Defining the immunological basis of cerebral pathology during murine experimental cerebral malaria and understanding the basis of infection induced resistance

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

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Contents

Contents ........................................................................................................................................... 1
List of figures ...................................................................................................................................... 5
List of supplementary movies ........................................................................................................ 7
List of supplementary tables .......................................................................................................... 7
Abstract ........................................................................................................................................... 8
Declaration ......................................................................................................................................... 9
Copyright statement ...................................................................................................................... 9
Acknowledgements ....................................................................................................................... 10
Abbreviations .................................................................................................................................. 11

1. Chapter 1: General Introduction............................................................................................. 13

1.1. Malaria burden ....................................................................................................................... 14
1.2. *Plasmodium* parasites .......................................................................................................... 15
  1.2.1. Plasmodium life cycle ...................................................................................................... 15
  1.2.2. Plasmodium species ...................................................................................................... 16
1.3. Uncomplicated malaria ........................................................................................................... 17
1.4. Severe malaria ......................................................................................................................... 18
  1.4.1. Severe anaemia ................................................................................................................ 18
  1.4.2. Respiratory distress ........................................................................................................ 19
  1.4.3. Cerebral malaria ............................................................................................................ 20
1.5. Naturally acquired immunity to malaria ................................................................................ 24
  1.5.1. Antibody responses ........................................................................................................ 26
  1.5.2. Cell mediated responses ................................................................................................. 28
1.6. Experimental cerebral malaria ............................................................................................... 29
  1.6.1. Pathogenesis of experimental cerebral malaria .......................................................... 30
1.7. CNS surveillance ...................................................................................................................... 35
1.8. Aims and objectives ................................................................................................................ 37
2. **Chapter 2: Perivascular arrest of CD8⁺ T cells is a signature of experimental cerebral malaria.** ................................................................. 49

Abstract ......................................................................................................................................................... 50

Introduction .................................................................................................................................................... 51

Results ............................................................................................................................................................ 55

Parasitaemia, neurological symptoms and BBB disruption during infection with *Pb* ANKA ............................................................................................................................ 55

Transcranial two-photon imaging of pRBCs .............................................................................................. 56

T cells are perivascularly located within the brain but display different behaviors in *Pb* ANKA and *Pb* NK65 infected mice ................................................................................................. 58

Comparable recruitment and activation of CD8⁺ T cells in the brains of mice infected with ECM-causing and non-ECM-causing *P. berghei* infections ................................................................. 62

Perivascular T cells within the subarachnoid space of mice with ECM form stable interactions with CX₃CR1⁺/GFP cells ................................................................................................................. 64

CX₃CR1⁺/GFP cells with an activated phenotype accumulate within the brain during infection with ECM-causing and non-ECM-causing *P. berghei* infections ......................................................... 65

CX₃CR1⁺ cells are dispensable for induction of ECM .................................................................................... 68

Parasite-specific CD8⁺ T cells recapitulate ECM pathology in resistant TCR Tg mice .................................................................................................................................................. 69

ECM induces increased apoptosis of brain endothelial cells but not sufficiently to account for widespread BBB disruption ........................................................................................................... 71

Discussion ...................................................................................................................................................... 73

Materials and Methods ............................................................................................................................. 79

References ....................................................................................................................................................... 88

Supplementary Figures .............................................................................................................................. 98

Supplementary Videos ............................................................................................................................... 102
3. Chapter 3: Repeated parasite exposure induces resistance to experimental cerebral malaria by modifying intracerebral expression of inflammation-related genes concomitant with antibody-dependent suppression of CTL activity and remodelling of the splenic DC compartment. ......................................................... 101

Abstract ......................................................................................................................... 102

Introduction .................................................................................................................... 103

Results ......................................................................................................................... 107

Repeated cycles of infection and drug-cure protects mice against ECM............. 107

Parasite exposure-induced resistance to ECM is associated with a distinct brain transcriptional signature and altered intracerebral leukocyte compartment .... 109

Exposure-induced resistance to ECM is associated with reduced transcription of inflammation-related pathways and corresponding attenuated intracerebral CD8+ T cell pathogenic functionality ................................................................. 113

Repeated parasite infection does not lead to long term maintained changes in brain transcriptional signature, or altered intracerebral leucocyte response. ............ 116

Exposure-induced resistance to ECM in WT mice is associated with suppression of splenic T cell activation .............................................................. 118

Splenic Treg subsets are not expanded in parasite exposure-induced ECM resistant mice ........................................................................................................ 120

Plasmacytoid dendritic cells are expanded within the spleens of parasite exposure-induced ECM resistant mice ................................................................. 122

An expanded splenic DCs population is not maintained between malaria infections ............................................................................................................. 125

Secreted antibody is required for the expansion of resistance-associated pDCs and attenuation of CD8+ T cell responses in the spleen and brain...................... 127

IgMi KO mice develop accelerated ECM during a fourth infection/Secreted antibody is necessary for the development of parasite exposure-induced ECM resistance ........................................................................................................ 130

Discussion .................................................................................................................. 132

Materials and Methods ......................................................................................... 139

References ............................................................................................................... 143
4. Chapter 4: The sub-cellular location of OVA in Plasmodium blood stages influences the magnitude of T-cell responses ................................................................. 151

Abstract .................................................................................................................. 152
Introduction .............................................................................................................. 153
Results ....................................................................................................................... 156
  Generation of different transgenic P. berghei parasite lines expressing OVA .......... 156
  Expression and subcellular location of OVA in the different transgenic P. berghei lines ........................................................................................................... 158
  Induction of OVA-specific T cell responses in the spleen during blood stage infections with the different OVA-expressing P. berghei ANKA lines ................. 162
  Development of OVA-specific T cell responses in the brain during blood stage infections with the different OVA-expressing P. berghei ANKA lines ............ 167
Discussion ................................................................................................................. 171
Materials and Methods .......................................................................................... 175
References ............................................................................................................... 183
Supplementary Information ..................................................................................... 186

5. Chapter 5: General Discussion ........................................................................... 194

Word count: 56,411
List of figures

Figure 1.1 Erythrocytic stages of the malaria life cycle.................................................. 16
Figure 1.2 Heterogeneous cerebral histopathology in fatal cases of cerebral malaria....21
Figure 1.3 Age associated incidence of severe, mild and asymptomatic malaria infections in *P. falciparum* endemic regions................................................................. 25

Figure 2.1 ECM with associated late stage BBB disruption............................................ 55
Figure 2.2 pRBCs make transient adhesive contact with endothelial cells and are deposited within the perivascular space of the meninges of mice with ECM. ...................... 57
Figure 2.3 T cells exhibit equivalent perivascular compartmentalisation but distinct behaviours during *Pb* ANKA and *Pb* NK65 infections.............................................................. 60
Figure 2.4 Parasite specific CD8+ T cells are comparably activated within the brains of mice infected with *Pb* ANKA and *Pb* NK65 parasites. ................................................. 63
Figure 2.5 Perivascular T cells form long-lasting interactions with CX3CR1^{+GFP} in the brains of mice infected with *Pb* ANKA............................................................................. 65
Figure 2.6 The intracerebral CX3CR1^{+GFP} cellular response is comparable during *Pb* ANKA and *Pb* NK65 infections.................................................................................. 67
Figure 2.7 Depletion of systemically and perivascularly located phagocytic cells from day 5 p.i. does not prevent ECM....................................................................................... 69
Figure 2.8 Parasite specific OT-I CD8+ T cells that directly cause ECM are perivascular and are highly arrested in the brain................................................................. 70
Figure 2.9 Apoptotic endothelial cells in contact with CD8+ T cells are observed in the brains of mice with ECM but are too rare to account for the extensive BBB disruption and vascular leakage observed during ECM......................................................... 72
Figure 2.10 Hypothetical model for the CD8+ T cell-dependent development of ECM. .... 78

Figure S2. 1 Infection with LCMV induces petechial hemorrhages............................. 94
Figure S2. 2 Infection with *Pb* NK65 does not cause ECM development.................... 94
Figure S2. 3 Few DsRed+ T cells are found within the brains of mice on day 5 p.i. with *Pb* ANKA.................................................................................................................. 95
Figure S2. 4 hCD2-DsRed T cells from isolated meningeal vessels of *Pb* ANKA infected mice on day 7 p.i. are mainly CD8+ ................................................................. 95
Figure S2. 5 CD45^{hi}CD11b^{hi} monocytes and macrophages are enriched within the meninges compared with the bulk brain................................................................. 96
Figure S2. 6 Parasite specific OT-I CD8+ T cells are highly arrested in the brains of infected wild type and P14 hosts. ................................................................. 96
Figure S2. 7 Elimination of high frequency speckled noise using a multiscale, -undecimated "A Trous" wavelet transform. ................................................................. 97
Figure S2. 8 Masking of intravascular cells for specific tracking of perivascular cells. ..... 97

Figure 3.1 Susceptible mice are made resistant to ECM development by three preceding infections. ................................................................................................. 108
Figure 3.2 ECM resistant mice have a distinct intracerebral gene expression profile and leukocyte composition. .............................................................. 112
Figure 3.3 ECM resistant mice show constrained up-regulation of genes and T cell markers associated with inflammation. ......................................................... 115
Figure 3.4 Uninfected ECM resistant mice have a comparable gene expression profile and intracerebral leukocyte composition as uninfected mice. ...................... 117
Figure 3.5 ECM resistant mice have enlarged spleens containing CD8+ T cells with an attenuated activation phenotype. ......................................................... 119
Figure 3.6 ECM resistant is not associated with an enhanced T_reg population........ 121
Figure 3.7 ECM resistant mice have an altered DC compartment.......................... 124
Figure 3.8 Splenic pDCs are not expanded prior to a fourth infection.................... 126
Figure 3.9 Repeated infection does not induce splenic pDC expansion in IgMi mice .129
Figure 3.10 Repeated infection does not protect IgMi mice from ECM................ 131

Figure S3. 1 Intracerebral leukocyte composition in uninfected, ECM affected and ECM resistant mice................................................................. 148
Figure S3. 2 Full defence response and regulation of apoptosis heat maps............ 149
Figure S3. 3 IFNγ and IL-6 signalling networks are expressed at lower levels in brains of ECM resistant mice................................................................. 150

Figure 4.1 Different transgenic P. berghei ANKA lines expressing OVA.............. 157
Figure 4.2 Subcellular locations of OVA in of different transgenic P. berghei ANKA lines. ................................................................................................. 161
Figure 4.3 The location of OVA in blood stage parasites influences the development of OVA-specific T cell responses in the spleen. ......................................................... 166
Figure 4.4 The location of OVA in blood stage parasites influences the magnitude of the OVA-specific T cell response in the brain during ECM. ........................................... 169

Figure S4. 1 Schematic of the generation of DNA-construct used to generate the line OVA::Hep17_17 .......................................................................................................................................................................................... 187

Figure S4. 2 Different transgenic P. berghei NK65 lines expressing OVA. ...................... 188

Figure S4. 3 The course of blood stage infection (parasitaemia and ECM) in mice infected with the different P. berghei ANKA lines. ............................................................... 189

Figure S4. 4 The location of OVA expression in blood stage parasites influences the activation of OVA-specific T cells during early stage of infection. ................................. 191

Figure S4. 5 Comparison of the endogenous splenic T cell responses in mice infected with the different P. berghei ANKA lines................................................................. 192

Figure S4. 6 Comparison of the endogenous intracerebral T cell responses in mice infected with the different P. berghei ANKA lines................................................................. 193

List of supplementary movies on compact disc

Supplementary Video 2.1. Transient cytoadhesion of intravascular Pb ANKA-pRBCs

Supplementary Video 2.2. Occurrence of perivascular pRBCs

Supplementary Video 2.3. Infection with LCMV induces petechial hemorrhages

Supplementary Video 2.4. Perivascular location and arrested behavior of T cells during Pb ANKA infection

Supplementary Video 2.5. Perivascular location and dynamic behavior of T cells during Pb NK65 infection

Supplementary Video 2.6. Perivascular DsRed T cells form stable interactions with CX3CR1+/GFP cells in the brains of mice infected with Pb ANKA

Supplementary Video 2.7. Perivascular DsRed T cells make contact, but do not form stable interactions, with CX3CR1+/GFP cells in the brains of mice infected with Pb NK65

Supplementary Video 2.8. Perivascular DsRed T cells form stable interactions with CX3CR1+/GFP cells in the brains of mice infected with Pb ANKA

Supplementary Video 2.9. Perivascular DsRed antigen-specific T cells are mostly arrested in the brains of mice infected with Pb ANKA

List of tables

Table S4. 1 Primers used in this study .............................................................................. 186
Abstract
Tovah N. Shaw
Defining the immunological basis of cerebral pathology during murine experimental cerebral malaria and understanding the basis of infection induced resistance.
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The University of Manchester
2015

Malaria affects 200 million people annually, resulting in 584,000 – 1,238,000 deaths. The majority of these deaths occur in children, less than 5 years of age, in sub-Saharan Africa and are due to cerebral malaria (CM), a neuropathology induced primarily by the species *Plasmodium (P.) falciparum*. The pathogenesis of CM remains poorly understood and the mechanisms involved in acquired protection against the syndrome in malaria-endemic regions are undefined.

Utilising the well characterised *P. berghei* ANKA experimental infection model of cerebral malaria (ECM), results presented in this thesis show that the development of ECM is associated with the accumulation and arrest of pathogenic CD8⁺ T cells within the perivascular spaces of the brain. Accumulation of activated CD8⁺ T cells, without arrest, was observed in the perivascular spaces of the brains of mice infected with the non-ECM causing *P. berghei* NK65 strain. These data show that the behaviour of intracerebral CD8⁺ T cells specifies their pathogenic function during malaria infection. The development of ECM was associated with extensive disruption to the BBB, which developed in the absence of extensive CD8⁺ T cell-dependent endothelial cell apoptosis.

We modified the ECM model, establishing an infection-drug cure strategy, to investigate the immunological basis of parasite exposure-induced resistance to ECM development. Three rounds of infection-drug cure promoted resistance to ECM, which was associated with reduced intracerebral expression of genes involved in defence response, regulation of apoptosis, chemotaxis, CTL activity, antigen processing and presentation and cell adhesion, compared with ECM susceptible mice. Additionally, CD8⁺ T cell activation was suppressed in exposure-induced resistant mice and was associated with the antibody dependent expansion of a splenic plasmacytoid DC population, with a regulatory phenotype. The infection-induced protection against ECM was critically dependent upon secreted antibody production.

A long standing problem in studying the immune response to malaria infection has been the inability to track parasite-specific CD4⁺ T cell responses. To address this, we generated and validated new transgenic *P. berghei* parasites expressing the model antigen, ovalbumin (OVA), either in the parasite cytoplasm or on the parasitophorous vacuole membrane (PVM). We found that cellular location and expression level of the antigen influence the induction and magnitude of parasite-specific T-cell responses. These parasites thus provide knowledge on the factors that influence the recognition of parasite antigens by the immune system and represent useful tools to study the development and function of antigen-specific T-cell responses during malaria infection.

The results in this thesis improve our understanding of the events that lead to the development of CM, and the host immune responses that develop following parasite exposure to protect against it. The results should contribute towards the rational development of adjunctive therapies and effective vaccines for human CM.
Declaration

The following portions of this work referred to in the thesis have been submitted to New York University in support of an application for the degree of Doctor of Philosophy by Phillip J Stewart Hutchinson: - Figure 2.1, Figure 2.2, Figure 2.8, and Figure 2.9, Supplementary Figure 2.1, Supplementary Figure 2.6, Supplementary Video 2.1, Supplementary Video 2.2, Supplementary Video 2.3 and Supplementary Video 2.9.

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Thank you all.
**Abbreviations**

- **Ag**  Antigen
- **APC**  Antigen presenting cell
- **BBB**  Blood brain barrier
- **CCL**  CC chemokine ligand
- **CCR**  CC-chemokine receptor
- **CD**  Cluster of differentiation antigen
- **CM**  Cerebral malaria
- **CNS**  Central nervous system
- **CSF**  Cerebrospinal fluid
- **CXCL**  CXC chemokine ligand
- **CX3CR**  CX3C chemokine receptor
- **EAE**  Experimental autoimmune encephalomyelitis
- **ECM**  Experimental cerebral malaria
- **EPRC**  Endothelial protein receptor C
- **GFP**  Green fluorescent protein
- **GPI**  Glycophosphatidylinositol
- **ICAM-1**  Inter-Cellular Adhesion Molecule 1
- **IFNγ**  Interferon-γ
- **IL**  Interleukin
- **I.p.**  Intra-peritoneal
- **Irgm3**  Immunity-related GTPase 3
- **I.v.**  Intra-venous
- **LT**  Lymphotoxin
- **mAbs**  monoclonal antibodies
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NAI</td>
<td>Naturally acquired immunity</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PbA/Pb ANKA</td>
<td>Plasmodium berghei ANKA</td>
</tr>
<tr>
<td>PfEMP1</td>
<td><em>Plasmodium falciparum</em> erythrocyte membrane protein 1</td>
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<tr>
<td>Pb NK65</td>
<td>Plasmodium berghei NK65</td>
</tr>
<tr>
<td>Py</td>
<td>Plasmodium yoelii</td>
</tr>
<tr>
<td>pRBC</td>
<td>Parasitised red blood cells</td>
</tr>
<tr>
<td>qRT PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>SA</td>
<td>Subarachnoid</td>
</tr>
<tr>
<td>SMA</td>
<td>Severe malaria anaemia</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper 1</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>T_{reg}</td>
<td>Regulatory T cell</td>
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<tr>
<td>Var</td>
<td>Variant</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VSA</td>
<td>Variant surface antigen</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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1. Chapter 1: General Introduction
1.1. Malaria burden

Protozoan parasites of the Plasmodium genus were identified as the causative agent of malaria in 1880 (Cox, 2010). Despite this discovery being made well over 100 years ago malaria remains a significant global health problem with 198 million cases, resulting 584,000 deaths, reported in 2013 (World Health Organization, 2014). Due to global variations in the methods of monitoring and reporting infectious disease it has been suggested that these figures underestimate the true burden of disease, which may be up to 50% higher (Snow et al., 2005, Murray et al., 2012).

Plasmodium species are transmitted by female mosquitos of the Anopheles genus. Global burden therefore depends on a climate and habitat that supports the survival of the vector as well as on human interventions (Lieshout et al., 2004). Malaria transmission is currently restricted to tropical and sub-tropical regions, with the highest disease burden and mortality seen in Africa (Lieshout et al., 2004, Murray et al., 2012). These regions are also some of the poorest in the world and limited resources are available for vector control and disease treatment (Sachs and Malaney, 2002, Basu, 2002, Miller et al., 2002). Added to this is the problem of increasing drug resistance of malaria parasites to existing parasiticidal chemotherapies, currently used to treat disease (Gamo, 2014). Improvements in our basic understanding of how this parasite causes disease will inform the development of new disease prevention and treatment strategies.
1.2. *Plasmodium* parasites

1.2.1. *Plasmodium* life cycle

The life cycle of *plasmodium* species is split between the mosquito vector and human host. The life cycle begins when an infected female mosquito takes a blood meal, injecting ~50 sporozoites into the skin of its host (Beier et al., 1991, Vanderberg and Frevert, 2004). Some sporozoites may become trapped and mature in the skin, or drain to the local skin draining lymph node (Gueirard et al., 2010, Radtke et al., 2015). However, the majority of sporozoites rapidly migrate to the liver of their human host, where they infect hepatocytes and differentiate into merozoites (Ishino et al., 2004). In a process lasting a minimum of 5 ½ days, hepatic merozites undergo extensive schizogony to produce thousands of further merozoites which are released into the blood where they infect red blood cells (Ménard et al., 2013). These early events are known as the pre-erythrocytic stage and are clinically silent. Infection with certain *Plasmodium* species, such as *P. vivax*, can lead to the formation of hypnozoites in the liver, which can reactivate weeks or years post infection, leading to delayed and repeated blood stage cycles (Miller et al., 2013).

During the erythrocytic stages, depicted in **Figure 1.1**, parasites pass through asexual developmental stages of, trophozoite, schizont and merozoite formation. A single invading merozoite gives rise to ~8-32 merozoites, released into the blood when the host RBC ruptures. These merozoites infect further RBCs, perpetuating the infection. Synchronous rupturing of infected RBCs and release of immunogenic material, in humans, gives rise to associated cycles of fever, every 24-72 hours depending on the species (Ménard et al., 2013, Langhorne et al., 2008, Ferreira et al., 2004). A small number of parasites develop into sexual erythrocytic stages, forming male and female gametocytes within the human host. When ingested by a mosquito taking a blood meal,
gametocytes develop through the stages of gamete, zygote, ookinet, oocyst and sporozoite, ready for delivery in to the human host (Ménard et al., 2013).

**Figure 1.1 Erythrocytic stages of the malaria life cycle**
Asexual developmental stages of *Plasmodium* parasite development resulting in propagation of the infection and clinical symptoms. A small number of merozoites develop into gametocytes, able to infect feeding mosquitos. Figure adapted from Gazzinelli et al., 2014 (Gazzinelli et al., 2014).

1.2.2. *Plasmodium species*

Five main species of *Plasmodium* parasite are infectious to humans; *vivax*, *malariae*, *ovale*, *knowlesi*, and *falciparum*. Infections with *vivax*, *malariae* and *ovale* species can cause morbidity but are rarely associated with complications leading to mortality (Jangpatarapongsa and Chootong, 2008, Garcia, 2010). Infection with *knowlesi*, and *falciparum* species, however can rapidly lead to serious complications and death (White, 2008, Marsh et al., 1995). *Plasmodium knowlesi* remains a zoonotic infection, with transmission from macaques to humans via mosquito vector limiting the number of cases (Kantele and Jokiranta, 2011). *Plasmodium falciparum* infection, on the other hand, is transmitted from human to human via a mosquito vector and is responsible for the majority of malaria related deaths (World Health Organization, 2014). *P.*
*Plasmodium falciparum* is more virulent than the other *Plasmodium* species due to its ability to invade all mature RBCs (Malleret et al., 2015) and its capacity to remodel the membrane of infected RBCs to promote cytoadherence to vascular endothelium (Maude et al., 2014).

### 1.3. Uncomplicated malaria

Infection with *Plasmodium falciparum* parasites is associated with production of IL-1β, IL-6, IL-12, TNFα and IFNγ (Walther et al., 2006), production of which has been attributed, in part, to innate cells including monocytes, macrophages, NK cells and γδ T cells (Stevenson and Riley, 2004). Following activation, which takes at least 48hrs, αβ CD4⁺ T cells then become the major source of IFNγ during malaria infection (Horowitz et al., 2010). Production of theses inflammatory cytokines is part of the normal response to the release of malaria antigens, including glycoposphatidylinositol (GPI) and hemazoin, during rupturing of pRBCs containing mature merozoites (Corrigan and Rowe, 2010). The malaria pathogen associated molecular patterns can be recognised by a number of pathogen recognition receptors, including TLRs 2, 4, 9, CD36 and STING, but the individual contribution of each of these pathways to the overall immune response to the parasite requires elucidation (Gazzinelli et al., 2014). Production of the regulatory cytokines IL-10 and TGFβ, as well as development/expansion of Foxp3⁺ Tregs, is also elicited by *Plasmodium* infection and is thought to be a self-regulating response to limit host mediated pathology (Walther et al., 2006, Walther et al., 2005). Heterogeneity in the amount and combination of the cytokines released by previously naïve subjects in response to experimental challenge with *P. falciparum* sporozoites has been reported and linked to variation in parasitaemia and clinical signs (Walther et al., 2006).
Release of pro-inflammatory cytokines in response to malaria antigens underlies the classical fever associated with uncomplicated malaria infections and serves to promote parasite control (Riley, 1999, Corrigan and Rowe, 2010). Dysregulation of this, usually beneficial, response has been implicated in the development of severe malaria complications with twice as much circulating TNFα detected in cases of CM and ten times as much in cases of CM with a fatal outcome, compared with uncomplicated cases (Kwiatkowski et al., 1990).

1.4. Severe malaria

Approximately 1% of the nearly 200 million annually reported malaria infections develop severe and potentially life threatening complications (Crompton et al., 2014). Factors determining whether or not complications develop are not completely understood but are likely to include parasite (such as infecting species/strain), host (genetics and level of pre-existing immunity), and environmental (early diagnosis and treatment) components (Miller et al., 2002). The vast majority of severe malaria cases are the result of infection with Plasmodium falciparum species and occur in sub-Saharan Africa (World Health Organization, 2014). Severe malaria is an umbrella term for three syndromes which may occur independently or in combination; severe anaemia, respiratory distress and cerebral malaria.

1.4.1. Severe anaemia

The most common form of severe malaria is severe malaria anaemia (SMA), defined by a haemoglobin concentrations <5g/dL in the presence of detectable parasitaemia (Griffin et al., 2015, Marsh et al., 1995). Young children are particularly at risk, with a case presentation rate of up to 55% in infants, declining to 21% by 10 years of age (Griffin et al., 2015). Although a significant contributor to malaria associated
morbidity, SMA on its own does not cause high mortality rates (1-5%) (Griffin et al., 2015, Marsh et al., 1995). The mechanisms that contribute to SMA are incompletely understood but are known to include the destruction of paristised and un-parasitised RBC, and dyserythropoiesis (Menendez et al., 2000, Awah et al., 2009). Individuals experiencing SMA typically have very high ratios of circulating TNF to IL-10, which is believed to lead to over activation of red pulp macrophages and suppression of erythropoiesis (Lamikanra et al., 2007, Keller et al., 2006).

1.4.2. Respiratory distress

Respiratory distress accompanies ~10% of malaria infections (Griffin et al., 2015) and may be the result of metabolic acidosis, (pH below 7.35 due to build-up of CO2 and lactate acidosis), and/or pulmonary pathology, including acute lung injury and acute respiratory distress (Taylor et al., 2012). Patients presenting with respiratory distress alone, symptoms of which include deep and laboured breathing, have an associated mortality rate of ~25% (Griffin et al., 2015, Marsh et al., 1995). Respiratory distress, however, most commonly accompanies other severe malaria syndromes (Marsh et al., 1995) and increases the mortality rates of both SMA and cerebral malaria compared with those seen in patients with only one of these syndromes (Marsh et al., 1995, Griffin et al., 2015). Parasite sequestration to, and obstruction of, pulmonary microvessels is a common feature of post mortem histopathology from patients with respiratory distress. Large numbers of activated monocytes and neutrophils containing the malaria pigment, hemozoin, are also commonly observed (Milner et al., 2013). The pathogenesis of respiratory distress associated with severe malaria is not well defined but is thought to be multifactorial, involving both parasite and host contributions (Taylor et al., 2012).
1.4.3. **Cerebral malaria**

1.4.3.1. Definition and outcome

Cerebral malaria (CM) is a neuropathology defined by the presence of seizures and unarousable coma, not attributable to other causes, in the presence of detectable parasitaemia (World Health Organisation, 2000). It is graded using the Blantyre coma scale in children and the Glasgow coma scale in adults, both of which measure eye movement, motor and verbal responses. Due to the high rate of incidental parasitaemia in endemic regions, misdiagnosis of CM has been reported to occur in 23% of cases (Taylor et al., 2004). Assessment of CM associated retinopathy has been shown to increase the specificity of CM diagnosis (Maude et al., 2009).

CM is the most severe form of severe malaria, with a case presentation rate of 2-14%, and an associated mortality rate of 10-20% (Griffin et al., 2015, Marsh et al., 1995, Snow et al., 2005). It principally affects children less than 5 years of age in malaria endemic regions of sub-Saharan Africa (Struijk and Riley, 2004). As well as causing acute morbidity and death, CM is also responsible for neurological deficits in ~12% of surviving children, 25% of which are long lasting (Idro et al., 2005, Brewster et al., 1990).

A significant reason for the high rates of CM related morbidity and mortality is our lack of understanding of the sequence of events that lead to, and are necessary for, its development. As a result therapeutics are limited to parasiticidal chemotherapies, such as intravenous artemesinin, administered late in the course of infection, after CM development. Unfortunately, such late stage and narrowly targeted intervention is not sufficient to prevent death in all CM cases (Dondorp et al., 2005, Dondorp et al., 2010).
1.4.3.2. Histopathology

A key feature of CM post-mortem histopathology is sequestration of parasitized RBC (pRBCs) in the microvasculature of the brain (Milner et al., 2014, MacPherson et al., 1985, Haldar et al., 2007, Patnaik et al., 1994). Significantly higher numbers of pRBC congested vessels are observed in brains from individuals with fatal CM compared with non CM severe malarial cases. It has been calculated that over 80% of cerebral blood vessels may show signs of pRBC sequestration during CM (Ponsford et al., 2012). Recently, it has been reported that patients with confirmed CM can be subdivided into two groups at post-mortem, CM1 and CM2. CM1 cases made up 20-25% of fatal CM cases and were defined by the presence of sequestered pRBCs in the absence of other signs of cerebral pathology. 75-80% of fatal CM cases were classified as CM2, with additional microvascular pathology, including oedema, extravascular ring petechial haemorrhages, intravascular thrombi and intravascular margination of hemozoin containing leukocytes, which include monocytes and macrophages (Dorovini-Zis et al., 2011, Taylor et al., 2004, Medana and Turner, 2006). Differences in CM1 and CM2 histopathology are shown in Figure 1.2.

Figure 1.2 Heterogeneous cerebral histopathology in fatal cases of cerebral malaria
Examples of histopathological features in the brains of fatal cerebral malaria cases classified as (A) CM1, with parasite packed microvessels in the absence of other signs of cerebral pathology or (B) CM2, with extravascular ring petechial haemorrhages associated with parasite packed microvessels. Figure adapted from Taylor et al., 2004 (Taylor et al., 2004).
1.4.3.3. Pathogenesis

Observations of widespread post-mortem pRBC sequestration in the cerebral microvasculature, leading to luminal narrowing and occlusion, led to the hypothesis that CM was the consequence of decreased oxygen delivery and a build-up of waste products, ultimately leading to a comatose state (MacPherson et al., 1985, Berendt et al., 1994). In support of this hypothesis, it has recently become clear that certain *P. falciparum* strains (genotypes) have high affinity for and are able to bind to brain blood vessel endothelial cells (Moxon et al., 2011). The binding of *P. falciparum* parasites to brain endothelial cells is mediated by the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) molecule, which is expressed on the surface of mature infected RBCs. PfEMP1 is encoded by variant (*var*) genes. There are 60 different *var* genes within the parasite genome and they can be split into three groups, A, B and C, according to chromosome location and upstream promoter sequence (Tembo et al., 2014, Melcher et al., 2010). Only 1-3 *var* variants are expressed at a time on a pRBC and each PfEMP1 molecule, due to structural differences, is able to bind to different host receptors with different affinity (Joergensen et al., 2010). As endothelial cells in different tissues express different repertoires of receptors, this results in *var*-dependent parasite tropism for specific tissue vascular beds (Tembo et al., 2014).

It has been shown that parasites expressing group A PfEMP1 variants that contain specific domain cassettes (termed DC8 or 13), bind strongly to brain endothelial cells compared with other organ-derived endothelial cells. Consequently, infection with *P. falciparum* parasites that express DC8 or DC13 containing PfEMP1 molecules is associated with severe malaria and CM in particular (Claessens et al., 2012, Avril et al., 2013, Turner et al., 2013). The host receptor on brain endothelial cells that mediates sequestration of parasites expressing DC8 or DC13 containing PfEMP1 molecules has been identified as endothelial protein receptor C (EPRC), the function of which is
impaired by parasite binding. EPCR activates Protein C and thus normally has a host protective role in maintaining endothelial cell function and integrity and reducing inflammation (Turner et al., 2013). It has also been demonstrated that *P. falciparum* parasites that express PfEMP1 molecules containing a DC4 region bind primarily to ICAM-1 expressed on brain endothelial cells (Bengtsson et al., 2013). As such, infection with these specific parasite strains is also associated with a higher risk of CM (Ochola et al., 2011). A recent study has further associated expression of group A encoded PfEMP1 proteins with CM2 type histopathology in particular and group B encoded PfEMP1 proteins, which bind CD36, with CM1 type histopathology (Tembo et al., 2014).

There is, therefore, significant evidence associating infection with specific *P. falciparum* strains that can bind to brain endothelial cells with higher probability of development of CM. However, this sequestration hypothesis, is challenged by the low correlation between parasitaemia and sequestration of pRBC with the development of CM and the rapid reversibility of the coma in some patients, contrary to the symptoms seen in stroke patients that provide a model of true vessel obstruction (van der Heyde et al., 2006).

An alternative hypothesis for the pathogenesis of CM, the inflammation hypothesis, proposes that systemic inflammation causes up-regulation of adhesion molecules including ICAM-1 and VCAM-1 on endothelium in the brain. This allows parasites and leukocytes to adhere to the activated endothelium, creating a localised inflammatory milieu, leading to leukocyte activation and rupturing of the blood brain barrier (BBB), neuronal disruption and coma (Clark and Rockett, 1994). In support of this hypothesis, as described above, plasma TNF levels are significantly higher in individuals with fatal CM compared with uncomplicated malaria or non-CM complications (Kwiatkowski et al., 1990). The inflammation hypothesis is, however, challenged by the fact that high
levels of inflammatory cytokines circulate in patients infected with the non-CM causing *Plasmodium vivax* parasite (Karunaweera et al., 1992) and by reports that adjunctive treatment of CM patients with anti-inflammatory dexamethasone is deleterious to recovery (Warrell et al., 1982).

Both hypotheses have caveats and more recently, through the recognition of various phenomena that do not vindicate either hypothesis on its own, it has been recognised that they are both likely to contribute to the pathogenesis of CM, along with other factors such as altered haemostasis, low nitric oxide availability and resultant endothelial dysfunction, and thrombocytopenia (van der Heyde et al., 2006, Cox and McConkey, 2010).

The pathogenesis of CM remains controversial and the relative contribution of each of the identified contributing factors, and the sequence in which they occur, remain to be determined.

**1.5. Naturally acquired immunity to malaria**

Naturally acquired immunity (NAI) to malaria develops gradually and in stages, dependent on the level of exposure, inextricably linked to age in endemic regions (Struik and Riley, 2004). Immunity to SMA is acquired after a small number of infections, such that in endemic areas SMA is confined to infants less than 2 years of age (Gupta et al., 1999, Struik and Riley, 2004). In endemic regions, both the development of, and protection from, CM appears to depend on previous exposure, such that peak incidence occurs in children between 2 and 5 years of age (Struik and Riley, 2004). By adulthood, infections in endemic areas are predictably clinically silent, with low parasite density (Struik and Riley, 2004). The age related incidence of severe, mild and asymptomatic infections is depicted in Figure 1.3. However, the exposure dependent, rather than age dependent, basis of NAI to malaria is demonstrated by the
fact that unexposed adults remain susceptible to CM (Riley, 1999); though a role for age associated physiological changes has been recognised in determining which severe malaria syndrome develops (Griffin et al., 2015). Of note, NAI appears to initially develop in the absence of significantly enhanced parasite control, as individuals protected against severe disease can exhibit extremely high parasite burdens (Gonçalves et al., 2014). Effective anti-parasitic immunity is only observed in older adults after multiple exposures (Struik and Riley, 2004).

Figure 1.3 Age associated incidence of severe, mild and asymptomatic malaria infections in *P. falciparum* endemic regions
The age associated incidence of severe, mild and asymptomatic infections in endemic regions is shown as the percent of maximum cases for each population index. Figure reproduced from Langhorne et al., 2008 (Langhorne et al., 2008).

Development of NAI has been well documented but attempts to recapitulate it through vaccination have largely failed. Despite years of development the leading vaccine candidate offers protection against clinical malaria and severe malaria in only 46% and 34% of children respectively. Vaccine efficacy was lower in infants and was found to wane in both age groups after 18 months, despite administration of three doses (RTSS
Clinical Trials Partnership, 2014). These statistics highlight that the immune responses elicited in response to repeated exposure and the mechanisms mediating immunity to SMA, CM and paraitaemia are currently poorly understood.

1.5.1. *Antibody responses*

Antibody mediated protection against blood stage parasitaemia and severe disease was demonstrated by passive transfer experiments in the 1960s (Cohen et al., 1961). However, identification of the target for protective antibody and the mechanisms by which they work remain elusive. High antibody titres against MSP-1 and AMA-1 are similarly found in children with complicated and uncomplicated malaria infections (Osier et al., 2008, Marsh et al., 1989) and are best used as a measure of exposure, rather than protection. Vaccine trials have similarly borne out the lack of correlation between antibody titre and protection against severe disease (Moormann and Stewart, 2014, Wykes, 2013).

It has been suggested that the large malaria genome, in combination with its complex life cycle involving the expression of different genes during various stages of development, is responsible for the lack of correlation between magnitude of antibody response and protection (Riley and Stewart, 2013). Furthermore, most of the immunogenic malarial antigens are highly polymorphic and differ between parasite isolates, such that many of the generated antibody responses are largely ineffectual against subsequent heterologous parasite infections (Riley and Stewart, 2013). Therefore, the breadth, rather than magnitude, of antibody responses has been proposed to be crucial in determining the development of protection. This hypothesis has been supported by recent reports that the memory B cell compartment expands gradually with repeated infections (Weiss et al., 2010) and that the breadth, but not, magnitude, of
circulating memory B cell responses increases with age and exposure (Nogaro et al., 2011).

Whilst the identity of specific parasite molecules which need to be targeted by protective antibodies remains ill defined, some progress has been made using newly available techniques such as protein microarray. Using this method, Crompton et al. have identified 49 malaria proteins which give rise to antibodies found only in children with asymptomatic infection. Of note, four of these proteins are already being developed as vaccine candidates but none of the current lead candidate proteins were found to discriminate between children with asymptomatic and symptomatic infections (Crompton et al., 2010)

One group of antibodies that has been identified as potentially important in mediating functional protection against disease include those against PfEMP1 molecules (Dodoo et al., 2001). Due to high levels of clonal variation and genetic polymorphism in these var genes, it has been suggested that protection against disease is only achieved when a broad antibody repertoire to a wide range of these var gene encoded proteins is achieved. Indeed, particular severe malaria syndromes are thought to be the result of ‘missing’ antibody responses against particular var gene encoded proteins (Recker et al., 2008).

Blocking of pRBC binding and sequestration by antibody binding presents one feasible mechanism of antibody mediated protection. Opsonising antibodies, promoting increased phagocytic activity, have also been positively correlated with immunity to clinical malaria (Chiu et al., 2015) and FcγRIIa polymorphisms have been associated with severe malaria (Cooke et al., 2003), though these mechanisms have not been well investigated.
1.5.2. **Cell mediated responses**

As discussed in 1.3, an appropriately balanced cytokine response is required to control parasitaemia and limit host mediated pathology. Mechanisms of IFN$_\gamma$ control of parasitaemia include promotion of phagocytosis of pRBCs and parasiticidal NO production by macrophages, and provision of help to B cells for production of optimal antibody responses (McCall and Sauerwein, 2010). However, to avoid excessive host mediated damage the IFN$_\gamma$ response must be limited and eventually turned off, once parasitaemia has been brought under control. Diversion of the immune response away from potentially harmful pro-inflammatory responses to give immunity against severe disease has been proposed to be mediated by adaptive changes within the T cell compartment (Riley et al., 2006). The ratio of IFN$_\gamma$ and IL-10 production by CD4$^+$ T cells, and the regulated switch from one to the other during the course of infection is thought to be critical to the development of an appropriate, but self-limiting, immune response (Walther et al., 2009). In support of this, children with severe malaria were found to have lower proportions of IL-10 producing Th1 cells than children with uncomplicated malaria, in combination with higher IFN$_\gamma$ and TNF$\alpha$ (Walther et al., 2009). The source of this IL-10 during infection is reported to be Th1 CD4$^+$ cells, lacking expression of the T$_{reg}$ marker FOXP3 (Walther et al., 2009).

Although T$_{reg}$s have been shown to expand during acute infection and limit control of parasitaemia (Walther et al., 2005), their number and function were found not to differ between children with severe and uncomplicated disease (Walther et al., 2009), and are therefore thought not to play a major role in determining the outcome of acute disease. There is, however, increasing evidence for a role for infection derived T$_{reg}$s in the suppression of subsequent memory Th1 responses, implicating them in the development of exposure dependent anti-disease immunity (Walther et al., 2009).
Subversion of the NAI response by *P. falciparum* to allow its continued transmission has been proposed to explain its gradual and incomplete development. Evidence of atypical memory B cell development and T cell exhaustion in response to chronic parasite exposure has been described (Illingworth et al., 2013), though the biological relevance of these observations has not yet been demonstrated.

### 1.6. Experimental cerebral malaria

Progress in defining the critical events that lead to the development of CM is hindered by the inaccessibility of the affected site for detailed study until post-mortem, scarcity of samples and lack of comparative samples from non-fatal cases. Consequently much effort has been put into the development of a suitable animal model that can be used as a tool to elucidate the pathogenesis of human CM and to identify and investigate the action of therapeutic candidates.

Infection of susceptible strains of mice, such as C57BL/6, with Plasmodium berghei ANKA (PbA) results in the development of a serious neurological syndrome, experimental cerebral malaria (ECM), 6-8 days after infection (Haque et al., 2011, Hearn et al., 2000). As in human CM, accumulation of pRBC and leukocytes in blood vessels, breakdown of the blood brain barrier and micro-haemorrhages are observed within the cortex and cerebellum regions during PbA infection resulting in signs of disease including ataxia, seizures and coma (Hearn et al., 2000, Randall et al., 2008, Lou et al., 2001). As such, the ECM model represents a potentially powerful tool for dissecting the pathogenesis of human CM; it enables various interventions and alterations to the course of infection to be assessed at the site of pathology preceding and following the onset of ECM. Furthermore, infections of susceptible strains of mice with non-ECM inducing strains of murine Plasmodium berghei, such as Pb NK65, and
infection of resistant strains of mice, such as BALB/c, with PbA, which does not result in the development of ECM, provide useful comparative models to identify the processes that are essential for the development of ECM.

1.6.1. Pathogenesis of experimental cerebral malaria

1.6.1.1. Parasite contribution

To date parasite sequestration in cerebral microvessels has not been observed at the same level in the ECM model compared to human CM and consequently it has been suggested that the pathologies of human and murine cerebral malaria differ, with the immune response more important in the murine model (White et al., 2010). Importantly, no parasite binding ligands, analogous to PfEMP1, have been identified for *P. berghei* strains (Franke-Fayard et al., 2004). A number of studies using bioluminescence imaging and qRT PCR have, however, demonstrated the occurrence of, and dependence upon, accumulation of pRBC and/or parasites in the brain for the development of ECM (Franke-Fayard et al., 2004, Amante et al., 2010, McQuillan et al., 2011, Baptista et al., 2010). El-Assad et al. (El-Assaad et al., 2013) have additionally provided direct evidence for the ability of RBC infected with PbA to cytoadhere to microvascular endothelial cells under flow conditions, *in vitro*, to a higher level than those infected with non-ECM causing PbK173. These results seem at odds with the recent intravital imaging results from Nacer et al. showing that parasites and pRBCs do not arrest within cerebral microvessels of ECM affected mice (Nacer et al., 2012). Further studies are therefore required to clarify whether these discrepant results are due to regional variations in parasite/pRBC accumulation, not detected by imaging methods restricted to the subarachnoid space and upper layers of the cerebral cortex used by Nacer *et al* or to differences in detection methods able to discriminate between
parasite material versus whole parasites. Important information on the precise nature of parasite/pRBC accumulation, its location and proximity to other important phenomena associated with ECM pathogenesis, such as inflammatory leukocyte infiltration and haemorrhage still remains to be determined. It also remains to be determined whether this accumulation occurs before or after leucocyte infiltration into the cerebral vasculature, whether it is necessary for the development of ECM and whether it can occur without causing pathology. A better understanding of these events may help to understand the differences and similarities of the ECM model compared with human CM and how best to use this model for extrapolation to the human disease.

1.6.1.2. Host T cell contributions

A role for the host immune system in the pathogenesis of ECM has long been demonstrated (Lou et al., 2001) with pro-inflammatory cytokine and chemokine changes observed both systemically (Lacerda-Queiroz et al., 2010), and within the local environment of the brain during PbA infection (Belnoue et al., 2008, Engwerda et al., 2002). Pro-inflammatory changes, and the development of ECM, are associated with local accumulation of leukocytes, including macrophages, T cells and neutrophils, within the brains of mice with ECM (Belnoue et al., 2002) and activation of brain resident macrophages, which may contribute to the pathogenesis of this syndrome (Pais and Chatterjee, 2005). Monocytes have been shown to play a role in the recruitment of T cells to the brain during ECM; however, the relative importance of this event in development of cerebral pathology remains undefined (Pai et al., 2014).

T cells are now widely recognised as major mediators of ECM associated pathology (Renia et al., 2006). CD8+ T cells are found to accumulate, by day 6 of infection, within the brains of mice infected with PbA, and their depletion, as late as 1 day before the onset of ECM, provides protection from this syndrome in 100% of otherwise susceptible
mice (Belnoue et al., 2002, Baptista et al., 2010, Nitcheu et al., 2003). In contrast CD4⁺ T cells may play a more important role in the induction phase of ECM, as early, but not late, depletion prevents the development of ECM (Amante et al., 2010, Belnoue et al., 2002, Yanez et al., 1996, Yanez et al., 1999). Pathogenic CD8⁺ T cells are primed primarily by CD8a⁺, Clec9A⁺ DCs within the spleen during PbA infection (deWalick et al., 2007, Lundie et al., 2008, Piva et al., 2012).

CD8⁺ T cells mediate cerebral pathology in a perforin and granzyme B dependent manner (Haque et al., 2011, Nitcheu et al., 2003), believed to result in damage to the local endothelium and breakdown of the BBB (Potter et al., 2006). Targeting and disruption of the BBB by pathogenic CD8⁺ T cells has been proposed to depend on local cross presentation of parasite antigen by cells within cerebral microvessels, putatively MHC I expressing endothelium (Howland et al., 2013). Cross presentation of antigen to CD8⁺ T cells was found not to occur in infections with non-ECM causing malaria parasites, Pb NK65 and Py 17X, thereby providing a possible mechanism underlying the development of E(CM) in infection with some parasites but not others (Howland et al., 2013). Although interesting, the biological relevance of these in vitro observations remain to be fully demonstrated. Supporting a role for antigen dependent in situ signalling to CD8⁺ T cells in the development of ECM is the finding that antigen-specific CD8⁺ T cells migrate to the brain, but do not induce ECM until a critical antigen threshold is reached within the brain (Haque et al., 2011). Despite clear evidence demonstrating a role for CD8⁺ T cells in the pathogenesis of ECM, their precise location within the brain and the in situ signals necessary to cause ECM development are not fully defined.

CD4⁺ T cells are believed to contribute to the development of ECM through the release of IFNγ (Villegas-Mendez et al., 2012), which is up-regulated during PbA infection.
(Grau et al., 1989), and is known to promote the migration of pathogenic CD8\(^+\) T cells, from the spleen, to the brain (Belnoue et al., 2008) by inducing CXCL10 expression in the brain (Nie et al., 2009). Results from infections with non-lethal \textit{Plasmodium} strains have also highlighted a role for CD4\(^+\) T cells in the control of parasitaemia and the development protective immune responses initially by activating macrophage and later by providing help to B cells for Ab production (Langhorne et al., 1990). Additionally, CD4\(^+\) T cells are the major producers of host-protective IL-10 during infection with murine malaria parasites (Freitas do Rosário et al., 2012). The varied roles played by CD4\(^+\) T cells in different infections, and their changing mechanism of action throughout infection, highlights some of the challenges involved in understanding this complex pathology. Whether all of these actions are mediated by the same population of CD4\(^+\) T cells or by different sub-populations remains to be determined. Such detailed investigation of malaria specific CD4\(^+\) T cell responses has been hampered by the lack of known malaria antigen epitopes or model antigens able to drive strong CD4\(^+\) T cell responses (Lundie et al., 2010). Identification or development of such tools would enable informative experiments involving adoptive transfer and long term tracking of malaria specific CD4\(^+\) T cells.

1.6.1.3. Host cytokine and chemokine responses

As discussed in 1.6.1.2, the trafficking of effector T cells, from the spleen, to the CNS has been highlighted as a key event in the pathogenesis of ECM and this has been shown to be mediated by IFN\(\gamma\) (Grau et al., 1989, Belnoue et al., 2008). IFN\(\gamma\) acts to modify the expression of chemokines and their receptors on both the T cells and in the brain, thereby controlling the accumulation of these cells to their target organ. Thus, the expression of CCR1, CCR2 and CXCL10 is reduced within the brain of IFN\(\gamma\)R1
deficient mice and this is correlated with impaired intracerebral accumulation of CD8⁺ T cells and resistance to ECM (Belnoue et al., 2008).

Studies, using knock-out mice and depleting monoclonal antibodies (mAbs), have demonstrated important roles for CXCL10 (Campanella et al., 2008, Nie et al., 2009) in the pathogenesis of ECM and have excluded a role for CCR1 and CCR2 (Belnoue et al., 2008, Belnoue et al., 2003).

Although CXCR4 and its ligand CXCL12 have been shown to control the movement and localisation of CD4⁺ and CD8⁺ T cells in the perivascular space during EAE and West Nile Virus (Siffrin et al., 2009, McCandless et al., 2006, McCandless et al., 2008) their role in the pathogenesis of ECM remains little explored, despite the expression of CXCL12 being down-regulated in the brain during PbA infection (Van den Steen et al., 2008).

ECM, like any inflammatory condition, has been associated with the altered expression of many cytokines including the up-regulation of IFNγ, TNF and LT (Hunt and Grau, 2003). These pro-inflammatory cytokines are responsible not only for inducing chemokine production, as described above, but also for the up-regulation of adhesion molecules on brain endothelium, such as ICAM1 and VCAM1. Both of these molecules have been suggested to facilitate cytoadhesion of pRBC (Hunt and Grau, 2003, Lou et al., 1998).

As well as pro-inflammatory cytokines, a role for anti-inflammatory cytokines in the pathogenesis of ECM has been suggested, with exogenously administered (Kossodo et al., 1997) and endogenously produced IL-10 (Specht et al., 2010) being found to provide protection from ECM. IL-10 may limit ECM pathology through a number of mechanisms as it is known to inhibit both the production of pro-inflammatory cytokines and chemokines (Moore et al., 2001). Though a role for IL-10 in ECM pathogenesis
has been identified its cellular sources, both at the site of T cell priming and of pathology, remain to be determined.

1.7. CNS surveillance

At present we do not know exactly where CD8\(^+\) T cells accumulate within the brain during ECM or how they get there. Under normal physiological conditions the CNS is subject to a reduced level of immunosurveillance compared to other organs (Galea et al., 2007a). The rigid structure of the skull necessitates that its contents be protected from excessive inflammation to avoid swelling in a confined space that would ultimately be fatal. On the other hand the ability of pathogens to enter the brain necessitates some level of immunosurveillance (Wilson et al., 2010).

In the absence of a conventional lymphatic system it is thought that memory cells are selectively recruited to pass from the blood through the choroid plexus into the CSF which then circulates around the subarachnoid space (between the arachnoid and pial membranes) and Vichrow Robin spaces (surrounding the tops of meningeal vessels vertically entering the parenchyma from the cortical surface) before draining back into the venous blood or the cervical lymph nodes via the arachnoid villi and the cribriform plate respectively (Ransohoff et al., 2003, Wilson et al., 2010).

An alternative route of entry into the subarachnoid and Vichrow Robin spaces involves crossing the BBB of meningeal capillaries and postcapillary venules. This route of entry is thought to be utilised under inflammatory conditions when integrins and adhesion molecules are up-regulated on endothelium and leukocytes, facilitating the rolling and adhesion of leukocytes prior to extravasation (Wilson et al., 2010).

Under inflammatory conditions, such as those experienced during EAE, T cells within the subarachnoid and Vichrow Robin spaces may encounter Ag presented by microglia,
perivascular macrophages or CD11c+ dendritic cells (Becher et al., 2006). Recognition of Ag by CD4+ T cells in the subarachnoid and Vichrow Robin spaces is considered to be an important factor determining whether these cells cross the glial limitans into the parenchyma or remain in the subarachnoid and Vichrow Robin spaces (Owens et al., 2008). Initial transmigration of CD4+ T cells across the blood vessel endothelium has also been reported to depend on MHC class II expression, though the location of its expression was not determined (Archambault et al., 2005). In line with a role for Ag recognition in mediating T cell entry into the parenchyma, the pathology associated with EAE is abrogated by the absence of MHC class II (Archambault et al., 2005, Greter et al., 2005).

Less is known about the trafficking of CD8+ T cells into the brain under inflammatory conditions but under non-inflammatory conditions their entry into the parenchyma is controlled by the recognition of Ag, presented by MHC class I molecules, on the luminal side of vascular endothelium (Galea et al., 2007b). Whilst there is little evidence that leukocytes infiltrate the parenchyma in ECM (Miu et al., 2008) suggesting that its associated pathology is caused by different mechanisms compared with EAE and other conditions, whether the T cells mediate all of their effects during ECM within the lumen of blood vessels or if they enter the subarachnoid space or perivascular spaces, and how this may contribute to neuropathology, remains to be examined.
1.8. Aims and objectives

This project aimed to utilise the PbA ECM model combined with established immunological protocols and novel techniques to address a number of important questions, leading to a significant increase in our understanding of neuropathogenesis during malaria infection.

The overall aims of my PhD were, therefore, to further our understanding of i) the underlying mechanisms and aetiology of CM and ii) the basis of infection induced resistance.

The specific questions to be addressed were: -

i) Where are the pathogenic CD8⁺ T cells located within the brain during ECM and how do they mediate disruption of the BBB?

ii) What cells and molecular pathways mediate the development of parasite exposure induced resistance to ECM?

iii) Does the subcellular location of a model antigen in the Plasmodium parasite influence the magnitude of T cell responses during PbA infection?

1.9. Expected outcomes from PhD:

Addressing the above questions will contribute significantly to our basic understanding of the underlying mechanisms and aetiology of ECM, and by association, CM. Novel imaging methods, when used in combination with a range of established immunological techniques, will provide new knowledge about T cell and parasite behaviour that will reveal the dynamic nature of ECM pathogenesis. The generation of ‘memory’ mice by repeatedly drug curing and re-infecting them will provide a model for investigating the gradual acquisition of disease resistance and identification of the cells and pathways
mediating this resistance. Clarification of the sequence of events that lead to the development of, and protection against, ECM may then provide more focused approaches to human anti-malaria vaccine and adjunctive drug development programs.
1.10. References


2. Chapter 2: Perivascular arrest of CD8⁺ T cells is a signature of experimental cerebral malaria.

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All work was carried out by T.N.S, with the exception of Figures 2.1C, 2.1D, 2.2, 2.8 and 2.9, Supplementary Figures 2.1, 2.6 and Supplementary Videos 2.1, 2.2, 2.3 and 2.9.

(P.J.S-H)
Abstract

There is significant evidence that brain-infiltrating CD8⁺ T cells play a central role in the development of experimental cerebral malaria (ECM) during *Plasmodium berghei* ANKA infection of C57BL/6 mice. However, the mechanisms through which they mediate their pathogenic activity during malaria infection remain poorly understood. Utilising intravital two-photon microscopy combined with detailed *ex vivo* flow cytometric analysis, we show that brain-infiltrating T cells accumulate within the perivascular spaces of brains of mice infected with both ECM-inducing (*P. berghei* ANKA) and non-inducing (*P. berghei* NK65) infections. However, perivascular T cells displayed an arrested behavior specifically during *P. berghei* ANKA infection, despite the brain-accumulating CD8⁺ T cells exhibiting comparable activation phenotypes during both infections. We observed T cells forming long-term cognate interactions with CX₃CR1-bearing antigen presenting cells within the brains during *P. berghei* ANKA infection, but abrogation of this interaction by targeted depletion of the APC cells failed to prevent ECM development. Pathogenic CD8⁺ T cells were found to colocalise with apoptotic cells, primarily endothelial cells, within the brain during ECM but the rarity of endothelial cell apoptosis did not correspond with the extensive disruption to the BBB observed during ECM. In summary, our data show that the arrest of T cells in the perivascular compartments of the brain is a unique signature of ECM-inducing malaria infection and implies an important role for this event in the development of the ECM-syndrome.
Introduction

Malaria remains a significant global health problem with 207 million cases, resulting in 584,000 – 1,238,000 deaths, annually (Murray et al., 2012, World Health Organization, 2014). A high proportion of these deaths are due to cerebral malaria (CM), a neuropathology induced primarily by the species *Plasmodium falciparum* (World Health Organization, 2014). Current treatment of cerebral malaria is limited to parasiticidal chemotherapies, typically administered late in the course of infection. These traditional and narrowly targeted interventions are ineffective in many cases, and the mortality rate of CM, even after treatment, remains at 10-20% (Oluwayemi et al., 2013, Dondorp et al., 2005, Dondorp et al., 2010). A greater understanding of the parasitological and immunological events leading to the development of CM would aid the development of improved therapeutic options to treat the condition.

Infection of susceptible strains of mice with *Plasmodium berghei* ANKA (Pb ANKA) results in the development of a serious neurological syndrome, termed experimental cerebral malaria (ECM), which recapitulates many of the clinical and pathological features of CM (Amante et al., 2010, Hearn et al., 2000, Lou et al., 2001, Randall et al., 2008, Yanez et al., 1996). Susceptible mice typically develop neurological signs of disease including ataxia, convulsions, paralysis and coma between 6 and 8 days post infection (Hearn et al., 2000, Haque et al., 2011). Histologically visible haemorrhages, widespread disruption of the blood brain barrier (BBB) and accumulation of leukocyte subsets are observed within the brain concomitant with the onset of signs of disease, (Renia et al., 2006, Nacer et al., 2012, Amani et al., 2000). The reason why Pb ANKA causes ECM while other strains of *P. berghei*, such as *P. berghei* NK65, do not is an area of active research.
investigation. However, the differing virulence of *P. berghei* parasites does not appear to be due to extensive genetic polymorphisms between strains (Ramiro et al., 2012, Otto et al., 2014).

Multiple cell types, including monocytes, macrophages, NK cells and CD8$^+$ T cells accumulate within the brain at the onset of ECM (Belnoue et al., 2002, Hansen et al., 2007, Pai et al., 2014, Nacer et al., 2014). However, to date, only CD8$^+$ T cells have been identified as playing an unequivocal role in the development of cerebral pathology; protection from ECM is afforded by their depletion as late as one day prior to the development of neurological signs (Renia et al., 2006, Belnoue et al., 2003, Pai et al., 2014, Yanez et al., 1996). The pathogenic parasite-specific CD8$^+$ T cells are primed in the spleen by CD8$\alpha^+$ dendritic cells (DCs) (Lundie et al., 2008) before migrating to the brain through homing dependent upon IFN$\gamma$-stimulated CXCL10 production in the CNS (Nie et al., 2009). Monocytes play a role in recruitment of the pathogenic CD8$^+$ T cells to the brain during ECM; however, the relative importance of this event in development of cerebral pathology remains undefined (Pai et al., 2014). It has previously been shown that parasite-specific CD8$^+$ T cells mediate ECM development through perforin- and granzyme B-dependent mechanisms (Haque et al., 2011, Potter et al., 2006, Nitcheu et al., 2003), yet where the CD8$^+$ T cells localize within the brain to cause ECM has remained unclear.

Parasite-specific CD8$^+$ T cells appear to require *in situ* antigen-dependent stimulation within the brain to program their pathogenic activity necessary for ECM development (Haque et al., 2011). To date, however, the identity of the putative antigen cross-presenting
cells that interact with pathogenic CD8\(^+\) T cells during ECM is unknown. Recently, it has been shown that parasite specific CD8\(^+\) T cells can specifically interact with antigen cross-presenting microvessel cells obtained from mice experiencing ECM (Howland et al., 2013), but the relevance of this interaction for development of ECM \textit{in vivo} is undefined.

In other models of neuroinflammatory diseases, such as experimental autoimmune encephalomyelitis (EAE), it has been demonstrated that professional antigen presenting cells (APCs) within the subarachnoid (SA) and perivascular spaces of the central nervous system (CNS) present antigen to T cells, instructing their pathogenic function (Kivisakk et al., 2009, Greter et al., 2005, Polfliet et al., 2002, Bartholomaus et al., 2009, Kawakami and Flugel, 2010). Whether interaction of CD8\(^+\) T cells with brain-resident or infiltrating APC types is a canonical event in ECM development is largely unexplored and may represent a hitherto unexplored mechanism in the development of ECM.

In this study, we have attempted to reveal, \textit{in vivo}, the mechanisms through which brain-infiltrating CD8\(^+\) T cells cause ECM. Using transcranial intravital two-photon microscopy, we report that T cells are recruited to, and accumulate perivascularly within, the SA and perivascular spaces of mice infected with both ECM-inducing and non-ECM-inducing \textit{Plasmodium berghei} strains. However, a high proportion of perivascular T cells exhibited arrested behavior, consistent with immunological synapse formation (Mempel et al., 2006, Boissonnas et al., 2007, Beal et al., 2008), in the meninges specifically during ECM-inducing malaria infection. These arrested perivascular T cells formed cognate interactions with cells expressing CX\(_{3}\)CR1, comprising inflammatory monocytes, macrophages and
dendritic cells, but this event was redundant for ECM development. Pathogenic CD8$^+$ T cells co-localised with apoptotic endothelial cells in brains of mice with ECM, but endothelial cell apoptosis was a rare event in relation to the extensive disruption to the BBB observed during ECM. Combined, our results support a model where CD8$^+$ T cells mediate ECM via direct recognition of cognate antigen on target cells without the need for additional in situ secondary activation in the brain by professional APCs and without causing apoptosis.
Results

Parasitaemia, neurological symptoms and BBB disruption during infection with Pb ANKA

To investigate the immunopathological events that contribute to the development of ECM, we used the well-characterized Pb ANKA infection of C57BL/6 mice. Infected mice developed fatal neurological symptoms of ECM on day 6-7 post infection (p.i.) (Figure 2.1A) with a peak peripheral parasitaemia of around 15% (Figure 2.1B). The brains of symptomatic mice (day 6 p.i) displayed extensive BBB disruption, as assessed by Evans blue leakage, with diffuse blue coloration throughout the brain along with a few intense blue foci, which identify sites of petechial haemorrhage (Figure 2.1C). In contrast, brains from uninfected mice showed no discolouration (Figure 2.1C). Spectrophotometric quantification of Evans blue extravasation due to disruption of the BBB revealed this to be a late occurring phenomenon, coinciding with the onset of ECM (Figure 2.1D).

Figure 2.1 ECM with associated late stage BBB disruption.
C57BL/6 (n=14) mice were intravenously infected with $10^6$ Pb ANKA-pRBCs. (A) Survival and (B) peripheral parasitemia ± SD were monitored daily during development of ECM (grey area). (n=14, pooled from 2 experiments). (C) Representative example of Evans blue leakage in the brain of an uninfected mouse and a mouse with ECM (day 6 p.i). (D) Quantification by spectrophotometry of Evans blue leakage in the brains of infected mice on days 4-7 p.i. Dashed line indicates baseline Evans blue signal (no leakage) from uninfected brains. (n=23, pooled from 4 experiments).
Transcranial two-photon imaging of pRBCs

A pathological hallmark of human CM is sequestration, or cytoadhesion, of parasitized RBCs (pRBCs) within the blood vessels (Taylor et al., 2004, Ho et al., 2000). In contrast, we observed low frequencies of pRBCs adhering to vascular endothelial cells in mice with advanced symptoms of ECM (Figure 2.2A and 2.2B). This interaction was weak, and the pRBCs were quickly removed by the shear force of blood flow (Video S1). These results are consistent with those recently obtained by Nacer et al. (Nacer et al., 2012). Surprisingly, we also observed an increase in the number of pRBCs located in the perivascular space in mice with advanced symptoms of ECM (Figure 2.2C and 2.2D, Video S2). In fact, on day 6 p.i., when the majority of mice developed ECM (9/14), perivascular pRBCs (~3 pRBC/mm²) were found more often than adherent luminal pRBCs (~2 pRBC/mm²). In those mice that developed ECM on day 7 p.i., there was a further significant increase in numbers of perivascular pRBCs (~22 pRBC/mm²), which represents a 10-fold increase that is substantially more than that of peripheral parasitaemia (1.5-fold increase), or adherent luminal pRBCs (no increase), observed between days 6 and 7 p.i.. In contrast to LCMV encephalitis, another CD8+ T cell-dependent immunopathological model, we did not observe any evidence of petechial hemorrhages during intravital imaging of meninges of mice with ECM. Thus, it is unlikely that the accumulation of pRBCs within the perivascular space during ECM was simply due to formation of and carriage within hemorrhages. (Figure S1 and Video S3). These results show that, at the point of ECM development, perivascularly located pRBCs are at least as common as briefly adherent intravascular pRBCs (Figure 2.2E). The interaction of pRBCs and parasite-derived material with cells behind the BBB within the perivascular space may, therefore, be an important event in the development of ECM.
Figure 2.2 pRBCs make transient adhesive contact with endothelial cells and are deposited within the perivascular space of the meninges of mice with ECM.

CFP and DsRed mice showing blue and orange endothelial cells, respectively, were infected with $10^6$ Pb ANKA-GFP pRBCs and monitored for symptoms of ECM. Transcranial two-photon microscopy of the meninges was performed on days 5 (n=2), 6 (n=7) and 7 p.i. (n=3). Circulatory blood flow was visualized by intravenous injection of Evans blue (red) prior to two-photon imaging. (A) Example of rare GFP$^+$ pRBCs (green) in contact with the luminal side of endothelial cells (orange, left panel or blue, right panel) of DsRed and CFP mice with ECM. (B) Quantification of adherent intraluminal pRBCs in cortical pial microvessels on days 5, 6 and 7 p.i.. (C) Orthogonal (left) and maximum intensity projection (right) examples of GFP$^+$ pRBCs (green) located within the perivascular space surrounding the pial microvessels (orange) of a DsRed mouse with ECM. (D) Quantification of pRBCs located within the perivascular space surrounding the pial microvessels on days 5, 6 and 7 p.i. with Pb ANKA. Endothelial cells are identified by expression of CFP (blue) or DsRed (orange) (E) Proportion of pRBCs found either adhering to the luminal vessel wall or located within the perivascular space on day 6 p.i (n=4). Results are pooled from 2 experiments. Bars represent mean number ± SD. Scale bars: 5 μm. **p<0.01 (unpaired t test with Welch’s correction)
T cells are perivascularly located within the brain but display different behaviors in *Pb* ANKA and *Pb* NK65 infected mice

CD8⁺ T cells are known to play a central role in the development of ECM (Belnoue et al., 2002, Haque et al., 2011), yet the mechanisms through which they promote cerebral pathology during malaria infection remains poorly understood. To address this, we performed intravital two-photon microscopy through the thinned skull to study the compartmentalization and dynamics of T cells within the brain of hCD2-DsRed transgenic B6 mice during *Pb* ANKA infection. Importantly we contrasted the T cell response during *Pb* ANKA infection with that in the brains of mice during *Pb* NK65 infection, a strain of malaria that causes similar peripheral parasite burdens but does not cause signs of cerebral dysfunction (Figure S2A-C). Few DsRed⁺ T cells were identified within the brains of naïve mice (Figure 2.3A) or on day 5 p.i. with *Pb* ANKA (Figure S3), confirming the late accumulation of T cells in the brain during infection. Characterization of hCD2-DsRed T cells from isolated meningeal vessels of infected mice on day 7 p.i. showed them to be mainly CD8⁺ (>70%) (Figure S4). Quantification of single movie frames revealed significantly higher numbers of T cells within the brains of *Pb* ANKA infected mice compared with *Pb* NK65 infected mice (day 7 p.i.) (Figure 2.3B). Surprisingly, the majority of T cells were compartmentalized to the perivascular side of the blood vessels during both *Pb* ANKA and *Pb* NK65 infections (70.5±10.3 vs 59.2±21.9% respectively) (Figure 2.3C). Interestingly, the distribution of T cells was not homogenous around the vessels imaged, with large clusters of T cells observed preferentially around particular vessels (Figure 2.3D), which are most likely post-capillary venules (Nacer et al., 2014).

It is becoming evident that the behavior of effector T cells, such as interaction with antigen presenting cells or responsiveness to chemokines and adhesion molecules,
determines the local activity and function of the cells within the tissue (Harris et al., 2012, Honda et al., 2014). Thus, as T cells, of which greater than 70% are CD8+, were observed in comparable compartments of the brain during Pb ANKA and Pb NK65 infections, we next examined whether the cells displayed disparate behavior in the two infections, underlying their pathogenic activity specifically during Pb ANKA infection. Tracking DsRed+ cells revealed that perivascular T cells within brains of mice infected with Pb ANKA (Video S4) exhibited a more arrested phenotype than those in mice infected with Pb NK65 (Video S5). These differences were reflected in a lower mean track speed (8.7±4.4 v 15.7±5.6 µm/min), higher arrest coefficient (0.18±0.21 v 0.05±0.1) and lower confinement ratio (0.35±0.23 v 0.46±0.24), reflecting greater confinement (Figure 2.3E).

The more constrained movement of perivascularly located T cells in brains of mice infected with Pb ANKA compared with Pb NK65 parasites is graphically illustrated by plotting 2D projections of tracks over 18 minutes that were then fixed to a common origin (Figure 2.3F). Thus, a major correlate of ECM is the perivascular arrest of CD8+ T cells.
Figure 2.3 T cells exhibit equivalent perivascular compartmentalisation but distinct behaviours during *P. b. ANKA* and *P. b. NK65* infections.

hCD2-DsRed C57BL/6 mice were infected with $10^5$ *P. b. ANKA* or *P. b. NK65* pRBCs or left uninfected. Transcranial two-photon microscopy of the meninges was performed on days 5 p.i. (*P. b. ANKA*), and 7 p.i. (*P. b. ANKA* and *P. b. NK65*). (A) Maximum intensity projections from intravital two-photon microscopy movies (283x283x30 µm) showing Qtracker® non-targeted quantum dot-labeled blood vessels (cyan) and DsRed T cells (red) within the meninges of infected mice on day 7 p.i. with *P. b. ANKA* and *P. b. NK65* and in uninfected mice. (B) Mean number ± SD of hCD2-DsRed T cells (including luminal and perivascular) in imaged tissue sites in uninfected control mice and on day 5 (*P. b. ANKA*, n=2) and 7 p.i. (*P. b. ANKA*, n=7; and *P. b. NK65*, n=5). (C) Mean proportion ± SD of hCD2-DsRed T cells located perivascularly in the meninges of uninfected and *P. b. ANKA* or *P. b. NK65* infected (day 7 p.i.) mice. (D) Representative tile scanned images showing the total imaging field (1415x1415 µm) of brains from *P. b. ANKA* and *P. b. NK65* infected mice (day 7 p.i.). (E) Quantification of average perivascular T cell speeds, arrest coefficient (proportion of time points when instantaneous velocity is <2 µm/min) and confinement ratio (track displacement/track length) from individual three-dimensional T cell tracks. Each point represents an individual DsRed T cell tracked in 4 mice, from 2 experiments, for both groups. (F) Graphical illustrations summarizing the XY movement of individual perivascularly located T cells from normalized starting positions in the brains of mice on day 7 p.i. with *P. b. ANKA* and *P. b. NK65* parasites. Scale bars: (A) 30 µm, (C) 150 µm. *p ≤ 0.05, ****p<0.0001 (Student’s unpaired t test)
Comparable recruitment and activation of CD8$^+$ T cells in the brains of mice infected with ECM-causing and non-ECM-causing *P. berghei* infections

We hypothesized that the more arrested behavior of perivascular T cells in brains of mice infected with *Pb* ANKA compared with *Pb* NK65 was due to intrinsic differences in the phenotype and activation status of the T cells in the two infections. To test this hypothesis, we isolated leukocytes from the brains of infected and uninfected mice and characterized them via flow cytometry. Similar frequencies and numbers of CD8$^+$ T cells within the brains of mice infected with *Pb* ANKA and *Pb* NK65 on day 7 p.i. were observed via flow cytometry (Figure 2.4A-B). Comparable frequencies of CD8$^+$ T cells recruited to the brains of mice infected with *Pb* ANKA and *Pb* NK65 expressed high CD11a (Figure 2.4C), a marker of antigen experience (Lau et al., 2011, Rai et al., 2009). Moreover, the intracerebral CD8$^+$CD11a$^{\text{high}}$ parasite-specific T cells displayed comparable activation in *Pb* ANKA and *Pb* NK65 infections, as evidenced by effector status (CD44$^+$CD62L$^-$) and increased expression of CD69, ICOS, KLRG1, CXCR3, and granzyme B (Figure 2.4D). Combined, these results show that CD8$^+$ T cells recruited to the brains of mice infected with either ECM-causing or non-ECM causing parasites are similarly activated to cause cerebral pathology and that, although necessary, their presence alone is not sufficient for ECM development. The discrepancy in T cell counts between the two-photon microscopy and whole brain analysis may reflect preferential meningeal T cell localization, diluted out by whole brain homogenization along with failure to recover, as single cells, the *Pb*-specific T cells that are more arrested and perhaps tightly bound to target cells under ECM conditions.
Figure 2.4 Parasite specific CD8+ T cells are comparably activated within the brains of mice infected with Pb ANKA and Pb NK65 parasites.

C57BL/6 mice were infected with $10^4$ Pb ANKA or Pb NK65 pRBCs. (A) Representative flow cytometric plots and calculated percentages showing frequencies of CD8+ T cells (gated on live leukocytes) within the brains of uninfected and infected (day 7 p.i.) mice (n=5). (B) Absolute numbers of CD8+ T cells within the brains of uninfected and infected (day 7 p.i.) mice (n=19-22). (C) Percentage of CD8+ T cells expressing high levels of the surrogate antigen specificity marker, CD11a (n=5). (D) Activation phenotype of CD8+CD11a$^{high}$ T cells from uninfected and infected (day 7 p.i.) mice (n=5). Results are representative of two independent experiments (A, C and D) or four combined experiments (B). Bars represent mean number ± SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (one-way ANOVA with Tukey's multiple comparisons test).
Perivascular T cells within the subarachnoid space of mice with ECM form stable interactions with CX3CR1+/GFP cells

The comparable activation status of CD8+CD11a\textsuperscript{high} parasite specific T cells in the brains of mice infected with *Pb* ANKA and *Pb* NK65 parasites on day 7 p.i. suggested that the disparate behavior, and pathogenic activity, of the cells in the two infections was driven by differential sensing of the local environment by the T cells in the two infections. Consequently, we next assessed the interaction of T cells with APC populations in the brains of mice infected with *Pb* ANKA and *Pb* NK65 utilizing hCD2-DsRed X CX3CR1+/GFP dual reporter mice, where GFP is expressed by subsets of monocytes, macrophages and DCs, and all microglia (Jung et al., 2000). We found that perivascular T cells within brains of mice infected with either *Pb* ANKA or *Pb* NK65 were closely associated with CX3CR1+/GFP cells and made frequent interactions with their cellular processes (Videos S6 and S7). Consistent with results from the single-reporter hCD2-DsRed mice, arrested perivascular T cells were more numerous in mice infected with *Pb* ANKA than *Pb* NK65, and many were stably bound to CX3CR1+/GFP cells (Figure 2.5A-C, Video S8). Combined with CD8\textsuperscript{T} T cell recruitment results, this raised the possibility that differences within the CX3CR1+/GFP population may determine the behavior, but not activation state, of perivascularly located T cells that enter the brain subsequent to full activation in the spleen during *Pb* infection.
Figure 2.5 Perivascular T cells form long-lasting interactions with CX3CR1+/GFP in the brains of mice infected with Pb ANKA.

hCD2-DsRed X CX3CR1^GFP/GFP dual reporter mice were infected with 10^4 Pb ANKA (n=5, from 3 experiments). (A) Maximum intensity projections from intravital two-photon microscopy movies (283x283x30 µm) showing interaction of perivascular T cells (red) with CX3CR1^+GFP cells (green) in brains of mice on day 7 p.i. Blood vessels (cyan) were visualized by i.v. injection of Qtracker® non-targeted quantum dots. GFP^+ pRBCs (green) can also be seen within the lumen of the vessels. White arrows highlight selected perivascular T cells forming stable interactions with CX3CR1^+GFP cells. (B) Selected cropped frames from a time-lapse movie showing an perivascular T cell [number one in (A)] in contact with a CX3CR1^+GFP cell over a 15-minute period. (C) Three dimensional section showing the same T cell and CX3CR1^+GFP cell interacting in the XY, XZ and YZ planes. Scale bars: (A) 30 µm, (B) 5 µm, (C) 10 µm.

CX3CR1^+/GFP cells with an activated phenotype accumulate within the brain during infection with ECM-causing and non-ECM-causing *P. berghei* infections

We hypothesized that the different nature of cognate interaction between T cells and CX3CR1^+/GFP cells in the brains during *Pb* ANKA and *Pb* NK65 infections was due to alterations in the composition and/or activation of the brain CX3CR1^+/GFP population during infection with *Pb* ANKA and *Pb* NK65. Thus, to more specifically identify and characterize the CX3CR1^+/GFP cells present within the brain during the two infections, we isolated GFP^+ leukocytes from the brains of infected and uninfected mice and characterized them for expression of phenotypic and functional markers. The frequencies of GFP^+ cells (out of total leukocytes) in the brain increased comparably during infection with both *Pb* ANKA and *Pb* NK65 (Figure 2.6A). We subsequently sub-gated GFP^+ cells into three
subsets based on expression of CD11b and CD45; R1 = CD45^{int}CD11b^{hi} microglia, R2 = CD45^{hi}CD11b^{hi} meningeal and perivascular macrophages and inflammatory monocytes and R3 = a mixed population of CD45^{hi}CD11b^{int} leukocytes (Pais and Chatterjee, 2005, Mutnal et al., 2011) (Figure 2.6B). The majority of GFP^+ cells in brains from uninfected mice were microglial cells (73.7±7.9%). During both infections, the proportion of microglia within the GFP^+ population decreased (52.3±12.2% for Pb ANKA, 47.2±14.1% for Pb NK65), likely due to other GFP^+ cells infiltrating the brain (Figure 2.6C). CD45^{hi}CD11b^{int} leukocytes were mainly CD11c^+ DCs (Pb ANKA 82.8±10.3%, Pb NK65 74.5±8.6%) and CD45^{hi}CD11b^{hi} leukocytes were mainly Ly6C^{hi} inflammatory monocytes (Pb ANKA 84.4±2.5%, Pb NK65 83.8±2.5%). Lack of CD11c and Ly6C expression on the CD45^{int} CD11b^{hi} population confirmed their identity as microglia (Figure 2.6D). These results demonstrate that the composition of the CX3CR1^{+/GFP} population changed within the brain during malaria infection, and that it changed comparably during ECM-causing and non-ECM-causing malaria infection. Furthermore, in contrast to our hypothesis, infection with both Pb ANKA and Pb NK65 caused largely comparable activation of all three CX3CR1^{+/GFP} populations with the most striking up-regulation of co-stimulatory (CD40 and CD80) and antigen presenting molecules (MHC-I) occurring in the CD45^{hi}CD11b^{hi} population (Figure 2.6E). Changes in T cell motility correlating with ECM could not be attributed to different T cell or myeloid cell surface phenotypes and, thus, in vivo motility represents a distinct parameter of value in assessing T cell function.
**Figure 2.6** The intracerebral CX3CR1+GFP cellular response is comparable during Pb ANKA and Pb NK65 infections.

CX3CR1+/GFP mice were infected with 10^4 Pb ANKA or Pb NK65 pRBCs. Brains from uninfected and infected (day 7 p.i.) mice were analyzed by flow cytometry. (A) Representative plots and calculated percentages of CX3CR1+/GFP cells (gating on live leukocytes) within the brains of uninfected and infected (day 7 p.i.) mice (n=6). (B) Representative plots showing the subdivision of GFP+ cells into: R1 - CD45hiCD11bhi microglia; R2 - CD45hiCD11bhi meningeal, perivascular macrophages and inflammatory monocytes; R3 - CD45hiCD11bint leukocytes. (C) Calculated percentages of R1, R2 and R3 sub-gated populations within the brains of uninfected and infected (day 7 p.i.) mice (gating on live GFP+ leukocytes) (n=15-17). (D) Representative dot plots showing the expression of phenotypic markers CD11c and Ly6C on the gated R1, R2 and R3 GFP+ populations from brains of uninfected and infected (day 7 p.i.) mice. (E) Representative histograms and calculated geometric means of CD40, CD80 and MHC I expression on the gated R1, R2 and R3 GFP+ populations from brains of uninfected and infected (day 7 p.i.) mice (n=6). Results are representative of at least two independent experiments (A, B, D and E) or are pooled from three combined experiments (C). Bars represent mean number ± SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (one-way ANOVA with Tukey's multiple comparisons test).

**CX3CR1+ cells are dispensable for induction of ECM**

The CD45hiCD11bhi monocyte and macrophage containing population displayed the most activated phenotype during infection and were also found to be enriched within the meninges, the site of imaging, compared with the whole brain (Figure S5A and S5B). Accordingly, we addressed the contribution of these cells, and the importance of T cell interactions with them, for the development of ECM. Unexpectedly, targeted depletion of the cells through combined intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) injection of clodronate liposomes (C.L.) adapted from Galea et al. (Galea et al., 2005), from day 5 p.i. failed to protect mice against development of ECM (Figure 2.7). Our results, therefore, indicate that although stable interaction of T cells with CX3CR1+/GFP APCs is a frequent event, specifically during Pb ANKA infection, this activity is redundant for the development of ECM.
Parasite-specific CD8$^+$ T cells recapitulate ECM pathology in resistant TCR Tg mice

To further analyze the mechanisms through which CD8$^+$ T cells mediate ECM, we developed a tractable Ag-specific model, where CD8$^+$ T cells with bona fide pathogenic activity can be visualized and tracked. Adoptive transfer of $10^6$ SIINFEKL-specific OT-I CD8$^+$ T cells into otherwise ECM-resistant P14 TCR transgenic mice, in which most CD8$^+$ T cells express the receptor specific for the gp33 epitope of LCMV (Brandle et al., 1991), led to the robust development of ECM when mice were subsequently infected with a GFP-SIINFEKL-expressing strain of Pb ANKA parasite (Pb-TG) (Figure 8A). Consistent with results obtained when examining the polyclonal T cell response (Figure 3), DsRed-expressing OT-I CD8$^+$ T cells were recruited to the brain of P14 hosts in response to infection with Pb-TG (Figure 8C) and were predominantly found to be perivascularly located (Figure 8C). Perivascular OT-I CD8$^+$ T cells were, however, more highly arrested than the polyclonal T cells with a mean speed of $2.36\pm2.32 \mu m/min$ and a mean arrest
coefficient of 0.829±0.256 (Figure 8D, Video S9). Transferred OT-I CD8+ T cells behaved comparably in infected wild type and P14 hosts, indicating that their highly arrested behavior was not an artifact of the transgenic recipient (Figure S6). As our results argue against the requirement for secondary activation by brain APCs to endow brain-infiltrating CD8+ T cells with pathogenic activity during Pb ANKA infection (Figure 4), it is likely that the arrested T cells are attached directly to antigen-presenting target cells, and that such interaction contributes to ECM pathogenesis. These results further emphasize that perivascular arrest of antigen-specific CD8+ T cells is a consistent signature of ECM.

**Figure 2.8 Parasite specific OT-I CD8+ T cells that directly cause ECM are perivascular and are highly arrested in the brain.** C57BL/6 (n=9) and P14 (n=10) mice were infected with 10^6 SIINFEKL-expressing Pb-TG pRBCs. Prior to infection, 10^6 naïve DsRed-expressing OT-I CD8+ T cells were adoptively transferred into CFP+ P14 host mice (n=6). Development of ECM (grey area) was monitored by assessing (A) peripheral parasitaemia ± SD and (B) survival. (C) Maximum intensity projections from an intravital two-photon microscopy movie showing accumulation of DsRed+CD8+ OT-I T cells (orange) within the brain of a P14 recipient on day 6 p.i. infection. Endothelial cells (blue) were visualized by CFP expression. (D) Proportion ± SD of CD8+ OT-I T cells located perivascularly on day 6 p.i. (n=8). (E) Quantification of average perivascular T cell speeds, arrest coefficient and confinement ratio from individual three-dimensional T cell tracks. Results are pooled from two experiments.
ECM induces increased apoptosis of brain endothelial cells but not sufficiently to account for widespread BBB disruption.

It has recently been proposed that CD8$^+$ T cells can directly interact with antigen expressing endothelial cells, and that this interaction may be a proximal event in ECM development (Howland et al., 2013, Potter et al., 2006). We, therefore, used an active-caspase3/7-reporter molecule, CellEvent, to examine the amount of endothelial cell apoptosis within the brains of mice with ECM, and we assessed the proportion of these apoptotic cells in contact with pathogenic CD8$^+$ T cells. We did not observe any CellEvent-positive cells by transcranial microscopy, suggesting that cellular apoptosis is a rare event in the brain during ECM. This was confirmed by examining thick coronal sections (Figure 9A), with only 11.2 cells/mm$^3$ apoptotic cells found in the vasculature of the cortex of brains of mice with ECM, compared with 1.36 cells/mm$^3$ in naïve brains (Figure 9B). Importantly, ~72% of the apoptotic cells were in immediate contact with parasite-specific CD8$^+$ T cells, suggesting that the CD8$^+$ T cells were mediating the cellular apoptosis (Figure 9C). Approximately half of the apoptotic cells were endothelial cells, as defined by CD31 expression (Figure 9D). Nevertheless, cellular apoptosis was rare in comparison to the extensive disruption to the BBB and vascular leakage observed in ECM (Figure 9E). Thus, our results indicate that CD8$^+$ T cells do not damage the BBB primarily by causing endothelial cell apoptosis; rather, our results support a model in which perforin and granzyme B release, by perivascular parasite-specific CD8$^+$ T cells, induces opening of intercellular junctions of the endothelium.
Figure 2.9 Apoptotic endothelial cells in contact with CD8+ T cells are observed in the brains of mice with ECM but are too rare to account for the extensive BBB disruption and vascular leakage observed during ECM.

10⁶ DsRed’CD8+ OT-I T cells were adoptively transferred into CFP+ P14 host mice, which were subsequently infected with 10⁶ SIINFEKL-expressing Pb-TG pRBCs (n=4) or left uninfected (n=5). Mice were intravenously injected with CellEvent Caspase-3/7 Detection Reagent when displaying signs of ECM (day 6 p.i.). Brains were subsequently isolated and processed for histological examination. (A) Representative snapshots showing apoptotic cells (green) in contact with parasite-specific CD8+ OT-I T cells (red). Endothelial cells (blue) were visualized by CFP expression. (B) Quantification ± SD of apoptotic cells per mm³ in brains from infected and uninfected control mice. (C) Proportion ± SD of apoptotic cells in direct contact with parasite-specific CD8+ OT-I T cells (n=2). (D) Brain sections were additionally stained with anti-CD31, a marker of endothelial cells. Representative snapshots showing examples of CellEvent+ cells that were also CD31+ (left column) or do not express CD31 (right column). (E) Brains from infected (day 6 p.i.) mice that had received intravenous injections of both CellEvent and Evans blue were examined for colocalization of vascular leakage and apoptotic cells. Representative tile scan showing CellEvent+ apoptotic cells (white asterisk) in relation to Evans blue leakage within the brain of a mouse with ECM. Scale bars: (A,C) 25 µm, (E) 1 mm. Results are pooled from mice infected in one experiment.
Discussion

In this study, we have shown that CD8$^+$ T cells accumulate at high levels in the perivascular space of the brain during malaria infection. The perivascular compartment of the brain is a hitherto understudied location in the study of ECM pathogenesis, even though, consistent with our results, leukocytes have previously been observed in this space during ECM using electron microscopy (Lackner et al., 2006, Ampawong et al., 2014). Although, our two-photon studies examined the superficial regions of the brain, perivascularly located CD8$^+$ T cells were also observed around vessels deeper in the brain during ECM (not shown) suggesting that this phenomenon is not unique to pial vasculature. Our results extend the study of Nacer et al, which used acute labeling with intravenous fluorescent antibodies to detect intravascular CD8$^+$ T cells during ECM (Nacer et al., 2014). We have utilized a genetically encoded fluorescent protein, expressed in all T cells, coupled with the phenotypic analysis of T cells in the CNS to reveal a large population of extravasated, but still perivascular, CD8$^+$ T cells that were previously unappreciated.

Interestingly, we observed accumulation of CD8$^+$ T cells in the perivascular spaces of the brain during both ECM-inducing and non-inducing infections, indicating that the presence of perivascular T cells per se is insufficient to cause ECM. Instead, dynamic time-lapse movies revealed distinct behaviors of these cells in the perivascular compartment during the different infections, suggesting that it is T cell motility in the brain that correlates with their pathogenic activity during malaria infection. Infection with ECM-causing parasites resulted in a higher proportion of perivascular T cells, including Ag-specific CD8$^+$ T cells that directly contribute to ECM, exhibiting behavior consistent with immunological synapse formation, including lower mean speed, higher mean arrest coefficient and lower mean confinement ratio. These findings demonstrate the utility of two-photon microscopy.
in revealing information on important dynamic cellular behaviors of leukocytes that contribute to disease and that can be missed using histological and anatomically insensitive flow cytometry techniques.

Perivascular CD8$^+$ T cells were observed forming stable interactions with CX$_3$CR1$^{+/GFP}$ cells, specifically during infection with ECM-causing $Pb$ ANKA parasites. Such interactions between perivascular APCs and T cells, leading to secondary in situ reactivation, are known to be essential for the development of pathology in other neuro-inflammatory models such as EAE (Owens et al., 2008, Bechmann et al., 2007). However, perturbation studies using clodronate liposomes and preliminary data from the CX$_3$CR1/iDTR system (Buch et al., 2005) (not shown) to deplete CX$_3$CR1$^+$ cells demonstrated that this interaction is not critical for development of ECM. This difference between EAE and ECM is perhaps unsurprising when the location of disease-associated tissue damage is considered. Whilst in EAE, perivascular T cells must receive further stimulation to cross the glia limitans into the parenchyma, where the disease-associated pathology occurs (Gimenez et al., 2004, Jallow et al., 2009, Kivisakk et al., 2009), in malaria the ECM-associated pathology is focused primarily to the cerebral vasculature (Nacer et al., 2012), which is accessible to both luminal and perivascular CD8$^+$ T cells. However, in contrast to luminal cells, it is also possible that arrested perivascular CD8$^+$ T cells may exert a secondary activity during ECM by interacting with the glia limitans. This may induce signals into the parenchyma of the brain, contributing to the activation and injury of brain-resident cells including astrocytes, microglia and neurons, which is observed during ECM (Szklarczyk et al., 2007, Lackner et al., 2007).
Thus, the critical questions are 1) why do T cells behave differently in the brains of ECM-inducing and non-inducing malaria infections, and 2) why can they specifically cause cerebral pathology only during Pb ANKA infection? We found that intracerebral CD8+ T cells possess an equally activated phenotype in mice with ECM-causing and non-ECM causing infections, indicating that upon migration to the brain, CD8+ T cells possess the intrinsic ability to mediate cerebral pathology, as long as they receive the necessary tissue signal. This result is in agreement with Howland et al who reported that parasite specific CD8+ T cells with cytolytic potential (Howland et al., 2013) are found within the brains of mice infected with non-ECM causing parasites. Howland et al suggested that the ability of CD8+ T cells to mediate pathology specifically during Pb ANKA infection is the presentation of parasite antigen by microvessel cells in the brain during this infection. This hypothesis is consistent with Haque et al.’s report that antigen-specific CD8+ T cells migrate to the brain, but do not induce ECM until a critical antigen threshold is reached within the brain (Haque et al., 2011).

The source of malarial antigen and its mode of presentation to pathogenic T cells specifically during ECM-inducing malaria infections has been a subject of intense investigation. A number of studies have shown that, despite similar peripheral parasitemia in the blood, parasite accumulation within the brain is higher during infection with ECM-causing parasites than non-ECM causing parasites (Baptista et al., 2010, Howland et al., 2013). Similarly, greater parasite accumulation is found in the brains of ECM-susceptible strains of mice than resistant strains (Baptista et al., 2010). Whilst these data support a major role for parasite accumulation in the brain in ECM-pathogenesis, in this study, consistent with Nacer et al (Nacer et al., 2012), we failed to observe widespread adhesion
of luminal parasites to brain vascular endothelium during \textit{Pb} ANKA infections. Nevertheless, we did observe accumulation of pRBCs within the perivascular space during \textit{Pb} ANKA infection, the location where arrested pathogenic CD8$^+$ T cells are observed. Thus, it is possible that parasite presence in this compartment may provide a source of parasite antigen for cross-presentation by endothelial cells or associated microvessel cells during \textit{Pb} ANKA infection, enabling perivascular CD8$^+$ T cells to mediate their pathogenic activity. In addition, it is possible that ECM-inducing parasites preferentially produce antigen-containing microparticles, which can induce CM-like pathology in the brain and lung of recipient mice (El-Assaad et al., 2014). The properties and features of \textit{Pb} ANKA parasites that cause them to reproducibly promote ECM, unlike other \textit{Pb} isolates, require further investigation, especially as there appears to be little genetic polymorphism between them (Otto et al., 2014, Ramiro et al., 2012).

Irrespective of how CD8$^+$ T cells encounter antigen within the brain during \textit{Pb} ANKA infection, the question remains as to the mechanism through which they mediate ECM pathology. The requirement for perforin and granzyme B in CD8$^+$ T cell-mediated breakdown of the BBB, leading to ECM, has previously led to the hypothesis that CD8$^+$ T cells directly induce apoptosis of endothelial cells within the brain (Potter et al., 2006, Haque et al., 2011, Nitcheu et al., 2003). Our results do not support this contention. Although increased in ECM mice, remarkably few apoptotic endothelial cells were observed in the brain when compared with uninfected mice. Critically, although the majority of apoptotic cells were co-localized with pathogenic CD8$^+$ T cells, they appeared insufficient in number to account for the extensive BBB disruption, assessed by Evans blue leakage that occurs during ECM. Instead, our results support the emerging alternative
theory that BBB disruption during ECM occurs at the level of the interendothelial tight junctions without, or long before, causing endothelial cell death (Nacer et al., 2012). Supporting this hypothesis, Suidan et al. have demonstrated the ability of intracerebral antigen-specific CD8+ T to initiate BBB disruption through a perforin-dependent mechanism that down-regulates tight junction proteins 12-24 hours before activation of the apoptotic caspase cascade (Suidan et al., 2008).

In summary, we have presented data that highlights the perivascular space as a site of significant CD8+ T cell accumulation during murine malaria infections. This site was additionally found to be a site for pRBC accumulation. Together, our results suggest that pathogenically relevant interactions between CD8+ T cells and their target cells may occur at the perivascular aspect of affected vessels, shifting the focus from intraluminal events to include this previously underappreciated location. We, therefore, propose that infection with malaria parasites results in accumulation of perivascular antigen-specific CD8+ T cells and antigen-dependent in-situ active engagement of the T cell receptor, only during Pb ANKA infection, leading to alteration of tight junction proteins, increased vascular permeability and death before the widespread occurrence of endothelial cell apoptosis (Figure 10).
Figure 2.10 Hypothetical model for the CD8$^+$ T cell-dependent development of ECM.

1) *Pb* ANKA infection leads to the upregulation of adhesion molecules and cross-presentation of parasite antigen by MHC-I on brain microvascular endothelial cells (Weiser et al., 2007, Monso-Hinard et al., 1997, Turner et al., 1994). (2) This promotes transient interaction of *Pb* ANKA-pRBCs and rolling of activated CD8$^+$ T cells on the luminal aspect of the brain microvessel endothelial cells. Beginning one day prior to signs of ECM, parasite-specific CD8$^+$ T cells are recruited to the perivascular space, either via direct diapedesis across the endothelium, or migration via the highly permissive choroid plexus. In the perivascular space, parasite-specific CD8$^+$ T cells form immune synapses with (3) parasite Ag-expressing APCs and (4) the basolateral membrane of cross-presenting endothelial cells. Perivascular APCs may acquire parasite antigen as a result of either transport of material across the BBB preceding generalized breakdown of the barrier or subsequent to breach of the BBB. (5) The interaction between CD8$^+$ T cells and the basolateral membrane of endothelial cells leads to targeted release of cytotoxic perforin and granzyme molecules in the perivascular space that down regulate intercellular tight junction proteins, damaging the BBB and causing vasogenic edema. (6) Through an undefined mechanism, perivascular CD8$^+$ T cells also communicate across the glia limitans to induce astrocyte and microglial activation, further amplifying cerebral inflammation and dysfunction. In non-ECM malarial infections, brain endothelial cells do not cross-present malaria antigen and perivascular CD8$^+$ T cells fail to recognize their cognate antigen, restricting their pathogenic activity and preventing ECM development.
Materials and Methods

Ethics

Animal work in New York was carried out in strict accordance with the recommendation in the Guide for the Care for the Care and Use of Laboratory Animals of the Public Health Service (National Institutes of Health) and was approved by New York University School of Medicine Institutional Animal Care and Use Committee (IACUC). Animal work in the U.K. was approved following local ethical review by the Universities of Manchester and Glasgow Animal Procedures and Ethics Committees and was performed in strict accordance with the U.K. Home Office Animals (Scientific Procedures) Act 1986 (approved H.O Project Licenses 70/6995 and 70/7293).

All surgery was performed under anesthesia: ketamine (50 mg/kg), xylazine (10 mg/kg), acepromazine (1.7 mg/kg); or isoflurane (2% in O₂ at 0.2 L/min).

Mice

The following mice were used in the study: At Skirball Institute Animal facility C57BL/6 (H-2b) from the National Cancer Institute or Taconic Labs. OT-I (Hogquist et al., 1994), P14 (Brandle et al., 1991), CFP (Hadjantonakis et al., 2002), DsRed (Vintersten et al., 2004). Both CFP and DsRed were expressed under the control of a chicken β-actin promoter and CMV enhancer cassette; At the Universities of Glasgow and Manchester, C57BL/6 mice from Harlan and Charles River, UK, hCD2-DsRed (Kirby et al., 2009), CX₃CR₁<sup>GFP/GFP</sup> (Jung et al., 2000), CX₃CR₁<sup>GFP/GFP</sup> X C57BL/6 (F1) and CX₃CR₁<sup>GFP/GFP</sup> X hCD2-DsRed mice<sup>+/+</sup> (F1) In all cases transgenic mice were fully backcrossed to a
C57BL/6 background and were used between 6 and 12 weeks of age. Mice were maintained in specific pathogen-free conditions.

**Parasites**

GFP-expressing *P. berghei* ANKA parasites (GFP expressed under control of elogation factor 1a [eEF1a] promoter) were a kind gift from Chris Janse (Leiden University Medical Center) (Franke-Fayard et al., 2004). GFP/SIINFEKL-expressing *P. berghei* ANKA (Pb-TG) parasites were a kind gift from William Heath (University of Melbourne) (Lundie et al., 2008). *P. berghei* NK65 parasites expressed GFP under control of the circumsporozite promoter (Natarajan et al., 2001). Parasites were maintained in liquid nitrogen and passaged through naive mice prior to being used to infect experimental animals. Experimental infections were initiated by i.v. inoculation with $10^4$ or $10^6$ pRBCs, depending upon the experiment, and infected mice were monitored for neurological symptoms (paralysis, ataxia, convulsions, and coma occurring between day 6 and 10 post-infection). Parasitemia was measured daily from day 3 p.i. by either examination of Giemsa-stained thin blood smears or by flow cytometric detection of DAPI stained GFP+ parasites.
Classification of experimental cerebral malaria

The induction and severity of ECM was assessed using the following well-defined grading system (Villegas-Mendez et al., 2012): 1: no signs; 2: ruffled fur/and or abnormal posture; 3: lethargy; 4: reduced responsiveness to stimulation and/or ataxia and/or respiratory distress/hyperventilation; 5: prostration and/or paralysis and/or convulsions. Stages 2/3 were classified as prodromal signs of ECM and stages 4/5 were classified as ECM.

Adoptive Transfer

Splenic DsRed^{+/+}OT-I CD8^{+} T cells were purified (>95% purity) from a naïve DsRed^{+/+}OT-I TCR Tg mouse using a Dynal Mouse CD8^{+} Negative Isolation Kit (Invitrogen). 10^4 and 10^6 DsRed^{+/+}OT-I CD8^{+} T cells were transferred i.v. into C57BL/6 and P14 TCR Tg recipients, respectively, one day prior to infection with 10^6 Pb-TG-pRBCs, as described above.

Detection of BBB disruption

BBB leakage was detected as described previously, with some modifications (Steinwall and Klatzo, 1966). Briefly, 50 μL 3% Evans blue/PBS (w/v) was injected i.v. into anesthetized mice and allowed to circulate for 5-6 hours. 100-150 μL blood was collected just before perfusion for serum sample. Mice were exsanguinated under KXA anesthesia by intracardial perfusion with 20 mL ice cold PBS. Each brain was removed, weighed, deposited in 500 μL formamide and incubated in darkness at 37° C for 48 hours to extract Evans blue. Formamide was then aspirated and Evans blue absorbance at 610 nm was measured for serum and brain samples with EnVision 2104 Multilabel Reader plate reader.
(PerkinElmer). Leakage was calculated as Evans blue concentration(brain) multiplied by extraction volume (500 µL), divided by Evans blue concentration(serum), divided by brain mass, divided by hours of circulation, yielding µL serum/g brain/hr.

**Intravital 2-photon microscopy**

Non-recoverable intravital transcranial imaging was performed utilizing adapted published protocols (Fumagalli et al., 2011, Myburgh et al., 2013, Yang et al., 2010). Infected mice were imaged on days 5, 6 or 7 post infection. For the imaging of mice with ECM, mice were selected only when they scored 3 or above using the grading system described above. Mice imaged on day 5 post infection showed no signs of ECM. The following numbers of mice were imaged to assess the polyclonal T cell response within the subarachnoid and perivascular spaces: 2 mice infected with Pb ANKA on day 5 p.i., before ECM development, 4 mice with ECM (score > 3) on day 7 p.i., 4 mice infected with Pb NK65 on day 7 p.i. and 4 uninfected mice. To visualize blood vessels, mice were injected (i.v.) with either 20 ng Evans blue in PBS or 10 µL Qtracker® 705 non-targeted quantum dots (Invitrogen) in PBS prior to imaging. After exposure of the skull by removal of the scalp and periosteum, mice were immobilized in a stereotaxic apparatus. An imaging window (~3-mm diameter) was created on the right parietal bone 2-3 mm lateral and posterior to bregma by thinning the bone with a micro-drill, under a dissecting microscope. Mice were maintained under anaesthesia throughout surgical and transcranial imaging procedures. Core body temperature was maintained at 37 °C, thermostatically controlled by a rectal temperature probe. Perfusion of the cranial window with an isotonic solution was maintained throughout the imaging and provided a meniscus for the dipping lens objective. Preparation and imaging of an individual animal lasted up to 3 hours. Individual movies
lasted between 15 and 35 minutes for uninfected and *Plasmodium* infected mice. For LCMV infected mice, movies lasted up to 1 hour.

Two-photon transcranial microscopy was performed with either a LSM-7 MP or LSM-710 system (Zeiss), using a 20x W Plan-Apochromat water immersion objective (NA 1.0, Zeiss). Excitation wavelengths between 910 and 940 nm were generated by a tunable Ti-sapphire femtosecond pulsed laser. Fluorescent emission signals were detected using a combination of non-descanned fluorescence detectors. Emission signals were sequentially separated by dichroic mirrors and bandpass filters arranged in two configurations: 1) 740 and 625 dichroic mirrors (Qtracker® 705), 490-nm dichroic mirror with a 485-nm shortpass (SHG), and 593-nm dichroic mirror in combination with 525/25 (GFP) and 585/22 (DsRed) bandpass filters (Semrock). 2) 442/45 (SHG) filter, 465-nm dichroic mirror with 483/35 (CFP/GFP) filter, 505-nm dichroic mirror with 538/36 (GFP/CellEvent) filter, 555-nm dichroic mirror with 610/70 (DsRed) filter and 660-nm dichroic mirror with 710/100 (Evans blue-albumin) filter (Chroma or Semrock). CFP and GFP signals were distinguished by assessing the ratio of 483/35 signal to 538/36 signal.

**Two-photon Image Processing and Analysis**

Two-photon time-lapse sequences acquisition (283x283x30 μm, 2 μm Z step) (Zen software, Zeiss) was performed with low laser power and short pixel dwell time. As a result, significant dark current shot noise was present in some of our images, which had a detrimental impact on our chosen automated tracking software. In order to eliminate this high frequency speckled noise, a multiscale, -undecimated "A Trous" wavelet transform
(Holschneider et al., 1990) based on a 3x3x3 linear kernel was applied on each volume of interest. Each channel and each time-point were decomposed separately into 7 additive layers plus final residual layer. Noise was mostly contained in the first layer, and cells were best detected in the 4th layer (Figure S7). By selecting the layers containing only cells, we produced time-lapse sequences which were devoid of noise and therefore suitable for cell tracking. Similarly, the blood vessel stacks were filtered by summing layers 3 to 7 of the wavelet decomposition, and thresholded using a fix value. These binary stacks (False outside the vessel, True inside) were used to discard any cells inside blood vessels (Figure S8). Imaris (Bitplane) or Volocity (Improvision) software packages were used track cells using a combination of automated and manual processing. Motility analyses were subsequently exported and processed in Excel. Mean speed was calculated as path length/time (µm/min). Mean instantaneous speed was calculated by determining speed of a tracked cell at each consecutive time-point. Arrest coefficient of a cell was defined as the percentage of time points when its instantaneous speed was <2 µm/min. The confinement ratio was calculated as track displacement (distance between start and end point)/track length. Analyses were performed for cells with tracks of at least 5 time-points.

Flow Cytometry

Brain sequestered leukocytes were isolated from PBS perfused mice as previously described (Villegas-Mendez et al., 2011). Isolated brain leukocytes were surface stained with α-mCD8 (53-6.7), CD45 (30-F11), CD3 (17A2), CD11a (M17/4), CD11b (M1/70), CD69 (H1.2F3), CXCR3 (CXCR3-173), KLRG1 (2F1), CD44 (IM7), CD62L (MEL-14), ICOS (15F9), CD40 (3/23), CD80 (16-10A1), MHC I (28-8-6). Intracellular staining for granzyme B (GB11) was performed for 1 hr, after treatment with fix-perm (eBioscience).
Dead cells were excluded using LIVE/DEAD® Fixable Blue Dead Cell Stain Kit (Life Technologies). Fluoresce minus one controls were used to set gates. Cells were analysed with a BD LSR II (Becton Dickinson) using BD FACSDiva software (Becton Dickinson), and data was analyzed with FlowJo (Tree Star Inc.). All antibodies were from eBioscience and Biolegend.

**Systemic and Intraventricular Depletion of Macrophages**

Macrophages were depleted during infection by the administration of clodronate-liposomes. Systemic depletion was performed from day 5 p.i. by injection (i.p.) of 300 µL clodronate liposomes. Depletion of perivascular macrophages, which are present behind the BBB and are not depleted by systemic administration of clodronate liposomes (Getts et al., 2008), was performed on day 5 p.i. by i.c.v. injection of 8 µL clodronate liposomes into the right lateral ventricle, adapted from Galea et al. (Galea et al., 2005). Briefly, animals were anesthetized with isoflurane (2%) in O₂ (0.2 L/min) and N₂O (0.4 L/min) and craniectomy performed. i.c.v. injection was performed using a glass microneedle (co-ordinates from bregma: anterior–posterior -0.22 mm, lateral −1.0 mm, ventral −2 mm. Sub-cutaneous buprenorphine was prophylactically administered at 0.1 mg/kg. The i.c.v. protocol was optimized and the injection placement within the ventricle confirmed by assessing the circulation of Evans blue throughout the perivascular compartments (results not shown).
Histology

Mice were exsanguinated under KXA anesthesia by intracardial perfusion with 20 mL ice cold PBS supplemented with 10 U/mL heparin. For immunohistofluorescent staining, brains were embedded in Optimum Cutting Temperature compound (O.C.T., Tissue-Tek) and frozen in a dry ice/isopropanol bath. 6-8 µm thick coronal sections were cut with a Leica CM3050 S cryotome sectioning system and mounted onto SuperFrost slides. Mounted sections were fixed in acetone at -20°C for 5 min, allowed to air dry for at least 5 hours, followed by rehydration, in TBS (20 mM Tris, 150 mM NaCl, pH 7.5) with orbital shaking for 10 min at RT. Sections were permeabлизed by soaking in TBST (0.05% Tween-20/TBS) with orbital shaking for 15 min at RT, then blocked by addition of 5% BSA, 10% mouse serum, TBS for 30 min at RT. Following washing in TBST sections were stained in the dark for 1 hour at RT with α-CD31-AlexaFluor647 (MEC13.3, Invitrogen) antibodies diluted in TBST, washed with TBST and mounted with glycerol and coverglass, and sealed with nail polish. For unstained histology, each brain was removed and fixed in 4% PFA/PBS (w/v) at 4°C overnight. Brains were washed with PBS 3 times for 5 min each. 500-µm thick coronal sections were cut with a Pelco100 vibratome sectioning system (Ted Pella Inc.) and mounted onto Superfrost slides with glycerol and sealed with nail polish. Samples were imaged with a 25x LD LCI Plan-Apochromat objective (NA 0.8, Zeiss) and analysed using Photoshop CS5 (Adobe).

Apoptosis Detection

10 µL 2 mM CellEvent Caspase-3/7 Green Detection Reagent (Invitrogen) was injected i.v. into anesthetized mice and allowed to circulate for 30-60 minutes. Z stacks were captured by two-photon microscopy spanning a surface area of 4-5 mm² and a depth of ~150 µm.
CellEvent-positive cells were counted and their number divided by the volume of brain tissue imaged.

**Statistical Analysis**

All statistical analyses were performed using GraphPad PRISM (GraphPad Software, USA). Comparison between two groups was made using unpaired t tests, with Welch’s correction where needed. Comparison between multiple groups was made using a one-way ANOVA with Tukey’s test for multiple comparisons. Differences in survival were analysed using the Mantel-Cox log-rank test.
References


Supplementary Figures

Figure S2. 1 Infection with LCMV induces petechial hemorrhages. C57BL/6 mice were intracranially infected with $10^4$ PFU LCMV-Armstrong and transcranial two-photon microscopy of the meninges was performed on day 6 p.i. LCMV encephalitis induces sporadic petechial hemorrhages in the meninges, visualized with Evans blue-stained blood (red). As indicated in these time-lapsed orthogonal images, such hemorrhages (white arrow) are usually repaired quickly. Such hemorrhages were not observed in any movies of symptomatic ECM mice, which could otherwise explain the perivascular deposition of pRBCs.

Figure S2. 2 Infection with Pb NK65 does not cause ECM development. C57BL/6 mice were intravenously infected with $10^7$ Pb ANKA or Pb NK65 pRBCs. Peripheral parasitaemia ± SD (A) and development of ECM (B) were monitored daily. (C) Representative examples of Evans blue leakage in the brains from mice infected with Pb ANKA and Pb NK65 (day 7 p.i).
Figure S2. 3 Few DsRed+ T cells are found within the brains of mice on day 5 p.i. with Pb ANKA.

hCD2-DsRed C57BL/6 mice were infected with 10^4 Pb ANKA. Transcranial two-photon microscopy of the meninges was performed on days 5 p.i. Maximum intensity projections from intravital two-photon microscopy movies showing few DsRed+ T cells (red) within the brains of infected mice on day 5 p.i. infection with Pb ANKA. Blood vessels (cyan) were visualized by i.v. injection of Qtracker® non-targeted quantum dots prior to imaging. Scale bar: 30 μm.

Figure S2. 4 hCD2-DsRed T cells from isolated meningeal vessels of Pb ANKA infected mice on day 7 p.i. are mainly CD8+.

hCD2-DsRed C57BL/6 mice were infected with 10^4 Pb ANKA or left uninfected. Meningeal vessels were removed from the whole brains of uninfected and infected (day 7 p.i.) mice and processed for flow cytometry. Representative flow cytometric plots showing frequencies of CD4+ and CD8+ T cells (gated on live leukocytes).
Figure S2. 5 CD45<sup>hi</sup>CD11b<sup>hi</sup> monocytes and macrophages are enriched within the meninges compared with the bulk brain.

CX<sub>3</sub>CR1<sup>+</sup>GFP mice were infected with 10<sup>4</sup> Pb ANKA or left uninfected. Meningeal vessels were separated from the whole brains of uninfected and infected (day 7 p.i.) mice and both parts processed for flow cytometry. Representative flow cytometric plots showing frequencies of R1 - CD45<sup>int</sup>CD11b<sup>hi</sup> microglia; R2 - CD45<sup>hi</sup>CD11b<sup>hi</sup> meningeal, perivascular macrophages and inflammatory monocytes; R3 - CD45<sup>hi</sup>CD11b<sup>int</sup> leukocytes (gated on live GFP<sup>+</sup> leukocytes) within the meninges (A) and bulk brain (B).

Figure S2. 6 Parasite specific OT-I CD8<sup>+</sup> T cells are highly arrested in the brains of infected wild type and P14 hosts.

10<sup>6</sup> naïve DsRed-expressing OT-I CD8<sup>+</sup> T cells were adoptively transferred into C57BL/6 and P14 mice, which were infected with 10<sup>6</sup> SIINFEKL-expressing Pb-TG pRBCs. Quantification of (A) average perivascular T cell speeds, (B) arrest coefficient (proportion of time points when instantaneous velocity is <2 µm/min) and (C) confinement ratio (track displacement/track length) from individual three-dimensional T cell tracks, collected from mice with ECM on day 6 p.i. (n=3).
Figure S2. 7 Elimination of high frequency speckled noise using a multiscale, undecimated "A Trous" wavelet transform.

"A trous" wavelet decomposition of a 512x512x16 volume (time point 0) using a 3x3x3 linear kernel. A single Z slice is shown through 7 decomposition layers and residual low pass layer.

Figure S2. 8 Masking of intravascular cells for specific tracking of perivascular cells.

Blood vessels (channel 1) were filtered by summing "A trous" layers 3 to 7 and thresholded to create a binary mask. DsRed T cells (channel 2) and GFP CX3CR1 cells (channel 3) were band-pass filtered using "A trous" layer 4 and pixel values below the set threshold were clipped. Cells within the blood vessel binary mask were ignored for specific tracking of perivascular cells.
Supplementary Videos

Supplementary Video 2.1. Transient cytoadhesion of intravascular Pb ANKA-pRBCs.
A representative example (at ~15 min) of a Pb ANKA-pRBC (green, highlighted by white circle) briefly adhering to the DsRed\(^+\) endothelium (orange) of an Evans blue-labeled (red) capillary, located just underneath the pia mater/glia limitans. Scale bar: 50 µm. Movie length: 31 min.

Supplementary Video 2.2. Occurrence of perivascular pRBCs. A representative example of an unmoving Pb ANKA-pRBC (green, highlighted by white circle) just next to the DsRed\(^+\) endothelium (orange) of an Evans blue-labeled blood (red) vessel. Scale bar: 20 µm. Movie length: 16 min.

Supplementary Video 2.3. Infection with LCMV induces petechial hemorrhages. LCMV-specific CFP\(^+\) P14 T cells were adoptively transferred into C57BL/6 mice, then intracranially infected with \(10^4\) PFU LCMV-Armstrong and transcranial two-photon microscopy of the meninges was performed on day 6 p.i. A representative example of a petechial hemorrhage (at ~45 min) of an Evans blue-labeled blood (red) vessel, in the context of P14 T cell (blue) movement. Scale bar: 50 µm. Movie length: 57 min.

Supplementary Video 2.4. Perivascular location and arrested behavior of T cells during Pb ANKA infection. A representative maximum intensity projection time-lapse sequence showing accumulation and arrested behavior of DsRed T cells (red) around the Pb ANKA-pRBC (green)-containing and Qtracker\(^®\) non-targeted quantum dot-labeled blood (cyan) vessels in the meninges of Pb ANKA-infected hCD2-DsRed C57BL/6 mice (day 7 p.i.). Movie length: 17 min. Z step 2 µm.
Supplementary Video 2.5. Perivascular location and dynamic behavior of T cells during Pb NK65 infection. A representative maximum intensity projection time-lapse sequence showing accumulation and dynamic behavior of DsRed T cells (red) around the Qtracker® non-targeted quantum dot-labeled blood (cyan) vessels in the meninges of Pb NK65-infected hCD2-DsRed C57BL/6 mice (day 7 p.i.). Movie length: 17 min. Z step 2 µm.

Supplementary Video 2.6. Perivascular DsRed T cells form stable interactions with CX3CR1+/GFP cells in the brains of mice infected with Pb ANKA. A representative maximum intensity projection time-lapse sequence showing stable interactions between perivascular DsRed T cells (red) and CX3CR1+/GFP cells (green) in the meninges of Pb ANKA infected dual-reporter mice (day 7 p.i.). Movie length: 17 min. Z step 2 µm.

Supplementary Video 2.7. Perivascular DsRed T cells make contact, but do not form stable interactions, with CX3CR1+/GFP cells in the brains of mice infected with Pb NK65. A representative maximum intensity projection time-lapse sequence showing brief contact made between perivascular DsRed T cells (red) and a CX3CR1+/GFP cell (green) in the meninges of Pb NK65 infected dual-reporter mice (day 7 p.i.). Movie length: 17 min. Z step 2 µm.

Supplementary Video 2.8. Perivascular DsRed T cells form stable interactions with CX3CR1+/GFP cells in the brains of mice infected with Pb ANKA. A representative 3D cropped time-lapse sequence showing a stable interaction between a perivascular DsRed T cell (red) and a CX3CR1+/GFP cell (green) in the meninges of a Pb ANKA infected dual-reporter mouse. Movie length: 17 min. Z step 2 µm.
Supplementary Video 2.9. Perivascular DsRed antigen-specific T cells are mostly arrested in the brains of mice infected with *Pb* ANKA. A representative time-lapse sequence showing stably arrested DsRed OT-I T cells (orange) surrounding a subarachnoid Evans blue-labeled blood (red) vessel, and several of which are in direct contact with endothelial cells (blue). Scale bar: 50 μm. Movie length: 33 min.
3. Chapter 3: Repeated parasite exposure induces resistance to experimental cerebral malaria by modifying intracerebral expression of inflammation-related genes concomitant with antibody-dependent suppression of CTL activity and remodelling of the splenic DC compartment.

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Abstract

Cerebral malaria (CM) remains a significant health problem, resulting in up to 500,000 deaths annually. Epidemiological evidence suggests that the age associated susceptibility to CM in malaria endemic regions is due to the evolution of the anti-parasitic immune response following repeated parasite exposure. The immunological basis of this infection-induced disease resistance is not currently well understood. In this study we have adapted the well characterised *P. berghei* ANKA experimental infection model of cerebral malaria (ECM) to replicate the repeated parasite exposure that occurs in children in malaria endemic regions. We show that three rounds of infection and drug-cure induces resistance to the development of ECM in C57BL/6 mice during a subsequent fourth infection. Transcriptional profiling performed on whole brains showed that exposure-induced ECM resistance was associated with reduced expression of genes involved in defence response, regulation of apoptosis, chemotaxis, CTL activity, antigen processing and presentation and cell adhesion, compared with ECM susceptible mice. Altered transcriptional profiles in the brains of ECM resistant mice were not maintained between infection rounds. Complementary immunophenotyping of leukocyte populations within the brain and spleen demonstrated that CD8$^+$ T cell activation was suppressed in exposure induced resistant mice, which was associated with the expansion of a splenic plasmacytoid DC (pDC) population with a regulatory phenotype. Utilisation of IgMi mice, which lack secreted antibody, demonstrated that infection-induced expansion of the pDC population, suppression of CD8$^+$ T cell activation, and protection against ECM, was dependent on secreted anti-malaria antibody production. Improvements in our basic understanding of the mechanisms involved in protection against ECM could enhance the rational development of adjunctive therapies and effective vaccines for human CM.
Introduction

Globally, 3.2 billion people are at risk of infection with malaria each year (World Health Organization, 2014). The majority of malaria-related morbidity and mortality are due to *P. falciparum* infection and are attributable to a number of severe complications that include severe anaemia, cerebral malaria and respiratory distress (Miller et al., 2002). In particular, cerebral malaria has a high case fatality rate of 10-15%, causing 300,000-500,000 deaths each year (de Souza et al., 2010). In endemic regions children less than 5 years of age are disproportionately susceptible to severe malarial disease: children under two years of age are particularly at risk of developing severe anaemia, whereas children aged 2-5yrs of age are most sensitive to cerebral malaria (Struik and Riley, 2004). In high transmission areas children aged over 5 years of age develop gradual resistance to malaria infection, first evidenced by protection against severe disease and subsequently by ability to control parasite burdens (Struik and Riley, 2004). Consequently, in high transmission areas adults typically have low density infections and rarely develop severe disease (Struik and Riley, 2004).

There is evidence that the age-associated protection against severe malarial disease in endemic regions is driven by infection-induced programming of the immune response, rather than age-dependent selection of virulent parasite strains, or natural evolution of protective immune components in the maturing host immune system. Indeed, adults infected for the first time are at increased risk of developing CM compared with children (Griffin et al., 2015, Struik and Riley, 2004, Riley, 1999). Thus, adults remain susceptible to severe malarial disease in non-endemic malarial areas and in regions of unstable transmission (de Souza et al., 2010). Notably, children resistant to severe malarial disease can still harbour parasite burdens comparable with those observed in children with severe disease (Gonçalves et al., 2014). Consequently, protection against cerebral malaria and severe malaria disease is not simply, or initially, a consequence of
improved control of parasite numbers (Gonçalves et al., 2014). Despite substantial research, the nature and identity of the immune responses that develop following natural exposure to prevent severe malaria are poorly understood (Marsh and Kinyanjui, 2006, Marsh, 1992). Moreover, whether the immune responses that protect against severe disease and those that reduce parasite burdens are of similar nature, or are highly distinct, remains to be elucidated (Riley and Stewart, 2013).

It has been known for many years that antibody is protective during both liver and blood stage malaria infection (Portugal et al., 2013). Antibodies may mediate protection by preventing invasion of new red blood cells (RBCs), blocking cytoadhesion of parasitized RBCs (pRBCs) to microvessel walls – a putatively critical event in cerebral malaria pathogenesis, neutralising proinflammatory malaria antigens or toxins, and opsonising pRBC for phagocytosis by monocytes and macrophages (Beeson et al., 2008). To date it has, however, been extremely difficult to identify antibody responses that protect against symptomatic malaria and those that provide protection. As such, associations between antibody responses and protection against severe malaria disease have been inconsistent (Fowkes et al., 2010), and in some cases high antibody levels have predicted susceptibility to infection and cerebral malaria (Dobaño et al., 2008). This is largely because antibody responses can be a measure of parasite exposure rather than protection per se (Dobaño et al., 2008, Greenhouse et al., 2011). Consequently, the identity of the parasite molecules targeted by protective antibodies is not well defined (Riley and Stewart, 2013, Portugal et al., 2013). Moreover, whether malaria infection generates atypical and short lived memory B cell compartments remains to be resolved (Portugal et al., 2013).

Individuals experiencing severe malaria also frequently exhibit high pro-inflammatory to regulatory plasma cytokine ratios compared with individuals with uncomplicated malaria (Kwiatkowski et al., 1990, Keller et al., 2006), suggesting that acquired
resistance may be established through improved immune homeostasis during infection (Walther et al., 2009). However, whether this snapshot within the plasma truly reflects the status of the immune response within tissues, such as the brain, which is the primary organ affected during cerebral malaria, has yet to be resolved. Foxp3+ regulatory T cells, IL-10 secreting T cells and tolerance to malaria toxins, such as GPI and hemozoin, have all been postulated to play important roles in the spatiotemporal establishment of immune balance during malaria infection, but their relative contribution to protection and how these responses develop is poorly understood (Langhorne et al., 2008, Riley and Stewart, 2013, Walther et al., 2009). Conversely, T cell dysfunction and exhaustion, which is associated with T cell expression of PD-1 and LAG-3, has been shown to inhibit effective parasite control (Butler et al., 2012), highlighting the importance of establishment of an appropriate balance between inflammation and regulation/suppression during infection. CD4+ T cells, principally through IFNγ production are required during malaria infection to optimise phagocyte activity and to instruct B cell responses and antibody production (Crompton et al., 2014, Riley and Stewart, 2013).

The inability to assess, within tissues, the immune responses that promote or protect against severe malarial disease in humans led to the development of the tractable Plasmodium berghei ANKA (PbA) murine model of cerebral malaria. Primary-infected C57BL/6 mice typically develop a neurological syndrome termed experimental cerebral malaria (ECM) between days 6 and 8 p.i. that recapitulates many of the clinical and pathological features of human cerebral malaria (Hearn et al., 2000, Lou et al., 2001). Utilisation of myriad transgenic animals and comparisons between susceptible and resistant (for example BALB/c) mice have demonstrated the importance of immune mediators including IFNγ producing NK cells, Th1 cells and granzyme B and perforin expressing CD8+ T cells in the development of cerebral pathology (Villegas-Mendez et
al., 2012, Haque et al., 2011, Nitcheu et al., 2003, Mohan et al., 1997, Baptista et al., 2010, Hanum P et al., 2003). To date, however, this ECM-model has been underused in the study of immune pathways that provide protection against severe malaria disease and only very recently has the model been developed to examine the mechanisms of exposure-induced protection against ECM (Bao et al., 2013), mimicking that observed in humans following *P. falciparum* infections.

In this study we have examined whether exposure-induced resistance against ECM is established by reprogramming of systemic immune responses against the parasite and/or whether it is imparted through altered conditioning and refractoriness of the brain environment.

We show, comparable to Bao et al. (Bao et al., 2013), that three rounds of infection and drug-cure can induce almost 100% resistance to the development of ECM during a subsequent fourth infection. We demonstrate that exposure-induced resistant mice display a significantly altered brain transcriptional signature compared with susceptible primary-infected mice, with lower expression levels of genes involved in antigen processing and presentation, IFNγ dependant pathways, chemokine pathways and T cell mediated cytotoxicity. In agreement, exposure-induced resistant mice exhibited attenuated inflammatory T cell responses within the brain and spleen, associated with significant remodelling of the splenic dendritic cell compartment. Finally, we show, utilising IgMi mice, that infection-induced protection against ECM, and modulation of anti-parasite innate and adaptive inflammatory immune pathways, are orchestrated by antibody-dependent mechanisms. The results in this study provide further evidence for the importance of antibody in protection against severe malarial disease and highlight novel pathways that can potentially be exploited to promote resistance to malaria-induced cerebral pathology.
Results

Repeated cycles of infection and drug-cure protects mice against ECM

To recapitulate the natural repeated malaria infections experienced by humans in endemic regions we adapted the established PbA model of ECM. C57BL/6 mice were infected with PbA and treated with anti-malarial drugs prior to the development of fulminant ECM. This cycle of infection-drug cure was repeated up to three times, with a minimum interval of 30 days between cessation of drug treatment and reinfection, as depicted in Figure 3.1. After each cycle the susceptibility of mice to ECM, and their ability to control parasite levels, was assessed by infecting mice with PbA (without drug cure) and monitoring the course of disease. As expected, 100% of mice infected for the first time developed ECM by day 8 p.i. and mice experiencing a second infection developed accelerated ECM, with 100% of mice developing ECM by day 7 p.i. (Figure 3.1B). In contrast, mice experiencing a third infection displayed intermediate resistance to ECM with 44% developing ECM whereas mice experiencing a fourth infection were completely resistant to late stage ECM (Figure 3.1B). Very few signs of pathology, such as haemorrhage or blocked vessels were observed in brains from mice experiencing a fourth infection, in contrast to that observed in primary infected mice (Figure 3.1C). Mice experiencing a second infection exhibited a minor reduction in peripheral parasite burdens on day 7 compared with primary infected mice (Figure 3.1D). Mice infected for a third time showed a slight delay in parasite patency; however, 89% of mice that survived the ECM-window period developed hyperparasitaemia and anaemia between days 14 and 30 p.i. (Figure 3.1B, D and results not shown). Mice infected for a fourth time exhibited reduced parasite burdens compared with other infected groups up to day 9 p.i. At day 13 p.i., 67% of mice infected for a fourth time developed hyperparasitaemia, which was brought under control again in 83% of mice by day 24 p.i. 80% of mice infected for a fourth time
survived past day 30, with earlier terminations due to the development of hyperparasitaemia and anaemia (Figure 1B, D). This model, therefore, mimics the gradual parasite-exposure dependent resistance to cerebral malaria seen in humans. As such it is a powerful system to investigate the infection-induced immune mechanisms that protect against severe malarial disease.

Figure 3.1 Susceptible mice are made resistant to ECM development by three preceding infections. C57BL/6 mice were infected with Pb ANKA (10^4 pRBCs i.v.) or left uninfected. (A) Four groups of infected mice were generated and defined as follows: - R1. Mice infected for a first time, later referred to as ECM affected mice. R2. Mice infected, treated with chloroquine and artesunate on days 6-12 p.i. and re-infected for a second time. R3. Mice generated as R2, treated with chloroquine and artesunate on days 5-11 p.i. and re-infected for a third time. R4.
Mice generated as R3, treated with chloroquine and artesunate on days 5-11 p.i. and re-infected for a fourth time, later referred to as ECM resistant mice. Re-infections were performed after a minimum interval of 30 days between cessation of drug treatment. (B) Kinetics of ECM development shown as percentage of survival of mice (n=8-10 from two independent experiments). (C) Representative histological examination of brains from an R1 and R4 mouse. Arrow highlights an area of haemorrhage. (D) Parasitaemia (% of pRBCs) ± SD in tail blood from mice infected for a first, second, third or fourth time. Comparison of parasitaemia on day 7 p.i. by one-way ANOVA with Tukey's multiple comparisons test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

Parasite exposure-induced resistance to ECM is associated with a distinct brain transcriptional signature and altered intracerebral leukocyte compartment

To assess the basis of infection-induced resistance to ECM we contrasted the whole genome transcriptional profiles of whole brains from uninfected mice, mice with signs of ECM (first infection: day 8) and brains from mice that exhibited parasite exposure-induced resistance to ECM (fourth infection: day 8). The brains from the three different groups exhibited distinct transcriptional signatures, as shown by principal component analysis (PCA) (Figure 3.2A). 1639 genes were differentially expressed (fold change -1.5 < or > 1.5 and q value <0.05) in brains of ECM affected mice, but not in ECM resistant mice, compared with brains from uninfected mice. 297 genes were differentially expressed within the brains of both ECM affected and ECM resistant mice compared with uninfected mice and only 21 genes were differentially expressed specifically in brains from ECM resistant mice, but not ECM affected mice, compared with uninfected mice (Figure 3.2B). Genes differentially expressed in brains of ECM affected or ECM resistant mice compared with brains from uninfected mice were clustered by K-means into 5 clusters and ranked by hierarchal clustering (Figure 3.2C). Pathways enriched in cluster 1, which were down-regulated in both infected groups, included synaptic vesicles. Pathways involved in metal ion binding, zinc ion binding and transcription were down-regulated specifically in brains from ECM affected mice (cluster 2) (Figure 3.2C). Multiple pathways involved in GTP binding and innate and adaptive immunity were preferentially enriched in clusters 4 and 5, being upregulated in
brains of both ECM affected and ECM resistant mice compared with brains from uninfected mice (Figure 3.2C).

The transcriptional perturbations observed in ECM affected and ECM resistant mice could have been entirely due to changes in the magnitude and composition of the intracerebral leukocyte responses in the two different groups. To examine if this was the case we immunophenotyped the intracerebral cell response in ECM affected, ECM resistant and uninfected mice (Figure 3.2D). The dominant leukocyte population within the brain changed from microglia (54.2±15.2%) in uninfected mice to CD8+ T cells in mice affected with ECM (49.3±4.8%). Whilst, CD8+ T cells were also the dominant leukocyte population within the brains of ECM resistant mice, they constituted a significantly smaller proportion of the total leukocytes (37.5±7.5%) compared with ECM affected mice. Notably, CD4+ T cells constituted a significantly higher proportion of the total brain leukocyte response in ECM resistant mice (26.2±5.5%) compared with mice with ECM affected mice (8.0±3.2) and naïve mice (7.0±4.1%) (Figure 3.2D). The numbers of brain accumulating CD8+ T cells was significantly increased in ECM affected mice, but not ECM resistant mice, compared with naïve mice (Figure 3.2E) whereas the numbers of brain accumulating CD4+ T cells were significantly increased in ECM resistant mice compared with uninfected mice (Figure 3.2F). The frequencies of other identified brain infiltrating leukocytes, including CD11b-CD11c+, CD11b+CD11c+, Ly6C+CD11b+ and Ly6C-CD11b+ did not significantly vary between the three groups of mice, with the exception of Ly6C+CD11b+ cells which were more frequent in ECM affected mice than naïve mice and Ly6C-CD11b+ cells which were reciprocally decreased (Figure S3.1). Combined these results show that infection-induced resistance to ECM is associated with a restructuring of the intracerebral leukocyte population. However, the magnitude and compositional changes in the intracerebral immune response appear insufficiently
different between ECM resistant and ECM affected mice to wholly explain the significant alterations in the infection-induced brain transcriptional response.
Figure 3.2 ECM resistant mice have a distinct intracerebral gene expression profile and leukocyte composition.
C57BL/6 mice were infected (10^4 pRBCs i.v.), or not, and treated as described in Figure 1. Whole brains were removed for RNA extraction and flow cytometric analysis when infected mice developed ECM signs (day 8 p.i.). (A) Principal components analysis of individual exposure-induced ECM resistant mice (purple), ECM affected mice (blue) and uninfected mice (red). X-axis (PCA1) and Y-axis (PCA2) are principal component 1 and 2, respectively, of the gene expression profiles obtained by DNA microarray (n=6, from 2 independent experiments). (B) Venn diagrams of intracerebral gene expression profiles of exposure-induced ECM resistant mice, ECM affected mice and uninfected mice, showing the intersection between genes differentially expressed in ECM affected mice v uninfected mice, and ECM resistant mice v uninfected mice. (C) Dendogram of genes (right of matrix) represent hierarchical clustering of differentially expressed genes in ECM resistant mice, ECM affected mice and uninfected mice. Each probe-set expression level was normalised to the naïve average. Colour scale: green low, black average, red high. Enriched pathways are detailed left of the matrix. (D) Pie charts showing the relative contribution of specific cellular populations to the total CD45⁺ population in the brains of ECM resistant mice, ECM affected mice and uninfected mice (n=4-5). Pie charts are scaled to represent relative number compared with numbers from uninfected mice. (E) Total numbers ± SD of intracerebral CD8⁺ T cells (n=4). (F) Total numbers ± SD of intracerebral CD4⁺ T cells (n=4). Flow cytometry results are representative of 2 independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (one-way ANOVA with Tukey’s multiple comparisons test).

Exposure-induced resistance to ECM is associated with reduced transcription of inflammation-related pathways and corresponding attenuated intracerebral CD8⁺ T cell pathogenic functionality

The differentially expressed genes in brains of the infected mice compared with naïve mice (defined in Figure 3.2C) were further filtered to identify the genes also differentially expressed between brains of ECM affected mice and ECM resistant mice. Using DAVID bioinformatics database it was found that many of these differentially expressed genes were enriched within immunological pathways involved in defence responses, regulation of apoptosis, chemotaxis, CTL activity, antigen processing and presentation and cell adhesion (Figure 3.3A and Figure S3. 2). In general, the majority of the pro-inflammatory genes were expressed at higher levels in brains from ECM affected mice compared with ECM resistant mice (Figure 3.3A). In agreement with these observations, cytotoxicity of T lymphocytes was predicted to be reduced in brains of ECM resistant compared with ECM susceptible mice using Ingenuity network
analysis (Figure 3.3B). Moreover, IFNγ and IL-6 were identified as two major gene hubs that were expressed at lower levels in brains of ECM resistant mice compared with ECM susceptible mice (Figure S3. 3).

Consistent with the nature of the gene pathways down-regulated in the brains of ECM resistant mice, using flow cytometry we observed significantly lower frequencies of CD4+ T cells expressing granzyme B and Ki67 in the brains of ECM resistant mice compared with ECM affected mice. Although increased compared with naïve mice, expression of ICOS and KLRG1 was, however, comparable on CD4+ T cells from the brains of ECM resistant and ECM affected mice (Figure 3.3C). In addition, CD8+ T cells displayed a less activated phenotype in the brains of ECM resistant mice compared with ECM affected mice, as evidenced by lower expression of granzyme B, ICOS and Ki67. CD8+ T cells were, however, similarly terminally differentiated in both groups of mice, as highlighted by KLRG-1 expression (Figure 3.3C). Combined, these results show that exposure-induced resistance to ECM is associated with suppression of key proliferative and cytotoxic activities of brain accumulating T cells. As CD8+ T cells play a central role in ECM pathology (Belnoue et al., 2002), these changes likely attenuate the establishment of cerebral pathology in these mice.
Figure 3.3 ECM resistant mice show constrained up-regulation of genes and T cell markers associated with inflammation.

C57BL/6 mice were infected (10⁴ PRCs i.v.), or not, and treated as described in Figure 1 to generate ECM affected and ECM resistant mice. Whole brains were removed for RNA extraction and flow cytometric analysis when ECM affected mice developed ECM signs (day 8 p.i.). (A) Heat maps of filtered genes differentially expressed in brains of ECM affected mice and ECM resistant mice, grouped by the biological functions indicated. Only selected genes from the defence response and regulation of apoptosis genes clusters are shown. Colour scale: green low, black average, red high (n=6, from 2 independent experiments). (B) Ingenuity® bioinformatics network of genes involved in cytotoxicity of T lymphocytes identified as being expressed at reduced (green) and increased (red) levels in brains from ECM resistant mice compared with ECM affected mice. (C) Activation phenotype of intracerebral CD4⁺ and CD8⁺ T cells from ECM resistant, ECM affected and uninfected mice (n=4). Flow cytometry results are representative of 2 separate experiments. Bars represent mean number ± SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (one-way ANOVA with Tukey's multiple comparisons test).
Repeated parasite infection does not lead to long term maintained changes in brain transcriptional signature, or altered intracerebral leucocyte response. The protection against ECM, and the associated modulation of intracerebral inflammatory responses, observed in repeatedly infected animals could have resulted from stable infection-induced programmed changes in the brain that modified the intrinsic response of the brain to subsequent parasite exposures. To investigate this, we assessed the whole genome transcriptional signature and intracerebral leukocyte responses in brains from mice that had undergone three rounds of PbA infection and drug cure but did not receive a further infection. Surprisingly brains from these uninfected-resistant mice displayed a transcriptional signature that was almost identical to that in brains from naïve mice, as defined by PCA analysis (Figure 3.4A) and scatterplot comparison between mean gene expression in brains of uninfected CM resistant and uninfected mice (Figure 3.4B). Furthermore, the magnitude and composition of the leukocyte population (Figure 3.4C), and T cell response (Figure 3.4D and E), were also largely comparable in the brains of uninfected ECM resistant and naive mice. These results suggest parasite exposure does not lead to long-term and maintained alterations in gene expression or intracerebral immune responses in the brain. Consequently, the resistance of repeatedly infected mice to ECM is unlikely to be due intrinsic modifications that protect the brain against malaria-induced pathology.
Figure 3.4 Uninfected ECM resistant mice have a comparable gene expression profile and intracerebral leukocyte composition as uninfected mice. C57BL/6 mice were infected (10^4 pRBCs i.v.), or not, and treated as described in Figure 1 to generate R3 mice that did not receive a fourth infection (uninfected resistant mice). Whole brains were removed for RNA extraction and flow cytometric analysis at least 30 days after cessation of drug treatment. (A) Principal components analysis of individual uninfected ECM resistant mice (green) and uninfected mice (red). X-axis (PCA1) and Y-axis (PCA2) are principal component 1 and 2, respectively, of the gene expression profiles obtained by DNA microarray (n=6, from 2 independent experiments). (B) Scatter plot comparing gene expression between uninfected ECM resistant and uninfected mice. Each point represents the mean expression level of a gene from 6 individual mice, from 2 experiments. Red dots represent...
differentially expressed genes. (C) Pie charts showing the relative contribution of specific cellular populations to the total CD45+ population in the brains of uninfected ECM resistant mice and uninfected mice (n=4). Pie charts are scaled to represent relative number compared with numbers from uninfected mice. (D) Total numbers ± SD of intracerebral CD4+ T cells (n=4). (F) Total numbers ± SD of intracerebral CD8+ T cells (n=4). Flow cytometry results are representative of 2 independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (one-way ANOVA with Tukey’s multiple comparisons test).

Exposure-induced resistance to ECM in WT mice is associated with suppression of splenic T cell activation

The lack of any apparent maintained alterations in the brain of parasite-exposed animals led us to examine whether the resistance of mice to ECM during the fourth infection was due to the active reprogramming of immune responses in other locations. Specifically, we hypothesised that infection-induced resistance during the fourth infection was the result of modulated T cell immunity in the spleen, the major site of T cell priming in malaria infection (Curfs et al., 1989). Significant splenomegaly was observed in ECM resistant mice, evidenced by increased total CD45+ leukocyte numbers compared with ECM affected and uninfected mice (Figure 3.5A). Increased numbers of CD4+ T cells (Figure 3.5B) and CD8+ T cells (Figure 3.5C) were found to contribute to this splenomegaly. The splenic CD4+ T cell response was marginally altered in ECM resistant mice, with reduced frequencies of GrB+ CD4+ T cells and higher frequencies of ICOS+ CD4+ T cells compared with ECM affected mice (Figure 3.5D). As opposed to ECM affected mice, the frequencies of splenic KLRG-1+ and Ki67+ CD4+ T cells were not significantly higher in ECM resistant mice compared with uninfected mice (Figure 3.5D). In contrast, the CD8+ T cell response was significantly attenuated in the spleens of ECM resistant mice compared with ECM affected mice. Significantly lower frequencies of GrB+, ICOS+ and Ki67+ splenic CD8+ T cells were observed in ECM resistant mice compared with ECM affected mice (Figure 3.5E). The frequencies of splenic CD8+ T cells expressing KLRG1 were, however, comparable in ECM resistant and ECM affected mice (Figure 3.5E). These results indicate that the
altered T cell response observed in the brains of ECM resistant mice is likely driven by parasite-exposure induced changes in T cell activation and differentiation within the spleen.

Figure 3.5 ECM resistant mice have enlarged spleens containing CD8+ T cells with an attenuated activation phenotype. C57BL/6 mice were infected (10^4 pRBCs i.v.), or not (n=3), and treated as described in Figure 1 to generate ECM affected (n=3) and ECM resistant mice (n=3). Spleens were removed for flow cytometric analysis when ECM affected mice developed ECM signs (day 8 p.i.). (A) Total numbers of splenic CD45+ leukocytes. (B) Total numbers of splenic CD4+ T cells. (C) Total numbers of splenic CD8+ T cells. (D) Activation phenotype of splenic CD4+ T cells from ECM resistant, ECM affected and uninfected mice. (E) Activation phenotype of splenic CD8+ T cells from ECM resistant, ECM affected and uninfected mice (n=3). Results are representative of 2 separate experiments. Bars represent mean number ± SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (one-way ANOVA with Tukey's multiple comparisons test).
Splenic T\textsubscript{reg} subsets are not expanded in parasite exposure-induced ECM resistant mice

Foxp3\(^+\) regulatory T cells (T\textsubscript{reg}) have been widely shown to dampen pro-inflammatory immune responses during a wide variety of infections (Hall et al., 2008). Consequently we examined if Treg expansion and activation was enhanced in ECM-resistant compared with ECM-susceptible mice, causing the observed attenuation of splenic and intracerebral T cell responses. In contrast to expectations, the percentage of total T\textsubscript{reg} was significantly reduced in ECM resistant mice compared with ECM affected mice (Figure 3.6A and B). Although the ratio of T\textsubscript{reg}: CD4\(^+\) T cells was increased in ECM resistant mice compared with the other groups, the ratio of T\textsubscript{reg}: CD8\(^+\) T cells was not significantly different in ECM affected and ECM resistant mice and was significantly lower in ECM resistant mice compared with uninfected mice (Figure 3.6C and D).

T\textsubscript{regs} may employ various different regulatory mechanisms and a number of T\textsubscript{reg} subsets have recently been identified that are specialised for regulating specific inflammatory immune responses (Campbell and Koch, 2011). In particular Tigit\(^+\) and CXCR3\(^+\) Foxp3\(^+\) T\textsubscript{regs} have been shown to be important for regulating Th1 responses (Koch et al., 2009, Hall et al., 2012, Joller et al., 2014). In general, apart from an increase in Tigit expression, T\textsubscript{reg} phenotype (CD25, CXCR3, CD103, CD127) and function (CTLA-4) were largely comparable in ECM resistant and ECM affected mice (Figure 3.6E and F). These results suggest that induction and specialised activation of T\textsubscript{regs} is not a major mechanism involved in the development of parasite exposure-induced ECM resistance.
Figure 3.6 ECM resistant is not associated with an enhanced T\textsubscript{reg} population.
C57BL/6 mice were infected (10^4 pRBCs i.v.), or not (n=3), and treated as described in Figure 1 to generate ECM affected (n=3) and ECM resistant mice (n=4). Spleens were removed for flow cytometric analysis when ECM affected mice developed ECM signs (day 8 p.i.). (A) Representative flow cytometric plots showing frequencies of Foxp3\textsuperscript{+} CD4\textsuperscript{+} T cells (gated on live CD45\textsuperscript{+} CD4\textsuperscript{+} leukocytes) within the spleens of uninfected, ECM affected and ECM resistant mice. (B) Percentages of Foxp3\textsuperscript{+} CD4\textsuperscript{+} T cells within the spleens of uninfected, ECM affected and ECM resistant mice. (C) Ratios of Foxp3\textsuperscript{+} CD4\textsuperscript{+} T cells and Foxp3\textsuperscript{+} CD4\textsuperscript{+} T cells within the spleens of uninfected, ECM affected and ECM resistant mice. (D) Ratios of Foxp3\textsuperscript{+} CD4\textsuperscript{+} T cells and CD8\textsuperscript{+} T cells within the spleens of uninfected, ECM affected and ECM resistant mice. (E) Representative histograms and calculated geometric means (F) of CTLA-4, Tigit, CD127, CXCR3, CD103 and CD25 on splenic Tregs of uninfected, ECM affected and ECM resistant mice. Results are representative of 2 separate experiments. Bars represent mean number ± SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (one-way ANOVA with Tukey's multiple comparisons test).

Plasmacytoid dendritic cells are expanded within the spleens of parasite exposure-induced ECM resistant mice
As the T\textsubscript{reg} response was only slightly modified in ECM resistant mice compared with ECM affected mice we explored other reasons for the attenuated T cell response during fourth infection. Notably, modulation of the DC compartment during primary malaria infection, in particular the depletion of CD8\textsuperscript{+} DCs, has been shown to promote resistance to ECM during primary malaria infection (deWalick et al., 2007, Piva et al., 2012). Moreover, the generation of tolerogenic DCs have also been shown to suppress inflammation during malaria infection (Wong and Rodriguez, 2008). Consequently, we hypothesised that the resistance of mice during a fourth infection may be due to alterations in T cell priming by the DC compartment. In agreement with this, we observed a dramatic and significant increase in both the percentage (Figure 3.7A-C) and number (Figure 3.7D) of CD11c\textsuperscript{+} MHC II\textsuperscript{+} cells in the spleens of ECM resistant mice compared with ECM susceptible mice. The CD11c\textsuperscript{+}MHCII\textsuperscript{+} cells in ECM resistant mice were largely CD8\textalpha{} and CD11b negative but expressed high levels of PDCA1, indicating they may be plasmacytoid DCs (pDC) (Figure 3.7E). To confirm this identification, as PDCA-1 expression can be promiscuous (Blasius et al., 2006), we
performed a more in depth phenotypic analysis of the PDCA-1\(^+\)CD11c\(^+\) population, gated on as shown in (Figure 3.7F and G). These cells were CD19\(^{hi}\) and Ly6C\(^{hi}\) in mice during primary and fourth infections (Figure 3.7H). However, as opposed to cells from primary infected mice, the PDCA-1\(^+\) CD11c\(^+\) cells expressed low levels of IgM and IgD in mice infected for a fourth time. In agreement, using an ImageStream, the PDCA\(^+\)CD11c\(^+\) cells were significantly larger and displayed morphology atypical of B cells (Figure 3.7I and J). Combined, this strongly indicated that the PDCA-1\(^+\)CD11c\(^+\) cells were pDCs in ECM resistant mice. The pDCs expressed low to intermediate levels of costimulatory markers CD40 and CD80 and high levels of inhibitory marker PDL1 and regulatory associated marker CD45RB during fourth infection, similar to that observed during primary infection (Figure 3.7H). Thus, repeated malaria infection leads to formation of a large splenic pDC population exhibiting a regulatory phenotype, concomitant with attenuation of T cell priming and protection against ECM.
Figure 3.7 ECM resistant mice have an altered DC compartment.
C57BL/6 mice were infected (10^7 pRBCs i.v.), or not (n=6), and treated as described in Figure 1 to generate ECM affected (n=8) and ECM resistant mice (n=8, pooled from 2 experiments). Spleens were removed for flow cytometric analysis when ECM affected mice developed ECM signs (day 8 p.i.). (A) Representative flow cytometric plots showing frequencies of CD11c<sup>+</sup> MHC II<sup>+</sup> cells (gated on live CD45<sup>+</sup> leukocytes) within the spleens of uninfected, ECM affected and ECM resistant mice. (B) Percentages and (C) total numbers of CD11c<sup>+</sup> MHC II<sup>+</sup> within the spleens of uninfected, ECM affected and ECM resistant mice. (D) Representative histograms of CD8α, CD11b and PDCA-1 expression on splenic CD11c<sup>+</sup> MHC II<sup>+</sup> cells from uninfected, ECM affected and ECM resistant mice. (E) Representative histograms of CD19, Ly6C, IgM, IgD, CD40, CD80, PDL-1 and CD45RB expression on splenic CD11c<sup>+</sup> PDCA-1<sup>+</sup> cells. (F) Representative flow cytometric plots showing the forward scatter (FSC) and side scatter (SSC) properties of splenic CD11c<sup>+</sup> PDCA1<sup>+</sup> cells (blue), compared to total live leukocytes (red), from uninfected, ECM affected and ECM resistant mice. (G) Representative ImageStream<sup>®</sup> images of a single B cell (CD45<sup>+</sup> CD3<sup>−</sup> CD11c<sup>−</sup> CD19<sup>+</sup>), a single CD11c<sup>+</sup> PDCA1<sup>−</sup> cell (CD45<sup>+</sup> CD3<sup>−</sup>) and a single CD11c<sup>+</sup> PDCA1<sup>−</sup> cell (CD45<sup>+</sup> CD3<sup>−</sup>) from the spleen of an ECM resistant mouse. Flow cytometric results are representative of 2 separate experiments. Bars represent mean number ± SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (one-way ANOVA with Tukey's multiple comparisons test).

An expanded splenic DCs population is not maintained between malaria infections
The expanded pDC population observed in mice during a fourth infection could potentially have been the consequence of stable changes imprinted by three rounds of PbA infection and drug cure. To assess if this was the case, we compared the splenic DC compartment of uninfected-resistant mice with naïve mice. We observed no differences in percentage (Figure 3.8A and B) or number of CD11c<sup>+</sup> MHC II<sup>+</sup> cells (Figure 3.8C) in spleens of uninfected-resistant mice compared with naïve mice.
Moreover, the DCs exhibited comparable activation, as assessed by CD40 and CD80 expression, in uninfected and uninfected resistant mice (Figure 3.8D). This suggests that the expanded pDC population observed in infected ECM resistant mice was induced actively during a fourth malarial infection in the context of parasite re-exposure.

Figure 3.8 Splenic pDCs are not expanded prior to a fourth infection. C57BL/6 mice were infected (10⁴ pRBCs i.v.), or not (n=5), and treated as described in Figure 1 to generate R3 mice that did not receive a fourth infection (uninfected resistant mice) (n=3). Spleens were removed for flow cytometric analysis at least 30 days after cessation of drug treatment. (A) Representative flow cytometric plots showing frequencies of CD11c⁺ MHC II⁺ cells (gated on live CD45⁺ leukocytes) from the spleens of uninfected, and uninfected ECM resistant mice. (B) Percentages and (C) total numbers of CD11c⁺ MHC II⁺ within the spleens of uninfected and uninfected ECM resistant mice. (D) Representative histograms of CD40 and CD80 expression on splenic CD11c⁺ MHC II⁺ cells. Results are representative of 2 separate
Secreted antibody is required for the expansion of resistance-associated pDCs and attenuation of CD8\(^+\) T cell responses in the spleen and brain

As the DC splenic compartment did not appear to be stably modified in mice between repeated malaria infections, we questioned which immune memory components may lead to reprogramming of the innate response during the fourth infection, subsequently affecting the adaptive T cell response. Antibody has been shown to be important for parasite control and protection against severe during malaria infection in humans and in mice (Crompton et al., 2014, Perez-Mazliah and Langhorne, 2014). Moreover, pDCs express Fc receptors and antibody complexes can lead to their activation (Guilliams et al., 2014, Björck et al., 2008). Consequently to examine the importance of Abs in orchestration of the resistant phenotype in repeatedly infected mice we used IgMi mice, that are unable to make secreted antibody (Waisman et al., 2008), in our infection-drug cure model.

As anticipated, IgMi mice developed a significantly reduced splenic CD11c\(^+\)MHC-II\(^+\) response during fourth infection compared with corresponding WT mice (Figure 3.9A-C). In addition, as opposed to results in WT mice, the CD11c\(^+\)MHC-II\(^+\) cells did not acquire pDC characteristics, including PDCA-1 expression, in four time-infected IgMi mice. (Figure 3.9D).

We next assessed how the alteration in the DC compartment in IgMi mice during fourth infection impacted on the nature of the T cell response. The splenic and intracerebral CD4\(^+\) T cell responses were largely unaltered in IgMi mice during a fourth infection (Figure 3.9E), as assessed by GrB, ICOS, Ki67 and KLRG-1 expression, compared with corresponding repeatedly infected WT mice. This was consistent with the minor
alteration in CD4⁺ T cell immunity observed in repeatedly infected WT mice compared with primary infected WT mice (Figure 3.5). However, in agreement with a putatively important role for pDCs in suppressing pathogenic T cell responses in WT mice during a fourth infection, the frequencies of CD8⁺ T cells expressing granzyme B, ICOS, Ki67 and KLRG1 were significantly increased in both the spleen and brains of repeatedly infected IgMi mice compared with WT ECM resistant mice (Figure 3.9F). Together these results suggest that the presence of secreted anti-malaria antibody is necessary for the expansion of pDCs and concomitant suppression of CD8⁺ T cell activation following repeated parasite infection.
Figure 3.9 Repeated infection does not induce splenic pDC expansion in IgMi mice

C57BL/6 (n=4) and IgMi (n=3) mice were infected (10^4 pRBCs i.v.), or not (n=2), and treated as described in Figure 1 to generate mice infected for a fourth time. Spleens were removed for flow cytometric analysis on day 7 p.i. (A) Representative flow cytometric plots showing frequencies of CD11c^+ MHC II^+ cells (gated on live CD45^+ leukocytes) from the spleens of WT ECM resistant mice and 4X infected IgMi mice. (B) Percentages and (C) total numbers of CD11c^+ MHC II^+ within the spleens of WT ECM resistant mice and 4X infected IgMi mice. (D) Representative histograms of CD8α, CD11b and PDCA-1 expression on splenic CD11c^+ MHC II^+ cells from WT ECM resistant mice and 4X infected IgMi mice. (E) Activation
phenotype of splenic CD4+ cells and (F) CD8+ T cells from WT ECM resistant mice and 4X infected IgMi mice. Results are representative of 2 separate experiments. Bars represent mean number ± SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (one-way ANOVA with Tukey’s multiple comparisons test).

IgMi KO mice develop accelerated ECM during a fourth infection - Secreted antibody is necessary for the development of parasite exposure-induce ECM resistance

Finally, we examined whether lack of antibody, and abrogated development of pDCs and dysregulated T cell responses, in repeatedly infected IgMi mice impacted on the development of infection-induced resistance to ECM. IgMi mice displayed equivalent susceptibility to WT mice during a first infection, with 85.7% (6/7) developing ECM by day 8 p.i (Figure 3.10A). These data are consistent with lack of pathogenic role for B cells or antibody in ECM development (Yanez et al., 1996). Crucially, in contrast to WT mice, repeated infection failed to protect IgMi mice against ECM, and IgMi mice infected for a fourth time exhibited accelerated ECM development with 100% (8/8) developing ECM between days 6 and 7 p.i. (Figure 3.10A). Primary infected IgMi mice tended to develop increased peripheral parasite burdens compared with primary infected WT mice (Figure 3.10B). In addition, IgMi mice developed elevated parasite levels during fourth infection compared with correspondingly infected WT mice (Figure 3.10B). However, parasite burdens in IgMi mice was reduced during fourth infection compared with primary infection (Figure 3.10B). Combined these results indicate that infection-induced resistance to ECM is antibody dependent. Furthermore, antibody is also important for control of peripheral parasite burdens during PbA infection, but a degree of antibody-independent parasite control can be generated.
Figure 3.10 Repeated infection does not protect IgMi mice from ECM.
C57BL/6 and IgMi mice were infected (10^4 pRBCs i.v.), or not, and treated as described in Figure 1 to generate R1 mice (n=6-8, pooled from 2 experiments), infected for a first time and R4 mice (n=7-8, pooled from 2 experiments), infected for a fourth time. (A) Kinetics of ECM development shown as percentage of survival of mice. (B) Parasitaemia (% of pRBCs) ± SD in tail blood from mice infected for a first or fourth time. Comparison of parasitaemia on day 7 p.i. by one-way ANOVA with Tukey's multiple comparisons test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
Discussion

In this study, we have shown that multiple rounds of PbA parasite exposure lead to the incremental development of ECM resistance in otherwise susceptible C57BL/6 background mice. Resistance against ECM was exemplified by absence of neurological signs, absence of cerebral haemorrhage and suppressed activation of CD8\(^+\) T cells within the brain. Protection was also associated with gradually acquired control of peripheral parasite burdens, although sterile immunity was not achieved. It is highly likely that protected mice also exhibited reduced parasite burdens in the brain, as parasite accumulation within the brain has been shown to be necessary for ECM development (Baptista et al., 2010, Franke-Fayard et al., 2004, Amante et al., 2010). Whilst longitudinal studies investigating the development of resistance to CM in humans are lacking, our results appear to recapitulate the development of naturally acquired resistance in endemic regions of malaria. Of note, it is unlikely that chloroquine and artesunate administration directly promoted the development of resistance against ECM in our experiments as IgMi mice, which received comparable drug regimes to WT mice in our infection-drug cure model, were completely susceptible to ECM during a fourth infection.

Transcriptional profiling of the brains from ECM affected and ECM resistant mice showed altered expression of a wide range of genes involved in homeostasis and inflammation pathways compared with uninfected mice. Notably very few genes were differentially expressed specifically in ECM resistant brains compared with uninfected mice. In contrast, a number of genes, many of which are related to brain function and ion binding were specifically down-regulated in brains of ECM affected mice compared with uninfected mice. The expression profile of many of these genes likely reflected the established neurological disturbances and cerebral pathology in these mice, rather than representing causal processes in formation of the syndrome. The majority of the genes
relating to immune function, such as defence response, chemokine activity, regulation of apoptosis, cell adhesion, antigen processing and presentation and CTL mediated immune responses were upregulated in brains of both groups of infected mice compared with uninfected mice. However, up-regulation was to a significantly lower level in ECM resistant mice compared with ECM affected mice. This suggested that the inflammatory response to a fourth infection was constrained in its magnitude, if not type, in comparison to primary ECM-inducing infection. Ongoing studies will validate differential gene expression using NanoString® technology.

The transcriptional changes observed in the brains of infection-induced ECM resistant mice compared with ECM affected mice largely recapitulated those observed in earlier studies that compared the transcriptional signatures in brains of resistant and susceptible strains of mice infected with PbA (Lovegrove et al., 2006), as well as those comparing profiles in ECM and non-ECM infections (Miu et al., 2008, Oakley et al., 2008). Thus, infection-induced dampening of intracerebral immune responses appears to mirror processes that specify genetic resistance to ECM. Central to this appears to be the inhibition of apoptotic pathways and IFN response networks. The importance of IFNγ in development of ECM is well characterised (Hunt et al., 2014) and roles for type 1 interferons in establishment of cerebral pathology has also been reported (Ball et al., 2013, Palomo et al., 2013). Furthermore, roles for a number of IRFs have now been defined (Gun et al., 2014, Berghout et al., 2013). Although we also identified IL-6 as a gene hub that is down regulated in ECM resistant mice, neutralisation of IL-6 did not protect primary infected mice against ECM (results not shown). The datasets generated in this study will be useful resources to identify other gene candidates that control resistance to ECM during PbA infection.

The transcriptional profiling data predicted that CD8+ T cell responses would be repressed in the brains of ECM resistant mice during a fourth infection. This was
verified by flow cytometry, which demonstrated that the activation of CD8+ T cells was significantly reduced in brains of ECM resistant mice compared with ECM affected mice. To our knowledge, this is the first report that development of parasite exposure-induced resistance to ECM is directly associated with the attenuation of intracerebral CD8+ T cell responses. Nonetheless, the importance of CD8+ T cell activation status and activity in the brain, rather than their presence per se, have previously been defined as important factors in development of ECM during primary infection (Haque et al., 2011, Baptista et al., 2010). The activation of brain accumulating CD8+ T cells during primary infection has been suggested to depend upon antigen load (Haque et al., 2011). Thus, in addition to the differentially expressed pathways identified in the arrays, it is possible that reduced parasite load led to restricted in situ cross presentation by CNS microvessels (Howland et al., 2013), inhibiting pathogenic CD8+ T cell activity in the repeatedly exposed mice. Further work will be required to assess whether the CD8+ T cells compartmentalise and behave differently in the brains of ECM affected and ECM resistant mice and whether simply increasing parasite antigen levels would endow the cells with pathogenic function.

Surprisingly, transcriptional profiles and leukocyte populations within the brain were not altered in resistant mice prior to a fourth infection, compared with naïve mice. This was surprising given the frequency of long term neurological dysfunction observed in humans and in mice post (E)CM, implying that the syndrome leads to significant alterations in brain activity (Reis et al., 2010). These observations led us to hypothesise that the transcriptional changes and altered intracerebral CD8+ T cell responses were actively driven during infection, most likely by systemic and/or splenic immune responses. In agreement, we found that infection-induced resistance was associated with the suppression of splenic CD8+ T cell activation. Interestingly, however, the splenic CD4+ T cell response was only marginally affected in ECM resistant compared
with ECM resistant mice. These observations were not entirely predictable as infection-induced resistance could have been associated with a deviation in splenic T cell immune response towards enhanced parasite control or towards enhanced regulation. For example, it has previously been shown that enhancement of splenic T cell responses, by either increased retention of parasite specific T cells by neutralisation of IP-10, or inhibition of Treg activity, can enhance parasite control and inhibit ECM development during primary infection (Nie et al., 2009, Amante et al., 2007). Conversely, enhancement of Treg responses can blunt pro-inflammatory immune responses and also prevent ECM formation during primary infection (Haque et al., 2010). Of note, the splenic T\textsubscript{reg} ratio and their activation were not majorly altered in ECM resistant compared with ECM affected mice. Together these results suggest that parasite exposure-induce resistance was likely due to attenuated CD8\textsuperscript{+} T cell activation, rather than enhanced CD4\textsuperscript{+} T cell or T\textsubscript{reg} responses.

Splenic CD8\textsuperscript{+} T cell priming by DCs, and particularly CD8\textsuperscript{+} and clec9\textsuperscript{+} DC subsets is critical to the development of ECM (Lundie et al., 2008, Piva et al., 2012). Consequently, it was unsurprising that the CD11c\textsuperscript{+} MHC II\textsuperscript{+} compartment was found to be dramatically changed in ECM resistant mice compared with ECM affected mice. We found that the expanded CD11c\textsuperscript{+} MHC II\textsuperscript{+} population in ECM resistant mice comprised largely of a pDC-like subset, which exhibited a regulatory signature. Very few pDCs were observed in the spleen of ECM affected mice during primary infection. Furthermore, the expanded CD11c\textsuperscript{+} MHC II\textsuperscript{+} population was absent in uninfected resistant mice. Combined, these data imply that pDCs are actively induced during repeated parasite infections by a component of the adaptive memory immune response.

At present the function of pDCs during malaria infection is unclear (Wykes and Good, 2008). pDCs are not believed to play major role in priming of pro-inflammatory CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells during malaria infection (Wykes, 2012, Voisine et al., 2010).
Although pDCs are known to be a source of type-1 interferon, they are not believed to be the major source of type-1 interferon during malaria (Haque et al., 2014). Irrespective of this, as type-1 interferon can promote the maturation of CD8+ T cells (Ball et al., 2013), it is unlikely that pDCs inhibit splenic CD8+ T cell responses in ECM resistant mice via this mechanism. pDCs have also been proposed as a major source of IL-10 during malaria infection (Wykes, 2012) but neutralisation of IL-10 failed to induce ECM development in WT mice experiencing a fourth infection (results not shown). It is possible that pDCs suppress T cell responses in ECM resistant mice through expression of the enzyme IDO-1, and inhibition of tryptophan (Baban et al., 2005). Inhibition of IDO-1 did not protect mice against ECM during primary PbA infection ((Miu et al., 2009) but the effect of overexpression of this pathway during PbA infection has yet to be examined. Ongoing studies are being performed to identify the transcriptional changes that occur in the spleen, as well as the differential expression of cytokines and chemokines in the plasma, of ECM resistant and ECM affected mice. These studies will further resolve the immunological changes instructed by previous parasite exposure that attenuate pathogenic CD8+ T cell responses and promote resistance to ECM. Moreover, assessment of splenic gene expression in uninfected resistant mice will additionally help to determine any stable infection-induced programmed changes that occur at this site that may account for the development of ECM resistance.

Anti-malaria antibodies are known to be key to the development of disease resistance (Cohen et al., 1961, Perez-Mazlia and Langhorne, 2014) and their repertoire is developed by repeated parasite exposure (Nogaro et al., 2011). We therefore hypothesised that immune-complex signalling through Fc receptors on pDCs may orchestrate their expansion during a fourth infection, explaining both their absence in a first infection and in uninfected resistant mice. Indeed, we found that splenic pDC
expansion was abrogated in IgMi mice, which lack secreted antibody, during a fourth infection. Crucially, we found that IgMi mice failed to develop ECM resistance in response to three previous PbA exposures, and instead developed ECM more rapidly than WT mice experiencing a first infection. In agreement, splenic and intracerebral CD8+ T cells from IgMi mice experiencing a fourth infection displayed a hyper-activated phenotype compared with IgMi and WT mice experiencing a first infection, or WT mice experiencing a fourth infection. As expected, control of peripheral parasitaemia was also impaired in IgMi mice, although it was reduced in IgMi mice experiencing a fourth infection compared with those experiencing a first infection. Taken together, these results demonstrate that peripheral parasitaemia is controlled during PbA infection by anti-malaria antibody in conjunction with antibody-independent mechanisms. Furthermore, an effective antibody response is necessary, not only to control peripheral parasitaemia, but also to control host-mediated immunopathology. Studies are currently being performed to demonstrate during which round of infection ECM-preventing anti-malaria antibodies are produced, and whether repeated exposure promotes alterations in both magnitude and isotype of antibody response. Furthermore, passive transfer experiments will help to determine whether antibody alone, from ECM resistant mice, is sufficient to induce expansion of protective pDCs in a first infection.

In summary, in this model of repeated parasite exposure we have shown that infection-induced resistance to ECM is critically dependent upon secretion of anti-parasite antibody. Antibody appears, possibly in combination with other memory immune components, to modify the innate compartment during parasite re-exposure, which subsequently specifically attenuates splenic CD8+ T cell priming. This leads to migration of non-pathogenic CD8+ T cells to the brain, and, possibly coupled with attenuated parasite antigen presentation, leads to lack of ECM development. Our results
have significant implications for studying the basis of infection-induced resistance to severe malarial disease in humans.
Materials and Methods

Ethics

Animal work was approved following local ethical review by the Universities of Manchester Animal Procedures and Ethics Committees and was performed in strict accordance with the U. K Home Office Animals (Scientific Procedures) Act 1986 (approved H.O Project Licenses 70/6995 and 70/7293).

Mice

The following mice were used in the study: C57BL/6 mice from Charles River, UK, IgMi mice (Waisman et al., 2008) form the University of Manchester. Mice were maintained in specific pathogen-free conditions.

Parasites

GFP-expressing \textit{P.berghei} ANKA parasites (GFP expressed under control of elogation factor 1a [eEF1a] promoter) were a kind gift from Chris Janse (Leiden University Medical Center) (Franke-Fayard et al., 2004). Parasites were maintained in liquid nitrogen and passaged through naive mice prior to being used to infect experimental animals. Experimental infections were initiated by i.v. inoculation with $10^4$ pRBCs, and infected mice were monitored for neurological symptoms (paralysis, ataxia, convulsions, and coma occurring between day 6 and 10 post-infection). Parasitemia was measured from day 3 p.i. by examination of Giemsa-stained thin blood. Radical drug cure was achieved by six daily i.p. injections of 30mg/kg chloroquine combined with 30mg/kg artesunate in PBS.
RNA isolation

Brains were isolated from PBS perfused mice and stored at -80°C until use. RNA isolation from whole brains was performed by homogenising brains in Trizol and using lipid tissue RNA easy kits according to the manufacturer’s instructions (Qiagen). Isolated RNA was DNAse treated to remove genomic DNA prior to QC analysis and use in microarray analysis.

Microarray and gene expression analysis.

The global gene expression of brains from uninfected, uninfected ECM resistant mice, ECM resistant mice and ECM affected mice were compared probed using the GeneChip® Mouse Genome 430 2.0 microarray containing 34,000 genes. Raw data were background corrected using Using dChip software (Harvard School of Public Health, Boston, USA), and subjected to gcRMA quantile normalisation. Quality assurance testing was performed through Microarray Suite version 5.0 (Affymetrix) and dChip. Following log2-transformation, data was statistically analysed through systematic limma testing, performed using R software (Technische Universität Wien, Vienna, Austria).

Venn diagrams and cluster maps were created as follows: - 1957 probe-sets were included which passed a filter of paired test qvalue < 0.05 and fc < or > 1.5 (ECM affected vs aged uninfected or ECM resistant vs aged uninfected). Each probe-set expression level was normalised to the naive average (in log scale the naive average expression was calculated and subtracted from each expression level) and then the standard deviation was normalised to 1 (expression level was divided by the standard deviation). Probe-sets were ranked by clustering the mean expression levels of each condition (aged uninfected, ECM affected, ECM resistant, normalised by z-
transformation in log scale i.e. mean set to zero and standard deviation set to 1) by k-means clustering into 5 clusters and then ranking the probe-sets by hierarchical clustering.

Differentially expressed genes were further analysed using DAVID bioinformatics database and Ingenuity Pathway Analyser.

Flow Cytometry and ImageStream analysis

Spleens were removed from naïve, and malaria-infected mice on day 4 and day 6 or 7 post infection (p.i.). Single cell suspensions were generated by homogenizing tissue through a 70 µm cell sieve (BD Biosciences). Brains were isolated from PBS perfused mice and chopped into small pieces, aspirated through a 10ml syringe, and incubated in HBSS containing 2% FCS with Collagenase (final concentration 1mg/ml) (Sigma) for 45 min on a tube roller at room temperature. The resulting suspension was filtered through a 70 µm cell sieve, layered on a 30% Percoll gradient and centrifuged at 2000g for 10 minutes. The supernatant was discarded and the pellet collected. For both the spleen and brain preparation, RBCs were lysed using RBC lysing buffer (BD Biosciences). Absolute cell numbers were determined by microscopy using a haemocytometer and live/dead differentiation was performed using the trypan blue exclusion cell viability assay (Sigma).

Isolated leukocytes were surface stained for 20 minutes with α-mCD3 (17A2), CD4 (GK1.5), CD8 (53-6.7), CD45 (30-F11), KLRG1 (2F1), ICOS (15F9), CD40 (3/23), CD80 (16-10A1), CTLA-4 (UC10-4B9), Tigit (GIGD7), CD127 (A7R34), CXCR3 (CXCR3-173), CD103 (2E7), CD25 (PC61.5), CD11b (M1/70), Ly6C (HK1.4), IgM (RMM-1), IgD (11-26c.2a), PDL-1 (MIH5), CD5RB (C363.16A), MHC II (M5/114.15.2). Intracellular staining for granzyme B (GB11), Foxp3 (FJK-16s) and
Ki67 (SolA15) was performed for 45 minutes, after treatment with Foxp3 fixation/permeabilisation buffer (eBioscience). Dead cells were excluded using LIVE/DEAD® Fixable Blue Dead Cell Stain Kit (Life Technologies). Fluoresce minus one controls were used to set gates. Cells were analysed with a BD LSR II (Becton Dickinson) using BD FACSDiva software (Becton Dickinson) or an ImageStream ImageStream X mk II (Amnis). Data was analyzed with FlowJo (Tree Star Inc.) or IDEAS (Amnis). All antibodies were from eBioscience and Biolegend.

**Histology**

Brains were isolated from mice perfused with PBS followed by 4% pfa. Brains were stored in 20% sucrose/4% pfa for 24hrs at (4°C) before being transferred to 20% sucrose/PBS for a further 24hrs (4°C). Coronal sections were subsequently cut using a sledge-microtome at a thickness of 10 µm. Sections were mounted and stained with Haematoxylin/Eosin.

**Statistical Analysis**

All statistical analyses were performed using GraphPad PRISM (GraphPad Software, USA). Comparison between multiple groups was made using a one-way ANOVA with Tukey’s test for multiple comparisons.
References


145


Supplementary Figures

Figure S3. 1 Intracerebral leukocyte composition in uninfected, ECM affected and ECM resistant mice.
C57BL/6 mice were infected (10^4 pRBCs i.v.), or not, and treated as described in Figure 1. Whole brains were removed for flow cytometric analysis when infected mice developed ECM signs (day 8 p.i.). Percentages ± SD of various intracerebral leukocyte populations. Results are representative of 2 independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (one-way ANOVA with Tukey’s multiple comparisons test).
**Defence Response**

1491015 ultr / Tbp1 / transporter 1, ATR-binding cassette, sub-family...
146625 ultr / Sarp1 / serine (or cysteine) peptidase inhibitor, clade...
147706 ultr / Clqbc / complement component 1, q subcomponent, beta ps...
147726 ultr / Cd14 / CD14 antigen
147726 ultr / Tnfra1a / tumor necrosis factor receptor superfamily, mem...
147743 ultr / Nfkbiz / nuclear factor of kappa light polypeptide gene ...
147787 ultr / Fcgr1 / Fc receptor, IgG, high affinity I
148021 ultr / Cbx1 / complement component 4B (C4b, b2m, blood group) III...
148313 ultr / Gm2olf / SAM domain and HD domain 1
148825 ultr / Irng / immunity-related GTPase family M member 1
1491909 ultr / Sarpma7 / serine (or cysteine) peptidase inhibitor, clade...
149367 ultr / Ppp2r2a / protein phosphatase 2A catalytic subunit 1a
149764 ultr / Chic3 / chitinase 3-like 3
149037 ultr / Idol / indoleamine 2,3-dioxygenase 1
149131 ultr / Tnaps1 / U07310:1 / complement component 4B (C4b, b2m, blood group) III...
149210a ultr / Tif7 / toll-like receptor 7
149251 ultr / Chic3 / chitinase 3-like 3
149257 ultr / Mibg / interferon gamma
149257 ultr / Bcl1 / interferon-induced with helicase C domain 1
149657 ultr / St8a3 / signal transducer and activator of transcription 3
149657 ultr / Hdaa8 / histone deacetylase 9 // histone deacetylase 9...
149579 ultr / Fcgr2b / Fc receptor, IgG, low affinity IIb
149598 ultr / Rsc2d / radical S-adenosylmethionine domain containing 2
149997 ultr / In1ap / interleukin 1 receptor accessory protein 1
149901 ultr / Csr1 / complement component 5a receptor 1
148453 ultr / Ifh8 / interferon regulatory factor 8
149820 ultr / Fcgr1 / Fc receptor, IgG, high affinity II
149184 ultr / Pglyrp1 / polypeptide recognition protein 1
145029 ultr / B6 / interleukin 6
148149 ultr / Dhx58 / DEXH-box RNA helicase 58
145060 ultr / Mc1 / myosin, light chain 1
145689 ultr / Ddx58 / DDX58 (DEAD [Asp-Glu-Ala-Asp] box polypeptide 58)
145753 ultr / Tif13 / toll-like receptor 13

**Regulation of Apoptosis**

149451 ultr / Pm01 / myeloid cell leukemia sequence 1
149629 ultr / Tdp53ip1 / transformation-related protein 53 inducible nucl...
147130 ultr / Angpt1 / angiopoietin-1
147796 ultr / Ada / adenosine deaminase
149214 ultr / Ugp2 / UDP-galactosylceramide beta 1,4-galactosyltransfer...
149004 ultr / B2a2 / B cell leukemia/lymphoma 2 related protein A2a...
149603 ultr / It204 / interferon activated gene 204
149712 ultr / Nfac1 / nacrin receptor 1
149691 ultr / Chil1 / Otp cyclin-dependent kinase 1
149019 ultr / Sgk3 / serine/threonine-protein kinase 3
142192 ultr / Bcl3 / bcl-2 homologous anti-apoptotic gene
142179 ultr / Cdkn1a / cyclin-dependent kinase inhibitor 1A (p21)
142166 ultr / Fcgr1 / nuclear receptor subfamily 5, group C, member 1
142356 ultr / Agt / angiotensinogen (serpin peptidase inhibitor, cl...
142442 ultr / Myc / myelocytomatosis oncogene
142521 ultr / Tic22d3 / TSC22 domain family, member 3
142599 ultr / Lnk / lymphoid tyrosine kinase
142652 ultr / Krl / kit ligand
142672 ultr / Neurod1 / neurogenic differentiation 1
143764 ultr / Cc17 / CCAAT/enhancer binding protein (C/EBP), beta
142984 ultr / Nuk2 / NUK kinase, Nuk2-like kinase, 2
143460 ultr / Erbb3 / v-erb-b2 erythroblastic leukemia viral oncogene...
143708 ultr / Atrx1 / ataxia-telangiectasia homolog 1 (Drosophila)
143954 ultr / Ndufs4 / NDUFS4 / NDUsf4
143963 ultr / Zbtb16 / zinc finger and BTB domain containing 16
143979 ultr / Serpnb9 / serine (or cysteine) peptidase inhibitor, clade...
143980 ultr / Pold / polymerase (DNA directed), beta
144061 ultr / Pck7 / p21 protein (Cdk4/cdk2)-activating kinase 7
144218 ultr / Veg1 / VEGF, vascular endothelial growth factor
144422 ultr / Foxoa3 / forkhead box O3
144785 ultr / Rn4g2 / nuclear receptor subfamily 4, group A, member 2
144898 ultr / Hmx1 / homeobox 1
144925 ultr / Casp1 / caspase 1
144931 ultr / Cflar / CASP8 and FADD-like apoptosis regulator
144934 ultr / Sypcp3 / synaptoplasmic complex protein 3
145218 ultr / Rbm6 / RNA binding motif protein 5
145366 ultr / Cpsd / calsequestrin
145480 ultr / Bmdm / BCL2 modulating factor
145547 ultr / Tmpr / tissue inhibitor of metalloproteinase 1
146075 ultr / Xpa / xeroderma pigmentosum, complementation group A
146079 ultr / Rock1 / Rho-associated coiled-coil containing protein k...

**Figure S3. 2 Full defence response and regulation of apoptosis heat maps.**

C57BL/6J mice were infected (10^7 pRBCs i.v.), or not, and treated as described in Figure 1 to generate ECM affected and ECM resistant mice. Whole brains were removed for RNA extraction and microarray analysis when ECM affected mice developed ECM signs (day 8 p.i.). Heat maps of filtered genes differentially expressed in brains of ECM affected mice and ECM resistant mice, grouped by the biological functions indicated. Colour scale: green low, black average, red high (n=6, from 2 independent experiments).
**Figure S3.** 3 IFNγ and IL-6 signalling networks are expressed at lower levels in brains of ECM resistant mice.

C57BL/6 mice were infected (10^4 pRBCs i.v.), or not, and treated as described in Figure 1 to generate ECM affected and ECM resistant mice. Whole brains were removed for RNA extraction and microarray analysis when ECM affected mice developed ECM signs (day 8 p.i.). Ingenuity® bioinformatics network of genes involved in IFNγ and IL-6 signalling identified as being expressed at reduced (green) and increased (red) levels in brains from ECM resistant mice compared with ECM affected mice.
4. Chapter 4: The sub-cellular location of OVA in Plasmodium blood stages influences the magnitude of T-cell responses

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All work was carried out by T.N.S, with the exception of Figures 4.1, 4.2 and Supplementary Figures 4.1 and 4.2 (JW.L)
Abstract

Model antigens are frequently introduced into pathogens to study determinants that influence T-cell responses to infections. To address whether an antigen’s subcellular location influences the nature and magnitude of antigen-specific T-cell responses, we generated *Plasmodium berghei* parasites expressing the model antigen, ovalbumin (OVA), either in the parasite cytoplasm or on the parasitophorous vacuole membrane (PVM). For cytosolic expression, OVA alone or conjugated to mCherry was expressed from a strong constitutive promoter (*OVA*<sub>hsp70</sub>; *OVA*:mCherry<sub>hsp70</sub>); for PVM-expression OVA was fused to HEP17/EXP1 (*OVA*:Hep17<sub>hep17</sub>). Unexpectedly, OVA expression in *OVA*<sub>hsp70</sub> parasites was very low; however, when OVA was fused to mCherry (*OVA*:mCherry<sub>hsp70</sub>) it was highly expressed. OVA expression in *OVA*:Hep17<sub>hep17</sub> parasites was strong but significantly less than *OVA*:mCherry<sub>hsp70</sub> parasites. These transgenic parasites were used to examine the effect of antigen subcellular-location and expression-level on the development of T-cell responses during blood-stage infections. While all OVA-parasites induced activation and proliferation of OVA-specific CD8<sup>+</sup> T-cells (OT-I) and CD4<sup>+</sup> T-cells (OT-II), the level of activation varied; *OVA*:Hep17<sub>hep17</sub> parasites induced significantly stronger splenic and intracerebral OT-I and OT-II responses than *OVA*:mCherry<sub>hsp70</sub> parasites, but *OVA*:mCherry<sub>hsp70</sub> parasites promoted stronger OT-I and OT-II responses than *OVA*<sub>hsp70</sub> parasites. Despite lower OVA-expression levels, *OVA*:Hep17<sub>hep17</sub> parasites induced stronger T-cell responses than *OVA*:mCherry<sub>hsp70</sub> parasites. These results indicate that unconjugated cytosolic OVA is not stably expressed in *Plasmodium* parasites and, importantly, its cellular location and expression level influence both the induction and magnitude of parasite-specific T-cell responses. These parasites represent useful tools to study the development and function of antigen-specific T-cell responses during malaria infection.
Introduction

T cells play a central role in the immune response to malaria and can help control blood stage infections (Langhorne et al., 2008, Hafalla et al., 2011). For example, in humans and rodent malaria infections, effector CD4$^+$ T cells promote anti-parasitic antibody production and regulate macrophage-based anti-parasitic effector responses (Langhorne et al., 2008, Hafalla et al., 2011). However, it is also clear that pro-inflammatory T cell responses can, if not regulated appropriately or when present in the wrong environment, contribute to the development of immunopathology during malaria infection (Finney et al., 2010, Spence and Langhorne, 2012). Thus, understanding how malarial proteins are recognised by the immune system to initiate adaptive T cell responses, and identifying the antigen-specific T cell responses involved in protection and pathology during infection, has significant importance for vaccine development and for identification of predictive immunological biomarkers for severe malarial disease.

Difficulties in identifying endogenous T cell epitopes within blood stage malaria parasites has hampered the investigation of parasite-specific adaptive T cell responses, necessitating the generation and use of transgenic parasites expressing model antigens. Transgenic Plasmodium parasites expressing OVA in the cytoplasm have been used successfully to examine parasite-specific CD8$^+$ responses during both blood and liver stages of infection (Miyakoda et al., 2008, Lundie et al., 2008, Kimura et al., 2013). These parasites do not, however, induce strong OVA-specific CD4$^+$ T cell responses in vivo (Lundie et al., 2010). One potential explanation for the dichotomy in ability of these parasites to prime OVA-specific CD4$^+$ T cell and OVA-specific CD8$^+$ T cell responses is because different antigen processing and presentation pathways exist for the presentation of antigens by MHC class I and MHC class II molecules (Vyas et al., 2008) and OVA expressed from the cytoplasmic location does not effectively enter the MHC class II
antigen processing pathway. In support of this, it has been reported in a variety of different models, such as *Trypanosoma cruzi* and *Toxoplasma gondii*, which secreted proteins induce stronger T cell activation than cytoplasmic proteins (Garg et al., 1997, Gregg et al., 2011). These data indicate that the subcellular location of antigen expression significantly influences how it is recognised by the immune system during protozoal infections. These observations likely have relevance to endogenous malarial proteins, as it has been shown that not all endogenous *Plasmodium* antigens induce strong T cell responses, but that a select number of malarial antigens are preferentially recognized by the immune system and initiate superior T cell responses (Hafalla et al., 2011, Howland et al., 2013).

In the present study we have directly compared the extent to which the expression level of a protein, compared with its subcellular location, in blood stage malaria parasites influence the development of antigen-specific T-cell responses. We have generated transgenic *P. berghei* parasites expressing OVA either in the cytoplasm, under the control of the Heat Shock Protein 70 (HSP70) promoter, or on the parasitophorous vacuole membrane (PVM), through fusion of OVA to the PVM-protein EXP1/HEP17 (exported protein 1, hepatocyte erythrocyte protein 17kD) (Simmons et al., 1987, Kara et al., 1988, Graewe et al., 2011). We found that while both cytoplasmic and PVM-anchored OVA could activate OVA-specific CD8⁺ T-cells (OT-I) and CD4⁺ T-cells (OT-II), OVA fused to the PVM induced the strongest antigen-specific T cell responses. This is despite OVA being expressed at higher levels in parasites when it was present in the parasite cytoplasm. These results demonstrate that both secreted and intracellular antigens can be cross-presented within the immune system and that the subcellular location of antigens affects parasite-specific T cell response. These data increase our understanding of the parasite-specific features that influence activation and expansion of T-cells during a malaria infection. The transgenic-OVA parasites described in this study are therefore valuable
reagents to examine site-specific immune responses and immunopathology during a malarial infection.
Results

Generation of different transgenic P. berghei parasite lines expressing OVA

Using the GIMO transfection method, we generated three different transgenic P. berghei ANKA lines, each expressing the reference antigen, chicken ovalbumin (OVA) (Figure 4.1A). In the first two lines, OVA\textsubscript{hsp70} and OVA::mCherry\textsubscript{hsp70}, OVA is expressed under the control of the strong and constitutive hsp70 promoter (PBANKA\_{071190}). When transgenes such as GFP and mCherry are encoded under the control of this promoter, it results in strong protein expression in the parasite cytosol (Amino et al., 2008, Ishino et al., 2006, Kooij et al., 2012). The OVA\textsubscript{hsp70} line expresses an unmodified full-length OVA, whereas OVA::mCherry\textsubscript{hsp70} line encodes full-length OVA that is C-terminal-tagged with the fluorescent protein, mCherry. In the third line, OVA::Hep17\textsubscript{hep17}, full-length OVA is fused to HEP17/EXP1 (PBANKA\_{092670}) (Simmons et al., 1987, Kara et al., 1988), which is a protein present on the parasitophorous vacuole membrane (PVM) (Sturm et al., 2006). The PVM is the structure that separates the parasite from the inside of the infected hepatocyte or red blood cell (Lingelbach and Joiner, 1998). In this transgenic line, the expression of this fusion protein (OVA::HEP17) is under the control of the 5’UTR (promoter) and 3’UTR (terminator) regions of the hep17 gene. To ensure correct trafficking of OVA to the PVM, we placed the full length CDS of OVA directly after the signal peptide of hep17 and before the remainder of the hep17 CDS (Fig.4.S1).

In addition to the OVA-expressing P. berghei ANKA lines, we generated two transgenic lines in the GIMO mother line of P. berghei NK65 (GIMO\textsubscript{NK65}); These two NK65 lines also express OVA under the control of the hsp70 promoter and were generated using the same constructs used to generate P. berghei ANKA OVA\textsubscript{hsp70} and OVA::mCherry\textsubscript{hsp70} (Fig.4.S2A). Genotyping by Southern analysis of separated PFG-chromosomes of all these transgenic lines confirmed that all the different OVA-constructs
were correctly integrated into the \textit{230p locus} after GIMO-transfection (Figure 4.1A and Fig.4.S2B).

\textbf{Figure 4.1 Different transgenic \textit{P. berghei} ANKA lines expressing OVA.} (A) Schematic representation of the 230p locus in the reference GIMOPbANKA motherline and in 3 OVA-expressing transgenic lines (left panel). The GIMOPbANKA has the positive and negative selectable marker cassette (hdhfr::yfcu, black box) inserted into 230p. The OVA-expression cassettes were introduced into 230p by double cross-over homologous recombination at the target regions (hatched boxes) using the method of GIMO transfection. Line 1 (1988cl1; OVAhsp70) encodes full-length ovalbumin (ova; blue box) under the control of 5’ (promoter)- and 3’ (terminator)-UTRs of hsp70 gene (grey boxes); Line 2 (2027cl1; OVA::mCherryhsp70) encodes OVA which is C-terminally fused to mCherry (red box) under the control of 5’-UTR of hsp70 and the 3’-UTR of dhfr/ts (white box); Line 3 (2030cl1; OVA::Hep17hep17) encodes OVA flanked by the N-terminal signal peptide of hep17 (1-81bp, yellow hatched box) and the remainder of the hep17 ORF (82-726bp, yellow box), under the control of 5’- and 3’-UTR hep17 gene (light yellow boxes). The direction of transcription is indicated by the arrows. Southern analyses of PFG-separated chromosomes (right panel) confirm the integration of the constructs into the 230p locus on chromosome 3 (Chr), resulting in the removal of the hdhfr::yfcu selection cassette. Hybridization was performed with a mixture of two probes, a control probe recognizing p25 on chromosome 5 and a hdhfr probe recognizing hdhfr::yfcu. (B) Northern blot analysis of OVA-transgene transcription in purified schizonts of the 3 transgenic lines. Blots were hybridized using a PCR
probe recognizing the ova (primers 6466/6467). As a loading control, an oligonucleotide probe L644R was used that recognizes the large subunit (lsu) rRNA. Quantification of the hybridization signals shows that OVAhsp70 (OVA) and OVA::mCherryhsp70 (OVA::mC) parasites had comparable levels of OVA transcripts, while in OVA::Hep17hep17 (OVA::Hep17) transcript levels were lower. The relative intensity of the hybridization signals was quantified using Image J. wt, wild-type P. berghei ANKA parasites. (C) Western analysis of OVA-transgene expression levels in purified schizonts in the 3 transgenic lines (arrowheads on long exposure image indicate the OVA products of expected sizes). Blots were stained with anti-OVA antibodies. Anti-HSP70 antibody staining was used as a loading control. Quantification of the staining signals shows hardly detectable OVA expression in OVAhsp70 parasites. In OVA::mCherryhsp70 an OVA product of expected size (2nd black arrow) is detected in addition to a smaller (truncated) product (white arrow). The relative intensity of OVA signals (long exposure) was quantified using Image J.

Expression and subcellular location of OVA in the different transgenic P. berghei lines

Northern and Western analyses were performed to analyse the expression level of OVA antigen in equivalent numbers of infected RBC of the different transgenic lines. Abundant OVA transcripts were present in OVAhsp70 and OVA::mCherryhsp70 blood-stage ANKA and NK65 parasites (Figure 4.1B and Fig.4.S2C, respectively). OVA transcript levels were substantially lower in the OVA::Hep17hep17 (expressed under the hep17 promoter) parasites than the other 2 lines expressing OVA under hsp70 promoter (Figure 4.1B). Unexpectedly, Western analysis using antibodies against OVA revealed very low levels of OVA protein in the OVAhsp70 line compared with the OVA::mCherryhsp70 ANKA line (Figure 4.1C). This very low, but specific, level of OVA expression was observed in 2 independent P. berghei ANKA OVAhsp70 lines (Fig.4.S2E), and moreover it was also reproduced in the equivalent NK65 parasites (Fig.4.S2D). These observations indicate that unconjugated/unmodified cytosolic OVA is unstable (and/or rapidly degraded) in blood stage P. berghei parasites. The OVA::mCherryhsp70 parasites expressed high levels of full-length OVA::mCherry fusion protein (ca 75 kDa) and also (less of) a ‘truncated’ (ca 55 kDa) form of OVA::mCherry protein recognised by anti-OVA antibodies (Figure 4.1C). When we used anti-mCherry antibodies against protein material derived from OVA::mCherryhsp70 parasites
we observed both the full-length fusion protein and weaker ~20kDa band, indicating that the truncation of OVA::mCherry fusion protein was occurring due to a specific cleavage within the mCherry reporter protein and that the T-cell epitopes within OVA remained intact (Fig.4.S2E). In the OVA::Hep17_{hep17} parasites we observed a single protein product of the expected 62 kDa size corresponding to OVA::HEP17 fusion (Figure 4.1C). The OVA protein levels in purified mature schizonts of OVA::Hep17_{hep17} parasites were lower than in mature schizonts of OVA::mCherry_{hsp70} parasites, but still significantly higher than observed in OVA_{hsp70} parasites, (Figure 4.1C). To analyse OVA expression levels during blood stage development, we collected different (synchronized) blood stages of OVA::Hep17_{hep17} and OVA::mCherry_{hsp70} parasites. Western analyses on these samples revealed that OVA expression in both lines was present in trophozoites and that it increased as the parasite matured into schizonts. Moreover, in all stages OVA expression was greater in OVA::mCherry_{hsp70} than in OVA_{hsp70} parasites (Fig.4.S2G).

We next performed immunofluorescence analyses to determine the subcellular location of OVA in the different lines. These analyses confirmed the low levels of OVA protein expression in OVA_{hsp70} parasites compared to the other two lines (Figure 4.2B and C). In addition it revealed, as expected, a cytosolic location of OVA in both OVA_{hsp70} and OVA::mCherry_{hsp70} blood-stage parasites (Figure 4.2A and B). In OVA::Hep17_{hep17} blood stage parasites, a clear circumferential staining was observed in both trophozoites and schizonts, indicative of a PVM location (Figure 4.2A,C) and is similar to the staining of the PVM proteins HEP17/EXP1 (PBANKA_092670) in wild-type blood stage (Figure 4.2C). The circumferential staining in schizonts with fully formed merozoites strongly suggests that OVA was not located at the parasite plasma membrane, although we cannot exclude its presence in the PV. Consequently, to confirm the PVM subcellular location of OVA in the OVA::Hep17 line, we analysed its expression in liver stages. *Plasmodium* liver
stages are larger, and the boundaries between the different membranes that surround the parasite in the host cell are more clearly defined and detailed (Graewe et al., 2011). As such, immunofluorescence analyses of the subcellular location of different PVM-located proteins have been previously performed in *P. berghei* liver stages (Graewe et al., 2011) (Figure 4.2A). OVA was clearly localised in the parasite cytosol of maturing *OVA::mCherry* liver stage parasites (48 h post sporozoite infection), with anti-OVA antibody staining closely resembling that of the cytoplasmic *P. berghei* protein, HSP70 (Figure 4.2D). In contrast, in maturing *OVA::Hep17* liver stage parasites, anti-OVA antibody staining provided characteristic PV/PVM staining, closely resembling the staining of the PVM proteins HEP17/EXP1 (PBANKA_092670) and UIS4 (PBANKA_050120) (Figure 4.2E). Combined our analyses demonstrate strong OVA expression in the *OVA::mCherry* and *OVA::Hep17* parasites; with OVA located (in both blood and liver stages) in the parasite cytoplasm of *OVA::mCherry* line and on the PVM in the *OVA::Hep17* line.
Figure 4.2 Subcellular locations of OVA in different transgenic *P. berghei* ANKA lines. Transgenic *P. berghei* parasites express full-length OVA lines either under the control of constitutive hsp70 promoter, unconjugated or fused to mCherry (OVA and OVA::mC, respectively), or under the control of hep17 promoter where OVA is fused to the parasitophorous vacuole membrane (PVM) protein, HEP17/EXP1 (OVA::Hep17). (A) The schematic shows the expected localization of OVA in the 3 transgenic lines during blood (RBC) and liver stage (hepatocyte) development (N, nucleus; parasite nucleus in the RBC and the hepatocyte nucleus in the liver cell). (B) Immunofluorescence analysis of blood (schizont) stages stained with anti-OVA antibodies (red) show a low, cytoplasmic expression in OVA parasites, whereas in OVA::mC-parasites a very strong cytoplasmic expression of OVA is observed. Trophozoites and schizonts are shown (single and multi-nucleated cells, respectively). Nuclei were stained with Hoechst-33342 (blue). (C) In OVA::Hep17-parasites, anti-OVA staining reveals a circumferential pattern at the periphery of the parasites indicative of a localisation at the PVM, which is very similar to PVM pattern observed in wild-type (wt) blood-stages using antibodies against HEP17/EXP1. A
trophozoite (single nuclei) and a fully segmented schizont (with individual merozoites indicated by arrow heads) are shown surrounded by OVA with a pattern indicative of the PVM. A multiply infected erythrocyte (bottom panel) is also shown with each individual parasite inside the red blood cell also surrounded by an OVA stained structure again indicating the PVM signal. (D) Immunofluorescence analysis of cultured Huh7 hepatocytes infected with OVA::mC (40 h post sporozoite infection; hpi) with anti-OVA antibodies (red) reveals a cytoplasmic expression for OVA. Intra-cellular wild-type parasites (at 40 hpi) stained with anti-HSP70 antibody show a similar cytoplasmic localisation pattern. (E) Immunofluorescence analysis of cultured Huh7 hepatocytes infected with OVA::Hep17 parasites at 48 hpi using anti-OVA antibodies (red), reveals a clear circumferential staining around the periphery of parasites. This staining pattern is very similar to PVM pattern observed in wild-type liver-stages (also at 48 hpi) using antibodies against the PVM markers EXP1 and UIS4 (PBANKA_050120). Nuclei were stained with Hoechst-33342 (blue). BF, bright field. Scale bar (B,C) 5 µm, (D,E) 10 µm.

**Induction of OVA-specific T cell responses in the spleen during blood stage infections with the different OVA-expressing *P. berghei* ANKA lines**

To address how the magnitude and location of OVA expression in malaria parasites influences the development of antigen-specific T cell responses during a blood stage infection, we adoptively transferred both CD45.1+ OT-I and OT-II cells into congenic (CD45.2+) C57BL/6 mice one day prior to infection with the various OVA-expressing transgenic parasite lines. To control for non-Ag-specific bystander activation of OT-I and OT-II cells, we also infected recipient mice with the non-OVA-expressing GIMOANKA (wild-type) parasites. The peripheral parasitaemia and the kinetics and incidence of ECM development were largely similar in the various infections (**Fig.4.S3A and B**), suggesting that the course of blood stage infections with the different parasite lines were comparable.

To assess the early stages of OVA-specific T cell activation during infection with the different OVA-expressing parasite lines, we first examined the splenic T cell response to infection on day 4 of infection. The spleen is the major site of T cell priming during the early stages of blood-stage malaria infection (Ferrer et al., 2014). The adoptively transferred OVA-specific T cells were distinguished from endogenous T cells based upon CD45.1+ expression and OT-I and OT-II cells were differentially identified based upon
their CD8- and CD4-receptor expression, respectively (Fig.4.S4A). In preliminary experiments we determined that adoptively transferred OT-I and OT-II cells do not down-regulate the CD8- and CD4-receptors during the course of blood stage *P. berghei* infections in C57BL/6 mice (results not shown). On day 4 of infection, we observed OT-II cells, but not OT-I cells, in spleens of all groups of mice (Fig.4.S4A A and B). The OT-II cells had not yet expanded in mice infected with the transgenic OVA-expressing parasites, when compared with mice infected with GIMOANKA or uninfected mice (Fig.4.S4A and B). Interestingly, however, significantly higher frequencies of splenic OT-II cells expressed the activation markers CD69 (early lymphocyte activation) and CD25 (alpha chain of the IL-2 receptor) and the proliferation marker Ki67, in the *OVA::mCherry hsp70* and *OVA::Hep17 hep17* -infected mice compared with *OVA hsp70* and GIMOANKA-infected mice (Fig.4.S4C). Moreover, early changes in CD44 (adhesion molecule and marker of effector T cell maturation) and CD62L (L-selectin, involved in cell adhesion) expression by splenic OT-II cells (forming effector CD44^+CD62L^- cells) were observed in the mice infected with *OVA::mCherry hsp70* and *OVA::Hep17 hep17* parasites, but not in the mice infected with *OVA hsp70* parasites. (Fig.4.S4C). These observations indicate that *OVA::mCherry hsp70* and *OVA::Hep17 hep17* parasites, but not *OVA hsp70* parasites, promote early activation of splenic OVA-specific CD4^+ T cells. As OT-I cells were not identified in the spleen at day 4 of infection, due to the lack of expansion from the relatively small population of transferred OT-I cells, it was not possible to determine their activation status at this early time point.

We next quantified expansion and numbers of OVA-specific T cells in the spleen on day 6 of infection, when T cells have acquired full effector status during *P. berghei* infection (Villegas-Mendez et al., 2011), and at the time of ECM development (Fig.4.S3B). Adoptively transferred OT-II cells were, again, identified in the spleen of all groups of mice on day 6 of infection. In addition, OT-I cells were clearly visible in the spleens of *OVA hsp70*.
OVA::mCherry$_{hsp70}$ and OVA::Hep17$_{hep17}$-infected mice at this later time point (Figure 4.3A). Importantly, the highest frequencies and numbers of OT-I and OT-II cells were observed in the spleens of mice infected with OVA::Hep17$_{hep17}$ parasites, compared with mice infected with GIMO$_{ANKA}$, OVA$_{hsp70}$ and OVA::mCherry$_{hsp70}$ parasites (Figure 4.3A and B). Unsurprisingly, based upon the low level of OVA protein expression (Figure 4.1C), the lowest frequencies and numbers of splenic OT-I and OT-II cells (out of the three groups of OVA-expressing parasite infections), were observed in mice infected with OVA$_{hsp70}$ parasites (Figure 4.3B).

We next expanded upon the above analyses and examined the activation status and maturation of the splenic OVA-specific T cell response on day 6 of infection with the different OVA-expressing parasites. Significantly higher frequencies of mature effector (CD44$^+$CD62L$^-$) OT-II and OT-I cells were observed in the spleens of mice infected with OVA::Hep17$_{hep17}$ and OVA::mCherry$_{hsp70}$ parasites compared with mice infected with OVA$_{hsp70}$ parasites (Figure 4.3C); however, interestingly, the maturation of the OT-I and OT-II cells, as defined by CD44$^+$CD62L$^-$ phenotype, were largely similar in spleens of mice infected with OVA::Hep17$_{hep17}$ and OVA::mCherry$_{hsp70}$ parasites (Figure 4.3C). The expression of Ki67, CD25 and CD69 by OT-II and OT-I cells were also largely similar in the spleens of mice infected with OVA::Hep17$_{hep17}$ and OVA::mCherry$_{hsp70}$ parasites, with the exception of CD25 expression on OT-I cells which was higher in OVA::Hep17$_{hep17}$ than OVA::mCherry$_{hsp70}$ parasites (Figure 4.3C). Thus, these results suggest that the location of OVA-expression with blood stage malaria parasites influences the size of the antigen-specific T cell population, whilst the magnitude of OVA expression within blood stage malaria parasites influences the maturation kinetics of the antigen-specific response.

Importantly, the kinetics, activation and magnitude of the endogenous (polyclonal) splenic T cell responses (both CD4$^+$ and CD8$^+$ T cells) were largely comparable in mice
infected with the different OVA-expressing and non-OVA-expressing parasite (Figure S4. 5). This shows that different OVA-expressing and non-OVA-expressing parasites do not inherently differ in their capacity to initiate and maintain parasite-specific T cell responses and verifies that the disparities in splenic OT-I and OT-II responses observed in mice infected with the different parasite lines results from differences in the magnitude and location of OVA expression.
Figure 4.3 The location of OVA in blood stage parasites influences the development of OVA-specific T cell responses in the spleen.

10,000 naïve CD45.1\(^{+}\) OT-I and 250,000 naïve CD45.1\(^{+}\) OT-II cells were adoptively transferred into CD45.2\(^{-}\)C57BL/6 mice one day prior to infection (10\(^{4}\) pRBC i.v.) with different *P. berghei* ANKA lines, GIMO\(_{ANKA}\) (control), OVA\(_{hep70}\) (OVA), OVA::mCherry\(_{hep70}\) (OVA::mC), OVA::Hep17\(_{hep17}\)
(OVA::Hep17). (A) Representative flow cytometric plots of total and donor CD4$^+$ and CD8$^+$ T cells in spleens of mice infected with different *P. berghei* ANKA lines at day 6 after infection. Hierarchal gating of total splenic CD4$^+$ and CD8$^+$ T cells (top row); host CD45.1 CD4$^+$ T cells and donor OVA-specific CD45.1$^+$CD4$^+$ OT-II cells (middle row); host CD45.1$^+$CD8$^+$ T cells and donor OVA-specific CD45.1$^+$CD8$^+$ OT-I cells (bottom row). (B) Percentages and absolute numbers (mean +/- SEM.) of total and donor (OT-I/OT-II) T cells in spleens of mice (n=4) infected with different *P. berghei* ANKA lines at day 6 after infection. The magnitude of the total splenic T cell responses was comparable in mice infected with the different parasite lines (left 4 graphs). Stronger OVA-specific T cell responses are observed in mice infected with OVA::Hep17 parasites compared with mice infected with OVA::mC and OVA parasites (right 4 graphs). The results are representative of 2 separate experiments. ND= Not detected *= p<0.05; **= p<0.01; ***= p<0.001. (C) Percentages and absolute numbers (mean +/- SEM) of donor (OT-I/OT-II) T cells in spleens of mice (n=4) infected with different *P. berghei* ANKA lines at day 6 after infection. The activation of CD4$^+$CD45.1$^+$ OT-II cells (top row) and CD8$^+$CD45.1$^+$OT-I cells (bottom row) was determined by examining the expression of CD69, CD25, Ki67, CD44 and CD62L. The results are representative of 2 separate experiments. *= p<0.05; **= p<0.01; ***= p<0.001, one-way ANOVA with Tukey’s post hoc analysis.

Development of OVA-specific T cell responses in the brain during blood stage infections with the different OVA-expressing *P. berghei* ANKA lines

ECM in *P. berghei* ANKA infected mice is a T cell driven-neuropathology characterized by migration to and accumulation of pathogenic CD4$^+$ and CD8$^+$ T cells in the brain (Renia et al., 2006, de Souza et al., 2010). To address whether differences in the location and magnitude of malarial antigen expression in blood stages significantly affects the functionality of the antigen-specific T cell response during infection, we therefore quantified the accumulation and activation status of OT-I and OT-II cells within the brain of mice infected with the different OVA-expressing parasite lines. As expected, we observed a significant increase in the frequencies and numbers of total (transferred CD45.1$^+$ and recipient CD45.1$^+$) CD4$^+$ T cell and CD8$^+$ T cells in the brains of mice showing signs of ECM (day 6 after infection), as compared with naïve mice (Figure 4.4A and B). Importantly, however the frequencies, numbers and activation of total, and endogenous (CD45.1$^+$), CD4$^+$ T cells and CD8$^+$ T cells were not significantly different within the brains of mice infected with the different parasite lines, on day 6 of infection.
These data verify that there were no differences in generalized brain-derived T cell migratory cues or activating signals in mice infected with the different OVA-expressing and non-OVA-expressing parasites.

Populations of OT-I and OT-II cells were observed in the brains of mice infected with all three OVA-expressing parasites (day 6 of infection). However, significantly higher frequencies and numbers of OT-I and OT-II cells were found in the brains of mice infected with OVA::Hep17_hep17 parasites, as compared with mice infected with OVA::mCherry_hsp70 and OVA_hsp70 parasites (Figure 4.4A and B). Moreover, consistent with the data in the spleen, significantly fewer OT-I and OT-II cells were found in the brains of mice infected with the OVA_hsp70 parasites compared with mice infected with the OVA::Hep17_hep17 parasites (Figure 4.4A and B). Of note, despite the heterogeneity in recruitment/accumulation of OT-I cells to the brain of mice infected with the different transgenic OVA-expressing parasites, the intracerebral OT-I cells displayed comparable activation profiles, as determined by CD69, CD25, Ki67, CD44 and CD62L expression in the different groups of mice, at the time of ECM development (day 6 of infection; Figure 4.4C). The low level of OT-II cell recruitment and accumulation within the brains of infected mice precluded the assessment of their activation status. Combined, our results show that the magnitude and location of OVA-expression within blood stage malaria parasites influences the level, but not activation state, of the cerebral antigen-specific T cell response that develops during ECM development.
Figure 4.4 The location of OVA in blood stage parasites influences the magnitude of the OVA-specific T cell response in the brain during ECM.

10,000 naïve CD45.1^OT-I and 250,000 naïve CD45.1^OT-II cells were adoptively transferred into CD45.2^C57BL/6 mice one day prior to infection (10^4 pRBC i.v.) with different P. berghei ANKA lines, GIMO\textsubscript{ANKA} (control), OVA\textsubscript{hsp70} (OVA), OVA::mCherry\textsubscript{hsp70} (OVA::mC), OVA::Hep17\textsubscript{hsp70}
(OVA::Hep17). (A) Representative flow cytometric plots of total and donor CD4\(^+\) and CD8\(^+\) T cells in brains of mice infected with different *P. berghei* ANKA lines at day 6 after infection. Hierarchical gating of total brain CD4\(^+\) and CD8\(^+\) T cells (top row); host CD45.1\(^-\) CD4\(^+\) T cells and donor OVA-specific CD45.1\(^-\) CD4\(^+\) OT-II cells (middle row); host CD45.1\(^-\) CD8\(^+\) T cells and donor OVA-specific CD45.1\(^-\) CD8\(^+\) OT-I cells (bottom row). (B) Percentages and absolute numbers (mean +/- SEM) of total and donor (OT-I) T cells in brains of mice (n=4) infected with different *P. berghei* ANKA lines at day 6 after infection. The magnitude of the total intracerebral T cell responses was comparable in mice infected with the different parasite lines (left 4 graphs). Stronger OVA-specific T cell responses are observed in mice infected with OVA::Hep17 parasites compared with mice infected with OVA::mC and OVA parasites (right 4 graphs). The results are representative of 2 separate experiments. *= p<0.05; **= p<0.01; ***= p<0.001. (C) Percentages and absolute numbers (mean +/- SEM) of donor (OT-I) T cells in brains of mice (n=4) infected with different *P. berghei* ANKA lines at day 6 after infection. The activation of CD8\(^+\) CD45.1\(^-\) OT-I cells was determined by examining the expression of CD69, CD25, Ki67, CD44 and CD62L. The results are representative of 2 separate experiments. ND= Not detected *= p<0.05; **= p<0.01; ***= p<0.001, one-way ANOVA with Tukey’s post hoc analysis.
Discussion

In other infections, such as *T. cruzi*, *T. gondii* and *Leishmania*, it has been shown that the subcellular location of antigens is a defining characteristic that dictates the development of antigen-specific T cell responses (Garg et al., 1997, Gregg et al., 2011, Bertholet et al., 2006). In the case of *T. gondii*, model antigen that is secreted by the parasite initiates superior antigen-specific CD4\(^+\) and CD8\(^+\) T cell responses compared to model antigen that is restricted to the parasite cytosol (Pepper et al., 2004, Gregg et al., 2011). In this study we have similarly found that the location of model antigen expression by blood stage malaria parasites influences how the antigen is recognized by the immune system. Specifically, we determined that OVA expressed on the PVM (fused to HEP17/EXP1) induced stronger splenic OVA-specific CD4\(^+\) and CD8\(^+\) T cell responses during blood stage infection than OVA located in the cytoplasm. This is despite OVA being expressed at higher absolute levels in the cytoplasm of *OVA::mCherry\textsubscript{hsp70}* parasites compared to PVM-associated OVA in the *OVA::Hep17\textsubscript{hep17}* parasites. The *OVA::Hep17\textsubscript{hep17}* parasites also promoted the highest level of OT-I and OT-II cell migration to, and accumulation within, the brain at ECM development, as compared with *OVA\textsubscript{hsp70}* and *OVA::mCherry\textsubscript{hsp70}* parasites. It is very unlikely that the differences in the magnitude of OVA-specific T cell responses to *OVA::Hep17\textsubscript{hep17}* parasites compared with *OVA::mCherry\textsubscript{hsp70}* parasites is due to the indirect effect of the different OVA processing and epitope generation. We expressed full length OVA (385 amino acid residues) in the transgenic parasites, with the OT-II and OT-I epitopes being located well within the protein (at residues 323—339 and 257—264 respectively), and have shown that in all lines OVA remains intact, therefore the amino acids flanking the epitopes, which provide the specificity for enzymatic cleavage, will remain unaffected by fusion of OVA to either mCherry or HEP17.
Importantly, we found that the polyclonal endogenous T cell responses, in terms of priming within the spleen and migration to and accumulation within the brain, were comparable during infection with the different parasite lines. This implies that the splenic and brain environment and, central nervous system migratory cues, are comparable during infections with the different OVA-expressing parasites. Thus, supporting the conclusion that the variations in OVA-specific T cell responses during the infections with the different OVA-expressing parasites is directly determined by the nature of OVA expression, and not by other inherent differences in the capacity of the parasites to influence T cell activity. Consequently, whilst it is has been reported that OT-I cells can be recruited to the brain during infections with OVA-expressing *P. berghei* ANKA parasites (Lundie et al., 2008, Miyakoda et al., 2008), our study is the first to show that the level of antigen-specific T cell recruitment to the brain can be largely governed by the magnitude of splenic T cell priming to a specific antigen, rather than secondary differences in brain-localized T cell migratory cues.

Why the subcellular location of a protein within blood stage malaria parasites is a determining characteristic that influences the induction of an antigen specific T cell response during malaria infection is not immediately obvious. In contrast to *T. gondii*, *Leishmania* (Bertholet et al., 2006) and *M. tuberculosis* (Einarsdottir et al., 2009), which can invade and reside within professional antigen presenting cells, the malaria merozoite invades circulating RBC and within these cells, creates and resides in a PV (Graewe et al., 2011). As mature RBCs neither display MHC-I nor MHC-II molecules, nor have antigen-processing machinery, an adaptive T cell response can, theoretically, only be generated when classical DCs phagocytise infected RBCs or free merozoites. However, an explanation is that the location of protein expression within blood stage parasites influences their incorporation into microparticles that are derived from infected RBCs. It is known that
such microparticles are produced when schizonts mature and that these microparticles influence the activation of innate immune responses (Couper et al., 2010, Mantel et al., 2013, Mantel and Marti, 2014). Current understanding suggests that at schizogony both PV and the RBC membranes rupture releasing microparticles containing RBC and PV membrane-associated proteins. In contrast, parasite proteins that are protected by the parasite plasma membrane, such as those located in the parasite cytosol, are less likely to become incorporated into such microparticles. Thus, OVA expressed on the PVM (OVA::Hep17_{hep17} parasites) is potentially recognised by the immune system in a different manner and at a different level than OVA expressed in the parasite cytoplasm (OVA::mCherry_{hsp70} and OVA_{hsp70} parasites). It would therefore be of interest to generate further transgenic lines expressing OVA in other subcellular locations (e.g. secreted into the RBC cytoplasm, on the RBC membrane) and characterise their capacity to stimulate T-cell and/or APC activation.

Of note, we unexpectedly found that very weak OVA protein expression occurred in OVA_{hsp70} parasites, in which unconjugated OVA was produced in the cytosol. This was despite strong OVA transcription levels in these parasites. When OVA was fused to mCherry and expressed under the control of the same promoter in OVA::mCherry_{hsp70} parasites, we observed strong OVA expression, and the levels reflected the transcription levels. Thus, our results show that unconjugated cytosolic OVA is unstable in blood stage malaria parasites, which is an important finding with implications for researchers involved in making transgenic reporter parasites. The exact reason for unconjugated OVA instability is unclear; other unconjugated heterologous proteins (such as fluorescent and luminescent proteins) are highly expressed in transgenic Plasmodium parasites using the hsp70 promoter (Hliscs et al., 2013). It may be possible that unconjugated OVA, which is a phosphorylated-glycoprotein (Suzuki et al., 1997), is rapidly degraded because it is
incorrectly post-translationally modified in *Plasmodium* whereas stability to OVA is conferred when it is fused to another protein. Irrespective of the exact reasons for the dichotomous expression of OVA in these two parasite lines, when we examined the T-cell responses to the parasites, we observed that *OVA*<sub>hsp70</sub> parasites induced significantly inferior OT-I and OT-II immune responses compared with *OVA::mCherry*<sub>hsp70</sub> parasites. Thus, these data indicate that the absolute expression level of an antigen, in addition to its location, is an important factor in determining antigen-specific immune responses during malaria infection.

In summary, these results demonstrate that the sub-cellular location of a model antigen in a transgenic parasite influences its effectiveness in initiating different T cell responses. The transgenic *P. berghei* ANKA and NK65 reporter parasites we have generated are useful tools for further study how parasite antigen expression and location can modulate site-specific immune responses, both in blood and the liver stage of infection. Indeed, in future work the comparing OVA transgenic parasites that induce ECM (*P. berghei* ANKA) with those that do not (i.e. *P. berghei* NK65) can be used to study the pathways orchestrating development and differentiation of pathogenic T cell responses during malaria infection. Additionally, *P. berghei* NK65 lines can be used to examine how the location and magnitude of OVA expression differentially influences Ag-specific T cell responses in more chronic malarial infections. More generally, the transgenic OVA parasite lines described may provide information on the types of endogenous malaria proteins that are most likely to be recognized by the immune system potentially informing decisions about which malarial proteins should be incorporated into vaccines.
Materials and Methods

Experimental animals and reference *P. berghei* lines

For generation of the transgenic parasite lines, female Swiss OF1 mice (6—8 weeks; Charles River/Janvier) were used. For the induction of experimental cerebral malaria (ECM) and the immunological assays, the following mouse strains were used: C57BL/6 (CD45.2⁺), RAG-1 OT-I x Pep3 (CD45.1⁺) F1 mice and RAG-1 OT-II x Pep3 (CD45.1⁺) F1 mice. All mice (6—10 weeks) were purchased from Charles River, UK or bred in house at the University of Manchester, UK.

All animal experiments performed at the LUMC were approved by the Animal Experiments Committee of the Leiden University Medical Center (12042). The Dutch Experiments on Animal Act were established under European guidelines (EU directive no. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes). Animal experiments performed at the University of Manchester (UoM) were approved following local ethical review by UoM Animal Procedures and Ethics Committee and was performed in strict accordance with the U. K Home Office Animals (Scientific Procedures) Act 1986 (approved H.O Project License and 70/7293).

To generate the transgenic lines two reference *P. berghei* lines were used. These lines are GIMO-mother lines of *P. berghei* ANKA (GIMO\(_{\text{PbANKA}}\); line 1596cl1) (Lin et al., 2011) and *P. berghei* NK65 (GIMO\(_{\text{PbNK65}}\); line 1995cl2; described below) that have been generated to rapidly introduce transgenes without drug-selectable markers (see below). In addition we used the reference cl15cy1 line of *P. berghei* ANKA (Janse et al., 2006) and the reference NK65 Edinburgh line.
Generation of GIMO\textsubscript{NK65} mother line

The GIMO\textsubscript{PbNK65} (line 1995cl2) was generated similar to the generation of the GIMO\textsubscript{PbANKA} line (Lin et al., 2011). Briefly, the DNA-construct pL1603 (Lin et al., 2011) was used to introduce the positive/ negative selectable marker cassette (SM, \textit{hdhfr::yfcu}) by double cross-over homologous recombination into the ‘silent’ \textit{230p} gene locus (PBANKA\textsubscript{030600}) of NK65 Edinburg parasites. The \textit{hdhfr::yfcu} marker is expressed under the control of the \textit{eef1\alpha} promoter and is a fusion gene of the positive SM human \textit{dihydrofolate reductase} (\textit{hdhfr}) and the negative SM which is a fusion gene of yeast \textit{cytosine deaminase} and \textit{uridyl phosphoribosyl transferase} (\textit{yfcu}). Transfection, selection and cloning of mutants were performed by standard procedures described for transfection of \textit{P. berghei} (Janse et al., 2006). Correct integration of the constructs was verified by diagnostic PCR analysis and Southern blot analysis of pulse-field gel (PFG) electrophoresis-separated chromosomes as described (Lin et al., 2011).

Generation of different transgenic \textit{P. berghei} (ANKA and NK65) lines expressing OVA

To generate transgenic mutants expressing ovalbumin (OVA) under the control of the strong, constitutive \textit{hsp70} promoter we generated two DNA constructs. For the first construct (pL1812), the complete coding sequence (CDS) of OVA was amplified from plasmid pENTRY201-OVA (kindly provided by K. Franken, Department of Infectious Diseases, Leiden University Medical Center) using the primers 6464/6465 (see Table S1 for primer sequences). This fragment was subsequently subcloned into restriction sites BamHI/SgrAI of construct pL1694, thereby replacing mCherry with OVA between the \textit{hsp70} promoter and the \textit{hsp70} 3’UTR (untranslated region). PL1812 was linearized with restriction enzyme SacII prior to transfection. For the second construct (pL1838), the CDS (without stop codon) of OVA was PCR-amplified from plasmid pENTRY201-OVA using
primers 6466/6467 (Table S1) and cloned into restriction sites XhoI/BamHI of pL1809, resulting in placement of OVA between the hsp70 promoter region and the mCherry CDS. PL1838 was linearized with restriction enzymes HindIII and NdeI prior to transfection.

To generate a transgenic mutant with OVA fused to HEP17 (hepatocyte erythrocyte protein 17-kDa, PBANKA_092670) (Graewe et al., 2011), the DNA construct pL1884 was generated (see Fig. S1). First, the 5’UTR of hep17 (1.3 kb upstream of the start codon) and signal peptide (SP) sequence of hep17 (1—81bp) was amplified from wild-type P. berghei ANKA genomic DNA using primers 6659/6660 and subcloned into the pCR2.1-TOPO vector (TOPO TA Cloning Kit, Invitrogen). Secondly, 2 pieces of DNA sequences were subsequently cloned into this vector: 1) the rest of hep17 CDS after the SP (82—726bp) along with ~700bp 3’UTR, which was amplified using primers 6661/6662 and 2) CDS of OVA which was amplified from pENTRY201-OVA using primers 6920/6467, into sites BamHI/KpnI and NdeI/BamHI, respectively, resulting the intermediate construct encodes a fusion protein of OVA::HEP17. Thirdly, the 5’- and 3’- targeting regions (TR) of 230p (PBANKA_030600) were amplified from wild-type P. berghei ANKA genomic DNA using primers 6838/6839 and 5587/6840 and subcloned into the above intermediate vector, using sites XhoI/EcoRI and KpnI, respectively, resulting in the final construct pL1884 (See Table S1 for primer sequences). This construct was linearized using SacII sites before transfection (Fig. S1).

Transfection was performed using parasites of the P. berghei mother lines GIMO_PbANKA (line 1596c11; (Lin et al., 2011)) and GIMO_PbNK65 (line 1995c12, see above) and transfected parasites were selected using the GIMO-method of negative selection as described previously (Lin et al., 2011). Negative selection was performed by treating mice infected with transfected parasites with drug 5-fluorocytosine (5-FC) in the drinking water (2 mg/mL). Clonal parasite lines were obtained by the method of limiting dilution (Janse et
Correct integration of DNA constructs was verified by Southern analyses of chromosomes separated by pulsed-field gel electrophoresis (Janse et al., 2006). Hybridization was performed with a mixture of two probes, a control probe recognizing p25 on chromosome 5 and a hdhfr probe recognizing hdhfr::yfcu SM (Lin et al., 2011). See Table S1 for PCR primers used to generate the different probes.

**Northern and Western analyses of OVA expression level in transgenic parasite lines**

Transcription was analysed by standard Northern blot analyses. Total RNA was isolated from *in vitro* cultured and purified schizonts (Janse et al., 2006), 2006 #51 of *P. berghei* ANKA (cl15cy1) and the different transgenic lines. Northern blots were hybridised with probes specific for the ova CDS, which was PCR-amplified from plasmid pENTRY201-OVA (see above) using primers 6920/6467 (Table S1). As a loading control, Northern blots were hybridized with the oligonucleotide probe L644R that recognizes the *large subunit* (*lsu*) *ribosomal rna* unit (van Spaendonk et al., 2001). For Western blot analysis of OVA expression, total protein-extracts from *in vitro* cultured and purified mature schizonts and from cultured trophozoites and immature schizonts obtained from synchronized infections in mice (Janse and Waters, 1995) were separated on 12% SDS-PAGE and transferred to nitrocellulose membranes by electroblotting. OVA expression was detected by incubation of membranes with polyclonal rabbit IgG anti-OVA antibody (kindly provided by M. Camps, Department of Immunohematology and Blood Transfusion, LUMC) followed by incubation with HRP-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen). Polyclonal goat IgG anti-DsRed (mCherry) antibodies (Santa Cruz Biotechnology) were used to confirm OVA::mCherry expression, followed by HRP-conjugated rabbit anti-goat IgG secondary antibody (Invitrogen). Immuno-stained protein complexes were visualized by enhanced chemiluminescence (Amersham). As a loading
control, the membranes were stained with anti-PbHSP70 antibody (Mueller et al., 2005), followed by incubation with HRP-conjugated goat anti-mouse IgG secondary antibody. The relative signal intensity of bands on Northern and Western blots were quantified using Image J software and graphed using GraphPad Prism.

Immunofluorescence analysis of the cellular localization of OVA

For the immunofluorescence assays (IFA) on blood stages, parasites were cultured using standard in vitro culture conditions (Janse et al., 2006) except for a lower culture temperature (34°C). Infected erythrocytes collected from these cultures contain both maturing trophozoites and schizonts. The infected erythrocytes on slides were fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.5% Triton-X 100 in PBS for 10 min. The parasites were stained with anti-OVA or anti-EXP1 (Sturm et al., 2006) antibodies followed by Alexa-Fluor® 594 anti-rabbit or Alexa Fluor® 488 anti-chicken antibodies (Invitrogen). Nuclei were stained with Hoechst-33342 (2 µmol/L, Sigma, NL) for 15 min. Slides were mounted in Vectashield (Vector Laboratories Inc) and examined using a DM-IRBE Flu Leica fluorescence microscope.

For immunofluorescence analysis on in vitro EEF (exo-erythrocytic form), 5×10⁴ sporozoites were added to a monolayer of Huh7 cells on coverslips in 24 well plates in ‘complete’ RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum (FBS), 1% (vol/vol) penicillin-streptomycin, 1% (vol/vol) GultaMAX (Invitrogen), and maintained at 37°C with 5% CO₂. At 48 h after infection, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X 100 in PBS, blocked with 10% FBS in PBS, and subsequently stained with primary and secondary antibodies for 2h and 1h, respectively. Primary antibodies used were anti-PbEXP1 (raised in chicken (Sturm et al., 2006)) and anti-UIS4 (raised in rabbit (Mueller et al., 2005), both detecting the PVM-
resident proteins; anti-PbHSP70 (raised in mouse (Mueller et al., 2005)), detecting the cytoplasmic heat shock protein 70 (PBANKA_081890) and anti-OVA antibodies (raised in rabbit, see above). Anti-mouse, -chicken and -rabbit secondary antibodies, conjugated to Alexa Fluor® 488 and 594, were used for visualization (Invitrogen). Nuclei were stained with Hoechst-33342. Cells were mounted in Vectashield and examined using a DM-IRBE Flu Leica fluorescence microscope.

**Blood stage infections and induction of experimental cerebral malaria (ECM)**

Cryopreserved OVA\textsubscript{hsp70}, OVA::\textit{mCherry}\textsubscript{hsp70}, OVA::Hep17\textsubscript{hep17} and GIMO\textsubscript{ANKA} parasites were passaged once through C57BL/6 mice before being used to infect experimental animals. Mice were infected by intravenous (i.v.) injection of \(10^4\) parasitized red blood cells (pRBCs). Peripheral parasitaemia was monitored by examination of Giemsa stained thin smears of tail blood. The induction and severity of ECM was assessed using the following well-defined grading system (Villegas-Mendez et al., 2012): 1: no signs; 2: ruffled fur/and or abnormal posture; 3: lethargy; 4: reduced responsiveness to stimulation and/or ataxia and/or respiratory distress/hyperventilation; 5: prostration and/or paralysis and/or convulsions. All animals were euthanized when observed at stage 4 or 5. Stages 2/3 were classified as prodromal signs of ECM and stages 4/5 were classified as ECM. Animals in stages 4 or 5 invariably show signs of cerebral pathology, including blocked vessels, haemorrhages, oedema, perivascular cuffing and disruption and damage to cerebral vasculature endothelial linings following histological examination (Villegas-Mendez et al., 2012).
Flow cytometry

Spleens were removed from naïve, and malaria-infected mice on day 4 and day 6 or 7 post infection (p.i.). Single cell suspensions were generated by homogenizing tissue through a 70 µm cell sieve (BD Biosciences). Brains were chopped into small pieces, aspirated through a 10ml syringe, and incubated in HBSS containing 2% FCS with Collagenase (final concentration 1mg/ml) (Sigma) for 45 min on a tube roller at room temperature. The resulting suspension was filtered through a 70 µm cell sieve, layered on a 30% Percoll gradient and centrifuged at 2000g for 10 minutes. The supernatant was discarded and the pellet collected. For both the spleen and brain preparation, RBCs were lysed using RBC lysing buffer (BD Biosciences). Absolute cell numbers were determined by microscopy using a haemocytometer and live/dead differentiation was performed using the trypan blue exclusion cell viability assay (Sigma).

Identification by flow cytometry of adoptively transferred OT-I and OT-II cells and the activation of endogenous (CD45.1+) and transferred T cells (CD5.1+) was performed by surface staining with the following antibodies: anti-mouse CD45.1 (A20), anti-mouse CD4 (GK1.5), anti-mouse CD8 (53-6.7), anti-mouse CD69 (H1.2F3), anti-mouse CD25 (PC61.5), anti-mouse CD44 (IM7) and anti-mouse CD62L (MEL-14). All antibodies were obtained from eBioscience or Biolegend. To determine the proliferative status of the T-cell populations, the cells were first stained within antibodies to surface markers, washed and subsequently fixed and permeabilised by incubation with the Foxp3 fixation/permeabilisation buffer (eBioscience). The cells were then washed and incubated for 30 minutes with anti-mouse Ki67 (SolA15) before flow cytometry analysis.

All flow cytometry analyses were performed using an LSR II (BD Systems, UK). Subsequent data analyses were performed using Flowjo Software (Treestar Inc, OR, USA). Fluorescence minus one controls were utilized to validate flow cytometric data.
Adoptive transfer of OVA-specific T cells

Splenic single cell suspensions were prepared from naive RAG-1 OT-I x Pep3 (C57BL/6 CD45.1+) F1 mice and RAG-1 OT-II x Pep3 F1 mice, as described above. Splenic CD8+ and CD4+ T lymphocytes were negatively selected from RAG-1 OT-I x Pep3 (C57BL/6 CD45.1+) F1 mice and RAG-1 OT-II x Pep3 F1 mice, respectively, using anti-PE midiMACS beads, following surface staining with PE labelled anti-mouse CD19 (eBio1D3), F4/80 (BM8), MHCII (M5/114.15.2) and either anti-CD4 (GK1.5) or anti-CD8 (53-6.7), respectively, according to the manufacturer’s instructions (Miltenyi Biotec). 1 × 10^4 RAG-1 OT-I x Pep3 CD8+ T cells and 2.5 × 10^5 RAG-1 OT-II x Pep3 CD4+ T cells, were adoptively transferred, together, into recipient mice by intravenous injection on the day before (-1) infection with the different P. berghei ANKA transgenic and wild-type lines. The purity and phenotype of positively selected T cell populations was assessed by flow cytometry prior to adoptive transfer. CD4+ and CD8+ T cells were approximately 95% TCR αβ+ and CD3+, and were predominantly naïve (CD44-CD62L+).

Statistical analysis

Statistical significance was determined using a one-way ANOVA with Tukey’s post hoc analysis. All statistical analyses were performed using Graphpad Prism. In all cases results were classified as significantly different when P <0.05.
References


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Supplementary Information

### Table S4. Primers used in this study

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<th>No.</th>
<th>Primer sequences</th>
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</table>

| **Primer for probe generation** | | | |
| 886 | GGAAGATCTATGTTGCTACCTAAACATGCGATCG | h3hf, R |
| 887 | GGAAGATCTATGTTGCTACCTAAACATGCGATCG | h3hf, F |
| 1462 | CATGCCATGGATGATATCTACTATACGATCG | p25, F |
| 1463 | CGGAAATCTCTTAAAGTATTTGTTGTTGTTGTTGTTGTTG | p25, R |

*F* = forward primer, *R* = reverse primer

Red, restriction sites
Figure S4. 1 Schematic of the generation of DNA-construct used to generate the line OVA::Hep17_hep17.

Schematic representation of the hep17 gene locus and the generation of the DNA construct used to generate OVA::Hep17_hep17. OVA (blue) is fused to hep 17 coding sequence (bright yellow box;signal peptide, yellow hatched box) under the control of the hep 17 5’-UTR promoter and 3’ terminus (light yellow boxes). The two 230p targeting sequences (hatched boxes) flank the OVA expression cassette (see also Material and Methods). The position of hep17 gene relative to start codon (+/- bp), primers (Table S4.1) and restriction sites used are shown. SacII sites (red) were introduced to linearize the vector pL1884 prior to transfection.
Figure S4. 2 Different transgenic P. berghei NK65 lines expressing OVA.
(A) Schematic representation of the 230p locus in the reference P. berghei NK65 (GIMO\textsubscript{PbNK65}) motherline and the two OVA-expressing transgenic lines. The GIMO\textsubscript{PbNK65} has the positive and negative selectable marker cassette (hdhfr::yfcu, black box) inserted into P. berghei NK65 230p locus. The OVA-expression cassettes were introduced into 230p by double cross-over homologous recombination using the gene targeting regions (hatched boxes) using GIMO transfection method. The OVA\textsubscript{hsp70} line (2169cl1) encodes unconjugated full-length ovalbumin (ova; blue box) under the control of 5'-(promoter) and 3'-(terminator) UTRs of the P. berghei hsp70 gene (grey boxes); The OVA::mCherry\textsubscript{hsp70} (2170cl1) encodes OVA which is C-terminally fused to mCherry (red box) under the control of 5'-UTR of hsp70 and the 3'-UTR of dhfr/ts (white box). The direction of transcription is indicated by the arrows.
(B) Southern analyses of PFG-separated chromosomes confirm the integration of the constructs into the 230p locus on chromosome 3 of the two transgenic NK65 lines, resulting in the removal of the hdhfr::yfcu selection cassette. Hybridization was performed with a mixture of two probes, a control probe recognizing p25 on chromosome (Chr.) 5 and a hdhfr probe recognizing hdhfr::yfcu on chromosome 3.
(C) Northern blot analysis of OVA-transgene transcription in purified schizonts of the two NK65 lines. Blots were hybridized using a PCR probe recognizing ova (primers 6466/6467, Table S4.1). As a loading control an oligonucleotide probe L644R was used that recognizes the large subunit (lsu) rRNA. wt, wild-type P. berghei NK65 parasites.
(D) Western analysis of OVA-transgene expression in purified schizonts in the two transgenic NK65 lines. Blots were stained with anti-OVA antibodies. Anti-HSP70 antibody staining was used as a loading control. wt, wild-type *P. berghei* NK65 parasites.

(E) Western analysis of OVA-expression in purified schizonts of two different clones (cl.1 and cl.2) of transgenic *P. berghei* ANKA *OVA*<sub>hsp70</sub> parasites (OVA). Blots were stained with anti-OVA antibodies and anti-HSP70 antibody as a loading control.

(F) Western analysis indicates that a fraction of fusion protein OVA::mCherry expressed in Pb ANKA *OVA*<sub>mCherry</sub><sup>hsp70</sup> line (OVA::mC) is truncated because of a specific cleavage within mCherry. Western blots were prepared from proteins extracted from RBC infected with schizonts of OVA::mC or OVA::Hep17 (*OVA*<sub>Hep17</sub><sup>hsp70</sup>) lines, and stained with anti-OVA (left) and anti-mCherry (right) antibodies. Black arrows indicate full-length OVA::mCherry (~75kDa) recognised by both anti-OVA and anti-mCherry antibodies. Anti-OVA antibody also recognised a truncated version of OVA::mC protein (~55kDa, white arrow). A ~20kDa band (red arrow) showing the remainder of the OVA::mC protein was recognised by anti-mCherry antibody indicating that in a fraction of the fusion protein a specific cleavage had occurred within mCherry. OVA::Hep17 line is included as a control and single full length band (~62kDa) is visible only when the blot was probed with anti-OVA antibodies.

(G) The level of OVA-expression in synchronised blood stages of *OVA*<sub>Hep17</sub><sup>hep17</sup> and *OVA*<sub>mCherry</sub><sup>hsp70</sup> parasites. Trophozoites (Troph; 12-16 hours post invasion) and immature schizonts (Imm. Schiz; 18-22 hours post invasion) were obtained from synchronised infections. Western blots were stained with anti-OVA antibodies and anti-HSP70 antibodies as a loading control. Relative OVA expression level was calculated as the ratio between Western staining intensities of OVA and HSP70, which were quantified using Image J.
Figure S4. 3 The course of blood stage infection (parasitaemia and ECM) in mice infected with the different *P. berghei* ANKA lines.

(A) Parasitemia (% of infected erythrocytes) in tail blood of mice infected with $10^4$ infected parasitized RBC (pRBC) of different *P. berghei* ANKA lines, GIMO<sub>ANKA</sub> (wilt-type control), OVA<sub>hsp70</sub> (OVA), OVA::mCherry<sub>hsp70</sub> (OVA::mC), OVA::Hep17<sub>hep17</sub> (OVA::Hep17). The results are the mean +/- SEM of a minimum of two separate experiments with a minimum total of 8 mice per group. (B) Kinetics of development of experimental cerebral malaria (ECM) shown as percentage of survival of mice infected with $10^4$ pRBC of different Pb ANKA lines. ECM was defined as stage 4 or 5 of the clinical scale of ECM symptoms (see Materials and Method section). The results are from two separate experiments with a minimum total of 8 mice per group.
Figure S4.4 The location of OVA expression in blood stage parasites influences the activation of OVA-specific T cells during early stage of infection.

10,000 naïve CD45.1+ OT-I and 250,000 naïve CD45.1+ OT-II cells were adoptively transferred into CD45.2+C57BL/6 mice one day prior to infection (10⁷ PRBC i.v.) with different *P. berghei* ANKA lines, GIMOANKA (control), OVA*hsp70* (OVA), OVA::*mCherry*<sub>hsp70</sub> (OVA::mC), OVA::*Hep17*<sub>hep17</sub> (OVA::Hep17).
(A) Representative flow cytometric plots of total and donor CD4⁺ and CD8⁺ T cells in the spleens of mice infected with the different *P. berghei* ANKA lines at day 4 after infection. Hierarchical gating of total splenic CD4⁺ and CD8⁺ T cells (top row); host CD45.1 CD4⁺ T cells and donor OVA-specific CD45.1⁺CD4⁺ OT-II cells (middle row); host CD45.1 CD8⁺ T cells and donor OVA-specific CD45.1⁺CD8⁺ OT-I cells (bottom row). (B) Percentages and absolute numbers (mean +/- SEM) of total and donor (OT-I/OT-II) T cells in spleens of mice (n=4) infected with different *P. berghei* ANKA lines at day 4 after infection. There was no significant expansion of total splenic T cells (left 4 graphs) nor OVA-specific OT-I or OT-II cells during infection with any of the *P. berghei* ANKA lines (right 4 graphs). The results are representative of 2 separate experiments. (C) Percentages and absolute numbers (mean +/- SEM) of donor (OT-II) T cells in spleens of mice (n=4) infected with different *P. berghei* ANKA lines at day 4 after infection. The activation of CD4⁺CD45.1⁺ OT-II cells was determined by examining the expression of CD69, CD25, Ki67, CD44 and CD62L. The results are representative of 2 separate experiments. *= p<0.05; **= p<0.01; ***= p<0.001.

**Figure S4. 5** Comparison of the endogenous splenic T cell responses in mice infected with the different *P. berghei* ANKA lines.
10,000 naïve CD45.1 \textsuperscript{+} OT-I and 250,000 naïve CD45.1 \textsuperscript{+} OT-II cells were adoptively transferred into CD45.2\textsuperscript{+}C57BL/6 mice one day prior to infection with the different \textit{P. berghei} ANKA lines (10\textsuperscript{5} pRBC i.v.), GIMO\textsubscript{ANKA} (control), \textit{OVA}hsp70 (OVA), \textit{OVA}::m\textit{Cherry}\textsubscript{hsp70} (OVA::mC), \textit{OVA}::\textit{Hep17}\textsubscript{hep17} (OVA::Hep17). The mean percentages of activated endogenous (recipient CD45.1) T cells was calculated in the spleens of mice (n=4) infected with different \textit{P. berghei} ANKA lines on day 4 and day 6 of infection. T cell activation was assessed by examining the expression of CD69, CD25, CD44 and CD62L and proliferation was assessed through expression of Ki67. The results are the mean +/- SEM of the group with 4 mice per group and are representative of 2 separate experiments. *= p<0.05; **= p<0.01; ***= p<0.001.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figureS4.jpg}
\caption{Comparison of the endogenous intracerebral T cell responses in mice infected with the different \textit{P. berghei} ANKA lines.}
\end{figure}

10,000 naïve CD45.1\textsuperscript{+} OT-I and 250,000 naïve CD45.1\textsuperscript{+} OT-II cells were adoptively transferred into CD45.2\textsuperscript{+}C57BL/6 mice one day prior to infection with the different \textit{P. berghei} ANKA lines (10\textsuperscript{5} pRBC i.v.), GIMO\textsubscript{ANKA} (control), \textit{OVA}hsp70 (OVA), \textit{OVA}::m\textit{Cherry}\textsubscript{hsp70} (OVA::mC), \textit{OVA}::\textit{Hep17}\textsubscript{hep17} (OVA::Hep17). The mean percentages of activated endogenous (recipient CD45.1) T cells was calculated in the brains of mice (n=4) infected with different \textit{P. berghei} ANKA lines on day 6 of infection, when infected mice developed ECM. T cell activation was assessed by examining the expression of CD69, CD25, CD44 and CD62L and proliferation was assessed through expression of Ki67. The results are the mean +/- SEM of the group with 4 mice per group and are representative of 2 separate experiments. *= p<0.05; **= p<0.01; ***= p<0.001.
5. Chapter 5: General Discussion
Due to its high levels of morbidity and mortality, understanding the pathogenesis of cerebral malaria (CM) remains a key global health objective (World Health Organization, 2014). Our lack of detailed understanding of the events that lead to the development of CM, and the host responses that protect against it, have resulted in inadequate adjunctive therapy to treat the condition and largely ineffectual vaccine strategies to prevent it. Current treatments for CM patients rely solely on parasiticidal chemotherapies, such as artemisinin based drugs. Treatments aimed at modifying the host physiologic state or immune response could decrease mortality and reduce the occurrence of long-term sequelae (Miller et al., 2013). Consequently, a mortality rate of 10-20% persists even after rapid chemotherapy administration (Dondorp et al., 2005, Dondorp et al., 2010). Efforts to develop a prophylactic vaccine against severe malaria have proved even more disappointing. The current lead vaccine candidate, RTS,S, offers protection against clinical malaria and severe malaria in only 46% and 34% of children respectively. Vaccine efficacy is even lower in infants and wanes in both age groups after 18 months, despite administration of three doses (RTSS Clinical Trials Partnership, 2014).

Progress in understanding the spatio-temporal events leading to CM development has, in part, been hindered by the inaccessibility of the affected site for detailed study until post-mortem, scarcity of samples and lack of comparative samples from non-fatal cases. To further our understanding of the events that lead to CM, a murine model has been developed that recapitulates many of the clinical and pathological features of CM (Hearn et al., 2000, Lou et al., 2001). This model has led to an increased understanding of splenic priming of pathogenic CD8⁺ T cells (deWallick et al., 2007, Lundie et al., 2008, Piva et al., 2012), molecular pathways leading to their intracerebral recruitment (Nie et al., 2009, Van den Steen et al., 2008, Belnoue et al., 2008) and mechanisms leading to alterations in BBB integrity (Nacer et al., 2014, Nacer
et al., 2012). Despite these mechanistic insights, our understanding of the proximal events leading to development of the condition remains incomplete. Moreover, due to its naturally acute and fatal outcome, this model has been wholly underutilised in the study of naturally acquired immunity to severe disease, through repeated infections.

The overall aims of my PhD were, therefore, to further our understanding of i) the underlying mechanisms and aetiology of CM and ii) the basis of infection induced resistance.

To address these aims I utilised murine models of malaria infection in conjunction with novel transgenic parasites, transgenic mice and innovative methodologies.

A role for CD8$^+$ T cell mediated cerebral pathology in ECM is well established (Renia et al., 2006, Howland et al., 2015). Very few studies, however, have been conducted to determine the trigger for, and target of, their perforin and granzyme B release, or where precisely they are located in the brain when they mediate their pathogenic effect. Nor have many studies been conducted comparing the phenotype and function of intra-cerebrally accumulated CD8$^+$ T cells in ECM and non-ECM inducing malaria infections.

In Chapter 2, we specifically examined the activation status and dynamic behaviour of intracerebral CD8$^+$ T cells in ECM-inducing and non-ECM inducing malarial infections. Both infections promoted the recruitment of activated CD8$^+$ T cells to the subarachnoid and perivascular spaces of the brain, demonstrating that ECM development depends on more than the presence of this population per se. Unifying reports of the presence of intracerebral pathogenic CD8$^+$ T cells with reports of their absence from the parenchyma (Hunt et al., 2014), our results, therefore, provide an understanding of where these pathogenic T cells accumulate and mediate their effect, and why they have hitherto been underreported. Indeed, the fragility and small size of
this compartment does not lend itself to being easily sampled, or preserved for detailed study. Overall, our work highlights the subarachnoid and perivascular spaces as, potentially key locations in the brain, where events leading to ECM development occur. It will now be important to examine whether compartmentalisation of CD8$^+$ T cells within the perivascular space of the meninges is a generalised phenomenon throughout different regions of the brain displaying ECM-pathology, such as the olfactory bulb, cerebellum, cortex and thalamus (results not shown). Further studies involving perturbation of the localisation of pathogenic T cells to, and function from within, the perivascular space will also be required to demonstrate the proposed functional relevance of this compartment to ECM development. If relevant, then localised administration of MHC I blocking antibodies would be expected to abrogate the development of ECM in mice infected with Pb ANKA and localised administration of antigen into this space would be expected to promote the development of ECM in mice infected with Pb NK65.

Differences in CD8$^+$ T cell motility within the cortical perivascular spaces of brains during ECM-inducing and non-inducing infections were found to correlate with their pathogenic activity during malaria infection. These results suggest that it is the engagement of these activated CD8$^+$ T cells with their cognate target, within the perivascular compartment, that determines infection outcome. These results, therefore, point to an inherent difference in the ability of different parasites to cause antigen presentation within this compartment. In support of this hypothesis, Howland et al. (Howland et al., 2013) have recently reported that microvessels isolated from the brains of mice infected with ECM-inducing parasites could cross-present parasite derived antigen for interaction with parasite-specific CD8$^+$ T cells, whereas those isolated from brains of mice infected with non-ECM-inducing parasites (which shared the epitope recognised by the CD8+ T cells) could not. The reasons for the differences in
endothelial cell cross presentation of ECM-inducing and non-ECM inducing parasite species/strains are not obvious as there appears to be little genetic polymorphism between ECM-inducing and non-inducing parasite strains (Otto et al., 2014, Ramiro et al., 2012).

It is possible that the presence of parasites and parasite material in the perivascular spaces of the brain specifically during PbA infection may contribute to the activation of endothelial cells, as material would not rapidly diffuse and circulate away as in the lumen of the vessels. However, as we and others (Nacer et al., 2012) have not observed significant levels of parasite sequestration during PbA infection, whether differences in binding of ECM-inducing and non-ECM-inducing parasites to brain endothelial cells contributes to differences in parasite entry into the perivascular compartments and subsequent development of ECM is unclear and requires further investigation. Indeed, whilst differences in expression of parasite molecules, including BIR-family antigens, on the surface RBCs infected with ECM-inducing and non-ECM inducing parasites have been reported (Pasini et al., 2013), it is also possible that endothelial cells cross present merozoite, food vacuole or pRBC-microparticle-derived antigens, rather than infected RBCs, to promote ECM. Whether merozoites, parasite food vacuoles or pRBC-derived microparticles from ECM-inducing and non-ECM inducing parasite species and strains exhibit disparate properties remains to be ascertained.

At present it is also unclear if ECM-inducing and non-inducing parasites express different repertoires or levels of pathogen associated molecular patterns, which may influence endothelial cell activation and/or phagocytic and cross presentation capacity. Understanding the physical, molecular and behavioral properties that specify the virulence of ECM-inducing and non-inducing parasites is a critical area of research that
may provide insights into events and processes that can be therapeutically targeted to prevent ECM development and progression.

We would therefore propose an evolution of the previously proposed model (Haque et al., 2011), and our starting hypothesis, that ECM-inducing CD8\(^+\) T cells must be activated in a two-step process, with initial priming in the spleen and \textit{in situ} secondary activation by perivascular APCs in the brain. Instead, we propose that ECM-inducing CD8\(^+\) T cells are fully activated within the spleen and attach directly to perivascular antigen-cross-presenting endothelium in the brain, and that such interaction contributes to ECM pathogenesis. We therefore argue it would be of value to examine more closely the perivascular space in post-mortem CM samples.

Fortunately, only a minority of \textit{Plasmodium falciparum} infections develop into CM (Dorovini-Zis et al., 2011). In endemic areas, the majority of the at risk population develop immunity to severe disease, including CM, by late childhood and anti-parasitic disease by early adulthood (Griffin et al., 2015). The necessity for multiple infections in the induction of this protection, and the absence of sterilising immunity, has led to the hypothesis that malaria, in contrast to other infections, induces a defective adaptive memory response (Struik and Riley, 2004). Difficulty inducing disease immunity has similarly hindered the development of an efficacious vaccine (Riley and Stewart, 2013). Improved understanding of the mechanisms underlying infection induced disease resistance could provide more rationally designed and effective vaccines.

In \textbf{Chapter 3}, we examined the basis of infection induced resistance to ECM. We found that parasite exposure-induced ECM resistance was associated with attenuated CD8\(^+\) T cell activation, rather than a reduction in magnitude of intracerebral T cell response; and that this in turn was critically dependent on the production of secreted anti-malaria antibody. Importantly, changes conferring ECM resistance were
found to be controlled by altered splenic T cell priming during a fourth infection, rather than by maintained changes in the brain imprinted by repeated infection. The absence of maintained transcriptional changes in the brain after infection is in contrast to the long-term neurological deficits reported in drug cured mice (Serghides et al., 2014) and in up to 25% of humans (Idro et al., 2010) surviving an episode of (E)CM. It is possible that whole brain analysis is not sensitive enough to detect very subtle or regionally maintained changes underlying such deficits. Indeed, regional differences within the brain, associated with physiological function, have been reported in mice (Yeh et al., 2009), rats (Stansberg et al., 2007) and humans (Ernst et al., 2007). Dissection and individual gene expression profiles of different functional regions of the brain may provide important information on whether specific regions of the brain undergo longer term damage post-ECM, related to neurological sequelae, than others.

Interestingly, the transcriptional alterations in the brain associated with parasite exposure-induced ECM resistance, when compared with ECM affected mice, were similar to those observed when comparing brain transcriptomes in primary PbA infected ECM resistant and ECM susceptible mice (Delahaye et al., 2006, Lovegrove et al., 2006, Oakley et al., 2008). Similar to infected ECM resistant BALB/c mice, infected exposure-induced ECM resistant C57BL/6 mice showed reduced expression of genes involved in defence and immune responses compared with ECM affected C57Bl/6 mice. These results suggest that regardless of a starting point of genetic susceptibility, resistance can be induced which mimics that seen in naturally resistant hosts. Associated with the development of ECM resistance, was the development of increased parasite control. As expected, repeated infection of IgMi mice demonstrated that this was largely mediated by an anti-malaria antibody response, although a contribution from antibody independent mechanisms, most likely CD4\(^+\) T cells, were also observed. Whilst quantification of the antibody response during each round of infection remains to
be performed (sample are to be stored and analysed), Bao et al. (Bao et al., 2013) report that at least two prior infections are required to achieve consistently higher anti-malaria antibody titres throughout infection, compared with mice that had one or no prior infections. These results recapitulate reports in humans that antibody levels drop quickly after an infection episode and that multiple infections are required to induce memory B cells and long lived plasma cells which maintain a basal level of protective circulating antibody (Weiss et al., 2010). The targets and mechanisms of action of the anti-malaria antibody produced in exposure-induced ECM resistant mice remain to be fully elucidated but could involve neutralisation and opsonisation of malaria antigen. Reducing the antigen load available for i) splenic T cell activation and differentiation to effector cells with pathogenic potential and ii) intracerebral cross presentation by endothelial cells are two possible, but not mutually exclusive, mechanisms by which anti-malaria antibody could mediate parasite exposure-induced resistance.

An additional indirect mechanism for antibody dependent suppression of CD8⁺ T cell activation was also highlighted by this work. Immune-complex mediated FcR signalling to expand a regulatory splenic pDC population represents a previously uninvestigated mechanism of inducing protection from ECM development. pDCs express FcγRII and FcγRIII (Guilliams et al., 2014, Björck et al., 2008) and have been shown be activated, and to acquire antigen for presentation to CD4⁺ and CD8⁺ T cells, by immune-complex opsonisation (Björck et al., 2008). The resulting CD8⁺ T cell responses could be polarised towards IL-10 production or cytolytic activity in the absence and presence of LPS, respectively (Björck et al., 2008). It is therefore conceivable that immune-complex mediated antigen presentation to CD8⁺ T cells, in the absence of pro-inflammatory cytokines, in repeatedly infected mice may divert CD8⁺ T cell differentiation away from a cytotoxic towards an anti-inflammatory effector function.
Reduced levels of pro-inflammatory cytokines have been linked to impaired development of CD8$^+$ T cell effector function and associated protection from ECM in immunity-related GTPase 3 (Irgm3$^{-/-}$) mice. Protection from ECM was afforded despite normal levels of peripheral parasitaemia and splenic T cell proliferation, and only modestly reduced intracerebral CD8$^+$ T cell accumulation, confirming the relevance of in situ effector function over accumulation per se (Guo et al., 2015). Quantification of circulating cytokines in exposure-induced ECM resistant mice, as well as nanostring analysis of splenic gene expression of various inflammatory and regulatory molecules, will provide further information on alterations in ‘signal 3’ cytokines that may influence the differentiation of malaria specific CD8$^+$ T cells (to be performed).

In addition to FcγRII and FcγRIII expression, pDCs (exclusively in humans) express TLR9 which recognises the parasite waste product, hemozoin (Coban et al., 2005, Wagner, 2010). TLR9 mediated hemozoin signalling has been shown to regulate the development of cytotoxic effector function and antibody responses to malaria infection (Gowda et al., 2012). These results support a role for pDCs as shapers of the adaptive response, sensing malaria antigens through a number of pathways which converge to determine the quality of CD8$^+$ T cell response.

Further work is needed to examine the functional relevance, and mechanisms of action, of antibody-dependent regulatory pDCs in exposure-induced ECM resistance. Passive transfer of antibody from exposure-induced ECM resistant mice into mice infected for a first time to look for the induction of resistance associated pDCs would confirm this as a potential mechanism in the development of anti-disease immunity. Determination of the antibody specificities required for putative FcγR signalling to pDC is also required. Repeated infection and drug cure of mice lacking pDCs to confirm their role in exposure-induce ECM resistance is also highly desirable, along with studies
to determine after which round of infection they are induced. Whether this population is present in humans with exposure-induced resistance to CM also needs to be determined. In the event that this population is found to be protective against ECM, and is associated with parasite exposure in humans, it will present a highly desirable correlate of immunity which can be used to identify vaccine candidates for progression to clinical trial.

Much attention has been given to the role of CD8$^+$ T cells in ECM but their relevance to human CM, and even the utility of the ECM mouse model as a whole has been intensely debated (White et al., 2010, de Souza et al., 2010). A recent publication, however, reporting that the proportion of activated blood circulating CD8$^+$, but not CD4$^+$, T cells in Kenyan children with severe malaria correlates with disease severity, provides the first support for a role for CD8$^+$ T cells in the pathogenesis of human CM (Guermonprez et al., 2013).

Although not thought to be involved in the effector phase of ECM, CD4$^+$ T cells have been shown to contribute to the induction of ECM in a primary infection (Belnoue et al., 2002, Villegas-Mendez et al., 2012). They are additionally known to be important in the control of peripheral parasitaemia and development of anti-malaria antibodies (Langhorne et al., 1990) which we, and others (Raj et al., 2014), have shown to be necessary for the development of ECM resistance. Currently, no target epitopes for these cells have been identified and investigation of malaria specific primary and memory responses therefore relies on the use of model antigens inserted into transgenic parasites and complementary transgenic T cells (Lundie et al., 2008). Although such tools have provided useful insights into ECM pathogenesis, mediated by CD8$^+$ T cells (Lundie et al., 2008, Haque et al., 2011), equivalently detailed investigation of CD4$^+$ T
cells has been hindered by a lack of model antigens able to drive strong *in vivo* CD4⁺ T cell responses (Lundie et al., 2008). In **Chapter 4**, we therefore developed and tested a number of new transgenic parasites able to drive the activation and expansion of both CD4⁺ and CD8⁺ transgenic T cells.

We report that both the absolute expression level and the subcellular location of the model antigen ovalbumin (OVA) determine the induction and magnitude of parasite-specific T-cell responses and identify parasites expressing OVA at the parasitophorous vacuole membrane as the best for driving activation and expansion of transgenic CD4⁺ OT II cells. These new parasites can now be used to study malaria specific CD4⁺ T cell responses.

**Together, data generated for my PhD have contributed to a better understanding of the aetiology of ECM, identified a potential new cellular correlate and mechanism of anti-disease immunity and, developed new tools for the further investigation of malaria specific CD4⁺ T cell responses.**
References


