Carbon monoxide and brain sequestration of Plasmodium berghei ANKA in experimental cerebral malaria

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Carbon monoxide and brain sequestration of *Plasmodium berghei* ANKA in experimental cerebral malaria

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ..................................................................................................................... I

ABBREVIATIONS .................................................................................................................................. II

1. RESUMO ........................................................................................................................................... 1

2. ABSTRACT ......................................................................................................................................... 6

3. INTRODUCTION............................................................................................................................... 8

3.1. Malaria: a worldwide burden ........................................................................................................ 8

3.2. *Plasmodium* life cycle in the mammalian host .............................................................................. 8

3.2.1. Liver stage - The exo-erythrocytic cycle .................................................................................. 9

3.2.2. Blood stage - The intra-erythrocytic cycle .............................................................................. 10

3.2.3. Sexual stage ................................................................................................................................ 11

3.3. Cerebral malaria (CM) .................................................................................................................. 11

3.4. Experimental cerebral malaria (ECM): the benefit of rodent models........................................ 12

3.5. Pathogenesis of CM: sequestration, inflammation and hemostasis dysfunction .............................................. 13

3.5.1. Sequestration of iRBCs ............................................................................................................ 13

3.5.2. Sequestration of iRBCs in the murine model ............................................................................ 14

3.5.3. Inflammation ............................................................................................................................. 15

3.5.3.1. Pro-inflammatory cytokines: tumor necrosis factor-α (TNF-α), lymphotoxin-α (LT-α) and interferon-γ (IFN-γ) .............................................................................................................. 16

3.5.3.2. Leukocyte accumulation in the brain: the importance of CD8+ T cells ..................................... 17

3.5.3.3. Adhesion molecules: intercellular adhesion molecule-1 (ICAM-1) ........................................ 18
3.5.4. Hemostasis dysregulation ................................................................. 19
3.5.5. The integration of events: blood-brain barrier (BBB) damage ... 20

3.6. Heme-oxygenase-1 (HO-1) and carbon monoxide (CO) ................. 21
  3.6.1. Pivotal role of HO-1 and CO in the protection against ECM ...... 22

3.7. CO-releasing molecules (CO-RMs) .................................................... 25
  3.7.1. CO-releasing molecule-2 (CORM-2): vasorelaxing, anti-
         proliferative, anti-ischaemic, anti-oxidant and anti-inflammatory
         properties .......................................................................................... 26

4. AIMS OF THE PROJECT ................................................................. ERRO! MARCADOR NÃO DEFINIDO.

5. RESULTS ................................................................................................................. 31
  5.1. CO inhalation prevents parasite accumulation/sequestration in the
       brain and neuroinflammation ................................................................. 31
  5.2. CORM-2 protects against ECM development ..................................... 36
  5.3. CORM-2 protective effects is dependent on CO release but does not
       involve COHb formation ........................................................................ 39
  5.4. CORM-2 prevents parasite accumulation/sequestration in the brain
       and neuroinflammation - CORM-2 mimics CO inhalation ............... 41
  5.5. CORM-2 changes the profile of intra-erythrocytic stages of P.berghei
       ANKA ........................................................................................................ 44
  5.6. CORM-2 does not alter reticulocytosis significantly ....................... 45
  5.7. CORM-2 treated mice show a reduced hematocrit and platelet volume
       ............................................................................................................. 47
  5.8. CORM-2 treatment increases the number of phagocytic cells in
       circulation .............................................................................................. 49
5.9. Spleens from CORM-2 treated mice have increased weights .......... 50
5.10. CORM-2 prevents loss of RBC deformability................................. 51

6. DISCUSSION................................................................................................. 53

7. CONCLUDING REMARKS AND FUTURE WORK ................................ 63

8. MATERIALS AND METHODS ....................................................................... 65
  8.1. Mice .......................................................................................................... 65
  8.2. Parasites, infection and disease assessment ........................................ 65
  8.3. CO exposure ............................................................................................ 65
  8.4. Visualization and quantification of bioluminescence in organs ex vivo ................................................................. 66
  8.5. Quantitative Real-Time PCR ................................................................. 66
  8.6. CO-releasing molecules administration .............................................. 67
  8.7. Quantification of $O_2$Hb, and MetHb and COHb ................................ 67
  8.8. BBB permeability ................................................................................ 68
  8.9. Flow cytometry analysis ....................................................................... 68
  8.10. Hematocrit and Hemogram ................................................................. 69
  8.11. RBC deformability .............................................................................. 70
  8.12. Statistical analysis ............................................................................... 70

9. REFERENCES .................................................................................................. 71
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Most of all, to my family, for being the dearest reason of my life.
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CCL5</td>
<td>CC ligand-5</td>
</tr>
<tr>
<td>CCR5</td>
<td>chemokine receptor-5</td>
</tr>
<tr>
<td>CM</td>
<td>cerebral malaria</td>
</tr>
<tr>
<td>COHb</td>
<td>carboxyhemoglobin</td>
</tr>
<tr>
<td>CXCR3</td>
<td>CXC chemokine receptor-3</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>experimental cerebral malaria</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>Hb</td>
<td>hemoglobin</td>
</tr>
<tr>
<td>HO-1</td>
<td>heme oxygenase-1</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenously</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide</td>
</tr>
<tr>
<td>IP-10</td>
<td>IFN-γ-inducible protein-10</td>
</tr>
<tr>
<td>iRBC</td>
<td>infected red blood cells</td>
</tr>
<tr>
<td>LPS</td>
<td>lypopolisaccharide</td>
</tr>
<tr>
<td>LT-α</td>
<td>lymphotoxin-α</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MetHb</td>
<td>methemoglobin</td>
</tr>
<tr>
<td>MIG</td>
<td>monokine induced by IFN-γ</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-κb</td>
<td>nuclear factor κ-light-chain-enhancer in B cells</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>O₂Hb</td>
<td>oxyhemoglobin</td>
</tr>
<tr>
<td>p.i.</td>
<td>post-infection</td>
</tr>
<tr>
<td>PbA</td>
<td><em>Plasmodium berghei</em> ANKA</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cells</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylate cyclase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular adhesion molecule-1</td>
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1. Resumo

A malária é uma das doenças infecciosas mais importantes em todo o mundo, afectando cerca de 200-300 milhões de pessoas todos os anos. A infecção por malária é causada por um protozoário do filo Apicomplexa do género Plasmodium. Estes parasitas possuem um ciclo de vida complexo que requer um mosquito Anopheles como vector e um hospedeiro vertebrado. Em mamíferos, o ciclo de vida do parasita inclui uma fase hepática, assintomática, seguida de uma fase eritrocítica, onde surgem os sintomas de malária e a patologia. Na fase eritrocítica, o parasita no estádio de merozoito, oriundo da fase hepática, entra na circulação sanguínea e invade um glóbulo vermelho (GV) onde se replica assexuadamente. O desenvolvimento intra-eritrocítico do parasita é constituído por vários estádios que incluem um forma inicial em anel, seguida de uma fase de trofozoito maduro que por sua vez se desenvolve em esquizonte. Durante a maturação do esquizonte ocorre um processo de reprodução assexuada designado esquizogenia através do qual são originados vários merozoitos, que levam à ruptura do glóbulo vermelho. Após a ruptura, os merozoitos libertados re-iniciam o ciclo intra-eritrocítico, invadindo novos glóbulos vermelhos. Este ciclo de ruptura e re-invasão é responsável pela hemólise e consequente anemia que se verifica durante uma infecção por malária.

Apesar da infecção por malária geralmente não originar um perfil clínico grave, alguns casos resultam em patologia severa, responsável pela morte de aproximadamente 1 milhão de pessoas anualmente. Em humanos, a malária severa ocorre sobretudo devido a infecções por Plasmodium falciparum. A malária cerebral (MC) é uma das formas mais letais de malária severa ainda sem tratamento e afectando sobretudo crianças com idades inferiores a 5 anos. A patogénese da MC é muito complexa, e os mecanismos que levam ao desenvolvimento da doença ainda são pouco conhecidos. No entanto, a patogénese da MC tem sido explicada por três processos principais: sequestração, inflamação e disfunção hemostática.

Uma melhor compreensão da patogénese da MC é fundamental para o desenvolvimento de novas estratégias terapêuticas contra este síndroma. O modelo de malária cerebral experimental (MCE) em murganhos C57BL/6 infectados com P. berghei ANKA (PbA), tem contribuído de forma decisiva para este conhecimento. Este
modelo apresenta várias características histopatológicas e imunológicas semelhantes à neuropatologia humana. No entanto, uma das principais diferenças é o tipo de células envolvido no processo de sequestração. Nos humanos, sabe-se que a sequestração de glóbulos vermelhos infectados (GVis) na microvasculatura do cérebro é um processo crucial para o desenvolvimento da patologia. Contudo, em murganhos, os leucócitos são as células que maioritariamente sequestram no cérebro. Esta observação tem lançado dúvidas sobre a importância da sequestração de GVis no cérebro no modelo de murganho, e contribuído para a controvérsia existente relativamente ao uso deste modelo como um modelo adequado ao estudo da MC. Desta forma, é importante compreender o papel da sequestração de glóbulos vermelhos infectados no cérebro e o seu papel no desenvolvimento da MCE em murganhos C57BL/6 infectados com PbA.

Para além do processo de sequestração, também a inflamação e a disfunção do sistema hemostático têm sido envolvidos na patogênese da MC. Pensa-se que uma resposta imune exacerbada contra o parasita poderá desencadear eventos patogênicos que se sobrepõem à função protectora do sistema imunitário, entre os quais a neuroinflamação será um processo crítico. Neste contexto, várias citocinas pró-inflamatórias têm sido implicadas na MC, como o factor de necrose tumoral-α (TNF-α), o interferon-γ (IFN-γ) e a linfotoxina-α (LT-α). A expressão no endotélio vascular cerebral de moléculas de adesão celular, como a molécula de adesão intercelular-1 (ICAM-1), parece também ser um processo importante para a patologia, mediando a sequestração de leucócitos e GVis no cérebro. Para além disto, o recrutamento de linfócitos T, em particular de linfócitos T CD8⁺, para a microvasculatura cerebral desempenha um papel crucial na patogênese da MCE. Nesse sentido, a expressão no cérebro de quimiocinas, i.e. moléculas quimiotácticas que dirigem a migração de leucócitos, tais como a proteína induzida pelo IFN-γ-10 (IP-10), foi demonstrada contribuir para o estabelecimento da patologia em murganhos. Por outro lado, pensa-se que o desequilíbrio hemostático também desempenhe um papel importante na patogênese da MC. A existência de um estado pro-coagulante e o potencial papel das plaquetas na sequestração de GVis e na congestão vascular parecem ser intervenientes importantes neste processo. A orquestração dos processos de sequestração, inflamação e desequilíbrio hemostático parecem culminar na lesão da barreira hemato-encefálica, um processo que parece decisivo na manifestação clínica da MC e cuja extensão poderá ditar sequelas neurológicas permanentes ou mesmo a morte.
O conhecimento mais aprofundado dos factores e mecanismos implicados na MC tem mostrado que várias moléculas do hospedeiro participam no desenvolvimento da doença, quer com uma acção patogénica, quer com uma função essencialmente protectora. Foi demonstrado recentemente que a expressão da heme oxigenase-1 (HO-1) do hospedeiro tem um papel preponderante em determinar a susceptibilidade à MC. De facto, foi provada uma importante acção protectora da enzima contra o desenvolvimento da MCE, que parece ser mediada sobretudo pela produção de monóxido de carbono (CO), um produto final da actividade da HO-1. Surpreendentemente, verificou-se que a administração de CO por inalação a murganchos C57BL/6 infectados com PbA, impedía o desenvolvimento de MCE em 100% dos casos. O efeito protector do CO manifestava-se na inibição da congestão microvascular cerebral, da neuroinflamação e do aumento da permeabilidade da barreira hemato-encefálica. A inibição da neuroinflamação reflectia-se na redução da expressão no cérebro de TNF-α, IFN-α, LT-α e ICAM-1 e inibição do recrutamento de células T CD8+ para o cérebro. O efeito terapêutico exercido pelo CO foi sugerido depender da supressão da oxidação da hemoglobina (Hb) em circulação, através da ligação do CO à Hb, formando carboxihemoglobina. Isto impediria a conversão da Hb em metahemoglobina e a libertação de heme, uma molécula com efeitos inflamatórios e oxidantes nocivos.

Neste trabalho pretendeu-se aprofundar o mecanismo protector do CO contra a MCE, em particular no que respeita ao seu efeito na sequestração de glóbulos vermelhos infectados no cérebro. Utilizou-se um método desenvolvido recentemente que permite revelar em tempo real a presença do parasita, por detecção de bioluminescência. Os murganchos foram infectados com um parasita PbA transgénico que expressa luciferase, o que permitiu a sua detecção nos órgãos por emissão de bioluminescência. Verificou-se que apesar do cérebro ser o órgão com menor acumulação de GVIs em murganchos C57BL/6 infectados com PbA, a presença de parasita neste órgão é nitidamente detectável por este sistema. De especial importância é o facto de se verificar que a acção protectora do CO parece estar associada com uma diminuição evidente da sequestração de GVIs no cérebro. Estes resultados, para além de indicarem que o mecanismo protector do CO inclui a redução da sequestração de parasita no cérebro, reforçam que o processo de sequestração cerebral de GVIs é fundamental para o
desenvolvimento da MCE, reforçando a relevância do modelo de murganho C57BL/6 infectado com PbA como um modelo muito semelhante à patologia humana e, por isso, adequado ao seu estudo. Adicionalmente, confirmou-se que o CO tem uma actividade anti-inflamatória e uma acção anti-quimiotáctica, uma vez que inibe a expressão de IP-10 no cérebro. Isto parece contribuir para uma menor migração de células T CD8+ para o cérebro, processo crucial para a patologia no murganho.

O segundo objectivo deste trabalho foi estudar a bio-actividade e o potencial terapêutico das moléculas libertadoras de CO (CO-RMs) no modelo experimental de malária cerebral. De facto, tem sido demonstrado que estas moléculas reproduzem os efeitos anti-oxidantes e anti-inflamatórios do CO em vários modelos de patologia, afigurando-se como potenciais novos fármacos. Uma das vantagens destas moléculas é a possibilidade de exercerem os efeitos benéficos do CO sem o perigo de toxicidade associado ao CO por inalação.

Surpreendentemente, observámos que a administração de CORM-2 com uma concentração de 20 mg/kg de peso corporal, por via intravenosa, duas vezes por dia, do dia 2 ao 3 após infecção, confere uma protecção de 100% contra o desenvolvimento de MCE. Adicionalmente, verificou-se que o CORM-2 não produz carboxihemoglobina, indicando que não induz a mesma toxicidade que o CO inalado, o que tem especial relevância para futuras aplicações terapêuticas. Tal como para o CO administrado por inalação, verificou-se uma diminuição nos níveis de metahemoglobina em circulação em murganhos tratados com CORM-2, reforçando o papel benéfico do CO no controlo do stress oxidativo causado pela infecção. Os resultados mostram também que o CORM-2 diminui a sequestração de Gvis no cérebro e a neuroinflamação em murganhos infectados com PbA, tal como anteriormente demonstrado para o tratamento com CO inalado. Adicionalmente, a administração de CORM-2 inibe a agregação plaquetária e a perda de deformabilidade eritrocitária durante a infecção. Estas observações são importantes porque durante a infecção o desequilíbrio do sistema de coagulação parece contribuir para a patogénese da MC e, por outro lado, a diminuição da deformabilidade eritrocitária representa um forte biomarcador de severidade em malária humana. O CORM-2 parece contribuir assim para o restabelecimento do equilíbrio hemostático no modelo C57BL/6-PbA de CM, e, deste modo, para o não desenvolvimento da MCE. Contrariamente ao que se observa com o CO inalado, o tratamento com CORM-2
mantém constante, durante os dias 5 e 8 após infecção, a percentagem de glóbulos vermelhos infectados. No entanto, a relevância desta observação para a protecção conferida pelo CORM-2 no desenvolvimento da MCE deverá ser melhor estudada em experiências futuras.

No seu conjunto, estes resultados indicam que as CO-RMs podem representar uma nova classe de compostos com potencial terapêutico na protecção do hospedeiro contra o desenvolvimento da malária cerebral.

**Palavras-chave:**
Malária Cerebral, CO, CO-RMs, *Plasmodium berghei* ANKA, Sequestração de glóbulos vermelhos infectados
2. Abstract

Malaria is a major infectious disease worldwide, causing ~1 million deaths each year due to severe complications, one of the most lethal being cerebral malaria (CM). Thereby, understanding CM pathogenesis is of vital importance for developing effective therapies against it.

The experimental cerebral malaria (ECM) model of C57BL/6 mice infected with *P. berghei* ANKA (PbA) shares many similarities with human CM. However, whereas in humans a critical event is sequestration of *Plasmodium*-infected red blood cells (iRBCs) in the brain microvasculature, in rodents is mostly leukocyte sequestration that occurs. Thereby, the pathologic significance of iRBCs brain sequestration during ECM is controversial and remains to be clarified.

Recently, it was shown that heme-oxygenase-1 (HO-1) plays a crucial role in protection against ECM, which appears to be mediated by carbon monoxide (CO) production, an end-product of its enzymatic activity. In fact, administration of CO by inhalation rescues all C57BL/6 PbA-infected mice from developing ECM.

The present study shows that CO protection comprises the reduction of iRBC brain sequestration in infected mice, supporting the importance of this process in ECM pathogenesis and the relevance of C57BL/6 PbA-infected mouse model to study CM. Moreover, our results indicate that CO has a therapeutic potential as a molecule with anti-inflammatory and anti-chemotaxis effects.

We also demonstrate that CORM-2, a CO-relasing molecule (CORM-2), mimics CO protection against ECM, suppressing neuroinflammation and parasite sequestration in the brain. Importantly, CORM-2 does not induce formation of carboxyhemoglobin, circumventing CO inhalation toxicity. Moreover, CORM-2 inhibits platelet aggregation and loss of RBC deformability, which likely contributes to prevent disease development. Additionally, CORM-2 also leads to an arrest in parasite load, which origin and relevance is not clear.
Altogether, these results indicate that CO-RMs seem to represent a novel class of drugs with therapeutic potential to protect the host from cerebral malaria.

**Key-words:**
Cerebral malaria, CO, CO-RMs, *Plasmodium berghei* ANKA, Sequestration of infected red blood cells
3. Introduction

3.1. Malaria: a worldwide burden
Malaria is one of the most important infectious diseases worldwide, affecting ~200-300 million individuals each year, over more than 100 countries, principally in sub-Saharan Africa (Fig. 1) (1). Along with the serious health problem, malaria has devastating economic consequences, standing as a main cause of poverty in many affected countries (2). Although most malaria infections only manifest as a relatively benign febrile illness, in many cases severe complications can arise from the disease, leading to nearly 1 million deaths every year, mainly among non-immune children under 5 years of age (1). Severe malaria comprises several clinical manifestations such as cerebral malaria, severe anemia, hypoglycemia and acute respiratory distress, often occurring in combination within the same patient (3).

3.2. Plasmodium life cycle in the mammalian host
Malaria infection is caused by apicomplexan protozoa of the genus Plasmodium. The parasites have a complex life cycle that requires two hosts: a mosquito vector and a vertebrate host, which can be a mammalian, a bird or a lizard, depending on the Plasmodium species. There are five parasite species reported to infect humans, namely P. falciparum, P. vivax, P. malariae, P. ovale and, most recently, P. knowlesi (4). P. falciparum is by far the most widespread and dangerous of all parasites, standing as the major responsible for severe illness and mortality associated with malaria (1, 3). The entire life cycle as well as the morphology of the different developmental stages is

Figure 1 Estimated incidence of malaria per 1000 population, on 2006 (adapted from WHO, 2008, ref. 1)
conserved between mammalian parasites, including those infecting rodents, namely *P. berghei*, *P. chabaudi*, *P. yoelli* and *P. vinckei*, which have been extensively used in experimental malaria models.

### 3.2.1. Liver stage - The exo-erythrocytic cycle

Malaria parasites are transmitted to a mammalian host through the bite of a female *Anopheles* spp. mosquito during a blood meal, inoculating the first infective form of the parasite, the sporozoite, into the host dermis. Further on, sporozoites move from the skin and enter the blood circulation, travelling readily to the liver where they will initiate asexual replication. In the liver, the sporozoites transverse several hepatocytes before invading and developing in a final hepatocyte. Between 2 to 6 days later, according to the *Plasmodium* species (for human *Plasmodium* species from 6-16 days later), the infected hepatocytes rupture and thousands of merozoites per invading sporozoite are released into the bloodstream. This stage is the so-called liver stage of the parasite and is totally asymptomatic ([Fig. 2](#)) (reviewed in ref. 5).

![Plasmodium life cycle in the human host](image-url)

**Figure 2** *Plasmodium* life cycle in the human host (adapted from an image from Malaria Unit at Instituto de Medicina Molecular)
3.2.2. Blood stage - The intra-erythrocytic cycle

In contrast to the clinically silent liver stage, the blood stage of infection is when the malaria-associated pathology occurs. Each merozoite released from the liver invades a red blood cell (RBC) where asexually replicates during 24-72h, depending on the *Plasmodium* species (human *Plasmodium* species have a replication cycle of 48-72h), to produce merozoites. The replication cycle ends with the burst of infected RBCs (iRBCs) and the release of merozoites, which in turn will infect other RBCs and initiate a new cycle of asexual replication (Fig. 2) (reviewed in ref. 6). The typical waves of fever and chills observed during a malaria infection correspond to the cycles of erythrocyte rupture and re-infection by the parasites. Some *Plasmodium* species show a preference for invading reticulocytes, i.e. immature RBCs, whether others invade both mature RBCs and reticulocytes. In humans, *P. vivax* and *P. ovale* predominantly infect reticulocytes, as well as *P. berghei* and *P. yoelli* in rodents. During the asexual intra-erythrocytic cycle, parasites mature through several developmental stages. Inside the RBC, the parasite first establishes as a ring-stage, surrounded by a vacuolar membrane (parasitophorous vacuolar membrane) created from the invagination of the RBC plasma membrane that occurs during the invasion step. The ring-stage is characterized by a large vacuole where hemoglobin starts to be digested to form hemozoin or the so-called “malarial pigment”, the characteristic brown pigment of *Plasmodium*. Parasites feed on host RBC hemoglobin to obtain free amino acids and iron. However, digestion generates a noxious by-product, the free heme. To overcome heme toxicity the parasite converts it into hemozoin, which is an inert crystal (7). The young ring parasite further grows into a trophozoite, which displays a different shape and a larger cell size, and where DNA replication begins. Next, the parasite enters the schizont stage where nuclear division occurs. During schizogony the parasite replicates its DNA and divides its nuclei several times, forming a syncytial cell. Finally, as the schizont matures, cytoplasm divides, the

![Figure 3 Asexual blood stages of *Plasmodium falciparum* (adapted from Coatney, G.R. *et al.* 1971, ref. 10).](image-url)
red blood cell rupture and 16 to 32 merozoites egress to invade fresh erythrocytes and perpetuate the cycle (Fig. 3) (reviewed in ref. 8, 9).

3.2.3. Sexual stage
The sexual life cycle of Plasmodium proceeds when some merozoites invade RBCs and undergo sexual differentiation (in response to stress or other stimuli) into gametocytes (Fig. 4), which can be taken up by mosquitoes during a blood meal. In the mosquito midgut, where fertilization occurs, the gametocytes fuse to produce a zygote denominated ookinete that develops into an oocyst in which thousands of sporozoites are formed. Finally, the sporozoites migrate into the mosquito’s salivary glands where they remain ready to be transmitted (Fig. 2) (reviewed in ref. 6).

3.3. Cerebral malaria (CM)
Cerebral malaria is the most severe neurological complication and one of the most life-threatening occurring from Plasmodium falciparum infection. CM affects mainly children and manifests clinically as motor and behaviour abnormalities, fever, consciousness impairment, convulsions and coma, ultimately leading to neurological sequelae or death (3). The usual treatment with anti-malaria drugs, like quinine and artemisin derivatives, is not sufficient to rescue CM patients from death or cognitive deficits. Along with anti-parasitic therapy, the patients of cerebral malaria must receive supportive treatment, such as exchange transfusion, ventilation support, treatment of renal failure, correction of acid-base imbalance and administration of anti-convulsants. These treatments are not always easily available in the sub-developed countries where malaria is endemic. Other adjunctive treatments have been suggested however none of them have shown proved benefits and some are reported as deleterious (reviewed in ref. 11, 12). Regardless of the efforts to improve CM treatment, there is still no successful therapy and the disease continues to exhibit high mortality and morbidity rates. The threat of this health problem is underlined by the observation that in endemic areas ~ 20% of children suffering from CM still succumb to the disease (13). A clear understanding of the mechanisms underlying CM pathogenesis is hampered by the complexity and multi-factorial...
dependence of the pathology. Besides the parasite transmission, virulence and drug resistance characteristics, many nutritional, genetic and immunological host factors have been implicated in the disease (14). In this way, despite the important insights already provided, many aspects underlying CM etiology remain unclear and subject of debate. Novel studies are crucial to shed light on the pathogenic mechanisms behind it and improve our knowledge of the disease in order to develop new therapeutic strategies that effectively combat it.

3.4. Experimental cerebral malaria (ECM): the benefit of rodent models

Studying CM pathology in humans is obviously not simple. Many of the studies rely on post-mortem analyses which do not provide insights into the full pathogenic events leading to the disease and hinder a direct correlation with clinical symptoms. Besides, the presence of other severe malaria complications or non-malaria pathologies often brings difficulties to the correct diagnosis of CM and the establishment of CM-specific associations. Therefore, experimental animal and in vitro models have been invaluable tools to study this pathology (reviewed in ref. 15). The most widely used and best characterized animal model of experimental cerebral malaria (ECM) is Plasmodium berghei ANKA (PbA) infection in C57BL/6 mice. In this ECM model 80-100% of C57BL/6 mice develop neurological signs identical to human CM (mono-, hemi-, para- or tetraplegia, tendency to roll over on stimulation, ataxia, convulsions and coma) and die between days 6 and 12 after inoculation with P. berghei ANKA iRBCs. Parasitemia, the percentage of iRBCs in circulation, is generally low at the time of death. ECM resistant strains of mice such as BALB/c mice are also used for comparative purposes. Only 0 to 20% of BALB/c mice infected with the same dose of P. berghei ANKA iRBCs develop ECM while the remaining BALB/c mice die 2 to 3 weeks after infection with severe anemia and hyperparasitemia but without neurological signs (16, 17).

The ECM model of C57BL/6 mice infected with P. berghei ANKA shares many similarities with the human pathology, including the referred neurological symptoms, development of brain petechial hemorrhages, edema, disruption of blood-brain barrier (BBB), systemic increase of pro-inflammatory cytokines, up-regulation of adhesion molecules in the brain endothelium, changes in the brain metabolism and establishment of a pro-coagulant state (18-20). The main distinction argued between the mouse and human CM is that whereas in humans are mainly iRBCs that sequester in the brain microvasculature and that this is a key event for the human disease, in rodents is mostly
leukocyte sequestration that takes place. Hence, the pathologic importance of iRBCs sequestration in the brain vasculature during ECM has long been a matter of controversy and remains to be clarified. This issue will be discussed further on.

3.5. Pathogenesis of CM: sequestration, inflammation and hemostasis dysfunction
Until know there are three main hypothesis to explain CM pathogenesis: the sequestration (or mechanical), the inflammation and the hemostasis hypothesis. Currently, it is accepted that these three main processes do not act independently in the development of CM but there is an intricate interplay between them, responsible for the complexity of the disease (21).

3.5.1. Sequestration of iRBCs
Since the first studies about severe malaria that the presence of infected and uninfected sequestered RBCs in the microvasculature of organs such as brain, heart, lungs, liver, kidney, spleen, intestines, adipose tissue and placenta has been a persistent histological finding in patients suffering from severe pathology (22-24). Given the constancy of this observation, erythrocyte sequestration is generally accepted as a hallmark and crucial event for the pathogenesis of severe malaria. The distribution of parasite sequestration in the organs varies and tends to reflect the clinical outcome of disease. Indeed, it has been reported that patients with CM show an increased RBC sequestration in the brain microvasculature, when compared with other organs (25). Sequestration of both non-infected and infected RBCs at later erythrocytic developmental stages, namely mature trophozoites and schizonts (22), occurs possibly via different possible mechanisms. The sequestration hypothesis, proposed as early as 1894 by Marchiafava and Bignami (26), establishes sequestration as the primary cause of severe malaria. According to it, the cytoadherence of iRBCs to endothelial cells (ECs) of capillaries and post-capillary venules results in blood flow obstruction that leads to petechial hemorrhages, metabolic dysfunction, hypoxia and deficient removal of waste products, such as lactate, giving rise to disease (reviewed in 27). In one hand, the iRBCs might adhere to the ECs via *P. falciparum* proteins expressed at the erythrocyte surface, principally through *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) (28). Antigenic variability of PfEMP-1 molecules is a major mechanism used by the parasite to evade host immune responses (reviewed in 29). PfEMP-1 binds to receptors up-regulated during infection in the host vascular endothelium, namely intercellular adhesion molecule-1 (ICAM-1),
vascular adhesion molecule-1 (VCAM-1), CD36, E-selectin, P-selectin, thrombospandin (TSP) or chondroitin sulphate A (CSA) carbohydrates, the last one particularly associated with placental malaria (reviewed in ref. 30, 31). On the other hand, sequestration can be amplified through the adhesion of iRBCs to other iRBCs (autoagglutination) or to non-infected RBCs (rosetting), or through platelets bound to iRBCs (platelet-mediated clumping) (32, 33). In addition to the iRBCs cytoadherence capacity, during a *P. falciparum* infection both infected and uninfected RBCs become less deformable (34) and consequently exhibit a higher tendency to plug vessels, further contributing to microcirculation obstruction (35). Cytoadherence of iRBCs is a process thought to confer survival advantage to parasite by providing a microaerophilic venous environment that is better suited for its maturation and by escaping clearance by the spleen, which recognizes iRBCs loss of deformability (34, 36) and opsonisation with antibodies and/or complement components (37).

### 3.5.2. Sequestration of iRBCs in the murine model

As stated before it is still a matter of debate whether the murine model closely resembles the pathogenesis of human CM, particularly in what concerns iRBCs sequestration in the brain microvasculature, a hallmark of CM. The sequestration phenomena appear to be a common denominator in human and murine CM, although the main cell type sequestered in the host organs differs between human and murine pathology. It has been shown that parasitized RBCs accumulate in the brains of different CM-susceptible mouse strains infected with *P. berghei* ANKA at the time of neurological symptoms (38-41). Nevertheless, leukocytes are the principal cell type consistently found to sequester in the mouse (38, 39, 42, 43) rather than iRBCs, as seen in humans (22). Given this fact, the pathological relevance of iRBC sequestration in the murine model has been discussed. Recently, Frank-Fayard and colleagues have investigated iRBC sequestration in the C57BL/6 murine model making use of a real-time *in vivo* imaging system and a transgenic luciferase-expressing *P. berghei* ANKA parasite. The work indicates that iRBC sequestration in mice is largely mediated by CD36, and occurs mainly in the lungs but also in the adipose tissue, however is negligible in the brain. The study also shows that parasite sequestration does not appear to correlate with pathology since CD36$^{-}$ mice develop ECM (44). On the contrary, other studies using the same mouse model and imaging system show an association between cerebral pathology and increased iRBCs accumulation in the brain (45, 46). Also, quantitative PCR data
suggests that significantly higher numbers of *P. berghei ANKA* parasites sequester in the brain and other organs of ECM-susceptible mice, when compared with the organs of resistant mice (15). Moreover, a very recent work by Baptista et al. strongly sustains that the concomitant presence of iRBCs and CD8+ T lymphocytes are a prerequisite for ECM onset in *P. berghei ANKA*-infected mice (47, submitted). CD8+ T cells are key mediators of ECM that will be considered in more detail further on. Nevertheless there is still no consensus, in the malaria research field, about the relevance of PbA iRBCs sequestration in ECM. Other parasite species and mouse strains have been suggested as alternative models to study iRBCs sequestration, although none of them shares as many similarities with human CM as the PbA infection in C57BL/6 mice model. One example is the lethal (17XL) strain of *P. yoelli* which induces ECM in Swiss mice. In this model, a prominent intravascular sequestration of iRBCs occurs but with few or any leukocyte accumulation in the brain, resembling human CM histopathology. However, this strain causes a virulent infection that gives rise to very high levels of parasitemia (48). Also, *P. chabaudi* AS iRBCs exhibit a significant cytoadherent capacity, sequestrating mainly in the liver and to a lesser extent in the spleen and brain of CBA mice. Nonetheless, the *P. chabaudi* AS infection self-resolves and do not develop into severe disease (49).

In fact, although iRBCs sequestration is widely accepted as a key feature of human CM, it has become evident that alone can not explain the pathogenesis of the disease. The sequestration process during *P. falciparum* is a common event in patients with severe illness or asymptomatic malaria. For this reason parasite sequestration in the tissues is not sufficient to induce a specific pathology (13). Indeed, some studies defend that brain sequestration does not occur in all patients who have succumbed from clinically diagnosed CM (50, 51) and, similarly to what is observed in mouse models, brain intravascular infiltrates with leukocytes and platelets have also been associated with human cerebral pathology (52-55). These findings approach mouse and human pathology and suggest other mechanisms underlying CM pathology apart from RBC sequestration.

### 3.5.3. Inflammation

Now, it is generally accepted that host immune responses also play an essential role in the malaria-associated pathology. According to the inflammation theory, the inflammatory processes triggered by infection are important for parasite control and
clearance; however, the immune system might set up an exacerbated inflammatory response that leads to multi-organ failure and death (reviewed in ref. 56). The inflammatory cascade is complex, probably including different players of the innate and adaptive immune system and the sequence of events is still far from being completely elucidated. Studies have been focused on identifying the crucial regulators of the inflammatory state responsible for CM pathology. These include pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), lymphotoxin-α (LT-α) and interferon-γ (IFN-γ), cellular adhesion molecules, like ICAM-1, and T lymphocytes, particularly CD8⁺ T lymphocytes, which have been closely associated with the pathogenesis of the disease and used as markers of murine cerebral pathology (reviewed in ref. 57).

3.5.3.1. Pro-inflammatory cytokines: tumor necrosis factor-α (TNF-α), lymphotoxin-α (LT-α) and interferon-γ (IFN-γ)

TNF-α is generally considered an essential element in the immunopathogenesis of CM. Increased levels of the cytokine in circulation and brain expression have been commonly associated with human and murine pathology (58-61). In the CBA murine model of CM, depletion of the cytokine prevents development of the disease (42). However, the abrogation of ECM development in TNF-α-LT-α double gene-knockout mice not only supports a role for TNF-α but also for LT-α, a molecule related to TNF-α family, in the pathogenesis of CM (62). In fact, LT-α⁻/⁻ mice infected with PbA were shown to be completely protected against ECM whereas TNF-α⁻/⁻ showed the same incidence of ECM as control C57BL/6 wild-type mice, suggesting that LT-α, and not TNF-α, is a principal cytokine mediator of pathology in this murine model (63). TNF receptor 2 (TNFR2) also appears crucial for the pathogenesis of the disease since TNFR2⁻/⁻ mice are significantly resistant to ECM development. The central role of TNFR2 would probably lie on the shared usage of this receptor by TNF-α and LT-α (64). Recently, also LT-α signalling mediated by lymphotoxin-β receptor (LTβR) has been shown to be an essential pathway in ECM (65, 66).

Other pro-inflammatory cytokine, IFN-γ, has been considered a key mediator in CM pathogenesis. Elevated concentrations of IFN-γ are also observed in circulation during human acute malaria (67, 68) and a recent study reports that heterozygotes for an IFN-γ receptor (IFN-γR) polymorphism had lower incidence and mortality from CM (69). The central role of IFN-γ in the cerebral pathology is strongly corroborated in the murine
3.5.3.2. Leukocyte accumulation in the brain: the importance of CD8$^+$ T cells

As aforementioned, brain accumulation of leukocytes occurs in both murine and human CM although leukocytes accumulate more than RBCs in the murine model. During ECM, brain intravascular leukocytes are mainly constituted of monocytes/macrophages, neutrophils and T lymphocytes (38, 39, 41, 72). Several studies using neutralising antibodies and T-cell deficient mice, demonstrated the requirement of CD4$^+$ and CD8$^+$ T cells for the cerebral pathology of *P. berghei* ANKA-infected mice, among which CD8$^+$ T cells were proved to play a central role (71-77). There is a selective increase in brain CD8$^+$ T cells in mice that developed ECM compared with mice that do not develop ECM. Moreover antibody depletion of CD8$^+$ T cells on the day before the development of neurological symptoms totally abrogates ECM (72, 74). The crucial involvement of CD8$^+$ T cells as direct effectors in ECM pathology is further supported by the observation that their recruitment to the brain just precedes the onset of neurological signs, demonstrating their pivotal role on the late stages of the disease (78). These results were corroborated by adoptive transfer experiments where spleen CD8$^+$ T cells, since the spleen is the main organ of CD8$^+$ T cell activation, of mice with ECM, adoptively transferred to T-cell deficient mice (RAG-2 deficient mice) were found to migrate to the brain and lead to ECM (76). CD8$^+$ T cells are essential effector cells of the adaptive immune system directed for killing of cells invaded by pathogens or malignant cells. Still, these cells have to be tightly regulated in a way to prevent immunopathogenesis. Thus, CD8$^+$ T cell-mediated apoptosis of neurovascular ECs and consequent BBB damage is thought to underlie the role of these cells during ECM (78). Granzyme B and perforin are molecules produced by activated CD8$^+$ T cells that mediate their cytotoxic action by inducing apoptosis through caspase activation. The pathogenic significance of EC killing by activated CD8$^+$ T cells is strongly supported by the observation that these molecules are highly up-regulated in the brain during ECM and that perforin-deficient mice do not develop the pathology (76, 78, 80). Of special importance is a recent work showing that, albeit the importance of CD8$^+$ T cells in ECM pathology, these cells are not sufficient per se to induce ECM unless iRBCs are also present in the brain microvasculature. Thereby, the simultaneous recruitment of both cells to the brain is required for the murine cerebral pathology to occur (47, submitted). As mentioned before, also CD4$^+$ T cells have been...
shown to participate in ECM pathogenesis of *P. berghei* ANKA-infected mice. However, these cells seem to be involved at an induction phase of the disease rather than exerting a direct action as CD8\(^+\) T cells (72, 73, 75). Besides T lymphocytes, also monocytes/macrophages and neutrophils accumulate in the brain microvasculature during ECM. These phagocytic cells are part of the innate immune response and seem more implicated in the early establishment and amplification of the inflammatory state, both systemically and locally in the brain, by producing high levels of pro-inflammatory cytokines, such as IFN-\(\gamma\) and TNF-\(\alpha\), and secreting chemokines, i.e. chemotactic molecules that trigger leukocyte migration, like CC ligand-5 (CCL5), IFN-\(\gamma\)-inducible protein-10 (IP-10) and monokine induced by IFN-\(\gamma\) (MIG). Indeed, depletion of macrophages or neutrophils early in infection prevents the development of ECM, down-regulating the expression of pro-inflammatory cytokines in the brain and markedly decreasing the sequestration of leukocytes (81, 82).

In fact, due to their chemotaxis function of leukocytes, chemokines have been investigated as possible mediators in CM pathogenesis. Mice deficient in the CC chemokine receptor-5 (CCR5), the receptor of CCL5, have been reported to be significantly resistant against ECM development or exhibit a delayed onset of the pathology, probably due to impaired CD8\(^+\) T cell trafficking to the brain (76, 83). Recent studies have drawn more attention over CXC chemokine receptor-3 (CXCR3) and its ligands IP-10 and MIG. These chemokines are up-regulated in response to PbA infection and mice knock-out for CXC3, IP-10 or MIG genes are significantly protected against disease, clearly implicating these molecules on ECM pathogenesis. Moreover, splenic T cells from CM susceptible mice but not resistant mice, induce the expression of CXCR3, indicating a correlation between CXCR3 expression and disease. In fact, the pathogenic contribute of CXCR3 during ECM was correlated with lymphocyte recruitment since CXCR3 deficient mice show a marked impairment of CD8\(^+\) T cell migration to the brain (46, 84-86). Importantly, IP-10 was found to be a biomarker of CM-associated mortality in *P. falciparum* malaria (87, 88). These studies suggest that chemotactic factors such as IP-10, and their respective receptors, play an important role in cerebral pathogenesis, at least in part by mediating CD8\(^+\) T cell trafficking to the brain.

3.5.3.3. Adhesion molecules: intercellular adhesion molecule-1 (ICAM-1)

Other key event that appears crucial to CM pathogenesis is the stimulation of a pro-adhesive phenotype in the vascular endothelium, through up-regulation of adhesion
molecules. Pro-inflammatory cytokines such as TNF-α, LT-α and IFN-γ, induced during infection, can activate the vascular endothelium inducing the expression of adhesion molecules such as ICAM-1, VCAM-1 and P-selectin. This in turn promotes sequestration of parasitized erythrocytes and leukocytes to brain microvasculature, which likely contributes to neurovascular endothelial damage and BBB derangement (65, 71, 89, 90). ICAM-1 has been an adhesion molecule strongly associated with CM pathogenesis. An high up-regulation of the molecule in the brain correlates with cerebral pathology in humans and rodents (91-93) and ICAM-1−/− mice were shown to be fully protected against the pathology (94).

3.5.4. Hemostasis dysregulation
Besides iRBC sequestration and inflammation, dysfunction of hemostasis system is another process argued to significantly contribute to CM pathogenesis. Supporting this hypothesis is the persistent observation that patients suffering from P. falciparum malaria exhibit dysregulation of the coagulation system including prolonged bleeding times and prothrombin times, decreased levels of anti-coagulants, platelet hyperaggregability, thrombocytopenia (low levels of circulating platelets), generation of pro-coagulant microparticles and occurrence of microthrombi and haemorrhages in brain vessels (95, 96). Several evidences during murine and human CM indicate an important role for platelets, major players of the hemostasis system, in the microvascular injury occurring during pathology (reviewed in ref. 97). Platelets can cooperate with iRBC to amplify sequestration by mediating iRBCs clumping, i.e. aggregation, and iRBCs adhesion to brain endothelium (33, 98). As a consequence, vascular obstruction and damage to brain ECs would be enhanced. In fact, the thrombocytopenia observed during severe malaria and particularly in CM is hypothesized to be a host protective mechanism against platelet-mediated clumping of iRBCs (99). On the other hand, platelets are also immune effector cells, that could augment the pro-inflammatory environment associated with CM. A recent study supporting such an immuno-modulatory role for platelets during pathology, demonstrates that platelet activation by iRBCs induces the production of platelet factor 4 (PF4), a platelet-derived chemokine, which acts as an important immune activator and mediator of T cell recruitment to the brain during ECM (100). In addition, microparticles, other elements of the hemostasis system, have been suggested as relevant effectors in the pathogenesis of the disease. Microparticles are submicrometer elements resulting from the shedding of the plasma membrane from various cell types,
which circulate in the peripheral blood and have been implicated in thrombosis, inflammation and vascular activation (20, 101).

3.5.5. The integration of events: blood-brain barrier (BBB) damage

The integrated action of the pathogenic mechanisms of sequestration, inflammation and hemostasis deregulation is generally accepted to culminate in the dysfunction and, ultimately the breakdown of BBB, during both murine and human CM (39, 102, 103). BBB is a highly specialised interface between the intravascular space and the central nervous system that function as a selective diffusion barrier which tightly controls molecular and cellular trafficking into the brain, maintaining homeostasis. The structural and functional integrity of BBB depends on endothelial cells (ECs) with characteristic tight and adherens cell junctions, astrocytes, pericytes, perivascular macrophages, neurons and microglia i.e. resident macrophages in the brain (104). During cerebral pathology, activation of microglia, damage and apoptosis of astrocytes appear as crucial events in BBB disruption (105-109). Moreover, injury and apoptosis of microvasculature ECs were also implicated in the BBB damage, and associated with CD8\(^+\) T cell perforin-mediated cytotoxicity, as already mentioned (76, 79, 80, 110). Additionally, CD8\(^+\) T cells can also impair BBB tight junctions without inducing apoptosis, though in a perforin-dependent manner (111). Furthermore, neuronal apoptosis, which is observed during ECM, is likely to play a role in human CM and mediate the irreversible neurological sequelae that can occur in non-lethal CM cases (112, 113).

In summary, although the precise sequence of events leading to CM is unknown, we know now many events involved in the pathology that allow us a more extended view of the mechanisms that lead to CM. The comprehensive knowledge of these processes is fundamental to discover new drug targets and develop novel treatment strategies against the pathology. Scientific work has made evident the involvement of different host factors in the progression of CM and keeps extending the range of cells and host molecules implicated either in pathogenesis or protection against the disease. In this way, recently, heme-oxygenase-1 (HO-1) and carbon monoxide (CO), one of its products, were identified as molecules playing a critical role in the host protection against CM in mice (114).
3.6. Heme-oxygenase-1 (HO-1) and carbon monoxide (CO)

Heme-oxygenase (HO) is the rate limiting enzyme in the catabolism of free heme (iron protoporphyrin IX). HO breaks down the porphyrin ring of heme rendering equimolar amounts of biliverdin, free iron (Fe$^{2+}$) and carbon monoxide (CO) (115). In mammals, biliverdin is rapidly converted by biliverdin reductase into bilirubin (116). Heme is a prosthetic group of many proteins, for this reason called hemoproteins, such as hemoglobin (Hb), cytochromes, peroxidases (117), as well as cyclooxygenase-2 (COX-2) (118), inducible nitric oxide synthase (iNOS) (119) and indoleamine 2,3-dioxygenase (IDO) (120), which are likely implicated in host protective response against severe malaria. Besides, heme is also directly involved in many cellular processes such as gene modulation, cell differentiation, cell proliferation and immune stimulation. However, when excessive concentrations of heme are present in the free state, this leads to oxidative damage of cells and tissues (reviewed in 117). Three isoforms of HO enzyme have been identified, respectively HO-1, HO-2 and HO-3, codified by different genes with different expression patterns. While HO-3 expression profile is still not characterized, HO-2 expresses constitutively and HO-1 expression is inducible (121, 122). HO-1 expression is up-regulated as part of the host protective response against stress conditions, and can be triggered by a wide range of agents particularly those that increase oxidative stress such as heavy metals, bacterial lipopolysaccharides (LPS), hypoxia, hyperoxia, heat shock, ischemia, UV radiation, H$_2$O$_2$, cytokines, nitric oxide (NO), and its substrate heme. HO-1 not only plays a role in important biological functions such as vasodilatation and neurotransmission, but also exerts a remarkable cytoprotective action in different pathological conditions due to its anti-inflammatory, anti-apoptotic, anti-oxidative and anti-proliferative effects. Such activities result both from degradation of excessive free heme by the enzyme and from the biological actions of the three products generated (reviewed in ref. 123). From these products, CO is the best studied and the one that more closely mimics HO-1 effects when administered exogenously, sustaining the notion that HO-1 protective actions are principally mediated by CO production (117). The biological significance of the molecule is reinforced by the observation that CO functions as a signalling mediator in important physiological processes such as vasorelaxation and neurotransmission (124). Similarly to HO-1, CO anti-apoptotic, anti-proliferative, anti-oxidative and anti-inflammatory properties and capacity to inhibit platelet aggregation have been associated with its ability to ameliorate cardiac, lung, liver and vascular injuries, including protection against experimental allo- and xenotransplantation.
rejection, oxidative damage, ischemic injury, thrombosis and sepsis (125-134). Although the direct molecular targets of CO responsible for its bioactivity remain to be indentified, its effects are known to be mediated by at least two major pathways: the soluble guanylate cyclase (sGC)/cyclic guanosine monophosphate (cGMP) system and mitogen-activated protein kinases (MAPKs) (123). Other molecules seem to be involved in CO signalling like stress-responsive transcription factors including nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), peroxisome proliferator-activated receptor-γ (PPAR-γ) and hypoxia-inducible factor-1α (HIF-1α). Also, the affinity of CO for metal atoms supports that metalloproteins could act as CO biological sensors, in particular heme-containing proteins such as hemoglobin, myoglobin, sGC, COX-2, cytochromes, iNOS and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (123, 124). Recently, also CO-induced production of reactive oxygen species (ROS) has been defended to mediate CO signalling (135). The CO pleiotropic effects thus rely on modulation of different pathways, depending on tissue and cell types and the biological effect exerted.

3.6.1. Pivotal role of HO-1 and CO in the protection against ECM

Reinforcing the function of HO-1/CO system as a host defence against pathological conditions, a recent work by Pamplona and colleagues (114), revealed that HO-1 plays a crucial role in the control of CM progression in *P.berghei* ANKA-infected mice. Upon infection, the enzyme is up-regulated at lower levels in the ECM-susceptible strain of C57BL/6 mice, which develop ECM, compared with the ECM-resistant strain of BALB/c mice that do not develop the pathology. The involvement of HO-1 in the susceptibility to ECM was further corroborated by showing that BALB/c resistance to disease was lost by the majority of mice due to inhibition of HO-1 activity or HO-1 deficiency. HO-1 protective effect appears to be mediated by CO production since exogenous administration of CO, by inhalation, rescues 100% of C57BL/6 infected mice from developing ECM, whereas biliverdin treatment failed to do so. The protection against ECM development conferred by HO-1 induction and CO exposure was not related with parasitemia alterations, but rather with abolishment of brain microvasculature congestion with leukocytes and RBCs, brain hemorrhages and prevention of BBB disruption. Importantly, the CO protective effect correlated with inhibition of neuroinflammation in the C57BL/6-infected mice, verified by reduction of brain expression of TNF-γ, LT-α, ICAM-1 and VCAM-1 and prevention of activated CD8+ T cell recruitment to the brain (114). The HO-1/CO
protective roles during ECM were shown to rely, at least in part, on reducing free heme in circulation. During a malaria infection extensive hemolysis occurs, due to rupture of iRBCs as a consequence of parasite replication, and to decreased life-span of uninfected RBCs (136). This originates the release of considerable amounts of hemoglobin (Hb) into circulation. In the presence of ROS, hemoglobin, HbFe$^{2+}$, is readily oxidized to methemoglobin (MetHb), HbFe$^{3+}$, which is highly unstable and promptly releases free heme. Thereby, the homeostasis of these molecules must be tightly regulated by the organism in order to protect cells and tissues from heme-induced inflammation and oxidative damage. Mammals possess several defense mechanisms to maintain Hb/MetHb/heme homeostasis including the RBC MetHb reductase system, HOs and the scavengers haptoglobin, hemopexin and albumin (137, 138). However, in situations of hemolysis, such as observed during a malaria infection, the physiological mechanisms of defence may become exhausted and insufficient to avoid excessive free heme release. In line with this, a significant increase in free heme in the plasma was shown to correlate with ECM onset in C57BL/5 PbA-infected mice, whereas BALB/c PbA-infected mice or C57BL/6 P.berghei NK65-infected mice, which do not developed ECM, had significantly lower levels of free heme in circulation (114). Moreover, CO protection against ECM was shown to be mediated by inhibition of free heme generation since C57BL/6 P. berghei ANKA-infected mice protected by CO exposure have reduced free heme in the plasma and, reversely, CO protection against ECM can be eliminated by heme administration in these mice. Importantly, it was also shown that MetHb plasm levels were significantly increased in ECM-developing C57BL/6 infected mice, while CO protected mice had MetHb levels similar to those observed in uninfected mice. Altogether, the results point towards an essential role of free heme in the pathogenesis of ECM. Moreover, the protective action of HO-1/CO system against pathology does not target the parasite but is hypothesized to result, at least partially, by preventing Hb oxidation to MetHb and subsequent heme release. This is probably attained through the CO binding to Hb, producing carboxyhemoglobin (COHb) (114, 138). Noteworthy, CO is also able to inhibit CD8$^+$ T cell recruitment to the brain, preventing neuroinflammation and BBB disruption. The interdependence of these events is not established but together, they seem to underlie the protective action of CO against CM in the murine model (114). These results give an important contribution for the better understanding of CM pathogenesis, which could lead to the development of novel therapeutic strategies, possibly by targeting the Hb/MetHb/heme system. Moreover, an important implication
from these observations is the hypothesis that measurement of free heme or MetHb in the blood could serve as a prognostic tool of CM (138). Additionally, an interesting link between CO and NO, another gaseous endogenous messenger, could be drawn, supporting the involvement of cell-free Hb in CM pathogenesis. NO and CO share many similarities including vasodilation, neurotransmission, anti-inflammatory properties (139). Evidences suggesting a protective function of NO in CM further close the two molecules. Actually, despite the fact that NO is produced as part of the inflammatory response against the parasite, low, rather than high NO bioavailability is associated with human severe malaria and with ECM onset in the C57BL/6 Pba-infected mice (140, 141). Strikingly similar to CO, exogenous NO administration markedly protected mice against disease development, without changes in parasitemia, by decreasing pro-inflammatory markers in the blood, BBB permeability and brain hemorrhages (141). NO low bioavailability was presumed to result from hypoargininemia (deficiency in arginine, the substrate for NO synthesis by nitric oxide synthase, NOS), by low blood and RBC nitrite production and, importantly, by the increase of free Hb in the blood, which scavanges NO (141). What is more, a significant correlation between cell-free Hb, low NO bioavailability, endothelial dysfunction and inflammation was also established for severe malaria in humans (142). It is possible that CO, by binding to Hb, prevents NO scavenging and this in turn would lead to increased NO bioavailability, and thus contribute to suppression of ECM. On the other side, NO is a potent inducer of HO-1 and thereby its protective action against disease might be mediated by stimulating CO production (114).

Besides prevention against ECM, HO-1/CO protective role was also demonstrated by our lab in another model of severe malaria. The *P. berghei*-ANKA-infected DBA/2 mice were shown to constitute a good model of malaria-associated acute respiratory distress syndrome (MARDS). During MARDS, lung injury is a central event, sharing histopathological similarities with BBB disruption during ECM, including increased permeability, hemorrhages and edema. Remarkably, HO-1 induction and CO exposure totally prevented MARDS incidence. Moreover, CO treatment blocked the increase in vascular endothelial growth factor (VEGF) in circulation, which appears crucial to the establishment of the respiratory syndrome (143, submitted).

Interestingly, although HO-1 and its product CO, have a pivotal role in preventing malaria pathology during the blood stage of infection, a work by our lab revealed that the anti-inflammatory activity of HO-1 and CO promotes the liver stage of infection. In fact,
Plasmodium sporozoite infection on the liver induces HO-1 expression, which in turn down-regulates the inflammatory response, protecting the infected hepatocytes, and leading to increased parasite loads in the liver (144). This suggests that HO-1 expression can play different regulatory roles during a malaria infection, depending on the tissue and parasite stage.

Discovery of CO signalling properties surprised the scientific community and attracted attention for a molecule formerly regarded as physiological insignificant and an air pollutant. In fact, CO was best known for its strong toxicity when present in high concentrations, by virtue of CO ability to tightly bind to the heme-dependent proteins haemoglobin (forming COHb), myoglobin and cytochromes, blocking oxygen delivery and leading to tissue hypoxia, ultimately causing death (145). However, it should be underlined that CO beneficial effects have been experimentally obtained in mice at concentrations (250 p.p.m, parts per million) far below its toxic levels (123).

In this way, while more studies to disclose the CO protective mechanisms are imperative, namely in the context of protection against severe malaria, a potential application of CO as a therapeutic agent, at low doses, has being increasingly predicted.

3.7. CO-releasing molecules (CO-RMs)

Indeed, the discovery that CO plays a significant role in life processes together with the observation that exogenous administration of CO gas could mimic the cytoprotective effects induced by HO-1 stimulation, prompted scientists to search for strategies aiming at CO application as a pharmacological agent. Major problems emerging from the potential use of CO gas therapy are the dangerous effects associated with its toxicity besides the fact that CO cellular targets responsible for its cytoprotective effects are still poorly known. Recently, to overcome these problems, the majority of research has been dedicated to the so-called CO-releasing molecules (CO-RMs), i.e. molecules that could carry and deliver CO in a more specific and controllable manner to cells and tissues, exerting the same protective effects of gaseous CO but overcoming CO toxicity. On the other hand, due to their more specific CO release, for instance through ligand substitution, these compounds would serve as important tools to identify the intracellular targets of CO and clarify the mechanism responsible for its bioactivity (148).

Transition metal carbonyl complexes, constituted by CO groups bounded to a transition metal centre, have been emerging as promising CO-releasing molecules (CO-RMs) (149), mimicking CO beneficial effects in different experimental models of pathology (148, 150). CO-releasing molecule-2 (CORM-2) i.e. tricarbonyldichlororuthenium (II) dimer ([Ru(CO)$_3$Cl$_2$]$_2$) is one of the compounds most studied. This molecule is constituted by two monomers, each with a ruthenium centre bounded to three carbonyl groups, linked by two chlorine atoms. CORM-2 only solubilises on organic solvents, and dimethyl sulfoxide (DMSO) has been the usual vehicle used to test the compound. During solubilisation, DMSO acts as a coordinated ligand to ruthenium, consequently promoting the separation of the monomers and CO liberation (151). Usually, the CO-dependent activity of CORM-2 is validated by comparison with the negative control compound, at equimolar concentrations. This compound contains a similar ruthenium centre but the does not contain carbonyl groups and thereby cannot release CO. In the first study characterizing CO-RMs properties, namely that of CORM-2, it was shown in vitro that the compound dissolved in DMSO acts as a fast CO-releaser (half-life $\approx 1$ min.) once in contact with heme-dependent proteins such as myoglobin. CO dissociation occurs by ligand substitution triggered by heme-iron, to which CO has strong affinity. Indeed, CORM-2 rapidly converts deoxymyoglobin to carbonmonoxy myoglobin, implying that CO is effectively liberated from the metal complex. CO liberation is concentration-dependent, each mole of CORM-2 solubilised in DMSO being estimated to release approximately 0.7 moles of CO (151). The study also assessed the biological properties of the compound, demonstrating in ex-vivo rat aortae that CORM-2 exerts a potent vasodilator activity and, in accordance, significantly attenuates acute hypertension in vivo in the rat model. Importantly, the fact that the negative control compound failed to promote the same vasorelaxing effects, sustained the involvement of CO in the bioactivity of CORM-2. The vasodilator action was mediated by activation of sGC/cGMP pathway, thus reproducing the vasorelaxing action of endogenous HO-1-derived CO. Noteworthy, no
differences in oxyhemoglobin (O$_2$Hb) saturation resulted from CORM-2 administration, indicating that the compound does not induce CO poisoning (151). These results encouraged the idea that CO-RMs could be potentially used as pharmacological agents in the treatment of pathological states where HO-1/CO system plays a beneficial role. Since then, CO-RMs have been proven to mimic the HO-1/CO vasodilator, anti-proliferative, anti-ischemic, anti-oxidant and anti-inflammatory bioactive properties and counteract diverse pathological conditions (148-150). CORM-2 vasorelaxing effect was already proven beneficial against hypertension in vivo (152). Furthermore, Kooli et al. demonstrated that the compound reproduces the HO modulation of vascular tone in the brain, exerting a significant sGC-dependent vasodilation action in the rat cerebral microvasculature (153). CORM-2 was also shown to prevent oxidative injury in rat cerebellar neurons treated with 3-nitropropionic acid, a toxin used to mimic oxidative stress-associated neurodegenerative pathologies. CORM-2 treated neurons are protected against ROS production and cell death induced by this toxin (154). Other studies report important cardioprotective effects of the CO-donor, particularly associated with the anti-oxidant and anti-inflammatory activities of CORM-2. On study shows CORM-2 prevents oxidative stress-induced response in vascular smooth muscle cells exposed to heme, suppressing the up-regulation of early growth response–1 (Egr-1). This molecule is a key regulator of cell proliferation and apoptosis suggested to act as an important trigger of inflammatory events involved in the progression of pathologies like atherosclerosis (155). CORM-2 was also observed to protect against oxidative injury induced by H$_2$O$_2$ in endothelial cells (156). Moreover, Importantly, CORM-2 rescues HO-1 deficient mice from vascular arterial thrombosis during murine aortic allotransplantation, preventing platelet aggregation and thrombi formation in the graft, reinforcing the capacity of the compound to mimic the HO-1-derived CO action (157). CORM-2 has also been shown to exert beneficial effects on renal pathophysiology. The compound significantly attenuates vasoconstriction of renal afferent arterioles, following NO synthase suppression, and thus might provide a protective mechanism of renal microcirculation during stress conditions where NO system is compromised, for instance during endothelial dysfunction (158). Furthermore, CORM-2 treatment decreases the levels of plasma creatinine and restricts renal injury in a mouse model of ischemia-induced acute renal failure (159). Noteworthy, several recent in vitro and in vivo studies have proved important anti-inflammatory actions of CORM-2. Treatment with CORM-2 decreases the inflammatory response in lipopolysaccharide (LPS)-activated murine
macrophages, markedly reducing the production of TNF-α, IL-1β and IFN-β and inhibiting iNOS and NO production in these cells (160-163). The reported anti-inflammatory effects of CORM-2 also include marked reduction of ROS formation in activated macrophages and neutrophils (164). Additionally, CORM-2 treatment prevents peroxynitrite-induced apoptosis (165). These findings are consistent with the known CO protective action against nitrosative and oxidative stress. Studies in human neutrophils and guinea pig mast cells also suggest that CORM-2 could be used in the treatment of allergic disease since the compound significantly attenuates the immunological release of histamine and the expression of CD203c, an activation marker of these cells (166). Importantly, the anti-inflammatory action of CORM-2 is extended to microglia, where the compound mimics the protective action of HO-1, induced by astrocytes as a response against brain inflammation. It was shown in rat microglia that the CO-donor attenuates the IFN-γ-elicited neuroinflammatory response, reducing nitrite formation (167). Likewise, CORM-2 prevents the inflammatory response induced by cytokines in human intestinal epithelial cells, down-regulating genes involved in intestinal inflammation and cancer progression, namely iNOS, IL-6, IL-8 and matrix metalloproteinase-7 (MMP-7) (168). Moreover, CORM-2 was shown to attenuate the pro-inflammatory response in LPS-stimulated human umbilical vein endothelial cells, reducing polymorphonuclear (PMN) leukocyte adhesion, ROS and NO production, and ICAM-1 and iNOS expression in these cells (169). Is of significant relevance that the anti-inflammatory properties of CORM-2 molecule have been confirmed in vivo using diverse models of inflammation-associated pathologies. In a mouse model of thermal injury, CORM-2 administered intravenously (i.v.) clearly inhibited the inflammatory response in the liver. Hepatocyte injury, accumulation and adhesion of PMN leukocytes to the ECs of liver sinusoids were significantly diminished in CORM-2 treated mice. The anti-inflammatory effect was mediated by a significant decrease of TNF-α and IL-1β levels and down-regulation of iNOS and EC expression of VCAM-1 and ICAM-1 adhesion molecules (170, 171). A similar anti-inflammatory effect of the CO donor has been verified in the lung, intestine and renal tissue of models of thermal or ischemia injury (172-175). Notably, CORM-2 was also shown to counteract in vivo the overwhelming systemic inflammatory response that occurs during sepsis (169). In fact, mice receiving CORM-2 treatment were clearly protected from lethal sepsis, exhibiting improved survival rates (160). Recently, CORM-2 was also confirmed to prevent inflammation and cartilage degradation in chondrocytes isolated from osteoarthritis patients, highlighting CORM-2 as a potential new drug
candidate against osteoarthritis pathogenesis (176, 177). CORM-2 anti-inflammatory beneficial effects carry on to skin protection. The topic application of skin lotion containing CORM-2 protects mice against the immunosuppressive effects of solar-simulated UV radiation and acts as an important anti-photocarcinogenic agent (178, 179). Besides the remarkable findings regarding CORM-2 action on mammalians, a recent study also extends CORM-2 activity against pathogenic microorganisms. In fact, this CO donor acts as a strong anti-microbial drug against the pathogenic bacteria Escherichia coli and Staphylococcus aureus. These authors suggest CO-RMs as a novel class of anti-microbial molecules, which could be in the future used as new therapeutic agents to combat antibiotic-resistant pathogens (180).

Although the beneficial effects of CO-RMs, and CORM-2 in particular, have been broadly confirmed, there are aspects that should be taken into consideration regarding a future pharmacological application of these compounds. In the case of CORM-2, there are studies reporting undesirable effects of the molecule, namely the activation of non-selective cation channels independently of CO release, and alterations of axonal conduction in spinal cord tissue (181, 182). Importantly, the molecule is insoluble in water and, similarly to other CO-RMs, its specific targets are poorly known. Therefore, considerable interest has been put on improving and designing new CO-RMs to overcome these drawbacks and enhance therapeutic applicability. CORM-3, tricarbonylchloro(glycinato)ruthenium II (Ru[CO]₃Cl(glycinate)) is a good example of a new and promising CO-RM, which already bears the advantage of water solubility due to its glycine group. Similarly to CORM-2, CORM-3 beneficial vasodilatory, anti-ischemic, anti-inflammatory and anti-microbial effects have been reported (148, 150, 180). Importantly, a new improved version of CORM-2 has also been synthesized, where solubility in water has been achieved through the coordination of an amino acid group, likewise to CORM-3. Bearing in mind the remarkable biological effects of CORM-2 we can envisage the high potentialities of this new CORM-2 improved compound, however, its biological properties have only now started to be investigated. Much work still needs to be conducted in order to unveil the mechanisms behind CO-RMs beneficial action. While the typical CO targets such as heme-containing sGC appear as important mediators, emerging evidences using CO-RMs more controlled delivery of CO, support an interaction of the gas with metal centres, specially transition metal moieties, of heme- or other metal-dependent proteins (183). CO-RMs research also adds further support regarding the downstream regulation of MAPKs signalling and transcription factors such
as NF-κB as crucial intervenient on the CO pleiotropic effects (184). Albeit these findings, the CO cellular targets remain largely unknown, and is reasonable to admit several hypothesis for the CO-effector mechanisms, if one takes into account the wide bioactivities of the molecule. Also, pharmakocinetic studies and data on CO-RMs tissue distribution and elimination are still expected. Despite that, research on these molecules has notably expanded in the past years, becoming clear that CO-RMs offer an attracting opportunity to exploit CO beneficial effects for therapeutic use. Indeed, the exciting results on CO-RMs biology, namely with CORM-2, anticipate these molecules as a new generation of pharmaceuticals against vascular and inflammatory diseases (148, 150).

4. Aims of the Project
The pathogenesis of CM is very complex. Three main processes, sequestration, inflammation, and hemostasis dysfunction have been implicated in this pathology.

This work has two main objectives: first, to study the CO protective mechanisms in ECM and, second, to study the bioactivity of CO releasing molecules (CORMs) in ECM.

In the first aim, we want to know if the protective effect of CO could interfere with parasite sequestration in the brain, as well as with neuroinflammation and chemotaxis processes.

In the second aim, we sought to address the bioactivity and therapeutic features of carbon monoxide releasing molecules (CO-RMs) in experimental cerebral malaria. These molecules may prove to be a very useful tool to explore the potential of CO as a therapeutic agent.
5. Results

5.1. CO inhalation prevents parasite accumulation/sequestration in the brain and neuroinflammation

Previous work demonstrated the protective effects of heme oxygenase-1 (HO-1) and carbon monoxide (CO) against the development of ECM in C57BL/6 infected with *P. berghei* ANKA (PbA), preventing BBB disruption and neuroinflammation (114). Similarly to what was shown, we verified that inhalation of CO (250 p.p.m) for 24h, from day 2 to day 3 after infection prevents ECM onset in all C57BL/6 infected mice.

Cerebral malaria is a severe neurological pathology associated to *P. falciparum* infection; in which *P. falciparum* infected red blood cells (iRBCs) sequestration in the brain microvasculature represents a critical event (22). It has been long argued that ECM does not reproduce in many aspects human CM. Particularly controversial has been the existence or not the sequestration of iRBCs in the brain microvasculature of mice. In the *P. berghei* ANKA-induced ECM it has been described a marked preference for leukocyte sequestration (38, 39, 42, 43) rather than for iRBCs, as seen in human CM (22). Hence, we aimed at assessing whether there is iRBCs accumulation/sequestration in the brain of infected mice and if the CO protective effects could inhibit this process.

For this, we used a recently described system of real-time imaging to detect iRBCs, based on the bioluminescence of transgenic luciferase-expressing parasites of the rodent malaria parasite *P.berghei* ANKA. The levels of parasite sequestration are assessed by the amount of bioluminescence detected by an Intensified-charge-coupled device (I-CCD) photon-counting video camera (44). We established infections in C57BL/6 (an ECM susceptible strain) and BALB/c (an ECM resistant strain) mice of PbA parasites that expressed luciferase in all blood stages.

Before organs collection and bioluminescence acquisition mice were perfused in order to avoid background signal from iRBCs in circulation that were not sequestered. The lung, spleen and fat tissue showed the highest bioluminescence signal in the PbA-infected C57BL/6 exposed to air and developing ECM (P < 0.05, comparing brain to the other organs; Fig. 1a,c) as well as in PbA-infected C57BL/6 mice treated with CO and PbA-infected BALB/c mice (data not shown), whilst the brain displayed the lowest.
Figure 1 CO inhalation prevents P. berghei ANKA (PbA) accumulation in the brain. Visualization and quantification of bioluminescence in ex vivo organs of mice infected with a PbA transgenic parasites that express luciferase in all blood stages. Bioluminescence detected in (a) organs (15 sec exposure) and (b) brain (300 sec exposure) of PbA-infected C57BL/6 mice exposed to air (I) (d6 p.i.) or to CO inhalation (I + CO) (d6-10 p.i.) and in PbA-infected BALB/c mice (l) (d7 p.i.). Image data are representative of the bioluminescence profile in each group of mice. Color scale indicates the image maximum and minimum values of average radiance. Bioluminescence quantification in the (c) organs and (d) brain at the exposures referred above. B – brain, F – fat tissue, K – kidney, L – lungs, LV – liver, S – spleen. C57BL/6: I (n = 5), I + CO (n = 7); BALB/c: I (n = 3). Error bars represent standard deviation.

This result indicates that sequestration/accumulation of iRBCs occurs mainly in the lung, spleen and fat; however it also occurs in the brain, though at a lower extent compared to the other organs. Hence, to increase the signal output, the brain bioluminescence was recorded separately for a longer exposure time (300 sec) than the other organs (15 sec). We observed clear reduction in parasite accumulation/sequestration both in the brains of infected C57BL/6 mice treated with CO and infected BALB/c mice by 64.8 ± 20.3% and 81.2 ± 12.8%, respectively, which did not develop ECM, at days (d) 6-10 post-infection (p.i.), compared with the infected C57BL/6 air-exposed controls when ECM symptoms developed at d6 p.i. (P < 0.05 for C57BL/6 + CO and P < 0.01 for BALB/c; Fig. 1b,d).
It is known that sequestration of iRBCs in the microvasculature of organs and tissues in human CM is carried out primarily by mature forms of the parasite, namely by schizonts (22). According to this we repeated the previous experiment using a *P. berghei* ANKA parasite that only expresses luciferase at the schizont stage (44). In this manner we would be able to further corroborate our results by avoiding the bioluminescence from earlier forms of the parasite, as well as improving the resolution by increasing the bioluminescence signal/background ratio.

The lung, spleen and fat tissue remained the organs with highest parasite accumulation/sequestration whereas the brain displayed the lowest levels of parasites accumulated and/or sequestered, similarly to what has been observed before (P < 0.05, brain compared with the other organs; Fig. 2a,c). The brains from infected C57BL/6 CO-treated mice and BALB/c mice showed a significant bioluminescence decrease, 88.1 ± 7.8 % and 91.8 ± 4.9 %, respectively, at d6-12 p.i., as compared with the air-exposed controls at d5-6 p.i., when ECM signs developed in these mice (P < 0.01; Fig. 2b,d).

This data confirms the results obtained in the previous experiment. However, using the parasite that expresses luciferase in the schizont stage, we can observe an enhancement in the bioluminescent signal which emphasizes the differences in brain iRBCs accumulation between mice groups. Therefore, this parasite seems more appropriate to detect variations in the accumulation/sequestration of parasites in the brain.

The accumulation/sequestration of *P. berghei* ANKA was further confirmed by quantitative RT-PCR (qRT-PCR) of mRNA expression of *P. berghei* 18S rRNA in the brains of the same mice from the experiment represented in Figure 1.

Parasite accumulation quantified by mRNA expression is clearly consistent with bioluminescence results, showing a reduction in the infected C57BL/6 CO-treated mice and BALB/c mice by 64.4 ± 26.6 % and 84.5 ± 2.8 %, respectively, versus the air-treated controls that developed ECM (P < 0.05 for C57BL/6 + CO and P < 0.01 for BALB/c; Fig. 3a).

Moreover, to confirm the ECM and ECM-protected phenotypes we analysed the brain neuroinflammatory status by assessing the mRNA expression of CD3 and CD8, in order to assess the recruitment of CD8+ T cells into the brain microvasculature, a key
pathological feature of ECM (72) shown to be inhibited upon CO treatment (114).

Additionally, the expression of granzyme B was also determined to assess the activation of CD8+ T cells. All these markers were significantly down-regulated in the infected C57BL/6 mice exposed to CO inhalation and BALB/c mice as compared to the infected C57BL/6 air-exposed controls, which developed all neurological signs of disease. We observed a reduction in CD3 expression by 33.5 ± 9.6 % and 56.3 ± 6.4 %, in CD8 expression by 74.5 ± 3.2 % and 83.2 ± 5.6 % and in granzyme B expression by 72.2 ± 12.9 % and 72.0 ± 5.8 % in C57BL/6 CO-exposed mice and BALB/c mice, respectively, as compared to air-exposed controls (Cd3, Cd8, Gzmb, P < 0.01 Fig. 3b-d).
Figure 3 CO inhalation inhibits parasite accumulation/sequestration and neuroinflammation. (a-f) Assessment, by qRT-PCR, of brain mRNA expression of PbA 18S rRNA (r18S), CD3 (Cd3), Granzyme B (Gzmb), CD8 (Cd8) IP-10 (Ip-10) and CXCR3 (Cxcr3) genes in C57BL/6 mice non-infected (NI), PbA-infected and exposed to air (I) (d6 p.i.), CO inhalation (I + CO) (d6-10 p.i.), and in PbA-infected BALB/c mice (I) (d7 p.i.). Expression is normalized to hprt expression and is shown as percentage of expression in control C57BL/6 infected mice exposed to air. C57BL/6: NI (n = 2), I (n = 5), I + CO (n = 3); BALB/c: I (n = 4) Error bars represent standard deviation.
In addition, the expression of the chemokine IP-10 and its receptor CXCR3 was also investigated, since these molecules have been recently implied in ECM by playing a role in CD8+ T lymphocytes migration to the brain microvasculature (84-86). IP-10 is clearly up-regulated in the brains of C57BL/6 air-exposed controls that developed ECM, at d6 p.i., and comparatively reduced by 70.4 ± 29.5 % in the C57BL/6 CO-treated mice, at d6-10p.i. (P < 0.01), while in BALB/c mice, at d7 p.i., IP-10 showed no significant differences (Fig. 3e). On the other hand, CXCR3 expression in the brain showed no differences between mice groups (P > 0.05; Fig. 3f).

5.2. CORM-2 protects against ECM development

We tested if the protection conferred by CO inhalation could be mimicked by the administration of CO-RMs. These compounds would bring the advantage of a more specific and controlled delivery of CO to cells and thereby overcome the toxicity of CO inhalation due to COHb production (150).

For this purpose, we tested the tricarbonyldichlororuthenium (II) dimer ([Ru(CO)3Cl2]2) i.e. CORM-2 (dissolved in DMSO 10% in PBS; vehicle) which has been proven to act beneficially in several mouse models of pathology by suppressing inflammation (169, 170, 175). C57BL/6 mice infected with P.berghei ANKA were administrated intravenously (i.v.) with CORM-2 at the indicated times after infection.

A concentration of 10 mg/kg of body weight of CORM-2, given at day 3 p.i. or at d3 and d4 p.i., proved to be ineffective, with all the infected C57BL/6 mice developing ECM (Fig. 4a,b). However, increasing the concentration of CORM-2 to 20 mg/kg and administration from d2 until d4 p.i., twice a day, prevented ECM symptoms in all C57BL/6 infected mice, while all the infected C57BL/6 controls administrated with the DMSO vehicle died at d6-7 p.i. from ECM (Fig. 4c). The CORM-2-treated mice die after 3 weeks of infection with hyperparasitemia However, contrary to what occurred with CO inhalation, we observed an arrest in parasitemia between d5 to 7 p.i in CORM-2 treated mice.
Parasitemia was significantly decreased in the CORM-2 treated mice at day 6 p.i., the day of ECM onset in DMSO controls (P < 0.05). After day 8 p.i. the levels of parasitemia start to increase rapidly, reaching a typical plateau around day 16 p.i. (Fig. 4d).

Thereafter, different schedules, concentrations and routes of administration (intraperitoneally, i.p., or i.v.) of CORM-2 were tested in P. berghei-ANKA infected C57BL/6 mice. We observed that CORM-2 (20mg/kg) administration at d2 and d3 p.i., twice a day was sufficient to prevent the development of ECM in 100% of the mice, while
the administration of CORM-2 one time per day at the same days was not. Though, if CORM-2 was given once a day but from d2-4 p.i., all mice were protected (Fig. 5a,b). Additionally, i.p. route of administration was tested since it has the advantage of being less invasive than i.v. In parallel we also wanted to know if CORM-2 given i.v. at a lower concentration, 10 mg/kg, was protective when administered at d2 and d3 p.i once a day. Nevertheless, we observed that neither CORM-2 given i.p. nor CORM-2 at a 10mg/kg concentration prevented ECM (Fig. 5c,d).

![Figure 5](image_url)

**Figure 5** CORM-2 abrogates ECM development. P.berghei-ANKA infected C57BL/6 mice. PbA-infected C57BL/6 mice were treated with CORM-2 (I + CORM-2) or DMSO vehicle (I + DMSO) (a, b) Effect on survival and mean parasitema of several schedules of administration of CORM-2 (20mg/kg): twice a day (2x) from d2-4 p.i. or from d2-3 p.i.; once a day (1x) from d2-4p.i. or from d2-3p.i. (c, d) Effect on survival and mean parasitemia of i.p. administration of CORM-2 (20 mg/kg) (2x) from d2-3 p.i. or i.v. administration of CORM-2 (10mg/kg) (1x) from d2-3 p.i. I + DMSO (n = 4-7); I + CORM-2 (n = 3-6). Error bars represent standard deviation.

Again, we observed an arrest in parasitemia between d5 to d7 p.i in CORM-2 treated mice that did not develop ECM (Fig. 5b). Parasitemia in these mice was significantly
reduced at d5 p.i. and d6 p.i., compared with DMSO controls that started to develop ECM (P < 0.05). No differences appear to occur on parasitemia during the non-protective schedules of CORM-2 administration. Whether this small but significant arrest in parasitemia for 3 days is important for the protective effect of CORM-2 is not known. For the following experiments CORM-2 was administered i.v. twice a day at d2 and d3 p.i.

5.3. CORM-2 protective effects is dependent on CO release but does not involve COHb formation

Furthermore, to confirm that CORM-2 protection was due to CO release and not due to other constituents of the compound, like the ruthenium centre of the molecule, we tested the effect of the negative control compound, generically named as ALF466, which is similar to CORM-2 however does not contain CO molecules.

Treatment with ALF466 revealed ineffective against ECM in all mice whereas CORM-2 prevented all infected C57BL/6 mice from developing the pathology. Again, we observed the parasitemia arrest from d4 to d8 p.i. in the CORM-2-protected mice (Fig. 6a,b) but not in the ALF466 treated mice. Because ALF466 does not contain any CO molecule,
this results demonstrates that CO is the responsible for CORM-2 protection against ECM.

Importantly, we also wanted to assess if CORM-2 treatment would induce the formation of carboxyhemoglobin, as observed for CO inhalation, or, on the contrary, is able to overcome the toxicity effect of CO inhalation.

Figure 7 CORM-2 treatment does not induce COHb production. COHb and O₂Hb measurement in whole blood of non-infected (NI) and PbA-infected C57BL/6 mice treated with DMSO vehicle (I + DMSO), CO (I + CO) or CORM-2 (I + CORM-2) (a) 15 min. after first CORM-2 administration, at d2 p.i.; (b) 3h after first CORM-2 administration, at d2 p.i. (c) and immediately after CO treatment / last CORM-2 administration, at d3 p.i. (d, e) O₂Hb, COHb and MetHb measurement in whole blood of non-infected, PbA-infected C57BL/6 mice treated with DMSO vehicle at the time of ECM development (at d6 p.i.), treated with CO (at d6-7 p.i.) or treated with CORM-2 (at d6-7 p.i.). NI (n= 4-9); I + DMSO (n = 3-4); I + CO (n = 4); I + CORM-2 (n = 5-6). Error bars represent standard deviation.
In fact, the levels of oxyhemoglobin (O$_2$Hb) and carboxyhemoglobin (COHb) revealed no significant differences in the whole blood of C57BL/6 infected mice treated with CORM-2 versus DMSO-treated controls, either 15 minutes or 3 hours after the first CORM-2 administration (P > 0.05; Fig. 7a,b). Moreover, we compared the levels of COHb at d3 p.i., immediately after CO treatment or after last CORM-2 administration. We observed that CO inhalation strongly induces COHb formation (P < 0.01) while CORM-2 does not induce COHb formation when compared with non-infected or infected DMSO-treated mice (Fig. 7c). Finally, at d6 p.i., methemoglobin (MetHb) is found significantly increased in the DMSO-treated controls showing signs of ECM whereas CO-treated and CORM-2-treated mice exhibit significant lower levels of MetHb (P < 0.01 and P < 0.05, respectively), similar to the non-infected mice (Fig. 7e).

5.4. CORM-2 prevents parasite accumulation/sequestration in the brain and neuroinflammation - CORM-2 mimics CO inhalation

Of special importance is the quantification of parasite accumulation/sequestration in the brain during the treatment with CORM-2. We assessed this through the same bioluminescence system used previously, with the P. berghei ANKA that expresses luciferase only at the schizont stage.

We obtained a significant reduction in brain bioluminescence by 76.3 ± 29.3 and by 97.2 ± 1.8 in the C57BL/6 infected mice treated with CORM-2 (P < 0.05) and in the BALB/c infected mice (P < 0.01), respectively, compared with C57BL/6 infected mice that received the control molecule ALF466, and that developed ECM (Fig. 8). This shows that CORM-2 mimics the effect of CO inhalation on parasite accumulation/sequestration in the brain and reinforces the importance of iRBCs accumulation/sequestration in the development of ECM in C57BL/6 P.berghei ANKA-infected mice. By qRT-PCR we obtained consistent results with the data obtained by bioluminescence acquisition. Indeed, we observed a reduction by 61.1 ± 17.4 % in parasite accumulation in the brains of mice that received CORM-2 treatment versus the DMSO treated controls that developed ECM (P < 0.05; Fig. 9a).
IFN-γ treatment also inhibited the recruitment of CD8+ T cells and the brain expression of IFN-γ and the adhesion molecule ICAM-1, known to play a critical role in the pathology (69, 92). IFN-γ and ICAM-1 have been already described to be down-regulated in the brains of mice infected with P. berghei ANKA schizonts the brain. Visualization and quantification of bioluminescence in ex vivo brains of mice infected with PbA parasites that express luciferase only in the schizonts. (a) Detection of bioluminescence (300 sec exposure) and (b) quantification in the brains of PbA-infected C57BL/6 mice treated with ALF466 (I + ALF466) (d6 p.i.) or with CORM-2 (I+ CORM-2) (d6-12 p.i.) and in PbA-infected BALB/c mice (d7 p.i.). Image data are representative of the bioluminescence profile in each group of mice. Color scale indicates the image maximum and minimum values of average radiance. C57BL/6: I + ALF466 (n = 3), I + CORM-2 (n = 5); BALB/c: I (n = 4). Error bars represent standard deviation.

Since blood-brain barrier (BBB) disruption and neuroinflammation are hallmarks of ECM, the occurrence of these two processes were assessed in the context of CORM-2 treatment to better characterize de protected phenotype. CORM-2 administration prevented BBB disruption in C57BL/6 PbA infected mice, as showed by the levels of Evans blue accumulation in the brain parenchyma, not significantly different from the non-infected controls. In contrast, C57BL/6 infected controls treated with DMSO vehicle and developing ECM showed a significant increase in BBB permeability. A 6 fold increase in Evans blue accumulation in the brain parenchyma was observed in these mice, when compared with non-infected mice, indicating BBB disruption (P < 0.05; Fig. 10).

We also assessed the brain mRNA expression of various genes associated to inflammation to confirm that CORM-2 protective effect, similarly to CO, was related with the modulation of inflammatory ECM-associated events. We confirmed if CORM-2 treatment also inhibited the recruitment of CD8+ T cells and the brain expression of IFN-γ and ICAM-1, known to play a critical role in the pathology (69, 92). IFN-γ and ICAM-1 have been already described to be down-regulated in the brains
of mice protected by CO inhalation (112). In C57BL/6 infected mice, CORM-2 reduced the brain CD3 and CD8 mRNA by 54.0 ± 16.7 % and 64.1 ± 17.0 %, respectively, in comparison with DMSO-administered controls suffering from ECM (P < 0.05, Cd3 and Cd8 expression; Fig. 9b,c).

Figure 9 CORM-2 treatment prevents parasite accumulation/sequestration and neuroinflammation. (a-e) Brain mRNA expression assessed by qRT-PCR of PbA 18S rRNA (r18S), CD3 (Cd3), CD8 (Cd8) IFN-γ (Ifng) and ICAM1 (Icam1) genes, in C57BL/6 non-infected (NI) and PbA-infected (I) mice treated with DMSO vehicle or CORM-2. Expression is normalized to that of Hprt and is shown as percentage of expression in control C57BL/6 infected mice exposed to air. NI (n = 2-3); I / DMSO (n = 3); I / CORM-2 (n = 3). Error bars represent standard deviation.
IFN-γ and ICAM-1 were also significantly down-regulated in the CORM-2-treated mice, by 72.1 ± 16.4 % and 45.2 ± 4.8 %, respectively, versus DMSO-treated controls (P < 0.01 *Ifng* and *Icam1* expression; Fig. 9d,e)

**Figure 10** CORM-2 prevents BBB disruption in *P.berghei ANKA* infected mice. BBB disruption was determined by Evans Blue (EB) quantification in C57BL/6 non-infected (NI) or PbA-infected (I) mice either treated with CORM-2 or DMSO vehicle. NI (n = 3); I / DMSO (n = 3); I / CORM-2 (n = 4). Error bars represent standard deviation.

**5.5. CORM-2 changes the profile of intra-erythrocytic stages of *P.berghei ANKA***

CORM-2 treatment leads to a delay in the progression of parasitemia, between d5 to d7 p.i. To attain some evidence about whether this effect resulted from impairment in the parasite intra-erythrocytic development, we looked at the percentage of rings and more mature parasite forms (trophozoites and schizonts), between C57BL/6 mice infected with *P.berghei ANKA* GFP-expressing parasites (PbA-GFP), that have received either CORM-2 or DMSO vehicle. We distinguished the developmental blood stages of the parasite based on the parasite GFP-fluorescence and DNA content, by staining with Hoechst DNA-specific fluorescent dye, as previously reported (185).

In the peripheral blood of CORM-2 treated mice, at day 6 p.i., we observed a significant reduction (19.8 ± 6 %; P < 0.01) in the percentage of ring forms, parasites at an early stage of infection. But in contrast, we observed a significant increase in the percentage of circulating trophozoites and schizonts, mature forms of the parasite, by 15.9 ± 5.2 % in the CORM-2 treated mice, as compared with controls receiving DMSO and in ECM (P < 0.01). Additionally, in CORM-2 treated mice we observed a significant 1.5 ± 0.6 % higher gametocyte fraction of parasites (P < 0.01; Fig. 11).
5.6. CORM-2 does not alter reticulocytosis significantly

Some *Plasmodium* species like *P. berghei* and *P. yoelli* 17XL show a preference for invading reticulocytes (young erythrocytes) over mature erythrocytes (186, 187). In fact, it has been established a correlation between reduction in *P. yoelli* 17XL parasite load in circulation and IFN-γ-induced suppression of erythropoiesis and reticulocytosis (187). Bearing this in mind, we sought to investigate whether CORM-2 treatment could be inhibiting erythropoiesis, thus reducing reticulocyte levels and by this way retarding parasitemia progression between day 5 and 8 p.i.

At day 3 p.i., reticulocytemia (the percentage of reticulocytes in the total population of red blood cells), assessed by flow cytometry, showed no differences between C57BL/6 mice non-infected or infected with PbA and receiving DMSO vehicle.
However, a slight but significant increase in the CORM-2 treated mice was observed, when compared with the DMSO-treated mice ($P < 0.05$). At day 6 p.i. all infected mice

Figure 12 CORM-2 treatment does not seem to induce significant alterations in reticulocytosis. Reticulocytemia was assessed by flow cytometry in C57BL/6 non-infected mice (NI) or in C57BL/6 PbA GFP-infected mice (I) treated with DMSO vehicle or CORM-2. Blood cells were stained for CD45 and CD71, and reticulocytes were gated in the CD45$^-$CD71$^+$ cell population (a) Dot plots as measured by flow cytometry, showing CD71 expression and GFP fluorescence intensity. Numbers in quadrants indicate adjacent percent cells in each. Data is representative of each group of mice. (b) Reticulocytemia, reticulocyte index (c) and absolute number of parasitized reticulocytes per volume of blood, at d3 p.i and d6 p.i. NI ($n = 2-3$); I + DMSO ($n = 3$); I + CORM-2 ($n = 5$). Error bars represent standard deviation.
showed a strong reduction in reticulocytosis, when compared with the non-infected mice. This is in accordance with previous observations reporting a decrease in circulating reticulocytes upon a *P. berghei* infection, due to the tropism of the parasite for reticulocytes (186), but should also result from the inhibition of erythropoiesis that typically occurs during a malaria infection (136). At this day, reticulocytemia also shows a small increase in the CORM-2 protected when compared with DMSO-treated control mice, that were showing signs of ECM (P < 0.05; **Fig. 12a,b**). In parallel, the number of infected reticulocytes per volume of blood was also determined by flow cytometry. In these results we noticed a high variability within groups, possibly caused by errors associated with cell counting method (beads). At d3 p.i. and d6 p.i CORM-2-treated mice seem to show increased numbers of parasitized reticulocytes when compared with control mice that received DMSO vehicle, although this was not significant (**Fig. 12c**). Importantly, we calculated the reticulocyte index, which has into account the hematocrit (the proportion of blood volume occupied by RBCs). This is a more accurate measure of reticulocytosis, particularly in situations where RBC number is decreased, such as hemolysis induced by a malaria infection. The reticulocyte index determined for d6 p.i. revealed no differences between mice receiving CORM-2 or DMSO vehicle (**Fig.12b**) suggesting that CORM-2 does not induce significant alterations in reticulocytosis.

### 5.7. CORM-2 treated mice show a reduced hematocrit and platelet volume

To further understand the effect of CORM-2 treatment in the peripheral blood, we looked to further hematological parameters, such as hematocrit, total number of RBCs per volume, hemoglobin concentration and platelet number. All these parameters were assessed for the DMSO-administered controls at day 6 p.i., the day of ECM onset in these mice.

C57BL/6 PbA-infected mice protected by CORM-2 treatment evidence a significant reduction of 14.3 ± 2.2 % in hematocrit, when compared with DMSO-treated control mice (P < 0.01) (**Fig. 13a**).
In straight agreement with this, RBC number and hemoglobin concentration were similarly decreased in the blood of CORM-2 treated mice, respectively, by 34.0 ± 4.9 % and 35.3 ± 4.7 % compared with DMSO controls (P < 0.01), while between non-infected and DMSO treated mice no differences appeared to occur (Fig. 13b,c).

Additionally, we assessed whether CORM-2 could be affecting platelets, which have been associated with human and rodent CM (95, 97). At day 6 p.i., the day of ECM onset in DMSO-treated controls, platelet counts appear decreased in all infected mice, when compared with non-infected ones, which was expected in *Plasmodium* infections. During malaria infection thrombocytopenia (the loss of platelets in circulation) in human and experimental malaria has been reported (95). However, the number of platelets showed no significant differences between CORM-2-treated mice and DMSO controls.

Figure 13 CORM-2 treated mice show a reduction in hematocrit. (a) Hematocrit, (b) number of RBCs per volume (c) and hemoglobin concentration were assessed in the peripheral blood of C57BL/6 mice non-infected (NI) or C57BL/6 PbA-infected mice (I) treated with CORM-2 (d6-7 p.i.) or DMSO vehicle (d6 p.i.). NI (n = 2); I + DMSO (n = 5); I + CORM-2 (n = 5). Error bars represent standard deviation.
Inversely, all infected mice seemed to show a larger platelet volume relatively to non-infected mice, but platelets from CORM-2 treated group have significant smaller average volumes when compared with DMSO-treated mice (P < 0.01; Fig 14b).

Figure 14 CORM-2 reduces average volume of platelets. (a) Platelet number per volume of blood (b) and average volume of platelets in the peripheral blood of C57BL/6 mice non-infected (NI) or infected with PbA (I) and treated with CORM-2 (d6-7p.i.) or DMSO vehicle (d6 p.i.). Note scale break. (n = 2); I + DMSO (n = 5); I + CORM-2 (n = 5). Error bars represent standard deviation.

5.8. CORM-2 treatment increases the number of phagocytic cells in circulation

Furthermore, to evaluate the CORM-2 effect in the immune response, leukocyte counts were assessed in the peripheral blood of DMSO-treated controls with ECM, at day 6 p.i., and CORM-2 treated groups at day 6-7 p.i. It was observed a significant increase in both circulating monocytes and neutrophils upon CORM-2 treatment, comparatively with the DMSO controls (P < 0.05; Fig. 15a,b). Lymphocyte numbers were not significantly altered between these groups (Fig. 15c).
5.9. Spleens from CORM-2 treated mice have increased weights

Spleen weights were also measured in C57BL/6 non-infected or PbA-infected C57BL/6 mice, treated with CORM-2 or DMSO vehicle. As expected, the spleens of infected mice are larger than non-infected (P < 0.01). Furthermore, we observed a significant increase in spleen weight in CORM-2-treated mice (P < 0.01) compared to DMSO controls, possibly related to and increased removal of RBCs (Fig. 16).

Figure 15 CORM-2 treatment increases the number of monocytes and neutrophils in peripheral blood of PbA-infected mice. The number of circulating (a) monocytes, (b) neutrophils and (c) lymphocytes, was determined in C57BL/6 mice non-infected (NI) or infected with PbA (I) and treated with CORM-2 (d6-7 p.i.) or DMSO vehicle (d6 p.i.). NI (n = 2); I + DMSO (n = 5); I + CORM-2 (n = 5). Error bars represent standard deviation.
5.10. CORM-2 prevents loss of RBC deformability

Reduced RBC deformability proved to be a strong predictor of human malaria severity and seems to occur principally in uninfected RBCs (35, 188). Loss of deformability is suggested to result from oxidative damage to membranes of RBCs by the release of heme products into circulation, such as hemozoin, during schizont rupture. Consequently, an increase of RBC rigidity may contribute to impairment of microcirculation and to microvasculature congestion (189, 190).

Based on this, we hypothesized that CORM-2 treatment may reduce deformability in RBCs during infection. The results support our hypothesis showing that C57BL/6 PbA-infected mice treated with CORM-2 exhibit a tendency for increased deformability, significantly higher for shear stress of 12 to 60 Pa, compared with DMSO-treated controls (P < 0.05). In fact, CORM-2 treatment seems to increase RBC deformability to the basal levels of non-infected control DMSO-treated, at shear stress stresses from 6-60 Pa, although we could not statistically validate this result due to a unique mouse in the non-infected group. Also, CORM-2 treatment seems to elevate RBC deformability in the non-infected mouse, at same range of shear stresses (6-60 Pa), comparatively with the non-infected mouse that received DMSO vehicle (Fig. 17).
Figure 17 CORM-2 prevents loss of RBC deformability during infection. RBC deformability was measured in the peripheral blood of non-infected C57BL/6 mice treated with CORM-2 (NI + CORM-2) or DMSO (NI + DMSO) and in PbA-infected C57BL/6 mice treated with CORM-2 (I + CORM-2) or DMSO (I + DMSO) at day 6 p.i. Results are indicated as mean elongation index, shown as percentage, measured over a range of 0.3-60 Pa of shear stress. NI (n = 1); NI + CORM-2 (n = 1); I + DMSO (n = 3); I + CORM-2 (n = 3). Error bars represent standard deviation.
6. Discussion

The pathogenesis of CM has been explained mainly by two processes, sequestration and inflammation, and more recently hemostasis dysfunction has been also implicated (21). Sequestration process has been considered a common feature in human and murine CM, although in murine CM leukocytes are the cells that predominantly sequester in the brain (38, 39, 42, 43) rather than iRBCs, as seen in human CM (22, 25). This observation has contributed to the controversy concerning the relevance of PbA-C57BL/6 model as a CM model. Nevertheless many authors have contributed for the elucidation of the importance of PbA iRBCs sequestration in ECM development. Using the same system of real-time imaging by bioluminescence detection, some authors report an irrelevant accumulation of iRBCs in the brain (44) whether others correlate ECM onset with increased parasite sequestration in the mouse brain (45, 46).

To address if the CO protective effect observed in ECM (114) could be due to a reduction in the parasite accumulation/sequestration in the brain of PbA-infected C57BL/6 mice, we used the same system of real-time imaging to detect parasite sequestration. We observed that the lung, spleen and fat tissue showed the highest bioluminescence signal, as previously reported (44), both in the PbA-infected C57BL/6 exposed to air and developing ECM, as well as in PbA-infected C57BL/6 mice treated with CO and PbA-infected BALB/c mice. Comparatively, the brain was the organ with lower bioluminescence signal indicating a lower parasite accumulation. Nevertheless, we could clearly detect the presence of parasite in this organ when higher camera exposition times were applied (300 sec). This could explain why in a previous report (44) the authors could not detect significant parasite sequestration in the brain, since the exposition times used were far to low (10-60 sec). We observed a clear reduction in the parasite accumulation/sequestration in the brains of infected C57BL/6 mice treated with CO and infected BALB/c mice, which did not develop ECM, relatively to the PbA-infected C57BL/6 that developed ECM (Fig. 1). Additionally, since mature trophozoites and schizonts are the parasite stages that sequester during human pathology (22) we infected mice with a strain of PbA that only expresses luciferase in schizonts in order to favor the bioluminescence signal produced by sequestering parasites. The results obtained with this PbA strain were in strict agreement with the data obtained previously, indicating a significant accumulation of PbA schizonts in the brain of C57BL/6 mice.
developing ECM, compared with CO-protected C57BL/6 mice or with BALB/c mice. In addition, we observed that the differences in parasite accumulation between mice groups were rendered more evident, most likely due to reduction in signal noise resultant from non-sequestering parasites (Fig. 2). This indicates that the PbA strain expressing luciferase only in schizonts is more appropriate to study parasite sequestration. Additionally, these results were further confirmed by qRT-PCR of mRNA expression of PbA 18S rRNA in the brains of the same mice used previously (Fig. 3a).

In preceding studies (45, 46), parasite sequestration was only assessed using the PbA parasite that expresses luciferase in all blood stages, and therefore it is probable that the bioluminescence results could have been affected by the presence of non-sequestering parasite forms, such as ring forms and trophozoites, accumulated in brain hemorrhages observed in mice that developed ECM. Moreover, in the previous reports, the authors could not exclude the hypothesis that the decrease of iRBC in the brains of mice protected against ECM resulted from reduced parasite load in circulation, since these mice had significant lower parasitemias than control C57BL/6 PbA-infected mice that developed cerebral pathology (45, 46). In our study this problem does not poses because CO-protected mice and BALB/c mice analyzed from day 6-12 p.i. did not have lower parasitemias compared with control mice. In addition, it should be noted that the method of imaging used does not allow a clear distinction between iRBCs accumulation and sequestration. However, if parasite sequestration was not accounting for the observed results, we would not expected that infection with PbA luciferase-expressing shizonts resulted in more pronounced differences in brain bioluminescence between mice that develop ECM and mice that are ECM-resistant, as observed.

It is true that CD36 was suggested to be the major adhesion receptor mediating iRBC sequestration in mice and it was shown that abrogation of this molecule does not prevent ECM development (44). However, the authors did not assess brain sequestration and it is probable that other adhesion molecules, such as ICAM-1 may play an important role in the sequestration process. Indeed, ICAM-1-mediated cytoadherence of iRBCs to brain microvessels has been correlated with CM (93). Moreover, ICAM-1 expression is required for ECM establishment, playing a role in leukocyte and iRBC vascular sequestration in the murine model (94). Therefore it is reasonable to presume that inhibition of iRBCs sequestration in the CO-treated mice
arises, at least in part, from ICAM-1 down-regulation in the brain, which was shown to occur in mice protected by CO inhalation (114). Thereby, although brain histological examinations should be done in the future to complement the data presented, the findings obtained in the present study strongly suggest that protection by CO inhalation against ECM comprises the reduction of iRBC sequestration in the brain of PbA-infected C57BL/6 mice, and that a reduced parasite sequestration is associated with a CM-resistant like phenotype, as verified with BALB/c mice.

In summary these results, altogether, reinforce once more the importance of iRBC sequestration in ECM development and consequently underline the relevance of C57BL/5 PbA-infected mouse model to study CM pathogenesis.

It has been demonstrated that CD4+ and CD8+ T cells are required for cerebral pathology of *P. berghei* ANKA-infected mice, of which CD8+ T cells play a crucial role (71-77). We show that recruitment of T cells and CD8+ T cells in particular, assessed by CD3 and CD8 expression, respectively, is reduced in the brain of C57BL/6 air-exposed controls and in BALB/c mice. Moreover, the cytolytic activity of CD8+ T cells in the brain, assessed by granzyme-B expression, is decreased in CO-treated C57BL/6 mice and in BALB/c mice (Fig. 3b-d). Thus, we show that CO treatment prevents the recruitment of activated CD8+ T cells to the brain, consistently to what was reported before (114). Moreover, we demonstrate that the protection conferred by CO against ECM consists of inhibiting essentially the presence of CD8+ T cells potentially cytotoxic to the brain endothelium. This was consistent with previous reports implicating perforin/granzyme B cytolytic pathway as a major mechanism employed by CD8+ T cells during ECM (80). Moreover, a survey of brain gene-expression patterns between ECM-resistant and ECM-susceptible mouse strains identified granzyme B up-regulation to be specifically associated with an ECM-susceptible phenotype (191).

In addition, the expression of the chemokine IP-10 and its receptor, CXCR3, was also assessed, since they have been shown to a play significant role in ECM by mediating leukocyte migration to the brain, in particular of CD8+ T cells (46, 84-86). Furthermore, recent studies have identified IP-10 as a biomarker associated with higher risk of CM-associated mortality during *P. falciparum* infections (87, 88). The expression of IP-10 was significant decreased in CO protected C57BL/6 mice but in BALB/c mice IP-10
showed no significant differences relatively to C57BL/6 air-exposed control mice that developed ECM (Fig. 3e). These results indicate that CO seems to have a therapeutic potential as a molecule with anti-chemotaxis effect in ECM. Accordingly, it has been suggested that inhibition of leukocyte trafficking through depletion of IP-10 could be used as a potential adjunctive therapy to improve treatment outcomes of CM (46). In fact, IP-10 down-regulation in the brain could explain the reduction in CD8+ T cell recruitment to the brain resulting from CO treatment. On the other hand, high expression of IP-10 in BALB/c mice is in agreement with previous work indicating that this chemokine is up-regulated in BALB/c mice after day 6 p.i., and consequently should not account for the ECM-resistant phenotype observed in this strain (86). On the other hand, CXCR3 expression in the brain showed no differences between the groups of mice (Fig. 3f). These results could be explained because, as has been previously shown, CXCR3 expression is required for early migration of activated CD8+ T cells from the spleen to the brain of infected mice (84, 86); and due to the redundancy of the chemokine system others have suggested that at later stages of disease, migration of CD8+ T cells by a CXCR3-independent process can be significant (84). However, given that previous reports demonstrated a clear induction of brain CXCR3 expression in response to PbA infection, further work is required to disclose why this was not observed in the present study.

A prospective therapy by CO administration seems attractive; however treatment by CO inhalation has practical limitations. CO reacts promptly with hemoglobin and other hemoproteins before reaching the target tissue and thus can potentially induce clinical toxicity. To overcome these problems, we tested if the protection conferred by CO inhalation against ECM could be mimicked by the administration of CO-releasing molecules, because these molecules are thought to act as more specific CO-donors to cells and tissues and have been shown to mimic the anti-oxidant and anti-inflammatory properties of CO in a variety of pathophysiological conditions. Due to this, CO-RMs have been defended as potentially good candidates for CO-based pharmaceuticals (148, 150).

Strikingly, we observed that administering CORM-2 at a concentration of 20 mg/kg of body weight, given i.v. two times per day, from day 2-3p.i. was sufficient to have 100% protection against ECM development in PbA-infected C57BL/6 mice, which eventually
died after 3 weeks of infection with hyperparasitemia (Fig. 5a,b). The total abrogation of disease development unequivocally reconstituted the effects of CO inhalation on mice survival, reported previously in the same ECM model (114). Nevertheless, we observed a significant arrest in parasitemia from day 5 p.i to day 7 p.i. only in protective schedules of CORM-2 administration (Fig. 4 and Fig 5), which has not been verified in mice treated by CO inhalation (114). Moreover, CORM-2 administered at half of the concentration (10 mg/kg) or by i.p. route failed to prevent ECM progression (Fig. 4a,b and Fig. 5d,e). Importantly, the CORM-2 protective effect was directly demonstrated to result from CORM-2-derived CO, since treatment with ALF 466, a molecule similar to CORM-2 but without CO groups, revealed ineffective against ECM in all mice tested (Fig. 6). Importantly, we also wanted to assess if CORM-2 treatment would induce the formation of carboxyhemoglobin, as observed for CO inhalation. Surprisingly, immediately after the CORM-2 administration the levels of oxyhemoglobin (O$_2$Hb) and carboxyhemoglobin (COHb) revealed no significant differences in the whole blood of C57BL/6 infected mice treated with CORM-2 versus DMSO-treated controls or non-infected mice (Fig. 7a,b). Moreover, by the end of the whole CO or CORM-2 treatment, we observed that CO inhalation strongly induced COHb formation, as expected, whereas CORM-2 treatment did not (Fig. 7c). Furthermore, at day 6 p.i., DMSO-treated controls that developed ECM displayed significantly elevated levels of MetHb, when compared with CO-exposed, CORM-2-treated and non-infected mice (Fig. 7e).

First, these data clearly demonstrates that contrary to CO inhalation, CORM-2 suppresses ECM without the formation of COHb. This is of extraordinary importance regarding potential future therapeutic applications. The observed high levels of MetHb in mice that developed ECM, together with an association between CO-prevention of pathology and inhibition of MetHb production is in accordance with previous observations (114). It has been postulated that the mechanism underlying CO protection against ECM consisted of preventing Hb oxidation by virtue of CO binding to Hb, generating COHb. This would lead to inhibition of conversion of Hb to MetHb and further heme release (114). MetHb and free heme are known to promote oxidative damage and inflammation. In fact, heme pathogenic activity in CM has been supported by demonstrating that free heme, in the presence of ROS, promotes disruption of BBB tight-junction function. The protective pheonotype conferred by CO in the murine model is reverted by heme administration (114). Given that CORM-2 treatment does not induce
COHb formation, we cannot argue the same mechanism proposed for CO inhalation to explain the protective action of CORM-2. Since CO donated by CORM-2 did not bind to Hb we should rule out the possibility of NO-CO interaction to suppress ECM pathogenesis, as had been hypothesized (114). This supposition was based on the observation that low NO bioavailability associated with ECM onset and appears to result in part from Hb scavenging of NO (141). Therefore, it was postulated that COHb formation could be limiting NO low bioavailability. However, MetHb levels were found significantly reduced in the CORM-2 treated mice, which can indicate that an antioxidant effect is also implicated in the therapeutic action of the compound.

We were also interested to assess whether CORM-2 could mimic the suppressing parasite accumulation/sequestration in the brain as seen for CO inhalation. We verified that, likewise to CO inhalation treatment, mice treated with CORM-2 were protected from ECM development and showed lower amounts of iRBCs in the brain, assessed using an real-time imaging system and confirmed by qRT-PCR (Fig. 8, 9a). These observations corroborate the preceding results obtained with CO-administration, further evidencing the significance of brain iRBC sequestration for ECM onset in the murine model. These results suggest that CO-mediated protection from CM seems to be dependent on the inhibition of parasite sequestration in the brain.

Furthermore, we assessed blood-brain barrier (BBB) disruption and neuroinflammation, to better characterize the protected phenotype ensuing from CORM-2 treatment. We confirmed by qRT-PCR that, like CO, CORM-2 conferred ECM resistance was associated with the inhibition of CD8+ T cell recruitment to the brain and suppression of IFN-γ and ICAM-1 expression in the brain (Fig. 9b-e), two molecules known to play a critical role in the cerebral pathology (70, 94), and known to be inhibited in response to CO exposure (114). Importantly, BBB barrier was not compromised in the CORM-2 treated mice (Fig. 10), similarly to what is observed upon CO treatment (114). Altogether these results support that CORM-2 protection against ECM pathogenesis result from the suppression of the same crucial pathogenic events as CO inhalation, namely parasite sequestration, neuroinflammation and BBB disruption.

On the other hand, during CORM-2 treatment we observed a significant delay in the progression of parasitemia between days 5 and 7 after infection, which does not occur
during CO administration by inhalation. In order to assessed whether this effect resulted from impairment in the parasite intra-erythrocytic development we looked at the proportion of the circulating blood stage forms at day 6 after infection, when all the mice treated with DMSO vehicle where showing signs of ECM. We observed in the blood of CORM-2 treated mice a significant reduction in the percentage of ring forms, and an increase in the presence of trophozoite and schizont forms as compared to the controls receiving DMSO and developing ECM. A decrease in the proportion of gametocytes was also verified in the mice receiving CORM-2 (Fig. 11). The biasing through middle-later stages of infection upon CORM-2 treatment suggests that CORM-2 might be arresting transitorily the parasite development inside the red blood cell, impairing the burst of shizonts and release of merozoites and/or affecting merozoite establishment of new infections. On the other hand, the presence of more trophozoites/schizonts in circulation observed in the CORM-2 protected mice can likely be a consequence of the suppression of parasite sequestration, namely in the brain, as we know that \textit{Plasmodium} parasites sequester principally at later stages of development, namely at mature trophozoite and schizont stages. A previous work supports this idea, reporting that in synchronous infections of \textit{P. berghei} ANKA, the disappearance of schizonts from circulation was associated with the sequestration of these forms in the microvasculature of organs in C67BL/6 mice (44).

\textit{P. berghei} shows a preference for reticulocyte invasion. Thus, we sought to test if CORM-2 was affecting erythropoiesis and consequently, the production of reticulocytes. If that would be the case, then the arrest in parasitemia could be easily explained by the unavailability of enough reticulocytes. In \textit{P. berghei} this preference has been postulated to be ~150-fold higher for reticulocytes than for mature RBCs, which were demonstrated to account for the reduced levels of reticulocytes, particularly at later stages of infection (186). As expected, in our experiments, infection led to a marked reduction in circulating reticulocytes at day 6 after infection. This decrease arises probably as a consequence of both hemolysis and insufficient erythropoiesis, observed during malaria infections (136), besides due to \textit{P. berghei} preference for reticulocytes. At day 6 p.i. the reticulocytemia showed small but significant differences between CORM-2 and DMSO-treated mice. We additionally calculated the reticulocyte index, which is a more appropriate measure of reticulocytosis, correcting for hematocrit values. Reticulocyte index did not show significant differences between mice receiving CORM-2 or DMSO vehicle, which
developed ECM (Fig. 12). These results suggest that CORM-2 treatment does not induce significant alterations in reticulocytosis and therefore, the parasitemia arrest observed possibly relies on a different explanation.

We further assessed the effect of CORM-2 in the hematological status of the treated mice. We observed an important reduction in hematocrit, consistent with a decrease in the number of circulating RBCs and the total concentration of Hb, in mice that were protected by CORM-2 administration (Fig. 13). The effect on hematocrit can result from an increased RBC destruction and/or removal of RBCs from circulation by the spleen, which can be conceived to lead to the observed arrest in parasitemia. In agreement with this, we observed larger spleens in the CORM-2 treated mice, as compared with the controls receiving DMSO, developing ECM, and the non-infected mice (Fig. 16). In fact, spleen has a crucial role during malaria infection, either by being a major site of damaged and infected erythrocyte clearance as well as a critical organ in the development of parasite-specific immune responses, through T and B cell activation/proliferation (192). Indeed, during P. falciparum infection, splenomegaly (abnormal spleen enlargement) is commonly observed and has been associated with an increased capacity of RBC filtration (36). Regarding the decrease in hematocrit, a hemolytic action of CORM-2 should not be excluded and should be clarified in the future. We should additionally say that the observed reduction in hematocrit could explain the reduced O₂Hb levels observed in the CORM-2 treated mice.

We also observed that there was significant increase in circulating monocytes and neutrophils upon CORM-2 treatment comparatively with the DMSO controls, while lymphocyte numbers were not significantly altered between these groups (Fig. 15). It is known that macrophages and neutrophils sequester in the brain microvasculature during cerebral pathology (72, 52). These cells contribute directly to the vascular congestion, where they are though to play an important role in attracting T cells to the brain through the release of chemokines. In fact, this initial involvement in the development of pathogenesis is suggested by the observation that depletion of macrophages or neutrophils early in infection prevents ECM onset (81, 82). Thereby, the increased presence of monocytes, which will differentiate in macrophages upon activation and recruitment to tissues, and neutrophils in circulation in mice treated with CORM-2 may
reflect a decreased recruitment of these cells to the inflamed tissues, namely to the brain, contrary to what occurs in the DMSO controls that develop ECM.

We did not characterize the splenic immune responses to infection that would be of significant interest. In fact, the spleen enlargement observed in the CORM-2 treated mice may reflect that CORM-2 treatment favors the retention of effector T cells in the spleen, which could enhance the anti-parasite immunity and explain the observed arrest in parasitemia. Supporting this view, a recent work demonstrates that likewise to CORM-2 treated mice, IP-10−/− mice are protected against ECM and show an arrest in parasitemia from day 5 p.i. to day 11 p.i. (46). Moreover, IP-10 deficiency confers a phenotype similar to that of CORM-2-treated mice, reducing leukocyte infiltration and iRBC sequestration in the brain microvasculature. Importantly, the parasitemia arrest observed in the IP-10−/− mice was correlated with an increased retention of T cells in the spleen and enhancement of their protective immune response against the parasite, particularly of CD4+ T cells. This led to the conclusion that suppression of T cell migration to the brain, resulting from lack of IP-10, reinforced the protective immune response mounted by the spleen over immunopathogenesis, resulting in parasite control. A similar process could be occurring with CORM-2 treatment since it also inhibits T cell recruitment to the brain and a similar arrest in parasitemia. Furthermore, we have shown that CO down-regulates IP-10 expression in the brain, and the same effect is presumable occurring with CORM-2 treatment. However, these hypotheses remain elusive and future work must be carried out to test them.

Whether the arrest in parasitemia could be contributing to the protection against ECM is not know. Nevertheless, a recent study indicates that this might not be the case. Baptista et al. (47, submitted) showed that treatment with an anti-malarial prior to the day of ECM onset strongly reduced the parasite in circulation, however did not protected mice from developing ECM later on, around day 14 p.i., when parasitemia was increased again. Although the experimental conditions are obviously not the same, this might elucidate that parasitemia control may be a consequence, as suggested before, than a cause of protection against ECM, during CORM-2 treatment.

Moreover, we also verified that the number of platelets in circulation showed no significant differences between CORM-2-treated mice and DMSO controls. Platelets
from CORM-2 treated group have significant smaller average volumes when compared with DMSO-treated mice, indicating that in CORM-2 protected mice there is a decrease in platelet aggregation (Fig 14). This effect is consistent with previous work showing that both CO and CORM-2 inhibit platelet aggregation (125, 157). Indeed, it is plausible that this effect is contributing to the protection against ECM, since platelets have been associated with both murine and human CM (95, 97). Among other processes, platelet aggregates associated with iRBCs (platelet-mediated clumping) may contribute to microvascular congestion during CM (33). Due to the potential pathogenic role of platelets, thrombocytopenia, which is commonly observed during human and experimental CM, has been suggested to make part of the host protective response against pathogenesis (99). Accordingly, we observed that thrombocytopenia is marked in all infected mice (Fig. 14a). These results suggests that CORM-2 protective effect against ECM relies not only in preventing neuroinflammation and parasite sequestration, but also in inhibiting hemostasis dysfunction, three processes invoked to contribute to CM (21).

Finally, we were also interested to investigate the effect of CORM-2 in RBC deformability. We observed that CORM-2 treatment inhibits the loss of deformability of RBCs during infection, comparatively to DMSO controls, at the day of ECM development in this later group (Fig. 17). The increased RBC deformability in CORM-2 treated mice might be playing a role in the CORM-2-associated protection against ECM. It is thereby important to repeat the assessment of RBC deformability to confirm the effect of CORM-2 in preventing loss of RBC deformability during infection.

Reduced RBC deformability is a strong predictor of human malaria severity (188, 189). It is proposed that an increased rigidity of both uninfected and RBCs significantly accounts for microvasculature congestion and contributes to impairment of microcirculatory blood flow (35). The loss of deformability has been suggested to result from oxidative damage of RBC membranes triggered by hemoglobin-derived products released into circulation during schizont rupture. In fact, it was shown that hemin directly induces RBC rigidity in a dose-dependent manner and supports the use of anti-oxidants as effective adjunctive therapy to prevent malaria mortality (190). Moreover, conversion of Hb to MetHb inside the RBC, elicited by ROS and NO produced during inflammation, leads to increased RBC rigidity (193). Indeed, CORM-2 treatment decreases MetHb levels in peripheral
Thereby CORM-2 enhancement of RBC deformability may reflect its capacity to ameliorate the pro-oxidant state that occurs during infection, and possibly contribute to the improvement of disease outcome.

7. Concluding Remarks and Future Work

In summary, the results obtained in the present work further revealed the mechanisms underlying CO protection against ECM and demonstrate that the administration of CO-releasing molecules, namely CORM-2, can mimic CO protection against the cerebral pathology in PbA-infected C57BL/6 mice. Our data strongly supports that CO protective effect against ECM is not only achieved by suppressing neuroinflammation and BBB disruption but also by inhibiting parasite sequestration in the brain. Moreover, inhibition of IP-10 mediated chemotaxis also seems to be implicated in CO protective effect in ECM.

CORM-2 administration reproduces the CO-induced protection observed in ECM, by reducing neuroinflammation, BBB disruption, parasite sequestration in the brain and MetHb formation, but does not induce the formation of COHb. Treatment with the CO donor is associated with an arrest in parasitemia. Its origin and importance for protection against ECM is not clear. Hemolysis due to CORM-2 molecule might be occurring in association with enhanced removal of RBCs by the spleen. It could also be that increased retention of T cells in the spleen improve anti-parasite immune responses and control parasite load. CORM-2 also limits platelet aggregation and increases RBC deformability and these effects are likely related with its protective action.

Altogether, the results indicate that CO protective mechanisms observed during *P. berghei* infection involve the reestablishment of hemostasis equilibrium and prevention of oxidative stress. Moreover, the results obtained with CORM-2 sustain that a potential CO-based therapy could be applied, circumventing the clinical toxicity of CO inhalation. Indeed, we have preliminary results showing that an improved version of CORM-2, with the advantage of being water soluble, prevents ECM development as well, but does not seem to affect parasite load. This molecule could constitute another interesting candidate for future pharmaceutical purposes.
In future work, we would like to understand the relevance of splenic immune responses and chemotaxis processes as well as the regulation of hemostasis in CORM-2 treatment during ECM. Furthermore, we would like to test different CO-RMs drug candidates, organometallic and organic compounds, in ECM as well as in other models of severe malaria such as the rodent model of malaria-associated respiratory distress syndrome.
8. Materials and Methods

8.1. Mice
C57BL/6 and BALB/c wild-type mice were bred in the pathogen-free facilities of the Instituto Gulbenkian de Ciência and housed in the pathogen-free facilities of the Instituto de Medicina Molecular. All protocols were approved by and conducted according to the Animal Care regulations of the Direcção Geral de Veterinária.

8.2. Parasites, infection and disease assessment
We used red blood cells infected with green fluorescent protein (GFP)-transgenic *P. berghei* ANKA (185), luciferase-transgenic *P. berghei* ANKA clone 676m1cl1 (194), which expresses luciferase constitutively during the whole life cycle, or *P. berghei* ANKA clone 354cl4, which expresses luciferase in schizonts (44) to infect mice. C57BL/6 or BALB/c mice with 6- to 8-week-old were infected by intraperitoneal (i.p.) injection of $10^6$ infected red blood cells (iRBCs), except in experiments using CO-treated mice infected with *P. berghei* ANKA clone 676m1cl1, where $10^5$ iRBCs were inoculated or CORM-2 treated mice infected with *P. berghei* ANKA clone 354cl4, were $5 \times 10^5$ iRBCs were inoculated. Infected mice were monitored daily for clinical symptoms of experimental cerebral malaria (ECM) including hemi- or paraplegia, head deviation, tendency to roll over on stimulation, ataxia and convulsions. Mice showing severe signs of ECM at day 5, 6 or 7 post-infection (p.i.) were sacrificed. Parasitemia was assessed by flow cytometry for mice infected with GFP-expressing *P. berghei* ANKA, using tail blood, as previously described (GFP ref.). For mice infected with luciferase-expressing *P. berghei* ANKA clone 676m1cl1 or *P. berghei* ANKA clone 354cl4 parasitemia was determined by Giemsa-stained blood smears, using tail blood, followed by microscopic counting. Mean parasitemia is expressed as percentage of infected red blood cells. Survival is expressed as percentage.

8.3. CO exposure
Mice were placed in a gastight 60-liter capacity chamber and exposed to CO at 250 p.p.m. for 24h between day 2 and 3 p.i., as described before (114). In brief, 1% CO (Aga Linde) was mixed with air in a stainless steel cylinder to obtain a final concentration of 250 p.p.m. CO was provided continuously at a flow rate of ~ 12 liter/min. We monitored CO concentration using a CO analyzer (Interscan Corporation).
8.4. Visualization and quantification of bioluminescence in organs ex vivo

Bioluminescence assessment of mice organs was performed for *P. berghei* ANKA-infected C57BL/6 mice when ECM symptoms developed (day 5-6 p.i.) and in *P. berghei* ANKA-infected C57BL/6 mice treated with CO or CORM-2 later on (d6-12 p.i.) in order to confirm that mice would not develop ECM. Bioluminescence was visualized and quantified in using an intensified-charge-coupled device (I-CCD) photon-counting video camera from the *in vivo* Imaging System IVIS Lumina (Xenogen), as described previously (44). The animals were injected i.p. with D-luciferin Firefly Potassium Salt (Caliper Life Sciences) dissolved in D-PBS 1x (Dulbecco’s Phosphate-Buffered Saline, without Ca2+ and Mg2+; Invitrogen) (150 mg/kg of body weight) and 10 min. after mice were sacrificed by CO2 inhalation and perfused intracardially with 10 ml of PBS 1x (Phosphate-Buffered Saline; Sigma-Aldrich) to remove cells in circulation. Organs were dissected, washed in PBS 1x and imaged immediately, with a 15-cm field of view, a small binning factor, and exposure times of 15 or 20 sec, for all organs, and 300 sec for the brains. Bioluminescence in the individual organs was quantified by using the software Living Image (Xenogen), applying a region-of-interest adjusted to the organs shape. The results are shown as mean average radiance (photons per second per cm2 per steradian; p/s/cm2/sr; x 10^4 or x 10^6).

8.5. Quantitative Real-Time PCR

Mice developing were sacrificed by CO2 inhalation and intracardially perfused with 10ml of PBS 1x. Brains were removed, minced and total RNA was extracted by using TRIzol (Invitrogen) and subsequently an RNAeasy Mini Kit (Quiagen) accordingly to the manufacturer protocols. RNA was converted to cDNA by a Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer instructions in a MyCycler thermocycler (BioRad). For quantitative PCR, cDNA was amplified in a total reaction volume of 25 µl with half of the volume (12.5 µl) of *Power* SYBR Green PCR Master Mix (Applied Biosystems) in a ABI Prism 7000 Sequence Detection System and by ABI Prism SDS 7000 software (Applied Biosystems). Each sample was assayed in duplicate. Thermal cycler program consisted in one cycle of 2 min at 50°C, followed by one cycle of 10 min at 95°C, 45 cycles of 15 sec at 95°C and 1 min at 60°C. Parasite brain accumulation/sequestration and neuroinflammation was assessed by the quantification of mRNA expression of *P. berghei* 18S ribosomal RNA (*r18S*) gene and several inflammatory genes related with ECM, respectively. The primer (Thermo Scientific)
sequences used are as follows: HPRT: Fwd: 5’–GTA ATG ATC GTC AAC GGG GGA C–3’; Rev: 5’–CCA GCA AGC TTG CAA CCT TAA CCA–3’; P. berghei r18S: Fwd: 5’–AAG CAT TAA ATA AAG CGA ATA C … TAC–3’; Rev: 5’–GGA GAT TGG TTT TGA CGT TTA TGT G–3’; CD3: Fwd: 5’–TCT CGG AAG TCG AGC ACA GT–3’; Rev: 5’–ATC AGC AAG CCC AGA GTG AT–3’; CD8 β: Fwd: 5’- GCT GGT AGT CTG CAT CCT GCT TC–3’; Rev: 5’–TTG GTA GCA GGC TAT CAG TGT TGT G-3’; Granzyme B: Fwd: 5’- CTA AAG CTG AAG AGT AAG GCC AAG-3’; Rev: 5’–GAT CCT TCT GTA CTG TCA GCT CAA-3’. CXCR3: Fwd: 5’ AAT GCC ACC CAT TGC CAG TAC-3’; Rev: 5’–AGC AGT AGG CCA TGA CCA GAA G-3’; IP-10: Fwd: 5’–GAC GGT CCG CTG CAA CTG-3’; Rev: 5’–GCT TCC CTA TGG CCC TCA TT-3’. RNA levels were normalized to the expression of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (hprt). Standard curves were obtained by serial dilutions of brain cDNA. Relative quantification of mRNA expression was determined using the Pfaffl method (195). The results are shown as percentage of mRNA mean expression in C57BL/6 P. berghei-ANKA infected mice, which developed ECM.

8.6. CO-releasing molecules administration
Tricarbonyldichlororuthenium (II) dimer (CORM-2) (Sigma-Aldrich) and ALF466 (ALFAMA) were solubilized in 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich) in PBS 1x. CORM-2 solution (10mg/kg or 20mg/kg of body weight) was administered intravenously (i.v.) according to the chosen schedules. ALF 466 was administered i.v. at equimolar concentrations relatively to CORM-2. A solution of 10% DMSO in PBS 1x administered i.v. was used as a vehicle control solution. The concentrations of CORM-2 used in the present study were based on previous reports (159, 171).

8.7. Quantification of $O_2$Hb, and MetHb and COHb
Blood was collected from mice tail in capillary tubes (VWR) with heparin (100 i.u./ml in PBS 1x; LEO Pharma Inc.) and transferred into AVOXimeter 4000 cuvettes (ITC) where the levels of carboxyhemoglobin (COHb), oxyhemoglobin ($O_2$Hb) and methemoglobin (MetHb) were measured in a portable AVOXimeter 4000 CO-oximeter (ITC). Results are shown as mean percentage of total hemoglobin species in circulation.
8.8. BBB permeability

Blood-Brain Barrier (BBB) permeability was assessed using the Evan’s blue technique, fundamentally as described before (196). Briefly, mice were i.v. with 0.2 ml of PBS 1–2% Evans blue (EB) (Sigma) when clinical symptoms of ECM were observed in mice treated with DMSO vehicle solution. Mice were killed 1 h thereafter, and brains were weighed and placed in 2 ml formamide (Merck) for 24 h at 37 °C to extract EB dye from the tissue. Absorbance was measured at $\lambda = 620$ nm (EB absorbance) in a DU 70 Spectrophotometer (Beckman). Evans blue concentration was calculated using a standard curve of EB dye. The data are expressed as mg of Evans blue per g of brain tissue.

8.9. Flow cytometry analysis

Blood from mice infected with *P. berghei* ANKA GFP-expressing parasites was collected from the tail into tubes with heparin (100 i.u./ml in PBS 1x).

To differentiate parasite blood stages, 10 µl of blood suspension was stained with the dsDNA vital dye Hoechst-33342 (Invitrogen) diluted in PBS 1x, for 30 min, at room temperature, in the dark, followed by FACS analysis. Red blood cells (RBCs) were selected on the basis of size (forward-scatter, FSC) and granulosity (side-scatter, SSC). Parasite ring forms (R) were gated on the Hoechst$^{\text{low}}$ GFP$^{\text{low}}$ RBC population. Trophozoites and schizonts (T/S) were gated on the Hoechst$^{\text{high}}$ GFP$^{\text{int}}$ RBCs and gametocytes (G) on the Hoechst$^{\text{int}}$ GFP$^{\text{high}}$ RBC population, based on previous work (183). low, int, high correspond to low, intermediate or high fluorescence intensity, respectively. Infected RBCs (iRBCs) were gated on the Hoechst$^{+}$ GFP$^{+}$ RBC population. Proportion of parasite blood stages are shown as mean percentage of total iRBCs.

To determine reticulocytemia and reticulocyte number, blood cells were stained for CD71 and CD45. CD71 corresponds to the transferrin receptor, which is expressed in reticulocytes but also in activated leukocytes. For this reason a staining for CD45, a tyrosine phosphatase expressed in all leukocytes, was also included to discriminate these cells. First, 2 µl of blood suspension were washed (200 µl of PBS 1x supplemented with 2% fetal bovine serum (FBS; Invitrogen)) in a 96-well plate, centrifuged (7 min, 4°C, 453 g) and supernatant discarded. Cells were incubated in 50 µl of FcBlock anti-mouse CD16/CD32 (clone 93; eBioscience) for 20 min, on ice, to block unspecific binding of antibodies to cells. Cells were further washed (150 µl PBS 1x with 2% FBS), centrifuged (7 min, 4°C, 453 g) and supernatant was discarded. Then, cells
were incubated with 50 µl of a mix of phycoerythrin (PE)-conjugated anti-mouse CD71 (clone RI7 217.1.4, Caltag) and allophycocyanin(APC)-conjugated anti-mouse CD45.1 (clone A20; eBioscience) antibodies, diluted in PBS 1x with 2% FBS, for 20 min, on ice, in the dark. After that, cells were washed (150 µl PBS 1x with 2% FBS), centrifuged (7 min, 4ºC, 453 g), resuspended (200 µl PBS 1x with 2% FBS) and analysed in the FACS. Cell number was determined using latex beads (10 µm; Beckman Coulter). Briefly, 2 µl of blood was diluted in 480 µl PBS 1x supplemented with 2% FBS, followed by addition of 20 µl of latex beads and analyses of the mixture in the FACS. As the concentration of beads is known and RBCs are acquired at the same time, it is possible to determine the number of RBCs equivalent to a certain number of beads, which, in turn, will indicate the correspondent volume acquired. This allows the determination of RBCs per volume (ml). RBCs were selected on the basis of size and granulosity (FSC and SSC) and additionally gated on the CD45⁻ population. Then, reticulocytes were gated in the CD71⁺ RBC population. Results are shown as mean percentage of reticulocytes in the total RBC population and mean absolute number of parasitized reticulocytes (CD71⁺GFP⁺ RBCs) per ml of blood (x10⁷). Reticulocyte index (%) was calculated using the formula: reticulocytes (%) x (hematocrit/hematocrit of uninfected mice). Hoechst stain and indicated antibodies were used at saturating concentrations. FACS analysis was performed on a FACS-Calibur instrument run with the CellQuest program (Becton and Dickinson). Data analysis was performed with FlowJo software (Tree Star).

8.10. Hematocrit and Hemogram

Mice were sacrificed by CO₂ inhalation, an blood was collected from the heart with a U-100 insulin 1 ml syringe (Terumo) with 20 µl heparin (100 i.u./ml in PBS 1x) and transferred to a 1 ml tube with EDTA (Normax). Samples were analysed by DNAtech. Hematocrit is shown as mean percentage of RBC volume in the total blood volume; RBC number is indicated as the mean number of RBCs per ml of blood (x 10⁹) and hemoglobin concentration is shown as mean g of hemoglobin per dl of blood. Platelet quantity is expressed as number of platelets per ml of blood (x 10⁶) and average volume of platelets is shown as mean µm. Results for absolute cell count of monocytes, neutrophils and lymphocytes are shown as mean number per ml of blood (x 10⁶)
8.11. RBC deformability
Mice were sacrificed by CO$_2$ inhalation and blood was collected from the heart to tubes with EDTA. Red blood cell deformability was measured as described previously (197). Briefly, deformability at different shear stress (0.30, 0.60, 1.20, 3.0, 12.0, 30.0, 60.0 Pascal) was determined by using the Rheodyn SSD shear stress diffractometer from Myrenne GMBH (Roentgen). RBC deformability is expressed as the elongation index (EI) in percentage. Rheodyn SSD diffractometer determines RBC deformability by simulating shear forces exerted by blood flow and vascular walls on erythrocytes.

8.12. Statistical analysis
Kolmogorov-Smirnov test was applied to confirm normal distribution of data. An unpaired Student’s T test was then applied to evaluate statistical significant differences between mean values. P < 0.05 was considered significant.
9. References

47. Baptista, F.G. et al. The recruitment of Plasmodium-infected red blood cells in the brain is crucial for the development of cerebral malaria in mice (submitted).


