Wilson, N.O., Huang, M.B., Anderson, W., Bond, V., Powell, M., Thompson, W.E., Armah, H.B., Adjei, A.A., Gyasi, R., Tettey, Y.*, et al.* (2008). Soluble factors from Plasmodium falciparum-infected erythrocytes induce apoptosis in human brain vascular endothelial and neuroglia cells. Molecular and biochemical parasitology *162*, 172-176.
Yanez, D.M., Manning, D.D., Cooley, A.J., Weidanz, W.P., and van der Heyde, H.C. (1996). Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. J Immunol *157*, 1620-1624.
Zhu, X., Pan, Y., Li, Y., Jiang, Y., Shang, H., Gowda, D.C., Cui, L., and Cao, Y. (2012). Targeting Toll-like receptors by chloroquine protects mice from experimental cerebral malaria. International immunopharmacology *13*, 392-397.

Chapter 2

Development of Pre-sensitization, Cell transfer Protocol

2.1 Author contribution

The protocol design and transfer experiments were conceived by myself and my supervisor Carlos Penha-Gonçalves. All experiments were carried out by me.

This pre-sensitization protocol has been accepted for publication in the Methods in Malaria Research Handbook: (http://www.mr4.org/Publications/MethodsinMalariaResearch.aspx) and will be published in the up and coming edition, (Appendix One).

2.2 Summary

Response to induction of Experimental Cerebral Malaria (ECM) in mouse strains can be classified as susceptible *vs* resistant. Current techniques exist for assessing mortality occurring by ECM, such as symptom onset, breakage of the blood brain barrier and presence of sequestered parasite in the brain tissue. However there is no reliable method to assess the role of individual cell types in the immunological responses underlying CM development.

This chapter describes the development of a novel pre-sensitization protocol we devised as a method to test potential induction and protection from ECM via select cell transfer techniques in ECM resistant mouse strains. We used this protocol to show that presence of IFNAR1 in $CD8^+$ cells is sufficient to restore ECM susceptibility in previously resistant *Ifnar1*$^{-/-}$ mice. Our design of an irradiation step within this protocol further uncovered the necessity of priming spleen $CD8^+$ cells for ECM induction. We suggest that this protocol be used for future experiments in denoting key cellular players of ECM development.

2.3 Introduction

The experimental field of CM has no consistent method for assessing individual cell type ability at induction of Experimental cerebral malaria (ECM) in mice models. Much work has been published for individual knockout mouse strains reporting that loss of activity such as *CD8*$^{-/-}$, CD4$^{-/-}$ (Claser et al., 2011) and or increased activity of specific cells such as dendritic cells (DC) (Tamura et al., 2011) is linked to ECM susceptibility or resistance. Individual focus on cellular expression of the chemokine receptor CXCR3; pro-inflammatory molecule TNF-α (Morrell et al., 2011); loss of function of GrB (Haque et al., 2011); as well as HO-1 protective capacities (Pamplona et al., 2007), have also led to ECM resistance. Therefore a specific technique for determining cellular involvement in pathogenesis is greatly needed to help test and define the key players in ECM development and prevention.

Work by (Nitcheu et al., 2003) has shown that ECM was induced in ECM resistant *Rag2*$^{-/-}$ mice through a transfer technique using total splenocytes from *PbA* infected C57BL/6 mice. However, in our hands we were unable to replicate these procedures without co-transfer of *PbA* infection to recipient mice. We developed therefore a novel cell transfer technique that could show how a particular cell type presence could lead to susceptibility *vs* resistance in select strains of mice.

We studied total spleen and purified CD8^{+} cell transfers between C57BL/6 (ECM susceptible) and *Ifnar1*$^{-/-}$, *Rag2*$^{-/-}$ and *CD8*$^{-/-}$ (ECM resistant) mouse strains. Through development of this transfer method we uncovered the necessity of a priming response, brought about via irradiating infected red blood cells (iRBC) taken from the spleen of donor mouse. This irradiation step proved

essential for inducing ECM in a previously resistant *Ifnar1*$^{-/-}$ mouse model.

We further established that exposing cell donor mice to infection before transfer of cells to recipient mice was instrumental for ECM induction, but established this protocol as a pre-sensitization not an immunization protocol. Although we could not produce sufficient ECM induction in *Rag2*$^{-/-}$ or *CD8*$^{-/-}$ mice, we show here preliminary results that our technique warrants further study in these mouse strains.

Together this work has uncovered the importance of primed $CD8^+$ cells in ECM development. Through this discovery we have established a new tool for ECM research which we believe will be essential for future ECM studies in the mouse model.

2.4 Materials and Methods

2.4.1 Animals

The protocol can use different combinations of males, 8-12 weeks old as cell donors and recipients. We performed criss-crossed transfer experiments using C57BL/6 mice or knockout mice backcrossed into the C57BL/6 background. iRBC donors were H2-compatible with the recipients. All procedures were in accordance with national regulations on animal experimentation and welfare as approved by the Instituto Gulbenkian de Ciência Ethics committee and the animal welfare national authority.

2.4.2 Parasite, infection and ECM disease assessment

All infections used 1×10^6 *P.berghei ANKA*-GFP parasitized iRBC (Franke-Fayard et al., 2004) given via intra-peritoneal (i.p)

injection. Frozen iRBC stocks were expanded in C57BL/6 mice prior to infection. Parasitemia was determined as the % of GFP^+ red blood cells, using flow cytometry analysis (FacScan Cell Analyser, Becton Dickinson, New Jersey) and non-infected RBCs as negative control. ECM development was monitored from day 5 post-infection (PI) as including; head deviations, paralysis, ataxia and convulsions (Pamplona et al., 2007). ECM susceptible mice developed symptoms at days 6-7 post-infection and died within 4-5 hours. Mice resistant to ECM died around days 22-25 from anemia and hyperparasitemia without displaying neurological symptoms.

2.4.3 Pre-Sensitization protocol

(Please see Figure 2.4 also as guidance).

For infected blood donor mice; blood was collected by mandibular vein puncture into an eppendorf containing 10 μl of Heparin (Heparina Leo, Leo Pharmaceutical products) from infected mouse at day 6 post-infection, upon display of ECM symptoms and when parasitemia was around 30%. Parasitemia was measured to calculate injection of 4×10^6 iRBC into cell donor mice. Collected blood was then placed on ice in a 50 ml falcon tube and irradiated at 20 k Rad (GAMMACELL 2000, MØLSGAARD Medical Denmark). Irradiated iRBC were then re-suspended in PBS and injected i.p into cell donor mice. At day 6 post irradiated-iRBC injection, cell donor mice were sacrificed and purified spleen cell populations were injected into recipient mice; recipient mice were left either non-infected (NI) or infected 1×10^6 *PbA* i.p one hour after transfer. Parasitemia and ECM development were monitored from day 5 post-infection. For control non-infected blood donor mice; mice were bled and an equivalent volume of non-infected

RBC were irradiated and diluted in PBS in the same manner as above.

2.4.4 Spleen cell preparations

Single cell suspensions were prepared from spleens of cell donor mice. For total spleen cell transfers; cells were washed 2 × in PBS, counted in a hemocytometer, re-suspended in PBS and injected either 3 or 5 ×10^6/ 100 μl into recipient mice.

For spleen $CD8^+$ cell transfers; cells were washed 2 × in PBS 2%FCS and incubated with anti-mouse CD8-alexa647 antibody (clone YTS169, in house production), for 30 min on ice. Stained cells were washed in PBS 2%FCS and sorted (FacsAria Multicolour Cell Sorter, Becton Dickinson, New Jersey) by combining lymphocyte morphological gating and relevant antibody labels. For $CD8^+$ transfers; cells were collected as purified $CD8^+$ cells and CD8- (depleted) groups of cells. Purified cell populations were washed in PBS and counted. Cells were injected intravenously (i.v) either 3 or 5 × 10^6/ 100 μl into recipient mice. Respective groups of recipient mice were then infected 1 × 10^6 iRBC/ 100 μl *PbA* one hour after cell transfer (as per standard infection, described previously). Mice were monitored for parasitemia and ECM development from day 5 post-infection.

2.5 Results

2.5.1 Total splenocyte cell transfers to *Rag2*$^{-/-}$ mice

We first investigated the process of transferring cell types between groups of knockout mice. Using this method we hypothesized that selected cell transfers from C57BL/6 and *Ifnar1*$^{-/-}$

mice into various mice strains, could lead to ECM induction *vs* ECM prevention.

We set to replicate the results seen in the paper (Nitcheu et al., 2003), where it was shown transfer of C57BL/6 total splenocytes to *Rag2*$^{-/-}$ mice caused 100% death from ECM, by day 8 post-infection. However we found we were unable to replicate these results. Figure 2.1 shows our initial findings, whereby transfer of total splenocytes from infected C57BL/6 mice *vs Ifnar1*$^{-/-}$ mice to *Rag2*$^{-/-}$ mice, led to a steady increase in parasitemia levels, but saw no significant differences between recipient *Rag2*$^{-/-}$ mice or either compared against control *Rag2*$^{-/-}$ mice that received infection only, and no cell transfer (Figure 2.1 A). Roughly all recipient mice died around day 30 post-infection, with control mice dying around day 40 post-infection (Figure 2.1 B). Recipient mice were kept for two weeks post transfer to allow for re-constitution of immune compartments.

This initial experiment was further repeated with recipient mice infected one hour post cell transfer (figure 2.1 C, D). Again we noted no significant difference between parasite loads in both groups of recipient mice (Figure 2.1 C), although parasite levels do not appear to reach as high as in Figure 2.1 A. We did find 25% of *Rag2*$^{-/-}$ mice that received total splenocytes from C57BL/6 mice, died within the time window for ECM (shadowed box in Figure 2.1 D). However, this did not match the previously reported 100% incidence, therefore we concluded that spleen cell populations, in our hands, proved not fully competent at inducing ECM in previously resistant *Rag2*$^{-/-}$ mice.

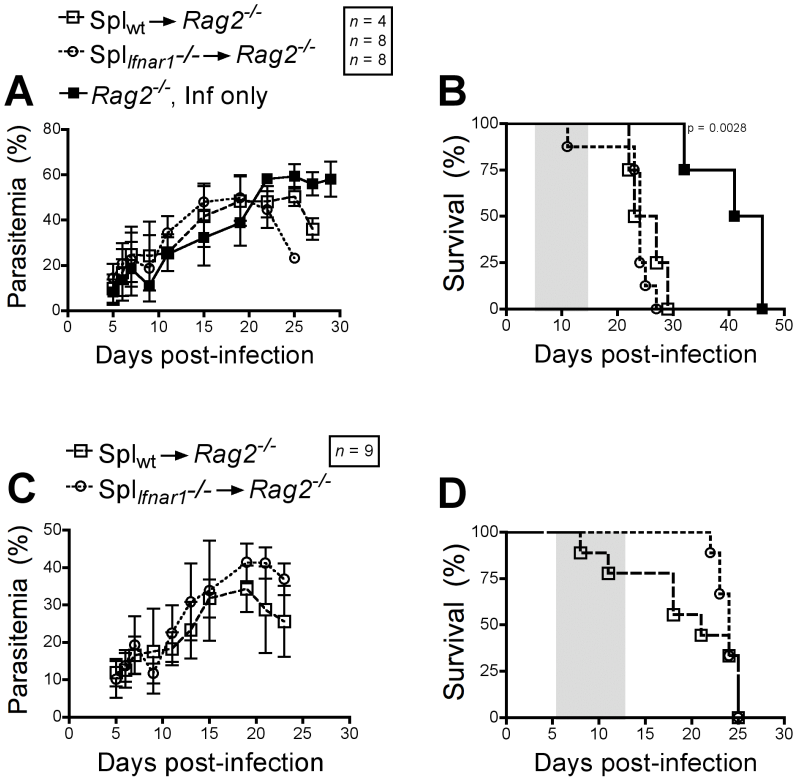


Figure 2.1. *Rag2*$^{-/-}$ mice partially resist to develop ECM upon transfer of C57BL/6 total splenocytes. Parasitemia and survival curves of *Rag2*$^{-/-}$ mice that received 5 × 10^6 total splenocytes from either C57BL/6 mice (open squares), or *Ifnar1*$^{-/-}$ mice (open circles), as compared to *Rag2*$^{-/-}$ mice that were infected only as a control (closed squares). Recipient mice were either infected 1 × 10^6 *PbA* (i.p) two weeks post splenocyte cell transfer (A, B), or one hour after cell transfer (C, D).

We continued our efforts to obtain a susceptible ECM phenotype in *Rag2*$^{-/-}$, mice, this time using sorted T cell transfers from lymph nodes of C57BL/6 *vs* *Ifnar1*$^{-/-}$ mice, and assessing whether these cells were sufficient for the ECM triggering in *Rag2*$^{-/-}$ mice. We hypothesized that transfer of naïve T cells from lymph

nodes, plus four weeks of reconstitution in *Rag2*$^{-/-}$ mice, might establish a larger pool of T cells within the recipient spleen and lead to an increased ability at triggering ECM in *Rag2*$^{-/-}$ mice. We found both groups of recipient mice developed parasitemia alike the previous total splenocyte transfers, with a slightly higher parasitemia being seen in recipient mice that received purified T cells from C57BL/6 mice compared to *Ifnar1*$^{-/-}$ transfer (Figure 2.2 A). But in contrast to our previous efforts, we found a 25% incidence of ECM in *Rag2*$^{-/-}$ mice that received C57BL/6 purified lymph node T cells. This was in comparison to a 40% incidence of ECM in mice that received *Ifnar1*$^{-/-}$ purified lymph node T cells (Figure 2.2 B). However as these results were not significantly different, and we were unable to reproduce a near 100% ECM incidence, we deemed this method of inducing *Rag2*$^{-/-}$ ECM insufficient to accomplish our goals.

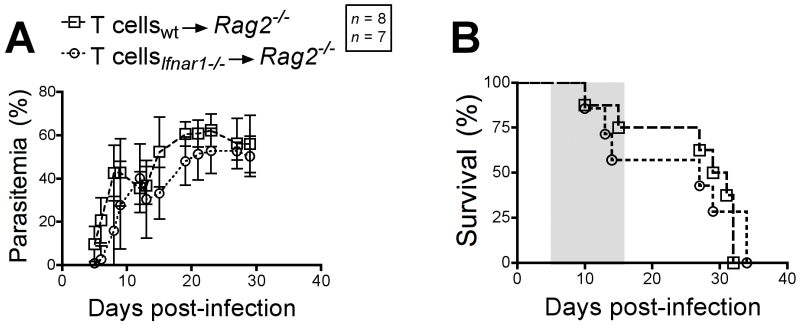


Figure 2.2. Purified lymph node T cells partially abrogate ECM resistance in *Rag2*$^{-/-}$ mice. Parasitemia (A), and survival curve (B) of *Rag2*$^{-/-}$ mice that received total T cells purified from lymph nodes of either C57BL/6 mice (open squares) or *Ifnar1*$^{-/-}$ mice (open circles).

2.5.2 ECM susceptibility in previously resistant *Ifnar1*$^{-/-}$ mice

We next attempted the possibility at restoring susceptibility to ECM in resistant *Ifnar1*$^{-/-}$ mice. Total splenocytes were transferred from previously infected C57BL/6 donor mice to *Ifnar1*$^{-/-}$ mice (as detailed above). Recipient mice were then divided into two groups, one group was infected 1 × 10^6 *PbA* one hour after infection; and the other group was left untreated (no infection). We found to our surprise that we were able to induce ECM in the previously resistant *Ifnar1*$^{-/-}$ mouse strain (Figure 2.3 B). However what was obscure is we found ECM incidence in both groups of recipient mice, even mice that were not infected post cell transfer. This was confirmed with both groups of mice developing parasitemia, albeit the untreated group from a lower starting percentage (Figure 2.3 A). In fact when we repeated this experiment by performing purified $CD8^+$ cell transfers from infected C57BL/6 donor mice to *Ifnar1*$^{-/-}$ recipients, we found the same incidence of ECM (Figure 2.3 D), and both recipients (post infected and untreated) developed parasitemia (Figure 2.3 C).

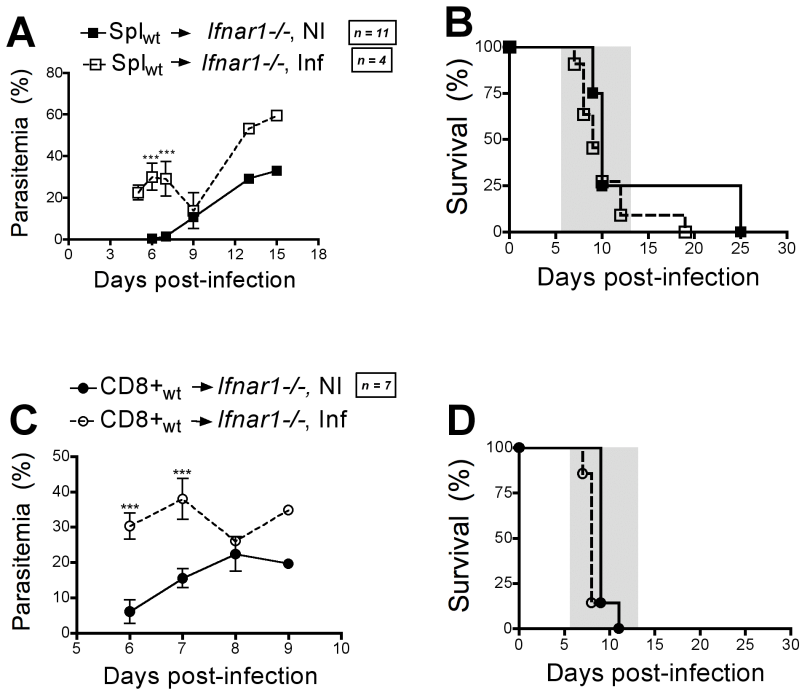


Figure 2.3 *Ifnar1*$^{-/-}$ mice succumb to ECM upon transfer of total splenocytes from previously infected C57BL/6 mice. Parasitemia and survival curves of *Ifnar1*$^{-/-}$ recipient mice that received either total splenocytes (A, B) or purified CD8^{+} cells (C, D) from C57BL/6 mice. Recipient mice were either infected one hour post cell transfer (open squares or circles), or left untreated (closed squares or circles). Statistics, parasitemia; unpaired *t*-test, ***P ≤0.001.

It seemed likely therefore that the ECM susceptibility we were recording was from the infected red blood cells (iRBC) being passed from the infected C57BL/6 donor mice. Therefore we aimed to establish a method of eliminating these invading parasites and to allow questioning whether *Ifnar1*$^{-/-}$ mice were susceptible to ECM due to the later infection after cell transfer.

2.5.3 Irradiated infected red blood cell passage

We began to explore the possibilities of irradiating parasites in the inoculum given to cell donor mice. We hypothesized that the process of irradiation would damage the DNA of the parasite preventing re-invasion of healthy RBCs within the cell donor mouse.

With reference to (de Souza and Playfair, 1983), we established that a previously infected mouse at 30% parasitemia could be used to obtain blood for passage into a new host, and would lead to host immune responses. The work of (Ocana-Morgner et al., 2003) was also referenced in establishing irradiation amount for cell populations. We set up a model using a day 6 post-infection C57BL/6 mouse, showing symptoms of ECM and parasitemia of 30%. Blood was taken, irradiated at 20 K rad, and re-injected 1 × 10^6 iRBC, into mice to be used as cell donors. The schematic of this protocol is shown in Figure 2.4.

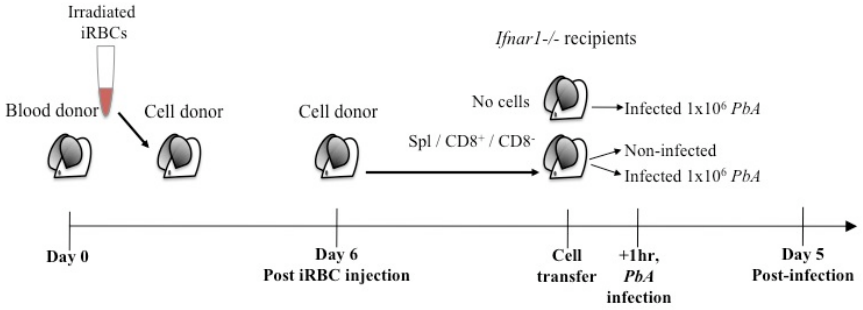


Figure 2.4. Schematic representation of pre-sensitization protocol. iRBCs are taken from an initial blood donor, irradiated and passed to cell donor mice. Six days post-injection, selected cell types are sorted from cell donor mice, and injected into *Ifnar1*$^{-/-}$ recipient groups. Recipients were then either, infected one-hour post transfer or left untreated.

To test the response of passage of irradiated iRBC, C57BL/6 mice received irradiated iRBC and were followed for development of parasitemia or ECM development. We found after two months observation, no incidence of parasitemia or ECM. Furthermore a second control was performed in which C57BL/6 mice received irradiated iRBC and were then infected 1 × 10^6 *PbA,* seven days after. We found all mice succumbed to ECM as per standard infection (Figure 2.5 B). We performed the same control experiment with *Ifnar1*$^{-/-}$ mice that received irradiated iRBC and seven days after were infected 1 × 10^6 *PbA*. All mice were resistant to ECM development, as compared to *Ifnar1*$^{-/-}$ mice that received infection only (Figure 2.5 B). We noted also *Ifnar1*$^{-/-}$ mice that received irradiated iRBC displayed lower parasitemia as compared to control *Ifnar1*$^{-/-}$ mice (Figure 2.5 A).

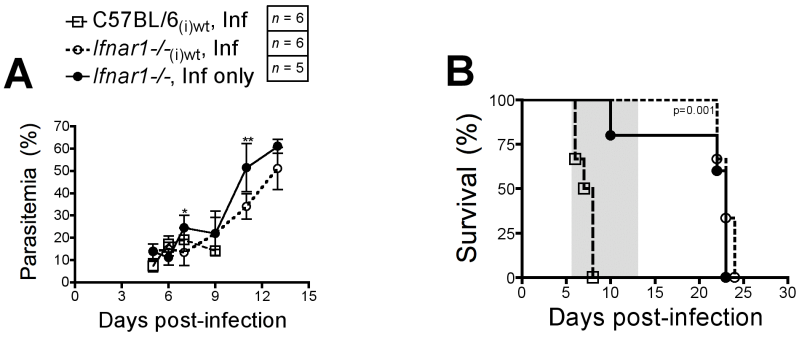


Figure 2.5. *Ifnar1*$^{-/-}$ mice show reduced parasitemia upon receiving irradiated iRBC and later infection. *Ifnar1*$^{-/-}$ mice received 1 × 10^6 irradiated iRBC (i.p) from donor C57BL/6 (wt) mice, and 6 days later 1 × 10^6 *PbA* standard infection (i.p). Statistics, parasitemia; unpaired *t*-test, *P ≤0.05, survival curves; Log-rank Test, open squares against open circles).

Overall we concluded that that this protocol was not an immunization protocol, thus meaning that exposure to irradiated iRBC from previously infected C57BL/6 mice did not induce in recipient C57BL/6 mice, a protective response from a later standard infection and furthermore did not alter the profile of ECM resistance in *Ifnar1*$^{-/-}$ mice.

2.5.4 Cell transfer experiments with *CD8*$^{-/-}$ mice

We hypothesized therefore that after successful induction of ECM in *Ifnar1*$^{-/-}$ mice, transfer of purified CD8^{+} cells from C57BL/6 mice could induce ECM susceptibility in previously resistant *CD8*$^{-/-}$ mice. The hypothesis we tested was that transfer of IFNAR1+ CD8^{+} T cells from C57BL/6 mice would induce ECM in *CD8*$^{-/-}$ mice, whereas transfer of IFNAR1- CD8^{+} T cells from *Ifnar1*$^{-/-}$ mice would maintain ECM resistance in *CD8*$^{-/-}$ mice.

We tested our now established protocol but were unable to obtain any conclusive results for ECM incidence in *CD8*$^{-/-}$ mice. We varied further our protocol set-up in the assumption that cell number and reconstitution time also factored in to ECM susceptibility. We altered, increasing the number of CD8^{+} T cells transferred, up to 5 × 10^{6} (Figure 2.6, open diamonds); increasing the time between cell transfer and infection of the *CD8*$^{-/-}$ mice, one hour and 24 hour post cell transfer (Figure 2.6 open squares *vs* open circles). We found a fraction of recipients developed ECM when transferring 5 × 10^{6} IFNAR1+ CD8^{+} cells (open diamonds), suggesting that the irradiation protocol is able to induce CD8^{+} cells and evoke ECM in a genetically resistance mouse. These early results implied these experiments warrant further exploration.

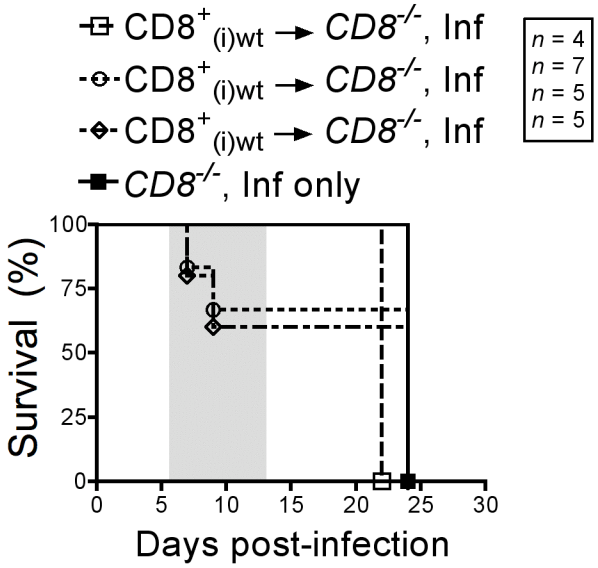


Figure 2.6. Partial abrogation of ECM resistance in *CD8*$^{-/-}$ mice upon CD8^{+} cell transfer. Survival curves of *CD8*$^{-/-}$ mice that received CD8^{+} splenic cells of previously exposed C57BL/6 mice either: 3 × 10^{6} cells and injected with *PbA* one hour after cell transfer (open squares); 3 × 10^{6} cells and infected 24 hours after cells transfer (open circles); 5 × 10^{6} cells and infected 24 hours after cell transfer (open diamonds). *CD8*$^{-/-}$ mice untreated and infected with *PbA* only were used as controls (closed squares). (i)wt indicates C57BL/6 mice that were exposed to irradiated iRBC. Time window for ECM susceptibility shadowed.

2.6 Discussion

These results show the development of a novel cell transfer protocol which highlights the introduction of irradiated iRBCs that led to 'priming' of splenic immune cells. Previous work has found that irradiating parasite sporozoites does not limit parasite antigen (Ag) synthesis but causes parasites to break open and shed their

Ag into surrounding cytoplasm of the infected cell (Suhrbier et al., 1990). We could suggest therefore that the irradiation step in our protocol halts parasite re-invasion whilst keeping parasite Ag available to activate immune system cells within the spleen. This step we prove does not grant immunity to susceptible mouse strains in a later infection, but instead primes potentially $CD8^+$ T cells for a secondary Ag encounter upon later infection time points.

Priming of $CD8^+$ T cells can result in the induction of a cytolytic (CTL) profile from recognition of a pre-existing Ag (Kohlmeier et al., 2010), thus allowing T cells a faster response upon Ag re-encounter. Inflammatory responses during priming can also act upon $CD8^+$ T cell responses (Badovinac et al., 2005; Joshi and Kaech, 2008). Extensive work by (Curtsinger et al., 2005) has shown that $CD8^+$ T cells need three signals to become fully activated: an Ag; co-stimulation and a third signal via a type I interferon. Furthermore the effects of type I IFN upon T cells is dependent on the continued exposure of T cells to type I IFN (Stewart, 2003), Therefore our work supports the notion that priming is required in $CD8^+$ T cells, to induce ECM onset.

It is difficult to explain however why we could not induce high incidence of ECM in *Rag2*$^{-/-}$ mice. Early work by (Grau et al., 1986) made use of an irradiation protocol, producing bone marrow transgenic CBA background mice, first irradiating to allow graft acceptance and then transferring selective cell populations. It could be suggested therefore that first establishing T cell populations within a resistant mouse strain, (as we tried for *Rag2*$^{-/-}$ mice), and then priming these mice with irradiated iRBC, might trigger a priming response in a now present $CD8^+$ population and lead to ECM triggering. It was interesting also that similar partial induction of 50% incidence of ECM was observed by other authors in

recipient *Rag1*$^{-/-}$ mice (Lundie et al., 2008). They could not explain the reasoning behind only 3/6 experiments working successfully, which corroborates with our data. The fact they also induced a priming step for spleen cells, points again to this as a key part in successfully inducing ECM in previously resistant mice strains.

We were able to induce low incidence of ECM in both *Rag2*$^{-/-}$ and *CD8*$^{-/-}$ mouse strains which warrants future work using higher number of transferred CD8^{+} cells, combined with initiating priming in these mice, suggests that our protocol could be further adapted for testing ECM resistant mouse strains. Furthermore work by (Prlic et al., 2006) suggests that CD8^{+} T cells need less than seven hours to receive all their necessary signals to become a full effector cell, and that 48 hours would be optimal for CD8^{+} T cell proliferation and survival. The same group also commented that exposure time to Ag dictated the accumulation of CD8^{+} T cells in the spleen. Therefore our future efforts could aim at introducing a larger number of parasite Ag and boost the priming challenge in *Rag2*$^{-/-}$ and *CD8*$^{-/-}$ mice, prior to later infections with live parasite.

It is important to conclude that the role of CD8^{+} T cells in ECM development is not completely resolved. It has been shown through wildtype CD8^{+} T cell transfers, specific components of CD8^{+} T cells that contribute to ECM induction (Campanella et al., 2008; Miu et al., 2008; Yanez et al., 1996) but there still remains no consistent method to perform these cell transfers. In fact the vast majority of reports collectively fail to show non-infected control CD8^{+} cell transfers, thereby exposing their work as potentially inducing ECM through passage of iRBC to their recipient mice. We believe our novel protocol fits well in the place of a uniformed method for specified cell transfers, and would be instrumental for future cell transfer experiments aiming at inducing ECM. With future

work dedicated to solving the induction of *Rag2*$^{-/-}$ and *CD8*$^{-/-}$ mice transfers, a mouse strain that has never actually been induced to ECM susceptibility, would be relevant in supporting our protocol.

2.7. Acknowledgements

This animal work was performed in the in-house animal facility of the Instituto Gulbenkian de Ciência (IGC), who bred and maintained the C57BL/6 and *Ifnar1*$^{-/-}$ mouse strains for use. The superb work of the IGC Cell Imaging unit contributed for purifying CD8^{+} cell populations also. I would also like to thank Bahtiyar Yilmaz for his initial advice in irradiating specimens.

2.8. References

Badovinac, V.P., Messingham, K.A., Jabbari, A., Haring, J.S., and Harty, J.T. (2005). Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination. Nature medicine *11*, 748-756.

Campanella, G.S., Tager, A.M., El Khoury, J.K., Thomas, S.Y., Abrazinski, T.A., Manice, L.A., Colvin, R.A., and Luster, A.D. (2008). Chemokine receptor CXCR3 and its ligands CXCL9 and CXCL10 are required for the development of murine cerebral malaria. Proceedings of the National Academy of Sciences of the United States of America *105*, 4814-4819.

Claser, C., Malleret, B., Gun, S.Y., Wong, A.Y., Chang, Z.W., Teo, P., See, P.C., Howland, S.W., Ginhoux, F., and Renia, L. (2011). CD8+ T cells and IFN-gamma mediate the time-dependent accumulation of infected red blood cells in deep organs during experimental cerebral malaria. PloS one *6*, e18720.

Curtsinger, J.M., Valenzuela, J.O., Agarwal, P., Lins, D., and Mescher, M.F. (2005). Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. J Immunol *174*, 4465-4469.

de Souza, J.B., and Playfair, J.H. (1983). Anti-lymphocyte antibodies in lethal mouse malaria. II. Induction of an autoantibody specific suppressor T cell by non-lethal P. yoelii. Clinical and experimental immunology *54*, 110-116.

Franke-Fayard, B., Trueman, H., Ramesar, J., Mendoza, J., van der Keur, M., van der Linden, R., Sinden, R.E., Waters, A.P., and Janse, C.J. (2004). A Plasmodium berghei reference line that constitutively expresses GFP at a high level throughout the complete life cycle. Molecular and biochemical parasitology *137*, 23-33.

Grau, G.E., Piguet, P.F., Engers, H.D., Louis, J.A., Vassalli, P., and Lambert, P.H. (1986). L3T4+ T lymphocytes play a major role in the pathogenesis of murine cerebral malaria. J Immunol *137*, 2348-2354.

Haque, A., Best, S.E., Unosson, K., Amante, F.H., de Labastida, F., Anstey, N.M., Karupiah, G., Smyth, M.J., Heath, W.R., and Engwerda, C.R. (2011). Granzyme B expression by CD8+ T cells

is required for the development of experimental cerebral malaria. J Immunol *186*, 6148-6156.
Joshi, N.S., and Kaech, S.M. (2008). Effector CD8 T cell development: a balancing act between memory cell potential and terminal differentiation. J Immunol *180*, 1309-1315.
Kohlmeier, J.E., Cookenham, T., Roberts, A.D., Miller, S.C., and Woodland, D.L. (2010). Type I interferons regulate cytolytic activity of memory CD8(+) T cells in the lung airways during respiratory virus challenge. Immunity *33*, 96-105.
Lundie, R.J., de Koning-Ward, T.F., Davey, G.M., Nie, C.Q., Hansen, D.S., Lau, L.S., Mintern, J.D., Belz, G.T., Schofield, L., Carbone, F.R., *et al.* (2008). Blood-stage Plasmodium infection induces CD8+ T lymphocytes to parasite-expressed antigens, largely regulated by CD8alpha+ dendritic cells. Proceedings of the National Academy of Sciences of the United States of America *105*, 14509-14514.
Miu, J., Mitchell, A.J., Muller, M., Carter, S.L., Manders, P.M., McQuillan, J.A., Saunders, B.M., Ball, H.J., Lu, B., Campbell, I.L., *et al.* (2008). Chemokine gene expression during fatal murine cerebral malaria and protection due to CXCR3 deficiency. J Immunol *180*, 1217-1230.
Morrell, C.N., Srivastava, K., Swaim, A., Lee, M.T., Chen, J., Nagineni, C., Hooks, J.J., and Detrick, B. (2011). Beta interferon suppresses the development of experimental cerebral malaria. Infection and immunity *79*, 1750-1758.
Nitcheu, J., Bonduelle, O., Combadiere, C., Tefit, M., Seilhean, D., Mazier, D., and Combadiere, B. (2003). Perforin-dependent brain-infiltrating cytotoxic CD8+ T lymphocytes mediate experimental cerebral malaria pathogenesis. J Immunol *170*, 2221-2228.
Ocana-Morgner, C., Mota, M.M., and Rodriguez, A. (2003). Malaria blood stage suppression of liver stage immunity by dendritic cells. The Journal of experimental medicine *197*, 143-151.
Pamplona, A., Ferreira, A., Balla, J., Jeney, V., Balla, G., Epiphanio, S., Chora, A., Rodrigues, C.D., Gregoire, I.P., Cunha-Rodrigues, M., *et al.* (2007). Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. Nature medicine *13*, 703-710.

Prlic, M., Hernandez-Hoyos, G., and Bevan, M.J. (2006). Duration of the initial TCR stimulus controls the magnitude but not functionality of the CD8+ T cell response. The Journal of experimental medicine *203*, 2135-2143.
Stewart, T.A. (2003). Neutralizing interferon alpha as a therapeutic approach to autoimmune diseases. Cytokine & growth factor reviews *14*, 139-154.
Suhrbier, A., Winger, L.A., Castellano, E., and Sinden, R.E. (1990). Survival and antigenic profile of irradiated malarial sporozoites in infected liver cells. Infection and immunity *58*, 2834-2839.
Tamura, T., Kimura, K., Yuda, M., and Yui, K. (2011). Prevention of experimental cerebral malaria by Flt3 ligand during infection with Plasmodium berghei ANKA. Infection and immunity *79*, 3947-3956.
Yanez, D.M., Manning, D.D., Cooley, A.J., Weidanz, W.P., and van der Heyde, H.C. (1996). Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. J Immunol *157*, 1620-1624.

Chapter 3

Article I

IFNAR1 Controls Progression to Cerebral Malaria in Children and $CD8^+$ T cell Brain Pathology in *Plasmodium berghei*-Infected Mice.

***IFNAR1* Controls Progression to Cerebral Malaria in Children and CD8$^+$ T cell Brain Pathology in *P.berghei* Infected Mice.**

Elizabeth Ann Ball[*], Maria Rosário Sambo[*, †, ‡], Madalena Martins[*, §], Maria Jesus Trovoada[*, ¶], Carla Benchimol[‡], João Costa[*], Lígia Antunes Gonçalves[*], António Coutinho[*], Carlos Penha-Gonçalves.[*]

Author affiliations

[*]Instituto Gulbenkian de Ciência, 2781-901 Oeiras, Portugal;

[†]Faculdade de Medicina, Universidade Agostinho Neto, Luanda, Angola;

[‡]Hospital Pediátrico David Bernardino, Luanda, Angola;

[§]Neurological Clinical Research Unit, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, 1649-028 Lisboa, Portugal;

[¶]Centro National de Endemias, São Tomé and Príncipe.

This work was supported by the *Fundação para a Ciência e a Tecnologia* (FCT) fellowship grants: SFRH / BD / 33564 / 2008 to EAB, SFRH / BPD / 29354 / 2006 to MM.

Paper accepted for publication in the Journal of Immunology, March 2013.

3.1 Author Contributions

All mouse experiments and analysis were designed by my supervisor Carlos Penha-Gonçalves and myself. All mouse experiments and analyses were performed by me.

Maria Rosário Sambo orchestrated human sample collections. Maria Rosário Sambo, Maria Jesus Trovoada, Lígia Antunes Gonçalves performed human sample processing and genotyping. Maria Rosário Sambo and Madalena Martins conducted the human genetic analysis. Manuscript was written by myself and Carlos Penha-Gonçalves, with contributions from Madalena Martins. António Coutinho and Carlos Penha-Gonçalves conceived the original human study design.

3.2 Summary

Development of cerebral malaria (CM), a severe and fatal form of clinical *Plasmodium falciparum* infection, results from a damaging cascade of vascular, inflammatory and immunological host responses that leads to brain injury. Progression to CM can be modified by host genetic factors. Our case-control study in Angolan children aimed at highlighting the role of Interferon (alpha, beta) Receptor 1 (*IFNAR1*) in progression to CM. We report here robust association between *IFNAR1* and CM protection, and detailed mouse studies showing analogous protection from Experimental CM in *Ifnar1*$^{-/-}$ mice infected with *Plasmodium berghei* ANKA-GFP. We developed a novel cell transfer protocol that enables spleen cell priming in absence of disease. This led to discover that IFNAR1 expression in CD8^{+} T cells is crucial, and can abrogate resistance to ECM in *Ifnar1*$^{-/-}$ mice. Splenic CD8^{+} T cells from *Ifnar1*$^{-/-}$ mice are functionally activated upon infection, yet are unable to mediate

ECM development within the brain tissue. Our findings prove that IFNAR1 signaling unleashes $CD8^+$ T cell effector capacity-vital for CM-and raises the hypothesis that the cohesive role of *IFNAR1* in both human and mouse CM, operates through $CD8^+$ T cell triggering.

3.3 Introduction

Cerebral malaria (CM) is a severe and often fatal, complicated form of *Plasmodium falciparum* malaria, affecting mostly children in endemic regions (1-3). While adhesion and sequestration of infected red blood cells (iRBCs) in brain microvasculature leads to blood vessel occlusion and is central to CM pathogenesis (4), immunological mechanisms operating during development of CM remain unclear.

Accessibility to human tissues limits research into understanding the progression and control of CM development in humans (5). Therefore usage of the mouse model, termed Experimental Cerebral Malaria (ECM) has enabled researchers to uncover cellular and molecular components occurring in CM. Recent papers addressed the relevance of the mouse model of ECM and emphasized the need of denoting consistent factors between the mouse model and human studies (6-9). As such factors are seldom confirmed, confounding lists of players in CM development has emerged as a result. One factor however remaining implicit are $CD8^+$ T cells (10, 11) although the exact mechanisms that drive $CD8^+$ T cell pathogenesis in CM remain unclear. Additionally, the role of cytokines in human CM development have been brought to light in recent genetic studies [reviewed by (12)]. Initial reports by Hill and co-workers began exploring the necessary role of Type I

interferon (Type I IFN) with CM progression, and found genetic variants in *IFNAR1* led to protection from CM development (13, 14). As IFNAR1 is the key signaling receptor mediating Type I IFN activity (15) and is expressed on virtually every cell surface (16, 17), the impact Type I IFN has on the immune response is vast. In particular Type I IFN has been shown to be involved in the priming of $CD8^+$ T cells (18, 19) and in the generation of a robust immune response to infection (20, 21). Recent publications address the actions of Type I IFN; through the IFNAR1 receptor, in response to hemozoin derivatives (22); and in controlling parasite burden upon *Plasmodium* infection(23). However, the exact caliber of *IFNAR1* action in CM development remains unexplored.

We performed a genetic study to analyze *IFNAR1* involvement in progression of CM *vs* uncomplicated malaria (UM). We selected children, the primary risk group of severe malaria, from an area of Angola where malaria transmission is perennial (24). We report here, variants of *IFNAR1* strongly associate with CM protection, which drove the use of *Ifnar1*$^{-/-}$ mice, to interrogate the action of *IFNAR1* in CM pathogenesis. We describe ECM protection in *Ifnar1*$^{-/-}$ mice and through design of a novel cell transfer protocol we expose the necessity of IFNAR1 expression in $CD8^+$ T cells during development of ECM.

3.4 Materials and Methods

3.4.1 Patient and control samples

A total of 272 children, living in Luanda and ranging from 6 months to 13 years of age were enrolled in the present study. Ethical permission for this study was granted by the Ethical Committee of the Hospital Pediátrico David Bernardino (HPDB) in

Luanda, appointed by the Angolan Ministry of Health. Written, informed consent was obtained from the parents or guardians of each child. Patients were selected from attendees to the HPDB. The details of the dataset have been described previously (25). Briefly, samples were collected from children aged 6-156 months, carried out between February 2005 to May 2007 and comprised of 130 patients with CM and 142 patients with UM. Malaria was diagnosed on the basis of a positive asexual parasitaemia detected on a Giemsa-stained thick smear (26). CM was defined according to the WHO criteria: a coma score < 3 on the Blantyre Scale for children < 60 months, or a coma score < 7 on the Glasgow Scale for children ≥ 60 months. Meningitis and encephalitis were ruled out by cerebrospinal fluid analysis after lumbar puncture. Exclusion criteria were different known aetiologies of encephalopathy and hypoglycaemia (glycaemia < 40 mg/dl). Patients with consciousness disturbances or with other diseases were also excluded from this group. The UM group represents patients with malaria diagnosis and febrile illness without any clinical finding suggestive of other causes of infection and with no manifestations of severe malaria.

3.4.2 Genotyping

Genomic DNA was extracted from whole blood using the Chemagen Magnetic Bead technology. DNA preparations were quantified using PicoGreen reagents according to the supplier instructions. 27 SNPs covering the *IFNAR1* region were initially genotyped using the Sequenom's iPlex assay (San Diego, USA) and the Sequenom MassArray K2 platform performed by the Genomics Unit of the Instituto Gulbenkian de Ciência.

Extensive quality control was performed using eight HapMap (http://hapmap.ncbi.nlm.nih.gov/) controls of diverse ethnicity, Hardy-Weinberg equilibrium (HWE) with $P > 0.01$, and a minimum of 90% call rate for each SNP. Genotype determinations were performed blinded to affection status. Only SNPs with minor allele frequency > 1% in both groups were analyzed for association. Nine SNPs in *IFNAR1* (rs224359, rs2252930, rs12626750, rs2834197, rs2226300, rs2254315, rs1012943, 2843996 and 2254305) did not meet the quality control criteria and were excluded. Samples with < 75% call rate and duplicates were excluded from analysis, a total of 33 samples were excluded. The final dataset used in the analysis consisted of 239 children comprising 110 CM cases (mean age 54.6 months) and 129 UM (mean age 49.1 months) patients.

3.4.3 Association testing

χ2 tests for HWE in both groups, allelic and haplotypic association of SNPs with progression to CM, and linkage disequilibrium (LD) plots were performed using Haploview 4.2 (27). Diagram with representation of allelic genetic association results was produced by the snp.plotter R package (28). Association analyses were performed with logistic regression and adjusted for potential confounding effects of age using the SNPassoc v.1.4-9 package (29) implemented in the R freeware (http://cran.r-project.org/). Results were considered suggestive below the conventional probability level of 0.05. Bonferroni corrections for multiple tests were carried out to exclude type I errors (the significance level for 18 tests is set at P value < 2.78 E−3).

3.4.4 Animals

Male, 8-12 weeks old C57BL/6, B6.Ly5.1, *Ifnar1*$^{-/-}$ and BALB/c mice were bred and used as provided by the Instituto Gulbenkian de Ciência in-house animal facility. *Ifnar1*$^{-/-}$ mice were originally a gift to the institute from Michel Aquet (*Ifnar1*$^{-tm\text{-}Agt}$) (30). *Ifnar1*$^{-/-}$ mice have been backcrossed into the C57BL/6 background, (98.7% - 99.36% C57BL/6 background outside of the congenic region, as confirmed by the animal facility at Instituto Gulbenkian de Ciência). All procedures were in accordance with national regulations on animal experimentation and welfare as approved by the Instituto Gulbenkian de Ciência Ethics committee.

3.4.5 Parasite, infection and ECM disease assessment

All infections used 1 × 10^6 *P.berghei ANKA*-GFP parasitized iRBC (31) given via i.p injection. Frozen iRBC stocks were expanded in C57BL/6 mice prior to infection. Parasitemia was determined as the % of GFP+ red blood cells, using flow cytometry analysis (FacScan Cell Analyser, Becton Dickinson, New Jersey) and non-infected RBCs as negative control. ECM development was monitored from day 5 post-infection (PI) as including; head deviations, paralysis, ataxia and convulsions (32). ECM susceptible mice developed symptoms at days 6-7 post-infection and died within 4-5 hours. Mice resistant to ECM died around days 22-25 from anemia and hyperparasitemia without displaying neurological symptoms.

3.4.6 Blood brain barrier (BBB) integrity

Upon display of ECM induced coma in C57BL/6 mice, the experimental groups of mice were successively injected with 100 μl

of 2% Evan's blue dye (Sigma), sacrificed one hour post injection and perfused intracardially with 15 ml ice-cold PBS. Brains were dissected, weighed and immersed in 2 ml formamide (Merck) covered at 37 °C for 48 h, to allow extraction of Evan's blue dye, images were taken after the 48 h period. Non-infected mice from each group were used as controls. Absorbance of dye was measured at wavelengths 620 nm and 740 nm using Spectrophotometry, (ThermoSpectronic, Helios Delta). Using a standard curve, data was calculated and expressed as μg of Evans blue dye/mg of brain tissue.

3.4.7 Histology

Infected C57BL/6 and *Ifnar1*$^{-/-}$ mice were sacrificed upon observation of clinical signs of ECM in C57BL/6 mice, around day 6 post-infection. Non-infected C57BL/6 and *Ifnar1*$^{-/-}$ mice were used as controls and sacrificed at the same time point. For hematoxylin and eosin (H&E) staining; brains were carefully removed, fixed in 10% neutral-buffered formalin, embedded, sectioned (4 μm) and mounted with entellan (Merck, Darmstadt, Germany) then finally stained with Hematoxylin and Eosin following standard procedures. CM and brain pathology were assessed by routine histopathology in coronal brain sections of the same anatomical location. Bright field images were captured utilizing a Leica DMD108 microscope (LeicaMicrosystems), 10 × (numerical aperture: 0.40) and 40 × (numerical aperture: 0.95) objectives were employed. Adobe Photoshop software was utilized to compose images and adjust the contrast (Adobe, USA).

3.4.8 Gene expression

C57BL/6 and *Ifnar1*$^{-/-}$ infected mice were sacrificed at days 5,

6, and 7 post-infection. *Ifnar1*$^{-/-}$ mice were sacrificed further at days 9 and 11 post-infection, C57BL/6 mice were not collected at these time points due to death from ECM. Non-infected C57BL/6 and *Ifnar1*$^{-/-}$ mice were used as controls and represent day 0. Brains were dissected, fast frozen in liquid nitrogen and stored at -80 °C. Brains were then homogenized and a final brain weight of 20 mg was used for total RNA extraction (RNeasy® Mini Kit, Qiagen). RNA quality was determined using Nanodrop and a final elution of 50 μl extracted. One microgram of total RNA was converted to cDNA (Transcriptor High Fidelity cDNA Synthesis Kit, Roche) using random hexamer primers and a final volume of 10 μl of the cDNA reaction was diluted 1:3 in RNAse-free water to be used for mRNA quantification. *Tnf-α*, *Il-10* mRNA was quantified using TaqMan® Gene Expression Assays from ABI (Mm00443258_m1 and Mm99999062_m1, respectively). *Plasmodium berghei ANKA* rRNA was quantified using specific primer sequences: Forward 5'-CCG ATA ACG AAC GAG ATC TTA ACC T-3', Reverse 5'-CGT CAA AAC CAA TCT CCC AAT AAA GG-3' and Probe 5'-ACT CGC CGC TAA TTA G-3' (FAM/MGB), all with Taqman® Universal PCR Master Mix. T lymphocyte (*Cd3e)* expression was quantified using specific primer sequence: Forward 5'-TCT CGG AAG TCG AGG ACA GT-3' Reverse 5'-ATC AGC AAG CCC AGA GTG AT-3' (33), using Power SYBR Green PCR Master Mix (Applied Biosystems). Gene expression quantification reactions were performed on an ABI Prism 7900HT system according to manufacturers' instructions, a total volume of 10 μl PCR reactions was amplified for 45 cycles. Relative quantification of specific mRNA was normalized to the mouse housekeeping gene *ACTB* (Mouse ACTB Endogenous Controls, ABI), used in multiplex PCR with the target genes and

calculated using the ΔΔCt method.

3.4.9 Pre-Sensitization protocol

For infected blood donor mice; blood was collected by mandibular vein puncture into an eppendorf containing 10 μl of Heparin (Heparina Leo, Leo Pharmaceutical products) from infected mouse at day 6 post-infection, upon display of ECM symptoms and when parasitemia was around 30%. Parasitemia was measured to calculate injection of 4×10^6 iRBC into cell donor mice. Collected blood was then placed on ice in a 50 ml falcon tube and irradiated at 20 k Rad (GAMMACELL 2000, MØLSGAARD Medical Denmark). Irradiated iRBC were then re-suspended in PBS and injected i.p into cell donor mice. At day 6 post irradiated-iRBC injection, cell donor mice were sacrificed and purified spleen cell populations were injected into recipient mice; recipient mice were left either non-infected (NI) or infected 1×10^6 *PbA* i.p one hour after transfer. Parasitemia and ECM development were monitored from day 5 post-infection. For control non-infected blood donor mice; mice were bled and an equivalent volume of non-infected RBC were irradiated and diluted in PBS in the same manner as above.

3.4.10 Spleen cell transfers

Single cell suspensions were prepared from spleens of cell donor mice. For total spleen cell transfers; cells were washed 2 × in PBS, counted in a hemocytometer, re-suspended in PBS and injected 5×10^6/ 100 μl into recipient mice. For spleen $CD8^+$ cell transfers; cells were washed 2 × in PBS 2%FCS and incubated with anti-mouse CD8-alexa647 antibody (clone YTS169, in house production), for 30 min on ice. For $CD8^+$Ly5.1/2+ cell transfers; cells

were incubated with anti-mouse CD8-alexa647 antibody (clone YTS169, in house production), anti-mouse CD45.1-FITC antibody (clone 820, BD Pharmingen) or anti-mouse CD45.2-PE antibody (clone 104.2, in house production) for 30 min on ice. Stained cells were washed in PBS 2%FCS and sorted (FacsAria Multicolour Cell Sorter, Becton Dickinson, New Jersey) by combining lymphocyte morphological gating and relevant antibody labels.

For $CD8^+$ transfers; cells were collected as purified $CD8^+$ cells and CD8- (depleted) groups of cells. For $CD8^+$Ly5.1/2 transfers; cells were gated for $CD8^+$ cells within which CD45.1+ or CD45.2+ populations were collected. Purified cell populations were washed in PBS and counted. Cells were injected intravenously (i.v) 3×10^6/ 100 μl into recipient mice. Respective groups of recipient mice were then infected 1×10^6 iRBC/ 100 μl *PbA* one hour after cell transfer. Mice were monitored for parasitemia and ECM development from day 5 post-infection.

3.4.11 Analysis of spleen cells

Single cell suspensions were prepared from spleens of C57BL/6 and *Ifnar1*$^{-/-}$ infected mice at day 5 post-infection, non-infected mice from both groups were used as control. Cells used for total cell counts and activation profile were prepared as single cell suspensions: from 5ml of total cells, 200 μl per sample was plated in a 96 round well plate, cells were centrifuged, pelleted and re-suspended in Fc Block for 20 min on ice, cells were then washed in PBS, 2%FCS, and re-suspended in either: anti-mouse CD8-alexa647 antibody (clone YTS169, in-house antibody) or CD4-alexa647 (clone GK 1.5, inhouse antibody) with CD44-FITC (clone IM7, BD Pharmingen 553133) and CD62L-PE (clone Mel 14, eBioscience 12-0621-85) for 20 min on ice. Cells were then

washed, resuspended in 100 μl PBS, 2%FCS, plus 20 μl propidium iodide (1μg/ml) (Sigma, P-4170), plus 10 μl 5×10^5 beads (Beckman Coulter ® nominal 10μm Latex Beads). For intracellular stainings: cells were then washed and re-suspended in red blood cell lysis, then washed and re-suspended in 100 μl RPMI medium supplemented with 10% FCS, 10 U/ml Pen-Strep, 1% sodium pyruvate, 1% HEPES 1M, 1% L-Glutamine, 0.01% β-Mercaptoethanol, centrifuged and finally re-suspended in 200 μl RPMI supplemented medium containing 10 μg/ml of Brefeldin A for fixation of cytokine production, 100 ng/ml of PMA and 500 ng/ml of Ionomycin for cell stimulation. Cells were stimulated at 37 °C, 5% CO_2 for 4 hours. Cells were then processed for extracellular staining; cells were centrifuged, pelleted and re-suspended in Fc Block for 20 min on ice, cells were then washed in PBS, 2%FCS, and re-suspended in anti-mouse CD8-alexa647 antibody (clone YTS169, in house antibody) for 20 min on ice. Cells were then washed and re-suspended in 2%PFA for fixation for 30min at room temperature (RT), then washed 2 × in PBS and re-suspended in 200 μl PBS, 2%FCS and stored at 4 °C overnight. The proceeding day, cells were pelleted, re-suspended in permeabilisation buffer (10% Saponin in PBS, 2%FCS) for 10 min at RT in preparation for intracellular staining. Cells were then washed and pelleted and incubated with anti-mouse GranzymeB-FITC (clone NGZB, eBioscience) and anti mouse IFN-γ-PE (clone XMG1.2, BD Pharmingen) for 30 min at RT. Cells were then washed 2 × in permeabilisation buffer and then 2 × in PBS, 2%FCS and re-suspended in 150 μl PBS, 2%FCS plus 5×10^5 beads (as before). All cell analysis was performed using FacsCalibur cell analyzer, and

FlowJo (version 7). Single staining preparations were used for control compensation of double staining analysis.

3.4.12 Analysis of brain-sequestered leukocytes

C57BL/6 and *Ifnar1*$^{-/-}$ infected mice were sacrificed at day 6 post-infection, perfused pericardially (as described above) and brains placed in 10 ml of HBSS supplemented with Collagenase VIII (Sigma-Aldrich), 0.2 mg/ml. Brains were then smashed and incubated for 30 min at 37 °C. Each sample was passed through a 100 μm strainer and centrifuged for 10 min at 1500 rpm. Supernatant was discarded and samples re-suspended in 10 ml PBS and centrifuged. Samples were re-suspended in 10 ml of 30% Percoll gradient (Percoll^{TM}, GE Healthcare, BioSciences) and centrifuged for 20 min at 2500 rpm at RT, without brake. The supernatant was carefully aspirated and pellet re-suspended in 50 ml of PBS and centrifuged. The supernatant was discarded and each sample pellet re-suspended to a final volume of 200 μl. Cells were centrifuged and re-suspended in 100 μl red blood cell lysis, incubated for 5 min at RT, washed 2 × in 200 μl PBS, 2%FCS and re-suspended in supplemented RPMI medium and proceeded for extra and intracellular staining and analysis as described above.

3.5 Results

3.5.1 *IFNAR1* variants are associated with protection against clinical progression to cerebral malaria

The *IFNAR1* gene has been associated to severe forms of malaria (13, 14). Here we made use of a cohort of Angolan children to identify *IFNAR1* genetic variants that specifically modify the risk

of progressing to CM syndrome from uncomplicated forms of malaria. We compared genotype frequencies of single nucleotide polymorphisms (SNPs) in infected children that developed CM (n = 110) *vs* children that presented clinically uncomplicated malaria (UM) (n = 129). After age correction, eight out of 18 *IFNAR1* SNPs showed suggestive association ($P < 0.05$) with progression to CM (Figure 3.1b) - possibly under a dominant mode of action (Appendix Two, Table S1). The two markers having the strongest association with progression to CM, rs2856968 (P = 1.33E−6) and rs2253923 (P = 5.77E−5), remained significantly associated after conservative Bonferroni correction for multiple testing (P < 2.78E−3) (Figure 3.1b). As the two SNPs are in strong linkage disequilibrium (LD) (r^2 = 0.77) this result suggests a single genetic effect in CM protection (Figure 3.1c).

Haplotypic association analysis comprising eight SNPs in the 5' region of *IFNAR1* (encompassing upstream regulatory region intron 1 and intron 2), identified one haplotype that shows robust association to CM protection (P_{hap} = 6.89E−7; OR_{hap} = 0.34). Conversely, the most frequent haplotype for the same eight SNPs shows an increased risk of CM progression (P_{hap} = 8.0E−4; OR_{hap} = 1.87).

Together this genetic evidence strongly suggests that specific minor frequency alleles of *IFNAR1* gene provide protection against CM. This proposes a determinant role of *IFNAR1* during the inflammatory response to malaria infection and specifically at dictating progression to CM.

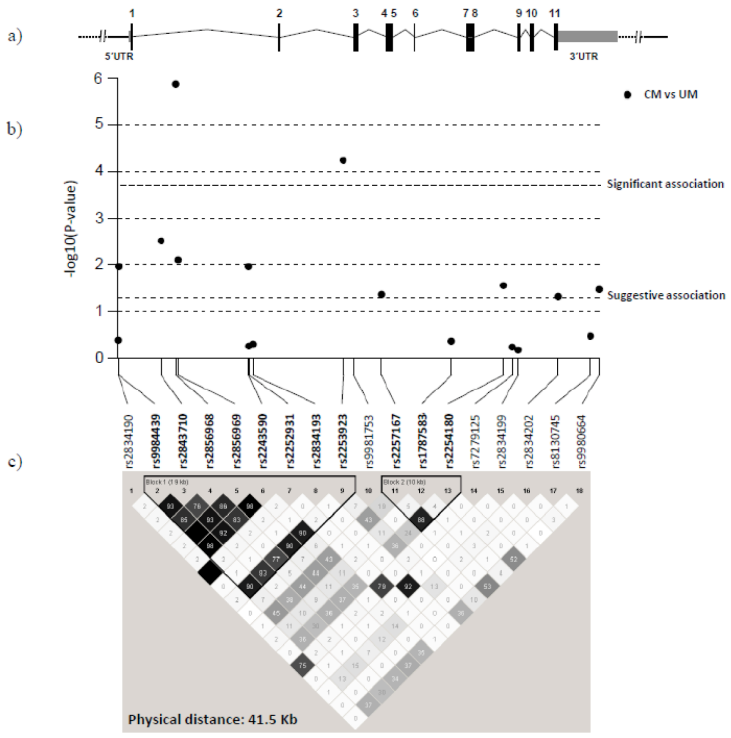


Figure 3.1 *IFNAR1* gene variants protect against malaria progression to cerebral malaria syndrome. Results of genotypic association tests under the dominant model at 18 *IFNAR1* SNPs, depicted at the bottom of the horizontal axis according to relative physical distance(a) Scaled diagram of the *IFNAR1* gene structure: exons are marked with their corresponding number; 5'UTR and 3'UTR are represented by gray boxes; introns are represented by black lines between exons. (b) Genotypic *P*-value of association tests were obtained by comparing 110 cases of cerebral malaria (CM) with 129 uncomplicated malaria patients (UM) using logistic regression and adjusted for age. Thresholds for suggestive association ($P = 0.05$) and for significant association after multiple testing Bonferroni correction ($P = 2.78\ E{-}3$) are represented. (c)

3.5.2 *Ifnar1*$^{-/-}$ mice are protected from development of Experimental Cerebral Malaria and brain pathology

To ascertain whether *IFNAR1* is involved in inflammation evoked by malaria infection we used the mouse model of ECM (6). In line with a recent report (22) we observed that *P.berghei* ANKA (*PbA*) infection in *Ifnar1*$^{-/-}$ mice led to strong resistance to ECM (75% ECM resistance, Figure 3.2a) in comparison to susceptible C57BL/6 control mice. *Ifnar1*$^{-/-}$ mice that resisted to ECM development, die between days 25-30 from anemia and hyperparasitemia and showed parasitemia comparable to C57BL/6 mice, up to day 5 post-infection (Figure 3.2b). This indicated that ECM resistance in *Ifnar1*$^{-/-}$ mice was not related to decreased peripheral parasite burden.

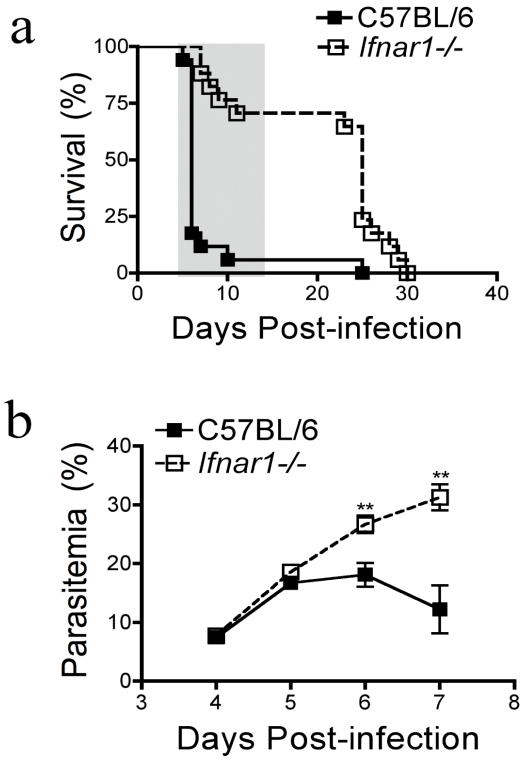


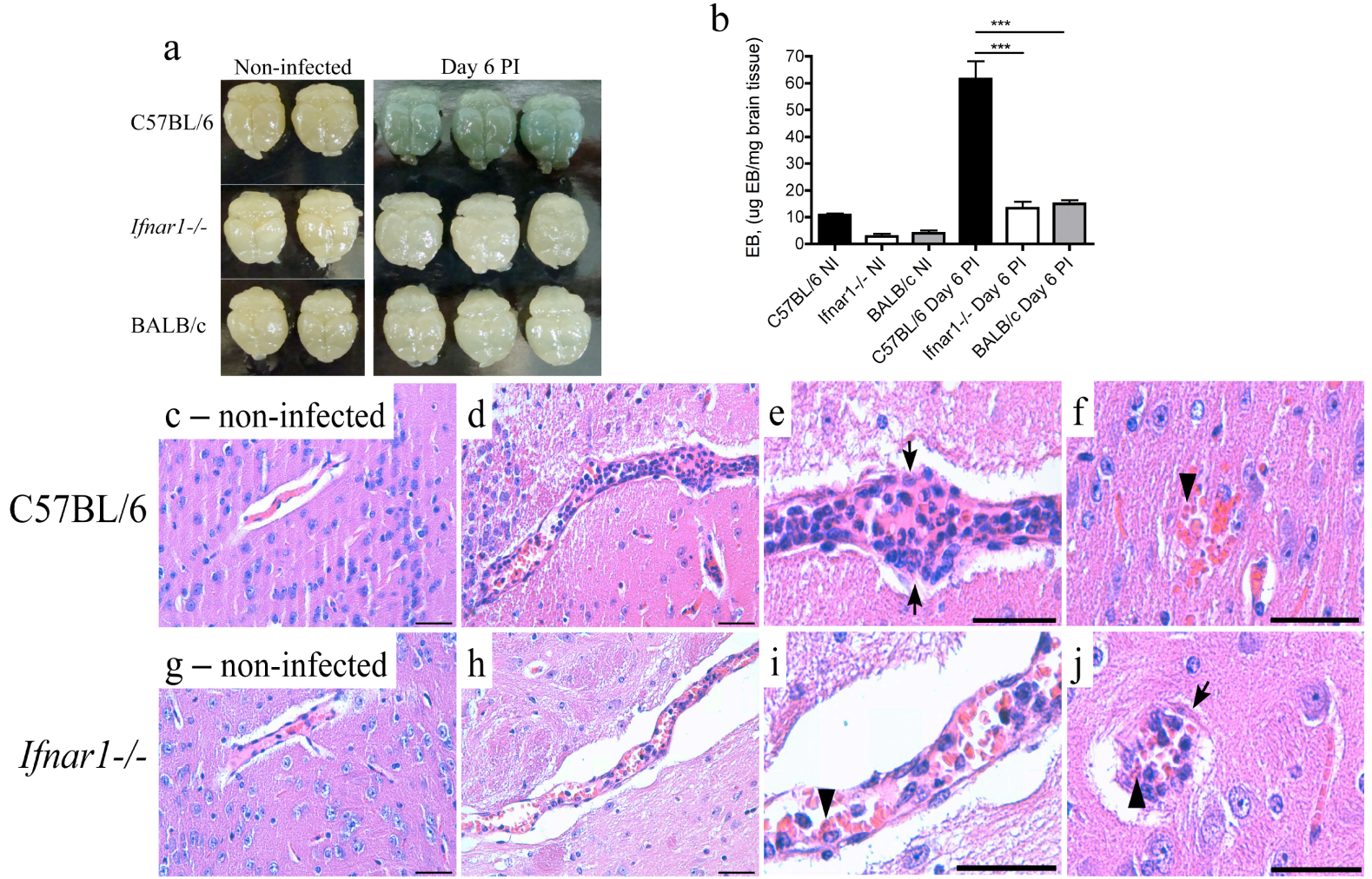
Figure 3.2 *Ifnar1*$^{-/-}$ mice are protected from ECM and show no decrease in parasite burden. C57BL/6 (filled squares) and *Ifnar1*$^{-/-}$ (open squares) mice were infected with 1 × 10^6 *P.berghei* ANKA-GFP (*PbA*) infected red blood cells (iRBC) via intraperitoneal (i.p) injection. (a) Survival curve; (*n* = 17, log-rank test). (b) Parasitemia progression; (*n* = 17, unpaired, two tailed *t*-test; day 6 post-infection (PI); ***P* = 0.0016, day 7 PI; ***P* = 0.0021). Data collective from at least four independent experiments. Time window of C57BL/6 ECM development is shadowed.

Disruption of the blood brain barrier (BBB) is a standard hallmark of ECM pathology (34). We analyzed infected and non-infected C57BL/6, *Ifnar1*$^{-/-}$ as well as ECM resistant BALB/c mice

for barrier breakage using Evans blue. Alike BALB/c mice, *Ifnar1*$^{-/-}$ mice display no BBB breakage (Figure 3.3a and b) as opposed to C57BL/6 mice that showed abundant dye leakage in the brain. Histopathological analysis of infected C57BL/6 brain sections exhibited: prominent mononuclear cell accumulations within brain micro-vessels (Figure 3.3d); evidence of disruption of vessel walls and endothelial cell destruction (Figure 3.3e); as well as parenchymal hemorrhagic lesions containing infected red blood cells (iRBCs) (Figure 3.3f). In contrast, ECM resistant *Ifnar1*$^{-/-}$ mice displayed: mild intravascular accumulation of mononuclear cells (Figure 3.3h); intact endothelial cells (Figure 3.3i) and absence of hemorrhagic lesions with iRBCs seen within the lumen of blood vessels only (Figure 3.3j). These results show that despite abnormal mononuclear accumulation within brain micro-vessels and presence of intra-luminal iRBCs, infected *Ifnar1*$^{-/-}$ mice do not show typical pathology of ECM.

Figure 3.3 *Ifnar1*$^{-/-}$ mice are protected from BBB breakage and show reduced mononuclear cell accumulation in brain microvasculature. C57BL/6, *Ifnar1*$^{-/-}$ and BALB/c mice were infected with 1×10^6 *PbA* iRBC i.p and analyzed at day 6 PI. (a) BBB integrity was assessed in all infected groups of mice by Evan's Blue perfusion. Non-infected mice from each group were used as controls; (n = 3-5 mice per group). Images representative of two independent experiments. (b) Quantification of Evan's Blue dye was measured as μg of dye/mg of brain tissue; (n = 3-4 mice per group, ANOVA, Tukey's Multiple Comparison Test; ***$P \leq 0.001$). Data representative of two independent experiments. (c-j) H&E staining of brain sections from C57BL/6 and *Ifnar1*$^{-/-}$ mice, (non-infected mice were used as controls). Mice were sacrificed upon display of ECM in C57BL/6 mice. Coronal sections of brain microvasculature and mononuclear cell accumulation in; non-infected C57BL/6 mice (c), infected C57BL/6 mice (d-f), non-infected *Ifnar1*$^{-/-}$ mice (g), infected *Ifnar1*$^{-/-}$ mice (h-j); magnification × 400, scale bar 30 μm. Coronal sections of infected C57BL/6 mice; showing absence of endothelial cells (e, arrows) and presence of hemorrhagic foci with

iRBCs (f, arrowhead). Sections of infected *Ifnar1*$^{-/-}$ mice; showing intact endothelial cells (j, arrow), and intra-luminal iRBCs (i and j, arrowhead); magnification × 1000, scale bar 30 μm. Images representative of three mice examined per group.
(Figure presented on the next page).


a
Non-infected
Day 6 PI
C57BL/6
Ifnar1-/-
BALB/c
b
EB, (ug EB/mg brain tissue)
70
60
50
40
30
20
10
0
***
***
C57BL/6 NI
Ifnar1-/- NI
BALB/c NI
C57BL/6 Day 6 PI
Ifnar1-/- Day 6 PI
BALB/c Day 6 PI
c – non-infected
d
e
f
C57BL/6
g – non-infected
h
i
j
Ifnar1-/-

3.5.3 *Ifnar1*$^{-/-}$ mice display a delayed brain inflammatory response upon infection

To further investigate the mechanisms underlying ECM protection in *Ifnar1*$^{-/-}$ mice, we evaluated cytokine production and parasite burden within the brain tissue of C57BL/6 and *Ifnar1*$^{-/-}$ infected mice. Comparison of *P. berghei* rRNA quantification showed no significant differences during the early time course of infection, strongly suggesting that ECM protection in *Ifnar1*$^{-/-}$ mice was not attributable to reduced brain parasite burden (Figure 3.4a). We also observed that kinetics of *Cd3* mRNA expression in the brain leading up to ECM manifestations was slightly lower in *Ifnar1*$^{-/-}$ mice but not significantly different from C57BL/6 mice. This suggests that T cell migration to the brain during infection was not impaired in *Ifnar1*$^{-/-}$ mice (Figure 3.4b). Nevertheless, we found that in the brain of *Ifnar1*$^{-/-}$ mice the expression of inflammatory markers, namely *Tnf* and *Il10* had lower induction in the period preceding ECM onset and showed delayed kinetics as compared to C57BL/6 infected mice. Overall, these results indicate that in spite the accumulation of iRBCs and presence of T cells, *Ifnar1*$^{-/-}$ mice show a diminished exacerbation in their inflammatory responses, deterring the development of brain pathology within the time window of ECM development.

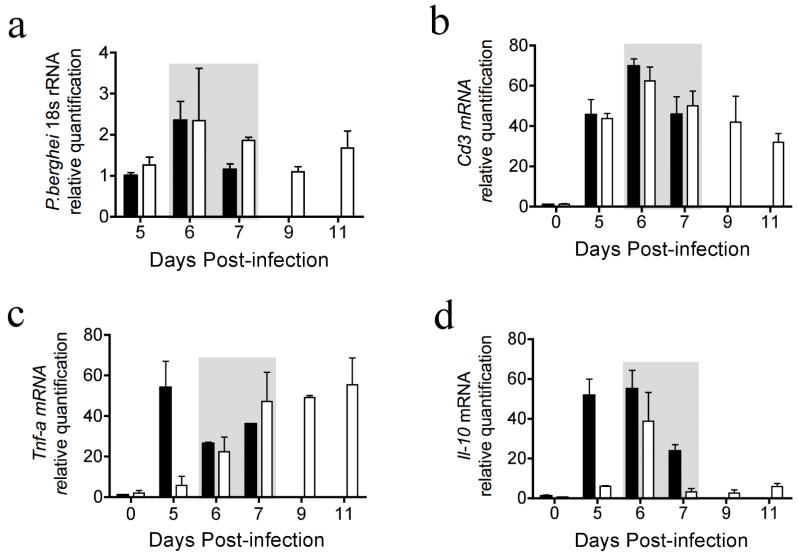


Figure 3.4 Parasite accumulation and brain inflammation markers in *Ifnar1*$^{-/-}$ infected mice. C57BL/6 (black bars) and *Ifnar1*$^{-/-}$ mice (white bars) were infected 1 × 10^6 *PbA* iRBC, i.p. and brain RNA extracted at indicated time points for RT-PCR quantification. (a) *P.berghei* rRNA relative quantification; (day 5 infected C57BL/6 mice used as calibrator), expression of (b) *Cd3*; (c) *Tnf-α* and (d) *Il-10*; (non-infected C57BL/6 mice used as calibrator, represented as day 0); (*n* = 3-4 mice per group/per day, non-parametric Mann Whitney test. Data presents means ± SD from one of two independent experiments. Time window of C57BL/6 ECM onset is shadowed.

3.5.4 Pre-sensitization for cell transfer

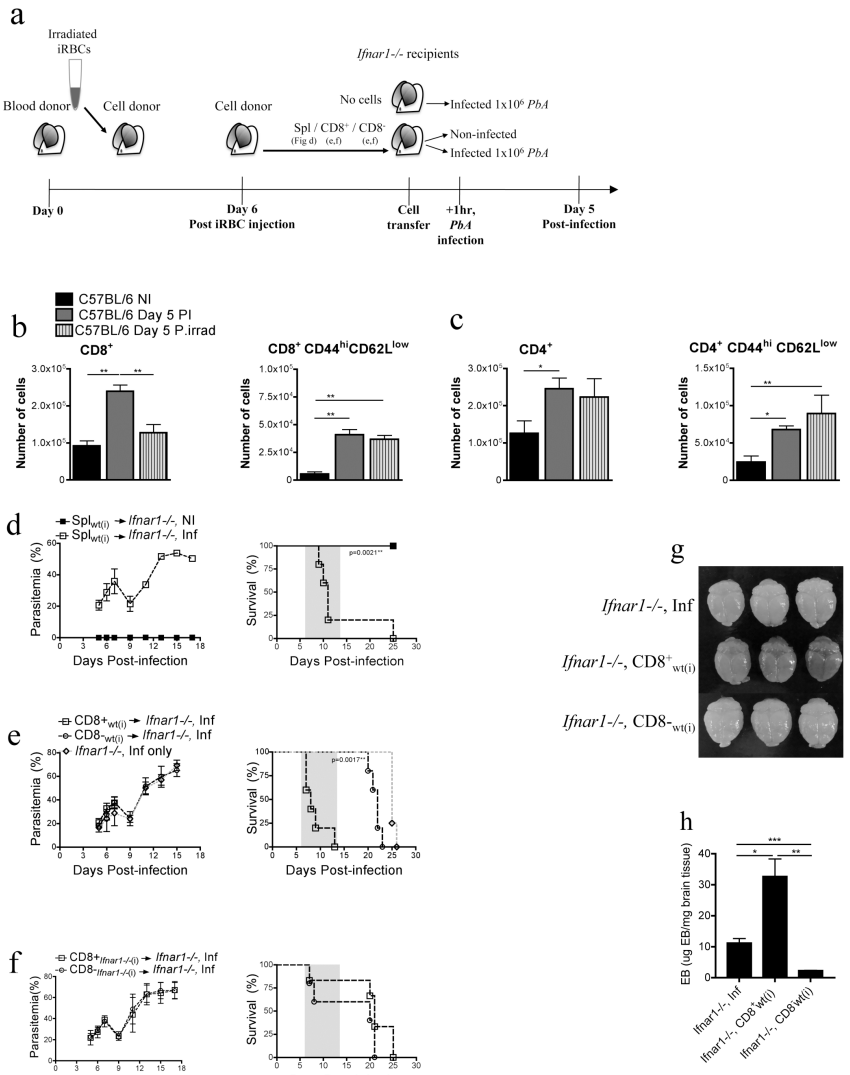
We questioned in which cell type IFNAR1 was fundamental to cause ECM development in mice. To this end we developed a novel cell transfer protocol to determine the ability of individual cell types at restoring ECM susceptibility in *Ifnar1*$^{-/-}$ mice (Figure 3.5a). Donor mice were exposed to irradiated iRBC allowing relevant

blood-stage parasite antigens (Ag) to prime *in vivo* immune system cells that were then used in transfer experiments. The irradiated parasite is unable to re-invade RBC, and therefore (exposed) donor mice do not develop parasitemia or ECM. We analyzed the T cell profiles of C57BL/6 mice that received iRBC against mice that received irradiated iRBC (Figure 3.5b, c). We found that $CD8^+$ T cells numbers of mice that received irradiated iRBC did not reach as high as that found in infected C57BL/6 mice, but showed similar numbers of activated $CD8^+$ T cells ($CD44^{hi}$ and $CD62L^{low}$) (Figure 3.5b). We noted that $CD4^+$ T cell numbers and activation profile were not significantly different between mice that received either irradiated iRBC *vs* iRBC (Figure 3.5c). Together this suggests that irradiated iRBC induces activation of both $CD8^+$ and $CD4^+$ T cells. A group of C57BL/6 cell donor mice were injected with irradiated iRBC and then infected with 1×10^6 non-irradiated *PbA* iRBC 7 days later, (as per normal infection); all mice subsequently developed ECM and died by day 7 post-infection (data not shown). We concluded that our protocol is not conferring protective immunity but is rather a protocol of immune cells pre-sensitization that occurs in absence of parasite expansion or disease development. We anticipate this method to be useful in investigating immuno-pathogenesis of malaria blood stage in future mouse models.

Figure 3.5 *Ifnar1*$^{-/-}$ mice succumb to ECM upon cell transfer of parasite-sensitized splenocytes; explicitly $CD8^+$ cells. (a) Schematic representation of pre-sensitization protocol: as described in Methods. (b) Total number and activation profile of C57BL/6 mice splenic $CD8^+$ and (c) $CD4^+$ T cells at 5 days post-challenge; non-infected NI (black bar), infected 1×10^6 *PbA* i.p (grey bar) or injected 4×10^6 irradiated iRBC i.p (lined bar); (n = 4-6 mice per group, non-parametric Mann Whitney test; $^*P \leq 0.05$, $^{**}P \leq 0.01$). Parasitemia and survival curves of *Ifnar1*$^{-/-}$ mice: (d) that received total spleen cells from C57BL/6 pre-exposed donor

mice, recipients were infected after transfer (open squares) or left non-infected (closed squares); (e and f) that received sorted pre-sensitized $CD8^+$ spleen cells (open squares) or sorted pre-sensitized $CD8^-$ depleted spleen cells (open circles) from C57BL/6 pre-exposed cell donor mice (e) or *Ifnar1*$^{-/-}$ pre-exposed cell donor mice (f), transfer control represents infected *Ifnar1*$^{-/-}$ mice that received no cells (diamonds); (*n* = 4-6 mice per group, parasitemia; unpaired *t*-test, survival curves; Log-rank Test, (e: open squares vs open circles); all significant differences displayed). Each plot represents one of at least two independent experiments. Time window of ECM, shadowed in survival plots. (g) Measurement of BBB breakage by Evans Blue perfusion in recipient *Ifnar1*$^{-/-}$ mice, as in (e); (*n* = 3-4 per group). (h) Quantification of Evan's Blue dye; (*n* = 3-4 per group, ANOVA, Tukey's Multiple Comparison Test; $^{**}P \leq 0.01$, $^{***}P \leq 0.001$). Data (g and h) represent one of two independent experiments.

(Figure presented on next page).



3.5.5 C57BL/6 spleen cells confer ECM susceptibility to *Ifnar1*$^{-/-}$ mice

Total splenocytes were transferred from C57BL/6 mice that had received irradiated iRBC (cell donors), to *Ifnar1*$^{-/-}$ mice. Upon

infection, recipient *Ifnar1*$^{-/-}$ mice showed 75% development of ECM, 6-12 days post-infection, within the ECM time window of susceptible C57BL/6 mice (Figure 3.5d). This demonstrates that C57BL/6 splenocytes pre-sensitized to irradiated iRBC are effective at restoring ECM susceptibility in *Ifnar1*$^{-/-}$ mice. In a control experiment, total splenocytes were transferred from C57BL/6 cell donor mice that had received non-infected irradiated RBC to *Ifnar1*$^{-/-}$ mice. When infected one hour post-transfer, recipient mice developed parasitemia alike *Ifnar1*$^{-/-}$ mice that received infection only (no cell transfer). Neither group of mice developed ECM, with all mice dying around day 25 post-infection from anemia and hyperparasitemia (Appendix Three, Figure S1). This shows that C57BL/6 splenocytes exposed to non-infected irradiated RBC are unable to induce ECM susceptibility in *Ifnar1*$^{-/-}$ mice indicating that RBC irradiation *per se* has no impact on ECM development in recipient mice. We then determined whether $CD8^+$ spleen cells induced ECM in *Ifnar1*$^{-/-}$ mice. Sorted $CD8^+$ or $CD8^-$ depleted cells, isolated from donor C57BL/6 mice that received irradiated iRBC, were transferred into *Ifnar1*$^{-/-}$ mice (Figure 3.5e). Recipient mice were then infected one hour after cell transfer. Parasitemia did not differ between the two groups of mice when compared against *Ifnar1*$^{-/-}$ mice that received infection only (and no cell transfer). In contrast, we found ECM development in only the group of *Ifnar1*$^{-/-}$ mice that received purified $CD8^+$ cells. This result indicate that transfer of pre-sensitized C57BL/6 $CD8^+$ cells is sufficient to restore susceptibility to ECM in *Ifnar1*$^{-/-}$ mice. Upon transfer of pre-sensitized *Ifnar1*$^{-/-}$ $CD8^+$ T cells to *Ifnar1*$^{-/-}$ mice, we found that recipient mice were not able to develop ECM, concluding that only $CD8^+$ IFNAR1+ cells, from C57BL/6 exposed mice were able to cause ECM development (Figure 3.5f). By assessing BBB integrity,

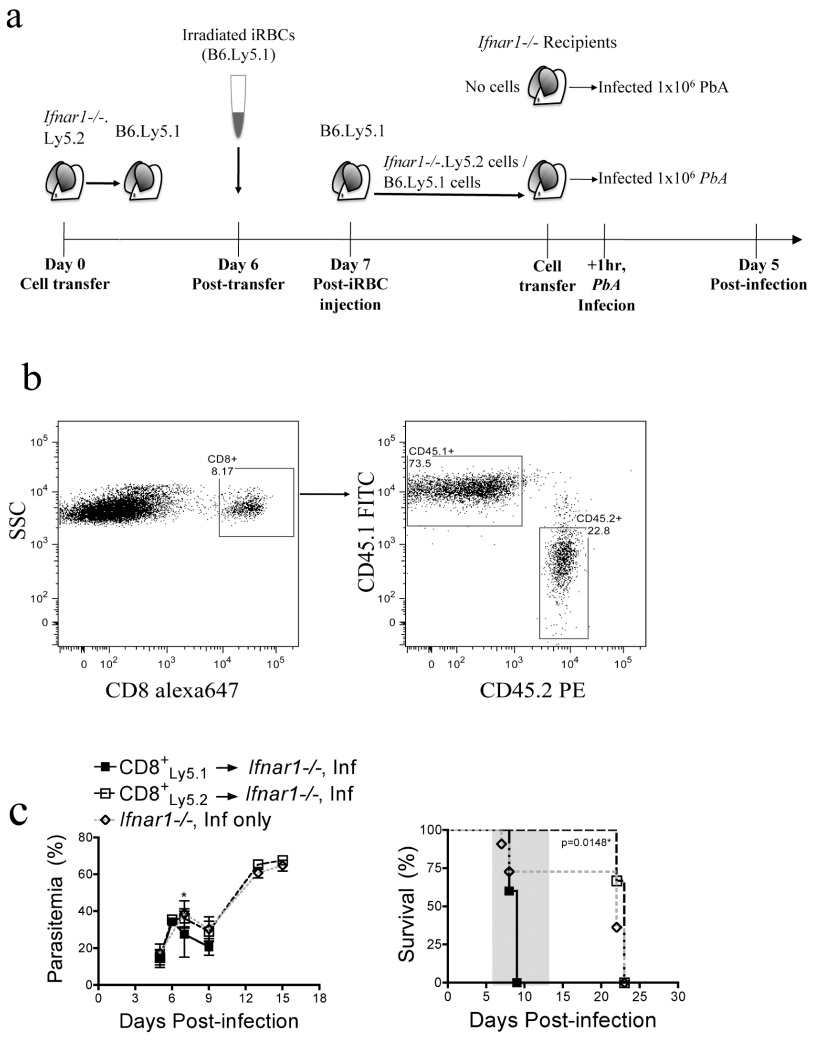
we confirmed the ability of pre-sensitized $CD8^+$ cells, isolated from C57BL/6 mice at inducing *bone fide* ECM. We observed BBB breakage only in *Ifnar1*$^{-/-}$ mice that received C57BL/6 $CD8^+$ cells, but not in $CD8^-$depleted cell recipient mice, (Figure 3.5g-h). Together these results show that IFNAR1 is critical for $CD8^+$ cell mediated abrogation of ECM resistance in *Ifnar1*$^{-/-}$ mice.

3.5.6 Intrinsic $CD8^+$ T cell properties trigger ECM development

To ascertain whether ECM induction was dependent on IFNAR1 expression in $CD8^+$cells we modified our cell transfer protocol (Figure 3.6a). $CD8^+$ cells were sorted from non-infected *Ifnar1*$^{-/-}$ .Ly5.2 mice and transferred to previously non-lethally irradiated C57BL/6.Ly5.1 mice; allowing for the discrimination of endogenous and transferred $CD8^+$ populations. Ly5.1/5.2 chimeric mice then received irradiated iRBCs from a B6.Ly5.1 mouse. Seven days later Ly5.1 $CD8^+$ and Ly5.2 $CD8^+$ cell populations were sorted (Figure 3.6b) and transferred to *Ifnar1*$^{-/-}$ recipient mice. No differences in parasitemia progression were observed in either group, as compared to *Ifnar1*$^{-/-}$ mice that received no cell transfer and infection only (Figure 3.6c).

Conclusively we found that only recipient *Ifnar1*$^{-/-}$ mice that had received Ly5.1 $IFNAR1^+$ $CD8^+$ cells developed ECM, showing 100% disease incidence, while recipients of Ly5.2 $IFNAR1^-$ $CD8^+$ cells maintained ECM resistance comparable to *Ifnar1*$^{-/-}$ mice that received no cell transfer and infection only. This strongly suggests that deficiency in IFNAR1 signaling in $CD8^+$ cells impairs their ability to induce ECM in *Ifnar1*$^{-/-}$ mice during *PbA* infection.

Figure 3.6. Intrinsic *Ifnar1*$^{-/-}$ CD8^{+} cell impairment protects from ECM.
(a) Schematic representation of experimental protocol: Non-infected *Ifnar1*$^{-/-}$ mice were used as donors of 5 × 10^{6} CD8^{+}Ly5.2+ spleen cells that were transferred to previously non-lethally irradiated B6.Ly5.1 recipient mice. Six days post transfer Ly5.1/Ly5.2 chimeric mice received 4 × 10^{6} irradiated iRBC, from a previously infected B6.Ly5.1 mouse. Seven days after iRBC infection, CD8^{+}Ly5.2+ and CD8^{+}Ly5.1+ spleen cells were FACS-sorted (b) as described in the methods section. The sorted CD8^{+} cell populations were injected back into *Ifnar1*$^{-/-}$ recipients, mice were then infected 1 × 10^{6} iRBC one hour after cell transfer. (c) Parasitemia and survival curves of *Ifnar1*$^{-/-}$ recipient mice that received from pre-exposed chimeras either: CD8^{+}Ly5.1+ (filled squares); CD8^{+}Ly5.2+ cells (open squares); or no cell transfer and infection only as control (open diamonds); (n = 3-12 mice per group, parasitemia; unpaired t-test between CD8^{+}Ly5.1+ and CD8^{+}Ly5.2+ recipient groups; *$P \leq 0.05$, survival curves; Log-rank Test, filled squares against open squares). Data pooled from three independent experiments. Time window of C57BL/6 ECM symptoms is shadowed in survival plot.
(Figure presented on next page).



3.5.7 *Ifnar1*$^{-/-}$ mice display differing activation states in spleen and brain tissue compared to C57BL/6 mice

To investigate CD8^{+} T cell impairments in *Ifnar1*$^{-/-}$ mice during *Pb*A infection we analyzed the activation and expansion of CD8^{+} T cells in the spleen at day 5 post-infection, prior to ECM

onset. We found a significant difference between non-infected C57BL/6 and *Ifnar1*$^{-/-}$ mice; that was attenuated upon infection. This indicates that infection-driven CD8^{+} T cell expansion was not impaired in *Ifnar1*$^{-/-}$ mice (Figure 3.7a). Similarly, splenic CD8^{+} T cells from *Ifnar1*$^{-/-}$ mice do not show impairments in Granzyme B (GrB) or Interferon gamma (IFN-γ) expression upon infection (Figure 3.7b and c). We also note that CD4^{+} T cell expansion and activation, as measured by IFN-γ, is not hindered in *Ifnar1*$^{-/-}$ mice upon infection, as compared to C57BL/6 mice (Appendix Four, Figure S2).

Likewise, we analyzed profiles of CD8^{+} T cells from the brain of C57BL/6 and *Ifnar1*$^{-/-}$ infected mice at day 6 post-infection, upon display of end stage ECM symptoms in control C57BL/6 mice. We found that total number of CD8^{+} T cells sequestered in the brain of *Ifnar1*$^{-/-}$ mice was significantly reduced, as compared to C57BL/6 mice (Figure 3.7d). Nevertheless, the vast majority of brain sequestered CD8^{+} T cells in both *Ifnar1*$^{-/-}$ and C57BL/6 mice were activated and showed a cytotoxic profile (Figure 3.7e and f). This indicates no faltering in sequestered *Ifnar1*$^{-/-}$ CD8^{+} T cells to exhibit a cytotoxic profile, as supported by mean fluorescence intensity analysis of GrB and IFN-γ in CD8^{+} T cells upon infection, as compared to C57BL/6 (Appendix Five, Figure S3).

We confirmed these results by analyzing sequestered CD8^{+} T cells at day 6 post-infection, from *Ifnar1*$^{-/-}$ mice that received either CD8^{+} T cells, (and were restored with ECM susceptibility) or CD8^{-} depleted cells. As expected, higher numbers of CD8^{+} T cells as well as GrB+ and IFN-γ+ CD8^{+} T cells were seen in the brain of mice that received CD8^{+} T cell transfer (Figure 3.7g-i). However these results were not significantly different, suggesting that induction of ECM in *Ifnar1*$^{-/-}$ mice is not strictly dependent on

accumulation of effector IFNAR1^{+} CD8^{+} T cells. Together these results suggest that ECM resistance in *Ifnar1*$^{-/-}$ mice operates through impaired IFNAR1 signaling in CD8^{+} T cells.

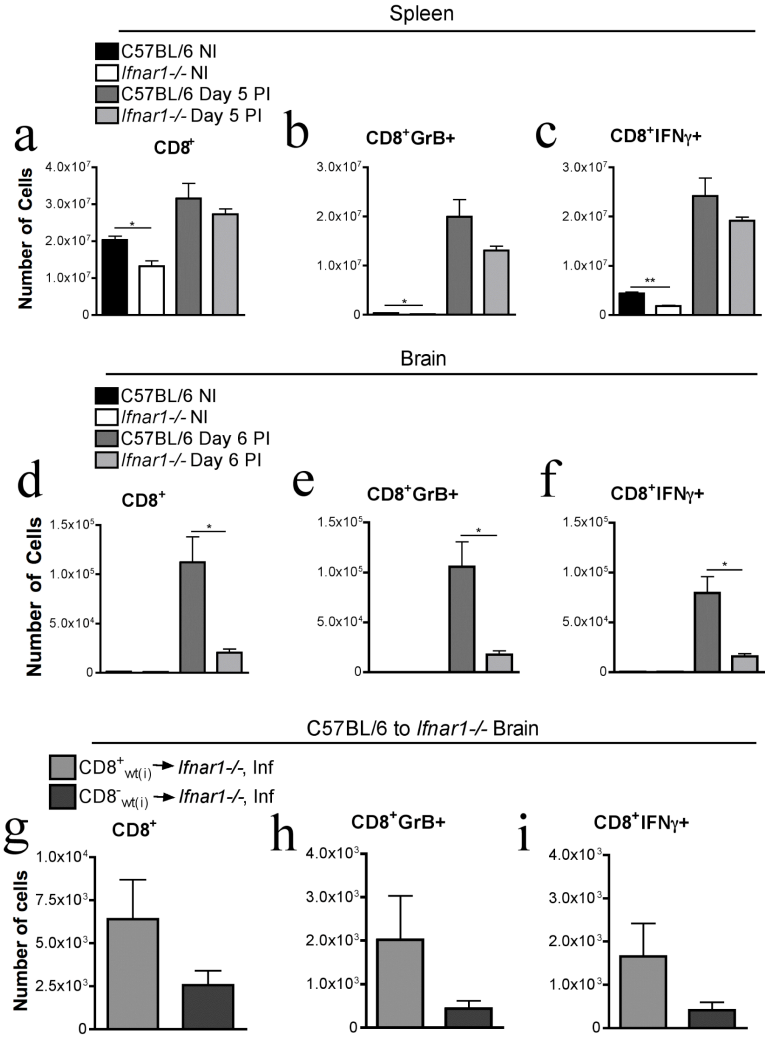


Figure 3.7 IFNAR1- $CD8^+$ T cells show activation upon infection but not accumulation in the brain. Numbers of $CD8^+$ T cells, GrB+ $CD8^+$ T cells, and IFN-γ+ $CD8^+$ T cells of infected and non-infected C57BL/6 and *Ifnar1*$^{-/-}$ mice: (a-c) in the spleen; (d-f) in perfused brains; (g-i) in perfused brains of *Ifnar1*$^{-/-}$ recipient mice that received sorted $CD8^+$ or $CD8^-$ depleted spleen cells from pre exposed C57BL/6 donor mice. Mice were analysed for $CD8^+$ T cells in the spleen on day 5 PI and in the brain on day 6 PI when C57BL/6 or *Ifnar1*$^{-/-}$ ECM induced-mice displayed symptoms of ECM development; (*n* = 3-5 mice per group, non-parametric Mann Whitney test; $*P \leq 0.05$, $**P \leq 0.01$; all statistically significant differences are shown). Data is representative of three (a-f) or two (g-i) independent experiments performed. (b: C57BL/6 non-infected group mean; 2.8×10^5, *Ifnar1*$^{-/-}$ non-infected group mean; 8.0×10^4).

3.6 Discussion

This work strengthens the role of Type I interferons/IFNAR1 signaling in human cerebral malaria and uncovers the actions of IFNAR1 in ECM development. We found that *IFNAR1* genetic variants showed protection against progression to CM in a population of Angolan children. In Gambian children, two SNPs were identified in the *IFNAR1* gene, in intron 3 and exon 4, that were associated with severe malaria and CM cases (13). However the study embarking Gambian, Kenyan and Vietnamese populations (14) highlighted positive associations in the 5' region of the *IFNAR1* locus. This was also found as a region of strong association in our study, suggesting that the 5' region of the *IFNAR1* gene-known to control transcriptional activity (35), represents a malaria susceptibility factor throughout diverse populations. Furthermore, the design established in our study proved to be advantageous, allowing us to evaluate specifically the

association of *IFNAR1* to disease progression, from uncomplicated malaria to cerebral malaria. *IFNAR1* has also been associated with AIDS progression (15) and clinical presentation of hepatitis B virus infection (36), this allows the inference that *IFNAR1* variants can impact on Type I IFN responses, and govern the course and progression of infectious diseases.

These results motivated the use of *Ifnar1*$^{-/-}$ mice to address the mechanisms underlying the pathogenesis of CM using the ECM mouse model. We found that protection from ECM in *Ifnar1*$^{-/-}$ mice was not controlled via a reduction in parasite burden. Conflicting results show no decrease in parasitemia, found when using *PbA* infection in *Ifnar1*$^{-/-}$ mice (22); *vs* a decrease in parasite burden, upon *PbA* GFP +luciferase infection (23). Although, it remains to be determined whether differing strains of *PbA* parasite dictate parasitemia burden in *Ifnar1*$^{-/-}$ mice infection. In our experimental system (*PbA*-GFP), Type I IFN appears to be dominant during other stages of ECM pathogenesis. Despite rapid and steep development of parasitemia throughout infection and parasite accumulation within the brain tissue-at comparable levels to susceptible C57BL/6 mice-this was not enough to provoke a sufficient inflammatory response for BBB breakage and mononuclear cell accumulation within brain micro-vessels. It is worth mentioning that recombinant human IFN-alpha evoked a striking reduction on parasite burden and protection from ECM in C57BL/6 mice, suggesting that the Type I IFN/IFNAR1 system has a role in parasite development (37).

Our data also points to Type I IFN/IFNAR1 controlling disease tolerance to malaria. Tolerance is a defense mechanism, that does not limit pathogen load (38), and this is in agreement with recent studies that illustrate *Ifnar1*$^{-/-}$ mice providing a survival advantage against *Plasmodium* infection (39). As a side note, Type I

IFN/IFNAR1 signaling system can also be regulated by heme oxygenase-1, a stress-response enzyme associated with CM pathogenesis in children, as we previously reported (25).

The development of a novel transfer protocol allowed focus on cellular mechanisms involved in ECM protection conferred by IFNAR1. These experiments uncovered that IFNAR1 in primed splenic $CD8^+$ T cells is unequivocally needed to abrogate ECM resistance in *Ifnar1*$^{-/-}$ mice. The cytotoxic profile of $CD8^+$ T cells in ECM induction has been shown using cell transfers, and identified perforin (40) and Granzyme B (41) as essential elements to produce cytotoxicity and effector $CD8^+$ T cell responses. As we saw no hindrance in the cytotoxic profile of splenic *Ifnar1*$^{-/-}$ $CD8^+$ T cells upon infection (Figure 3.7a-c), this suggests that priming in the spleen is not impaired. However, as we observed lower retention of activated $CD8^+$ T cells in the brain of *Ifnar1*$^{-/-}$ ECM resistant mice, Type I IFN could influence ECM via its action in the brain. Type I IFN has been shown as critical in maintaining $CD8^+$ T cells at the site of their effector function (42), this hints therefore that unresponsive *Ifnar1*$^{-/-}$ $CD8^+$ T cells may have impaired ability at accumulating within the brain tissue. We question whether enabling accumulation of IFNAR1$^-$$CD8^+$ T cells in the brain, could generate enough power to trigger an ECM response? We noted *Ifnar1*$^{-/-}$ mice that received primed $CD8^+$ splenocytes accumulated relatively low numbers of activated $CD8^+$ T cells within the brain tissue, yet developed ECM (Figure 3.7g-i). This supports the hypothesis that induction of ECM in transferred mice was not due to $CD8^+$ T cell accumulation, but rather by their expression of IFNAR1, allowing $CD8^+$ T cells to become re-activated by Type I IFN within the brain; unleashing their cytotoxic effector capacity.

Evidence has also shown that $CD8^+$ T cells require constant antigen presentation to remain at their effector site (43-45). Type I IFN has been shown to directly stimulate $CD8^+$ T cell responses during antigen cross-presentation (46). Therefore resistance of *Ifnar1*$^{-/-}$ $CD8^+$ T cells to induce ECM, could be due to their inability to respond to Type I IFN signals during antigen presentation within the brain tissue. Moreover, the exact nature of how $CD8^+$ T cells are presented in the brain is still unknown. We hypothesize that IFNAR1 impacts upon $CD8^+$ T cell stimulation at different stages of *Plasmodium* infection. We support the spleen as a primary site of parasite antigen presentation and propose that a secondary site of stimulation and presentation to $CD8^+$ T cells occurs within the brain tissue. In fact, transfer of *Ifnar1*$^{-/-}$ $CD8^-$ splenocytes caused a slight increase in susceptibility to ECM, possibly reflecting the effect of other immune cells, prominently activated $CD4^+$ T cells, in promoting ECM development (47), by an IFNAR1 independent mechanism.

Ifnar1$^{-/-}$ mice have also proved fundamental in uncovering the therapeutic use of Type I IFN in cancer (48), and in cases of Experimental autoimmune encephalomyelitis (17). However in support of our results (45), reviews how Type I IFN therapy has led to the induction of auto-immune diseases. Our results tempt the notion that blocking IFNAR1 would be therapeutic for CM protection, however this would have to done with extreme precision due to the vast array of cells expressing IFNAR1, and more importantly this would not directly mimic an *IFNAR1* gene polymorphism.

Our work collectively highlights the cohesive role of *IFNAR1* during CM development, conferring human CM protection and as an implicator of $CD8^+$ T cell pathology in mouse ECM. These

observations allow us to speculate that the impact of *IFNAR1* in human CM may operate through stimulation of $CD8^+$ T cells. Together these results classify responses of Type I IFN through IFNAR1 as vital components in CM pathogenesis.

3.6.1 Statistical Analysis

Non-parametric Mann Whitney test was used for analysis when comparing two groups of mouse data. One-way ANOVA was used in comparing three or more groups of data. Survival analysis was assessed by Log rank Test; all performed using Prism GraphPad 4. $*P \leq 0.05$ was considered statistically significant.

3.7 Acknowledgements

We are most grateful to the children that participated in this study and thank the staff of the Hospital Pediátrico David Bernardino, Drs. Filomena Silva, Emingarda Castelo Branco and Rosa Silva from the Instituto de Saúde Pública de Angola for the help on sample processing and Dr. Rui Pinto from the Clínica Sagrada Esperança de Luanda for sample collection materials. We thank Miguel Soares, (Instituto Gulbenkian de Ciência) for critical revision of the manuscript and use of reagents and Ivo Marguti for technical support and discussion. We thank Dr. Tânia Carvalho, resident pathologist at Instituto Gulbenkian de Ciência for analysis of pathology samples and the Cell Imaging Unit for expertise in cell sorting.

3.8 References

1. Issifou, S., E. Kendjo, M. A. Missinou, P. B. Matsiegui, A. Dzeing-Ella, F. A. Dissanami, M. Kombila, S. Krishna, and P. G. Kremsner. 2007. Differences in presentation of severe malaria in urban and rural Gabon. *Am J Trop Med Hyg* 77: 1015-1019.
2. Mackintosh, C. L., J. G. Beeson, and K. Marsh. 2004. Clinical features and pathogenesis of severe malaria. *Trends Parasitol* 20: 597-603.
3. Newton, C. R., and S. Krishna. 1998. Severe falciparum malaria in children: current understanding of pathophysiology and supportive treatment. *Pharmacol Ther* 79: 1-53.
4. Milner, D. A., Jr. 2010. Rethinking cerebral malaria pathology. *Curr Opin Infect Dis* 23: 456-463.
5. Carvalho, L. J. 2010. Murine cerebral malaria: how far from human cerebral malaria? *Trends Parasitol* 26: 271-272.
6. Craig, A. G., G. E. Grau, C. Janse, J. W. Kazura, D. Milner, J. W. Barnwell, G. Turner, and J. Langhorne. 2012. The role of animal models for research on severe malaria. *PLoS Pathog* 8: e1002401.
7. de Souza, J. B., J. C. Hafalla, E. M. Riley, and K. N. Couper. 2010. Cerebral malaria: why experimental murine models are required to understand the pathogenesis of disease. *Parasitology* 137: 755-772.
8. Renia, L., A. C. Gruner, and G. Snounou. 2010. Cerebral malaria: in praise of epistemes. *Trends Parasitol* 26: 275-277.
9. Riley, E. M., K. N. Couper, H. Helmby, J. C. Hafalla, J. B. de Souza, J. Langhorne, W. B. Jarra, and F. Zavala. 2010. Neuropathogenesis of human and murine malaria. *Trends Parasitol* 26: 277-278.
10. Belnoue, E., M. Kayibanda, A. M. Vigario, J. C. Deschemin, N. van Rooijen, M. Viguier, G. Snounou, and L. Renia. 2002. On the pathogenic role of brain-sequestered alphabeta CD8+ T cells in experimental cerebral malaria. *J Immunol* 169: 6369-6375.
11. Hermsen, C., T. van de Wiel, E. Mommers, R. Sauerwein, and W. Eling. 1997. Depletion of CD4+ or CD8+ T-cells prevents Plasmodium berghei induced cerebral malaria in end-stage disease. *Parasitology* 114 (Pt 1): 7-12.
12. Driss, A., J. M. Hibbert, N. O. Wilson, S. A. Iqbal, T. V. Adamkiewicz, and J. K. Stiles. 2011. Genetic

polymorphisms linked to susceptibility to malaria. *Malar J* 10: 271.

13. Aucan, C., A. J. Walley, B. J. Hennig, J. Fitness, A. Frodsham, L. Zhang, D. Kwiatkowski, and A. V. Hill. 2003. Interferon-alpha receptor-1 (IFNAR1) variants are associated with protection against cerebral malaria in the Gambia. *Genes Immun* 4: 275-282.
14. Khor, C. C., F. O. Vannberg, S. J. Chapman, A. Walley, C. Aucan, H. Loke, N. J. White, T. Peto, L. K. Khor, D. Kwiatkowski, N. Day, A. Scott, J. A. Berkley, K. Marsh, N. Peshu, K. Maitland, T. N. Williams, and A. V. Hill. 2007. Positive replication and linkage disequilibrium mapping of the chromosome 21q22.1 malaria susceptibility locus. *Genes Immun* 8: 570-576.
15. Diop, G., T. Hirtzig, H. Do, C. Coulonges, A. Vasilescu, T. Labib, J. L. Spadoni, A. Therwath, M. Lathrop, F. Matsuda, and J. F. Zagury. 2006. Exhaustive genotyping of the interferon alpha receptor 1 (IFNAR1) gene and association of an IFNAR1 protein variant with AIDS progression or susceptibility to HIV-1 infection in a French AIDS cohort. *Biomed Pharmacother* 60: 569-577.
16. Ito, T., H. Kanzler, O. Duramad, W. Cao, and Y. J. Liu. 2006. Specialization, kinetics, and repertoire of type 1 interferon responses by human plasmacytoid predendritic cells. *Blood* 107: 2423-2431.
17. Prinz, M., H. Schmidt, A. Mildner, K. P. Knobeloch, U. K. Hanisch, J. Raasch, D. Merkler, C. Detje, I. Gutcher, J. Mages, R. Lang, R. Martin, R. Gold, B. Becher, W. Bruck, and U. Kalinke. 2008. Distinct and nonredundant in vivo functions of IFNAR on myeloid cells limit autoimmunity in the central nervous system. *Immunity* 28: 675-686.
18. deWalick, S., F. H. Amante, K. A. McSweeney, L. M. Randall, A. C. Stanley, A. Haque, R. D. Kuns, K. P. MacDonald, G. R. Hill, and C. R. Engwerda. 2007. Cutting edge: conventional dendritic cells are the critical APC required for the induction of experimental cerebral malaria. *J Immunol* 178: 6033-6037.
19. Lundie, R. J., T. F. de Koning-Ward, G. M. Davey, C. Q. Nie, D. S. Hansen, L. S. Lau, J. D. Mintern, G. T. Belz, L. Schofield, F. R. Carbone, J. A. Villadangos, B. S. Crabb, and W. R. Heath. 2008. Blood-stage Plasmodium infection induces CD8+ T lymphocytes to parasite-expressed antigens, largely regulated by CD8alpha+ dendritic cells. *Proc Natl Acad Sci U S A* 105: 14509-14514.

20. O'Connell, R. M., S. K. Saha, S. A. Vaidya, K. W. Bruhn, G. A. Miranda, B. Zarnegar, A. K. Perry, B. O. Nguyen, T. F. Lane, T. Taniguchi, J. F. Miller, and G. Cheng. 2004. Type I interferon production enhances susceptibility to Listeria monocytogenes infection. *J Exp Med* 200: 437-445.
21. Stockinger, S., and T. Decker. 2008. Novel functions of type I interferons revealed by infection studies with Listeria monocytogenes. *Immunobiology* 213: 889-897.
22. Sharma, S., R. B. DeOliveira, P. Kalantari, P. Parroche, N. Goutagny, Z. Jiang, J. Chan, D. C. Bartholomeu, F. Lauw, J. P. Hall, G. N. Barber, R. T. Gazzinelli, K. A. Fitzgerald, and D. T. Golenbock. 2011. Innate immune recognition of an AT-rich stem-loop DNA motif in the Plasmodium falciparum genome. *Immunity* 35: 194-207.
23. Haque, A., S. E. Best, A. Ammerdorffer, L. Desbarrieres, M. M. de Oca, F. H. Amante, F. de Labastida Rivera, P. Hertzog, G. M. Boyle, G. R. Hill, and C. R. Engwerda. 2011. Type I interferons suppress CD4 T-cell-dependent parasite control during blood-stage Plasmodium infection. *Eur J Immunol* 41: 2688-2698.
24. e Pinto, E. A., and J. G. Alves. 2008. The causes of death of hospitalized children in Angola. *Trop Doct* 38: 66-67.
25. Sambo, M. R., M. J. Trovoada, C. Benchimol, V. Quinhentos, L. Goncalves, R. Velosa, M. I. Marques, N. Sepulveda, T. G. Clark, S. Mustafa, O. Wagner, A. Coutinho, and C. Penha-Goncalves. 2010. Transforming growth factor beta 2 and heme oxygenase 1 genes are risk factors for the cerebral malaria syndrome in Angolan children. *PloS one* 5: e11141.
26. Greenwood, B. M., and J. R. Armstrong. 1991. Comparison of two simple methods for determining malaria parasite density. *Trans R Soc Trop Med Hyg* 85: 186-188.
27. Barrett, J. C., B. Fry, J. Maller, and M. J. Daly. 2005. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21: 263-265.
28. Luna, A., and K. K. Nicodemus. 2007. snp.plotter: an R-based SNP/haplotype association and linkage disequilibrium plotting package. *Bioinformatics* 23: 774-776.
29. Gonzalez, J. R., L. Armengol, X. Sole, E. Guino, J. M. Mercader, X. Estivill, and V. Moreno. 2007. SNPassoc: an R package to perform whole genome association studies. *Bioinformatics* 23: 644-645.
30. Muller, U., U. Steinhoff, L. F. Reis, S. Hemmi, J. Pavlovic, R. M. Zinkernagel, and M. Aguet. 1994. Functional role of type

I and type II interferons in antiviral defense. *Science* 264: 1918-1921.

31. Franke-Fayard, B., H. Trueman, J. Ramesar, J. Mendoza, M. van der Keur, R. van der Linden, R. E. Sinden, A. P. Waters, and C. J. Janse. 2004. A Plasmodium berghei reference line that constitutively expresses GFP at a high level throughout the complete life cycle. *Mol Biochem Parasitol* 137: 23-33.
32. Pamplona, A., A. Ferreira, J. Balla, V. Jeney, G. Balla, S. Epiphanio, A. Chora, C. D. Rodrigues, I. P. Gregoire, M. Cunha-Rodrigues, S. Portugal, M. P. Soares, and M. M. Mota. 2007. Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. *Nature medicine* 13: 703-710.
33. Epiphanio, S., S. A. Mikolajczak, L. A. Goncalves, A. Pamplona, S. Portugal, S. Albuquerque, M. Goldberg, S. Rebelo, D. G. Anderson, A. Akinc, H. P. Vornlocher, S. H. Kappe, M. P. Soares, and M. M. Mota. 2008. Heme oxygenase-1 is an anti-inflammatory host factor that promotes murine plasmodium liver infection. *Cell Host Microbe* 3: 331-338.
34. Ferreira, A., I. Marguti, I. Bechmann, V. Jeney, A. Chora, N. R. Palha, S. Rebelo, A. Henri, Y. Beuzard, and M. P. Soares. 2011. Sickle hemoglobin confers tolerance to Plasmodium infection. *Cell* 145: 398-409.
35. Zhou, J., J. D. Huang, V. K. Poon, D. Q. Chen, C. C. Chan, F. Ng, X. Y. Guan, R. M. Watt, L. Lu, K. Y. Yuen, and B. J. Zheng. 2009. Functional dissection of an IFN-alpha/beta receptor 1 promoter variant that confers higher risk to chronic hepatitis B virus infection. *Journal of hepatology* 51: 322-332.
36. Song le, H., N. T. Xuan, N. L. Toan, V. Q. Binh, A. B. Boldt, P. G. Kremsner, and J. F. Kun. 2008. Association of two variants of the interferon-alpha receptor-1 gene with the presentation of hepatitis B virus infection. *Eur Cytokine Netw* 19: 204-210.
37. Vigario, A. M., E. Belnoue, A. C. Gruner, M. Mauduit, M. Kayibanda, J. C. Deschemin, M. Marussig, G. Snounou, D. Mazier, I. Gresser, and L. Renia. 2007. Recombinant human IFN-alpha inhibits cerebral malaria and reduces parasite burden in mice. *J Immunol* 178: 6416-6425.
38. Medzhitov, R., D. S. Schneider, and M. P. Soares. 2012. Disease tolerance as a defense strategy. *Science* 335: 936-941.

39. Erdman, L. K., and K. C. Kain. 2011. Taking the sting out of malaria. *Immunity* 35: 149-151.
40. Nitcheu, J., O. Bonduelle, C. Combadiere, M. Tefit, D. Seilhean, D. Mazier, and B. Combadiere. 2003. Perforin-dependent brain-infiltrating cytotoxic CD8+ T lymphocytes mediate experimental cerebral malaria pathogenesis. *J Immunol* 170: 2221-2228.
41. Haque, A., S. E. Best, K. Unosson, F. H. Amante, F. de Labastida, N. M. Anstey, G. Karupiah, M. J. Smyth, W. R. Heath, and C. R. Engwerda. 2011. Granzyme B expression by CD8+ T cells is required for the development of experimental cerebral malaria. *J Immunol* 186: 6148-6156.
42. Marrack, P., J. Kappler, and T. Mitchell. 1999. Type I interferons keep activated T cells alive. *J Exp Med* 189: 521-530.
43. Galea, I., M. Bernardes-Silva, P. A. Forse, N. van Rooijen, R. S. Liblau, and V. H. Perry. 2007. An antigen-specific pathway for CD8 T cells across the blood-brain barrier. *J Exp Med* 204: 2023-2030.
44. Miu, J., N. H. Hunt, and H. J. Ball. 2008. Predominance of interferon-related responses in the brain during murine malaria, as identified by microarray analysis. *Infection and immunity* 76: 1812-1824.
45. Stewart, T. A. 2003. Neutralizing interferon alpha as a therapeutic approach to autoimmune diseases. *Cytokine & growth factor reviews* 14: 139-154.
46. Le Bon, A., V. Durand, E. Kamphuis, C. Thompson, S. Bulfone-Paus, C. Rossmann, U. Kalinke, and D. F. Tough. 2006. Direct stimulation of T cells by type I IFN enhances the CD8+ T cell response during cross-priming. *J Immunol* 176: 4682-4689.
47. Villegas-Mendez, A., R. Greig, T. N. Shaw, J. B. de Souza, E. Gwyer Findlay, J. S. Stumhofer, J. C. Hafalla, D. G. Blount, C. A. Hunter, E. M. Riley, and K. N. Couper. 2012. IFN-gamma-producing CD4+ T cells promote experimental cerebral malaria by modulating CD8+ T cell accumulation within the brain. *J Immunol* 189: 968-979.
48. Johns, T. G., I. R. Mackay, K. A. Callister, P. J. Hertzog, R. J. Devenish, and A. W. Linnane. 1992. Antiproliferative potencies of interferons on melanoma cell lines and xenografts: higher efficacy of interferon beta. *Journal of the National Cancer Institute* 84: 1185-1190.

Chapter 4

General Discussion

This thesis work has shown through the development of a novel pre-sensitization protocol the essential role that type I IFN priming plays in $CD8^+$ T cell mediated ECM induction. Our work on understanding the intrinsic properties of $CD8^+$ T cell responses to type I IFN also suggests future experiments. Selective co-transfer experiments enabled information to be gained on understanding the necessary roles of type I IFN on surrounding cell types upon $CD8^+$ T cell expansion. Preliminary results show that $CD8^+$ T cells from C57BL/6 mice can be primed in an IFNAR1- environment and are still able to induce ECM in previously resistant *Ifnar1*$^{-/-}$ mice. This confirms that IFNAR1 expression on $CD8^+$ T cells is critical in ECM induction.

Future prospects for further exploration of type I IFN mediated CD8+ T cell properties in the context of malaria infection, could include the following aspects. As our work focused on day five post-infection for the time point of assessing T cell activation, supported by (Ferreira et al., 2011; Villegas-Mendez et al., 2011), it would be of interest to also assess a kinetics curve for proliferation of $CD8^+$ T cells and investigate activation states, such as CD62L and CD44 expression, which would enable us to assess migration of $CD8^+$ T cells from the spleen. This would allow us to ascertain timings of type I IFN action upon $CD8^+$ T cells during innate and adaptive immune responses across infection, and highlight any defects in cell migration to the brain tissue site.

Type I IFN also governs $CD8^+$ T cell clonal expansion and memory differentiation (Aichele et al., 2006; Thompson et al., 2006), with 5-10% of effector $CD8^+$ T cells developing into memory T cells (Kolumam et al., 2005). Therefore we could explore in depth $CD8^+$ T cell sub-populations upon parasite infection, and thus

determine whether type I IFN signaling affects expansion and differentiation.

Further it is of specific interest to also evaluate expression of chemokines in *Ifnar1*$^{-/-}$ *vs* C57BL/6 CD8^{+} T cells and it is of interest to perform kinetic studies of entry and maintenance of CD8^{+} T cells within the brain tissue.

In fact secondary memory CD8^{+} T cells, defined as repeatedly exposed to Ag, have increased expression of GrB and CTL activity, but show an exhaustive profile (Nolz and Harty, 2011; Ou et al., 2001), and have their capacity to kill worn out. This is an interesting idea that could warrant investigation in malaria infections, and would allow us to explore the different CD8^{+} T cell responses upon exposure to the parasite Ag at the spleen and brain site. This could be achieved by repeating our pre-sensitization step several times in wild-type mice and then scoring the power of CD8^{+} T cells to induce CM. Exhaustion of parasite specific CD8^{+} T cells could be monitored following CD8^{+} T cells activation status in the spleen and ultimately the exhaustion mechanism would offer an explanation for the high incidence of CM in young children. This would parallel to the accumulation of exhausted B cells and CD4^{+} T cells in individuals living in endemic regions of malaria infection (Illingworth et al., 2013), as a new concept to explain naturally acquired immunity.

The use of *Ifnar1*$^{-/-}$ mice has proved essential in understanding the system of type I IFN during ECM. *Ifnar1*$^{-/-}$ mice are reported by The Jackson Laboratory to have increased susceptibility to viral infections, as well as higher levels of myeloid lineage cells and a reduced response to immuno-stimulatory DNA and Lipopolysaccharide (LPS) via the TLR pathway (Smith et al., 2005).

It was also found that genes dependent on the NF-κB pathway, c-FLIP and iNOS, are further down regulated in the *Ifnar1*$^{-/-}$ mice (Huys et al., 2009). However as we saw increased proliferation of peripheral parasites within the *Ifnar1*$^{-/-}$ mice model during infection, presence of *PbA* and immune responses mediated through IL-10 and TNF-α for example, in the brain tissue, in absence of ECM, we can infer that a dampened down immune response in *Ifnar1*$^{-/-}$ mice represents a tolerance mechanism during a parasite mediated infection. It remains to be determined at which level such effects are exerted and whether they are exclusively due to absence of IFNAR1 expression in CD8^{+} cells, which could be addressed by using mouse models with conditional deletions of IFNAR1 in specific cells populations, for example CD8^{+}, CD4^{+} and DC, (Mizutani et al., 2012; Prinz et al., 2008). Thus, this raises the possibility that disease tolerance strategies (Gozzelino et al., 2012), can be achieved by deterring innate immunity mechanisms that enhance adaptive pro-inflammatory responses and impact on immune-mediated tissue destruction in malaria infections.

As we observed both presence and seemingly responsive IFNAR1- CD8^{+} T cells at both critical sites of malaria infection, spleen and brain, it is now of importance to exactly uncover where management of CD8^{+} T cells is lacking in the *Ifnar1*$^{-/-}$ mice shielding their pathogenic function. Of interest to us is the method by which CD8^{+} T cells are presented and carry out their pathogenic role within the brain tissue site during malaria infection. We began work into exploring the potential role of endothelial cells (EC) at this site and whether they could be targeted for APC ability. We hypothesize that parasite Ag could be presented by EC within brain microvasculature to CD8^{+} T cells. The close interaction between

$CD8^+$ T cells and EC is suggestive as a site of Ag presentation, however this would require the ability of ECs to act as APC.

Research has begun to examine the potential of EC as APCs (McDouall et al., 1996), showing that human umbilical EC can provide allogenic stimulation to $CD8^+$ T cells, due to their expression of MHC-I molecules on resting EC, and furthermore EC have been previously classified as "semi-professional" APCs (Epperson and Pober, 1994). In malaria infections, the role of EC has mostly been focused on their expression of ICAM-1 and its role in sequestering $CD8^+$ T cells within the micro-vessels; mice deficient in ICAM-1 are protected from ECM development (Sun et al., 2003). We believe that the role of EC during ECM needs to be shifted towards the perspective that EC may be one possible vehicle of Ag presentation to $CD8^+$ T cells during CM pathogenesis in the brain tissue, something that has been suggested but never proved in ECM models (Wheway et al., 2013).

Type I IFN has been shown to act upon human EC (da Silva et al., 2002; Erdmann et al., 2011; Mintzer et al., 1998), and as EC can produce type I IFN via STAT4 and 6 (Torpey et al., 2004), this shows their ability at producing and being responsive to type I IFN. As presence of type I IFN during *PbA* infection leads to up-regulation of genes for Ag processing and presentation (Miu et al., 2008), as well as up-regulation of components of the MHC I and II pathways during *PbA* infection (Hervas-Stubbs et al., 2011; Miu et al., 2008; Smith et al., 2005), this supports our hypothesis that in the context of malaria infection and type I IFN action, the EC could act as an APC within the brain tissue site of infection.

We believe this mechanism of Ag presentation could occur through the phenomenon of cross-presentation. Type I IFN is critical in coordinating DC activation and response in T cell

stimulation (Montoya et al., 2002), and it was reported that DCs can process and present extracellular Ag via 'cross-presentation' (Bevan, 1976; Lundie et al., 2008). This hypothesis would enable us to explain a puzzling result we have obtained for IFNAR1- $CD8^+$ T cells taken from the brain tissue. We see these cells express GrB; have migrated and accumulated in the brain tissue, (until day six post-infection); but no ECM pathogenesis is seen, as confirmed by no disruption of the blood brain barrier. A promising line of work would aim therefore to resolve whether deficiencies in Ag presentation are seen within *Ifnar1*$^{-/-}$ mice, and whether absence of IFNAR1 signaling in the context of Ag presentation results in $CD8^+$ T cells not being maintained within the brain tissue or not being able to perform effector killing functions. Another interesting aspect worth to investigate are the possible mechanisms used by EC to acquire parasite Ag during infection. Furthermore as type I IFN has been shown to act directly on DC, leading to effective cross-presentation of Ag, to Ag specific $CD8^+$ T cells (Le Bon et al., 2003; Le Bon et al., 2001), this reinforces a role of type I IFN acting upon EC to enhance its APC function.

4.1 Therapeutic targets for cerebral malaria

To date there are no therapies in action for specific treatment of CM. Work done by (Cabrales et al., 2010; Eisenhut, 2012; Polder et al., 1991), has suggested therapies aimed at triggering vaso-dilation would reduce the vessel restriction and occlusion that causes CM development. Work has also focused on down-regulating the inflammatory response via neutralizing IP-10 and *IP-10*$^{-/-}$ mice (ligand for CXCR3 chemokine), showed protection from development of ECM (Nie et al., 2009). TNF is also a dictating

factor in CM development, and has been targeted in CM therapy. However a clinical study for the use of anti-TNF on Gambian CM patients reported no effect for increasing survival (van Hensbroek et al., 1996).

Outside of malaria infection, an FDA approved treatment for multiple sclerosis (MS) has aimed at inhibiting T cell egress from lymph nodes in the lymphatic circulation, as MS is aggravated by T cells within the brain micro-vessels (Hla and Brinkmann, 2011). This drug treatment therefore may pose a potential candidate in prevention of ECM. In fact a recent study in Ugandan children showed lower plasma levels of S1P – the designated drug target - in CM *vs* uncomplicated malaria cases (Finney et al., 2011).

This Ph.D. thesis work points to a great potential in inhibiting the action of type I IFN, via hindering signaling through the IFNAR1 for CM protection. The action of type I IFN is vast, during both the innate and adaptive immune response, therefore silencing or shutting down the type I IFN response would need to be carefully co-ordinated. Manipulation of type I IFN actions has been proposed for beneficial outcomes (Smith et al., 2005). In fact, very recently clinical trials have found promising results for the inhibition of the IFNAR1 receptor in systemic sclerosis (SSc) patients (Wang et al., 2013). Increased type I IFN activity is associated with pathogenesis of SSc and Systemic lupus erythematous (SLE), where over-expression of type I IFN-inducible genes and plasmocytoid (p)DC are found in the skin and blood of patients (Tan et al., 2006; Yao et al., 2009). Furthermore actual induction of SLE or SSc has been seen in patients receiving type I therapy for chronic viral hepatitis, (Crow, 2010; Solans et al., 2004). This provides early evidence that the zealous effects of type I IFN during host immune responses can be controlled. This negative action of type I IFN has been put to use

as a therapy in targeting apoptosis in relapsing-remitting multiple sclerosis. The action of type I IFN can lead to apoptosis of the immune cells involved in the pathogenesis (Badovinac et al., 2005; Dhib-Jalbut and Marks, 2010; Zula et al., 2011) and eliminate these causative cell types. Therefore if it would be possible in our hands to selectively inhibit action of type I IFN at time points during malaria infection, we could aim at inhibiting type I IFN action upon T cells and suppress $CD8^+$ T cell mediated induction of CM pathogenesis.

However in strong disagreement with the results of this Ph.D. work and this hypothesis, are the following papers by (Morrell et al., 2011; Vigario et al., 2007), who found that treatment with recombinant-IFN-β blocked type I IFN production which led to decreased iRBC engagement and entry into the brain, delaying onset of CM in *PbA* infection.

Collectively these findings clearly reflect the plethora of actions type I IFN takes during immune responses, and how close these type I IFN therapeutic effects are in becoming harmful over protective. Together with the work presented here, these investigations definitely warrant future efforts for uncovering how IFNAR1 could be targeted for ECM protection in malaria infections, and how important selective cell candidates and tissue sites may be in this discovery.

4.2 Final Conclusions

This work collectively supports the vital role of type I IFN in CM development. Figure 4.1 represents the final hypothesis of this

thesis work and the role that type I IFN governs over the key players in ECM development.

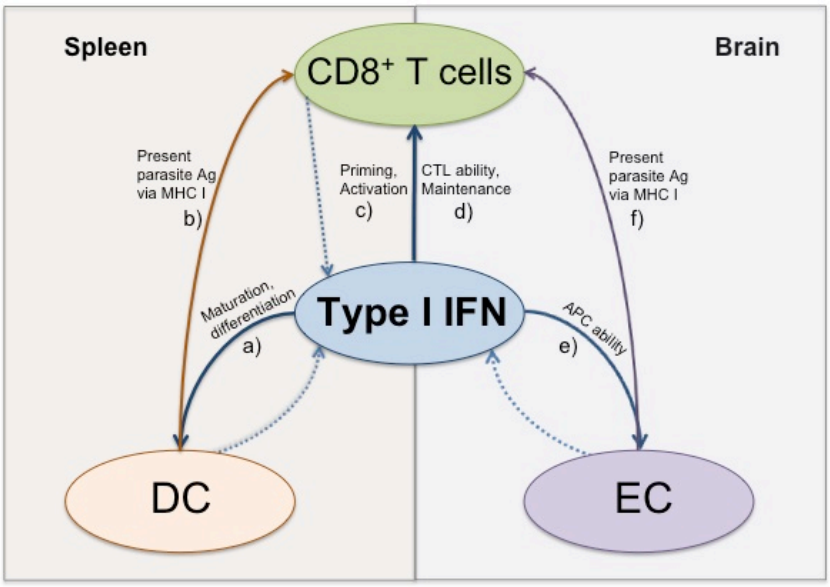


Figure 4.1. The Role of Type I IFN - IFNAR1 signaling in CM. Type I IFN can be seen as a central part in building an immune response to parasite invasion in CM development. Dendritic cells (DC), Endothelial cells (EC) and $CD8^+$ T cells all have the ability to produce and release type I IFN (blue dotted lines). In turn, during a primary immune response in the spleen, type I IFN can act upon DC (a) to allow them to present parasite Ag to $CD8^+$ T cells (b), whilst in turn type I IFN can act on $CD8^+$ T cells, priming them in preparation for secondary Ag presentation (c). In the brain tissue site, we propose type I IFN is vital in maintaining $CD8^+$ T cells at effector site (d), mediated through $CD8^+$ T cell expression of IFNAR1. Type I IFN may act upon EC, enabling APC capacity (e), which leads to presentation of parasite Ag to $CD8^+$ T cells (f). The action of type I IFN on these cell types is mediated through their expression of IFNAR1.

We propose that the novel protocol developed and explored in Chapter two be instrumental in future ECM research, and used as a

method for further individual cell type understanding of the mechanisms leading to ECM development.

It is important to close this work with the initial data that led to the beginning of this thesis. We must always remember the relevance of the mouse model and these discoveries to the human cases of CM. This work supports primary findings of a role of IFNAR1 in CM susceptibility in human cases, and the work presented here now supports revisiting these cases of CM. Together this could uncover therapeutic targets and preventative measure of CM development for the many countries still harrowed by malaria infection.

4.3 References

Aichele, P., Unsoeld, H., Koschella, M., Schweier, O., Kalinke, U., and Vucikuja, S. (2006). CD8 T cells specific for lymphocytic choriomeningitis virus require type I IFN receptor for clonal expansion. J Immunol *176*, 4525-4529.

Badovinac, V.P., Messingham, K.A., Jabbari, A., Haring, J.S., and Harty, J.T. (2005). Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination. Nature medicine *11*, 748-756.

Bevan, M.J. (1976). Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during in vivo priming. J Immunol *117*, 2233-2238.

Cabrales, P., Zanini, G.M., Meays, D., Frangos, J.A., and Carvalho, L.J. (2010). Murine cerebral malaria is associated with a vasospasm-like microcirculatory dysfunction, and survival upon rescue treatment is markedly increased by nimodipine. The American journal of pathology *176*, 1306-1315.

Crow, M.K. (2010). Interferon-alpha: a therapeutic target in systemic lupus erythematosus. Rheumatic diseases clinics of North America *36*, 173-186, x.

da Silva, A.J., Brickelmaier, M., Majeau, G.R., Lukashin, A.V., Peyman, J., Whitty, A., and Hochman, P.S. (2002). Comparison of gene expression patterns induced by treatment of human umbilical vein endothelial cells with IFN-alpha 2b vs. IFN-beta 1a: understanding the functional relationship between distinct type I interferons that act through a common receptor. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research *22*, 173-188.

deWalick, S., Amante, F.H., McSweeney, K.A., Randall, L.M., Stanley, A.C., Haque, A., Kuns, R.D., MacDonald, K.P., Hill, G.R., and Engwerda, C.R. (2007). Cutting edge: conventional dendritic cells are the critical APC required for the induction of experimental cerebral malaria. J Immunol *178*, 6033-6037.

Dhib-Jalbut, S., and Marks, S. (2010). Interferon-beta mechanisms of action in multiple sclerosis. Neurology *74 Suppl 1*, S17-24.

Diop, G., Hirtzig, T., Do, H., Coulonges, C., Vasilescu, A., Labib, T., Spadoni, J.L., Therwath, A., Lathrop, M., Matsuda, F.*, et al.* (2006). Exhaustive genotyping of the interferon alpha receptor 1 (IFNAR1) gene and association of an IFNAR1 protein variant with AIDS progression or susceptibility to HIV-1 infection in a French AIDS cohort. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie *60*, 569-577.
Eisenhut, M. (2012). The contribution of vasospasm in the pathophysiology of cerebral malaria. Future microbiology *7*, 1341-1343.
Epperson, D.E., and Pober, J.S. (1994). Antigen-presenting function of human endothelial cells. Direct activation of resting CD8 T cells. J Immunol *153*, 5402-5412.
Erdmann, J., Vitale, G., van Koetsveld, P.M., Croze, E., Sprij-Mooij, D.M., Hofland, L.J., and van Eijck, C.H. (2011). Effects of interferons alpha/beta on the proliferation of human micro- and macrovascular endothelial cells. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research *31*, 451-458.
Ferreira, A., Marguti, I., Bechmann, I., Jeney, V., Chora, A., Palha, N.R., Rebelo, S., Henri, A., Beuzard, Y., and Soares, M.P. (2011). Sickle hemoglobin confers tolerance to Plasmodium infection. Cell *145*, 398-409.
Finney, C.A., Hawkes, C.A., Kain, D.C., Dhabangi, A., Musoke, C., Cserti-Gazdewich, C., Oravecz, T., Liles, W.C., and Kain, K.C. (2011). S1P is associated with protection in human and experimental cerebral malaria. Mol Med *17*, 717-725.
Gozzelino, R., Andrade, B.B., Larsen, R., Luz, N.F., Vanoaica, L., Seixas, E., Coutinho, A., Cardoso, S., Rebelo, S., Poli, M.*, et al.* (2012). Metabolic adaptation to tissue iron overload confers tolerance to malaria. Cell host & microbe *12*, 693-704.
Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C., and Amigorena, S. (2002). Antigen presentation and T cell stimulation by dendritic cells. Annual review of immunology *20*, 621-667.
Hervas-Stubbs, S., Perez-Gracia, J.L., Rouzaut, A., Sanmamed, M.F., Le Bon, A., and Melero, I. (2011). Direct effects of type I interferons on cells of the immune system. Clinical cancer research : an official journal of the American Association for Cancer Research *17*, 2619-2627.

Hla, T., and Brinkmann, V. (2011). Sphingosine 1-phosphate (S1P): Physiology and the effects of S1P receptor modulation. Neurology *76*, S3-8.
Huys, L., Van Hauwermeiren, F., Dejager, L., Dejonckheere, E., Lienenklaus, S., Weiss, S., Leclercq, G., and Libert, C. (2009). Type I interferon drives tumor necrosis factor-induced lethal shock. The Journal of experimental medicine *206*, 1873-1882.
Illingworth, J., Butler, N.S., Roetynck, S., Mwacharo, J., Pierce, S.K., Bejon, P., Crompton, P.D., Marsh, K., and Ndungu, F.M. (2013). Chronic exposure to Plasmodium falciparum is associated with phenotypic evidence of B and T cell exhaustion. J Immunol *190*, 1038-1047.
Kolumam, G.A., Thomas, S., Thompson, L.J., Sprent, J., and Murali-Krishna, K. (2005). Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. The Journal of experimental medicine *202*, 637-650.
Le Bon, A., Etchart, N., Rossmann, C., Ashton, M., Hou, S., Gewert, D., Borrow, P., and Tough, D.F. (2003). Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. Nature immunology *4*, 1009-1015.
Le Bon, A., Schiavoni, G., D'Agostino, G., Gresser, I., Belardelli, F., and Tough, D.F. (2001). Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. Immunity *14*, 461-470.
Lundie, R.J., de Koning-Ward, T.F., Davey, G.M., Nie, C.Q., Hansen, D.S., Lau, L.S., Mintern, J.D., Belz, G.T., Schofield, L., Carbone, F.R., *et al.* (2008). Blood-stage Plasmodium infection induces CD8+ T lymphocytes to parasite-expressed antigens, largely regulated by CD8alpha+ dendritic cells. Proceedings of the National Academy of Sciences of the United States of America *105*, 14509-14514.
McDouall, R.M., Page, C.S., Hafizi, S., Yacoub, M.H., and Rose, M.L. (1996). Alloproliferation of purified CD4+ T cells to adult human heart endothelial cells, and study of second-signal requirements. Immunology *89*, 220-226.
Mintzer, R.J., Croze, E., Rubanyi, G.M., and Johns, A. (1998). Differential effects of IFN-beta1b on the proliferation of human vascular smooth muscle and endothelial cells. Journal of interferon & cytokine research : the official journal of the

International Society for Interferon and Cytokine Research *18*, 939-945.
Miu, J., Hunt, N.H., and Ball, H.J. (2008). Predominance of interferon-related responses in the brain during murine malaria, as identified by microarray analysis. Infection and immunity *76*, 1812-1824.
Mizutani, T., Neugebauer, N., Putz, E.M., Moritz, N., Simma, O., Zebedin-Brandl, E., Gotthardt, D., Warsch, W., Eckelhart, E., Kantner, H.P.*, et al.* (2012). Conditional IFNAR1 ablation reveals distinct requirements of Type I IFN signaling for NK cell maturation and tumor surveillance. Oncoimmunology *1*, 1027-1037.
Montoya, M., Schiavoni, G., Mattei, F., Gresser, I., Belardelli, F., Borrow, P., and Tough, D.F. (2002). Type I interferons produced by dendritic cells promote their phenotypic and functional activation. Blood *99*, 3263-3271.
Morrell, C.N., Srivastava, K., Swaim, A., Lee, M.T., Chen, J., Nagineni, C., Hooks, J.J., and Detrick, B. (2011). Beta interferon suppresses the development of experimental cerebral malaria. Infection and immunity *79*, 1750-1758.
Nie, C.Q., Bernard, N.J., Norman, M.U., Amante, F.H., Lundie, R.J., Crabb, B.S., Heath, W.R., Engwerda, C.R., Hickey, M.J., Schofield, L.*, et al.* (2009). IP-10-mediated T cell homing promotes cerebral inflammation over splenic immunity to malaria infection. PLoS pathogens *5*, e1000369.
Nolz, J.C., and Harty, J.T. (2011). Protective capacity of memory CD8+ T cells is dictated by antigen exposure history and nature of the infection. Immunity *34*, 781-793.
Ou, R., Zhou, S., Huang, L., and Moskophidis, D. (2001). Critical role for alpha/beta and gamma interferons in persistence of lymphocytic choriomeningitis virus by clonal exhaustion of cytotoxic T cells. Journal of virology *75*, 8407-8423.
Pichyangkul, S., Yongvanitchit, K., Kum-arb, U., Hemmi, H., Akira, S., Krieg, A.M., Heppner, D.G., Stewart, V.A., Hasegawa, H., Looareesuwan, S.*, et al.* (2004). Malaria blood stage parasites activate human plasmacytoid dendritic cells and murine dendritic cells through a Toll-like receptor 9-dependent pathway. J Immunol *172*, 4926-4933.
Polder, T.W., Jerusalem, C.R., and Eling, W.M. (1991). Morphological characteristics of intracerebral arterioles in

clinical (Plasmodium falciparum) and experimental (Plasmodium berghei) cerebral malaria. Journal of the neurological sciences *101*, 35-46.
Potter, S., Chan-Ling, T., Ball, H.J., Mansour, H., Mitchell, A., Maluish, L., and Hunt, N.H. (2006). Perforin mediated apoptosis of cerebral microvascular endothelial cells during experimental cerebral malaria. International journal for parasitology *36*, 485-496.
Prinz, M., Schmidt, H., Mildner, A., Knobeloch, K.P., Hanisch, U.K., Raasch, J., Merkler, D., Detje, C., Gutcher, I., Mages, J.*, et al.* (2008). Distinct and nonredundant in vivo functions of IFNAR on myeloid cells limit autoimmunity in the central nervous system. Immunity *28*, 675-686.
Renia, L., Potter, S.M., Mauduit, M., Rosa, D.S., Kayibanda, M., Deschemin, J.C., Snounou, G., and Gruner, A.C. (2006). Pathogenic T cells in cerebral malaria. International journal for parasitology *36*, 547-554.
Santini, S.M., Lapenta, C., Logozzi, M., Parlato, S., Spada, M., Di Pucchio, T., and Belardelli, F. (2000). Type I interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity in vitro and in Hu-PBL-SCID mice. The Journal of experimental medicine *191*, 1777-1788.
Santodonato, L., D'Agostino, G., Nisini, R., Mariotti, S., Monque, D.M., Spada, M., Lattanzi, L., Perrone, M.P., Andreotti, M., Belardelli, F.*, et al.* (2003). Monocyte-derived dendritic cells generated after a short-term culture with IFN-alpha and granulocyte-macrophage colony-stimulating factor stimulate a potent Epstein-Barr virus-specific CD8+ T cell response. J Immunol *170*, 5195-5202.
Smith, P.L., Lombardi, G., and Foster, G.R. (2005). Type I interferons and the innate immune response--more than just antiviral cytokines. Molecular immunology *42*, 869-877.
Solans, R., Bosch, J.A., Esteban, I., and Vilardell, M. (2004). Systemic sclerosis developing in association with the use of interferon alpha therapy for chronic viral hepatitis. Clinical and experimental rheumatology *22*, 625-628.
Sun, G., Chang, W.L., Li, J., Berney, S.M., Kimpel, D., and van der Heyde, H.C. (2003). Inhibition of platelet adherence to brain microvasculature protects against severe Plasmodium berghei malaria. Infection and immunity *71*, 6553-6561.

Tan, F.K., Zhou, X., Mayes, M.D., Gourh, P., Guo, X., Marcum, C., Jin, L., and Arnett, F.C., Jr. (2006). Signatures of differentially regulated interferon gene expression and vasculotrophism in the peripheral blood cells of systemic sclerosis patients. Rheumatology (Oxford) *45*, 694-702.
Thompson, L.J., Kolumam, G.A., Thomas, S., and Murali-Krishna, K. (2006). Innate inflammatory signals induced by various pathogens differentially dictate the IFN-I dependence of CD8 T cells for clonal expansion and memory formation. J Immunol *177*, 1746-1754.
Torpey, N., Maher, S.E., Bothwell, A.L., and Pober, J.S. (2004). Interferon alpha but not interleukin 12 activates STAT4 signaling in human vascular endothelial cells. The Journal of biological chemistry *279*, 26789-26796.
Urban, B.C., Ferguson, D.J., Pain, A., Willcox, N., Plebanski, M., Austyn, J.M., and Roberts, D.J. (1999). Plasmodium falciparum-infected erythrocytes modulate the maturation of dendritic cells. Nature *400*, 73-77.
van Hensbroek, M.B., Palmer, A., Onyiorah, E., Schneider, G., Jaffar, S., Dolan, G., Memming, H., Frenkel, J., Enwere, G., Bennett, S.*, et al.* (1996). The effect of a monoclonal antibody to tumor necrosis factor on survival from childhood cerebral malaria. The Journal of infectious diseases *174*, 1091-1097.
Vigario, A.M., Belnoue, E., Gruner, A.C., Mauduit, M., Kayibanda, M., Deschemin, J.C., Marussig, M., Snounou, G., Mazier, D., Gresser, I.*, et al.* (2007). Recombinant human IFN-alpha inhibits cerebral malaria and reduces parasite burden in mice. J Immunol *178*, 6416-6425.
Villegas-Mendez, A., de Souza, J.B., Murungi, L., Hafalla, J.C., Shaw, T.N., Greig, R., Riley, E.M., and Couper, K.N. (2011). Heterogeneous and tissue-specific regulation of effector T cell responses by IFN-gamma during Plasmodium berghei ANKA infection. J Immunol *187*, 2885-2897.
Voisine, C., Mastelic, B., Sponaas, A.M., and Langhorne, J. (2010). Classical CD11c+ dendritic cells, not plasmacytoid dendritic cells, induce T cell responses to Plasmodium chabaudi malaria. International journal for parasitology *40*, 711-719.
Wang, B., Higgs, B.W., Chang, L., Vainshtein, I., Liu, Z., Streicher, K., Liang, M., White, W.I., Yoo, S., Richman, L.*, et al.* (2013). Pharmacogenomics and Translational Simulations to Bridge

Indications for an Anti-Interferon-alpha Receptor Antibody. Clinical pharmacology and therapeutics.
Wheway, J., Obeid, S., Couraud, P.O., Combes, V., and Grau, G.E. (2013). The brain microvascular endothelium supports T cell proliferation and has potential for alloantigen presentation. PloS one *8*, e52586.
Yao, Y., Richman, L., Higgs, B.W., Morehouse, C.A., de los Reyes, M., Brohawn, P., Zhang, J., White, B., Coyle, A.J., Kiener, P.A.*, et al.* (2009). Neutralization of interferon-alpha/beta-inducible genes and downstream effect in a phase I trial of an anti-interferon-alpha monoclonal antibody in systemic lupus erythematosus. Arthritis and rheumatism *60*, 1785-1796.
Zula, J.A., Green, H.C., Ransohoff, R.M., Rudick, R.A., Stark, G.R., and van Boxel-Dezaire, A.H. (2011). The role of cell type-specific responses in IFN-beta therapy of multiple sclerosis. Proceedings of the National Academy of Sciences of the United States of America *108*, 19689-19694.

Appendix

Appendix One

Methods in Malaria Research

(Protocol Submission: Suggested to be included in section of Cellular Immunology)

Induction of Experimental Cerebral Malaria susceptibility by transfer of mature lymphocyte populations.

by ***Elizabeth Ball, Carlos Penha Gonçalves***
Instituto Gulbenkian de Ciência, Oeiras, Portugal
Email: cpenha@igc.gulbenkian.pt

We developed a novel cell transfer protocol to test individual cell population ability to induce susceptibility to Experimental Cerebral Malaria (ECM). This protocol is based on pre-sensitizing donor mice with irradiated blood stage parasites before cell transfer. The protocol has been tested to induce ECM susceptibility to genetically resistant mouse strains using purified $CD8^+$ T cell populations.
This protocol provides the means to activate immune cells by infected red blood cells (iRBC) in the absence of disease development.

Equipment

Centrifuge (15ml tube)
Irradiator (for 20Krad)
Facs Cell Analyzer
FacsAria Multicolour Cell sorter

Materials and reagents

PBS 1X
PBS 1X, FCS 2%
FACS buffer (PBS1X, 2%FCS, 2mM NaN_3)

Heparin (5000 UI/ml)
Eppendorf tubes
50ml + 15ml Falcon tubes
Surgical tools
Small petri dishes
Sterile gauze
Plasmodium berghei ANKA- GFP infected red blood cells (iRBC)

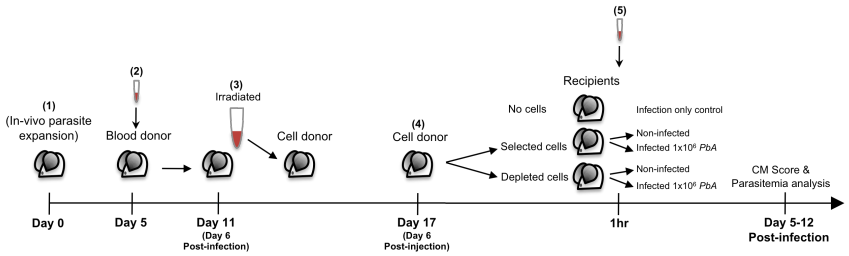


Figure 1. Timeline and schematic representation of the protocol

<u>Procedure</u>

(1) Infect initial mouse for parasite expansion* $1x10^6$ iRBC/100µl in PBS1X intraperitoneally (i.p) from frozen vial of *P.berghei* ANKA-GFP. Allow expansion of parasite and measure daily parasitemia percentage until mouse reaches between 1-5% iRBCs.

- To measure parasitemia percentage, take a drop of tail blood from infected mouse into 600µl FACS buffer. Using a Facs cell analyzer, measure parasite GFP fluorescence as indicator of iRBC percentage. Using this percentage of iRBC, calculate the volume of blood to collect from infected mouse to then infect blood donor mouse $1x10^6$ iRBC/100µl, in PBS1X.

(2) To collect blood, bleed infected mouse via mandibular vein puncture into an eppendorf containing 10µl Heparin, pipette up and down to prevent clotting. Take from this the previously calculated volume for iRBC and

resuspend in PBS1X. Inject iRBCs i.p into blood donor mouse. Allow parasite expansion for 6 days.

(3) On day 6 post-infection (day 11)/or upon display of ECM symptoms in blood donor mouse, measure parasitemia (as described). Calculate from parasitemia percentage the volume of blood to collect that will be irradiated and injected $4x10^6$ iRBC/100μl in PBS1X, per cell donor mouse.

- For irradiation, collect blood from blood donor mouse in an eppendorf (as described, with 10μl heparin, pipette well) and place immediately the eppendorf in a 50ml falcon tube containing ice (do not dilute blood in PBX1X in this step).
- Place 50ml falcon tube containing ice and eppendorf with collected blood in Irradiator and irradiate at 20,000rad. (Obs: Irradiation of parasite to prevent recipient development of ECM and parasitemia).
- After irradiation, take volume of blood and dilute in PBS1X (final concentration, $4x10^6$ iRBC/100μl per mouse) and inject i.p into cell donor mice.

(4) On day 6 post-injection (day 17) sacrifice cell donor mice and remove spleens.

- Place spleens in a petri dish on ice containing PBS1X, FCS2%.
- In a new petri dish smash each spleen in 2ml of PBS1X, FCS2% in-between sterile gauze using the tips of two sterile surgical forceps. Pipette cell suspension into a 15ml Falcon on ice, and wash petri dish with additional 2ml PBX1x, FCS2%. (2 spleens per falcon can be used).
- Proceed with a standard FACS staining protocol for the desired cell population and sorting for purified populations.
- After sorting, collect cells and wash in sterile PBS1X. Count cells and resuspend in PBS1X to a final concentration of $3x10^6$ cells/100μl, per mouse. Inject cells intravenously into recipient mice groups.

(5) Infect i.p appropriate recipient mice groups 1 hour after cell transfers with $1x10^6$ iRBC/100μl of *P.berghei* ANKA-GFP.

- Monitor Recipient mice for parasitemia and ECM development from day 5 PI.

  * All mice used throughout the protocol need to be carrying the same H-2 haplotype.

Appendix Two

Article I Supplemental Data

Supplemental Table 1. Collection dataset of Angolan children used for cerebral malaria and uncomplicated malaria case-control analyses (samples remaining after quality control).

Collection	*n*	Mean age (months)	Male	Female
CM	110	54,6	67	43
UM	129	49,1	69	60
Total	239		136	103

Abbreviations: CM, cerebral malaria; UM, uncomplicated malaria; *n*, number of individuals.

Appendix Three

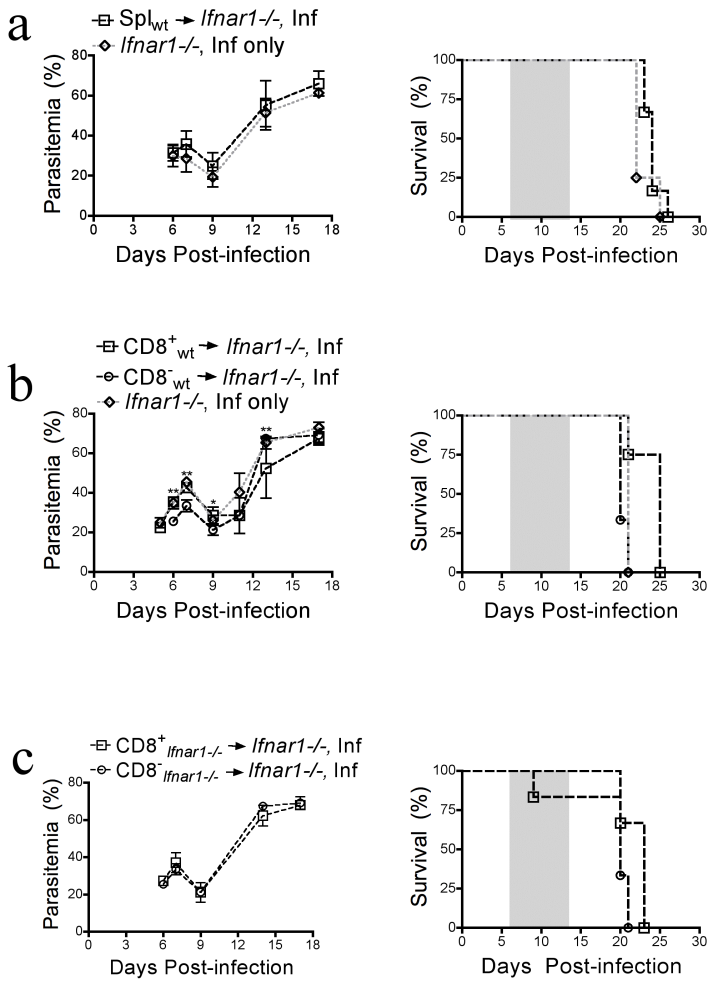


Figure S1. Transfer of non-exposed CD8^{+} C57BL/6 cells cannot induce ECM in *Ifnar1-/-*$^{-/-}$ mice. (a) *Ifnar1-/-*$^{-/-}$ mice that received total spleen cells (open squares) from C57BL/6 non-exposed mice; transfer control is represented by infected *Ifnar1-/-*$^{-/-}$ mice that received no cell transfer (diamonds). (b) *Ifnar1-/-*$^{-/-}$ recipient mice that received sorted CD8^{+} spleen cells (open squares) or sorted

CD8-depleted spleen cells (open circles) from C57BL/6 non-exposed mice; transfer control is represented by infected *Ifnar1-/-*$^{-/-}$ mice that received no cell transfer (diamonds). (c) *Ifnar1-/-*$^{-/-}$ mice that received sorted $CD8^+$ spleen cells (open squares) or sorted $CD8^-$ depleted spleen cells (open circles) from *Ifnar1-/-*$^{-/-}$ mice. (n = 3-6 mice per group, parasitemia; unpaired, two tailed t-test performed between $CD8^+$ and CD8-depleted cell transfer recipient groups; $*P \leq 0.05$, $**P \leq 0.01$, survival curves; Log-rank Test). Each plot represents one of at least two independent experiments performed. Time window of C57BL/6 ECM manifestations is shadowed in survival plots.

Appendix Four

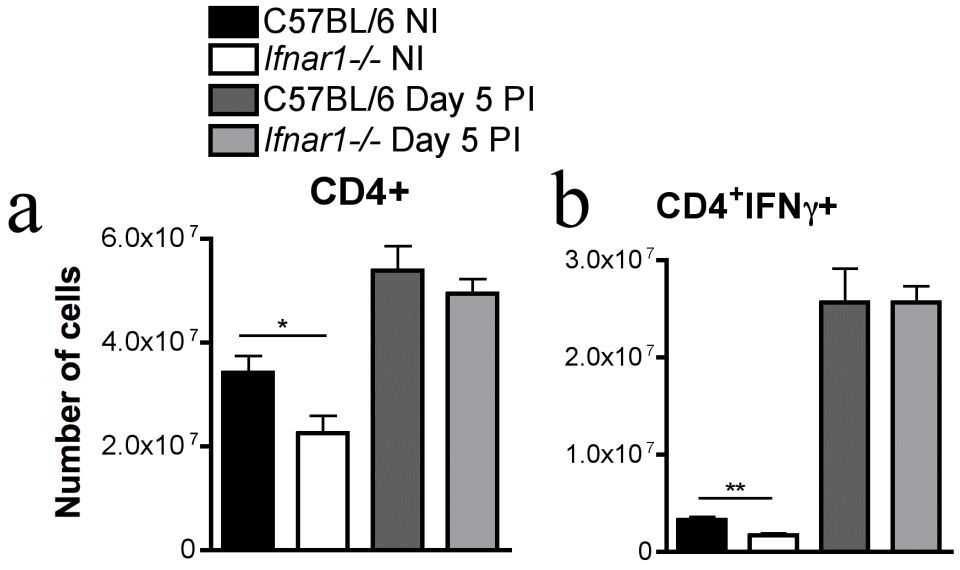


Figure S2. *Ifnar1-/-*$^{-/-}$ mice show no significant difference in CD4^{+} T cell numbers and activation profile compared to C57BL/6 mice upon infection. Total number of CD4^{+} T cells (a) and IFN-γ+ CD4^{+} T cells (b) in the spleen of non-infected (NI) and infected C57BL/6 and *Ifnar1-/-*$^{-/-}$ mice. Mice were infected 1×10^{6} *PbA*, analyzed on day 5 PI. (n = 3-5 mice per group, non-parametric two tailed Mann Whitney test; *$P \leq 0.05$, **$P \leq 0.01$). Data is representative of two independent experiments performed.

Appendix Five

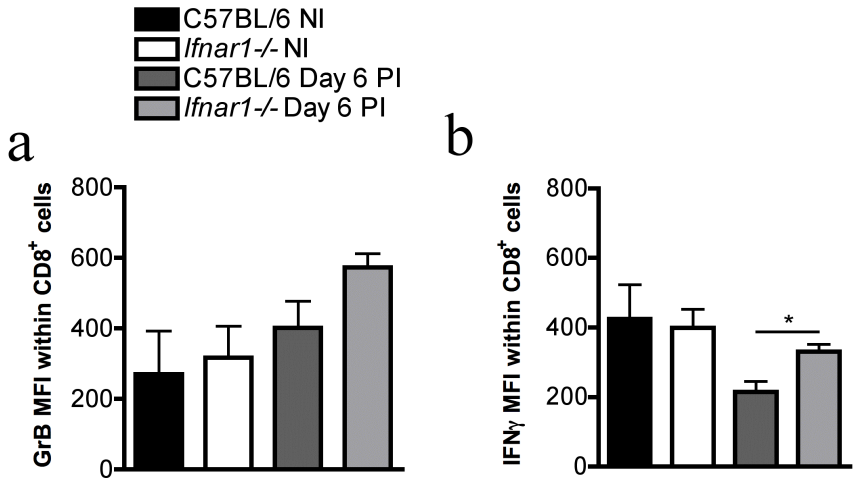


Figure S3. *Ifnar1*$^{-/-}$ CD8^{+} T cells show effector profile like that of C57BL/6 mice.

Mean fluorescence intensity (MFI) of GrB+ on CD8^{+} T cells (a) and IFN-γ+ on CD8^{+} T cells (b) in perfused brains of infected and non-infected C57BL/6 and *Ifnar1-/-*$^{-/-}$ mice, infected 1 × 10^{6} *PbA,* on day 6 PI when C57BL/6 displayed symptoms of ECM development. (*n* = 3-5 mice per group, unpaired, two tailed *t*-test; **P* ≤ 0.05. Data is representative of three independent experiments performed.