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ESTUDO DOS MECANISMOS ASSOCIADOS À DISFUNÇÃO COGNITIVA EM MODELO MURINO DE MALÁRIA CEREBRAL

Aline Silva de Miranda

Belo Horizonte

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Hermann Hesse- Demian

RESUMO

A malária é a principal e a mais grave doença parasitária da humanidade. A Malária cerebral (MC) tem sido definida como uma encefalopatia aguda, difusa, potencialmente reversível, caracterizada por coma e presença de formas assexuadas do *P. falciparum* em esfregaço sanguíneo, com exclusão de outras causas de encefalopatias. Apresenta uma fisiopatologia complexa e pouco elucidada, na qual alterações vasculares, imunológicas e metabólicas têm sido descritas. A taxa de mortalidade é alta e 10 a 20% daqueles que sobrevivem permanecem com transtornos cognitivos e comportamentais mesmo após a efetiva resolução da infecção por antimaláricos. Nesse contexto, estudos que investiguem os mecanismos celulares e moleculares envolvidos na patogênese da MC, bem como a eficácia de terapias adjuvantes em melhorar o prognóstico da MC são urgentemente recomendados. No presente estudo, utilizando o modelo experimental de MC por *Plasmodium berghei* ANKA (PbA), demonstramos que o aumento da concentração de citocinas inflamatórias (IL-6, IFN-γ e TNF-α) e da quimiocina CCL11 no hipocampo alterou a expressão de fatores neurotróficos, reduzindo a neurogenêse e induzindo a morte celular nessa região, o que contribuiu para os déficits cognitivos observados durante a fase aguda da infecção. A administração de uma única dose do antimalárico artesunato (32mg/kg) foi capaz de atenuar significativamente a produção de citocinas inflamatórias no hipocampo e no córtex frontal no quinto dia após a infecção, promovendo melhora significativa na sobrevida e nos sinais clínicos típicos da MC. O tratamento com o MK801 (0.5mg/kg), um antagonista de receptores glutamatérgicos do tipo NMDA, preveniu o desenvolvimento de transtornos cognitivos e comportamentais após a resolução da infecção com o antimalárico cloroquina (30mg/kg). Adicionalmente, o MK801 foi capaz de modular a resposta inflamatória TH1/TH2 e induzir a produção de fatores neurotróficos no hipocampo e no córtex frontal, conferindo assim um efeito neuroprotetor. Dessa forma, este estudo forneceu evidências sobre o papel dos mediadores inflamatórios em regular eventos como a neurogênese e a morte celular no hipocampo durante a fase aguda da MC que contribuem significativamente para o desenvolvimento dos déficits cognitivos. A ocorrência de sequelas cognitivas e comportamentais após a resolução do processo infeccioso depende da interação entre os sistemas imune e de neurotransmissores, indicando que intervenções direcionadas a modulação do sistema glutamatérgico podem constituir potenciais adjuvantes terapêuticos ao tratamento antimalárico.

Palavras-chave: Malária; Malária Cerebral; Transtornos cognitivos; Alterações comportamentais; Artesunato; Cloroquina; Glutamato; MK801; Citocinas; Fatores neurotróficos.

ABSTRACT

Malaria is the main and most serious parasitic disease of mankind. Cerebral Malaria (CM) has been defined as a potentially reversible diffuse encephalopathy characterized mainly by coma and the presence of asexual forms of P. falciparum parasites in peripheral blood smears in the absence of other causes of encephalopathy. This condition presents a complex and incompletely understood pathogenesis, in which vascular, immunological and metabolic changes have been described. The mortality rate is high and 10 to 20% those who survived remain with cognitive and behavioral deficits even after a successful resolution of infection by antimalarial compounds. In this scenario, studies to investigate the cellular and molecular mechanisms underlying CM pathogenesis as well as the efficacy of adjunctive therapies in improve CM outcome are urgently warranted. In the present study, using the *Plasmodium berghei* ANKA (PbA) model of CM, we demonstrated that the enhancement in inflammatory cytokines (IL-6, IFN-γ e TNF-α) and chemokine (CCL11) levels in the hippocampus influenced neurotrophic factors expression reducing neurogenesis and inducing cell death in this region contributing to the cognitive impairment observed during the acute phase of infection. A single dose of the antimalarial artesunate (32mg/kg) was able to significant attenuate the production of inflammatory cytokines in the hippocampus and frontal cortex on day 5 post-infection promoting a significant improve of survival and CM clinical signs. Furthermore, MK801 (0.5mg/kg), a non-competitive NMDA receptors antagonist, prevented cognitive and behavioral impairment following infection resolution by the antimalarial chloroquine (30mg/kg). Additionally, MK801 was also able to modulate the TH1/TH2 inflammatory response and to induce neurotrophic factors release in the hippocampus and frontal cortex exerting a neuroprotective effect. The current study provided further evidence regarding inflammatory mediators role in regulating neurogenesis and cell death in the hippocampus during the acute phase of CM, contributing significantly for cognitive deficits development. Cognitive and behavioral sequelae following infection resolution depends on immune and neurotransmitter system interactions indicating that interventions targeted modulation of the glutamatergic system may constitute potential therapeutic adjuvants to antimalarial treatment.

Keywords: Malaria; Cerebral Malaria; Cognitive deficits; Behavioral Changes; Artesunate; Chloroquine; Glutamate; MK801; Cytokines; Neurotrophic factors.

LISTA DE ABREVIATURAS E SIGLAS

AMPA – α-amino-3-hidroxi-5-metil-4-isoxazolpropiónico

ANOVA – Análise de variância

BHE – Barreira hematoencefálica

BSA – Soralbumina bovina (*Bovine Serum Albumine*)

Ca^{2+} – Cálcio

CEBIO – Centro de Bioterismo

CETEA – Comitê de Ética em Experimentação Animal

CSA – Sulfato de condroitina A

DP – Desvio padrão

dpi – Dias pós-infecção (*days post-infection*)

EDTA – Ácido etil-diamino-tetra-acético

ELISA – Ensaio imunoenzimático (*Enzyme Linked Immuno Sorbent Assay*)

HE – coloração Hematoxilina-Eosina

HPLC – Cromatografia líquida de alta eficiência

H_2O_2 – Peróxido de Hidrogênio

H_2SO_4 – Ácido sulfúrico

ICAM – Molécula de adesão intercelular (*Intercellular adhesion molecul*e)

IFN-γ – Interferon gamma

KCl – Cloreto de potássio

IL-1β – Interleucina 1

IL-4 – Interleucina 4

IL-10 – Interleucina 10

iNOS – Óxido nítrico sintase induzível

i.p. – Intraperitoneal

LPS – Lipopolissacarídeo

M – Molar

MC – Malária cerebral

μL – Microlitro

mg – Miligrama

mM – Milimolar

NaCl – Cloreto de sódio

Na_2HPO_4 – Fosfato dissódico

NK – Célula *Natural Killer*

nm – Nanômetro

NMDA – N-metil-D-aspartato

NO – Óxido nítrico

OMS – Organização Mundial de Saúde

OPD – O-fenilenodiamina

PbA – *Plasmodium berghei* ANKA

PBS – Tampão fosfato de sódio (*Phosphate buffered saline*)

PECAM-1 – Molécula de adesão celular plaquetária/endotelial-1

PfEMP1 – Proteína 1 de membrana do eritrócito do *P. falciparum*

PMSF – Fluoreto de fenilmetilsufonila

r.p.m. – Rotações por minuto

SHIRPA – SmithKline Beecham Pharmaceuticals; Harwell MRC Mouse Genome Centre and Mammalian Genetics Unit Imperial College School of Medicine at St Mary's Royal London Hospital; St Bartholomew's; Royal London School of Medicine; Phenotype Assessment

SNC – Sistema Nervoso Central

Th1 – Linfócito T *helper* tipo 1

Th2 – Linfócito T *helper* tipo 2

TNF- α – Fator de necrose tumoral alfa

VCAM-1 – Molécula de adesão celular vascular-1

SUMÁRIO

1- APRESENTAÇÃO

O presente texto tem como objetivo apresentar os resultados obtidos durante o doutorado, no qual foram avaliados possíveis mecanismos relacionados ao desenvolvimento de alterações comportamentais e cognitivas durante a fase aguda da malária cerebral (MC) experimental e após a resolução da infecção. O efeito de antagonistas de receptores de glutamato do tipo NMDA como potencial terapia adjuvante ao tratamento antimalárico na prevenção de sequelas cognitivas e comportamentais resultantes da MC, também foi investigado.

A tese foi estruturada em sete partes principais: i) Introdução ii) revisão da literatura; iii) Objetivos; iv) Métodos; v) Resultados, sob a forma de artigos publicados e dados adicionais ainda não publicados; vi) Discussão; vii) Anexos, contendo a descrição do trabalho realizado durante estágio sanduíche na *Cleveland Clinic* (Estados Unidos), bem como artigos publicados durante o doutorado, mas não relacionados diretamente a tese.

A revisão da literatura neste trabalho contém o artigo intitulado "*Improving cognitive outcome in cerebral malaria: insights from clinical and experimental research*", publicado na revista *Cent Nerv Syst Agents Med Chem (*Miranda et al., 2011). Trata-se de revisão da literatura sobre alterações cognitivas e comportamentais na MC e possíveis terapias imunomoduladoras e neuroprotetoras adjuvantes ao tratamento antimalárico.

No artigo 1 intitulado "*Evidence for the contribution of adult neurogenesis and hippocampal cell death in experimental cerebral malaria cognitive outcome*" publicado na revista *Neuroscience* (Miranda et al., 2015), demonstramos que alterações cognitivas na fase aguda da MC estão associadas a um desequilíbrio na produção de citocinas inflamatórias e fatores neurotróficos no córtex frontal e no hipocampo. Esse

desequilíbrio acarretaria redução da neurogênese no giro denteado e aumento da morte neuronal no hipocampo, regiões relacionadas a funções mnemônicas.

No artigo 2 intitulado "*Further evidence for an anti-inflammatory role of artesunate in experimental cerebral malaria*" publicado na revista *Malaria Journal* (Miranda et al., 2013), demonstramos que uma única dose do antimalárico artesunato foi capaz de diminuir significativamente a carga parasitária, reduzir a concentração de citocinas inflamatórias em regiões associadas a funções cognitivas como o córtex frontal e o hipocampo, amenizar a gravidade dos sintomas decorrentes da MC e reduzir a mortalidade.

No artigo 3 intitulado "*A neuroprotective effect of glutamate receptor antagonist MK801 in experimental cerebral malaria long-term cognitive and behavioral outcomes*", em preparação para submissão, demonstramos que o MK801, antagonista do receptor de glutamato do tipo NMDA, associado ao antimalárico cloroquina foram capazes de prevenir as sequelas cognitivas e comportamentais resultantes da MC.

2- INTRODUÇÃO

A malária é a principal e a mais grave doença parasitária da humanidade. Considerada uma condição negligenciada e um relevante problema de saúde pública em mais de 90 países, estima-se que aproximadamente 40% da população mundial está sob risco de transmissão da doença (Kappe *et al.*, 2010). No ano de 2013 foram registrados 198 milhões de casos em todo o mundo, dos quais 584 mil evoluíram para o óbito, sendo em sua maioria (78%) crianças menores de cinco anos de idade (Organização mundial de saúde 2014).

Na América Latina, o maior número de casos (99%) tem sido registrado na Amazônia brasileira, sendo que apenas no ano de 2013, o Brasil registrou 179.340 casos de malária (Organização mundial de saúde 2014). Além dos problemas associados à saúde, têm sido descritos importantes prejuízos sociais e econômicos em regiões endêmicas (Sachs and Malaney, 2002).

Transmitida pela fêmea do mosquito *Anopheles* infectado, a malária é causada pelo protozoário do gênero *Plasmodium*. Clinicamente manifesta-se por meio de uma diversidade de sintomas que podem ser mais ou menos graves dependendo de fatores associados ao parasito e ao hospedeiro, além de questões geográficas e sociais (Miller *et al.*, 2002). Nas infecções por *P. vivax*, *P. malariae* e *P. ovale*, predomina a forma não complicada da doença, caracterizada por febre intermitente, sudorese, mal estar e vômitos. As infecções por *P. falciparum* podem levar à forma complicada denominada malária grave, considerada uma doença multissistêmica capaz de afetar diretamente o sistema nervoso central (SNC), causando a malária cerebral (MC), além de anemia grave, insuficiência renal aguda, edema pulmonar, colapso circulatório e acidose metabólica (Mung'Ala-Odera *et al.*, 2004). Evidências recentes têm demonstrado que não apenas as infecções por P. *falciparum*, mas também aquelas causadas pelo *P. vivax*

podem afetar o SNC, acarretando prejuízos nas funções cognitivas de crianças residentes em regiões endêmicas como a Amazônia brasileira e o Siri Lanka (Fernando *et al.*, 2006; Vitor-Silva *et al.*, 2009).

De acordo com a Organização Mundial de Saúde (2000), a MC pode ser definida como uma encefalopatia aguda, difusa, potencialmente reversível, caracterizada por coma e presença de formas assexuadas do *P. falciparum* em esfregaço sanguíneo, com exclusão de outras causas de encefalopatias, principalmente meningite bacteriana e encefalites virais. Constitui a principal e a mais grave complicação resultante da infecção pelo *P. falciparum* (Idro *et al.*, 2005; Mung'Ala-Odera *et al.*, 2004). Estima-se que apenas no ano de 2013 ocorreram 455.520 mil óbitos em decorrência dessa condição (Organização mundial de saúde 2014). Além disso, é fundamental ressaltar que 10 a 20% dos que sobrevivem ao quadro de MC permanece com algum déficit cognitivo e/ou comportamental (Boivin *et al.*, 2007; Carter *et al.*, 2005; John *et al.*, 2008). Em adição, prejuízos sociais, econômicos e educacionais significativos têm sido descritos em áreas endêmicas como resultado das alterações cognitivas e comportamentais que permanecem após a resolução da MC (Fernando *et al.*, 2010).

A fisiopatologia da MC é complexa e envolve múltiplos mecanismos celulares e moleculares ainda pouco compreendidos (Hunt *et al.*, 2006; van der Heyde *et al.*, 2006). As alterações metabólicas causadas pelo bloqueio mecânico do fluxo sanguíneo cerebral decorrente do sequestro de eritrócitos parasitados, leucócitos e plaquetas na microvasculatura e a ativação do sistema imune em resposta à infecção, com consequente liberação de mediadores inflamatórios (citocinas e quimiocinas) pelo hospedeiro, têm sido descritos como os principais fatores envolvidos no desenvolvimento da MC (van der Heyde *et al.*, 2006).

O modelo experimental utilizando roedores é bem caracterizado e útil para a pesquisa da MC. A maioria dos estudos provém do modelo de infecção por *P. berghei* ANKA (PbA), que apresenta uma divisão clara entre linhagens de camundongos resistentes (BALB/c e A/J) e susceptíveis (C57Bl/6 e CBA). Há evidências de que os camundongos susceptíveis apresentam significativas alterações estruturais cerebrais, como ativação de células endoteliais e micróglia (de Souza and Riley, 2002; Desruisseaux *et al.*, 2008; Lackner *et al.*, 2006), e desenvolvem sinais neurológicos típicos da MC humana como paralisia, convulsão e coma, evoluindo para o óbito (Hunt and Grau, 2003).

Em um importante estudo conduzido por Lackner et al. (Lackner *et al.*, 2006) foram investigadas, por meio de uma bateria padronizada de testes (SHIRPA), diferentes alterações comportamentais e neurológicas em camundongos da linhagem C57BL/6 no 6º dia após a infecção com PbA. Utilizando a mesma bateria de testes, em estudo conduzido pelo nosso grupo, Lacerda-Queiroz et al. (Lacerda-Queiroz *et al.*, 2010) demonstraram que o aumento de mediadores inflamatórios (citocinas e quimiocinas) no tecido cerebral de camundongos infectados com PbA antecedeu as alterações neurológicas e comportamentais observadas. Demonstramos também, pela primeira vez, que alterações histopatológicas, incluindo sequestro de leucócitos na microvasculatura cerebral, estão associadas à produção exacerbada de citocinas inflamatórias no SNC, em especial IL-1β e TNF-α, durante a infecção, e contribuem para o desenvolvimento de sintomas específicos como ansiedade (de Miranda *et al.*, 2011). Corroborando achados prévios, camundongos que desenvolveram MC após infecção com a cepa PbA apresentaram déficits cognitivos significativos associados a áreas de inflamação e hemorragia em diferentes regiões cerebrais (Desruisseaux *et al.*, 2008).

A fisiopatologia dos déficits cognitivos associados à MC permanece pouco esclarecida. Nesse contexto, no presente trabalho tivemos como um dos objetivos testar a hipótese de que prejuízos de memória na MC estariam associados a desequilíbrio na produção de citocinas inflamatórias e fatores neurotróficos em regiões associadas a esta função cognitiva. Esse desequilíbrio acarretaria, por sua vez, redução da formação de novos neurônios na região do giro denteado do hipocampo, fenômeno conhecido como neurogênese, bem como aumento da morte de neurônios hipocampais, contribuindo para o desenvolvimento dos déficits cognitivos.

A MC sem tratamento constitui uma condição invariavelmente fatal (Mishra and Newton, 2009). Estudos têm demonstrado que o artesunato, composto semissintético derivado da artemisinina e considerado o fármaco antimalárico mais eficiente e seguro para o tratamento de pacientes com malária grave e resistentes à cloroquina, apresenta ação anti-inflamatória em diferentes condições patológicas como sepse, artrite, lúpus eritematoso sistêmico e asma (Cheng *et al.*, 2011; Ho *et al.*, 2012; Jin *et al.*, 2009; Li *et al.*, 2013). A administração de uma única dose de artesunato reduziu significativamente a parasitemia e preveniu o desenvolvimento de sintomas graves e a mortalidade associados aos últimos estágios da MC após a infecção por PbA (Clemmer *et al.*, 2011). A ação imunomoduladora do artesunato no SNC não foi previamente investigada durante o curso da MC. Assim, no presente estudo testamos a hipótese de que uma única dose de artesunato, além do efeito antiparasitário, poderia modular a resposta inflamatória no SNC, prevenir o desenvolvimento de alterações neurológicas e comportamentais e reduzir a mortalidade decorrente da infecção pelo PbA.

A permanência de déficits cognitivos mesmo após um tratamento antimalárico efetivo tem sido descrita em estudos recentes utilizando o modelo experimental de MC

(Dai *et al.*, 2010; Reis *et al.*, 2010). A permanência das alterações cognitivas decorrentes da MC pode estar associada a uma variedade de eventos metabólicos e patofisiológicos que persistem mesmo com o fim do quadro infeccioso. A ausência de déficits neurológicos em modelos sistêmicos de malária, após a eliminação do parasita pela administração de cloroquina, indica que o desenvolvimento desses transtornos decorrentes da MC está associado a alterações específicas no SNC e não apenas a resposta inflamatória sistêmica ou a presença do parasita (Reis *et al.*, 2010). Em estudo prévio desenvolvido pelo nosso grupo, demonstramos o envolvimento do sistema glutamatérgico, na patogênese da MC (Miranda *et al.*, 2010). No presente trabalho investigamos o efeito do antagonista não competitivo do receptor ionotrópico de glutamato do tipo N-metil-D-aspartato (NMDA), o MK801, como terapia adjuvante ao tratamento antimalárico no desenvolvimento de sequelas cognitivas e comportamentais decorrentes da MC.

Diante do exposto, o presente estudo investigou os mecanismos envolvidos no desenvolvimento de alterações cognitivas e comportamentais durante a fase aguda e após a resolução da MC experimental, bem como o efeito de antimaláricos e antagonistas de receptores de glutamato na prevenção dessas alterações.

3- REVISÃO DA LITERATURA

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Improving Cognitive Outcome in Cerebral Malaria: Insights from Clinical and Experimental Research

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Improving Cognitive Outcome in Cerebral Malaria: Insights from Clinical and Experimental Research

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Abstract: Cerebral Malaria (CM) is a clinical syndrome defined by the World Health Organization (WHO) as a potentially reversible diffuse encephalopathy characterized mainly by coma and the presence of asexual forms of *Plasmodium falciparum* parasites in peripheral blood smears in the absence of other causes of encephalopathy. A wide range of clinical manifestations follows the disease including cognitive, behavioral and motor dysfunctions, seizures and coma. The underlying mechanisms of CM pathogenesis remain incompletely understood although vascular, immunological and metabolic changes have been described. The classical treatment of CM is based on the administration of antimalarial drugs, especially chloroquine and artemisinin derivates as artesunate. Even with treatment, 15 to 20% of children with CM die and approximately 10 to 17% of those who survive remain with significant long-term cognitive impairment. In this context, neuroprotective and adjuvant therapies have been recently investigated in clinical and experimental studies of CM in an attempt to improve cognitive outcome. A poor understanding of pathophysiological mechanisms, properties of compounds used and patient selection have contributed to the lack of success of these interventions. This review discusses clinical aspects of cognitive sequelae, possible mechanisms involved in the brain injury, perspectives and limitations regarding the pharmacological strategies to improve cognitive outcome in CM.

Keywords: Adjuvant therapies, cerebral malaria, cognitive impairment, pharmacological strategies, neuroprotective interventions, malaria.

CEREBRAL MALARIA

Cerebral malaria (CM) is a severe complication resulting from *Plasmodium falciparum* infection [1]. According to the World Health Organization (WHO) criteria, CM is a clinical syndrome defined as a potentially reversible diffuse encephalopathy characterized mainly by coma and the presence of asexual forms of *P. falciparum* parasites in peripheral blood smears in the absence of other causes of encephalopathy, especially meningitis and viral encephalitis [2]. This condition is associated with at least 2.3 million deaths annually, from an estimated 400 million cases of malaria each year worldwide, and is the leading cause of hospitalization, mortality, and morbidity of children under 5 years of age in sub-Sahara Africa [3].

A wide range of clinical manifestations occurs in CM including cognitive, behavioral and motor dysfunctions, seizures and coma. Without treatment, this condition is invariably fatal [1]. However, even with appropriate antimalarial treatment, 15 to 20% of children with CM die and approximately 10 to 17% of those who survive remain with significant long-term cognitive impairment [4]. In addition, a significant social, economic and educational burden has been reported in endemic areas as a consequence of cognitive impairment following CM [5].

This review discusses clinical aspects of cognitive sequelae, possible mechanisms of brain injury, perspectives and limitations regarding the pharmacological strategies to improve cognitive outcome in CM.

PATHOPHYSIOLOGICAL MECHANISMS OF CEREBRAL MALARIA

The underlying mechanisms of CM pathogenesis remain incompletely understood although vascular, immunological and metabolic changes have been described [6]. Two main nonexclusive theories/hypothesis have been widely accepted to explain the pathological process of CM: i) the sequestration of infected red cells (or mechanical) and ii) the inflammatory response [7, 8].

The sequestration hypothesis, first proposed by Marchiava and Bignami in 1894, suggests that CM is a consequence of the adherence of parasitized red blood cells ($_P$RBCs) to the cerebral microvascular endothelium, leading

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to obstruction of blood flow, cerebral hypoxia, ischemia and decreased removal of waste products causing central nervous system (CNS) dysfunction [6, 9]. This complex phenomenon occurs as a consequence of the expression of parasite antigens, such as *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1), on the surface of $_P$RBCs which can bind to receptors, especially the intercellular adhesion molecule-1 (ICAM-1), expressed or up-regulated in the host endothelial cells during the infection [7, 10]. Although being an important feature, some evidences, including low correlation between parasitemia and mortality and an increasing number of case reports of CM by *P. vivax*, which does not present sequester, suggests that the sequestration hypothesis alone cannot fully explain CM development [11, 12].

The inflammation hypothesis was first proposed by Maegraith in 1948 and postulates that the brain damage found in CM is a result of a host exacerbate inflammatory response to the parasite in the CNS [8, 13]. Briefly, parasite antigens released in the blood after pRBC lysis can be recognized by pattern recognition receptors of the innate immune system and activate monocytes and neutrophils to secrete proinflammatory cytokines including tumor necrosis factor α (TNF-α), interferon γ (IFN-γ) and interleukin (IL)-1β that act by recruiting other immune cells like $CD4^+$ and $CD8^+$ T cells, activating metabolic changes in endothelial cells and up-regulating the production of chemoattractants, expression of adhesion molecules with subsequent sequestration of leucocytes, pRBC and platelets, leading to microvascular obstruction and hypoxia [6, 8, 14]. High levels of circulating and cerebral tissue proinflammatory cytokines, especially TNF-α, have been associated with CNS dysfunction found in human and experimental CM [15-19]. In addition, the release of anti-inflammatory cytokines, mainly IL-10, seems to have a host protective role by regulating the synthesis of proinflammatory cytokines in response to the parasite. However, this host-protective response might be deficient in individual that develop CM [13, 20], which present an imbalance in the release of proinflammatory and anti-inflammatory cytokines, mechanism seems to be crucial to avoid this disease [7, 20].

Recently, metabolic changes in the CNS have been demonstrated in experimental studies of CM including high brain concentration of lactate and alanine, alterations in the kynurenine pathway of tryptophan metabolism and increase of glutamatergic activity [21-23]. Indicating, that these metabolic changes seem to be involved in the induction of the CNS dysfunction found during malaria infection and appear to be relevant targets for therapeutic intervention [6].

Lactate and alanine are readily transported from neurons and glia and, under physiological cerebral flow, are removed via circulation to prevent the accumulation in these cells. In fact, during CM an increase of lactate and alanine concentration in the CNS may be a consequence of the sequestration of pRBC in the cerebral microvasculature leading to obstruction of blood flow and hypoxia [24]. There is evidence that high levels of lactate can cause cerebral edema with consequent glial and neuronal damage contributing to the CM development [25]. Previous studies have demonstrated a correlation between increased levels of lactate in the cerebrospinal fluid of children with CM and disease severity [26, 27].

The kynurenine pathway of tryptophan metabolism has also been implicated in the pathogenesis of cognitive dysfunction observed in human and experimental CM [21, 28]. During tryptophan metabolism a range of bioactive molecules is metabolized, especially the quinolinic and the kynurenic acid [13]. Quinolinic acid (QA) is an excitotoxin which acts through the excitatory amino acid site on the N-methyl-D-aspartate (NMDA) receptor and, at elevated concentration, can cause neuroexcitotoxic damage including mitochondrial dysfunction and increased permeability of the blood brain barrier to plasma proteins [29]. The kynurenic acid (KA) is a natural antagonist of the quinolinic acid and has a neuroprotective role since it acts through the glycine binding site of NDMA receptor to inhibit the excitatory actions of glutamate [29]. In this context, an imbalance in the QA:KA ratio and the consequent increase of QA concentration in the CNS have been associated to seizures and to neuroexcitotoxicity found in human and experimental CM [26, 28, 30].

Neuroexcitotoxic effects of QA are mediated by glutamate receptors [29]. Glutamate is the major excitatory neurotransmitter in the mammalian CNS, playing an important role in neuronal development, synaptic plasticity, learning and memory processes under physiological conditions [31]. However, high amounts of glutamate release in intersynaptic spaces can cause neuronal cell death and neurodegeneration via excitotoxicity processes [32], which plays an important role in many CNS diseases, including ischemia, trauma, and neurodegenerative disorders [33]. In CM, was demonstrated by Rae *et al.* [22] an increased levels of glutamate in the brain of mice, indicating a role for this neurotransmitter in the pathogenesis of this disease. Furthermore, recent studies have shown that inflammatory mediators, especially TNF-α, can modulate neurotransmitter activity indicating that the interaction of both systems (immune and neurotransmitter) could be relevant for the development of a wide range of pathological conditions [34-36]. The TNF-α, described as the main inflammatory mediator in CM pathogenesis, is also produced by glia cells during another CNS dysfunction such as ischemia, infectious diseases and trauma, was show to induce an increase glutamate release by astrocytes [37-39]. Suggesting, at least in part, this cytokine contributes to the increase of glutamate concentration in CNS during the *Plasmodium* infection [22]. However, only few studies have investigated glutamate involvement in CM pathogenesis [22, 30, 40]. Therefore, the mechanisms of action and the real contribution of this neurotransmitter for disease development remain poorly understood [22].

ANTIMALARIAL TREATMENT

Cerebral malaria is a medical emergency demanding urgent clinical assessment and treatment [41]. Emergency management aims to rapidly correct severely altered metabolic states, restoring vital physiological functions, and the administration of an effective and briskly anti-parasitic drug [1]. The preferred drugs are chloroquine, quinine (Cinchona alkaloid) and the first generation of artemisinin derivates as artemether and artesunate.

Due to the growing resistance to chloroquine observed in most endemic areas, severe malaria have been treated with

parenteral artemisinin derivative or quinine, and followed by a complete course of an effective artemisinin oral monotherapy as soon as the patient can take oral medications, as recommended by the *WHO Guidelines for the treatment of malaria* published in March 2010. Quinine management has been associated with faster clearance of parasitemia and resolution of fever and coma in severe malaria [1]. Artemisinin derivates have been described as the most potent antimalarial compound with a faster clearance time than quinine despite its shorter half-life in plasma. Due to the safety and simplicity in administration, and declining sensitivity to quinine in some areas, they supersede quinine as the treatment of choice for severe malaria. Generally, quinine is an acceptable alternative if parenteral artesunate is not available [42, 43].

Even with successful anti-parasitic treatment a significant number of children remain with long-term cognitive and neurological deficits [44, 45]. In this scenario, a combination of antimalarial drugs and adjunctive therapies has been proposed in order to prevent or minimize the cognitive outcome in CM [42, 46].

COGNITIVE OUTCOME IN PATIENTS WITH CM

Long-term cognitive impairment after CM resolution has been described in retrospective and prospective studies, especially in children [44, 45, 47, 48].

A previous study conducted by Carter *et al.* [47] demonstrated that cognitive deficits can persist for a long period (3 to 9 years) after a CM episode and become more evident as children grow and face more complex cognitive and linguistic demands, socially and educationally. The main cognitive domains affected in children following CM are attention, visual spatial skill, language and memory [49, 50].

Cognitive sequelae in CM were firstly described by retrospective studies (Table **1**). For instance, Boivin [51] investigated in Senegal 29 children aged 5–12 years with a history of CM and compared with 29 age and education-matched controls. Those with a history of CM performed significantly poorer on cognitive tasks assessing attention, memory, visual-spatial skills, speech and language abilities. They also found a significant correlation between duration of the coma state and attention performance in CM group, emphasizing the importance of arousal compromise on outcome.

In Kenya, 152 children aged 6–9 years with CM history were compared to 179 children without exposure to malaria [47, 52]. The performance of children previously exposed to CM was poorer than unexposed children on all the cognitive tasks evaluated: speech and language, attention, memory and nonverbal functioning. There were significant differences in higher-level language abilities and non-verbal functioning. Deficits in speech and language functions after CM have also been reported by other authors who suggested that language deficits may persist if early interventions are not carried out [53]. In a more recent article, Idro *et al.* [54] reported severe neurological, behavioral and cognitive sequelae in 23 Ugandan children who survived a CM episode. These sequelae included motor deficits, hearing and speech problems, blindness, attention deficit hyperactivity disorder (ADHD), aggressive, destructive and obsessive behaviors. They also demonstrated that the onset of these symptoms may vary, occurring immediately on discharge from hospital or later, months or years after the insult, indicating the existence of distinct mechanisms of brain injury. Despite the limitations regarding the small sample size and the methods applied to evaluate behavioral and cognitive symptoms, this study systematically described for the first time behavioral problems as one of the major sequelae of CM in children.

These retrospective studies broadened the understanding of the potential long-term morbidity of CM. However, assessment of potential confounding factors such as nutrition, home environment, and education level, which can change over time and may be related to long-term cognitive status, was not performed in these studies [45]. To better understand cognitive impairment following CM and to estimate the contribution of confounding risk factors, prospective studies have recently been conducted (Table **1**).

A prospective study conducted by Boivin *et al.* [44] demonstrated that 21.4% of children who were 5 to 12 years of age and had CM present cognitive deficits 6 months after their CM episode, as compared with 5.7% of unexposed children. In the same cohort of children, John *et al.* [45] reported that 2 years after CM episode, cognitive impairment was observed in 26.3% of children with CM in comparison with 7.6% of unexposed children, suggesting that CM is associated with long-term cognitive deficits. This study also showed that the cognitive impairment present at 6 months after CM episode is strongly associated with impairment 2 years after the index episode, demonstrating that children with CM and early cognitive deficits are likely to maintain the impairment on follow-up.

Kihara *et al.* [55] assessed "everyday memory" in three groups of children following recovery from malaria. The three groups were: i) patients with CM, ii) patients with malaria plus complex seizures and iii) those without exposure to malaria. Everyday memory was assessed with the Kilifi Creek Behavioral Memory Test for Children which evaluated the memory required to carry out activities of daily living. Children with CM had significantly lower scores in everyday memory compared to the other two groups, suggesting a non-specific hippocampal involvement. The impairment was more significant in recall (memory of past events) and recognition (recognition of previous exposures following clues) subcategories. In addition, on a multivariate analysis, schooling, nutrition and a diagnosis of CM were significantly associated with memory impairment. Despite growing interest in investigating the long-term cognitive impairment following CM, differences in the methodologies applied and in the criteria used to define the disease have made cross-study comparisons difficult [48, 50].

While there are many studies assessing cognitive impairment of children after malaria, the impact on adults is not addressed by systematic research [41, 56]. In adults, the incidence of neurological and cognitive impairment is lesser than in children (approximately 5%), but the range of sequelae seems much greater, including cranial nerve lesions, neuropathies, extrapyramidal syndromes, focal epilepsy, personality change, depression, and subclinical mixed anxiety-depression syndrome [57]. Sattar *et al.* [58] investigated neurological impairment in one hundred adult patients diagnosed

Table 1. Clinical Studies Which Investigated Cognitive Outcome Following Cerebral Malaria

Author / Year	Country	Population (Age Range)	Clinical Description	Diagnostic Criteria	Number of Patients	Number of Controls	Follow-up (Month)	Study Design	Cognitive Tests	Main results
Boivin [51]	Senegal	5-12	CM	W.H.O. criteria for CM	29	29	* ± 36	Case - control	Kaufman battery; Visual form of computerized test of variables of attention	Visual-spatial skills, memory and attention deficits; Speech and language disorder
Carter *et al.*, [53]	Kenya	8-9	CM	Blantyre coma score of <2	25	27	>24	Retrospective Cohort	Speech and language assessment battery	Speech and language deficits
Carter *et al.*, [47, 52]	Kenya	6-10	CM	Blantyre coma score of <2	152	179	>24	Retrospective Cohort	Rivermead behavioural memory test; Behaviour screening questionnaire	Speech and language disorder; Memory, attention and behavior deficits
Carter *et al.*, [60]	Kenya	6-10	CM	Blantyre coma score of <2	152	179	>24	Retrospective Cohort	Rivermead behavioural memory test; Speech and language assessment battery	Alterations in several domains of language and speech; Memory and attention impairment
Idro *et al.*, [61]	Kenya	6-9	CM	W.H.O. criteria for CM	143	179	20	Retrospective Cohort	Rivermead behavioural memory test	Memory, attention, behavior and motor impairment; Speech and language disorder
Boivin *et al.*, [44]	Ugandan	5-12	CM	W.H.O. criteria for CM	44	89	6	Prospective	Kaufman battery; Visual form of computerized test of variables of attention; Tactual performance test	Attention and working memory impairment
John *et al.*, [45]	Ugandan	5-12	CM	W.H.O. criteria for CM	44	89	24	Prospective	Kaufman battery; Visual form of computerized test of variables of attention; Tactual performance test	Attention, learning and working memory deficits
Kihara *et al.* [55]	Kenya	6-9	CM	Blantyre coma score of <2	152	179	>20	Retrospective Cohort	Kilifi creek behavioral memory test	"everyday memory" impairment, especially in recall and recognition subcategories
Idro *et al.*, [54]	Ugandan	1-6	CM	W.H.O. criteria for CM	23	_	6	A case series study based on hospital records	Clinical observation of child's abilities in the areas of play, communication, safety, self-care and school performance.	Cognitive and motor deficits; Hearing and speech problems; Blindness; ADHD; Aggressive, destructive and obsessive behaviors

CM: Cerebral malaria; W.H.O: World Health Organization; ADHD: Attention deficit hyperactivity disorder

* Retrospective study which CM patients where compared with age and education matched controls approximately 36 months after CM episode.

with CM. The commonest neurological feature observed was symmetrical upper motor neuron lesion founding in 61% of CM patients. Abnormal posturing including decerebrate rigidity (6%) and decorticate rigidity (4%) with or without opisthotonus and focal neurological deficit (5%) were also described. Regarding CM outcome, 80% of patients recovered without any persistent neurological sequelae at the time of discharge from hospital and 20% died. Similarly, a high mortality (23%) was also reported in a study with 526 adult patients with CM conducted in eastern India [59]. Further systematic studies are needed to clarify the impact of malarial infection on the cognition and behavior of adults [5].

COGNITIVE OUTCOME IN EXPERIMENTAL STUDIES OF CM

Animal models have been of great relevance in the study of the mechanisms involved in CM pathogenesis and in the development of therapeutic agents that might contribute to minimize the CNS dysfunction associated with this disease [7, 46]. Due to the high degree of reproducibility, easily manageable characteristics and development of typical neurological signs and histopathological changes of human CM, the murine model using the *Plasmodium berghei* strain ANKA (PbA) has been widely used to better understand this condition [62].

Our group has previously demonstrated that C57BL/6 mice infected with PbA presented a wide range of neurological and behavioral changes, assessed by a battery of tests called SHIRPA (SmithKline/Harwell/Imperial College/Royal Hospital/Phenotype Assessment), during the infection which were associated with leukocyte recruitment induced by increased levels of cytokines and chemokines in the brain tissue [19]. Similarly, Lackner *et al.* [63] also found significant behavioral changes in functional domains of the SHIRPA battery during PbA infection which were correlated with histopathological alterations in the brain of PbA-infected mice, especially the size of parenchymal haemorrhages. Furthermore, Desruisseaux *et al.* [64] studied cognitive impairment, assessed by the object recognition paradigm, in the acute phase of experimental infection with PbA in mice and tried to correlate it with *post mortem* histopathological findings. They found a significant impairment in visual memory of infected mice on day seven post-infection. Cognitive dysfunction correlated with histopathological changes (cell infiltration and hemorrhage) in many areas of the brain including thalamus, midbrain and cerebellum and also with microglial activation in key areas such as cerebral cortex and hippocampus. It was hypothesized that extensive microglial activation and inflammation of parenchyma in determined areas such as hippocampus and posterior parahippocampal region might contribute to the observed cognitive impairment.

Cognitive and behavioral dysfunctions have been extensively described during the acute phase of malaria infection. However, to the best of our knowledge only two studies have investigated cognitive outcome after successful antimalarial therapy in experimental CM. Recently, Reis *et al.* [46] demonstrated that PbA infected C57BL/6 mice rescued from CM with chloroquine had significant impairment in several cognitive domains at least 30 days after infection, indicating that cure of the parasitic infection does not prevent the development of late cognitive sequelae once CM is established. They also studied C57BL/6 mice infected with *Plasmodium chabaudi chabaudi*, these mice developed clinical signs of infection but failed to develop CM and cognitive symptoms, suggesting that cognitive impairment is not an unavoidable consequence of systemic malarial infection in C57BL/6 mice, but rather is associated with the development of clinically detected CM. In this same study, CM-resistant mice (BALB/c strain) infected with PbA did not present cognitive deficits after chloroquine treatment, reinforcing the view that cognitive impairment was not due to the general systemic infection. Similar findings were reported in a study performed by Dai *et al.* [65] in which C57BL/6 mice infected with PbA presented cognitive (visual and spatial memory) and motor deficits 10 days after successful elimination of parasitaemia by chloroquine therapy. These deficits persisted at least one month after the parasitological cure even with resolution of brain inflammation and hemorrhage. Therefore development of long-term deficits in CM may involve a variety of metabolic and physiological events that can persist after the end of infection. Despite demonstrating the occurrence of long-term cognitive impairment after CM resolution, these works did not evaluate factors involved in its development. Further studies are needed to investigate the pathogenesis of long-term cognitive impairment in CM and also possible strategies for effective therapy to prevent this complication. In fact, several aspects of the cellular and molecular mechanisms involved in CM pathogenesis as well as of its cognitive sequelae remains to be elucidated [48, 54, 65]. It is not clear whether neurologic and cognitive sequelae reflect the severity of the acute phase of CM or whether it is derived from a distinct pathologic process [61].

There is evidence that cognitive outcome in human CM depends on a range of risk factors which include history of previous seizures, raised intracranial pressure, severe malnutrition, deep and prolonged coma, hypoglycemia and multiple neurological deficits on hospital discharge [48, 61]. The variety of cognitive sequelae following CM and related risk factors suggest that different brain regions are affected and distinct pathogenic mechanisms may be responsible for long-term deficits [54, 61]. The extent of brain injury, neuronal death and consequently cognitive outcome may depend on causation of coma, degree of microvascular obstruction, host immune response, duration of infection exposure, metabolic and biochemical changes [6, 61].

In a clinical study with CM children and healthy controls, John *et al.* [66] demonstrated that high levels of TNF-α in the cerebrospinal fluid of CM children on hospital admission were positive correlated with neurological and cognitive (attention and working memory) deficits three and six months later. These findings indicate that elevated levels of TNF-α in the CNS during the acute infection adversely affect long-term cognitive outcome in CM. Furthermore, Reis *et al.* [46] found a significant increase in oxidative stress in the brain of C57BL/6 mice infected with PbA. They also demonstrated that combined treatment of antioxidant agents and chloroquine prevented cognitive damage in infected mice, pointing out a role for oxidative stress in the development of long-term cognitive dysfunction in experimental CM. The persistent cognitive deficits following CM resolution could be a consequence of the similar cerebral injury mechanisms

occurring during the acute period of the disease or, in the other hand, could result from complex mechanisms that are independent of those that cause neurologic injury and death during acute CM. Future investigations are aimed to clarify this point.

ADJUNCTIVE AND NEUROPROTECTIVE THERAPIES

Neuroprotective and adjuvant therapies have recently been investigated in clinical and experimental studies of CM in an attempt to improve cognitive outcome (Table **2** and **3**). It is worth mentioning that these therapies do not replace anti-parasitic treatment, being the combination of both strategies important to prevent CM symptoms [67].

As mentioned above, the role of immune system is critical in determining the outcome of malaria infection. In fact, the administration of immunomodulators in some models of this infection, demonstrated to be effective in preventing cerebral injury found in CM [67]. It is widely accepted that up-regulation of TNF-α and IFN-γ, is involved in the development of CM [6, 8], therefore, studies have investigated the efficacy of some agents to prevent the release of TNF-α or to interfere with its actions.

In a study conducted by Van Hensbroek *et al.* [68] in 610 Gambian children with CM, the monoclonal anti-TNF antibody (B-C7) therapy did not improve survival and was associated with a significant increase in neurological sequelae. A possible explanation was regarding to the capacity of monoclonal antibody in retain TNF-α within the circulation and thereby prolonging its effects on vascular endothelium leading to up-regulation of adhesion molecules with consequent increase of pRBC and leukocyte sequestration in the brain, thus aggravating neurological complications.

Attempts have also been made to block TNF-α using non-antibody molecules, such as pentoxifylline, a methyl xanthine which inhibits TNF-α synthesis in activated macrophages. On these line, Kremsner *et al.* [69] showed that pentoxifylline treatment prevented CM development in a susceptive mice strain (CBA/Ca) after PbA infection. They also demonstrated that pentoxifylline treated animals presented significantly lower serum levels of TNF-α in comparison with non-treated infected mice. Similar findings were observed in clinical studies that used pentoxifylline as a supportive therapy in human CM. Adults and children patients who received a combination of quinine and pentoxifylline (10 mg/kg/day) for the first 3 days presented a significant improve in coma resolution time and a rapid decline in circu-

Table 2. Studies Examining Adjunctive and Neuroprotective Therapies to Improve Cerebral Malaria Outcome in Humans

Author/ Year	Study Design	Antimalarial Treatment	Adjunctive Pharmacological Approach	Proposed Mechanism of Action	Major Findings
Hensbroek *et al.* [68]	Clinical (CM children)	Quinine or Artemether	Monoclonal anti-TNF antibody (B-C7) therapy	Neutralization of TNF activity	No improve in survival and increase of neurological sequelae
Di Perri *et al.*, [70]	Clinical (CM children)	Quinine	Pentoxifylline	Inhibits TNF-α synthesis	Improved coma resolution time and decrease mortality
Das *et al.*, [71]	Clinical (CM adults)	Quinine	Pentoxifylline	Inhibits TNF-α synthesis	Improved coma resolution time and decrease mortality
Casals-Pascual *et al.* [78]	Clinical (CM children)	Quinine	Erythropoietin	Inhibits the expression of proinflammatory cytokines, antagonize the cytotoxic effect of glutamate, reduce nitric oxide mediated formation of free radicals	Decreased in 80% the risk of developing neurological sequelae following CM
Gordeuk *et al.*, [81]	Clinical (CM children)	Quinine	Desferroxamine	Iron chelating agent with antioxidant properties which acts preventing free radicals formation	Reduced coma duration and improved full consciousness recovery time
Thuma *et al.*, [82]	Clinical (CM children)	Quinine	Desferroxamine	Iron chelating agent with antioxidant properties which acts preventing free radical formation	No beneficial effect on mortality
Watt *et al.*, [83]	Clinical (CM adults)	Quinine	N-acetylcysteine	Antioxidant agent which acts preventing free radicals formation, inhibiting TNF-α release and preventing cytoadherence	Quick normalization of serum lactate levels and early replace of intravenous therapy with oral therapy

CM: Cerebral malaria.

Table 3. Studies Examining Adjunctive and Neuroprotective Therapies to Improve Experimental Cerebral Malaria Outcome

Author/ Year	Study Design	Antimalarial Treatment	Adjunctive Pharmacological Approach	Proposed Mechanism of Action	Major Findings
Kremsner *et al.* [69]	Experimental (CBA/Ca mice infected with PbA)	——	Pentoxifylline	Inhibits TNF-α synthesis	Prevented experimental CM development
Wassmer *et al.*, [72]	*in vitro* (human brain-derived endothelial cells)	——	LMP-420	Inhibits transcription of mRNA for TNF-α in human cells	Inhibition of endothelial cell activation and of up-regulation of adhesion molecules, interfering with sequestration process
Vigario *et al.* [73]	Experimental (C57BL/6 mice infected with PbA)	——	Recombinant human IFN-α	Reduction of parasitaemia and cell sequestration	Prevented experimental CM death
Morrell *et al.* [74]	Experimental (C57BL/6 mice infected with PbA)	——	IFN-β	Anti-inflammatory activity, including decrease brain levels of CXCL9 and ICAM-1, down-regulation of serum TNF-α and reduction of brain T-cell infiltrates	Suppressed experimental CM development and prolonged mice survival
Lackner *et al.* [75]	Experimental(C57BL/6 mice infected with PbA)	——	Glatiramer acetate	Immunomodulatory effect, leading to a decrease of IFN-γ release within reduction of leukocytes sequestration in the brain	Decreased risk for developing experimental CM
Kaiser *et al.*, [79]	Experimental (CBA/J mice infected with PbA)	——	Recombinant human erythropoietin	Inhibition of IFN-γ and TNF-α mRNA overexpression in the brain	Decreased experimental CM mortality
Reis *et al.* [46]	Experimental (C57BL/6 mice infected with PbA)	Chloroquine	Combination of Desferroxamine-andN-acetylcysteine	Antioxidant agent which acts preventing free radicals formation, inhibiting TNF-α release and preventing cytoadherence	Prevented late cognitive impairment in experimental CM
Penet *et al.*, [84]	Experimental (CBA/J mice infected with PbA)	——	B5 complex provitamin pantethine	Decrease of circulating microparticles and preservationof the blood–brain barrier integrity	Prevented experimental CM development
Cabrales *et al.*, [85]	Experimental (C57BL/6 mice infected with PbA)	Artemether	Nimodipine	Calcium channel blocker which acts reversing calcium-dependent vasoconstriction	Improved survival and neurological outcome after CM
Clark *et al.* [86]	Experimental (C57BL/6 mice infected with PbA)	——	Ro-61-8048	Inhibition of the kynurenine pathway from tryptophan metabolism	Prevented experimental CM development and improved survival
Miu *et al.* [87]	Experimental (C57BL/6 mice infected with PbA)	——	Ro-61-8048	Inhibition of the kynurenine pathway from tryptophan metabolism	Partial protection for experimental CM development

CM: Cerebral malaria; PbA: Plasmodium berghei ANKA strain.

lating TNF-α levels. A lower mortality was also observed in comparison with patients who only received quinine treatment [70, 71].

In an *in vitro* model of CM using human brain-derived endothelial cells (HBEC-5i), TNF-α activity in the endothelial cells was inhibited by the administration of a anti-inflammatory compound named LMP-420 (2-NH2-6-Cl-9-(5-dihydroxyboryl)-pentyl purine). LMP-420 inhibits transcription of mRNA for TNF-α in a variety of human cell types including monocytes, T lymphocytes, neutrophils, adipocytes, and endothelial cells. When added before or concomitantly with TNF-α, LMP-420 inhibited endothelial cell activation and consequently the up-regulation of ICAM-1 and VCAM-1 on the HBEC-5i surface, interfering with sequestration process. Although experimental results *in vitro* are not necessarily predictive of potential efficacy in animal models or man, these data provide evidence for a strong *in*

vitro anti-inflammatory effect of LMP-420 and suggest that targeting host cell pathogenic mechanisms might provide a new therapeutic approach to improve the outcome of CM patients [72].

Due to the important role of IFN-γ in CM development, some immune interventions have also been conducted using different IFN treatments in the attempt to improve CM outcome. In a previous work performed by Vigario *et al.* [73], administration of a recombinant human IFN-α prevented death in an experimental CM model. Mice infected with PbA and treated with IFN-α showed a marked decrease in parasitemia, less sequestered parasites and leukocytes (especially $CD8^+$ T cells) in cerebral vessels, reduced up-regulation of ICAM-1 expression in brain endothelial cells and decreased levels of TNF-α in the circulation when compared with no treated infected mice. Similarly, Morrell *et al.* [74] demonstrated that IFN-β, due its anti-inflammatory properties, suppressed the development of experimental CM and prolonged the survival of mice infected with PbA. Among the protective effects of IFN-β treatment there were decrease of CXCL9 and ICAM-1 levels in the brain, reduction of T-cell CXCR3 expression, down-regulation of serum TNF-α and decrease of brain T-cell infiltrates.

In addition to the TNF-α and IFN interventions, other immunomodulatory agents have also been investigated in CM. Lackner *et al.* [75] reported that C57BL/6 mice infected with PbA which received glatiramer acetate treatment (used in multiple sclerosis) presented a significant lower risk for developing CM than no treated infected animals (57.7% versus 84.6%, respectively). The mechanism of action seems to be an immunomodulatory effect since glatiramer acetate suppressed the release of IFN-γ in the early course of the disease which also led to a lower number of sequestered leukocytes in the brain of treated animals.

Additionally, the neuroprotective properties of the cytokine erythropoietin have been described in experimental and clinical studies of CM. There is evidence that erythropoietin prevents inflammation by inhibiting the expression of proinflammatory cytokines, antagonizing the cytotoxic effect of glutamate, reducing nitric oxide mediated formation of free radicals, normalizing cerebral blood flow and promoting neuroangiogenesis, acting as an antiapoptotic and cytoprotective cytokine [76, 77]. Casals-Pascual *et al.* [78] demonstrated that high concentrations of erythropoietin in the plasma of CM children (>200 units/liter) were strongly associated with 80% less risk of neurological sequelae, suggesting that erythropoietin should be better investigated as an adjuvant therapy in CM. The effect of erythropoietin in CM outcome was assessed in a murine model, using a systemic injection of recombinant human erythropoietin at the beginning of the clinical manifestations. The results demonstrated that this treatment protected 90% of CBA/J mice infected with PbA from the development of CM and death. Inhibition of IFN-γ and TNF-α mRNA overexpression was also observed in the brain of infected mice treated with erythropoietin [79]. A recent phase I trial has demonstrated the safety of erythropoietin at high doses (1500U/Kg) combined with quinine in children with CM. However, a randomized, double blinded, multi-centre trial are required to provide definitive data on the potential use of erythropoietin as an adjunctive therapy in humans as well as the effect of the combination with anti-malarial compounds on CM outcome [77].

Reactive nitrogen (NO) and oxygen species (ROS) have been implicated in the pathogenesis of cognitive deficits found in CM [46]. During malarial infection, the host and parasites are under severe oxidative stress with increased production of ROS and NO by activated cells in the host. High levels of ROS and nitrogen intermediates may cause damage to the host tissue including the vascular endothelium which can determine increased vascular permeability and leukocyte and platelet adherence, important processes for CM development [80]. In this context, antioxidant agents such as N-acetylcysteine and desferroxamine could be of great relevance as an additive therapy. A previous randomized, double-blind, placebo-controlled trial assessed the efficacy of desferroxamine in combination with quinine treatment to improve CM outcome in 83 Zambian children. They found that desferroxamine treatment reduced coma duration and improved the full consciousness recovery time. As coma length predicts the development of cognitive sequelae, desferroxamine could decrease late cognitive deficits in CM [81]. A later study by the same group examined the effect of desferroxamine on mortality in CM. A total of 352 children with CM from Zambia were treated with desferroxamine (100 mg/kg/day infused for a total of 72 h) or placebo, both associated to a 7 day regimen of quinine that included a loading dose. However, there was no beneficial effect on the mortality of CM children treated with the combination of desferroxamine and quinine [82]. In a pilot study, adult patients diagnosed with severe *P. falciparum* infection were treated with a combination of N-acetylcysteine and quinine. Serum lactate levels normalized quicker in patients treated with N-acetylcysteine when compared to patients treated only with quinine. Besides this, intravenous therapy was replaced with oral therapy earlier in those patients. No data regarding patient's mortality were provided [83]. In a recent experimental study performed by Reis *et al.* [46], the administration of both antioxidants in combination, N-acetylcysteine and desferroxamine, as adjunctive treatment with chloroquine prevented late cognitive impairment in experimental CM, and reduced microvascular congestion and plugging in the cortex, hippocampus and cerebellum of infected mice. When administered separately these antioxidant compounds did not improve CM outcome. Due to the well-known pharmacological profile and side effects of N-acethylcysteine and desferoxamine in humans, these drugs should be examined as additive therapy with antimalarial drugs in more systematic clinical trials to investigate their potential to decrease or prevent cognitive impairment after CM.

As discussed before, sequestration of pRBC and consequently blood flow decrease with hipoxia are important features of CM pathogenesis [6, 9]. To prevent endothelial injury, reverse rossetting and improve blood flow can be effective additional strategies for CM treatment. The administration of a low molecular weight thiol, the B5 complex provitamin pantethine, prevented the development of CM in a murine model. Pantethine, which acts modulating the coagulation cascade, seemed to offer protection by down-regulating platelet reactivity and releasing microparticles

from the activated endothelium with preservation of the blood brain barrier integrity. This compound is well tolerated, and it has already been administered in other contexts to human with limited side effects. Therefore, clinical trials of pantethine treatment should be conducted to investigate its potential as an adjunctive therapy for CM [84]. Additionally, in a more recent article a coadministration of artemether and nimodipine, a calcium channel blocker which acts reversing calcium-dependent vasoconstriction, to C57Bl/6 mice infected with PbA markedly increased survival and improved neurological outcome after CM compared with artemether administration only. The primary beneficial effect of nimodipine can be attributed to its effect in inducing vasodilation and consequently increase cerebral blood flow. Therefore, calcium channel blockers have also been shown to decrease vascular inflammation and oxidative stress, all seen in CM. It is worth mentioning that nimodipine has been safely used in humans for many years. Considering its beneficial effect in improve CM outcome, this compound should urgently be investigated as a potential adjunctive therapy [85].

The kynurenine pathway from tryptophan metabolism is activated in macrophages and microglia by inflammatory stimuli and generates excitotoxic and neuroprotectant products which have been implicated in the pathogenesis of human and murine CM [21, 28]. In a previous study performed by Clark *et al.* [86], the administration of an inhibitor of the kynurenine pathway, Ro-61-8048 compound, prevented the development of experimental CM and improved the survival of PbA infected mice. The decrease of CM symptomatology and mortality after Ro-61-8048 treatment were associated with increased brain levels of neuroprotective metabolites (kynurenic acid and anthranilic acid) and/or low brain levels of picolinic acid (excitotoxic) and MIP-1α/CCL3. Similarly, Miu *et al.* [87] found a partial protection for CM development in C57BL/6 mice infected with PbA and treated with Ro-61-8048 which was associated with suppressed levels of picolinic acid in the brain. Together these works suggest that compounds which inhibit the kynurenine pathway may potentially be effective in the treatment and prevention of human CM and should be investigated in further clinical trials.

Due to the role of NMDA receptors in modulating neurotransmission, high levels of glutamate and quinolinic acid produced during CM infection may have long-term deleterious effects on cognitive function. Recently, our group demonstrated a significant increase of glutamate levels in the cerebral cortex and in the cerebrospinal fluid of mice infected with PbA in parallel to the development of neurological and behavioral symptoms, indicating a role for glutamate in CM pathogenesis [40]. The mechanisms by which glutamate contribute to the CNS dysfunction observed in CM remain poorly understood [22]. Additional insights regarding glutamate syntheses, release and reuptake process as well as glutamate receptors expression during CM could contribute to the understanding of the role of this neurotransmitter in CM development. Glutamatergic strategies should be investigated as a potential additive therapy in CM.

CONCLUSION

A poor understanding of the cellular and molecular mechanisms involved in CM pathogenesis has significantly slowed the development of effective neuroprotective pharmacological measures during the acute stage of illness. However, other limiting factors especially regarding the properties of compounds used, appropriate therapeutic time window, patient selection and small sample size have also contributed to the lack of success of adjunctive and neuroprotective interventions. Furthermore, multi-centre clinical studies to systematically investigate the efficacy of adjunctive therapies in improve CM outcome are urgently warranted.

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4- OBJETIVOS

4.1 - Objetivo geral

Investigar os mecanismos relacionados ao desenvolvimento de alterações cognitivas e comportamentais durante a fase aguda e após a resolução da MC, bem como o efeito de estratégias antimaláricas e glutamatérgicas na prevenção dessas alterações.

4.2 - Objetivos específicos

4.2.1 – Investigar transtornos cognitivos e comportamentais durante a fase aguda e após a resolução da MC, em modelo experimental por *P. berghei* ANKA.

4.2.2 – Caracterizar os processos inflamatórios localizados no SNC durante a fase aguda e após a resolução da MC, por meio da análise da expressão de citocinas e de fatores neurotróficos no hipocampo e córtex frontal de camundongos C57BL/6 infectados com *P. berghei* ANKA.

4.2.3 – Investigar a associação da resposta inflamatória, neurogênese e morte celular com o desenvolvimento de transtornos cognitivos durante a fase aguda e após a resolução da MC em modelo experimental por *P. berghei* ANKA.

4.2.4 – Investigar o efeito imunomodulador de uma única dose do antimalárico Artesunato no SNC de camundongos C57BL/6 infectados com *P. berghei* ANKA.

4.2.5 – Investigar o efeito do antagonista de receptores glutamatérgicos do tipo NMDA, o MK801, como potencial adjuvante terapêutico ao tratamento antimalárico.

5- MATERIAL E MÉTODOS

5.1 – Aspectos éticos

Este projeto foi submetido e aprovado pelo Comitê de Ética em Experimentação Animal (CETEA) da UFMG, sob o número de protocolo 105/2009. Todos os procedimentos específicos para a análise do hipocampo foram submetidos e aprovados pelo CETEA sob o número de protocolo 57/2010.

5.2 – Animais

No presente estudo foram utilizados camundongos da linhagem C57BL/6, fêmeas, com idade de seis a oito semanas, obtidos no Centro de Bioterismo do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais (CEBIO-ICB-UFMG). Os animais foram mantidos com água e ração *ad libitum*.

5.3 - Parasito e infecção dos animais

Os camundongos foram infectados, com a cepa *Plasmodium berghei* ANKA-GFP clone cl15cy1 (a qual expressa *Green Fluorescent Protein*, GFP, constitutivamente durante todo o ciclo de vida). A rota de infecção foi intraperitoneal (i.p.) com inóculo padronizado de 10^6 hemácias parasitadas/camundongo, em solução tampão fosfato estéril (PBS, 200 µl), para garantir um grau de infecção uniforme nos diferentes grupos (Grau *et al.*, 1986). Os eritrócitos parasitados (pRBC) usados para infectar os

camundongos dos grupos experimentais, foram obtidos por meio de uma passagem *in vivo* em camundongos C57/BL6 quando alcançaram um percentual de pRBC entre 5 e 8%. A parasitemia foi obtida através da análise da expressão de GFP por citômetro de fluxo. Os animais controle, sem infecção, receberam por via i.p., o mesmo volume de PBS ou do veículo especifico utilizado em cada tratamento.

5.4 – Determinação da parasitemia

Para determinação da evolução da parasitemia, foi coletada uma gota de sangue (~ 3 µl) em 2 ml de PBS estéril para análise por citometria de fluxo (FACScan – Becton Dickinson, San Jose, CA) Os dados foram analisados no programa FACS Diva (Becton Dickinson, San Jose, CA). Eritrócitos não infectados de C57/BL6 foram utilizados como parâmetro para identificar as propriedades específicas de tamanho x granulosidade (FSC/SSC). Os pRBC foram identificados por meio do aumento da fluorescência verde e expressos em percentual de pRBC. Um total de 100.000 eventos foi adquirido para cada amostra.

5.5 - Análise de parâmetros cognitivos e comportamentais

Os sintomas clínicos clássicos da MC foram analisados por meio de uma escala de avaliação rápida de coma e outros sinais (RMCBS) modificada da bateria SmithKline/Harwell/Imperial College/Royal Hospital/Phenotype Assessment - SHIRPA (Carroll *et al.*, 2010; Rogers *et al.*, 1997). Os parâmetros cognitivos e comportamentais foram avaliados por meio dos testes de Reconhecimento de novos objetos; Esquiva inibitória; Labirinto em cruz elevado; Nado forçado e Campo aberto. Antes dos testes, os animais foram conduzidos ao local do experimento para minimizar os efeitos da

transferência sobre o resultado dos testes. Os aparelhos foram devidamente limpos após a avaliação de cada animal, para evitar a influência de odores durante o teste (Takahashi *et al.*, 2006). A sobrevida, parasitemia, massa corporal bem como os testes clínicos, cognitivos e comportamentais foram realizados de acordo com cada desenho experimental proposto baseado nos objetivos específicos e descrito nas seções posteriores. Um sistema de captação de imagem foi utilizado para registrar o comportamento dos animais, com intuito de facilitar a avaliação dos parâmetros investigados. O *software Anymaze* (Stoelting Co., Wood Dale, IL, USA) foi utilizado, quando possível, para análise dos testes realizados.

5.5.1 – Escala rápida para análise de coma e outros sintomas específicos da MC (Rapid murine coma and behavior scale -RMCBS)

A RMCBS é uma escala objetiva e quantitativa que permite o acompanhamento rápido do curso natural da MC e foi realizada como previamente descrito por Carroll et al., (Carroll *et al.*, 2010). É composta por 10 parâmetros (marcha, equilíbrio, capacidade motora, posição corporal, força, escape ao toque, reflexo pineal, beliscada das patas, agressão e *grooming*) baseados nos componentes da bateria SHIRPA. Cada teste recebe uma pontuação que varia de 0 a 2, sendo 20 a pontuação máxima e indicativa da ausência de sintomas específicos da MC (Carroll *et al.*, 2010). No presente estudo foi utilizada para análise da gravidade da doença e para determinar o inicio do tratamento antimalárico.

5.5.2 – Teste de Reconhecimento de Novos Objetos

O teste de Reconhecimento de Novos Objetos é utilizado em modelos experimentais com roedores na avaliação de aspectos cognitivos, como a memória de curto e longo prazo. É baseado na tendência natural dos animais em explorar preferencialmente objetos desconhecidos (Desruisseaux *et al.*, 2008).

O teste foi realizado em um campo aberto de 40 x 60 cm delimitado por 4 paredes com 50 cm de altura, sendo 3 de acrílico fosco e uma transparente. No primeiro dia foi realizada a habituação, no qual o animal cuidadosamente colocado no quadrado do canto posterior esquerdo do aparelho explorou o ambiente por 5 minutos. No segundo dia o animal foi recolocado no aparelho, no qual estavam dois objetos iguais em forma, tamanho e cor (A e B). O animal pôde novamente explorar o ambiente por 5 minutos e o tempo gasto na exploração de cada objeto foi registrado. No dia seguinte 24 horas após a sessão de treino foi avaliada a memória de longa duração. O animal explorou novamente o ambiente na presença do objeto familiar (A) e de um novo objeto (C), diferente em forma, tamanho e cor. Foi considerada exploração do objeto pelo animal qualquer contato físico (tocar o objeto com o nariz ou patas) ou orientação óbvia para o objeto a uma distância de 3 cm. O tempo total que cada animal explorou o objeto foi registrado. Um índice de reconhecimento foi calculado para cada animal: tempo gasto para explorar o objeto novo / tempo gasto na exploração de ambos os objetos (novo e familiar). Uma porcentagem maior de tempo gasto na exploração do objeto novo indica normalidade da função cognitiva (Tuon *et al.*, 2008).

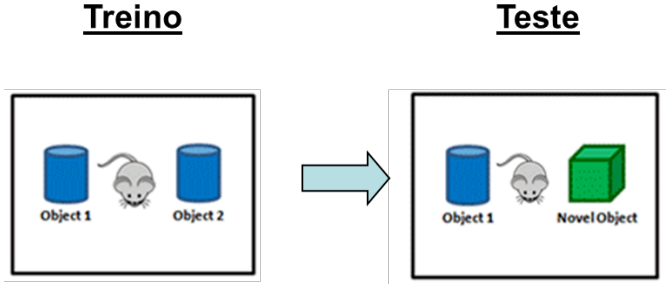

Figura 1: Representação ilustrativa do teste de reconhecimento novos objetos.

5.5.3 – Esquiva Inibitória

O teste de Esquiva Inibitória é utilizado em modelos experimentais para avaliar a memória aversiva (Tuon *et al.*, 2008).

Foi realizado em uma caixa de acrílico na qual o piso é formado por barras de metal paralelas (1mm de diâmetro), separadas entre si por espaços de 1cm. Uma plataforma com 7cm de largura e 2,5cm de comprimento é colocada junto à parede esquerda do aparelho. Na sessão de treino, os animais foram colocados sobre a plataforma e o tempo que cada animal gastou para descer com as quatro patas da plataforma foi registrado. Esse tempo é denominado latência. Imediatamente após descer da plataforma, o animal recebeu um choque de 0,2mA durante 2 segundos. Na sessão de teste, o animal foi novamente colocado na plataforma e o tempo que ele gastou para descer (latência) foi mensurado (tempo limite de 180 segundos), porém não foi administrado choque. A latência é um parâmetro clássico de retenção de memória. Os intervalos entre o treino e o teste foram 1,5 horas para avaliar a memória de curta duração e 24 horas para a memória de longa duração (Tuon *et al.*, 2008).

Figura 2: Representação ilustrativa do teste de esquiva inibitória.

5.5.4 – Teste do Nado Forçado

O teste do Nado Forçado foi utilizado para avaliar sintomas de depressão e foi realizado como descrito previamente por Haring et al., (Haring *et al.*, 2013). O teste foi realizado em um *beaker* de vidro (18 cm de diâmetro e 30 cm de altura) contendo água em temperatura de aproximadamente 25 ºC. O nível da água foi de aproximadamente 20 cm para evitar que os camundongos tocassem o fundo do *beaker*. Os animais foram cuidadosamente colocados na água e o comportamento deles foi registrado durante 6 minutos por meio de uma câmera digital (SONY, Tokyo, Japan). Os primeiros 2 minutos foram descartados e o comportamento de flutuação do animal foi analisado durante os 4 minutos restantes por um examinador experiente e cego quanto aos grupos. A flutuação foi definida pela imobilidade do animal e pelo movimento mínimo necessário para manter o equilíbrio corporal. O maior tempo de flutuação registrado em segundos indica os sintomas depressivos. Após cada sessão os animais foram devidamente secos e a água substituída.

Figura 3: Representação ilustrativa do teste do nado forçado.

5.5.5 – Labirinto em Cruz Elevado

Constitui um dos principais testes para avaliação da ansiedade em camundongos, sendo um método válido de investigação comportamental. Baseia-se no conflito entre o desejo de explorar novos ambientes e o medo de áreas abertas e desconhecidas, além da associação de elementos ansiogênicos tais como elevação e iluminação (File, 2001).

O labirinto em Cruz Elevado (Insight®, SP, Brasil) possui dois braços abertos e dois fechados, que emergem de uma plataforma central e estão dispostos em direções opostas formando uma cruz. A plataforma apresenta uma elevação da base (38,5 cm) e o aparato é iluminado por lâmpadas nos quatro braços e pela claridade do ambiente onde é aplicado o teste.

Inicialmente, cada camundongo foi colocado na plataforma central do labirinto com a cabeça direcionada para o braço aberto. A seguir, o animal percorreu livremente o labirinto durante cinco minutos (Walf and Frye, 2007). A frequência com que o camundongo entrou com as quatro patas nos braços aberto e fechado e o tempo total em que permaneceu em ambos os braços foram registrados pelo avaliador.

A medida da ansiedade é a porcentagem de entradas e de tempo gasto nos braços abertos (tempo ou entradas nos braços abertos/tempo ou entradas nos braços abertos e fechados X 100), sendo que baixas porcentagens indicam maiores níveis de ansiedade. O número absoluto de entradas nos braços fechados do labirinto tem sido descrito como uma medida da atividade locomotora (Walf and Frye, 2007).

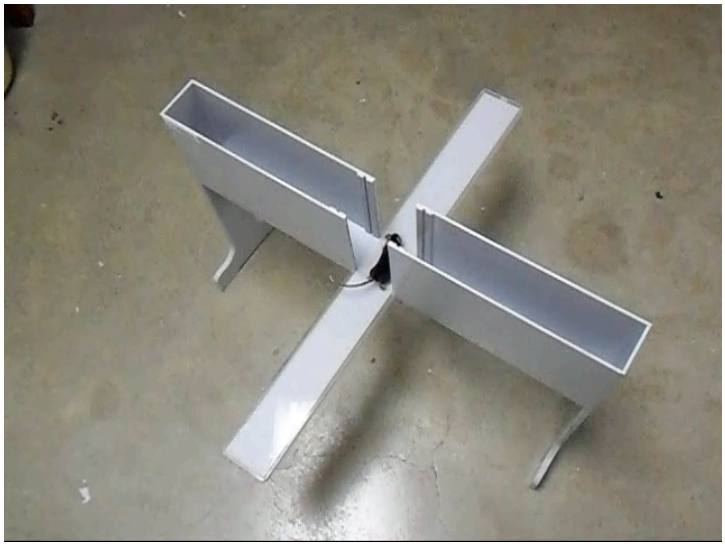

Figura 4: Representação ilustrativa do labirinto em cruz elevado.

5.5.6 – Campo Aberto

O teste comportamental denominado Campo Aberto (Open Field) é utilizado em estudos experimentais para investigar simultaneamente a atividade locomotora e exploratória bem como níveis de ansiedade (Podhorna and Brown, 2002). É um método válido e amplamente empregado na avaliação de parâmetros comportamentais em modelos experimentais de diversas doenças humanas (Takahashi *et al.*, 2006).

O Campo Aberto (Insight®, SP, Brasil) consiste em uma arena opaca (106 cm X 106 cm) dividida em quadrantes iguais. Para iniciar o teste cada camundongo é colocado no centro da arena e deve se mover livremente durante cinco minutos. Diferentes parâmetros como atividade global, movimentos estereotipados, locomoção, número de *rearings* e a porcentagem de tempo gasto no centro da arena (medida da ansiedade) são registrado por um *software* de rastreamento (ACTITRACK v2.7.13, Panlab, MA, USA).

Figura 5: Representação ilustrativa do campo aberto.

5.6 – Histologia

Para avaliar as alterações estruturais e patológicas durante a fase aguda da infecção e após a resolução com o tratamento antimalárico , estudos histopatológicos do tecido cerebral foram realizados. Os camundongos foram sacrificados por doses excessivas de anestésico (xilazina, Rompun®, Bayer, Leverkusen, Germany e quetamina, Laboratório Cristália, Campinas, SP, Brasil). Durante a necropsia, o tecido cerebral foi coletado e fixado por imersão em solução de formol tamponado a 10%. Após o período de fixação (12 horas), os tecidos foram recortados e seccionados transversalmente. A cada animal foi dado um código que apenas foi revelado ao final de todas as análises.

Os tecidos foram, em seguida, submetidos à desidratação, com a finalidade de remover a água presente nos mesmos. O processo de desidratação foi realizado em concentrações crescentes de álcool (70%, 80%, 90% e absoluto I, II e III), sendo que os fragmentos permaneceram imersos por um período de 30 minutos em cada álcool. Após a etapa de desidratação, foi realizado o processo de diafanização, que tem como

objetivo tornar o tecido translúcido. A diafanização consistiu em submeter os fragmentos a dois banhos de xilol com duração de 20 minutos cada. Posteriormente, os tecidos foram impregnados e incluídos em parafina.

Os blocos de parafina, contendo o fragmento do órgão, foram submetidos à microtomia, sendo obtidos cortes seriados com 4 μm de espessura. Os cortes obtidos foram corados pela técnica de hematoxilina-eosina (HE). As lâminas obtidas foram avaliadas ao microscópico óptico (Olympus, Japan, JP), para estudos histológicos.

5.7 – Medida dos níveis de citocinas e quimiocinas no cérebro, soro e baço por ELISA e CBA

5.7.1-*Preparo de homogenato de cérebro e baço de camundongo*

Retirou-se o hipocampo, o córtex frontal e o baço dos animais infectados e controles. O hipocampo e o córtex frontal foram, então, devidamente acondicionados e estocados a –80ºC. Posteriormente, as amostras foram pesadas (100 mg) e colocadas em 1,0 mL de solução inibidora de proteases para extração de citocinas [NaCl 0,4 M; Tween 20 0,05%; Albumina de soro bovino (BSA) 0,5%; Fluoreto de fenilmetilsufonila (PMSF) 0,1mM; cloreto de benzetônio 0,1 mM; EDTA 10 mM; 20 UI de aprotinina], preparada a partir de uma solução de tampão fosfato (NaCl 8 g, KCl 0,2 g e $Na_2HPO_4.12H_2O$ 2,89 g diluídos em 1 litro). As amostras foram maceradas por um homogenizador de tecidos (Power Gen 125, Fisher Scientific Pennsylvania, EUA) a uma velocidade ótima e a solução resultante foi centrifugada a 10.000 r.p.m, a 4ºC, por 10 minutos (Centrífuga BR4, Jouan, Winchester, VA, EUA). O sobrenadante foi recolhido, aliquotado e estocado a -80^0C até a sua utilização para detecção da

concentração dos mediadores inflamatórios por meio das técnicas de CBA (*Cytometric Bead Array*) e ELISA (Ensaio imunoenzimático: *Enzyme Linked Immuno Sorbent Assay*).

5.7.2 - Obtenção de soro de camundongos

Camundongos infectados, tratados e controle de cada grupo experimental sofreram exsanguinação através do plexo retro-orbital, e o sangue foi coletado em tubos individuais. Para obtenção do soro, as amostras foram mantidas durante uma hora em temperatura ambiente e uma hora a 4^0C. Posteriormente, o material foi centrifugado a 3000 r.p.m./10 min e o soro recuperado e estocado a -20^0C até o uso.

5.7.3- *Determinação da concentração de citocinas e fatores neurotróficos por ELISA e CBA*

A concentração das citocinas IL-2, IL-4, IL-6, IL-10, IL-17 TNF-α e IFN-γ no tecido cerebral , no baço e no soro foi analisada por meio do método CBA (Cytometric Bead Array). A concentração da citocina IL-1β, da quimiocina CCL11 e dos fatores neurotróficos BDNF, NGF e GDNF no tecido cerebral foi analisada pelo método ELISA. Os Kits de ELISA obtidos da R&D Systems (DuoSet) e os Kits de CBA TH1/TH2/TH7 obtidos da BD Biosciences, foram utilizados de acordo com os procedimentos previamente descritos pelo fabricante. A concentração dos mediadores inflamatórios foi determinada por leitura em espectofotômetro (Spectramax 190) no comprimento de onda de 450 nm no caso da técnica de ELISA e por leitura no instrumento FACSCanto (Becton Dickinson, San Jose, CA, USA) no caso da técnica de CBA.

5. 8 – Análise da expressão de fatores neurotróficos e citocinas por RT-PCR

A técnica de transcriptase reversa – reação de polimerização em cadeia (RT-PCR) foi utilizada para avaliação da expressão de fatores neurotróficos e citocinas no hipocampo e córtex frontal de animais controles e infectados. Inicialmente, todo o encéfalo foi removido e imerso em solução PBS gelada. Após dissecação, foram obtidas fatias do hipocampo e do córtex frontal, conforme método descrito por Hagihara et al., (Hagihara *et al.*, 2009). As amostras foram armazenadas a -80ºC. Foram analisados os níveis de expressão dos *primers* para as neutrofinas BDNF, TRK-B, NGF e para as citocinas TNF-α, IL-1β, IL-6 e IFN-γ. O PCR para 18S foi utilizado como controle para confirmar a proporção na quantidade de RNA e DNAc nos experimentos.

5.8.1 – Extração de RNA total do hipocampo de camundongo

As amostras foram homogeneizadas em Trizol, 100mg/ml, e incubadas por 15 minutos a 4°C. Em cada amostra foram adicionados 200µl de clorofórmio/ml de Trizol, seguido por homogeneização durante 15 segundos, em vórtex, e incubação de 2-3 minutos a 4°C. Posteriormente, a mistura foi centrifugada a 10.000 rpm/15min a 4°C. A fase aquosa foi, então, coletada e transferida para outro tubo contendo isopropanol 500µl/ ml de Trizol e, novamente, o material foi homogeneizado, incubado por 10min a 4°C e centrifugado a 10.000rpm/10min a 4°C. O sobrenadante foi cuidadosamente removido e o pellet lavado com 1 ml de etanol a 75% e submetido a uma nova centrifugação 5000rpm/5min a 4°C. O RNA, depois de seco, foi ressuspendido em 50µl de água (livre de RNAse). Para estabelecer a concentração do RNA extraído, foi utilizada a leitura em 260nm.

5.8.2 – Transcrição reversa do RNA para obtenção do cDNA

Em um tubo contendo 1µg de RNA (5µl) foi adicionada uma mistura contendo: 1µl de Oligo dT (500ng/ml); 0,2 µl de RNAsin (inibibor de RNase, 40U/µl); 1,8 µl de dNTPs (dATP, dTTP, dCTP, dGTP; 0,2mM cada, oligonucleotídeos para a formação da fita de DNA complementar); 2,5 µl do tampão Tris-HCl (50mM , pH 8,3, Gibco BRL); e 1,0µl da enzima transcriptase reversa (100U/µl). Esta enzima é uma DNA polimerase dependente de RNA que estende a fita de cDNA no sentido 5'→ 3', sem o domínio de clivagem do RNA, o que a torna mais eficiente. A reação ocorreu em um termociclador a temperaturas previamente ajustadas, e após a síntese do cDNA, este foi acrescido com 87,5µl de H2O estéril para um volume final de 100µl.

5.8.3 – Reação de polimerização em cadeia – PCR

A amplificação do material obtido foi realizada em microtubos a partir de 1µl de cDNA e uma mistura contendo: 0,8µl de dNTPs (0,2mM cada); 1,0 µl de tampão Tris-HCl (10mM, pH 8,3, Gibco BRL); 1,0 µl de cada par de primers ou iniciadores (senso e antisenso, 5pM/µl cada) específico para a molécula analisada; 0,05µl da enzima Taq DNA polimerase (5U/ml) e 6,15µl de água estéril. O material foi amplificado em ciclos de: desnaturação inicial (95°C/3min); segunda desnaturação (94°C/1min); anelamento (58°C/1min); polimerização da fita (72°C/2min) e uma extensão final (72°C/7min). O número de ciclos pode variar de acordo com o tamanho da molécula estudada. A separação do produto amplificado foi realizada através de eletroforese em gel de poliacrilamida em tambão borato-EDTA (TBE) 5×. Uma mistura contendo 5µl da amostra e 6µl do corante (azul de bromofenol 0,025%), foi aplicada no gel. Para uma

análise qualitativa, foi utilizado um padrão de peso molecular. O gel foi submetido a uma voltagem de 100V por 1 hora em tampão TBE 1X. Posteriormente o gel foi corado em solução de nitrato de prata, permitindo a visualização das bandas correspondentes.

Quadro 1: Sequência de *primers* (iniciadores) para neurotrofinas e citocinas.

Primer	Seqüência	
	Senso	**Antisenso**
BDNF	GGTATCCAAAGGCCAACTGA	CTTATGAATCGCCAGCCAAT
TKR-B	CGCCCTGTGAGCTGAACTCTG	CTGCTTCTCAGCTGCCTGACC-3
NGF	CAGACCCGGAACATCACTGTA	CCATGGGCCTGGAAGTCTAG
IFN-γ	CAGGATCCTTTGGACCCTCTGAC	GGCAGAATTAAGCTTATTGGGAC
TNF-α	ACAAGCTTGTAGCCCACGTCGTAGC	TGACTCGAGAGTAGACCTGCCCGG
IL-1β	CTACAGGCTCCGAGATGAACAAC	TCCATTGAGGTGGAGAGCTTTC
IL-6	TTC CAT CCA GTT GCC TTC TTG	TTG GGA GTG GTA TCC TCT GTG A
18S	CGT TCC ACC AAC TAA GAA CG	CTC AAC ACG GGA AAC CTC AC

5.9 – Análise da morte celular na região CA1 do hipocampo por microscopia confocal

A técnica de microscopia confocal foi realizada para análise de morte neuronal, decorrente do processo isquêmico presente na MC, no hipocampo de camundongos infectados com *Plasmodium Berghei* ANKA.

Camundongos C57BL/6, controles e infectados, foram decapitados e tiveram seus cérebros rapidamente removidos e mergulhados em solução gelada de ACSF (4ºC) contendo (em mM): 127 NaCl, 2 KCl, 10 glicose, 1.2 KH2PO4, 26 NaHCO3, 2 MgSO4, 2CaCl2, HEPES borbulhados com 95% O2/5% CO2. O hipocampo foi dissecado e cortado em fatias de 400μM. As fatias foram submersas em solução de 2-3 mm de ACSF borbulhada com 95% O2/5% CO2. Na fase final do experimento as amostras foram coradas com marcadores de viabilidade celular por 30 minutos (calceína

e etídio homodímero) que marcam os núcleos de células mortas e células vivas. Após o período de marcação o microscópio confocal (Leica TCS SP5) foi utilizado para identificar o grau de lesão isquêmica causada pela MC.

5.10 – Análise da neurogênese no giro denteado do hipocampo por imunoistoquimica

As fatias de hipocampo cortadas no criotótomo (30rmM) foram processadas como previamente descrito por Palazuelos et al. (Palazuelos *et al.*, 2009). Resumidamente, após uma hora de bloqueio com PBS, 0.25% Triton X-100 e 5% de soro de cabra, as fatias foram incubadas *overnight* a 4 ºC com o anticorpo primário anti-doublecortina (Santa Cruz Biotechnology, Dallas, TX). Em seguida foram incubadas durante uma hora em temperatura ambiente com o anticorpo secundário adequado (Invitrogen, Carlsbad, CA). A reatividade à doublecortina foi detectada pelo método avidina–biotina imunoperoxidase (Vectastain ABC kit; Vector Lab, Burlingame, CA) e o produto da reação foi revelado pela adição do cromógeno tetrahydroclorido 3,3-diaminobenzidina (Sigma Chemical, St. Louis, MO). Imagens foram adquiridas por microscopia confocal por meio do software e microscópio Leica TCS-SP2 (Wetzlar, Germany). *In vivo*, as células positivas para doublecortina foram quantificadas na zona subgranular do hipocampo em pelo menos cinco fatias com cortes coronais por animal. As células positivas foram normalizadas pela área do giro denteado determinada com uma objetiva X10. O número absoluto de células positivas foi calculado considerando o volume total do hipocampo. A reatividade à marcação com a doublecortina foi quantificada por meio de um sistema computadorizado de análise de imagems (ImagePro software, Media Cybernetics, Rockville, MD).

5.11 – Ressonância Magnética

5.11.1 – Aquisição e análise da imagem

As imagens foram adquiridas por meio do sistema 4.7T NMR system (Oxford Systems). Os camundongos (n= 4 *per group*) foram anestesiados por aproximadamente 50 minutos com halotano para a aquisição das imagens apresentando recuperação total após o procedimento. Máscaras de diferentes regiões cerebrais foram realizadas por meio de um sistema denominado *tablet driver* (Bamboo Tablet Driver, V5.2.5 WIN, WACOM Technology Corporation, USA) e pelo *software* MeVisLab (MeVis Medical Solutions AG, Fraunhofer). Adicionalmente, a densitometria foi realizada utilizando o programa MatLab® (Matrix laboratory, Novo México, México).

5.11.2 – Descrição da Densitometria

Após a segmentação, uma máscara binária com as mesmas dimensões da imagem original foi gravada no formato *.raw*. O volume é dado pelo número de *voxels* da máscara com valor diferente de 0, multiplicado pelo volume de cada *voxel.* Após a sobreposição da máscara à imagem original, a densidade foi dada pela soma das intensidades dos *voxels* dentro da região segmentada, dividido pelo volume segmentado.

5.12 – Medida da liberação cortical de glutamato

Esta técnica foi utilizada para mensurar a liberação de glutamato no hipocampo de camundongos controles e infectados, após tratamento antimalárico associado aos antagonistas de receptores glutamatérgicos, sendo realizada como previamente descrito por Prado et al. (Prado *et al.*, 1996). Resumidamente, os camundongos foram

sacrificados por meio de deslocamento cervical, pois a administração de anestésico interfere na obtenção do sinaptosoma. O cérebro foi retirado e o hipocampo separado e colocado em uma solução gradiente. Em seguida, as amostras foram maceradas por um homogeneizador de tecidos e o homogenato centrifugado a 3000rpm durante 10 minutos. Em seguida, o sobrenadante foi recolhido e colocado em gradiente de percoll (23%, 15%, 10%, 3%). As amostras foram novamente centrifugadas a 18000rpm por 15 minutos. Após a centrifugação houve a formação de diferentes fases na amostra das quais as fases 3 e 4 contêm o sinaptosoma. As fases 3 e 4 foram coletadas e foi realizada a lavagem do sinaptosoma com KRH. Em seguida, a solução foi centrifugada novamente a 18000rpm durante 15 minutos, obtendo-se assim o sinaptosoma purificado (Srinivasan *et al.*). A liberação de glutamato na fenda sináptica foi então mensurada por um espectrofluorímetro por meio da reação: Glutamato + NAD + GDH = α – Cetoglutarato + NADPH.

5.13 – Desenho Experimental 1

No presente estudo foi investigado a associação de mediadores inflamatórios (citocinas e fatores neurotróficos), neurogênese e morte celular com o prejuízo da memória de reconhecimento de novos objetos durante a fase aguda da MC experimental resultante da infecção pela cepa PbA. Camundongos C57BL/6 foram infectados com o inoculo padronizado de 10^6 eritrócitos parasitados como previamente descrito. Os animais controle, não infectados, receberam o mesmo volume de PBS. A parasitemia, a sobrevida e a presença dos sintomas clínicos da MC foram analisadas diariamente. No quinto dia após a infecção, os animais foram submetidos ao teste de reconhecimento de novos objetos para análise cognitiva e posteriormente foram sacrificados e o hipocampo

e córtex removidos para a análise de citocinas e fatores neurotróficos. A concentração das citocinas IL-2, IL-4, IL-6, IL-10, IFN-γ e TNF-α foi analisada por CBA enquanto que a concentração da quimiocina CCL11 e dos fatores neurotróficos BDNF e NGF foi avaliada por ELISA. A expressão de BDNF, do seu receptor TRKB e de NGF também foi mensurada pela técnica de RT-PCR. No mesmo período de infecção a proliferação de novos neurônios no giro denteado e a porcentagem de morte celular na região CA1 do hipocampo foram avaliadas por meio da técnica de microscopia confocal.

5.14 – Desenho Experimental 2

No presente estudo, inicialmente, foram investigadas diferenças no perfil de expressão de citocinas no hipocampo e córtex frontal, áreas fundamentais para a consolidação da memória e do aprendizado, durante a fase aguda da MC. Camundongos C57BL/6 foram infectados com o inoculo padronizado de 10^6 eritrócitos parasitados como previamente descrito. Os animais controle, não infectados, receberam o mesmo volume de PBS. No quinto dia após a infecção a memória aversiva foi mensurada por meio do teste de esquiva inibitória simples. Simultaneamente, a expressão das citocinas IFN-γ, IL-1β, IL-6 e TNF-α foi avaliada pela técnica RT-PC. Em seguida o efeito do antimalárico artesunato no SNC durante o pico da MC no quinto dia após a infecção foi investigado. Os animais infectados com a cepa PbA foram monitorados quanto à parasitemia, a massa corporal e os sinais clínicos. No quarto dia foram tratados com uma única dose do antimalárico artesunato administrada intraperitonealmente (32mg/kg diluído em solução salina 0.9% e bicabornato de sódio 5%) como previamente descrito por Clemmer et al. (Clemmer *et al.*, 2011). Após 24 horas da administração do artesunato, a parasitemia e os sinais clínicos foram novamente mensurados. Animais

controles infectados com a cepa PbA foram utilizados para assegurar o desenvolvimento adequado da doença. Animais controles sem infecção receberam o mesmo volume do veículo. No quinto dia após a infecção o hipocampo, o córtex frontal e o soro dos animais foram retirados. A concentração das citocinas IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ e TNF-α foi analisada por CBA e a concentração da citocina IL-1β por ELISA.

5.15 – Desenho Experimental 3

No presente estudo foi investigado o efeito da dizocilpina (MK801), um antagonista não competitivo de receptores de glutamato do tipo N-metil-D-aspartato (NMDA), na prevenção de déficits cognitivos e comportamentais após a resolução da infecção com o antimalárico cloroquina. Camundongos C57BL/6 foram infectados com o inoculo padronizado de 10^6 eritrócitos parasitados como previamente descrito. O antimalárico cloroquina foi administrado a todos os animais em associação ou não com o MK801. A cloroquina (30 mg/kg/dia) foi administrada por via oral (200 µl) durante 10 dias a partir do quinto dia de infecção. Os critérios para inicio do tratamento antimalárico foram parasitemia acima de 7%, perda de 2% da massa corporal e presença dos sinais clínicos da MC (Dai *et al.*, 2010). A primeira dose administrada foi de 60mg/kg para evitar a recidiva da parasitemia. O padrão de cura foi a negativação da parasitemia. O MK801 (0.5 mg/kg) foi administrado, a um dos grupos que recebeu cloroquina, por via intraperitoneal (200 µl) durante 12 dias a partir do 3º dia de infecção, período no qual ainda não houve morte neuronal. Durante e após o tratamento, a sobrevida, a parasitemia, a massa corporal e os sinais clínicos da MC foram monitorados. Animais controles, não infectados, receberam salina, cloroquina ou a combinação de MK801 com cloroquina. Dez dias após o fim do tratamento com o

antimalárico, associado ou não ao MK801, os animais foram submetidos aos testes de reconhecimento de novos objetos, esquiva inibitória, nado forçado e labirinto em cruz elevado para análise de sequelas cognitivas e comportamentais. Paralelamente, o hipocampo e o córtex frontal foram removidos para análise da concentração das citocinas IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ e TNF-α por CBA, e da citocina IL-β, da quimiocina CCL11 e dos fatores neurotróficos BDNF, NGF e GDNF por ELISA. O soro e o baço também foram coletados para análise por CBA da concentração sistêmica das citocinas citadas acima. Análises histopatológicas de todo o encéfalo foram realizadas após coloração com hematoxilina e eosina. Além disso, anormalidades na região do hipocampo foram investigadas por meio da técnica de ressonância magnética.

5.16 – Análise Estatística

A análise estatística foi realizada utilizando-se o programa estatístico Prisma 4.0 (GraphPad, La Jolla, CA, USA) e/ou o programa SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Os dados foram relatados de forma descritiva utilizando-se as medidas de tendência central média e desvio padrão e/ou mediana e erro padrão. Os resultados foram analisados quanto à distribuição normal pelo teste *Kolmorov-Smirnov*. As variáveis com distribuição normal foram comparadas por meio dos testes estatísticos *t-student,* ANOVA ou *Two-way* ANOVA. O pós-teste de Duncan foi utilizado quando necessário para múltiplas comparações. No caso de distribuição não normal, as variáveis foram comparadas por meio dos testes estatísticos *Mann-Whitney U* ou *Kruskal-Wallis*. O teste *log-rank* foi utilizado para análise da curva de sobrevida. O nível de significância foi estabelecido em $p<0,05$.

5.17 – Pesquisa e normalização bibliográfica

A pesquisa bibliográfica foi realizada a partir de consulta a base de dados *MedLine/PubMed* disponível na *internet*, por meio da busca de palavras chave relacionadas ao tema investigado.

As referências foram citadas e organizadas de acordo com o *International Committee of Medical Journal*. Os artigos foram escritos de acordo com as normas especificas estabelecidas pelos jornais aos quais foram ou serão submetidos.

6 – RESULTADOS

Os resultados serão apresentados na forma de três artigos científicos que foram ou serão submetidos em periódicos indexados.

6.1 – Artigo científico 1

Artigo publicado na revista *Neuroscience* em 2015.

de Miranda AS, Brant F, Campos AC, Vieira LB, Rocha NP, Cisalpino D, Binda NS, Rodrigues DH, Ransohoff RM, Machado FS, Rachid MA, Teixeira AL. Evidence for the contribution of adult neurogenesis and hippocampal cell death in experimental cerebral malaria cognitive outcome. **Neuroscience.** 2015; 284: 920-933. doi: 10.1016/j.neuroscience.2014.10.062.

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EVIDENCE FOR THE CONTRIBUTION OF ADULT NEUROGENESIS AND HIPPOCAMPAL CELL DEATH IN EXPERIMENTAL CEREBRAL MALARIA COGNITIVE OUTCOME

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Abstract—Cognitive dysfunction is a major sign of cerebral malaria (CM). However, the underlying mechanisms of CM cognitive outcome remain poorly understood. A body of evidence suggests that adult neurogenesis may play a role in learning and memory processes. It has also been reported that these phenomena can be regulated by the immune system. We hypothesized that memory dysfunction in CM results from hippocampal neurogenesis impairment mediated by the deregulated immune response during the acute phase of CM. C57Bl/6 mice were infected with *Plasmodium berghei* ANKA (PbA) strain, using a standardized inoculation of 10^6 parasitized erythrocytes. Long-term working memory was evaluated using the novel object recognition test. The mRNA expression of brain-derived neurotrophic factor (BDNF), tropomyosin-receptor-kinase (TRK-B) and nerve growth factor (NGF) in the frontal cortex and hippocampus was estimated by real-time polymerase chain reaction (PCR). The protein levels of cytokine interleukin-2 (IL-2), IL-4, IL-6, IL-10, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and CCL11 and neurotrophins BDNF and NGF were determined using a cytometric bead array (CBA) kit or enzyme-linked immunosorbent assay. Cell viability in the hippocampus was analyzed by Confocal Microscopy. Neurogenesis in the dentate gyrus was determined through quantification of doublecortin (DCX) positive cells. PbA-infected mice presented working memory impairment on day 5 post-infection. At this same time point, CM mice exhibited a decrease in DCX-positive cells in the dentate gyrus in parallel with increased cell death and elevated inflammatory cytokines (IL-6, TNF-α, IFN-γ and CCL11) in the hippocampus and frontal cortex. A significant reduction of BDNF mRNA expression was also found. IL-6 and TNF-α correlated negatively with BDNF and NGF levels in the hippocampus of CM mice. In summary, we provide further evidence that neuroinflammation following PbA-infection influences neurotrophin expression, impairs adult hippocampal neurogenesis and increases hippocampal cell death in association with memory impairment following CM course. The current study identified potential mediators of memory impairment in CM.

Key words: cerebral malaria, cytokines, neurotrophin, CCL11, cognitive dysfunction, neuroinflammation.

INTRODUCTION

Cerebral malaria (CM) is the most severe complication resulting from *Plasmodium falciparum* infection. Clinically, this condition is characterized by neurological and cognitive dysfunction, seizures and coma, ultimately leading to death (Idro et al., 2005). Even with appropriate antimalarial treatment, the mortality rate is high and approximately 10–20% of survivors experience long-term cognitive impairment (Boivin et al., 2007; John et al., 2008). As a consequence, a significant economic and educational burden has been reported in malaria endemic areas (Fernando et al., 2010).

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Abbreviations: BDNF, brain-derived neurotrophic factor; CBA, cytometric bead array; CM, cerebral malaria; CNS, central nervous system; DCX, doublecortin; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; IFN-γ, interferon-γ; IL-2, interleukin-2; IL-4, interleukin-4; IL-6, interleukin-6; IL-10, interleukin-10; IL-17, interleukin-17; LTM, long-term memory; NGF, nerve growth factor; PbA, *Plasmodium berghei* ANKA; p.i., post-infection; RMCBS, rapid murine coma and behavior scale; SHIRPA, SmithKline/Harwell/Imperial College/Royal Hospital/Phenotype Assessment; TNF, tumor necrosis factor; TRKB, tropomyosin-receptor-kinase.

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Experimental models of CM, particularly the murine model using the *Plasmodium berghei* ANKA (PbA) strain, have become a valuable tool to better understand neurological and cognitive outcomes associated with this condition (de Souza et al., 2010; de Miranda et al., 2011a). Desruisseaux et al. reported a significant impairment in visual memory, assessed by the object recognition paradigm, of infected mice in the acute phase of experimental infection with PbA. Cognitive dysfunction was associated with cell infiltration and hemorrhage in many areas of the brain (thalamus, midbrain and cerebellum) and also with microglial activation in key areas such as the cerebral cortex and hippocampus (Desruisseaux et al., 2008). Recently, we demonstrated that PbA-infected mice also presented significant deficit in short-term aversive memory in the step-down inhibitory avoidance test in parallel with enhanced mRNA expression of inflammatory cytokines in the frontal cortex and hippocampus (Miranda et al., 2013). Although extensively investigated, the underlying mechanisms of CM pathogenesis, including the related cognitive impairment, remain incompletely understood (Hunt et al., 2006).

Adult neurogenesis is a complex process that involves the proliferation of neural stem and progenitor cells and their subsequent differentiation, migration, functional integration into pre-existing circuitry along with a gradual increase of neuronal connectivity as well as changes in physiological neuronal properties (Ehninger and Kempermann, 2008). In adult brain, this phenomenon occurs in two specific regions: the subgranular zone of the hippocampus located between the granule cell layer and hilus of the dentate gyrus, and the subventricular zone located in the walls of the lateral ventricles (Deng et al., 2010). A great body of evidence supports a role for new neurons in learning and memory processes (Zhang et al., 2008; Clelland et al., 2009; Deng et al., 2009; Jessberger et al., 2009; Villeda et al., 2011).

It has been reported that the immune system, among other factors, can regulate neurogenesis. Overexpression of inflammatory mediators, including cytokines and chemokines, promotes a deleterious effect on adult neurogenesis by inhibiting new neuron survival, proliferation, differentiation and integration in pre-existing neuronal networks (Bastos et al., 2008; Jakubs et al., 2008; Fujioka and Akema, 2010; Belarbi et al., 2012). On the other hand, it has been suggested that anti-inflammatory cytokines like interleukin 4 (IL-4) and interleukin 10 (IL-10) could play a role in memory and learning processes at least in part by enhancing adult neurogenesis (Butovsky et al., 2006; Derecki et al., 2010). An imbalance between inflammatory and anti-inflammatory responses, as in CM, can disrupt neural processes such as neurogenesis, leading theoretically to cognitive deficits.

Neurotrophins such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are also critically involved in memory formation and neurogenesis (Heldt et al., 2007; Conner et al., 2009). Immune system activation, during disease or stressful conditions, can inhibit neurotrophin secretion and contribute to the deleterious effect of inflammatory response on adult neurogenesis and cognitive function (Tanaka et al., 2006; Bilbo et al., 2008; Taepavarapruk and Song, 2010). Enhanced production of inflammatory cytokines along with decreased expression of neurotrophins in the hippocampus, particularly BDNF, and neurogenesis impairment have been detected in depression and in stress-induced animal models of depression (Angelucci et al., 2005; Xu et al., 2006; Koo and Duman, 2008). Cognitive deficits, especially hippocampus-dependent learning and memory, have been also reported in those models (Mineur et al., 2007; Li et al., 2008).

Based on these pieces of evidence, in the present work we tested the hypothesis that memory impairment found in experimental CM could be associated with a decrease in adult hippocampal neurogenesis and increase of hippocampal cell death possibly due to local imbalance in cytokine and neurotrophin production.

EXPERIMENTAL PROCEDURES

Ethics statement

This study was carried out in strict accordance with Brazilian's ethical and animal experiment regulations. The animal ethics committee of the Universidade Federal de Minas Gerais CETEA/UFMG approved all experiments and procedures including euthanasia, fluid and organ removal (Permit Number: 105/09). All animal experiments were performed under i.p. injections of a mixture of Ketamine (150 mg/kg, Laboratório Cristália, Campinas, SP, Brazil) and Xylazine (10 mg/kg, Rompun®, Bayer, Leverkusen, Germany) anesthesia and were planned in order to minimize mouse suffering.

Animals

Female C57BL/6 mice (20–25 g), aged 6–8 weeks, were obtained from Animal Care Facilities of the Institute of Biological Sciences, Universidade Federal de Minas Gerais (ICB-UFMG), Belo Horizonte, Brazil. The animals were housed in groups of six mice per cage in a room controlled temperature (25 °C) with food and water *ad libitum*. Experiments were performed on day 5 post-infection (p.i.) when PbA-infected mice develop brain inflammation without motor impairment (Lacerda-Queiroz et al., 2010; de Miranda et al., 2011b).

Parasite and experimental infection

Blood stages of PbA strain constitutively expressing green fluorescent protein (*P. berghei* ANKA-GFP) (15cy1 clone), kindly provided by Dr Claudio Marinho (Universidade de São Paulo), were stored in liquid nitrogen (Clemmer et al., 2011). Mice were infected intraperitoneally (i.p.) with 10^6 PbA-infected red blood cells suspended in 0.2 mL PBS. Control animals received the same volume of vehicle.

The percentage of parasitemia was quantified by green fluorescent protein (GFP) frequency in whole

blood using flow cytometry. Briefly, a drop of whole blood tail from mice infected or not with PbA was collected directly into a polystyrene tube containing 2 mL of PBS for flow cytometry analysis (FACS CANTO II, Becton Dickinson, San Jose, CA, USA). GFP frequency was measured in each sample using argon laser (488 nm) and the acquisition was processed using the Diva software (Becton Dickinson, San Jose, CA, USA). Erythrocyte population was identified on the basis of their morphological characteristics in dot plot graphic (FSC × SSC) and was analyzed for their GFP + presence. A minimum of 100,000 gated events on erythrocytes population of each sample was acquired for analysis.

Behavioral and cognitive assessment

Mice were observed daily for parasitemia, body weight and clinical signs of CM (i.e. ataxia, paralysis, and coma). CM signs were evaluated using the rapid murine coma and behavior scale (RMCBS), a protocol based on the components of the SHIRPA (SmithKline/Harwell/Imperial College/Royal Hospital/Phenotype Assessment) score (Rogers et al., 1997; Carroll et al., 2010). The RMCBS consists of ten parameters (gait, balance, motor performance, body position, limb strength, touch scape, pinna reflex, toe pinch, aggression and grooming), and each item is scored from zero as the lowest, to two as the highest, with a maximum total score of 20. This scale is a quantitative and objective method that enables a rapid follow up of CM course (Carroll et al., 2010). This assessment was carried out on day 3 p.i. until day 5 p.i. and ten animals *per group* were used.

The object recognition task was performed to assess long-term working memory on day 5 p.i. (Schroder et al., 2003; Reis et al., 2010). Briefly, animals had the opportunity to explore the open field for 5 min (habituation phase). On the next day (day 4 p.i.), a training session was conducted by placing individual mice for 10 min in the center of the open field arena, in which two identical objects (object A1 and A2; Double Lego Toys) were positioned in two adjacent corners at 8 cm from the walls. In the long-term memory (LTM) test (24 h after training), on day 5 p.i., the mice explored the field for 5 min in the presence of the familiar (A) and different novel (B) objects. Objects were distinct in shape but were made by the same material (plastic). The exploratory preference was defined as the percentage of the total exploration time animal spent investigating the object A or the novel object (B) and calculated for each animal by the ratio TB /(TA + TB) [TA = time spent exploring the familiar object A; TB = time spent exploring the novel object B). The distance traveled in the apparatus arena was also recorded as a locomotor activity parameter. The Anymaze software (Stoelting Co., Wood Dale, IL, USA) was used for behavioral analysis. All tests were performed by the same investigator who was blinded to the animal status (control or infected). A total of ten animals per group was used.

Hippocampal and frontal cortex mRNA expression of neurotrophic factors

The mRNA expression of BDNF, tropomyosin-receptor-kinase (TRK-B) and NGF in the hippocampus and frontal cortex of PbA-infected mice and matched controls ($n = 5$ per group) was estimated by quantitative real-time PCR (polymerase chain reaction) at day 5 p.i.

RNA isolation was performed using Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The RNA obtained was resuspended in diethyl pyrocarbonate-treated water and stocked at −70 °C until use. Reverse transcription was performed using 2 μg of total RNA, 200 U of reverse transcriptase, RT buffer 5× (4 μl), 10 mM dNTPs (1 μl), RNAsin 10,000 U (0.2 μl) and oligo dT 15 50 μM (1.0 μl) in a final reaction volume of 20 μl. Resultant cDNA was used for qPCR. Real-time RT-PCR was performed on an ABI PRISM 7900 sequence-detection system (Applied Biosystems, San Francisco, CA, USA) by using SYBR Green PCR Master Mix (Applied Biosystems, San Francisco, CA, USA) after a reverse transcription reaction of 2 μg of total RNA by using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Specific primers were designed using Primer Express software (Applied Biosystems, San Francisco, CA, USA) and synthesized by integrated DNA technologies (IDT). The relative expression level of genes was determined by the $2^{-\Delta\Delta Ct}$ method and data were normalized by 18S ribosome subunit expression levels. Data were shown as fold increase over the negative control (non-infected) group. All reactions were replicated. Specific primer sequences are described in box 1 as following:

Box 1. Oligonucleotide primers for real-time quantitative PCR (SYBR).

Primer	Sequences	
	Forward	Reverse
BDNF	GGTATCCAAA GGCCAACTGA	CTTATGAATC GCCAGCCAAT
TRKB	CGCCCTGTGAG CTGAACTCTG	CTGCTTCTCAGC TGCCTGACC
NGF	CAGACCCGGAA CATCACTGTA	CCATGGGCCT GGAAGTCTAG
18S	CGTTCCACCAAC TAAGAACG	CTCAACACGGG AAACCTCAC

Cytometric bead array (CBA) and enzyme-linked immunosorbent assay (ELISA) analyses of neurotrophins and cytokines

Hippocampal and frontal cortex tissues of controls ($n = 10$ *per group*) and PbA-infected mice ($n = 10$ *per group*) were carefully removed on day 5 p.i. and homogenized in an extraction solution (100 mg of tissue per mL), containing 0.4 M NaCl, 0.05% Tween 20, 0.5%

BSA, 0.1 mM phenyl methyl sulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KIU aprotinin, using Ultra-Turrax. Lysates were centrifuged at 13,000*g* for 10 min at 4 °C, supernatants were collected and stocked at -70 °C until use.

Brain cytokine levels were determined using a mouse Th1/Th2 CBA kit (BD Biosciences, San Diego, CA, USA) in a FACS CANTO II flow cytometer (Becton Dickinson, San Jose, CA, USA). The following cytokines were measured: interleukin-2 (IL-2), IL-4, interleukin-6 (IL-6), IL-10, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). The protein quantities in lysates for CBA analysis were quantified using the Bradford assay reagent from Bio-Rad (Hercules, CA, USA).

The concentration of neurotrophins (BDNF and NGF) and chemokine CCL11 was determined by ELISA (R&D Systems, Minneapolis, MN, USA) in accordance to the manufacturer's instructions. Results are expressed as pg/100 mg of tissue. The detection limit of the ELISA assays was in the range of 5–10 pg/ml.

Cell viability analysis in the hippocampal slices by Confocal Microscopy

Cell viability assay in CA1 area of the hippocampus of control and PbA-infected mice ($n = 6$ *per group*) was performed (Agostini et al., 2011). Briefly, hippocampal slices were stained for 30 min in the dark with ethidium homodimer-AM (6 mol/L) and calcein-AM (4 mol/L), and with the Live-Dead cell assay kit (Molecular Probes, Eugene, OR, USA), and then washed at room temperature for 15 min in 2 mL of Krebs-Ringer bicarbonate bubbled with 95% O_2/5% CO_2. Viable cells showed the green fluorescence of calcein, whereas dead cells were indicated by the red fluorescence of ethidium homodimer. Slices were imaged by microscopy using a LEICA SP5 (Wetzlar, Germany) confocal equipped with a krypton–argon laser and Confocal Assistant software (PUCL, Lafayette, IN, USA) to combine consecutive optical sections (1024 31024) from a Z-series to form image reconstructions. For quantitative analysis, the images were enhanced by further processing using the median filter. The quantification of dead cells required identification of the nuclei that had been fluorescently stained with ethidium homodimer. In the present study, we defined nuclei as connected pixels that were above a certain intensity threshold calculated using the image histogram, and any pixels below that threshold were set to zero. Slices were selected from the threshold images and analyzed using the MetaMorph® Imaging System (Universal Imaging Corporation, Downingtown, PA, USA) to calculate the percentage of dead cells above the threshold in the image. The morphologic analysis of dead cells was performed in eight fields per slice from three different experiments in control (non-infected) or PbA-infected mice; 100 cells were counted per field.

Neurogenesis analysis in dentate gyrus of hippocampus by immunohistochemistry procedure

Adult coronal free floating brain sections (30 μm) were processed as described elsewhere (Campos et al., 2013). Briefly, after 1-h blockade with PBS supplemented with 0.25% Triton X-100 and 1% bovine serum albumin, brain sections were incubated overnight at 4 °C with the goat polyclonal anti-doublecortin (anti-DCX) primary antibody (Santa Cruz Biotechnology, Dallas, TX, USA), followed by incubation for 1 h at room temperature with the appropriate secondary antibodies (1:1000; Vector Lab, Burlingame, CA, USA). DCX immunoreactivity was detected by the avidin–biotin immunoperoxidase method (Vectastain ABC kit; Vector Lab, Burlingame, CA, USA) and the product of the reaction was revealed by adding the chromogen 3,3-diaminobenzidine tetrahydrochloride (Sigma Chemical, St. Louis, MO, USA). DCX-positive cells were quantified in the subgranular zone of the hippocampus in a minimum of five coronal sections per animal. A 1-in-10 series of hippocampal sections located between 1.3 and 2.5 mm posterior to the bregma were analyzed and positive cells were normalized to the dentate gyrus area determined with ×10 objective. The absolute number of positive cells was calculated considering the total hippocampal volume as determined by the sum of the areas of the sampled sections multiplied by the distances between them (Campos et al., 2013). DCX immunoreactivity was quantified using a computerized image analysis system (ImagePro software, Media Cybernetics, Rockville, MD, USA). A total of four animals per group (control or PbA-infected mice) were used and data are representative of two independent experiments.

Data analysis and statistical evaluation

Results obtained were presented as mean ± standard error of the mean (SEM). All data were tested for normality (Kolmogorov–Smirnov's test) and homogeneity of variances (Levene's test). For variables normally distributed and with compliance of homogeneity of variances, differences were compared by Student's *t* test. Pearson's correlation analyses were performed to examine the relationship among cytokines and neurotrophins. The significance level was set at as $p < 0.05$. Statistical analyses were performed using Prism 4 software (GraphPad, La Jolla, CA, USA) and SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

PbA-infected mice show LTM impairment in the object recognition test

PbA-infected mice, as expected, presented a significant increase in parasitemia during the course of infection. A significant decrease in body weight as well as in the RMCBS score was also found, confirming the development of CM following PbA infection (Fig. 1).

Fig. 2 shows the effect of PbA infection in the object recognition test. No significant difference was found in the total time exploring the objects in the training session, showing that both groups, infected and control, were equally able to explore the objects (Fig. 2A; $t = 1.745$; $df = 18$; $p = 0.0980$). Control mice presented a significant increase in the percentage of time exploring the novel object in the test session compared to

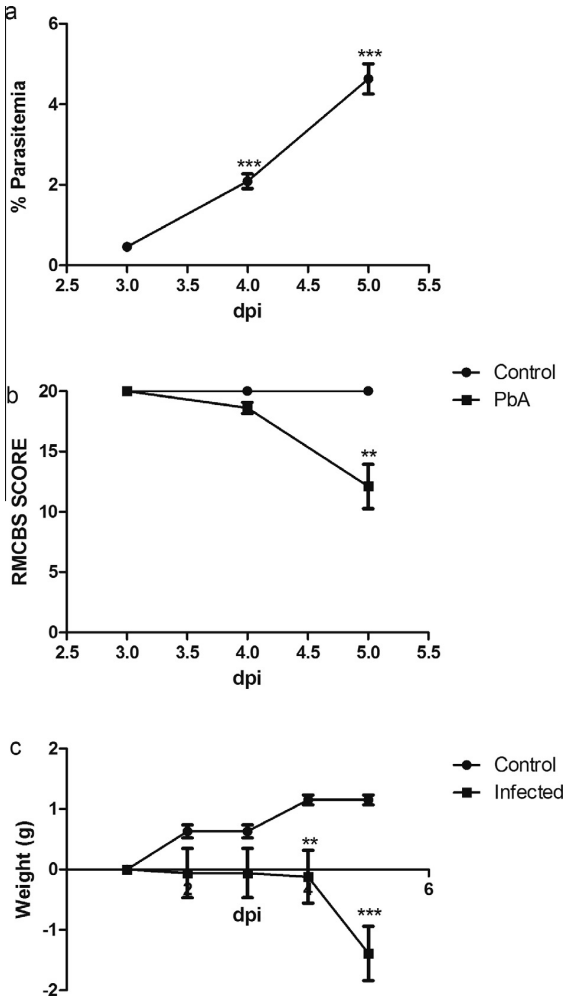

Fig. 1. Clinical signs of cerebral malaria. (a) Natural course of *P. berghei* ANKA (PbA) infection was determined by flow cytometric analysis; (b) clinical signs of cerebral malaria assessed by the RMCBS scale; (c) weight variation in PbA-infected mice compared with controls (non-infected). The data are representative of two independent experiments (n = 10 per group) and shown as the mean ± SEM. Asterisk(s) indicate statistical differences, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

the training session (Fig. 2A; $t = 4.572$; $df = 18$; $p = 0.0005$). No significant difference was found in PbA-infected group comparing the training session with the test session, indicating an impairment of recognition memory (Fig. 2A; $t = 0.09482$; $df = 18$; $p = 0.9265$). When compared with controls, PbA-infected mice also showed a significant decrease in the percentage of time exploring the novel object in the test session 24 h after training session, supporting the cognitive decline (Fig. 2A; $t = 3.233$; $df = 18$; $p = 0.0065$). There was no significant difference in the distance traveled between PbA-infected mice and control groups, indicating that at this time point CM induced long-term memory deficits without promoting changes on locomotor activity (Fig. 2B; $t = 1.722$; $df = 18$; $p = 0.1234$). Importantly, alterations in locomotor activity are accepted as a technically easy and high-throughput measure of sickness behavior, i.e. host nonspecific symptoms such as lethargy and depression (York et al., 2012). Therefore, the cognitive/behavioral findings do not seem to reflect sickness behavior.

Neurotrophin expression in hippocampus and frontal cortex of PbA-infected mice

At day 5 p.i., we analyzed the mRNA expression of BDNF, TRK-B, NGF and the protein levels of BDNF and NGF in the hippocampus and frontal cortex of controls and PbA-infected mice to assess the levels of neurotrophins in brain regions involved in memory formation and consolidation.

The mRNA expression of BDNF was significantly decreased in the frontal cortex ($t = 3.677$; $df = 8$; $p = 0.0104$) and hippocampus ($t = 21.79$; $df = 8$; $p < 0.0001$) of PbA-infected mice compared to controls, whereas a significant increase in TRKB expression (frontal cortex: $t = 17.12$; $df = 8$; $p < 0.001$ and hippocampus: $t = 3.747$; $df = 8$; $p = 0.0046$) was observed (Fig. 3). No significant differences were detected in the mRNA expression of NGF (Fig. 3) or in the protein levels of BDNF and NGF (Fig. 4) in the frontal cortex and hippocampus when comparing controls and PbA-infected animals.

PbA-infection promotes up-regulation of cytokines in hippocampus and frontal cortex

Variations in IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α, and CCL11 are associated with alterations in neurogenesis, and also in learning and memory processes (Hryniewicz et al., 2007; Baune et al., 2008; Derecki et al., 2010; Villeda et al., 2011). We evaluated the levels of these cytokines in the hippocampus and frontal cortex of non-infected and PbA-infected mice.

PbA-infected mice showed increased hippocampus levels of TNF-α ($t = 7.171$; $df = 18$; $p < 0.0001$), IFN-γ ($t = 6.863$; $df = 18$; $p < 0.0001$), IL-6 ($t = 6.874$; $df = 18$; $p < 0.0001$) and CCL11 ($t = 2.793$; $df = 18$; $p = 0.0175$) in comparison with control group (Fig. 5). An up-regulation of IL-10 was also found in the hippocampus of infected mice ($t = 2.704$; $df = 18$; $p = 0.0129$) while no significant differences were observed in IL-2 and IL-4 levels (Fig. 5). Moreover, IL-6 and TNF-α protein levels were negatively correlated with BDNF (IL-6 $r = -0.802$; $p = 0.005$; TNF-α $r = -0.782$; $p = 0.007$) and NGF (IL-6 $r = -0.725$; $p = 0.017$; TNF-α $r = -0.978$; $p < 0.0001$) in the hippocampus of PbA-infected mice (Fig. 6).

PbA-infected mice showed increased frontal cortex levels of TNF-α ($t = 3.413$; $df = 18$; $p = 0.006$), IFN-γ ($t = 4.648$; $df = 18$; $p = 0.0009$), IL-6 ($t = 3.799$; $df = 18$; $p = 0.0035$) and CCL11 ($t = 2.564$; $df = 18$; $p = 0.0208$) in comparison with control group (Fig. 7). No significant differences were observed in IL-2, IL-4 and IL-10 levels (Fig. 6). Moreover, IL-4 protein levels were positively correlated with BDNF (IL-4 $r = 0.700$; $p = 0.026$) in the frontal cortex of PbA-infected mice (Fig. 6).

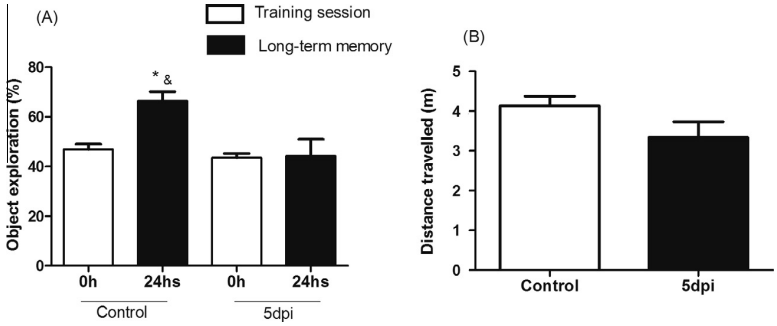

Fig. 2. Novel object recognition memory impairment without locomotor changes following *Plasmodium berghei* ANKA (PbA) infection. C57BL/6 mice (n = 10 *per group*) were intraperitoneally infected with 10^6 parasitized erythrocytes. As a control, non-infected animals received the same volume of PBS (n = 10 *per group*). On days 4 and 5 post-infection (dpi), all the animals were submitted to object recognition task training and test session, respectively. (A) Percentage of object exploration during the training session recorded on day 4 post-infection and during the test session on day 5 post-infection; (B) distance traveled were recorded on day 5 post-infection, 24 h after training session. Results are expressed as mean ± SEM and are representative of at least two independent experiments. Asterisks indicate statistical differences between training and test session in the same group (control or infected) and indicate statistical difference between groups comparing the test session $p < 0.05$.

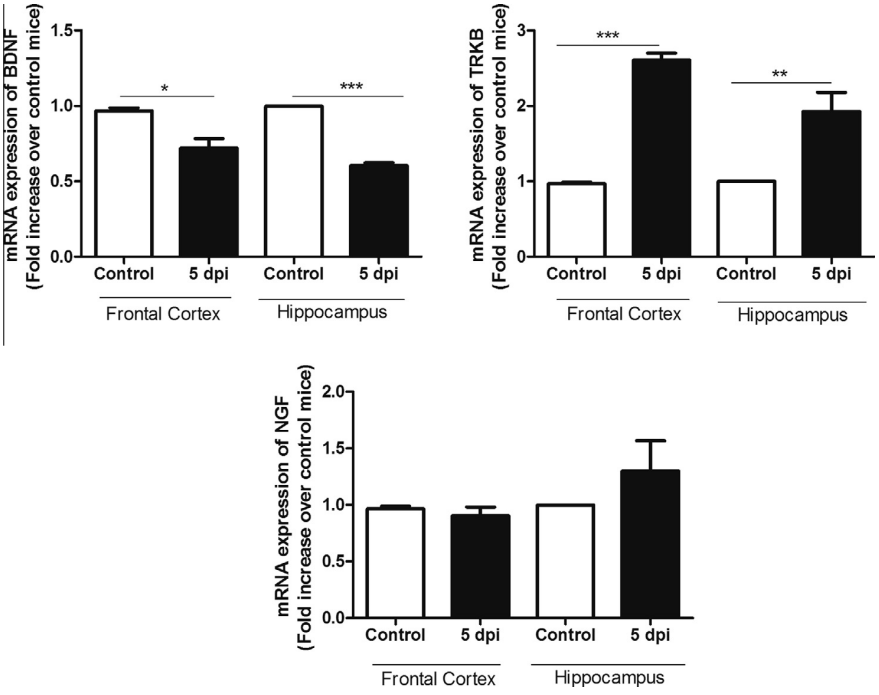

Fig. 3. Frontal cortex and hippocampus mRNA expression of BDNF, its receptor TRKB and NGF. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and controls or non-infected animals received the same volume of PBS (n = 5 *per group*). On day 5 post-infection (5 dpi) all the animals were culled and the frontal cortex and hippocampus were collected for real-time PCR analysis. Results use arbitrary units for the ratio of the target gene mRNA to the endogenous control, eukaryotic 18SmRNA. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Increased hippocampal neuronal death and decreased neurogenesis following PbA infection

To investigate hippocampus integrity following PbA infection, we evaluated cell death and neurogenesis using microscopy techniques. Images obtained from hippocampal slices of PbA-infected mice contained abundant dead (red) cells compared to controls, indicating a high percentage of cell death in the CA1 area (Fig. 8; t = 5.565; df = 10; $p < 0.0001$).

PbA-infected animals also presented a significant decrease in neurogenesis potential measured by the reduction in DCX-positive cells in the dentate gyrus of the hippocampus (Fig. 9; t = 3.781; df = 6; $p < 0.001$).

DISCUSSION

A poor cognitive outcome has been widely reported following human and experimental CM. However, the

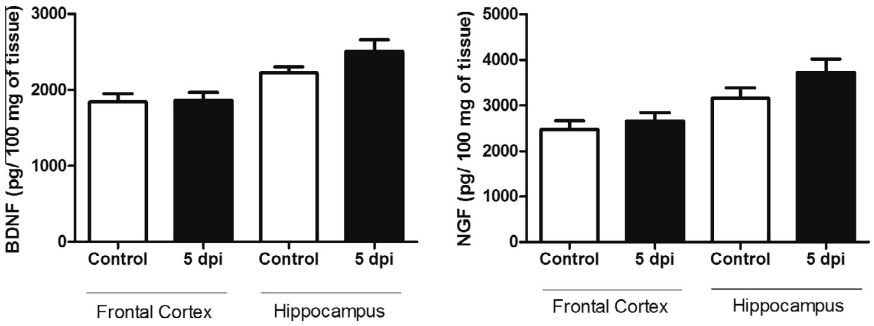

Fig. 4. Frontal cortex and hippocampus protein levels of the neurotrophins BDNF and NGF. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and controls or non-infected animals received the same volume of PBS ($n = 5$ *per group*). On day 5 post-infection (5 dpi) all the animals were culled and the frontal cortex and hippocampus were harvested, homogenized and neurotrophic factor concentration were measured by ELISA. Results are expressed as mean ± SEM.

underlying mechanisms of cognitive dysfunction remain unclear (Desruisseaux et al., 2008; John et al., 2008; Miranda et al., 2013).

In the current study, cognitive decline was evaluated by the novel object recognition test. The training session or memory acquisition phase was performed on day 4 p.i. when PbA-infected mice had a clinical score in the RMCBS equal to those found in control animals with no locomotor activity changes. It has been reported that synaptic plasticity changes associated with memory acquisition and consolidation, and induced by the object recognition test occur only a few hours (peak at 6 h) after the training phase (Clarke et al., 2010). Accordingly, the decrease in the percentage of time exploring the new object observed in the infected group 24 h after the training session would reflect the cognitive decline associated with the progression of CM rather than a deficit in the acquisition of memory in the training phase.

In the present study, we demonstrated that object recognition memory impairment was associated with a significant reduction in BDNF mRNA expression in the frontal cortex and hippocampus of PbA-infected mice. In agreement with our findings, Linares et al. demonstrated a progressive down-regulation of BDNF mRNA expression in several brain regions including the thalamus, hypothalamus, cerebellum and brainstem during the course of PbA infection (Linares et al., 2013). BDNF levels remained low in the hippocampus of CM mice even following full parasitemia clearance with chloroquine therapy which were associated with long-term cognitive deficits (Comim et al., 2012). A decrease in hippocampal BDNF levels correlated with memory impairment in different experimental models (Bilbo et al., 2008; Barichello et al., 2010; Jurgens et al., 2012). Moreover, genetic depletion of the BDNF released by microglia, under physiological conditions, resulted in deficits in multiple learning tasks and in motor-learning-dependent synapse formation, supporting the role of this neurotrophin in cognitive function (Parkhurst et al., 2013).

Interestingly, we showed for the first time an increase in TRKB mRNA expression in both the frontal cortex and hippocampus of PbA-infected mice. TRKB is the high-affinity receptor for BDNF and mediates BDNF signaling, regulating several aspects of neural plasticity including long term potentiation, neurogenesis and memory (Barnes and Thomas, 2008; Lu et al., 2008). The increase of TRKB expression may represent a compensatory mechanism following BDNF reduction as an attempt to protect the brain from damage. This assumption is supported by other studies. For instance, Li et al. reported that an up-regulation of TRKB in the rat brain 24 h after *Streptococcus pneumoniae* infection exerts a neuroprotective role, preventing neurological sequelae caused by pneumococcal meningitis (Li et al., 2011).

We also investigated protein levels of BDNF and NGF in the frontal cortex and hippocampus, but no significant differences between controls and PbA-infected groups were found. Corroborating this finding a recent study found similar protein levels of BDNF in the cerebellum and brainstem of control animals and of those with symptomatic stage of CM (Linares et al., 2013). The lack of diminished BDNF at the protein level could be explained by a down-regulation of proteasome subunits which delays protein turnover via the ubiquitin-proteasome pathway, masking BDNF mRNA reduction (Sommerfeld et al., 2000). The proteasome impairment could be mediated by an up-regulation of inflammatory cytokines such as TNF-α which are involved in CM pathogenesis (Groettrup et al., 2010).

In parallel with a down-regulation of BDNF mRNA expression we found a significant decrease of DCX-positive cells associated with higher levels of inflammatory cytokines (IL-6, IFN-γ and TNF-α) and the CCL11 chemokine in the hippocampus of infected mice. Moreover, an up-regulation of the anti-inflammatory cytokine IL-10 was found. In agreement with our findings, a recent work reported that mild malaria induced by *Plasmodium chabaudi adami* results in adult hippocampal neurogenesis inhibition associated with increased levels of inflammatory cytokines in the CNS even in the absence of a blood–brain barrier rupture (Guha et al., 2014). Moreover, the detrimental effects of inflammation on adult neurogenesis have been reported in other acute and chronic inflammatory models (Ekdahl et al., 2003; Monje et al., 2003; Jakubs et al., 2008; Fujioka and Akema, 2010; Das et al., 2011). For instance, in a murine model of Japanese encephalitis the resultant microglial activation and inflammatory cytokines (IL-6,

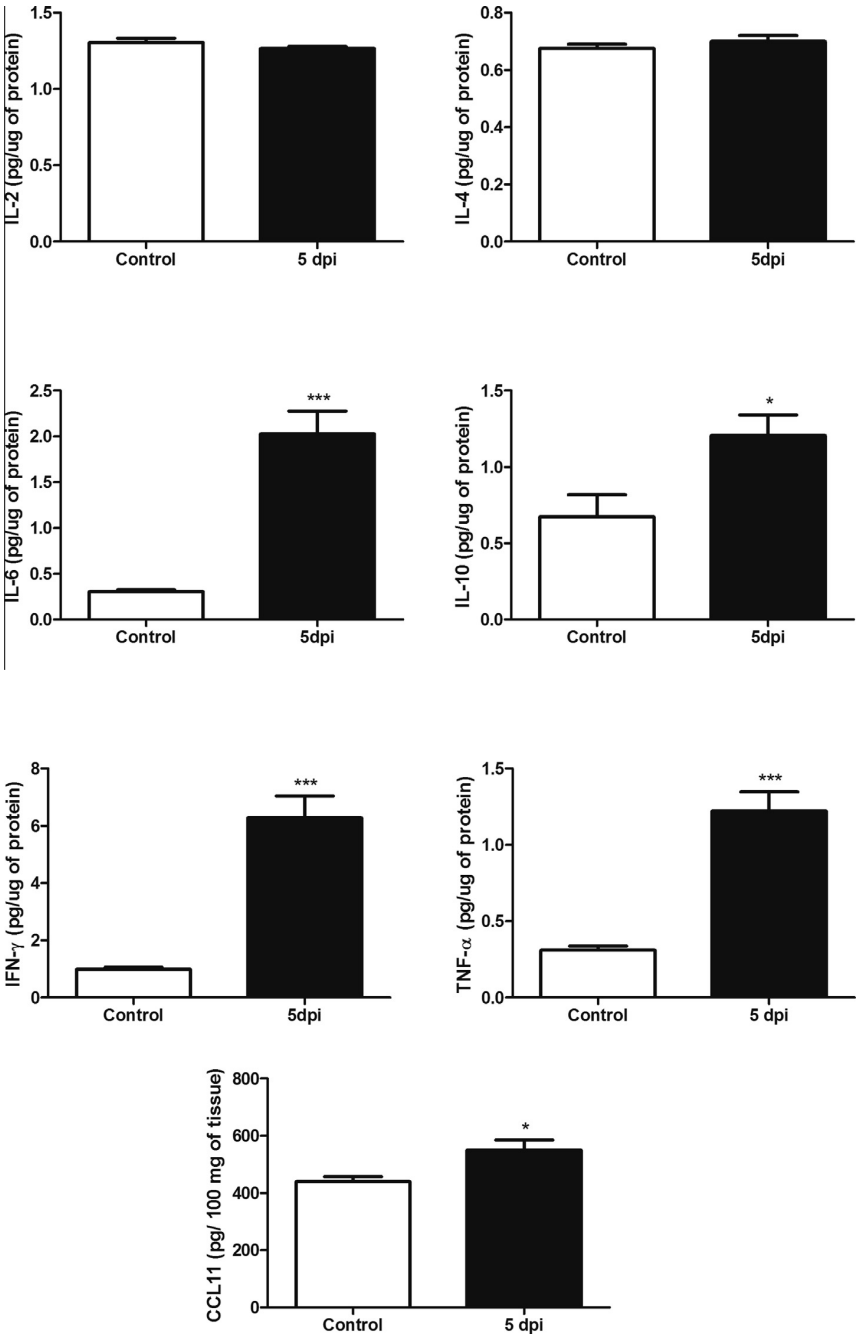

Fig. 5. Hippocampus protein levels of pro-inflammatory and anti-inflammatory cytokines following *Plasmodium berghei* ANKA (PbA) infection. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and controls or non-infected animals received the same volume of PBS ($n = 10$ *per group*). On day 5 post-infection (5 dpi) all the animals were culled and the hippocampus was harvested, homogenized and CCL11 concentration was measured by ELISA while IL-2, IL-4, IL-10, IL-6, IFN-γ and TNF-α levels were assessed by cytometric bead array (CBA). Results are expressed as mean ± SEM. Asterisk(s) indicate statistical differences where $^{*}p < 0.05$, $^{***}p < 0.001$.

IFN-γ and TNF-α) impair the proliferation and differentiation of new neurons in the subventricular zone (Das et al., 2011). A chronic inflammatory environment induced by intrahippocampal infusion of LPS impairs integration of new neurons into pre-existing neural networks and increases inhibitory signaling to new neurons (Jakubs et al., 2008). On the other hand, it has been reported that regulatory cytokines such as IL-4 and IL-10 may support neurogenesis. An enhancement in new cells survival, proliferation and neuronal differentiation was observed when

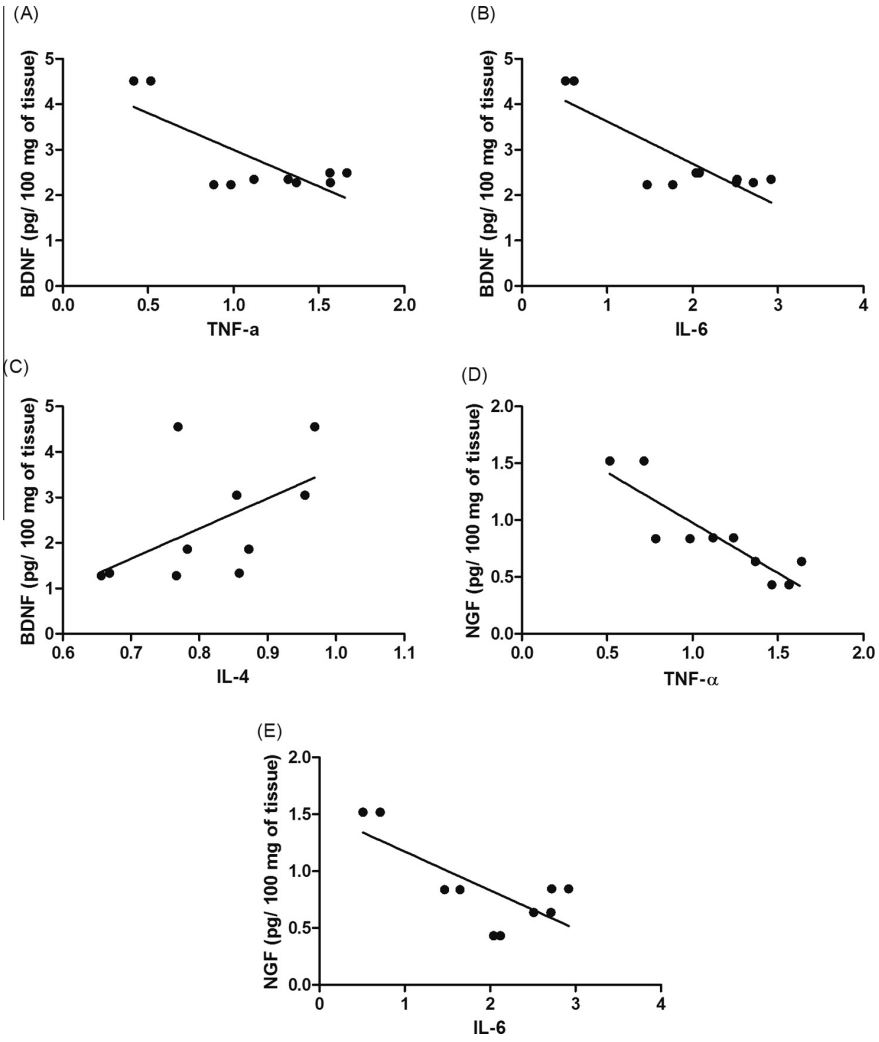

Fig. 6. Pearson correlation between cytokines (IL-4, IL-6, TNF-α) and neurotrophins (BDNF, NGF) in the hippocampus (A, B, D, E) and frontal cortex (C) of mice infected with *Plasmodium berghei* ANKA (PbA) strain ($n = 10$ *per group*).

neural progenitor cells were co-cultured with microglia stimulated with IL-4 or IL-10 (Butovsky et al., 2006; Cacci et al., 2008; Kiyota et al., 2012). IL10 elevation seems to be closely associated with IL-6 production, supporting the hypothesis of a counter-regulatory mechanism mediated by IL-10. Interestingly, an increase in IL-6 and IL-10 levels was found in the serum of CM children compared to those with severe malaria without neurological symptoms and healthy controls (Lyke et al., 2004). In this scenario, the up-regulation of IL-10 found in the present study may be regarded as a compensatory response to counterbalance the deleterious effects of the overexpression of inflammatory cytokines.

Apart from their role in neurogenesis impairment, inflammatory molecules, particularly TNF-α, can mediate apoptotic and/or necrotic processes (Clark et al., 2010; Arslan and Scheidereit, 2011). At the same time point of neurogenesis reduction, we also found a significant increase of cell death in the CA1 area of the hippocampus of PbA-infected mice. The imbalance in cell death and new neuron formation can impair hippocampus integrity function (Daval and Vert, 2004), potentially explaining, at least in part, CM cognitive deficits.

A growing body of evidence has suggested that hippocampal-cortical neural circuit may underlie learning and memory processes, with changes in this system leading to cognitive dysfunction (Barichello et al., 2009; Barichello et al., 2014; Evuarherhe et al., 2014; Mina et al., 2014). In the present study, PbA-infected mice also presented a significant increase of inflammatory cytokines (IL-6, IFN-γ and TNF-α) and CCL11 chemokine in the frontal cortex compared with controls. High levels of IL-6 and TNF-α in the frontal cortex of Wistar rats with *Klebsiella pneumoniae* meningitis were associated with aversive memory impairment (Barichello et al., 2014). Similar findings were recently reported in a sepsis model induced by cecal ligation and perforation (Mina et al., 2014). The authors also demonstrated that the

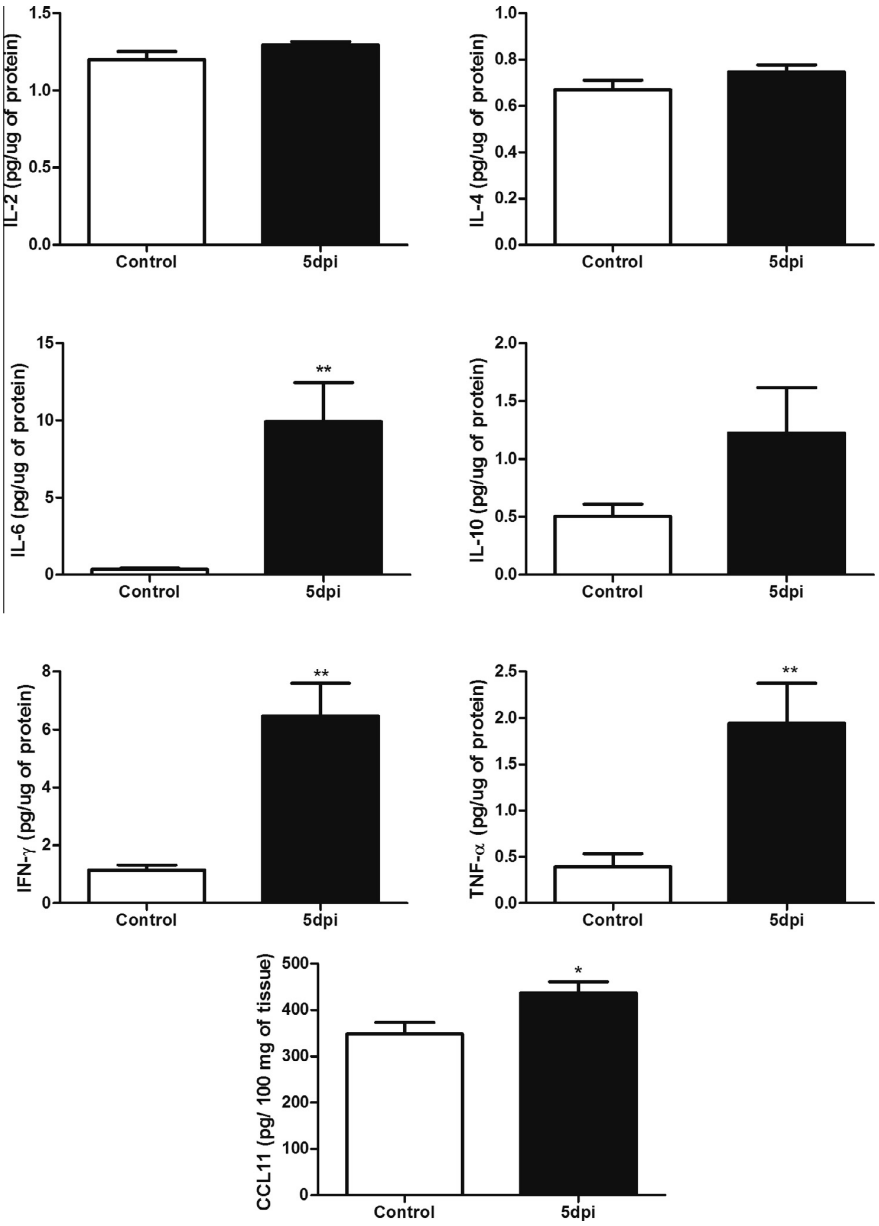

Fig. 7. Frontal cortex protein levels of pro-inflammatory and anti-inflammatory cytokines following *Plasmodium berghei* ANKA (PbA) infection. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and controls or non-infected animals received the same volume of PBS ($n = 10$ per group). On day 5 post-infection (5 dpi) all the animals were culled and hippocampus was harvested, homogenized and CCL11 concentration was measured by ELISA while IL-2, IL-4, IL-10, IL-6, IFN-γ and TNF-α levels were assessed by cytometric bead array (CBA). Results are expressed as mean ± SEM. Asterisk(s) indicate statistical differences where $^{*}p < 0.05$, $^{**}p < 0.01$.

administration of IL-1β receptor antagonist (IL-1ra) immediately after sepsis induction reverted the increase of inflammatory cytokines in the pre-frontal cortex and improved cognitive function (Mina et al., 2014). Taken together, these studies corroborate our findings indicating that neuroinflammation resulting from infection can influence the hippocampal-cortical circuit and contribute, at least in part, with the emergence of cognitive symptoms.

It has been reported that immune system can affect neurogenesis, and memory and learning functions by modulation of neurotrophin expression (Tanaka et al., 2006; Bilbo et al., 2008; Taepavarapruk and Song, 2010). We found a negative correlation between IL-6 and TNF-α with BDNF and NGF levels in the hippocampus of PbA-infected mice. In line with our findings, Jurgens et al. demonstrated that Influenza virus-infected mice exhibited cognitive deficits in parallel with increase of inflammatory cytokine expression and decrease of BDNF and NGF mRNA expression in the hippocampus. They also found that Influenza virus induced architectural

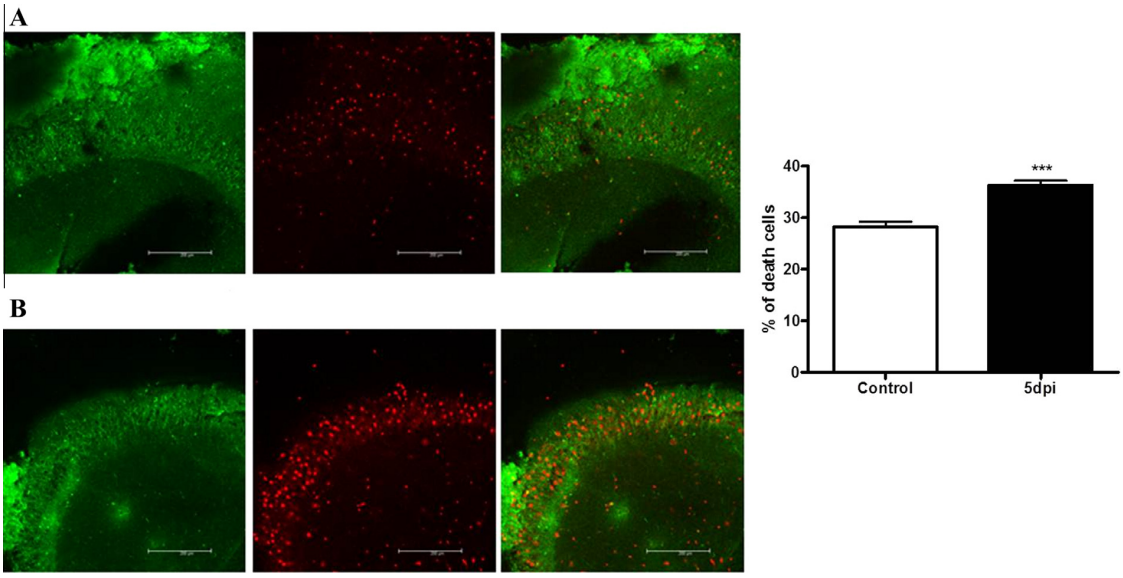

Fig. 8. High percentage of cell death in hippocampus CA1 area following *Plasmodium berghei* ANKA (PbA) infection. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and controls or non-infected animals received the same volume of PBS ($n = 6$ *per group*). On day 5 post-infection (5 dpi) all the animals were culled and hippocampal slices were stained for viable cells with calcein (green fluorescence) whereas dead cells were indicated by the red fluorescence of ethidium homodimer. Cell death in the hippocampal slices following calcein and ethidium homodimer staining were analyzed by Confocal Microscopy. In the representative image letter (A) are the controls and letter (B) are the PbA-infected mice. Results are expressed as mean ± SEM. Asterisk(s) indicate statistical differences where $^*p < 0.05$.

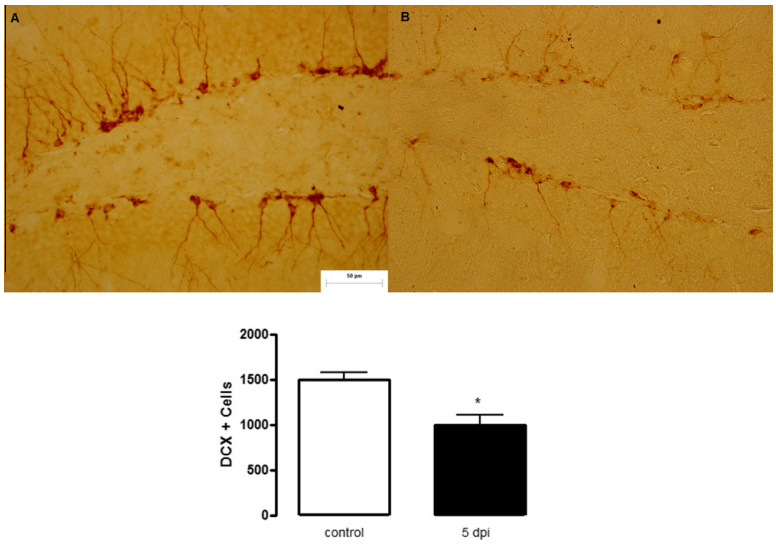

Fig. 9. Doublecortin (DCX)-positive cells' reduction in the subgranular zone following *Plasmodium berghei* ANKA (PbA) infection. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and controls or non-infected animals received the same volume of PBS ($n = 4$ *per group*). On day 5 post-infection (5 dpi) all the animals were culled and coronal free floating brain sections (30 μm) were processed and then incubated with the rabbit polyclonal anti-doublecortin primary antibodies. Doublecortin immunoreactivity was analyzed by Confocal Microscopy and used as a measure of neurogenesis in the subgranular zone of the hippocampus. In the representative image letter (A) are the controls and letter (B) are the PbA-infected mice. Results are expressed as mean ± SEM and are representative of at least two independent experiments. Asterisk(s) indicate statistical differences where $^*p < 0.05$.

changes to hippocampal neurons in the CA1 and dentate gyrus, providing evidence that neuroinflammation and changes in hippocampal structural plasticity may underlie cognitive dysfunction following Influenza infection (Jurgens et al., 2012). Moreover, transgenic mice over-expressing TNF-α in the CNS showed decreased levels of NGF and BDNF in the hippocampus associated with cognitive deficits, indicating that this cytokine significantly

influences BDNF and NGF synthesis, probably in a dose-dependent manner (Aloe et al., 1999; Fiore et al., 2000). IL-6 likely TNF-α also influences neurotrophic factor expression and its up-regulation has been associated with neurodegenerative conditions such as Alzheimer's disease along with a significant decrease in local BDNF transcripts (Peng et al., 2009; Spooren et al., 2011). There is evidence that BDNF down-regulation in Alzheimer's disease depends on beta-amyloid protein aggregation (Peng et al., 2009), a phenomenon mediated, among other factors, by IL-6 (Del Bo et al., 1995; Ringheim et al., 1998; Spooren et al., 2011). We also found a positive correlation between BDNF and IL-4 in the frontal cortex although no changes in their protein levels were observed. Both molecules have been associated with neuroprotective action, exerting beneficial effects on cognition (Choy et al., 2008; Derecki et al., 2010). Altogether these studies suggest that inflammatory cytokines can modulate neurotrophin activity, influencing their roles in the brain as well as the outcome of different CNS disorders. Herein we provided evidence on the involvement of cytokines and neurotrophin in CM-induced memory impairment.

Beyond their classical role in directing the migration of leukocytes, chemokines have been recognized as regulatory factors influencing the development of different tissues including the CNS (Ransohoff, 2009). In particular, some chemokines play a role in neurogenesis. Neural precursor cells express chemokine receptors and the involvement of CXCL12 and its receptors CXCR4 and CXCR7 in the development and functions of CNS has been well recognized (Krathwohl and Kaiser, 2004, Dambly-Chaudiere et al., 2007, Wang et al., 2011). Recently, Villeda et al. demonstrated an increase in CCL11 levels in the plasma of aging-mice associated with a decline in memory as assessed by contextual fear conditioning and water maze tasks. Interestingly, young mice (2–3 months) receiving old mice (18–22 months) plasma by heterochronic parabiosis or systemic administration presented a decrease in neurogenesis, synaptic plasticity and impaired learning and memory. After intraperitoneal injections of recombinant CCL11 into adult young mice a significant reduction in DCX-positive cells in the dentate gyrus was also observed while systemic or central neutralization of CCL11 rescued neurogenesis impairment (Villeda et al., 2011). This elegant paper proposed a major role for CCL11 in the age-related decline of hippocampal neurogenesis. Our finding of increased levels of CCL11 in the hippocampus of PbA-infected mice provides further evidence on the role of this chemokine in neurogenesis.

In summary, we provide further evidence that the exacerbated production of inflammatory cytokines in the CNS is associated with changes in neurotrophin expression, decreased hippocampal neurogenesis and increased hippocampal cell death, leading to memory impairment in CM.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTION

ASM participated in the experimental design, carried out behavioral, immunological and Confocal Microscopy assays, data analysis and drafted the manuscript. FB participated in the experimental design, carried out behavioral and immunological assays and revised the manuscript. ACC participated in neurogenesis assays, data analysis and revised the manuscript. LBV and NB carried out cell death assays, data analysis and revised the manuscript. NPR and DHR performed immunological assays. DC performed real-time PCR analysis. RMR was responsible for interpretation of data, revised and edited the manuscript. FSM and MAR participated in the design and coordination of the study. ALT designed the study and was responsible for the interpretation of experiments and editing the manuscript. All authors have read and approved the final version of the manuscript.

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6.2 – Artigo científico 2

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MALARIA JOURNAL

RESEARCH Open Access

Further evidence for an anti-inflammatory role of artesunate in experimental cerebral malaria

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Abstract

Background: Cerebral malaria (CM) is a clinical syndrome resulting from *Plasmodium falciparum* infection. A wide range of clinical manifestations follow the disease including cognitive dysfunction, seizures and coma. CM pathogenesis remains incompletely understood and without treatment this condition is invariably fatal. Artesunate has been accepted as the most effective drug for treating severe malaria. Besides its antiparasitic activity, an anti-inflammatory property has also been reported. In the current study, the immunomodulatory role of artesunate was investigated using a *Plasmodium berghei* ANKA model of CM, trough evaluation of behavioural changes and cytokines expression in hippocampus and in frontal cortex.

Methods: C57Bl/6 mice were infected with *P. berghei* by intraperitoneal route, using a standardized inoculation of 10^6 parasitized erythrocytes. Memory function was evaluated using the step-down inhibitory avoidance test. The mRNA expression of IFN-γ, IL-1β, IL-6 and TNF in the frontal cortex and hippocampus of control and infected mice on day 5 post-infection were estimated by quantitative real time PCR. *Plasmodium berghei* -infected mice also received intraperitoneally a single dose of artesunate (32 mg/kg) on day 4 post-infection, and 24 hours after treatment behavioural and immunological analysis were performed. The protein levels of cytokines IL-2, IL-6, IL-10, IL-17, IFN-γ, TNF in the serum, frontal cortex and hippocampus of controls and *P. berghei* -infected mice treated or not treated with artesunate were determined using a cytometric bead array (CBA) kit. The survival and neurological symptoms of CM were also registered.

Results: CM mice presented a significant impairment of aversive memory compared to controls on day 5 post-infection. A higher mRNA expression of pro-inflammatory cytokines was found in the hippocampus and frontal cortex of infected mice. A single dose of artesunate was also able to decrease the expression of inflammatory cytokines in the hippocampus and frontal cortex of *P. berghei*-infected mice. In parallel, a significant improvement in neurological symptoms and survival were observed in artesunate treated mice.

Conclusions: In summary, the current study provided further evidence that CM affects key brain areas related to cognition process. In addition, different patterns of cytokine expression during the course of CM could be modulated by a single administration of the anti-malarial artesunate.

Keywords: Malaria, Cerebral malaria, Memory impairment, Artesunate, Cytokines, Neuroinflammation

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Background

Cerebral malaria (CM) is a potentially reversible, diffuse encephalopathy characterized mainly by the presence of asexual forms of *Plasmodium falciparum* parasites in the peripheral blood smears, and altered level of consciousness in the absence of other causes of encephalopathy, especially meningitis and viral encephalitis [1]. This condition accounts for approximately 80% of all fatal cases of malaria, being the leading cause of hospitalization and mortality of children under five years of age in sub-Saharan Africa [2,3]. A series of residual symptoms may follow the resolution of CM including cognitive, behavioural and motor changes [4]. Moreover, a significant social, economic and educational burden has been reported in endemic areas of malaria [5].

Although vascular, immunological and metabolic changes have been implicated in CM pathogenesis, it remains largely unknown [6]. The obstruction of cerebral microvasculature by sequestered infected red blood cells and an exacerbation in host inflammatory response are the two main non-exclusive theories widely accepted to explain this pathological process [7].

The release of pro-inflammatory mediators, including tumor necrosis factor (TNF) and interleukin (IL)-1, and immune cells infiltration in the brain parenchyma have been associated with cognitive and behavioural alterations during the acute phase of experimental CM [8-10]. However, it was only recently that a study conducted by Linares *et al.* [11] suggested that the immune response evoked by CM can specifically affect key brain areas involved in the control of cognition and mnemonic process, such as the hippocampus and the frontal cortex.

Without treatment CM invariably leads to death. Emergency management is based on the correction of metabolic states, re-establishment of vital physiological functions severely affected by CM and the administration of an effective anti-parasitic drug [12].

Artesunate, a semi-synthetic derivative of artemisinin, has been accepted as the most effective and safe drug for treating severe and chloroquine-resistant malaria [13]. Besides its antiparasitic activity, artesunate has shown to exhibit a putative anti-inflammatory profile in different inflammatory models, including sepsis, arthritis, systemic lupus erythematous and allergic asthma [14-18]. Clemmer *et al.* [19] also demonstrated that artesunate was effective in rescuing mice in a late-stage CM and that artemether, another compound derivative of artemisinin, decreases leukocyte accumulation in brain vessels. However, no previous study has investigated the immunomodulatory properties of artesunate in the central nervous system (CNS) during the progression of CM.

The present study aimed to characterize the role of inflammatory mediated process within the hippocampus and frontal cortex in the behavioural changes (memory impairment and sickness behaviour) induced by CM. In addition, the putative immunomodulatory effect of a single dose of artesunate was also investigated in the experimental model of CM.

Methods

Ethics statement

This study was carried out in strict accordance with the Brazilian Government's ethical and animal experiments regulations. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of the Universidade Federal de Minas Gerais (CETEA/UFMG, Permit Protocol Number 105/09). All tissue-collected procedures was performed under ketamine/xylazine anesthesia and all efforts were made to minimize animal suffering.

Animals

Female C57BL/6 mice (20-25 g), aged six to eight weeks, were obtained from Animal Care Facilities of the Institute of Biological Sciences, Federal University of Minas Gerais (ICB-UFMG), Belo Horizonte, Brazil. The animals were housed in groups of six mice per cage in a room controlled temperature (24°C) with food and water *ad libitum*. Experiments were performed on day 5 post-infection (pi), when mice infected with *Plasmodium berghei* strain ANKA develop brain inflammation without motor impairment.

Parasite, experimental infection and artesunate treatment

Due to the high degree of reproducibility, easily manageable characteristics and development of histopathological and neurological signs typical of human CM, the murine model using the *Plasmodium berghei* strain ANKA has been widely used to better understand this condition [20]. In the present study, blood stages of *P. berghei* strain ANKA constitutively expressing green fluorescent protein (*P. berghei* ANKA-GFP) (15cy1 clone), kindly provided by Dr Claudio Marinho (Universidade de São Paulo), were stored in liquid nitrogen [19]. Mice were infected intraperitoneally (ip) with 10^6 *P. berghei*-infected red blood cells suspended in 0.2 mL PBS [21]. Artesunate (Sigma, St. Louis, MO, USA) was dissolved in 5% sodium bicarbonate in saline and a single dose (32 mg/kg) was administered ip in the morning of the fourth day pi [19,22]. Control animals received the same volume of vehicle.

The percentage of parasitaemia was determined by flow cytometry. Briefly, a drop of blood from the tail was collected directly into 2 ml of PBS for flow cytometry analysis. Each sample was run on a FACScalibur (Becton Dickinson, San Jose, CA, USA) flow cytometer with a 488 nm argon laser and DIVA software (Becton Dickinson, San Jose, CA, USA). Erythrocytes were identified on the basis of their specific forward (FSC) and

side (SSC) light-scattering properties and a total of 100,000 events were counted for each sample. Mice were observed daily for parasitaemia, body weight, survival, and clinical neurological signs of CM (i e, ataxia, paralysis and coma).

Step-down inhibitory avoidance test

The step-down inhibitory avoidance test was performed to assess short- and long-term aversive memory on day 5 and 6 post-infection (control and infected mice; n = 10 per group) as previously described by Reis *et al.* [23]. Briefly, in the training trial, animals were placed on the platform and their latency to step down on the grid with all four paws was measured. Immediately after the stepping down on the grid, the animals received a single mild foot shock (0.4 mA, 2.0 seconds). A retention test trial was performed 1.5 hour and 24 hours after the training section. The results were expressed as latency period to step down the platform, with a cut-off at 180 seconds. All tests were performed by the same investigator who was blinded to the animal status (control or infected).

Open field test

The open field test was performed as previously described by Barichello *et al.* [24] and conducted in order to evaluate the locomotor and exploratory activities on day 5 and 6 pi of control and infected mice (n = 5 per group). The apparatus is a 30 cm × 30 cm circular open field surrounded by 30 cm-high walls made of transparent plexiglass (Insight®, São Paulo, Brazil). The floor of the open field is divided in 12 rectangles by black lines. Briefly, animals were gently placed on the left rear quadrant, and then they were allowed to explore the arena for 5 minutes. The number of crossings (the number of times that animals crossed the black lines) and rearings (the exploration behaviour observed in rats subjected to a new environment) were recorded. Behavioural tests were performed by the same investigator who was blinded to the animal status (control or infected).

Rapid murine coma and behaviour scale (RMCBS)

Behavioural and functional parameters were evaluated using the RMCBS protocol as previously described by Carrol *et al.* [25]. The RMCBS is a quantitative and objective scale that enables an investigator to rapidly follow up the course of CM. The subjects were labelled as affected or not, and the levels of illness are correlated with neuropathological injury. This method intended to imitate the situation in the field and attempted to bring the animal model closer to the human disease [25].

The RMCBS consists of ten parameters (gait, balance, motor performance, body position, limb strength, touch scape, pinna reflex, toe pinch, aggression and grooming) based on the components of the SHIRPA (SmithKline/Harwell/Imperial College/Royal Hospital/Phenotype Assessment) score [26]. Each item is scored from zero as the lowest, to two as the highest, with a maximum total score of 20. The procedure was carried out on day 3 until day 5 pi. On day 5 pi the RMCBS were performed approximately 24 hours after artesunate treatment. A total of five animals per group were used. After the test all animals were sacrificed under deep anesthesia by ip injection of a mixture of Ketamine (150 mg/kg, Laboratório Cristália, Brazil) and Xylazine (10 mg/kg, Rompun®, Bayer, Germany), decapitated, frontal cortex and hippocampal dissected and serum collected for posterior cytokine expression assessment.

Hippocampal and frontal cortex mRNA expression of cytokines

The mRNA expression of IFN-γ, IL-1β, IL-6 and TNF in the hippocampus and frontal cortex of *P. berghei*-infected mice and matched controls (n = 5 per group) were estimated by quantitative real time PCR (polymerase chain reaction) at day 5 pi. RNA isolation was performed using Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The RNA obtained was re-suspended in diethyl pyrocarbonate-treated water and stocked at -70°C until use. Reverse transcription was performed using 2 μg of total RNA, 200 U of reverse transcriptase, RT buffer 5 × (4 μl), 10 mM dNTPs (1 μl), RNAsin 10,000 U (0.2 μl) and oligo dT 15 50 μM (1.0 μl) in a final reaction volume of 20 μl. Resultant cDNA was used for qPCR. Real-time RT-PCR was performed on an ABI PRISM 7900 sequence-detection system (Applied Biosystems, CA, USA) by using SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) after a reverse transcription reaction of 2 μg of total RNA by using M-MLV reverse transcriptase (Promega, Madison, Wisconsin-WI, USA). Specific primers were designed using Primer Express software and synthesized by integrated DNA technologies (IDT). The relative

Table 1 Oligonucleotide primers for real-time quantitative PCR (SYBR)

Primer sequences	Forward	Reverse
IFN-γ	CAG GAT CCT TTG GAC CCT CTG AC	GGC AGA ATT AAG CTT ATT GGG AC
TNF-α	ACG GCA TGG ATC TCA AAG AC	AGA TAG CAA ATC GGC TGA CG
IL-1β	CTA CAG GCT CCG AGA TGA ACA AC	TCC ATT GAG GTG GAG AGC TTT C
IL-6	TTC CAT CCA GTT GCC TTC TTG	TTG GGA GTG GTA TCC TCT GTG A
18S	CGT TCC ACC AAC TAA GAA CG	CTC AAC ACG GGA AAC CTC AC

levels of gene expression were determined by the ΔΔ Cycle threshold method as described by the manufacturer, in which data for each sample is normalized to 18S expression and data were shown as fold increase over the negative control (non-infected) group. Specific primers sequences are described in Table 1.

Cytometric bead array (CBA) and enzyme-linked immunosorbent assay (ELISA) analyses of cytokines

Hippocampal and frontal cortex tissues of controls (n = 5 per group) and *P. berghei*-infected mice treated or not treated (n = 5 per group) with a single dose of artesunate were carefully removed on day 5 pi and homogenized in a PBS-buffer extraction solution containing a protease-inhibitor cocktail. Lysates were centrifuged at 13,000 g for 10 minutes at 4°C and quantified using the Bradford assay reagent from Bio-Rad (Hercules, CA, USA). Analyses of serum and brain cytokine levels were determined using a mouse Th1/Th2/Th17 cytometric bead array kit (CBA; BD Biosciences, San Diego, CA, USA) and analysed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The following cytokines were measured:

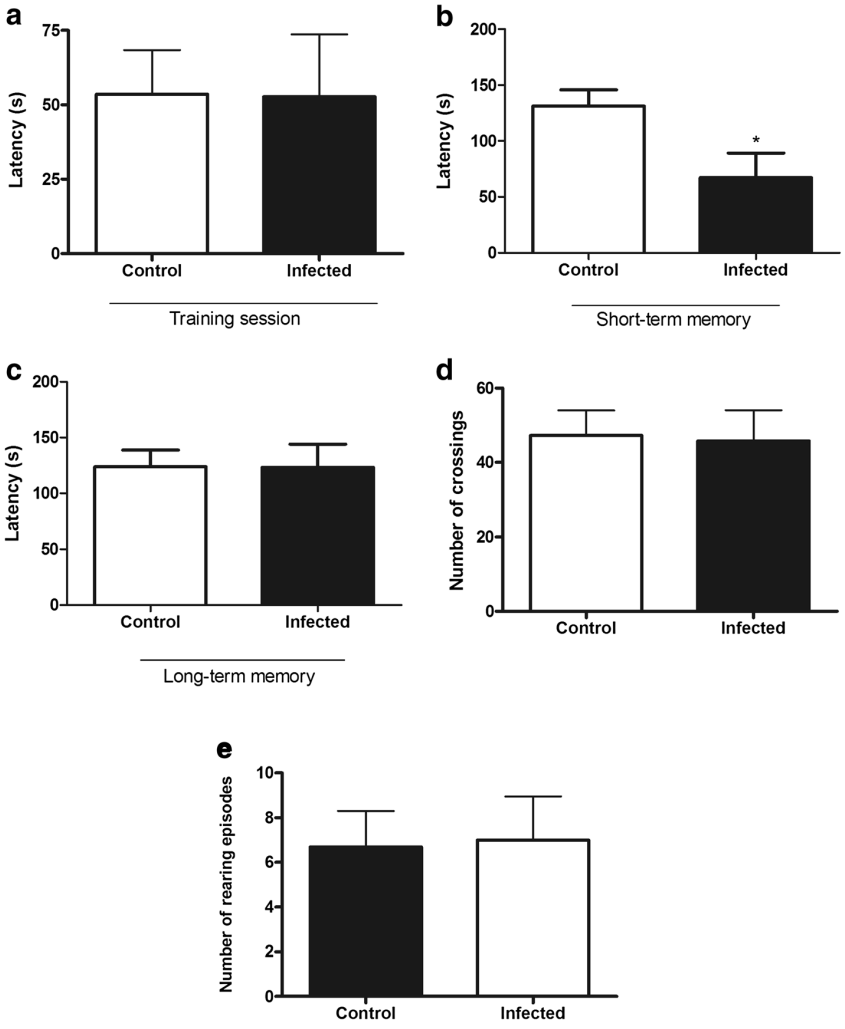

Figure 1 Short-term aversive memory impairment without locomotor and exploratory changes following *Plasmodium berghei* ANKA infection. Aversive memory, locomotor and exploratory activities measurement of *P. berghei*-infected mice on day 5 and 6 post-infection (pi) and of control group in the step-down inhibitory avoidance test **(a-c)** and in the open field **(d-e)**. **(a)** Latency in seconds during training session (5 pi) in the step-down inhibitory avoidance test; **(b)** Short-term memory analysed 1.5 hours after training session in the step-down inhibitory avoidance test; **(c)** Long-term memory analysed 24 hours (6 pi) after training session in the step-down inhibitory avoidance test; **(d)** number of crossings and **(e)** number of rearing episodes analysed on day 5 post-infection in the open field. Results are expressed as mean ± SEM and are representative of two independent experiments. Asterisk(s) indicate statistical differences, *$p < 0.05$.

interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-17 (IL-17), Interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF). The concentration of the cytokine IL-1β was determined by ELISA (R&D Systems, Minneapolis, MN, USA) in accordance with manufacturer's instructions.

Data analysis and statistical evaluation

Results obtained were presented as mean ± standard error of the mean (SEM). All data were tested for normality (Kolmorov-Smirnov's test) and homogeneity of variances (Levene's test). For variables normally distributed and with compliance of homogeneity of variances, differences were compared by using Student's t test, analysis of variance (ANOVA) or Two-way ANOVA. Duncan's post-test was used as needed for multiple comparisons. In the case of variables not normally distributed, differences were analysed by Kruskal-Wallis non-parametric test. When significant effects were detected by this latter test, post-hoc comparisons were performed using the Mann-Whitney U test. Differences between lethality curves were calculated using the Log rank test. The significance level was set at the level of $p < 0.05$. Statistical analyses were performed using Prism 4 software (GraphPad, La Jolla, CA, USA) and SPSS software version 17.0 (SPSS Inc, Chicago, IL, USA).

Results

Plasmodium berghei-infected mice exhibited short-term aversive memory impairment

Figure 1 shows the effect of *P. berghei* infection in the step-down inhibitory avoidance test and open field test. No difference in the step-down latency between *P. berghei*-infected mice and controls was found in the training session (Figure 1a). In the test session performed 1.5 hours after the training to evaluate the short-term aversive memory, infected mice presented a significant decrease in the step-down latency (Figure 1b; $p = 0.03$). No difference was found in the long-term aversive memory analysed 24 hours (day 6 pi) after training session (Figure 1c; $p = 0.99$).

In the open field test, there were no significant differences in the number of crossings (Figure 1d; $p = 0.89$) and rearings (Figure 1e; $p = 0.91$) in the *P. berghei*-infected group compared to the controls on day 5 pi, indicating no difference in motor and exploratory activities, respectively. However, on day 6 pi, the period when the long-term memory was assessed, *P. berghei*-infected

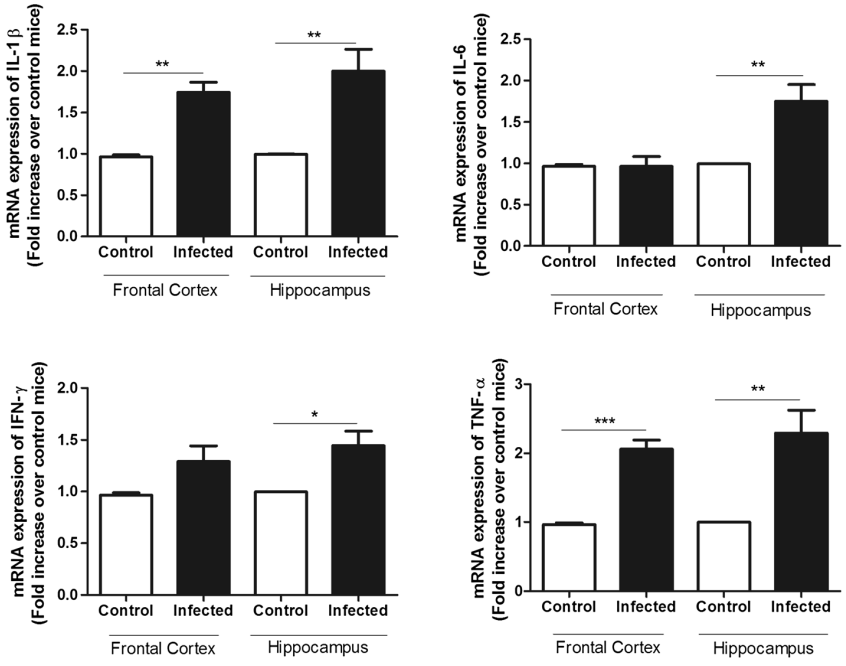

Figure 2 Frontal cortex and hippocampus mRNA expression of pro-inflammatory cytokines following *Plasmodium berghei* ANKA infection. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and control (non-infected) animals received the same volume of PBS (n = 5 *per group*). On day 5 post-infection (5 dpi) frontal cortex and hippocampus were collected for real time PCR analysis. Results use arbitrary units for the ratio of the target gene mRNA to the endogenous control, eukaryotic 18SmRNA. Statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

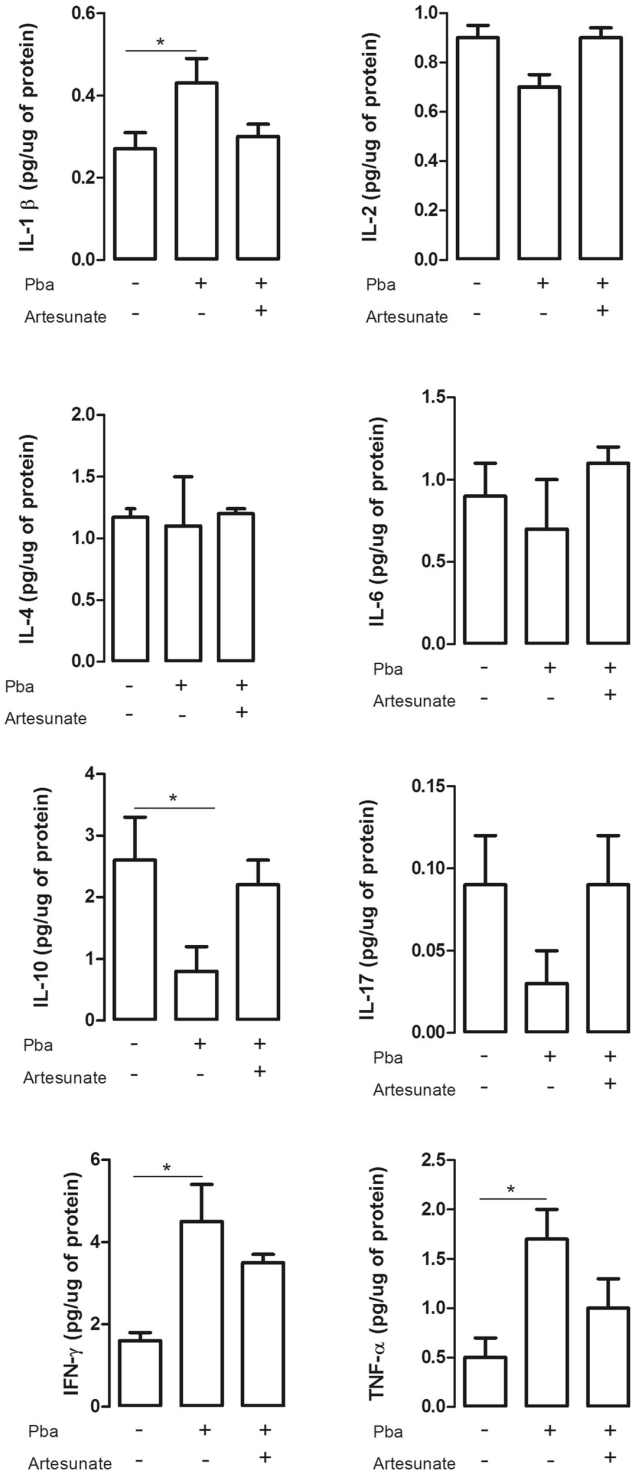

Figure 3 (See legend on next page.)

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Figure 3 Cytokine levels in the frontal cortex of controls and infected mice following artesunate treatment. C57BL/6 mice were intraperitoneally infected with 106 parasitized erythrocytes and control (non-infected) animals received the same volume of PBS (n = 5 *per group*). On day 4 post-infection *P. berghei*-infected (mice received a single dose of artesunate (ip 32 mg/kg). The frontal cortex was collected on day 5 post-infection, homogenized and IL-1β expression was measured by ELISA while IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10 and IL-17 levels were assessed by cytometric bead array (CBA). Results are expressed as mean ± SEM. Asterisk(s) indicate statistical differences, *p <0.05.

mice presented significant deficits in motor ($p = 0.001$) and exploratory ($p = 0.002$) activities (data not shown).

Inflammatory changes in hippocampus and frontal cortex of *P. berghei*-infected mice, key areas related to memory and learning

In order to evaluate inflammatory parameters in specific regions well known to participate in memory formation and consolidation, the mRNA expression of IFN-γ, TNF, IL-1β and IL-6 and the protein levels of IFN-γ, TNF, IL-1β, IL-2, IL-4, IL-6, IL-10 and IL-17 was analyzed in hippocampus and frontal cortex of controls and *P. berghei*-infected mice on day 5 pi.

The mRNA expression of TNF and IL-1β was significantly increased in the frontal cortex and hippocampus of *P. berghei*-infected mice compared to controls. A higher mRNA expression of IFN-γ and IL-6 was only observed in the hippocampus of *P. berghei*-infected mice (Figure 2).

Regarding protein levels, a significant increase of TNF, IL-1β and IFN-γ was found in the frontal cortex of infected animals, whereas a reduction of IL-10 was also observed (Figure 2). In the hippocampus higher levels of IL-1β, IL-6 and IFN-γ were found on day 5 pi in parallel with a decrease in IL-2 expression (Figure 3). No significant differences were detected in the levels of IL-2, IL-4, IL-6 and IL-17 in the frontal cortex, and in the levels of IL-4, IL-10, IL-17 and TNF in the hippocampus when comparing controls and *P. berghei*-infected animals (Figures 3 and 4).

A single dose of artesunate reduced cytokines levels in the hippocampus and frontal cortex of *P. berghei*-infected mice

The putative anti-inflammatory property of artesunate has been demonstrated in different conditions [17,18]. To investigate its effects in CM, the cytokine levels in frontal cortex, hippocampus and serum of controls and *P. berghei*-infected mice that received or not a single dose of artesunate were assessed.

PbA-infected mice presented an increase in frontal cortex levels of TNF ($F_{(2,12)} = 3.8$; $p < 0.05$), IFN-γ ($F_{(2,12)} = 7.1$; $p < 0.01$) and IL-1β ($F_{(2,12)=}$ 3.6; $p < 0.05$), and decreased levels of IL-10 ($F_{(2,12)} = 3.1$; $p < 0.05$) in comparison with controls (Figure 3). The levels of cytokines of infected mice treated with artesunate in the frontal cortex were similar to controls (Figure 3). *Plasmodium berghei*-infected mice showed an increase in hipoccampal levels of IFN-γ ($F_{(2,12)} = 7.7$; $p < 0.01$) and IL-6 ($F_{(2,12)} = 5.91$; $p < 0.05$), and decreased levels of IL-2 ($F_{(2,12)} = 8.6$; $p < 0.01$) in comparison with controls and treated *P. berghei*-infected mice (Figure 4). No significant difference was found in serum cytokine levels between treated and non-treated *P. berghei*-infected groups. When compared to the controls they exhibited higher serum levels of IL-6 ($\chi2(df = 2, n = 15) = 9.5$, $p < 0.05$, control *versus P. berghei*, $U = 0$; $z = -2.6$,$p < 0.05$; control *versus P. berghei* + artesunate $U = 0$; $z = 2.,6$; $p < 0.05$), IFN-γ ($\chi2(df = 2, n = 15) = 10.5$, $p < 0.05$, control *versus P. berghei*, $U = 0$; $z = -2.6$,$p < 0.05$; control *versus P. berghei* + artesunate $U = 0$;$z = 2.,6$, $p < 0.05$) and TNF ($\chi2(df = 2, n = 15) = 10.8$, $p < 0.05$, control *versus P. berghe*, $U = 0$; $z = -2.6$, $p < 0.05$; control *versus P. berghei* + artesunate $U = 0$; $z = 2.6$, $p < 0.05$) than controls (Figure 5).

A single dose of artesunate improved survival and clinical signs of cerebral malaria

Plasmodium berghei -infected mice treated with a single dose of artesunate presented a significant improvement in survival ($\chi2 = 8.5$, $p < 0.001$; Figure 6a) and a decrease in the percentage of parasitaemia on day 5 pi when compared with non-treated infected animals (Figure 6b). While *P. berghei*-infected mice presented significant changes in clinical parameters assessed by RMCBS, treated mice did not show clinical symptoms of CM (Figure 6c). Both treated and non-treated *P. berghei*-infected mice lost weight, but it was more significant in non-treated mice at day 5 pi (Figure 6d).

Discussion

In the present study, short-term aversive memory impairment was associated with increased levels of pro-inflammatory cytokines and decrease of regulatory mediators, such as IL-2 and IL-10, in the hippocampus and frontal cortex of *P. berghei*-infected mice. Therefore, different patterns of cytokines expression seem to be dependent on the region assessed, i e, hippocampus or frontal cortex. This study investigated the expression (mRNA and protein) of cytokines in memory-related areas during CM, and the results reinforce a role for inflammation in the development and persistence of cognitive changes.

Inflammatory processes in the CNS, including an up-regulation of pro-inflammatory cytokines, have been associated with cognitive and behavioural dysfunctions in clinical and experimental CM. For instance, a previous study demonstrated that *P. berghei*-infected mice exhibit

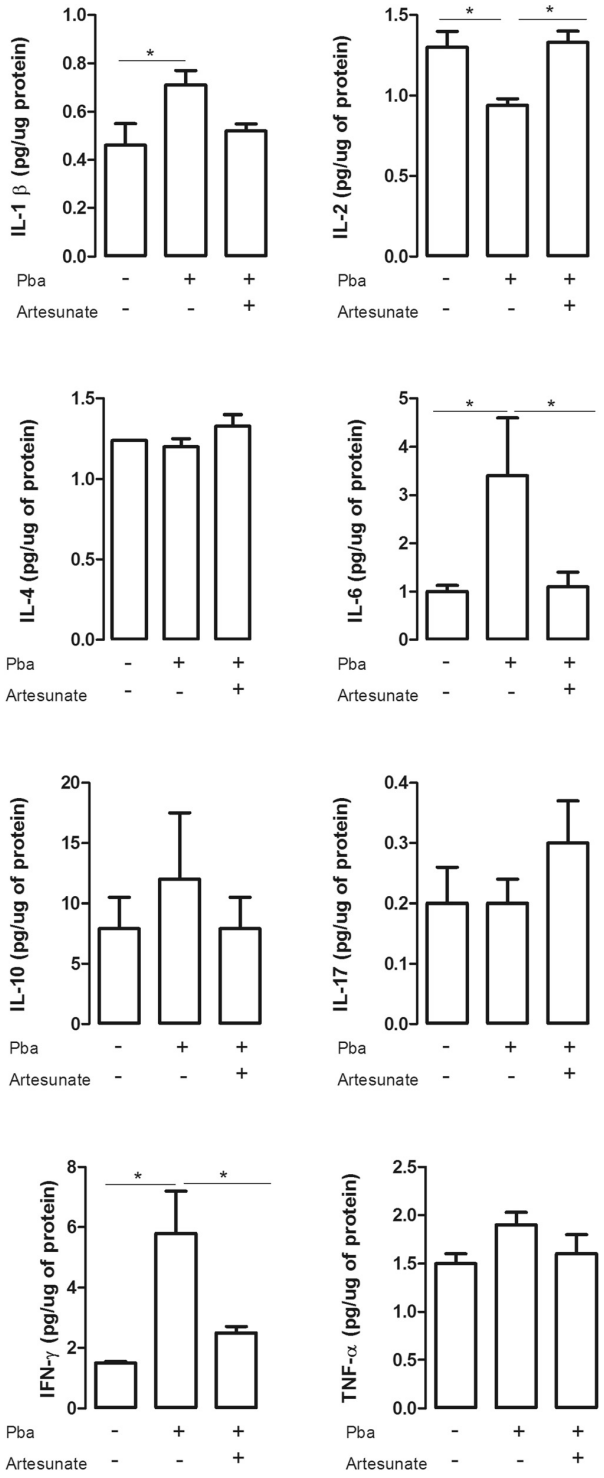

Figure 4 (See legend on next page.)

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Figure 4 Cytokine levels in the hippocampus of controls and infected mice following artesunate treatment. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and control (non-infected) animals received the same volume of PBS (n = 5 *per group*). On day 4 post-infection *P .berghei*-infected mice received a single dose of artesunate (ip 32 mg/kg). The hippocampus was collected on day 5 post-infection, homogenized and IL-1β expression was measured by ELISA while IFN-γ, TNF, IL-2, IL-4, IL-6, IL-10 and IL-17 levels were assessed by cytometric bead array (CBA). Results are expressed as mean ± SEM. Asterisk(s) indicate statistical differences where *p < 0.05.

anxiety-like symptoms associated with increased brain levels of IL-1β and TNF, and histopathological changes in brainstem, cerebrum and hippocampus [10]. Desruisseaux *et al*. [8] found a significant impairment in working memory of PbA-infected mice, which was associated with cell infiltration and haemorrhage in thalamus, midbrain and cerebellum and with microglial activation in the cortex and hippocampus. Additionally, John *et al*. [27] have suggested a negative correlation between cognitive (attention and working memory) deficits and cerebrospinal fluid (CSF) levels of TNF in children with CM. Although these experimental and human studies have provided evidence for the role of altered levels of cytokines in cognitive and behavioural changes found in CM, few studies investigated the expression of cytokines in specific brain areas, such as hippocampus and frontal cortex, involved in these functions.

A recent study conducted by Linares *et al*. [11] demonstrated an increase in IFN-γ and TNF mRNA expression in several brain areas of *P. berghei*-infected mice, including cortex, hippocampus, thalamus/hypothalamus and brainstem. The upregulation of these cytokines was found in an early or asymptomatic stage of CM and was associated with an increased expression of immunoproteasome subunits. The replacement of proteasome subunits for immunoproteasome factors is a brain-inflammatory response that inhibits normal protein turnover [28]. There is evidence that proteasome also participates in neuronal survival and plasticity [29]. The substitution of proteasome for immunoproteasome complex could lead to neuronal death contributing to the cognitive deficits through impairment of synaptic function [28,30]. The present study provides evidence that pro-inflammatory cytokines expression remain upregulated in the hippocampus and frontal cortex until symptomatic stage of CM, culminating with cognitive impairment. It is worth mentioning that no impairment was found in long-term aversive memory, probably due to significant locomotor and exploratory deficits observed on day 6 pi. In agreement, previous studies have been demonstrated that changes in motor activity during CM occurred on day 6 p.i. whereas anxiety like behaviour appeared earlier on day 5 pi [10,31]. Thus, it seems that behavioural disorders precede locomotor impairment during neuroinflammation caused by *P. berghei* ANKA infection.

CM is a clinical emergence that is invariably fatal without appropriate treatment [12]. Artesunate is a potent anti-malarial drug which has been widely accepted as the treatment of choice for severe malaria [13]. Apart from its well-known antiparasitic property, there is growing evidence suggesting an immunomodulatory activity of artesunate in infectious and autoimmune disorders [14-18]. Artesunate was able to protect mice subjected to sepsis by decreasing serum IL-6 and TNF levels via inhibition of Toll-like receptors expression and NF-κB activation in peritoneal macrophages [17,32,33]. In a rat model of arthritis, artesunate treatment significantly attenuated inflammation and cartilage damage by decreasing the levels of IL-1β, IL-17 and TNF in rat's hind paws. The anti-arthritic effect of artesunate was associated with NF-κB and mitogen-activated protein kinase signaling pathway suppression [18]. A decrease in cytokine levels and oxidative stress in lung tissue was also found in an allergic asthma model after artesunate treatment [14,15]. Altogether these studies provide strong evidence of artesunate capacity of modulating inflammatory response through the regulation of signaling pathways.

No previous study investigated the effect of artesunate in the CNS inflammation during CM. In the present study, a single dose of artesunate was able to decrease pro-inflammatory cytokines expression in the hippocampus and frontal cortex of *P. berghei*-infected mice in parallel with a significant reduction of parasitaemia and improvement in neurological symptoms and survival.

A single dose of artesunate was highly effective in clearing *P. berghei* parasitaemia at day 5 pi, rescuing mice in the late stages of CM. Reduction of the number of adherent leukocytes in brain vessels of *P. berghei*-infected mice was also observed 24 hours after a single dose of artemether [19]. These authors suggested that the decrease in brain inflammatory response could be a consequence of faster parasitaemia clearance associated with an intrinsic anti-inflammatory property presented by artemisinin derivatives [19]. Interestingly, a systemic release of pro-inflammatory cytokines during the acute phase of malaria is required for the control of parasitaemia [34]. Moreover, the serum levels of cytokines (IL-6, IFN-γ and TNF) in *P. berghei*-infected mice treated with artesunate were comparable to non-treated *P. berghei*-infected animals.

An *in vitro* study conducted by Lee *et al*. [35] showed that artesunate attenuates LPS-induced inflammatory responses in microglial BV2 cells by activating the Nrf2 transcription factor NF-E2-related factor-2 (Nrf2). This

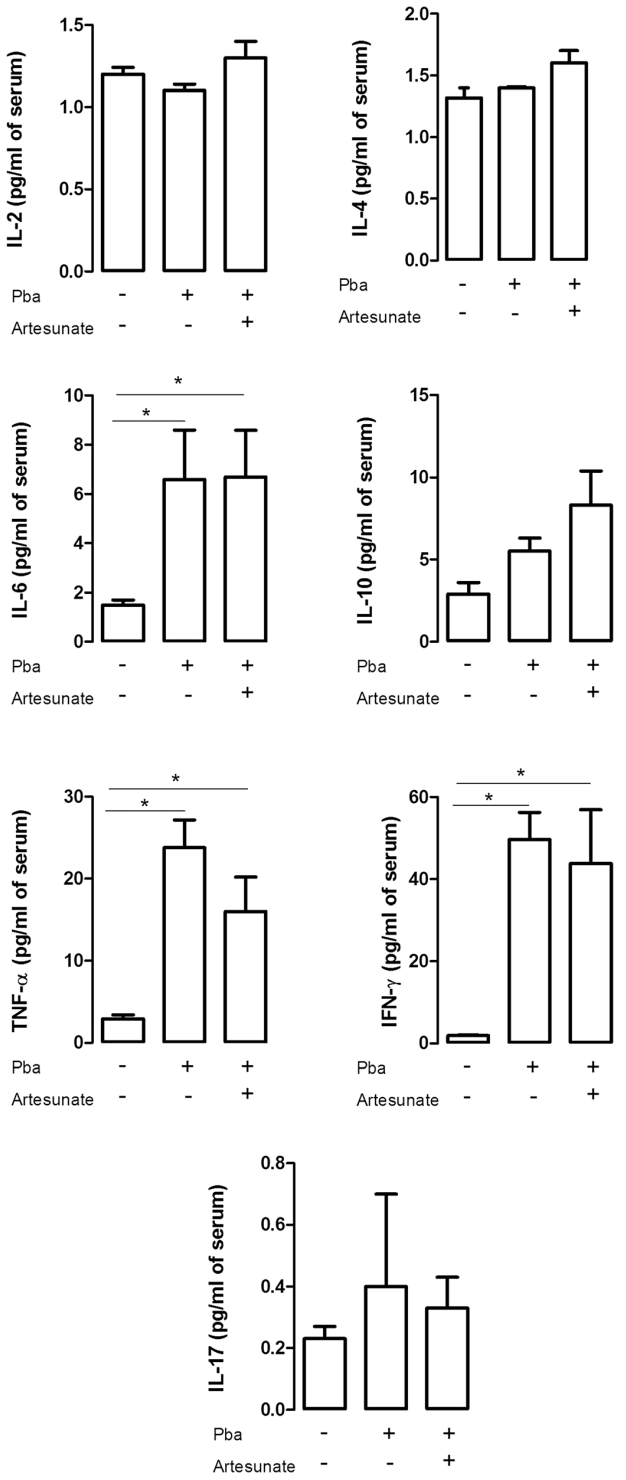

Figure 5 (See legend on next page.)

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Figure 5 Cytokine levels in the serum of controls and infected mice following artesunate treatment. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and control (non-infected) animals received the same volume of PBS (n = 5 *per group*). On day 4 post-infection *P. berghei*-infected mice received a single dose of artesunate (ip 32 mg/kg). The serum was collected at day 5 post-infection and IFN-γ, TNF, IL-2, IL-4, IL-6, IL-10 and IL-17 levels were assessed by cytometric bead array (CBA). Results are expressed as mean ± SEM. Asterisk(s) indicate statistical differences. *p < 0.05.

transcription factor regulates inflammatory responsiveness in the CNS by mediating the expression of important antioxidant and phase II detoxification genes, and leading to an increase in the level of haem oxygenase-1 [35]. There is evidence that haem oxygenase-1 suppress the pathogenesis of experimental CM by preventing blood-brain barrier disruption, brain microvasculature congestion and neuro-inflammation [36,37]. The current study provided evidence of an anti-inflammatory role of artesunate in the CNS of *P. berghei*-infected mice indicating that CM symptoms prevention was not only due to parasitaemia clearance.

Experimental cerebral malaria development is dependent of Plasmodium strains and mice of susceptible genetic background. In systemic models of malaria, infected mice did not present CNS inflammation as well as CM signs but rather a significant increase in parasitemia and anemia. The lack of cognitive and neurological impairment after parasitemia clearance by chloroquine in those models supports a specific CNS involvement in CM outcome rather than severe systemic disease or the presence of the parasite *per se* [23]. A recent study conducted by Reis *et al.* [38] demonstrated that the statin Lovastatin (which exhibits a significant anti-inflammatory effect) was capable of decreasing pro-inflammatory cytokine levels in CNS during the acute phase of CM without influencing parasitaemia. However, improvement in survival rate was only observed in association with the antimalarial chloroquine. Despite being highly effective in preventing mortality, chloroquine alone did not prevent CM cognitive and behavioral outcome. This indicates that limiting the inflammatory damage to the brain is necessary to improve CM outcome in addition to an effective control of the parasitic disease. *P. berghei* ANKA mice were also treated with sodium diclofenac or allopurinol as control anti-inflammatory agents and did not detect protective effects in CM. In fact, sodium diclofenac increased mortality in infected animals, suggesting that anti-inflammatory drugs could trigger distinct immunomodulatory effects which could be effective or not to improve CM outcome [38].

Conclusion

In summary, the present study provided further evidence that key areas related to memory, such as hippocampus and frontal cortex, presented different patterns of cytokine expression. Furthermore, it seems that the anti-malarial artesunate presented not only a systemic antiparasitic activity but could also regulate inflammatory responses in the CNS by decreasing local pro-inflammatory cytokines release during the course of CM. As this study is largely

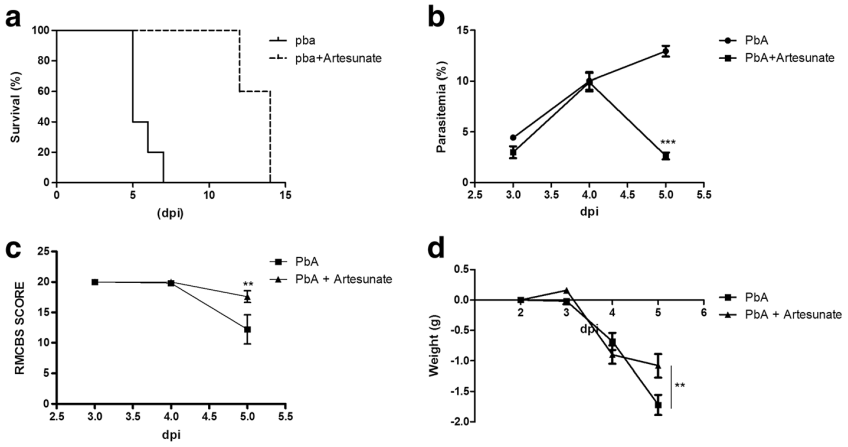

Figure 6 A single dose of artesunate improved survival and clinical signs of cerebral malaria. (a) Survival curve comparisons of *P. berghei*-infected mice treated or not treated with artesunate were expressed days after infection; **(b)** natural course of *P. berghei* infection was determined by flow cytometric analysis; **(c)** clinical signs of cerebral malaria assessed by the RMCBS scale; **(d)** weight variation in *P. berghei*-infected mice treated or not treated with artesunate. The data is representative of two independent experiments (n = 5 *per group*) and shown as the mean+/- SEM. Asterisk(s) indicate statistical differences, *p < 0.05.

descriptive, further studies are required to systematically investigate the immune/inflammatory pathways involved in the anti-inflammatory activity of artesunate in CM as well as its impact on cognitive outcome. Moreover, other symptoms typical of infection conditions such as fever should be also evaluated in association with parasitemia, body weight loss and clinical neurological signs of CM (ataxia, paralysis and coma) to determine the progression of the disease as well as to guide antimalarial treatment.

Abbreviations

CNS: Central nervous system; CM: Cerebral malaria; CBA: Cytometric bead array; ELISA: Enzyme-linked immunosorbent assay; IFN-γ: Interferon-γ; IL-1β: Interleukin-1 beta; IL-2: Interleukin-2; IL-6: Interleukin-6; IL-10: Interleukin-10; IL-17: Interleukin-17; RMCBS: Rapid murine coma and behaviour scale; SEM: Standard error of the mean; SHIRPA: SmithKline/Harwell/Imperial College/Royal Hospital/Phenotype Assessment; TNF: Tumor necrosis factor; WHO: World health organization.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ASM participated in the experimental design, carried out behavioural and immunological assays, data analysis and drafted the manuscript. FB participated in the experimental design, data analysis and carried out behavioural and immunological assays. NPR and DHR performed immunological assays. DC and DGS performed real time PCR analysis. FSM and MAR participated in the design and coordination of the study. ALT designed the study and was responsible for the interpretation of experiments and editing the manuscript. ACC participated in the experimental design and in the execution of experiments involving artesunate, immunological assays, data analysis and revised the manuscript. All authors have read and approved the final version of the manuscript.

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6.3 – Artigo científico 3

Artigo em preparação para submissão.

Title: A neuroprotective effect of glutamate receptors antagonist MK801 in experimental cerebral malaria long-term cognitive and behavioral outcomes.

A neuroprotective effect of glutamate receptor antagonist MK801 in experimental cerebral malaria long-term cognitive and behavioral outcomes

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Abstract

Cerebral malaria (CM) is a life-threatening complication of *Plasmodium falciparum* infection which can result in long-term cognitive and behavioral deficits despite successful anti-malarial therapy. Due to the substantial social and economic burden of CM outcome the development of adjuvant therapies is a scientific goal of highest priority. Apart from vascular and immune responses changes in glutamate system have been reported in CM pathogenesis suggesting a potential therapeutic target. We hypothesized that interventions with glutamate system induced by blockage of Nmethyl-D-aspartate (NMDA) receptors could attenuate experimental CM long-term cognitive and behavioral outcomes. Before development of evident CM symptoms, susceptible mice infected with *Plasmodium berghei* ANKA (PbA) strain initiated the treatment with Dizocilpine maleate (MK801, 0.5mg/kg), a noncompetitive NMDA receptors antagonist. On day 5 post-infection mice were treated orally with a ten-day course chloroquine (CQ, 30 mg/kg). Control mice (non-infected) also received saline, CQ or MK801+CQ therapy. After ten days of cessation of CQ treatment resonance magnetic images, behavioral and immunological assays were performed in order to assess the efficacy of MK801in improving CM outcome. Indeed, MK801 combined with CQ prevented long-term memory impairment and depressive-like behavior following successful PbA-infection resolution. In parallel, MK801 also modulate the immune system by promoting a balance of TH1/TH2 response and up-regulating neurotrophic factors levels in the frontal cortex and hippocampus. Moreover, hippocampus abnormalities observed by resonance magnetic images were quite prevented by MK801 treatment. In summary, we provided further evidence of the neuroprotective role of MK801 indicating that NMDA receptor antagonists could be a valuable adjuvant strategy for the management of CM long-term outcome.

Key-words: Cerebral malaria; memory impairment; depressive-like behavior; MK801; cytokines, neurotrophic factors; glutamate; Nmethyl-D-aspartate (NMDA) receptors.

1. Introduction

Cerebral malaria is a life-threatening complication of *Plasmodium falciparum* infection resulting in nearly 1 million annual deaths worldwide (de Souza et al., 2010). Despite successful anti-malarial therapy a significant number of children remain with long-term cognitive impairment (Boivin et al., 2007; Carter et al., 2005; John et al., 2008a). Prospective clinical studies demonstrated that these deficits could persist at least two years following cessation of anti-parasitic treatment (Boivin et al., 2007; John et al., 2008a) resulting in substantial economic and educational burden in endemic regions (Fernando et al., 2010). Apart from the impact of CM cognitive outcome emotional disorders including depression and anxiety has been also reported during the acute phase of CM as well as following its resolution (de Miranda et al., 2011; Dugbartey et al., 1998; Varney et al., 1997).

CM long-term cognitive and behavioral pathogenesis remains unclear (Boivin (Boivin et al., 2007; Idro et al., 2010; John et al., 2008b). The onset of those symptoms may vary, occurring immediately on discharge from hospital or later, months or years after CM, indicating the existence of distinct mechanisms of brain injury (Idro et al., 2010). Blood flow reduction in brain microvasculature by sequestered parasitized erythrocytes and an exacerbated host immune response are main hallmarks of CM (Hunt et al., 2006; van der Heyde et al., 2006) although metabolic changes including high brain concentration of lactate and increase of glutamatergic activity have been also described (Miranda et al., 2010; Parekh et al., 2006; Rae et al., 2004).

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS). Through mainly by the activation of Nmethyl-D-aspartate (NMDA) receptors plays an important role in neuronal development, synaptic plasticity,

learning and memory processes under physiological conditions (Meldrum, 2000; Schauwecker, 2010). Ionotropic NMDA receptors also regulate neuronal circuits critically involved in emotional behavior (Engin et al., 2009; Zarate et al., 2006). However, high amounts of glutamate release in intersynaptic spaces and consequent overactivation of NMDA receptors can cause neuronal cell death and neurodegeneration via excitotoxicity processes which includes increase release of intracellular calcium (Ca^{+2}) and other cellular ions (Bano and Nicotera, 2007; Wang and Qin, 2010). Excitotoxicity plays an important role in many CNS diseases, including ischemia, trauma, and neurodegenerative disorders (Lau and Tymianski, 2010). For instance, we previously demonstrated a significant increase of glutamate release in the central nervous system during the acute course of experimental CM, indicating a role for glutamate in the pathogenesis of this condition (Miranda et al., 2010). Due to the role of NMDA receptors in modulating neurotransmission (Yan et al., 2013), changes in glutamate syntheses, release and reuptake processes during CM may have long-term deleterious effects on cognitive and emotional functions. In this scenario, glutamatergic strategies, especially regarding to glutamate receptor antagonists, should be investigated as a potential additive therapy in CM.

Dizocilpine maleate (MK801) and memantine are noncompetitive NMDA receptor antagonists which are thought to protect neurons from excessive glutamate activity that results in excitotoxicity (Schauwecker, 2010; Tayeb et al., 2012). It has been reported that MK801 and memantine prevented neuronal damage and improve cognitive and behavioral deficits presenting a neuroprotective and potentially therapeutic activity in several pathological conditions including Alzheimer's disease, ischemia, spinal cord injury and sepsis (Bakiri et al., 2008; Cassol-Jr et al., 2011; Esposito et al., 2011; Salomone et al., 2012). An immunomodulatory property was also

reported following MK801 administration as a therapeutic strategy to improve experimental spinal cord injury (Esposito et al., 2011).

Adjunctive therapies including immunomodulators and antioxidant agents have been increasingly investigated in order to improve CM cognitive outcome (Casals-Pascual et al., 2008; Picot et al., 2009). However, a poor understanding of CM pathogenesis as well as other limiting factors especially regarding the properties of compounds used, appropriate therapeutic time window, patient selection and small sample size have contributed to the lack of success of these interventions (John et al., 2008a). Although recent works have demonstrated that lovastatin (Reis et al., 2012) and lithium (Dai et al., 2012) successfully prevented long-term cognitive deficits in experimental CM, the efficacy and safety of those compounds were not established yet by clinical trials. Thus, the development of new adjuvant strategies to improve CM outcome is a scientific goal of highest priority.

Based on the concept that CM pathogenesis could be related to glutamatergic alterations, we proposed to investigate the effect of NMDA receptor antagonists in the long-term cognitive and behavioral impairment following experimental CM as a neuroprotective therapy associated with the anti-parasitic treatment.

2. Material and Methods

Ethics statement

This study was carried out in strict accordance with the Brazilian Government's ethical and animal experiments regulations. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of the Universidade Federal de

Minas Gerais (CETEA/UFMG, Permit Protocol Number 105/09). All tissue collected procedures was performed under ketamine/xylazine anesthesia and all efforts were made to minimize animal suffering.

Animals

Female C57BL/6 mice (20-25g), aged 6-8 weeks, were obtained from Animal Care Facilities of the Institute of Biological Sciences, Federal University of Minas Gerais (ICB-UFMG), Belo Horizonte, Brazil. The animals were housed in groups of 6 mice per cage in a room controlled temperature (25◦C) with food and water *ad libitum*.

Parasite and experimental infection

Due to the high degree of reproducibility, easily manageable characteristics and development of histopathological and neurological signs typical of human CM, the murine model using the Plasmodium berghei strain ANKA has been widely used to better understand this condition (de Souza et al., 2010). In the present study, blood stages of *P. berghei* strain ANKA constitutively expressing green fluorescent protein (*P. berghei* ANKA-GFP) (15cy1 clone), kindly provided by Dr Claudio Marinho (Universidade de São Paulo), were stored in liquid nitrogen (Reis et al., 2012). Mice were infected intraperitoneally (i.p.) with 10^6 PbA-infected red blood cells suspended in 0.2 mL PBS (Grau et al., 1986).

The percentage of parasitemia was determined by flow cytometry. Briefly, a drop of blood from the tail was collected directly into 2 ml of PBS for flow cytometry analysis. Each sample was run on a FACScalibur (Becton Dickinson, San Jose, CA, USA) flow cytometer with a 488 nm argon laser and DIVA software (Becton

Dickinson, San Jose, CA, USA). Erythrocytes were identified on the basis of their specific forward (FSC) and side (SSC) light-scattering properties and a total of 100,000 events were counted for each sample.

Clinical assessment by Rapid murine coma and behavior scale (RMCBS)

Behavioral and functional parameters were evaluated using the RMCBS protocol as previously described by (Carroll et al., 2010). The RMCBS is a quantitative and objective scale that enables an investigator to rapidly follow up the course of CM. The subjects were labeled as affected or not, and the levels of illness are correlated with neuropathological injury. This method intent to imitate the situation in the field and attempts to bring the animal model closer to the human disease (Carroll et al., 2010).

The RMCBS consists of ten parameters (Gait, balance, motor performance, body position, limb strength, touch scape, pinna reflex, toe pinch, aggression and grooming) based on the components of the SHIRPA (SmithKline/Harwell/Imperial College/Royal Hospital/Phenotype Assessment) score (Rogers et al., 1997). Each item is scored from 0 as the lowest, to 2 as the highest, with a maximum total score of 20. Murine experimental CM was defined as the presentation of one or more of the following clinical signs of neurological involvement: ataxia, limb paralysis, poor righting reflex, seizures, roll-over and coma. Infected mice show presence of parasites as well as murine ECM signs on day 5 or 6 post-infection (Clemmer et al., 2011). The procedure was carried out on day 3 until the beginning of cognitive and emotional evaluation ten days after the cessation of treatment. Clinical signs of murine CM was evaluated and used for scoring disease severity and guide the start of antimalarial treatment.

N-methyl-D-aspartate (NMDA) receptor antagonist treatment and study design

The present study was designed to determine the efficacy of adjunctive therapy by blocking glutamate NMDA-receptor in improve cognitive and behavioral outcome following PbA infection. Mice were randomly assigned into five groups (Control + saline; Control + CQ; Control + CQ + MK801; PbA + CQ and PbA+ CQ+ MK801). Parasitemia and clinical signs of CM were assessed as previously described every day from day 3 post-infection until the start of CQ treatment and every 3 days until the beginning of cognitive and behavioral analysis. Baseline weight was recorded on the day of infection and then every day from day 3 post-infection. Survival was examined daily. The MK801 (0.5 mg/kg diluted in 200µl 0.9% saline) was administrated intraperitoneally in controls (non-infected) and PbA-infected mice and treatment was initiated at day 3 post-infection and was continued until the end of CQ therapy. This dose of MK801 was chosen as it was the highest dose administered, over several dose/response trials, which did not confer immediate neurological side effects (data not presented). All mice, control and infected, were treated orally with a ten-day course CQ (30 mg/kg diluted in 200µl of water). An attach dose (60 mg/kg) was given on first day of CQ treatment to avoid parasitemia recrudescence. CQ therapy was initiated on day 5 post-infection following CM diagnosis. CQ treatment was also guided by a parasitemia over then 7% and a loss of 2% baseline body weight (Dai et al., 2010). CQ treatment was initiated at day 5 post-infection in control mice, the earliest day when infected mice first reached treatment criteria. A control group with non-infected animals receiving only saline was also performed to exclude possible side effects of MK801 and CQ therapies in behavioral and immune assays. Each treatment administrations subsequent to the initial dose were given in the morning.

Cognitive and behavioral tasks were performed from 10 days after cessation of CQ treatment. Immediately following cognitive and behavioral assays brains were harvested for immunological and histopathological analysis. Magnetic resonance images were also performed 10 days after cessation of CQ treatment.

Glutamate release and measurement in the hippocampus

Synaptosomes were prepared as previously described by (Romano-Silva et al., 1993). Mice were decapitated and their hippocampus were removed and homogenized in 1:10 (w/v) 0.32 M sucrose containing 0.25 mM dithiothreitol and 1 mM EDTA. Homogenates were then submitted to low-speed centrifugation (1000 g/10 min) and isolated nerve terminals (synaptosomes) were purified from the supernatant by discontinuous Percoll density gradient centrifugation (Dunkley et al., 1988). The synaptosomes were resuspended in 400 μL Krebs-Ringer-HEPES buffer (124 mM NaCl, 4 mM KCl, 1.2 mM MgSO4, 10 mM glucose, 25 mM HEPES, pH 7.4), divided into 200-μL aliquots and stored on ice for later measurement of glutamate release. The glutamate release assay was performed using an RF5301PC spectrofluorometer (Shimadzu, Tokyo, Japan) monitoring the increase in fluorescence due to the production of NADPH+ in the presence of glutamate dehydrogenase and NADP+ (Nicholls et al., 1987). A total of 5 animals *per group* were used.

Cognitive and behavioral analysis

Novel object recognition task

The object recognition task was performed to asses long-term working memory as previously described by (Reis et al., 2010). Briefly, animals had the opportunity to explore the open field for 5 min. On the following day, a training session was conducted by placing individual mice for 5 min into the field in the center of the arena, in which two identical objects (object A1 and A2; Double Lego Toys) were positioned in two adjacent corners at 10 cm from the walls. In a long-term memory (LTM) test (24 h after training), the mice explored the field for 5 min in the presence of the familiar (A) and different novel (B) object. Objects had only distinction in shape. The exploratory preference was defined as percentage of the total exploration time animal spent investigating the object A or the novel object and calculated for each animal by the ratio TB /(TA+TB) [TA = time spent exploring the familiar object A; TB = time spent exploring the novel object B). The distance traveled in the apparatus arena was also recorded as a locomotor activity parameter. The Anymaze software (Stoelting Co., Wood Dale, IL, USA) was employed for behavioural analysis. All tests were performed by the same investigator who was blinded to the animal status (control or infected). A total of five animals per group were used. After the test all animals were sacrificed under deep anesthesia by i.p. injection of a mixture of Ketamine (150 mg/kg, Laboratório Cristália, Campinas, SP, Brazil) and Xylazine (10 mg/kg, Rompun®, Bayer, Leverkusen, Germany), decapitated and frontal cortex and hippocampal collected for posterior cytokine and neurotrophic factors levels assessment by Cytometric Bead Array (CBA) and enzyme-linked immunosorbent assay (ELISA) methods.

Step-down inhibitory avoidance test

The step-down inhibitory avoidance test was performed to assess long-term aversive memory 10 days after cessation of CQ treatment (n=7 per group), as previously described by Reis *et al*. (2010). Briefly, in the training trial, animals were placed on the platform and their latency to step down on the grid with all four paws was measured. Immediately after the stepping down on the grid, the animals received a single mild foot shock (0.4 mA, 2.0 seconds). A retention test trial was performed 24 hours after the training section. The results were expressed as latency period to step down the platform, with a cut-off at 180 seconds. All tests were performed by the same investigator who was blinded to the animal status (control or infected). A total of 7 animals per group were used.

Forced Swimming test (FST)

The FST was performed to evaluate depressive-like behavior as previously described by (Haring et al., 2013). The paradigm was performed in a round glass beaker (18 cm in diameter and 30 cm in height) filled with tap water at 25 ± 0.5 ºC. The water level was approximately 20 cm to prevent the animal from touching the bottom of the glass. The mouse was also unable to climb out of the beaker. The animal was carefully lowered into the water and videotaped by a digital camera (SONY, Tokyo, Japan) for 6 min. The first 2 min were not evaluated; however, floating behavior was scored for the following 4 min by an experimenter blind to genotype and treatment. Floating was defined by immobility of the animal and minimal movements to keep the body's balance. After the swim session, mice were dried and placed in a cage surrounded by a

heating pad. The water was changed between each animal. A total of 8 animals *per group* were used.

Elevated plus maze (EPM)

Anxiety-like behavior was assessed using the elevated plus maze (Insight®, Ribeirão Preto, SP, Brazil). The elevated plus maze (EPM) is a test of unconditioned anxiety-related behavior that involves a conflict between the rodent's desire to explore a novel environment and anxiogenic elements such as elevation and an unfamiliar open area (Lister, 1987). This is a widely used test for anxiety behavior of rodents (File, 2001; Walf and Frye, 2007). The EPM test was conducted as previous described by (Walf and Frye, 2007). Briefly, mice were placed in the center of the maze facing an open arm and were allowed to freely explore the EPM for 5 min. The animal placing all four paws onto the arm was considered to be in the arm, otherwise the animal was in the center of the maze. Behavior that was recorded when rodents were in the EPM included the time spent and entries made on the open and closed arms. The measures of anxiety were the percentage (%) of open arm entries and the percentage (%) of time spent on the open arms. The number of closed arm entries was considered as a locomotor measure. Decreased open arm activities indicate increased anxiety levels in EPM. After each trial, the maze was wiped clean with a damp sponge and dried with paper towels. Before behavioral assessment, animals were allowed to accommodate to their new environment for 2 days. The Anymaze software (Stoelting Co., Wood Dale, IL, USA) was employed for behavioural analysis. The EPM test was performed by the same investigator blinded to the animal status (control or infected; n= 8 *per group*).

Open Field

The open field test was conducted in order to evaluate locomotor and exploratory activities as well as anxiety-like behavior ten day after cessation of CQ treatment (n=8 *per group*). Briefly, mice were gently placed in an opaque plastic arena (106 cm X 106 cm square) and then they were allowed to free explore the arena for 5 minutes. The animals global activity, stereotype movements, locomotion, mean velocity, number of rearing episodes (the exploration behavior observed in rats subjected to a new environment) and percentage of time spent in the open field center area (an anxiety measure) was assessed with automated tracking software (ACTITRACK v2.7.13, Panlab, MA, USA). The open field test was performed by the same investigator who was blinded to the animal status.

Histopathology

We analyzed histopathological changes following PbA infection in the whole brain of all control animals and both treated PbA-infected mice groups.

After 10 days of cessation of CQ treatment all animals (n=4 *per group*) were anesthetized by i.p. injection of a mixture of Ketamine and Xylazine. Brains were carefully removed and postfixed in 10% buffered formalin. Sections of 5 µm at intervals of 10 µm were cut and mounted for routine hematoxylin-eosin (H&E) staining. The sections were examined at the optical level (Olympus, Japan, JP). Digital images were acquired for documentation using a ×400 planachromatic objective.

Cytometric Bead Array (CBA) and enzyme-linked immunosorbent assay (ELISA) analyses of cytokines and neurotrophic factors

Hippocampal, frontal cortex and spleen tissues of controls and treated PbA-infected mice (n=5 *per group*) were carefully removed and homogenized in an extraction solution (100 mg of tissue per mL), containing 0.4M NaCl, 0.05% Tween 20, 0.5% BSA, 0.1 mM phenyl methyl sulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KIU aprotinin, using Ultra-Turrax. Lysates were centrifuged at 13,000g for 10 min at 4ºC, supernatants were collected and stocked at -70ºC until use. Blood samples were also obtained, centrifuged at 10,000 x g for 15 minutes at 4ºC and the serum was collected and stocked at -70ºC until use.

Analyses of brain, spleen and serum cytokine levels were determined using a mouse Th1/Th2/Th17 CBA kit (BD Biosciences, San Diego, CA) and analyzed on a FACSCanto flow cytometer (Becton Dickinson, San Jose, CA). The following cytokines were measured: interleukin-2 (IL-2); interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10); interleukin-17 (IL-17); interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α).

The concentration of neurotrophins (BDNF, NGF and GDNF), of cytokine interleukin-1 beta (IL-1β) and of chemokine CCL11 in the hippocampus and frontal cortex was determined by ELISA (R&D Systems, Minneapolis, MN) in accordance to the manufacturer's instructions.

Results are expressed as pg/100 mg of tissue or pg/ml of serum. The detection limit of the CBA and ELISA assays was in the range of 5–10 pg/ml.

Resonance Magnetic Images (RMI)

Image acquisition

The MR image experiments were acquired using 4.7T NMR system (Oxford Systems) controlled by a UNITY Inova-200 imaging console (Varian). The imaging protocol consisted of coronal T2-weighted (TR = 3000 ms, TE = 50 ms) spin echo multislice scans, 20 contiguous 100 μm-thick slices. Mice (n= 20) were anesthetized with halothane (4% induction, 1.5% maintenance) and oxygen (1.5 %) delivered by a face mask in a head holder, to minimize artifact movements. Animals anesthetized for the duration of an imaging experiment (50 minutes) recovered with no apparent difficulty.

RMI Analysis

Brain masks, based on the anatomical scans, were done using a tablet driver (Bamboo Tablet Driver, V5.2.5 WIN, WACOM Technology Corporation, USA) and MeVisLab software (MeVis Medical Solutions AG, Fraunhofer). Additionally, densitometric analysis was done using MatLab® scripts and repeated measures ANOVA was used to compare densitometric values in different groups.

RMI Densitometry description

After segmentation, a binary mask with the same dimensions of the original image is saved in a .raw file. The segmented volume is given by the number of voxels in the mask with value different from 0, multiplied by the volume of each voxel. After overlapping the mask with the original image, the density is calculated as the sum of the intensities of all voxels inside the segmented region, divided by the segmented volume.

Data analysis and statistical evaluation

Results obtained were presented as mean ± standard error of the mean (SEM). All data were tested for normality (Kolmorov-Smirnov's test) and homogeneity of variances (Levene´s test). For variables normally distributed and with compliance of homogeneity of variances, differences were compared by using Student's t test, analysis of variance (ANOVA) or Two-way ANOVA. Duncan's post-test was used as needed for multiple comparisons. In case of variables not normally distributed, differences were analyzed by Mann-Whitney U test or Kruskal-Wallis non-parametric test. When significant effects were detected by this latter test, post-hoc comparisons were performed using the Mann-Whitney U test. Differences between lethality curves were calculated using the Log rank test. Pearson's or Spearman's correlation analyses were performed to examine the relationship of cytokines and neurotrophic factors with novel object recognition memory and of cytokines with neurotrophic factors. The significance level was set at $p < 0.05$. Statistical analyses were performed using Prism 4 software (GraphPad, La Jolla, CA, USA).

Results

Survival, parasitemia, clinical and histopathological signs of CM

Survival rate was similar in both MK801+CQ and only CQ treated mice following PbA infection (fig. 1 A). No mortality occurs in control groups indicating no toxicity of MK801 and CQ treatment (data not shown).

At the administered dose, MK801 did not confer any antiparasitic effect. PbA-infected mice presented similar parasitemia levels prior to the institution of CQ therapy with both groups peaking at day 5 post-infection (PbA+CQ: 7.76%±0.56; PbA+MK801+CQ: 7.26%± 0.74; p=0.6). Moreover, the parasite response to CQ therapy was similar in both treated infected mice groups. Parasitemia returned to 0% by 3 days after beginning of CQ treatment (fig. 1 B).

PbA-infected mice presented clinical signs of CM on day 5 post-infection indicated by a significant decrease in RMCBS score prior to the administration of CQ therapy. Interestingly, infected animals receiving MK801 presented a significant better RMCBS score on day 5 post-infection compared to PbA-infected mice not treated, suggesting a possible earlier neuroprotective effect. However, after CQ treatment institution both groups (PbA+ CQ and PbA+MK801+CQ) increased RMCBS score reaching the maximum by the time of cognitive and behavioral tasks (Fig. 1 C).

Infected animals demonstrated a gradual decrease in weight during the course of active infection with significant reductions (2% or greater) of original body weight occurring between 5 and 7 days post-infection. Weights of infected mice recovered to original levels by the time of testing following CQ therapy (data not shown).

CM is histopathologically characterized by substantial hemorrhage and leucocyte sequestration in several brain regions including those related to cognition such as hippocampus and cortex (Desruisseaux et al., 2008; Lackner et al., 2006). These histopathological changes were largely resolved after CQ and MK801+CQ treatment, as there were no significant differences in hemorrhage and inflammation in the cortex and hippocampus between controls and PbA infected mice (data not shown).

NMDA receptor antagonist MK801 increase hippocampus glutamate release

It has been reported that an effect of MK801 is to induce glutamate release in different brain areas (Roenker et al., 2012). In order to confirm the efficacy of MK801 treatment in our CM model we measured glutamate release from isolated hippocampus nerve terminals (synaptosomes) ten days after cessation of MK801+CQ treatment.

Non-infected animals (control) which received MK801+CQ treatment presented a significant increase in glutamate release in the hippocampus in comparison with control mice receiving only CQ or saline. A similar result was found when comparing PbA-infected mice treated with MK801+CQ with infected animals receiving only CQ. No significant difference was observed between non-infected and PbA-infected mice submitted to CQ therapy (Fig. supplementary 1).

NMDA receptor antagonist MK801 prevents long-term working memory impairment following PbA infection resolution

Figure 2 shows the effect of MK801 treatment in the novel object recognition test. Uninfected and PbA-infected mice were given either MK801 or saline from day 3

post infection until the cessation of a 10-day course of CQ treatment. Cognitive testing was performed 10 days after the cessation of CQ, and comparisons were made between each group. Memory loss was indicated by a significant reduction in the percentage of time exploring the novel object 24 hours after training session. PbA-infected mice treated with CQ presented an impairment of novel object recognition memory compared to controls. Moreover, MK801+CQ treatment of CM mice resulted in significant prevention of their cognitive deficits. No significant differences were found between control groups, suggesting that MK801 and CQ therapy do not interfere with cognitive measure (fig. 2 A).

There was no significant difference in the distance travelled between controls and PbA-infected mice treated with CQ or MK801+CQ, indicating no difference in motor activity (fig 2 B). Moreover, no significant difference in the step-down latency was found in the training session or in the long-term aversive memory analyzed 24 hours after training session by the step-down inhibitory avoidance test (Fig. supplementary 2A, B).

NMDA receptor antagonist MK801 effect on depressive and anxiety like behaviors

Depressive and anxiety like behaviors were evaluated 10 days after cessation of CQ therapy (n= 8 *per group*). PbA-infected mice receiving only CQ therapy presented a depressive-like behavior indicated by a higher immobility time in the swimming forced test. MK801+CQ treatment was effective in preventing depression following PbA infection resolution. Infected mice treated with MK801+CQ presented immobility time similar to controls. No significant differences were found between control groups,

suggesting that MK801 and CQ therapy do not interfere with depressive-like behavior measure (fig. 3 A).

Anxiety-like behavior was assessed in the EPM by the percentage of time spent and entries in the open arms of the maze (fig. 3 B-C). Moreover, the percentage of time spent in the center area of the open field was also used as an anxiety measure (fig. 3 D). No significant differences were found in anxiety-like behavior between controls and PbA-infected mice treated with CQ or MK801+CQ in the EPM neither in the open field (fig. 3 B-D).

Motor and exploratory activities following PbA infection resolution

Motor function is compromised during the active course of PbA infection (Dai et al., 2010) and could interfere with cognitive and behavioral analysis. We evaluated global activity, stereotype movements, motor and exploratory activities in the open field 10 days after cessation of CQ therapy (n= 8 *per group*). PbA-infected mice treated with CQ presented similar motor and exploratory behavior compared to PbA-infected mice receiving MK801+CQ therapy. Moreover, no significant differences were observed comparing to control groups (fig. 4 A-D).

NMDA receptor antagonist MK801 effect on cytokines concentration in the frontal cortex, hippocampus, spleen and serum following PbA infection

An imbalance in pro-inflammatory and anti-inflammatory cytokines in the CNS has been implicated in CM pathogenesis as well as in cognitive and behavioral outcomes (de Miranda et al., 2011; Hunt et al., 2006; John et al., 2008b). We evaluated

the effect of MK801 treatment in IL-1β, IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, TNF-α, and CCL11 levels in the frontal cortex and hippocampus of controls and PbA-infected mice 10 days after cessation of CQ. No significant differences in cytokine levels were found between uninfected mice receiving saline and non-infected animals treated with CQ or MK801+CQ indicating that the drugs used *per si* do not interfered in the CNS immune response (data not show).

PbA-infected mice treated with MK801+CQ presented increased frontal cortex levels of pro-inflammatory (IL-6, IL-17 and TNF-α) and anti-inflammatory (IL-2, IL-4 and IL-10) cytokines in comparison with treated matched controls (fig. 5). An up-regulation of IL-2, IL-4, IL-6, IL-10 and TNF-α was also observed compared with PbA-infected mice which received only CQ. No significant differences were found in IL-1β and IFN-γ concentrations. Moreover, infected mice treated with CQ presented higher levels of IL-2, IL-6, IL-17 and TNF-α compared with non-infected mice receiving CQ and of CCL11 compared with CM mice treated with MK801+CQ (fig. 5).

In the hippocampus, PbA-infected mice treated with CQ showed an up-regulation of IL-1β, IL-10 and CCL11 when compared with treated matched controls and infected mice which received MK801+CQ therapy. Higher levels of IL-1β and IL-6 were observed in the hippocampus of MK801+CQ treated mice compared to controls. Moreover, IL-4 was up-regulated whereas CCL11 was down-regulated in the hippocampus of those animals compared to PbA-infected mice receiving only CQ. No significant differences were observed in IL-2, IL-17, TNF-α and IFN-γ between controls and PbA-infected mice groups (fig. 6).

The pro-inflammatory cytokines IL-1β and TNF-α correlated negatively with novel object recognition memory in the frontal cortex of infected mice which received only CQ treatment (IL-1β: $r= -0.985$; $p<0.0001$; TNF-α: $r= -0.954$; $p<0.0001$).

Moreover, CCL11 levels correlated negatively with novel object recognition memory in the hippocampus of CM mice treated with CQ (r= -0.667; p= 0.035). Interestingly, a negative correlation between IL-4 and CCL11 was observed in the hippocampus of infected mice treated with MK801+CQ (r= -0.639; p= 0.046).

In order to analyze a systemic inflammatory response, we also evaluated cytokine levels in the spleen and serum of controls and PbA-infected mice 10 days after cessation of CQ. In the spleen, non-infected animals treated with MK801+CQ presented a significant decrease in IL-2, IL-4 and IL-6 levels in comparison with uninfected mice receiving saline. No significant differences were found in IL-10, TNF-α, IFN-γ and IL-17 concentrations. High levels of TNF-α was found in the spleen of control animals which received only CQ compared with saline controls, whereas no significant differences were observed in IL-2, IL-4, IL-6, IL-10, IFN-γ and IL-17 levels. CQ treated uninfected mice showed higher levels of IL-2, IL-4, IL-6, TNF-α and IL-17 compared with MK801+CQ non-infected animals. Regarding the infected groups, PbA-infected mice treated with CQ presented a significant reduction in IL-2, IL4, IL-6, IL-10, TNF-α and IL-17 levels in comparison with treated match controls and in IL-4 concentrations compared with PbA-infected animals treated with MK801+CQ. Moreover, higher levels of IL-14 were also observed in the spleen of PbA-infected mice treated with MK801+CQ in comparison with treated match controls (Fig. supplementary 3).

In the serum, non-infected mice treated with CQ or MK801+CQ showed a significant decrease in IL-6 and TNF-α concentrations in comparison with uninfected animals receiving saline. No significant differences were found in IL-2, IL-4, IL-10, IFN-γ and IL-17. Similar findings were observed comparing non-infected animals which received only CQ with those treated with MK801+CQ. A significant decrease in IL-2, IL-6, IL-10, TNF-α, IFN-γ and IL-17 concentrations was found in the serum of

PbA-infected mice receiving CQ compared with treated match controls. PbA-infected animals which received only CQ also presented a reduction in IL-2 levels compared with infected animals treated with MK801+CQ. Interestingly, no significant differences were observed in the levels of the cytokines evaluated when compared PbA-infected mice receiving MK801+CQ with treated match controls (Fig. supplementary 4).

NMDA receptor antagonist MK801 up-regulates neurotrophic factors in frontal cortex and hippocampus following PbA infection

Neurotrophic factors regulates neuronal survival and proliferation being involved in emotional, memory and learning processes (Angelucci et al., 2005; Conner et al., 2009; Heldt et al., 2007; Schmidt and Duman, 2007). We investigated MK801 effect in BDNF, NGF and GDNF levels in the frontal cortex and hippocampus of uninfected and PbA-infected mice 10 days following CQ therapy.

MK801+CQ treated infected mice presented a significant up-regulation of BDNF in the hippocampus compared to treated matched controls and PbA-infected animals receiving only CQ. The MK801+CQ also increase NGF and GDNF levels in the hippocampus of infected mice compared to those which received only CQ. Interestingly, PbA-infected mice which received only CQ showed a down-regulation of NGF and GDNF in the hippocampus compared to matched controls (fig. 7).

A significant enhancement in BDFN, NGF and GDNF levels was observed in the frontal cortex of infected mice following MK801+CQ treatment compared to CQ treated infected animals (fig. 8). A significant decrease in BDNF and NGF concentrations was also found in the frontal cortex of CM mice receiving only the anti-parasitic therapy compared to match controls (fig. 8).

A positive correlation was found between BDNF and novel object recognition memory in the hippocampus of PbA-infected mice treated with MK801+CQ (r=0.899; p=0.0004). Interestingly, GDNF correlated positively with TNF-α in the hippocampus and frontal cortex of those animals.

NMDA receptor antagonist MK801 effect in hippocampus density and volume measured by Resonance Magnetic Images (RMI)

Brain RMI abnormalities including edema and increased cerebral volume has been reported during the course of CM, especially in children (Jelicks et al., 2013; Penet et al., 2005; Potchen et al., 2012). We investigated a possible neuroprotective effect of MK801 in the hippocampus of control and infected mice (n= 4 *per group*) by MRI approximately 10 days after cessation of CQ treatment. PbA-infected mice receiving only CQ presented a significant decrease in hippocampus density, indicative of edema, associated with an increase in this region volume compared to controls and infected mice treated with MK801+CQ. The MK801 treatment of CM mice resulted in significant prevention of hippocampus abnormality measured by MRI (fig. 9).

4. Discussion

CM pathogenesis is multifactorial and apart from immune and vascular responses changes in glutamate system have been reported (Miranda et al., 2010; Parekh et al., 2006; Rae et al., 2004). We previously demonstrated a significant increase of glutamate levels in the cerebral cortex and in the cerebrospinal fluid of CM mice in parallel with the development of neurological and behavioral symptoms (Miranda et al., 2010). The finding that a high amount of glutamate was released in the CNS in response

to PbA infection suggests that treatments which modulate glutamate signaling such as MK801, could attenuate the CM outcome.

In the present study, we found a beneficial effect of MK801 during experimental CM and mainly following parasitic resolution by CQ. Firstly, we find an earlier neuroprotective effect of MK801 prior to CQ therapy institution on day 5 post-infection indicated by less CM symptoms in the RBMCS. However, no significant differences in survival and parasitemia were observed. Interestingly, an enhancement in glutamate levels was also found in the hippocampus of uninfected and PbA-infected mice which received MK801+CQ treatment. In agreement, a previous study showed that systemic administration of NMDA receptor antagonists elevates glutamate release in rat CNS (Roenker et al., 2012), indicating that it is an expected effect of MK801 treatment not associated to PbA-infection.

NMDA receptor antagonists could mimic schizophrenia symptoms in a dose dependent manner (Blot et al., 2013). However, growing evidence has been supporting a neuroprotective role rather than a detrimental effect in many conditions including Alzheimer's disease, ischemia, spinal cord injury and sepsis (Bakiri et al., 2008; Cassol-Jr et al., 2011; Esposito et al., 2011; Salomone et al., 2012). For instance, we demonstrated that a low dose of MK801 (0.5mg/kg) successfully rescued long-term object recognition memory 10 days after elimination of PbA infection by CQ therapy. In line with our finding, (Cassol-Jr et al., 2011) showed that MK801 prevented cognitive impairment in sepsis survivor rats ten days after cecal ligation and perforation (CLP) surgery. An inhibition of NMDA receptor activation by MK801 also attenuates seizure-induced cell death in mice hippocampus, a major brain region related to memory function, following kainate-induced status epilepticus (Schauwecker, 2010). Altogether these studies provided evidence that inhibiting glutamate excitotocity could

be a promise therapeutic strategy against CNS insult. It is worth mentioning that in line with previous studies (Dai et al., 2010; Reis et al., 2010) CQ treatment alone was not able to prevent CM long-term cognitive deficits.

It has been reported depressive and anxiety symptoms following successful resolution of CM by antimalarial therapy (Dugbartey et al., 1998; Varney et al., 1997). However, those emotional disorders are underestimated since more severe symptoms such as cognitive and motor dysfunctions are also frequently described. We showed, for the first time, that PbA-infected mice remain with long-term depressive-like behavior despite full clearance of parasitemia by CQ. The MK801 treatment successfully prevented this long-term symptom in CM mice. Interestingly, although we have previously demonstrated anxiety-like behavior during acute experimental CM (de Miranda et al., 2011) this symptom seems to resolve following PbA-infection resolution by CQ therapy. Overwhelming evidence provided by clinical and experimental studies supports an antidepressant and anxiolytic effect of NMDA receptor antagonists (Berman et al., 2000; Engin et al., 2009; Yang et al., 2013; Zarate et al., 2006). A fast-acting and long-lasting antidepressant response was induced by intravenous administration of Ketamine, a NMDA receptor antagonist, in patients suffering from major depressive disorder (Zarate et al., 2006). Moreover, NMDA receptor antagonists including MK801 also produced a fast-acting behavioral antidepressant-like effect, measured by predictive tasks such as the forced swim test, following chronic mild stress an animal model of depression (Autry et al., 2011). Importantly, no differences in locomotor and exploratory activities were observed indicating that cognitive and behavioral impairments were not related to peripheral organ or muscle dysfunction due to general systemic illness, or effects of CQ and MK801treatments.

Systemic inflammatory response associated to infectious conditions can lead to sickness behavior and might affect behavioral assessment. Physiological and behavioral changes including lethargy, fatigue, weakness, anorexia and sleep disorders associated to fever are typical symptoms of sickness behavior (Dantzer, 2001; Kelley et al., 2003; McCusker and Kelley, 2013). As lethargy is a core symptom of sickness behavior, locomotor activity can be used as a technically easy and high-throughput measure of sickness behavior (York et al., 2012). Moreover, inflammatory cytokines, especially TNF, can induce sickness behavior, in an attempt to control an evolving infectious disease. In the current study, PbA-infected mice treated with CQ or both CQ+MK801 presented no change or a reduction in cytokine levels in the spleen and serum compared to controls. Therefore, our cognitive/behavioral findings seem not to reflect sickness behavior.

Noninvasive imaging technologies such as MRI have become an important research toll in the study of the mechanisms that underlie CM pathogenesis as well as for evaluation of new therapeutic agents efficacy (Jelicks et al., 2013; Penet et al., 2005). In the present study, using the MRI technique, we provided further insights regarding hippocampus integrity following completed parasitic cure by CQ therapy and neuroprotection conferred by MK801. CM mice rescued by CQ treatment presented hippocampus edema associated with a significant increase in this region volume. Similar findings were reported by previous studies that used MRI to investigated brain injury during human and experimental CM (Penet et al., 2005; Potchen et al., 2012). The potential mechanisms involved in increased brain volume included a localized response to inflammatory factors and parasite sequestration with venous congestion (Potchen et al., 2012). Interestingly, in line with previously reported by (Dai et al., 2010) histopathological alterations such as hemorrhage and leukocyte sequestration

were largely resolved in PbA-infected mice receiving CQ therapy combined or not with MK801. Importantly, MK801 completed prevented the hippocampus abnormalities found in MRI. The ability of MK801 to control brain edema improving neurological deficits was previously demonstrated during experimental embolic stroke (Allahtavakoli et al., 2007).

It has been proposed that the immune system mediators, especially cytokines, under physiological conditions may regulate essential CNS functions such as memory and learning (McAfoose and Baune, 2009). However, the over-expression of pro-inflammatory cytokines, mainly IL-1β, IL-6 and TNF-α promotes a deleterious effect in the CNS and has been involved in the pathogenesis of different conditions including CM and Alzheimer's disease (John et al., 2008b; Linares et al., 2013; Rocha et al., 2012). In parallel with long-term cognitive deficits, depressive-like behavior and MRI brain abnormalities, PbA-infected mice which received only CQ exhibited an up-regulation of pro-inflammatory cytokines in frontal cortex and hippocampus. Novel object recognition memory also correlates negatively with IL-1β and TNF-α in the frontal cortex of those animals. Corroborating our findings, (John et al., 2008b) demonstrated that high levels of TNF-α in the cerebrospinal fluid of CM children in hospital admission was correlated negatively with age-adjusted scores for attention and working memory 6 months later. They provided relevant evidence that TNF-α remains up-regulated in the CNS following acute infection resolution and adversely affect long-term cognitive outcome in CM. Depressive-like behavior were also associated with high levels of TNF-α in the frontal cortex of rats ten days after pneumococcal meningitis induction (Barichello et al., 2010). Additionally, a release of regulatory cytokines, mainly IL-10, has also been reported during CM (de Souza (de Souza and Riley, 2002; Hunt and Grau, 2003; Kossodo et al., 1997). The enhancement in IL-10 levels observed

in the hippocampus of PbA-infected mice receiving only CQ could be a compensatory mechanism in an attempting to control the inflammatory response and prevent secondary brain injury.

Pro-inflammatory mediators, especially IL-1β and TNF-α could induce glutamate release by neurons and glial cells contributing to neurotoxicity mediated mainly through NMDA receptors over-activation (Bezzi et al., 2001; Chen et al., 2012; Ye et al., 2013). Besides preventing neuronal damage by directly blockage of NMDA receptors activity, growing evidence supports an immunomodulatory effect of MK801treatment during CNS insults including focal brain ischemia and spinal cord injury (Al-Amin et al., 2011; Esposito et al., 2011; Jander et al., 2000; Liu et al., 2011). In this scenario, we found a balance of TH1/TH2 response in frontal cortex and hippocampus of MK801 treated infected mice 10 days following cessation of CQ therapy. This balance is essential to maintain a restrained inflammatory environment in the CNS which contributes to mnemonic and emotional processes (Butovsky et al., 2006; Derecki et al., 2010). In agreement, blockade of NMDA receptors by MK801 attenuated experimental spinal cord injury severity mainly through modulation of immune response by inhibiting the inflammatory transcription factor NF-κB and apoptosis pathways as well as by decreasing cytokines release, oxidative stress and leukocytes infiltration (Esposito et al., 2011). An anti-inflammatory response was also observed following long-term exposure of cortical neurons to the NMDA receptor antagonist MK801 (Dobbertin et al., 2000).

Chemokines constitute a class of small inflammatory proteins mainly recognized by its ability to control the chemotaxis of leukocytes (Ransohoff, 2009). It has also been reported that chemokines regulate calcium transients and glutamate release in astrocytes (de Haas et al., 2007) and can affect neuronal activity through modulation of

neurotransmission (Gosselin et al., 2005; Skrzydelski et al., 2007). An up-regulation of chemokines has been described during human and experimental CM (Armah et al., 2007; Lacerda-Queiroz et al., 2010; Wilson et al., 2011). We provided further evidence that CCL11 remains up-regulated in the hippocampus of CM mice treated with CQ and correlated with novel object recognition memory impairment. A recent study conducted by (Villeda et al., 2011) demonstrated that high plasma levels of CCL11 inhibited adult hippocampal neurogenesis, synaptic plasticity and impaired learning and memory, supporting our findings. The exactly mechanism by which CCL11 affects neurogenesis and cognitive function remains unclear. A possible hypothesis is the suppression by CCL11 of the IL-4 ability to restrain the inflammatory functions of meninges myeloid cells which could account for neurogenesis reduction and cognitive deficits (Derecki et al., 2010; Stevenson et al., 2009). Interestingly, MK801 treatment induced an up-regulation of IL-4 in parallel with a reduction in CCL11 levels in the hippocampus of infected mice. A negative correlation between IL-4 and CCL11 was also observed. These findings could at least in part explain the MK801neuroprotective effect.

Neurotrophic factors are critically involved in mnemonic and emotional processes (Angelucci et al., 2005; Conner et al., 2009; Heldt et al., 2007; Schmidt and Duman, 2007). CM mice treated with CQ presented a significant decrease in BDNF, NGF and GDNF levels in the frontal cortex and hippocampus following PbA infection resolution. Recently, (Comim et al., 2012) demonstrated a reduction in BDNF concentration in the hippocampus of PbA-infected mice rescued from CM by CQ therapy which was correlated with cognitive deficits. A decrease in neurotrophic factors expression in the CNS has been also detected in animal models of depression (Angelucci et al., 2005) as well as in the brain of patients with depressive disorder (Michel et al., 2008). On the other hand, we showed for the first time that MK801

treatment was capable to up-regulates hippocampus and frontal cortex neurotrophic factors expression. High levels of BDNF in the hippocampus were also positively correlated with novel object recognition memory. Previous studies have been reported an enhancement in neurotrophic factors expression, especially BDNF, in different brain regions including hippocampus and limbic areas such as entorhinal and retrosplenial cortex following MK801 treatment (Al-Amin et al., 2011; Linden et al., 2000; Matsuki et al., 2001). In the CNS, neurotrophic factors are associated with neuronal development, differentiation and plasticity playing a crucial role in the maintenance of normal learning and memory functions (Conner et al., 2009; Lu et al., 2013; Pertusa et al., 2008). Additionally, NMDA receptor antagonists exert its rapid and long-lasting antidepressant effect by increasing BDNF hippocampus and cortex levels. Since those antagonists presented short half-lives (about 2–3 h), it has been proposed that the sustained antidepressant responses are probably due to synaptic plasticity rather than to persistent blockade of receptors (Autry et al., 2011).

It has been proposed a crosstalk between inflammatory cytokines and neurotrophic factors. However this interaction is complex and still not fully understood (Ji et al., 2011). For instance, TNF-α was able to induce the production of NGF and GDNF by astrocytes through binding with its receptor TNFR2 (Kuno et al., 2006). Therefore, NGF induced TNF-α expression in neurons through NFκB activation and then bound to TNFR2 activating AKT pathway and promoting neuronal survival (Takei and Laskey, 2008). These findings suggested a positive-feedback loop of TNF-α and neurotrophic factors which could protect brain from damage. In agreement, the positive correlation between TNF-α and GDNF found in the hippocampus and frontal cortex of infected mice following MK801 probably represented a positive-feedback loop which could account for MK801 neuroprotective role. On the other hand, an imbalance in

neurotrophic factors and pro-inflammatory cytokines release as we observed in the brain of CM mice treated with CQ have been described in the pathogenesis of cognitive deficits related to Alzheimer's disease as well as during the acute phase of CM (Ji et al., 2011; Miranda et al., 2015).

In summary, we provided further evidence regarding the interplay between NMDA-receptors, cytokines and neurotrophic factors in the pathogenesis of long-term cognitive and depressive symptoms following CM resolution. We also demonstrated that blocking NMDA receptors by MK801 modulates cytokines and neurotrophic factors expression preventing CM long-term outcomes, indicating that NMDA receptor antagonists could be a promise adjuvant therapy. Memantine, a noncompetitive NMDA receptor antagonist, already presented a well safety and established applicability in clinic, especially regarding cognitive outcome in Alzheimer's disease patients. Its neuroprotective effect has been investigated in ongoing experiments conducted by our group in order to further translate its possible therapeutic benefits to clinical trials.

Author's contribution

ASM participated in the experimental design, carried out behavioral and immunological assays, data analysis and drafted the manuscript. FB participated in the experimental design, carried out behavioral and immunological assays and revised the manuscript. LBV, FMR, and MVG carried out glutamate release assays, data analysis and revised the manuscript. NPR performed immunological assays. GHSR and MFDM performed MRI analysis. FSM and MAR participated in the design and coordination of the study. ALT designed the study and was responsible for the interpretation of experiments and editing the manuscript. All authors have read and approved the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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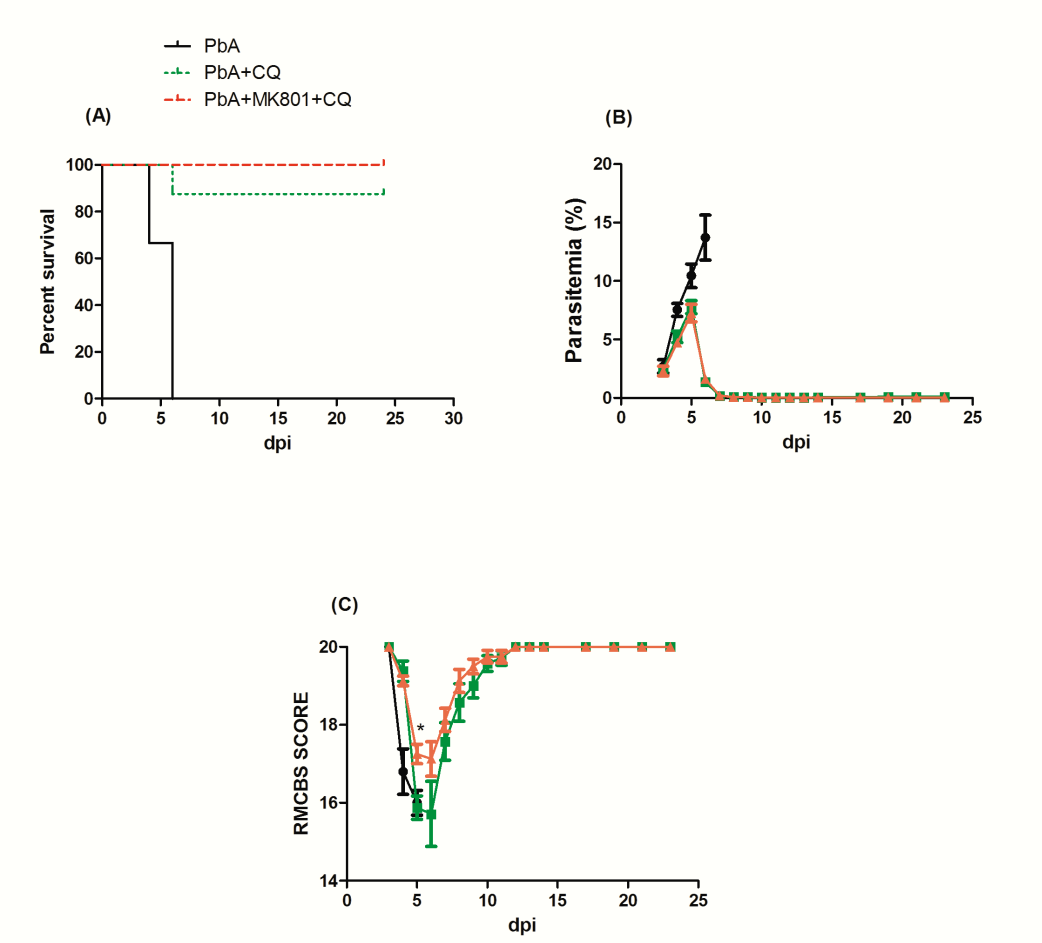

Fig. 1 Effect of MK801 on survival rate, parasitemia and clinical signs of cerebral malaria after infection with *P.berghei* ANKA (PbA). C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and then separated in three groups. On day 3 post-infection a group initiated the MK801 (i.p. 0.5 mg/kg) treatment which was continued until the end of CQ therapy. All mice were treated orally with a ten-day course CQ (30 mg/kg) from day 5 post-infection excepted one group which was used as control for successful PbA infection. Non-infected animals used as controls for glutamate release assay received saline, CQ or CQ+MK801. (A) Survival curve comparisons of PbA-infected mice treated with CQ or treated with MK801+CQ were expressed days after infection; (B) natural course of PbA infection was determined by flow cytometric analysis; (C) clinical signs of cerebral malaria assessed by the RMCBS scale. The data is representative of two independent experiments (n= 10 *per group*) and shown as the mean+/- SEM. Asterisk(s) indicate statistical differences where *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$.

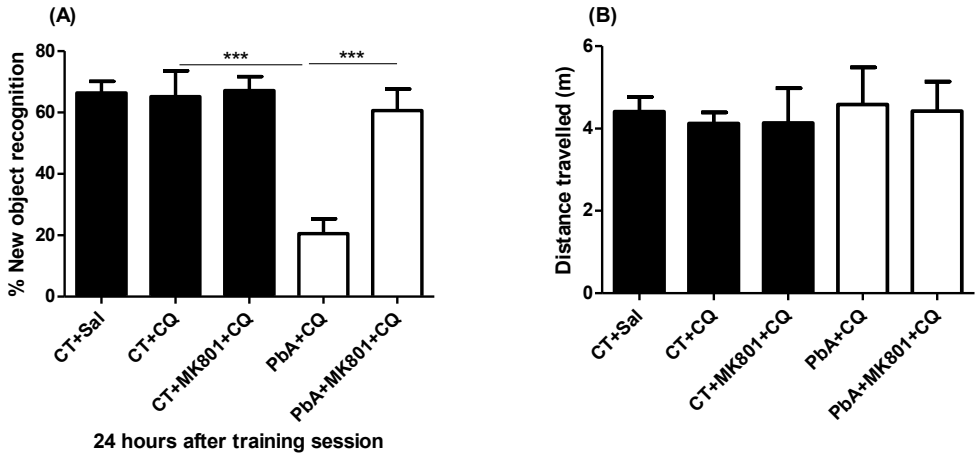

Fig. 2: The NMDA receptor antagonist MK801 prevents novel object recognition memory impairment following *Plasmodium berghei* ANKA (PbA) infection resolution by chloroquine (CQ) therapy. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and then separated in two groups. On day 3 post-infection a group initiated the MK801 (i.p. 0.5 mg/kg) treatment which was continued until the end of CQ therapy. All mice were treated orally with a ten-day course CQ (30 mg/kg) from day 5 post-infection. As controls, non-infected animals received the same volume of saline, CQ or CQ+MK801. From ten days after cessation of CQ treatment all mice were submitted to object recognition task training and test session. (A) Long-term memory and (B) distance travelled were recorded 24 hours after training session. Results are expressed as mean ± SEM and are representative of at least two independent experiments (n= 5 *per group*). Asterisk(s) indicate statistical differences where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

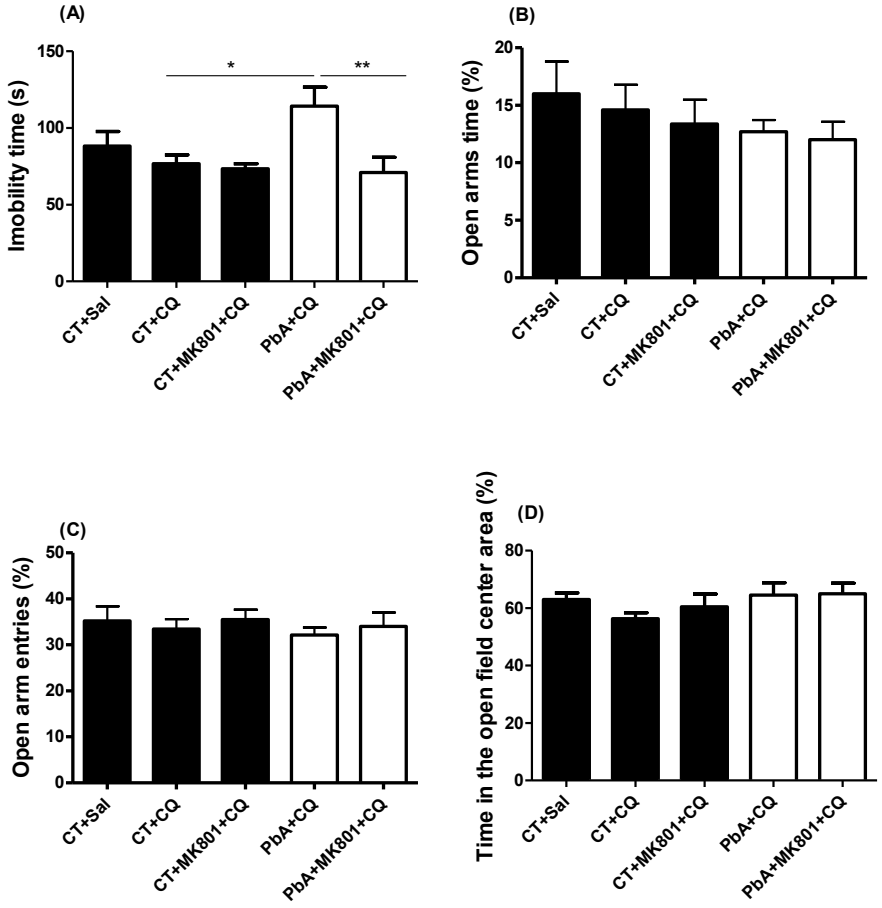

Fig. 3: Effect of MK801 in long-term depressive and anxiety like behaviors following *Plasmodium berghei* ANKA (PbA) infection resolution by chloroquine (CQ) therapy. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and then separated in two groups. On day 3 post-infection a group initiated the MK801 (i.p. 0.5 mg/kg) treatment which was continued until the end of CQ therapy. All mice were treated orally with a ten-day course CQ (30 mg/kg) from day 5 post-infection. As controls, non-infected animals received the same volume of saline, CQ or CQ+MK801. From ten days after cessation of CQ treatment all mice were submitted to the forced swimming test for depression analyses or to the elevated plus maze and to the open field to anxiety assessment. (A) MK801 prevented long-term depressive-like behavior in the forced swimming test indicated by a less immobility time in seconds. No significant differences were found in the percentage of time spent (B) and entries (C) in the open arms of the elevated plus maze. Similar result were observed in the open field as no difference was found in the percentage of time spent in the center area indicating no long-term anxiety-like behavior. Results are expressed as mean ± SEM and are representative of at least two independent experiments (n= 8 *per group*). Asterisk(s) indicate statistical differences where *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$.

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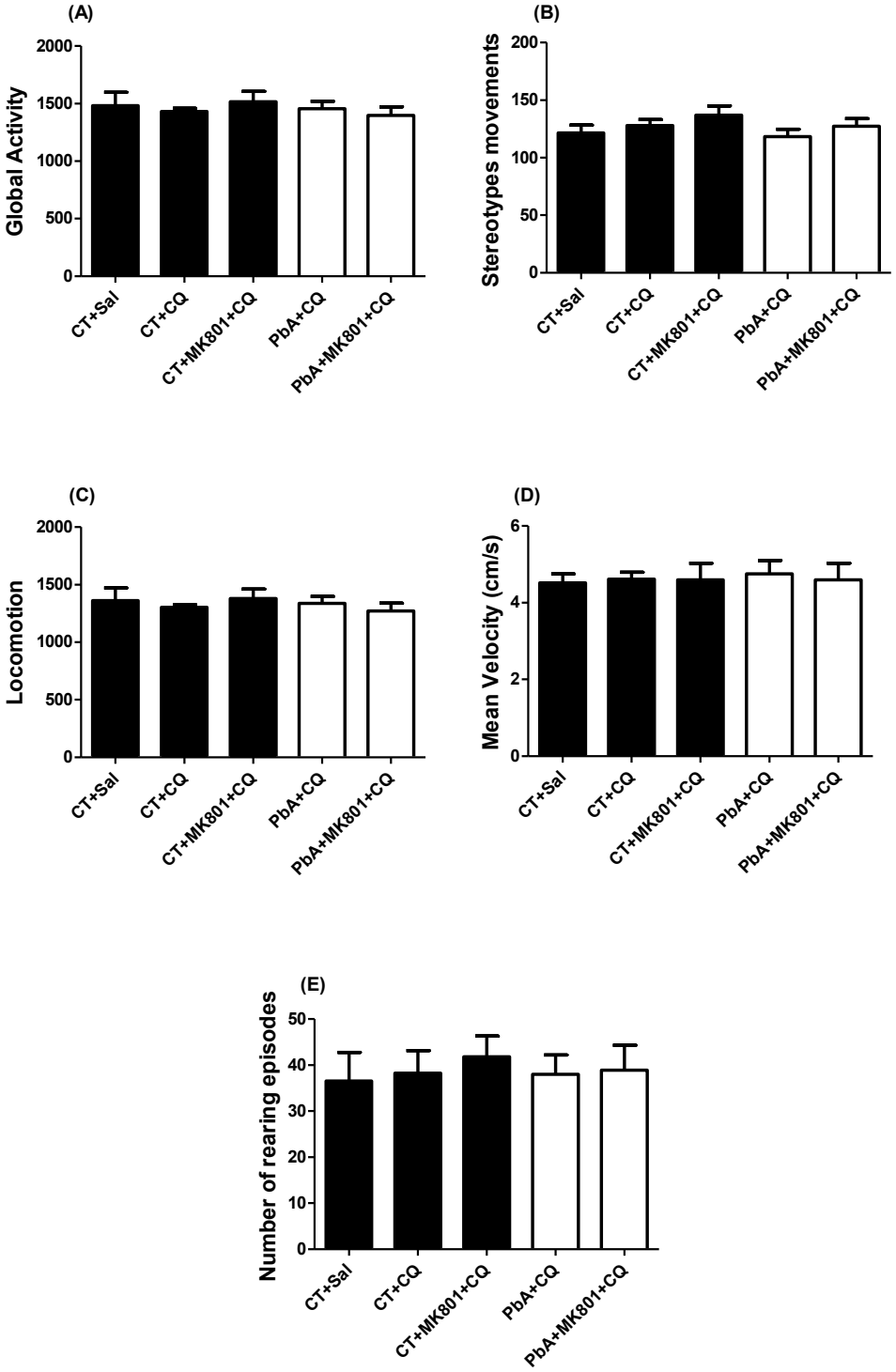

Fig. 4: Effect of MK801 in motor and exploratory activities following *Plasmodium berghei* ANKA (PbA) infection resolution by chloroquine (CQ) therapy. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and then separated in two groups. On day 3 post-infection a group initiated the MK801 (i.p. 0.5 mg/kg) treatment which was continued until the end of CQ therapy. All mice were treated orally with a ten-day course CQ (30 mg/kg) from day 5 post-infection. As controls, non-infected animals received the same volume of saline, CQ or CQ+MK801. From ten days after cessation of CQ treatment all mice were submitted to the open field task for general motor and exploratory activities analysis. No significant differences were found in (A) global activity, (B) stereotype movements, (C) locomotion, (D) mean velocity or (E) in the number of rearing episodes. Results are expressed as mean ± SEM and are representative of at least two independent experiments (n= 8 *per group*). Asterisk(s) indicate statistical differences where *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$.

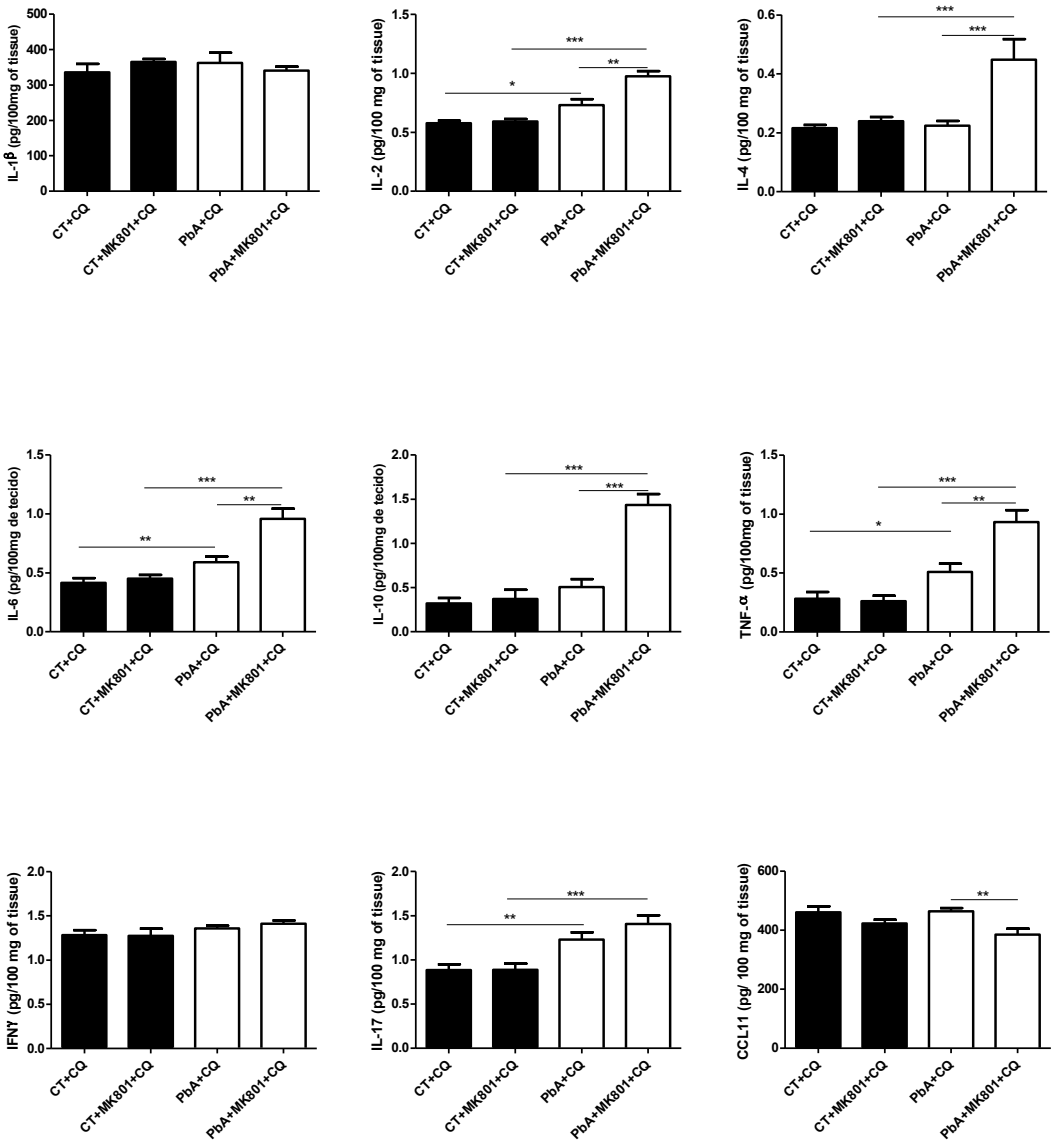

Fig. 5: MK801 modulates inflammatory response in the frontal cortex of CM mice following *Plasmodium berghei* ANKA (PbA) infection resolution by chloroquine (CQ) therapy. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and then separated in two groups. On day 3 post-infection a group initiated the MK801 (i.p. 0.5 mg/kg) treatment which was continued until the end of CQ therapy. All mice were treated orally with a ten-day course CQ (30 mg/kg) from day 5 post-infection. As controls, non-infected animals received the same volume of saline (data not show), CQ or CQ+MK801. Ten days after cessation of CQ therapy all animals were culled and frontal cortex was harvested, homogenized and IL-1β and CCL11 concentrations were measured by ELISA while IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α levels were assessed by Cytometric Bead Array (CBA). Results are expressed as mean ± SEM and are representative of at least two independent experiments (n= 5 *per group*). Asterisk(s) indicate statistical differences where *p < 0.05, **p < 0.01, ***p< 0.001.

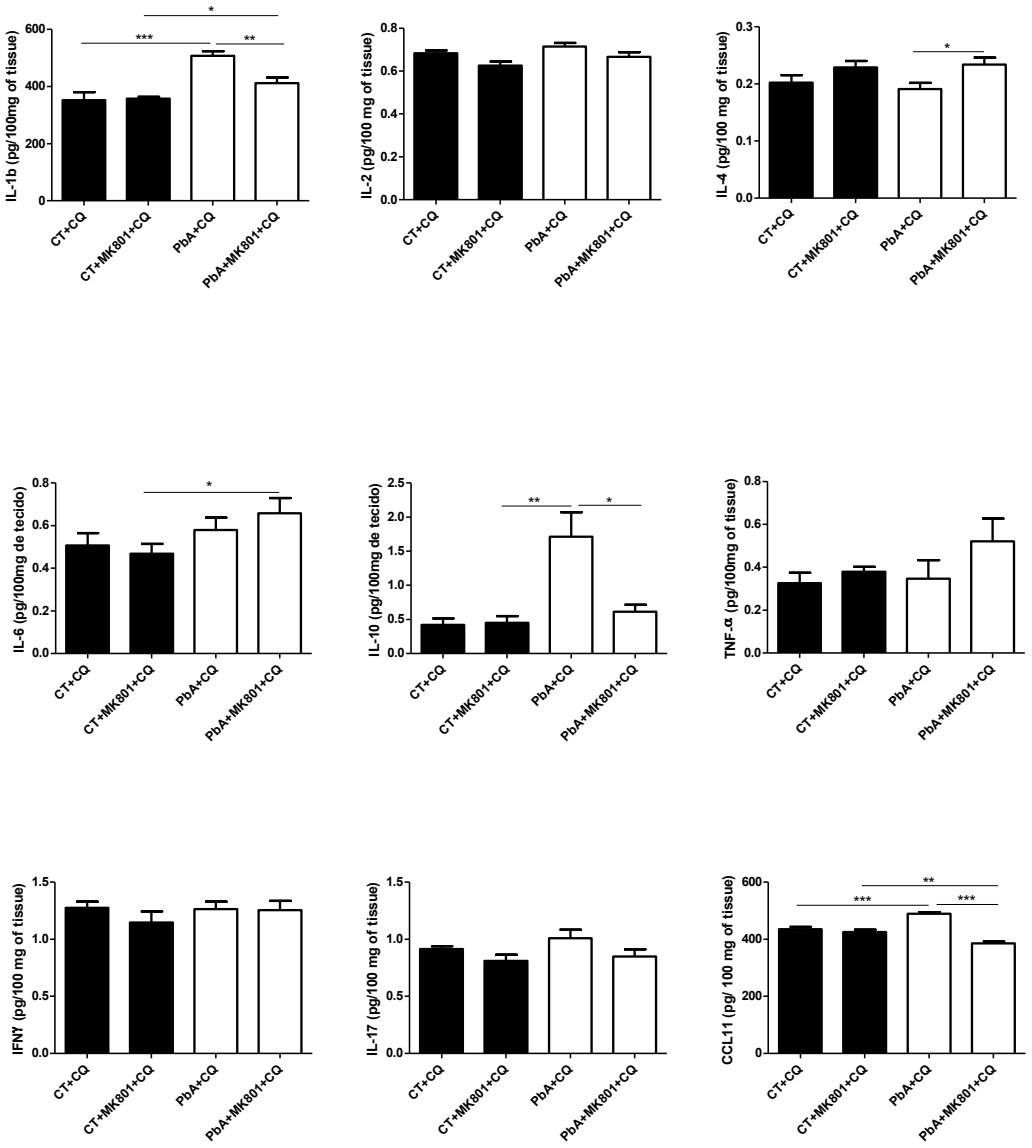

Fig. 6: MK801 modulates inflammatory response in the hippocampus of CM mice following *Plasmodium berghei* ANKA (PbA) infection resolution by chloroquine (CQ) therapy. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and then separated in two groups. On day 3 post-infection a group initiated the MK801 (i.p. 0.5 mg/kg) treatment which was continued until the end of CQ therapy. All mice were treated orally with a ten-day course CQ (30 mg/kg) from day 5 post-infection. As controls, non-infected animals received the same volume of saline (data not show), CQ or CQ+MK801. Ten days after cessation of CQ therapy all animals were culled and hippocampus was harvested, homogenized and IL-1β and CCL11 concentrations were measured by ELISA while IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α levels were assessed by Cytometric Bead Array (CBA). Results are expressed as mean ± SEM and are representative of at least two independent experiments (n= 5 *per group*). Asterisk(s) indicate statistical differences where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

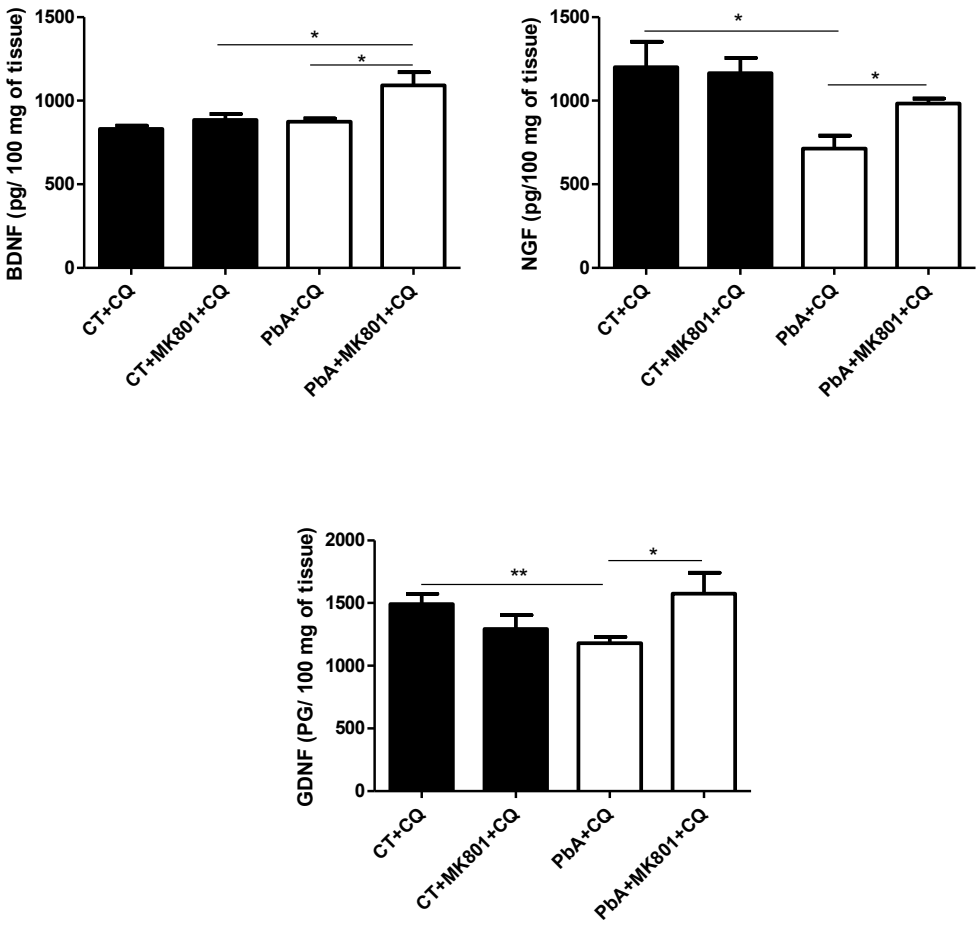

Fig. 7: MK801 up-regulates neurotrophic factors levels in the hippocampus of CM mice following *Plasmodium berghei* ANKA (PbA) infection resolution by chloroquine (CQ) therapy. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and then separated in two groups. On day 3 post-infection a group initiated the MK801 (i.p. 0.5 mg/kg) treatment which was continued until the end of CQ therapy. All mice were treated orally with a ten-day course CQ (30 mg/kg) from day 5 post-infection. As controls, non-infected animals received the same volume of saline (data not show), CQ or CQ+MK801. Ten days after cessation of CQ therapy all animals were culled and hippocampus was harvested, homogenized and BDNF, NGF and GDNF concentrations were measured by ELISA. Results are expressed as mean ± SEM and are representative of at least two independent experiments (n= 5 *per group*). Asterisk(s) indicate statistical differences where *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$.

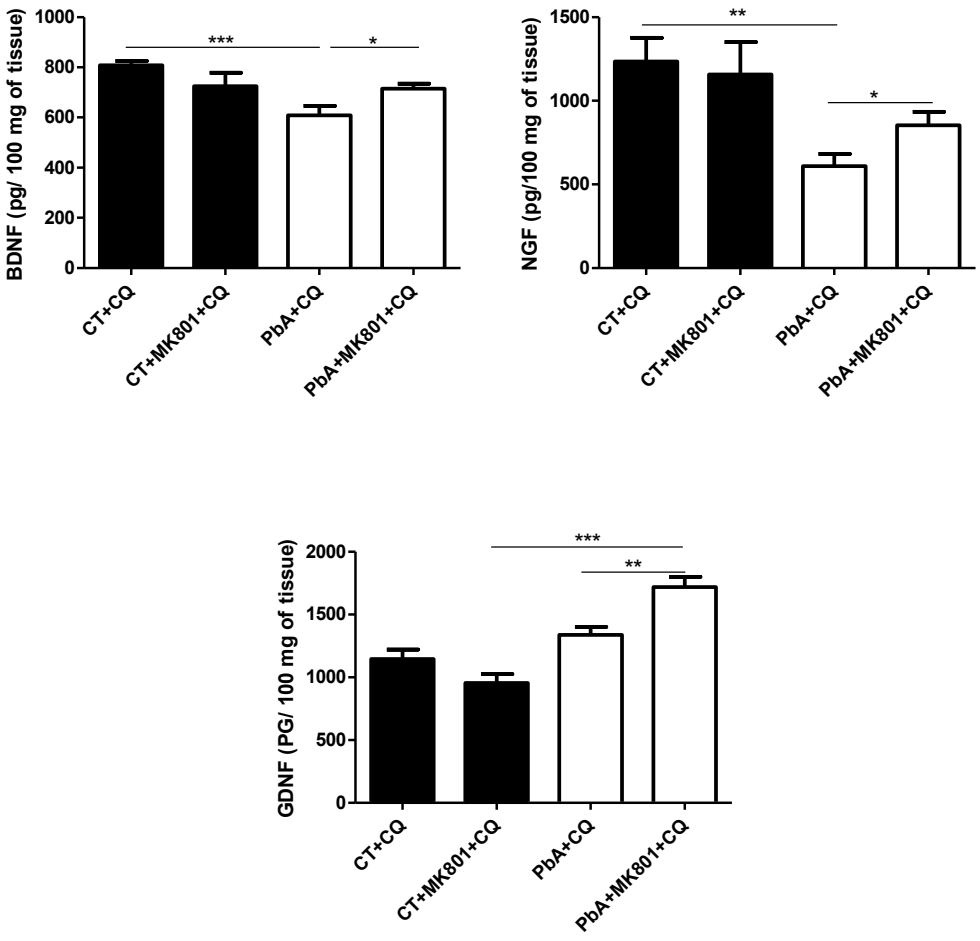

Fig. 8: MK801 up-regulates neurotrophic factors levels in the frontal cortex of CM mice following *Plasmodium berghei* ANKA (PbA) infection resolution by chloroquine (CQ) therapy. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and then separated in two groups. On day 3 post-infection a group initiated the MK801 (i.p. 0.5 mg/kg) treatment which was continued until the end of CQ therapy. All mice were treated orally with a ten-day course CQ (30 mg/kg) from day 5 post-infection. As controls, non-infected animals received the same volume of saline (data not show), CQ or CQ+MK801. Ten days after cessation of CQ therapy all animals were culled and frontal cortex was harvested, homogenized and BDNF, NGF and GDNF concentrations were measured by ELISA. Results are expressed as mean ± SEM and are representative of at least two independent experiments (n= 5 *per group*). Asterisk(s) indicate statistical differences where *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$.

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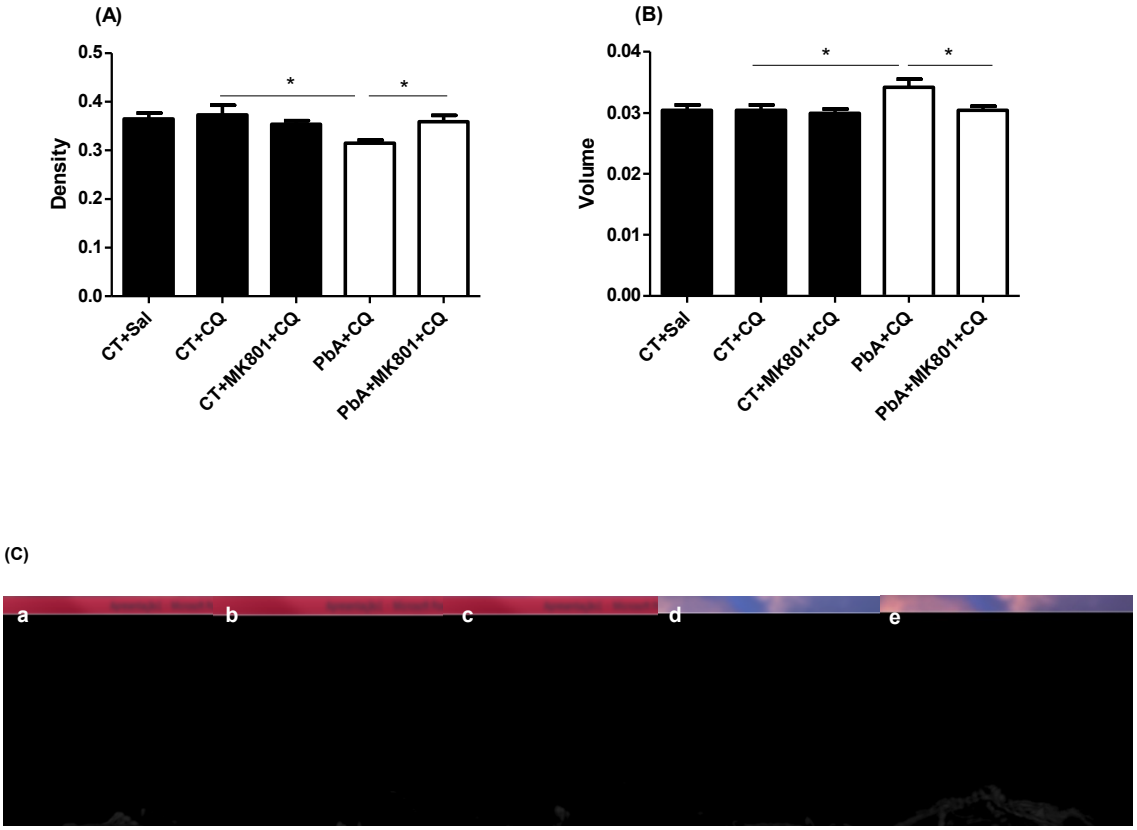

Fig. 9: MK801 prevents cerebral malaria long-term hippocampus abnormalities measured by RMI following *Plasmodium berghei* ANKA (PbA) infection resolution by chloroquine (CQ) therapy. C57BL/6 mice were intraperitoneally infected with 106 parasitized erythrocytes and then separated in two groups. On day 3 post-infection a group initiated the MK801 (i.p. 0.5 mg/kg) treatment which was continued until the end of CQ therapy. All mice were treated orally with a ten-day course CQ (30 mg/kg) from day 5 post-infection. As controls, non-infected animals received the same volume of saline, CQ or CQ+MK801. Ten days after cessation of CQ therapy all animals hippocampus were analyzed by MRI. PbA-infected mice receiving only CQ presented lower hippocampus density indicative of edema (A) associated with increased hippocampus volume (B) which were prevented by MK801 treatment. (C) Representative images of hippocampus (a: CT+Sal; b: CT+CQ; c: CT+MK801+CQ; d: PbA+CQ; e: PbA+MK801+CQ). Results are expressed as mean ± SEM (n= 4 *per group*). Asterisk(s) indicate statistical differences where *p < 0.05, **p < 0.01, ***p< 0.001.

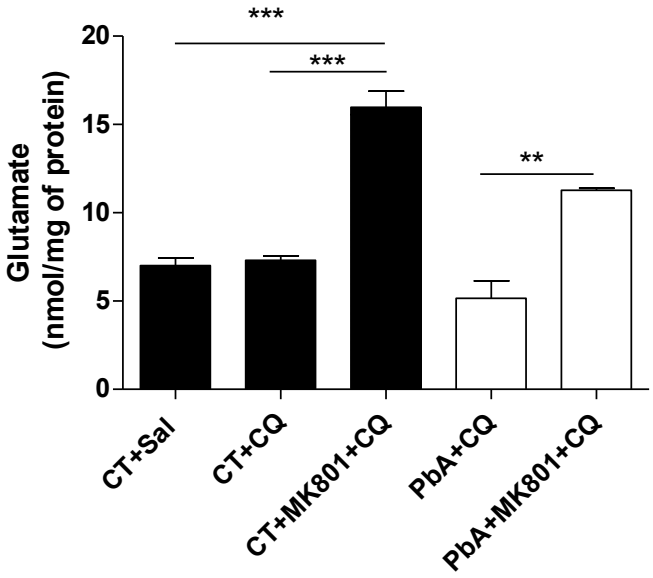

Fig. supplementary 1: Effect of MK801 on hippocampus glutamate release following *Plasmodium berghei* ANKA (PbA) infection resolution by chloroquine (CQ) therapy. C57BL/6 mice were intraperitoneally infected with 106 parasitized erythrocytes and then separated in two groups. On day 3 post-infection a group initiated the MK801 (i.p. 0.5 mg/kg) treatment which was continued until the end of CQ therapy. All mice were treated orally with a ten-day course CQ (30 mg/kg) from day 5 post-infection. As controls, non-infected animals received the same volume of saline, CQ or CQ+MK801. Ten days after cessation of CQ therapy all animals were culled, hippocampus was harvested, synaptosomes were prepared and glutamate release in the hippocampus was measured by spectrofluorometer. Results are expressed as mean ± SEM and are representative of two independent experiments (n= 5 *per group*). Asterisk(s) indicate statistical differences where *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$.

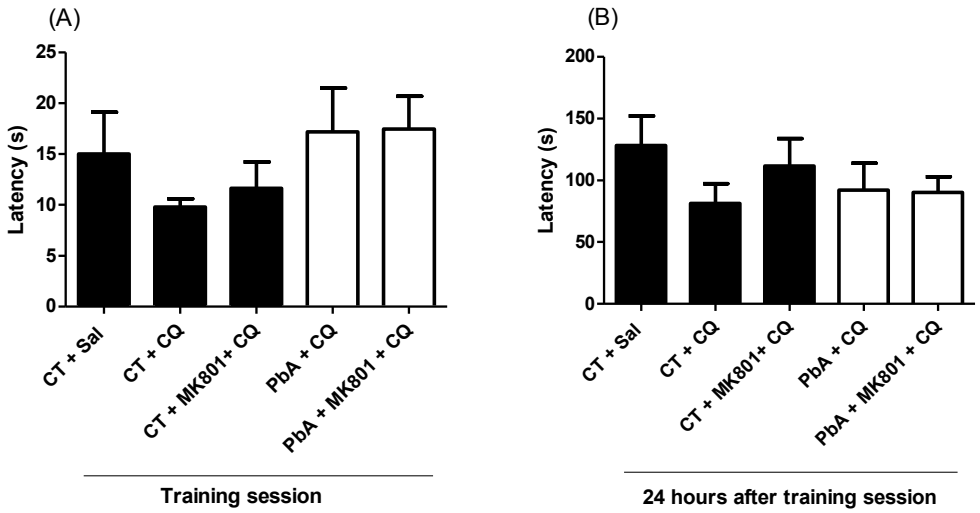

Fig. supplementary 2: Effect of MK801 in long-term aversive memory following *Plasmodium berghei* ANKA (PbA) infection resolution by chloroquine (CQ) therapy. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and then separated in two groups. On day 3 post-infection a group initiated the MK801 (i.p. 0.5 mg/kg) treatment which was continued until the end of CQ therapy. All mice were treated orally with a ten-day course CQ (30 mg/kg) from day 5 post-infection. As controls, non-infected animals received the same volume of saline, CQ or CQ+MK801. From ten days after cessation of CQ treatment all mice were submitted to the step-down inhibitory avoidance test. No significant difference in the step-down latency was found in the training session (A) or in the long-term aversive memory analyzed 24 hours after training session (B). Results are expressed as mean ± SEM and are representative of at least two independent experiments (n= 7 *per group*).

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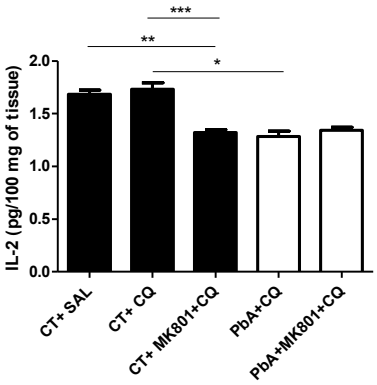

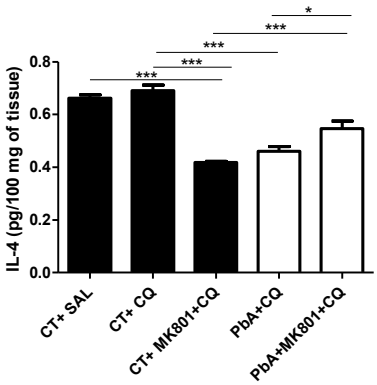

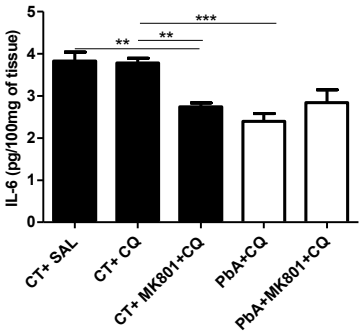

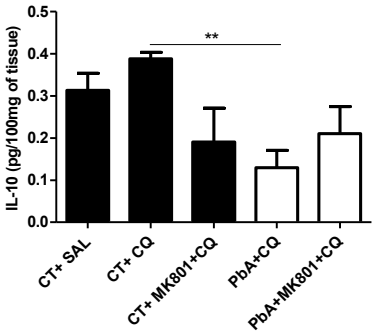

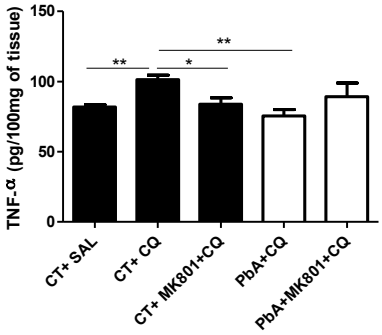

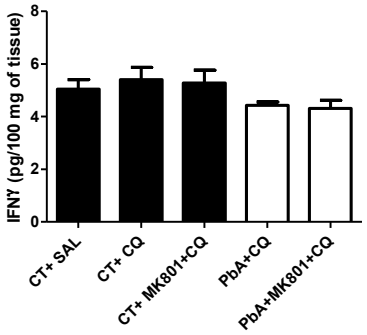

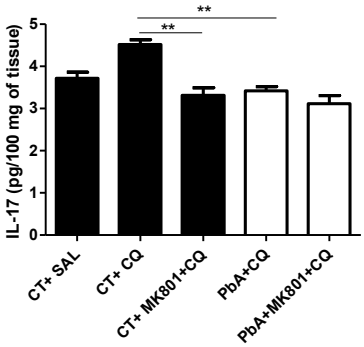

Fig. supplementary 3: MK801 effect in inflammatory response in the spleen of CM mice following *Plasmodium berghei* ANKA (PbA) infection resolution by chloroquine (CQ) therapy. C57BL/6 mice were intraperitoneally infected with 10^6 parasitized erythrocytes and then separated in two groups. On day 3 post-infection a group initiated the MK801 (i.p. 0.5 mg/kg) treatment which was continued until the end of CQ therapy. All mice were treated orally with a ten-day course CQ (30 mg/kg) from day 5 post-infection. As controls, non-infected animals received the same volume of saline, CQ or CQ+MK801. Ten days after cessation of CQ therapy all animals were culled and spleen was harvested, homogenized and IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α levels were assessed by Cytometric Bead Array (CBA). Results are expressed as mean ± SEM and are representative of at least two independent experiments (n= 5 *per group*). Asterisk(s) indicate statistical differences where *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$.

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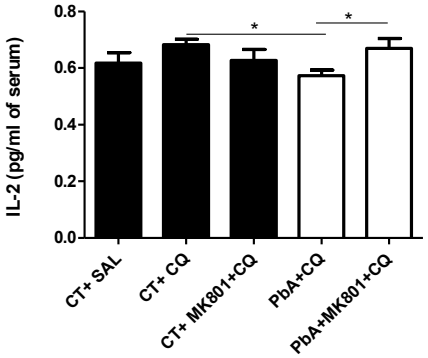

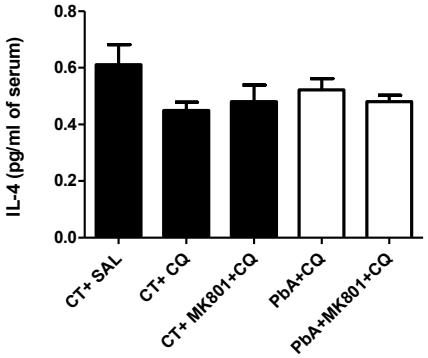

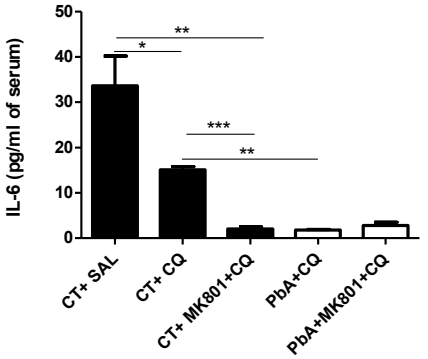

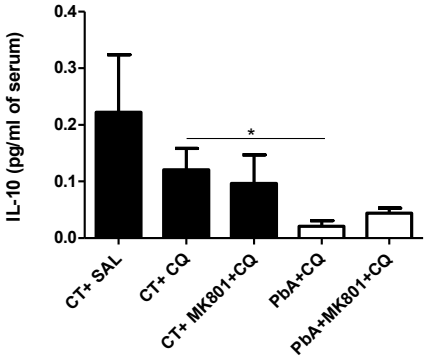

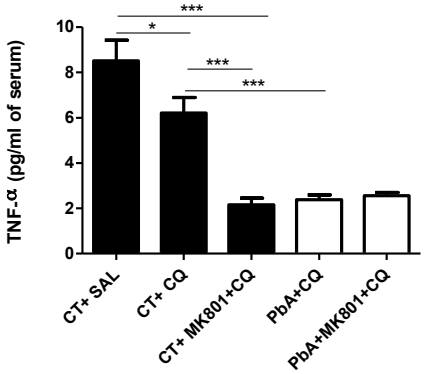

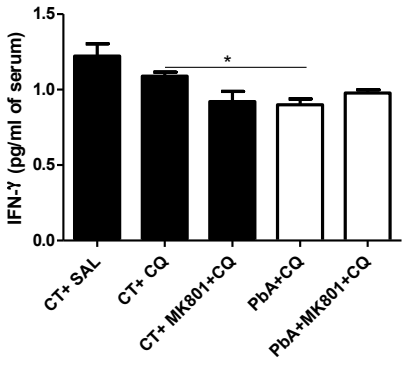

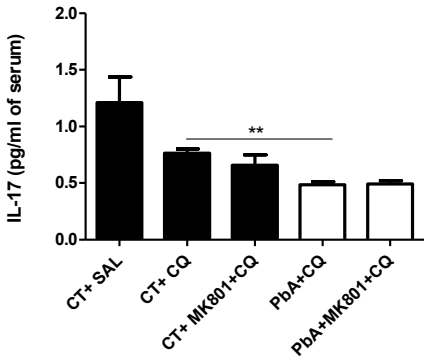

Fig. supplementary 4: MK801 effect in inflammatory response in the serum of CM mice following *Plasmodium berghei* ANKA (PbA) infection resolution by chloroquine (CQ) therapy. C57BL/6 mice were intraperitoneally infected with 106 parasitized erythrocytes and then separated in two groups. On day 3 post-infection a group initiated the MK801 (i.p. 0.5 mg/kg) treatment which was continued until the end of CQ therapy. All mice were treated orally with a ten-day course CQ (30 mg/kg) from day 5 post-infection. As controls, non-infected animals received the same volume of saline, CQ or CQ+MK801. Ten days after cessation of CQ therapy serum was collected and IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α levels were assessed by Cytometric Bead Array (CBA). Results are expressed as mean ± SEM and are representative of at least two independent experiments (n= 5 per group). Asterisk(s) indicate statistical differences where *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$.

7 – DISCUSSÃO

O presente estudo forneceu evidências de que há alterações na interação entre citocinas e fatores neurotróficos expressos no hipocampo e no córtex frontal que contribuem significativamente para o desenvolvimento dos transtornos cognitivos durante a fase aguda da MC e para a permanência dos mesmos após a resolução da infecção. Durante a fase aguda da MC, esses mediadores inflamatórios regulam fenômenos relacionados à cognição, como a neurogênese e a morte celular no hipocampo.

Uma única dose do antimalárico artesunato foi capaz de modular a resposta inflamatória no SNC, melhorando a sobrevida e os sinais clínicos típicos da MC. Os déficits de memória e sintomas de depressão que permaneceram após a eliminação da infecção pela administração da cloroquina foram completamente revertidos pelo tratamento com o antagonista do receptor NMDA, MK801. Este foi capaz de modular a resposta inflamatória TH1/TH2 e induzir a produção de fatores neurotróficos no hipocampo e no córtex frontal, conferindo assim um efeito neuroprotetor. Esses achados indicam que o sistema imune permanece ativado no SNC após a resolução da infecção. O presente estudo fornece, então, evidências de que interações entre os sistemas imune e nervoso contribuem para a ocorrência das sequelas cognitivas e comportamentais. Além disso, intervenções direcionadas à modulação do sistema glutamatérgico podem constituir potenciais adjuvantes terapêuticos ao tratamento antimalárico.

Durante o curso deste trabalho, um estudo conduzido por Dai et al., (Dai *et al.*, 2012) demonstrou que o tratamento com lítio associado ao antimalárico cloroquina preveniu a ocorrência de sequelas cognitivas após a eliminação do parasita. Apesar de o mecanismo de ação do lítio permanecer desconhecido, o seu efeito neuroprotetor parece

estar associado à regulação em neurônios da via de sinalização Akt/GSK3β relacionada a funções como metabolismo e sobrevida celular. Corroborando a hipótese proposta inicialmente pelo nosso estudo, a redução da excitotoxicidade mediada pelo glutamato, favorecendo a sobrevida neuronal, estaria dentre os efeitos protetores do lítio ao inibir a GSK3β (Dai *et al.*, 2012). Associada ao antimalárico cloroquina, a Lovastatina, um fármaco da classe das estatinas, geralmente utilizado no tratamento da hipercolesterolemia e que apresenta ação anti-inflamatória, foi capaz de reduzir a concentração de citocinas inflamatórias no SNC e de prevenir a persistência de alterações cognitivas após a MC. Esse resultado indicaria que o controle da resposta inflamatória no SNC poderia constituir estratégia terapêutica promissora na prevenção das sequelas cognitivas decorrentes da MC. Entretanto, nesse mesmo estudo, o uso do anti-inflamatório diclofenaco de sódio associado ao antimalárico cloroquina aumentou a mortalidade dos animais infectados com a cepa PbA, sugerindo que compostos com ação anti-inflamatória podem modular o sistema imune de formas distintas, promovendo efeitos que podem ser benéficos ou deletérios no curso da doença (Reis *et al.*, 2012).

Existem evidências de que o MK801, além de regular os efeitos excitotóxicos mediados pelo glutamato, possui propriedades anti-inflamatórias (Dobbertin *et al.*, 2000; Esposito *et al.*, 2011) com efeitos benéficos em modelos experimentais de lesão da medula espinhal, isquemia cerebral e sepse (Bakiri *et al.*, 2008; Cassol-Jr *et al.*, 2011; Esposito *et al.*, 2011). Assim, os antagonistas do receptor NMDA, como o MK801, podem constituir potenciais terapias adjuvantes ao tratamento antimalárico.

Entretanto, o presente trabalho permanece em um âmbito mais descritivo. As vias de sinalização imune envolvidas no desenvolvimento das sequelas cognitivas e comportamentais, bem como a modulação das mesmas pelo tratamento antimalárico

combinado ou não com os antagonistas de receptores de glutamato devem ser sistematicamente investigadas. Apesar do efeito anti-inflamatório observado após a administração de uma única dose de artesunato, a eficácia desse tratamento em prevenir o desenvolvimento dos déficits cognitivos durante a fase aguda da doença não foi avaliada. O papel do sistema glutamatérgico, incluindo a expressão de seus receptores e alterações nas vias de síntese e recaptação durante a infecção e após sua resolução não foi estabelecido, o que facilitaria a compreensão dos fatores envolvidos na proteção conferida pelo MK801. As vias pelas quais o sistema imune modula a ação do glutamato não foram investigadas.

A inibição da excitotoxicidade mediada pelo glutamato, por meio de antagonistas glutamatérgicos, contitui estratégia promissora como terapia adjuvante ao tratamento antimalárico. Dessa forma, se faz necessária à compreensão dos mecanismos relacionados à neuroproteção conferida por esses compostos durante a fase aguda bem como após a resolução da MC. Diante do exposto, como perspectivas, pretendemos investigar o papel dos receptores de glutamato ionotrópicos e metabotrópicos e do transportador glutamatérgico na patogênese dos transtornos cognitivos e comportamentais durante a fase aguda e após a resolução da infecção, com intuito de melhor compreender o envolvimento dos componentes associados à via glutamatérgica no curso da doença. Devido ao potencial papel da neurogênese e da morte neuronal hipocampal no desenvolvimento de déficits cognitivos, o efeito dos antagonistas de receptores de glutamato na regulação desses fenômenos será avaliado. A resposta de células da glia e neurônios após o tratamento antimalárico combinado com antagonistas de receptores glutamatérgicos será analisada para determinar a possível fonte de produção de mediadores inflamatórios mesmo após a resolução da infecção. Visando uma abordagem translacional, a eficácia da memantina em prevenir os transtornos

cognitivos e comportamentais durante a fase aguda bem como após a resolução do quadro infeccioso será também investigada. É fundamental ressaltar que a memantina é um inibidor não competitivo dos receptores de glutamato do tipo NMDA que apresenta segurança e eficácia clínica já estabelcida no tratamento dos danos cognitivos relacionados à doença de Alzheimer, o que poderia facilitar a tradução dos seus efeitos para estudos clínicos.

8 – CONCLUSÕES

- Déficits cognitivos durante a fase aguda da MC estão associados a desequilíbrio na produção de citocinas inflamatórias e fatores neurotróficos no córtex frontal e no hipocampo, assim como alterações na neurogênese e morte neuronal no hipocampo.

- A administração de uma única dose do antimalárico artesunato apresentou efeito imunomodulador no SNC durante a fase aguda da infecção.

- Associado ao antimalárico cloroquina, o antagonista do receptor de glutamato do tipo NMDA, o MK801, preveniu as sequelas cognitivas e comportamentais resultantes da MC.

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ANEXOS

ANEXO 1 - Pesquisa realizada durante o doutorado sanduíche

O doutorado sanduíche foi realizado, durante o período de Julho de 2013 a Agosto de 2014, na *Cleveland Clinic*, localizada no estado de OHIO nos Estados Unidos. A opção por realizar o doutorado sanduíche na *Cleveland Clinic*, sob a supervisão do professor Richard M Ransohoff se justificou pela excelência desse pesquisador no estudo das interfaces entre o sistema nervoso central e o sistema imune em diferentes condições patológicas. O grupo do professor Ransohoff tem trazido contribuições valiosas no campo da neuroimunologia em especial relacionadas ao desenvolvimento de técnicas inovadoras que permitem a análise detalhada da estrutura e função das células que compõem o sistema imune e o sistema nervoso central permitindo investigar as interações complexas que ocorrem entre esses sistemas. Acredito que a alta qualidade do conhecimento teórico e técnico apresentado pelo grupo do professor Ransohoff foi de extrema importância para a minha formação acadêmica e para o andamento do projeto de doutorado além de contribuir para a excelência das pesquisas desenvolvidas pelo nosso grupo no Brasil. A realização do doutorado sanduíche no laboratório do professor Richard M Ransohoff na *Cleveland Clinic,* possibilitou o aprendizado do modelo experimental de traumatismo craniano (TCE/TBI) bem como a utilização de camundongos geneticamente modificados, aplicação de técnicas de Microscopia Confocal com aquisição de imagens em 3D, citometria de fluxo e *sorting* para separação de células residentes no SNC (micróglia) e células imunes provenientes da periferia (monócitos) e análise da expressão gênica (*microarray gene expression analysis by Nanostring technology*) dessas células em condições de TCE/TBI e tratamento com o imunomodulador *Laquinimod*. Os resultados obtidos nesse modelo encontram-se descritos nas seções a seguir.

1.1 - Introdução e Justificativa: Traumatismo Cranioencefálico

O traumatismo cranioencefálico (TCE), do inglês *traumatic brain injury* (TBI), é definido como uma lesão cerebral aguda resultante da aplicação de uma força mecânica externa sobre o crânio. Dentre as principais causas pode-se citar uma rápida aceleração e desaceleração cranial na qual o encéfalo e a caixa craniana, por possuírem densidades e amplitudes de movimento diferentes, quando submetidos às mesmas forças inerciais respondem de forma desigual levando a uma lesão axonal difusa e a ruptura de vasos sanguíneos cerebrais. As lesões focais são decorrentes de forças de contato diretas que apresentam como principais causas acidentes automobilísticos, penetração ou impacto por projeteis bem como explosões (Maas *et al.*, 2008). Estima-se que ocorram anualmente em todo o mundo 10 milhões de mortes e/ou hospitalizações associadas diretamente a essa condição e que aproximadamente 57 milhões de pessoas já tenham sofrido algum tipo de trauma craniano. O TCE/TBI tem sido descrito como a principal causa de mortalidade e incapacidade entre indivíduos até 45 anos de idade acarretando em elevados custos aos sistemas de saúde. Além disso, importantes alterações cognitivas e comportamentais têm sido atribuídas a essa condição. Apenas nos Estados Unidos, estima-se que aproximadamente 10% dos indivíduos que sobrevivem a um TCE/TBI permanecem com sequelas cognitivas, neurológicas e comportamentais (Langlois *et al.*, 2006; Masel and DeWitt, 2010).

A fisiopatologia do TCE/TBI é complexa e os mecanismos celulares e moleculares envolvidos em sua patogênese não estão completamente elucidados. As alterações estruturais e funcionais decorrentes do TCE/TBI estão associadas a lesões denominadas como primárias e secundárias (Davis, 2000; Masel and DeWitt, 2010). A lesão primária é o resultado direto das forças mecânicas transmitidas ao crânio durante o impacto inicial e é caracterizada por contusões, hematomas (epidural, subdural,

intraparenquimatoso), hemorragias subaracnoideia e lesão axonal difusa. O tipo de lesão primária decorrente de um TCE/TBI depende da natureza e magnitude da força física aplicada, da sua duração e do local de aplicação (Davis, 2000; Gaetz, 2004). A lesão secundária pode ocorrer minutos ou mesmo meses após a lesão primária e está associada a alterações bioquímicas, metabólicas, celulares e moleculares que incluem excitotoxicidade, stresse oxidativo, neuroinflamação e edema com consequente neurodegeneração e atrofia (Bramlett and Dietrich, 2007).

O sistema imune parece desempenhar um importante papel na patogênese do TCE/TBI, principalmente em relação à gravidade e permanência dos sintomas associados a essa condição. O TCE/TBI induz uma resposta inflamatória complexa caracterizada pela rápida proliferação e migração de células do sistema imune residentes no CNS (micróglia) e periféricas (macrófagos circulantes) para o sítio de lesão com consequente produção de mediadores inflamatórios como citocinas e quimiocinas (Davalos *et al.*, 2005; Turtzo *et al.*, 2014). Estudos clínicos e experimentais têm demonstrado que micróglias permanecem em estado de ativação durante semanas ou até anos após o TCE/TBI, o que tem sido associado ao aumento da expressão de citocinas inflamatórias como IL-1β e TNF-α e a permanência de disfunções cognitivas (Johnson *et al.*, 2013; Loane *et al.*, 2014; Ramlackhansingh *et al.*, 2011; Smith *et al.*, 2013). Dessa forma, o estudo das alterações associadas ao TCE/TBI não deve ser limitado apenas à fase aguda uma vez que os processos bioquímicos e inflamatórios crônicos parecem influenciar o desenvolvimento dos distúrbios cognitivos e comportamentais a longo prazo (Loane *et al.*, 2014).

Apesar de as lesões secundárias ao TCE/TBI serem potencialmente reversíveis e tratáveis, atualmente não existem tratamentos eficazes disponíveis (Loane and Faden, 2010). Nesse sentido, estudos pré-clínicos e clínicos que investiguem sistematicamente

a eficácia das terapias neuroprotetoras e/ou imunomoduladoras em melhorar o prognóstico do TCE são urgentemente recomendados.

O agente imunomodulador *Laquinimod* tem demonstrado significativa eficácia terapêutica em estudos clínicos de esclerose múltipla em fase dois e três, sem relatos de imunossupressão ou toxicidade (Comi *et al.*, 2010; Comi *et al.*, 2008). Embora o mecanismo de ação do *Laquinimod* não esteja completamente elucidado, evidências provenientes de estudos conduzidos com o modelo experimental de esclerose múltipla (EAE), têm demonstrado que além de reduzir a desmielinização e a perda axonal, o composto foi capaz de inibir vias de sinalização inflamatórias como o NF-κB, reduzir os níveis de citocinas inflamatórias, aumentar a expressão de fatores neurotróficos (BDNF) bem como prevenir o infiltrado de células imune (macrófagos e células T) no SNC (Thone *et al.*, 2012; Wegner *et al.*, 2010; Yang *et al.*, 2004). Em estudo recente, o tratamento com *Laquinimod* no mesmo modelo experimental de esclerose múltipla, induziu uma subpopulação de células mielóides com perfil anti-inflamatório, caracterizado pela redução da expressão de citocinas inflamatórias (IL-6, IL-12/23 e TNF-α) e aumento dos níveis de IL-10 (Schulze-Topphoff *et al.*, 2012). Além disso, diminuição na expressão de via inflamatórias, como o NF-κB, tem sido também demonstrada em cultura de células mononucleares do sangue periférico (PBMC) de pacientes com esclerose múltipla, tratadas com o *Laquinimod* (Gurevich *et al.*, 2010). Nesse contexto, o *Laquinimod* pode constituir uma potencial estratégia terapêutica para o tratamento de outras condições patológicas que afetam o SNC e que estão associadas a alterações de componentes do sistema imune, como o TCE/TBI.

Diante do exposto, no presente estudo tivemos como objetivos investigar: 1) disfunções cognitivas e comportamentais associadas ao TCE/TBI; 2) potencial contribuição de células imunes residentes no SNC (micróglia) e periféricas (monócitos)

na patogênese do TCE durante a fase aguda (3 dias) e crônica (120 dias); 3) possíveis alterações no fenótipo dessas células induzidas pelo TCE/TBI, com e sem administração do *Laquinimod.* A nossa hipótese principal é de que células imunes inatas residentes no SNC e na periferia apresentam fenótipos distintos em resposta ao TCE/TBI que podem ser modulados pelo tratamento com o *Laquinimod* e influenciar o desfecho clínico associado a essa condição. Para testar essa hipótese animais geneticamente modificados (*red-green*) nos quais o receptor CX3CR1 (*Fractalkine)* essencialmente expresso em micróglia foi marcado com GFP (*green fluorescente protein*) e o receptor CCR2 expresso em monócitos foi marcado com RFP (*red fluorescente protein*) foram utilizados, permitindo a separação e coleta dessas células por meio das técnicas de citometria de fluxo e *sorting* para posterior análise do perfil gênico por meio de *microarray gene expression analysis by Nanostring technology*. As técnicas de imunohistoquímica e imunofluorescência também foram utilizadas para caracterização morfológica dessas células, quantificação em diferentes regiões cerebrais, análise da ativação microglial e neurogênese. Testes comportamentais como o *rota-rod* e o labirinto em Y foram utilizados para análise de parâmetros motores e cognitivos respectivamente, durante a fase aguda e crônica do TCE/TBI. O modelo de percussão fluídica unilateral foi utilizado para indução do TCE/TBI, 24 horas após realização da craniotomia na região do hemisfério cortical direito. Os animais considerados como SHAM foram àqueles submetidos apenas a craniotomia. O *laquinimod* foi administrado diariamente por gavagem, na dose de 25mg/kg (100µl/animal). Os animais controle receberam a mesma quantidade de veículo, no caso, água do tipo Mili-q (Water).

1.2- Resultados

Inicialmente, o modelo de TCE/TBI por percussão fluídica lateral foi realizado para induzir o trauma craniano nos animais geneticamente modificados (*red-green*). A técnica de citometria de fluxo foi realizada para análise do número de micróglia ($CD45^{Low}$ $CX3CR1^{High}$) e monócitos infiltrantes ($CD45^{high}$ $CX3CR1^{neg\text{-}lo}$ $CCR2^{+}$) nos dias 1, 3 e 5 após a indução do TCE/TBI. O *Dot-plot* representativo da análise realizada no FlowJo 7.6.5 para distinguir as células supracitadas encontra-se demonstrado na figura 1. Aumento significativo do número de micróglia e monócitos foi encontrado no terceiro dia (3dpi) após a indução do TCE/TBI quando comparado ao primeiro (1dpi) e quinto (5dpi) dias após a lesão e aos animais SHAM (submetidos apenas a craniotomia) em todos os períodos avaliados (figura 2). Baseado no resultado apresentado na figura 2, o 3dpi foi então determinado para as análises posteriores, por representar o período de maior reação microglial e infiltrado de monócitos após o TCE/TBI. Aumento do infiltrado de monócitos (células vermelhas-$CCR2^{+}$ *RFP*) foi também observado, por meio de imunofluorescência, no córtex cerebral ipsilateral (região da lesão) no terceiro dia (3dpi) após a indução do TCE/TBI (figura 3). Em relação à análise da expressão gênica (*Nanostring technology*) o TCE/TBI induziu o aumento significativo de 116 genes (tabela 1) e a redução de 40 genes (tabela 2) expressos por micróglia isoladas dos camundongos *red-green* submetidos ao TCE/TBI, quando comparados aos camundongos que não foram submetidos a esse procedimento (*Naive*). Os resultados preliminares sugerem que genes relacionados à manutenção funcional da micróglia (Atf3, Egr1, Jun, Junb) apresentaram redução significativa após a indução do TCE/TBI (tabela 2).

Após a análise do perfil inflamatório durante a fase aguda do TCE/TBI, foi então iniciado o tratamento com o agente imunomodulador *Laquinimod*. Os camundongos *red-green* foram tratados durante sete dias antes da indução do TCE/TBI e três dias após, totalizando dez dias de tratamento com o *Laquinimod* (25mg/kg/dia). A análise de expressão gênica em micróglia e monócitos, imunohistoquimica e imunofluorescência foram realizadas no terceiro dia (3dpi) após a indução do TCE/TBI. A densidade microglial foi analisada por meio de microscopia de fluorescência no córtex cerebral (região da lesão) e regiões CA1, CA3 e giro denteado no hipocampo (figura 4). Não foi encontrada diferença significativa entre os grupos tratados com o *Laquinimod* (LAQ) e tratados com o veículo (*Water*). Entretanto, foi observada uma tendência de aumento da densidade microglial na região ipsilateral do giro denteado dos animais tratados com *Laquinimod* (LAQ) em comparação com a região contralateral, o que será confirmado posteriormente com o aumento do número de animais analisados (figura 5). A ativação microglial no córtex cerebral (região da lesão- figura 6) e no hipocampo (giro denteado- figura 7) ipsilateral de camundongos *red-green* submetidos ao TCE/TBI tratados com *Laquinimod* (LAQ) ou com o veículo (*Water*) foi analisada pela co-localização do marcador TSPO (CY5) com micróglia (GFP) por meio de microscopia Confocal. Embora as imagens estejam em processo de quantificação e o número de animais em cada grupo será aumentado em novo experimento, os resultados preliminares indicam que o tratamento com o *Laquinimod* foi capaz de induzir aumento da expressão de TSPO por micróglia (figura 6-7). A formação de novos neurônios foi avaliada por meio de imunohistoquimica. O aumento de células com expressão positiva para a marcação com doublecortina no hipocampo (giro denteado) é indicativo de aumento da neurogênese. Embora as imagens estejam em processo de quantificação e o número de animais em cada grupo será aumentado em novo experimento, os resultados

preliminares sugerem um aumento na neurogênese no hipocampo ipsilateral de camundongos submetidos ao TCE/TBI tratados com *Laquinimod* comparado com aqueles tratados com o veículo (*Water*- figura 8). Em relação à análise da expressão gênica (*Nanostring technology*) o tratamento com o *Laquinimod* (25mg/kg/dia) sete dias antes e três dias após o TCE/TBI induziu o aumento significativo de 15 genes (tabela 3) e a redução de 43 genes (tabela 4) expressos por micróglia isoladas dos camundongos *red-green* submetidos ao TCE/TBI que receberam o tratamento com o *Laquinimod*, quando comparado aos camundongos tratados com o veículo, no caso água do tipo Mili-q. Os resultados preliminares sugerem que o tratamento com o *Laquinimod* preveniu a supressão de genes associados à manutenção funcional da micróglia (Atf3, Jun, Egr1, Fos, Chi3l3, Adamts1) observada após a indução do TCE/TBI (tabela 3).

O efeito do tratamento com o *Laquinimod* no modelo de TCE/TBI por percussão fluídica lateral foi também avaliado na fase crônica dessa condição (120 dias após o TCE/TBI). Neste desenho experimental, os camundongos *red-green* foram tratados com o *Laquinimod* ou com o veículo durante sete dias antes e sete dias após a indução do TCE/TBI, totalizando quatorze dias de tratamento. A coordenação motora dos animais foi analisada por meio do teste de *rota rod*, antes do inicio do tratamento (*baseline*), três dias após o inicio do tratamento, e nos dias 1, 3, 6, 30, 60 e 90 após a indução do TCE/TBI. Não foi observada diferença significativa na coordenação motora dos animais tratados com *Laquinimod* comparado com SHAM ou com aqueles tratados com o veículo submetidos ao TCE/TBI (figura 9A). O teste do labirinto em Y foi realizado antes do inicio do tratamento (*baseline*) e 30, 60 e 90 dias após a indução do TCE/TBI. Redução significativa no total de entradas nos braços do labirinto em Y foi observada em todos os grupos nos dias 30, 60 e 90 após o TCE/TBI quando comparado com o *baseline*, indicando que os animais apresentaram uma possível adaptação ao labirinto.

Não foi encontrada diferença significativa entre os grupos na porcentagem de alternância de entradas nos braços do labirinto durante os períodos avaliados, indicando que não houve déficit hipocampal significativo associado ao TCE/TBI (figura 9B,C). A coloração por Cresyl violeta demonstrou um aumento significativo no volume do ventrículo lateral dos camundos submetidos ao TCE/TBI e tratados com o veículo quando comparado àqueles tratados com o *Laquinimod* (figura 10 A,B). Entretanto, não houve diferença significativa no volume hipocampal 120 dias após a indução do TCE/TBI (figura 10 A,C). A ativação microglial no córtex cerebral (região da lesão- figura 11) e no hipocampo (giro denteado- figura 12) ipsilateral de camundongos *red-green* submetidos ao TCE/TBI tratados com Laquinimod (LAQ) ou com o veículo (Water) foi analisada pela co-localização do marcador TSPO (CY5) com micróglia (GFP) por meio de microscopia Confocal, 120 dias após a indução do TCE/TBI por percussão fluídica unilateral. Embora as imagens estejam em processo de quantificação e o número de animais em cada grupo será aumentado em novo experimento, os resultados preliminares sugerem que não há diferença significativa na expressão de TSPO em micróglias, entre os grupos nas regiões e no período (120 dias após TCE/TBI) avaliados (figuras 11-12).

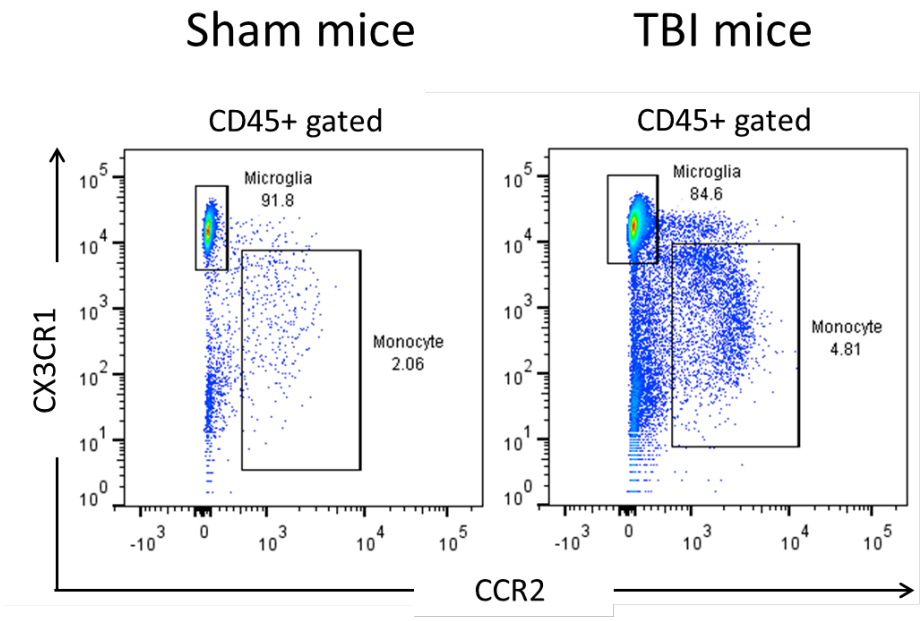

Figura 1: Exemplo ilustrativo de análise realizada no FlowJo 7.6.5. Dot-plot demonstrando a seleção dos tipos celulares de acordo com a expressão de CD45, CX3CR1, CCR2, no qual micróglia foi determinada por $CD45^{Low}$ $CX3CR1^{High}$ e monócitos infiltrantes por $CD45^{high}$ $CX3CR1^{neg\text{-}lo}$ $CCR2^{+}$ nos camundongos *red-green* SHAM (submetidos somente a craniotomia) e TBI-TCE (submetidos a craniotomia e ao trauma craniano por percussão fluídica).

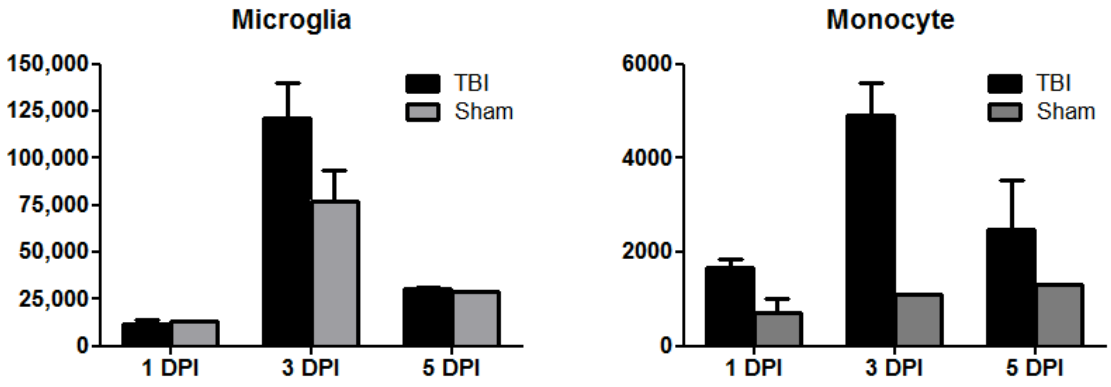

Figura 2: Número de micróglia e monócitos quantificados no tecido cerebral de animais submetidos ao TCE/TBI por percussão fluídica unilateral e camundongos SHAM (submetidos apenas a craniotomia), nos dias 1, 3 e 5 após o trauma craniano. Aproximadamente seis camundongos modificados geneticamente (*red-green)* foram utilizados por grupo e três experimentos distintos foram realizados.

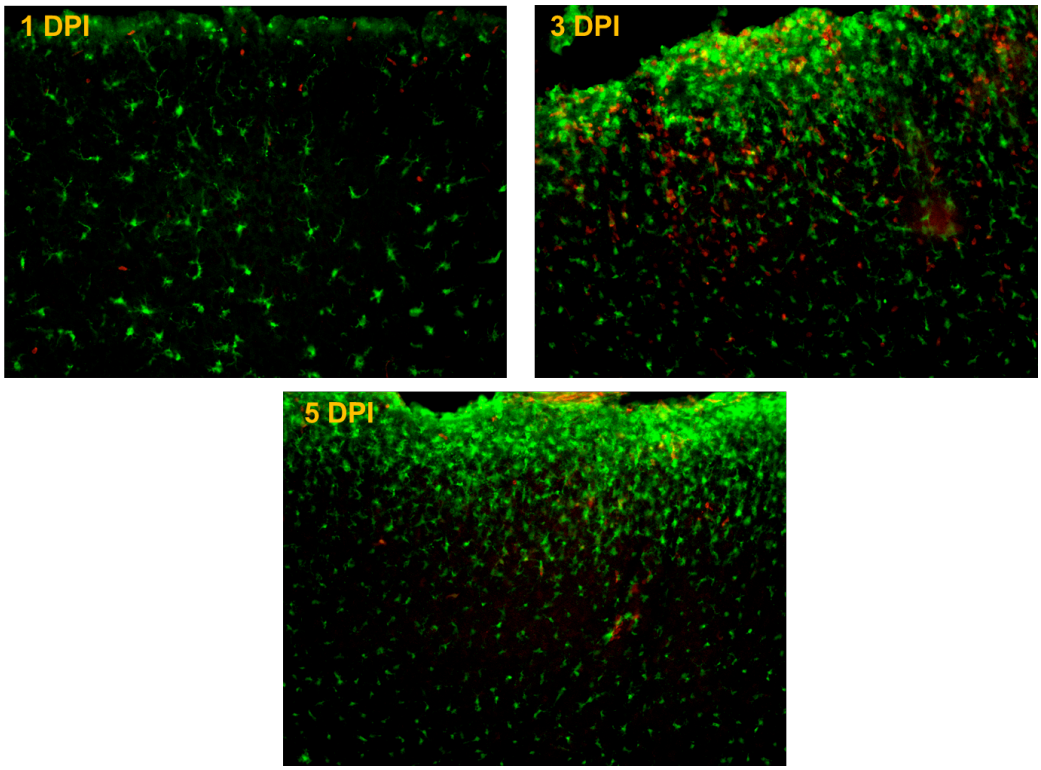

Figura 3: Representação por imunofluorescência de micróglia com expressão do receptor CX3CR1 marcado com GFP (verde) e do infiltrado de monócitos com expressão do receptor CCR2 marcado com RFP (vermelho), 1, 3 e 5 dias após TCE/TBI por percussão fluídica unilateral. Aproximadamente três camundongos modificados geneticamente (*red-green*) foram utilizados por grupo e três experimentos distintos foram realizados. Magnitude 10x

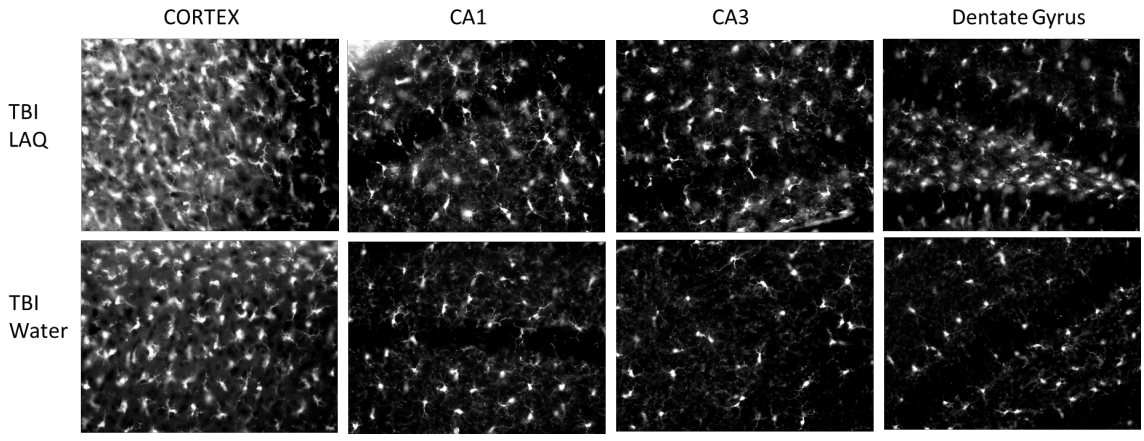

Figura 4: Representação da densidade microglial analisada por meio de microscopia de fluorescência no córtex cerebral (região da lesão) e regiões CA1, CA3 e giro denteado no hipocampo ipsilateral de camundongos *red-green* tratados com *Laquinimod* (LAQ- n=3) ou veículo (*Water*- n=3) no terceiro dia após a TCE/TBI por percussão fluídica unilateral. Magnitude 20x.

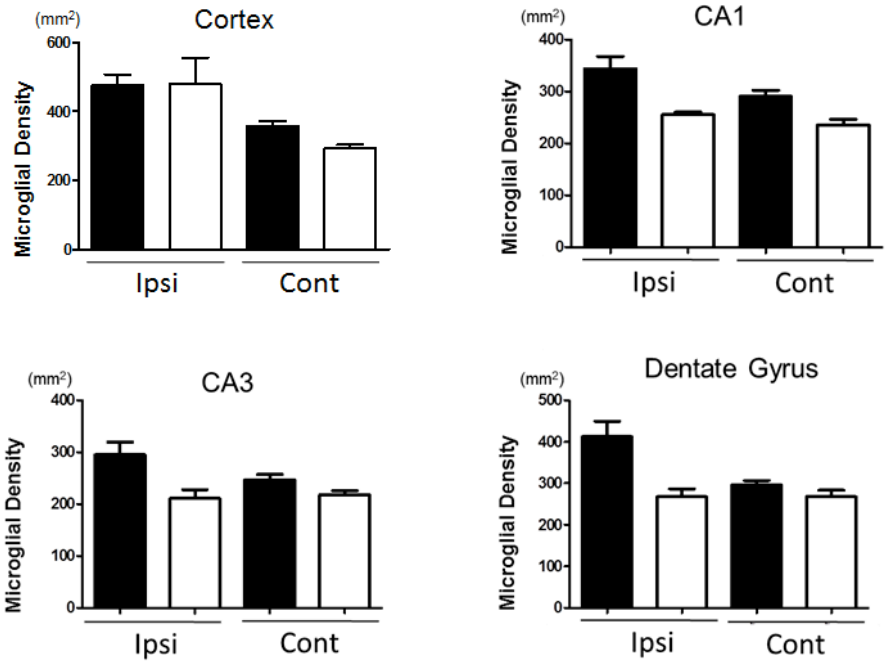

Figura 5: Análise da densidade microglial avaliada por meio de microscopia de fluorescência no córtex cerebral (região da lesão) e regiões CA1, CA3 e giro denteado no hipocampo ipsilateral e contralateral de camundongos *red-green* tratados com Laquinimod (Barras negras-LAQ- n=3) ou veículo (Barras brancas-Water- n=3) no terceiro dia após a TCE/TBI por percussão fluídica unilateral.

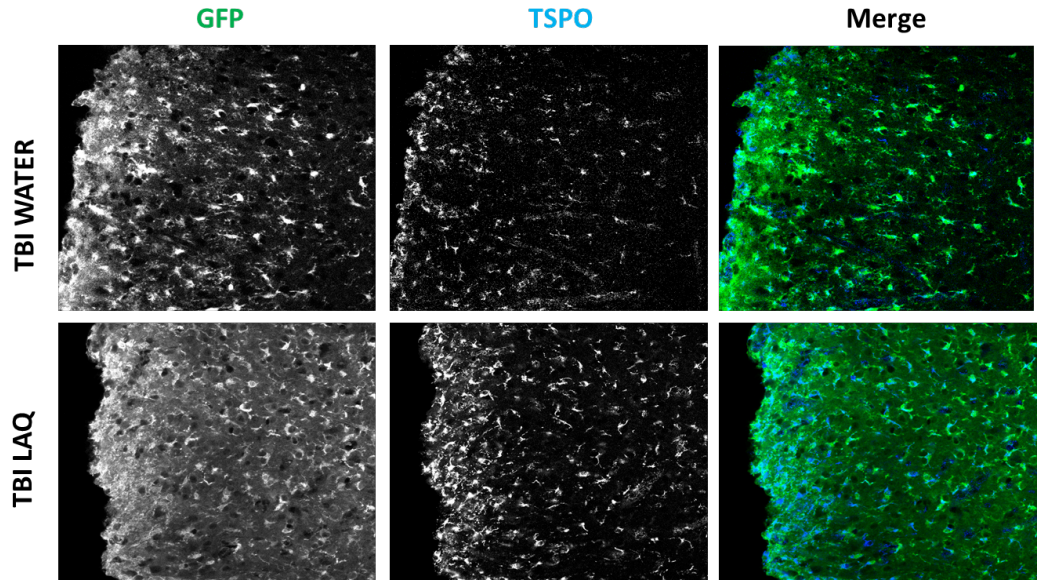

Figura 6: Representação da ativação microglial (expressão de TSPO) avaliada por meio de microscopia confocal no córtex cerebral ipsilateral (região da lesão) de camundongos *red-green* tratados com *Laquinimod* (LAQ- n=3) ou veículo (Water- n=3) no terceiro

dia após a indução do TCE/TBI por percussão fluídica unilateral. GFP (*Green Fluorescent Protein*), TSPO (Proteína translocadora 18-kDa). Magnitude 40X.

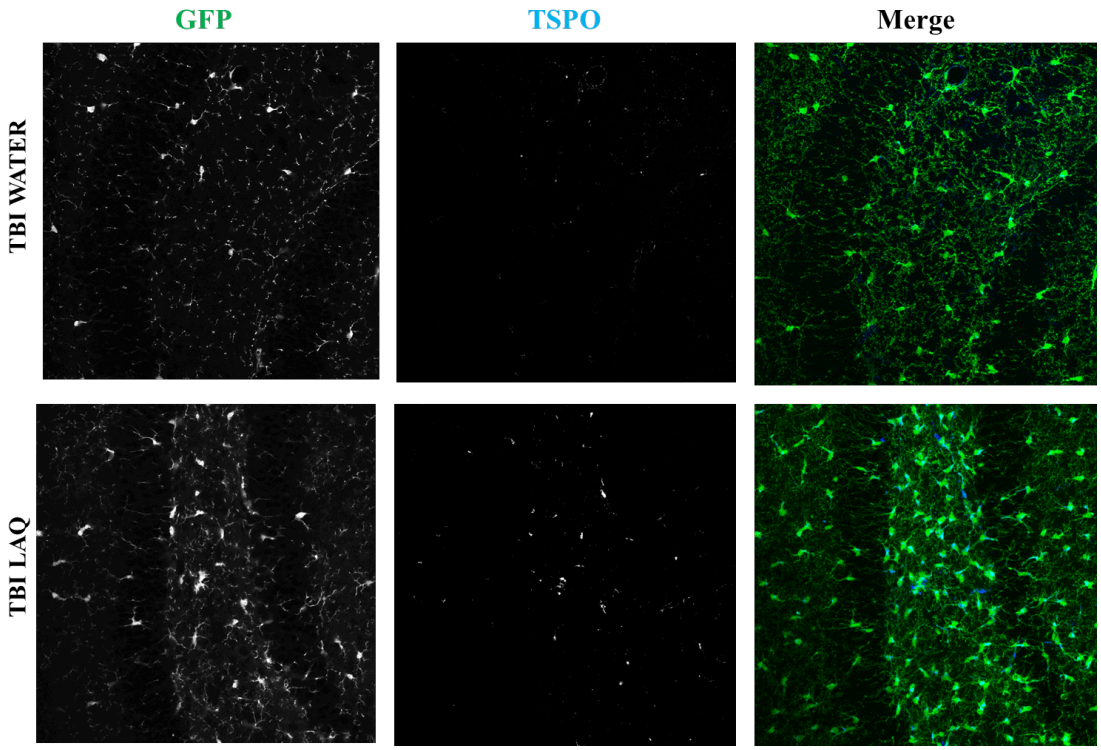

Figura 7: Representação da ativação microglial (expressão de TSPO) avaliada por meio de microscopia confocal no hipocampo ipsilateral (giro denteado) de camundongos *red-green* tratados com *Laquinimod* (LAQ- n=3) ou veículo (Water- n=3) no terceiro dia após a indução do TCE/TBI por percussão fluídica unilateral. GFP (*Green Fluorescent Protein*), TSPO (Proteína translocadora 18-kDa). Magnitude 40X.

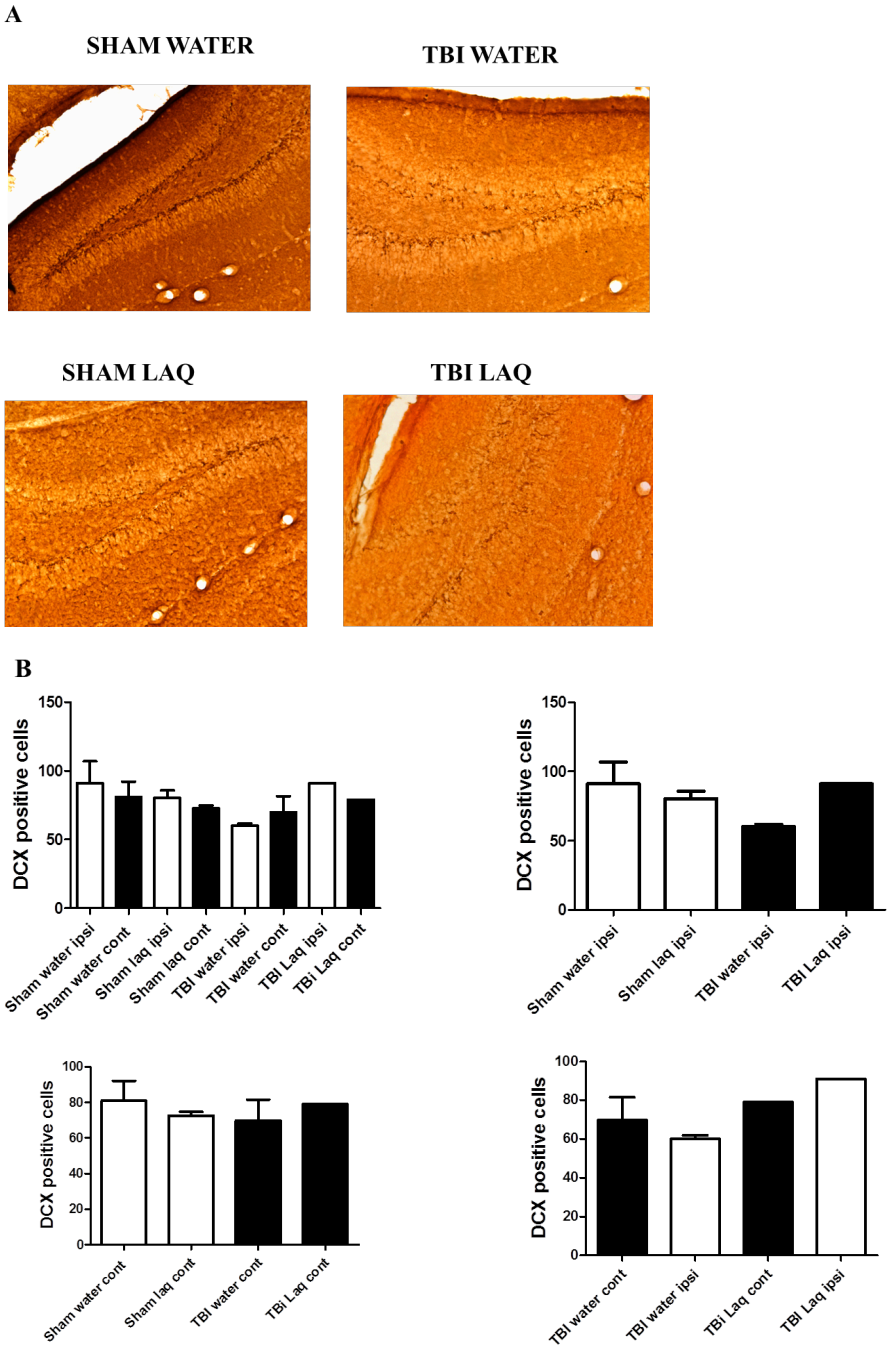

Figura 8: Representação (A) e quantificação (B) de neurônios positivos para a marcação com Doublecortina no hipocampo (giro denteado) de camundongos *red-green* tratados com *Laquinimod* (LAQ- n=3) ou veículo (Water- n=3) SHAM e no terceiro dia após a TCE/TBI por percussão fluídica unilateral.

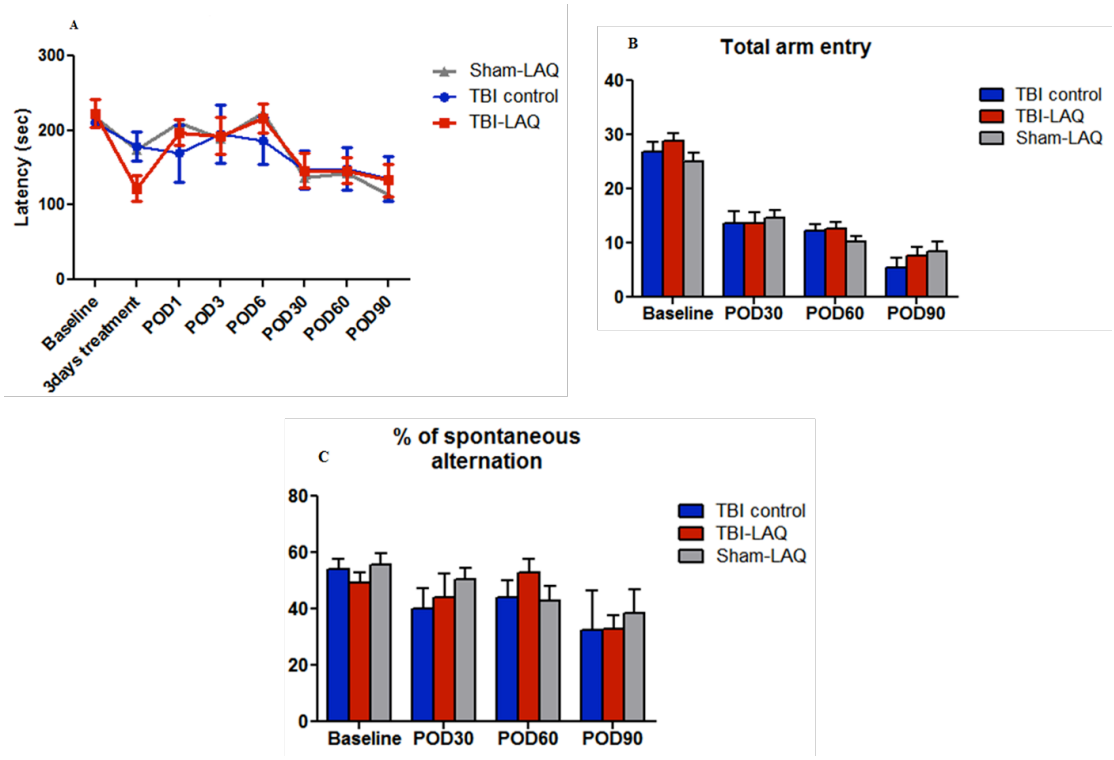

Figura 9: Análise da coordenação motora mensurada pela latência em segundos (s) no *Rota Rod* (A) e memória no labirinto em Y indicada pela entrada total (B) e porcentagem de alternância nos braços do labirinto (C). Aproximadamente nove camundongos *red-green* foram utilizados por grupo, ou seja, submetidos ao TCE/TBI e tratados com *Laquinimod* (TBI LAQ) ou veículo (TBI *Water*), ou apenas submetido a craniotomia e tratados com *Laquinimod* (SHAM-LAQ). *Baseline:* dia anterior ao inicio do tratamento; POD: dias após o tratamento.

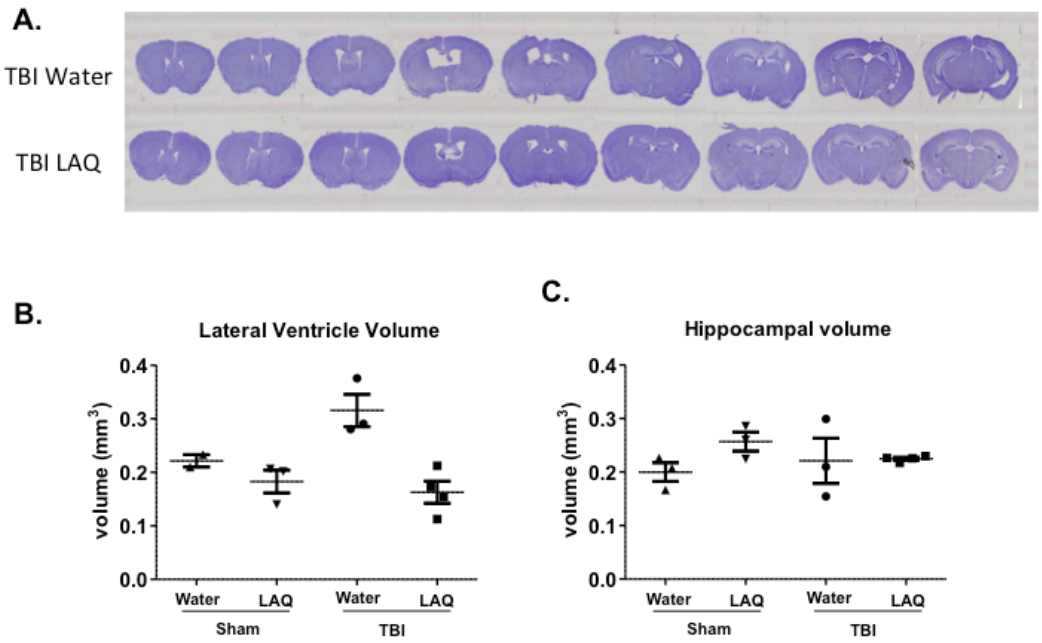

Figura 10: Representação e análise do volume do ventrículo lateral e da região do hipocampo de camundongos *red-green* tratados com *Laquinimod* (LAQ- n=3) ou veículo (Water- n=3), por meio da coloração de *Cresyl violeta*, 120 dias após a indução do TCE/TBI por meio de percussão fluídica unilateral.

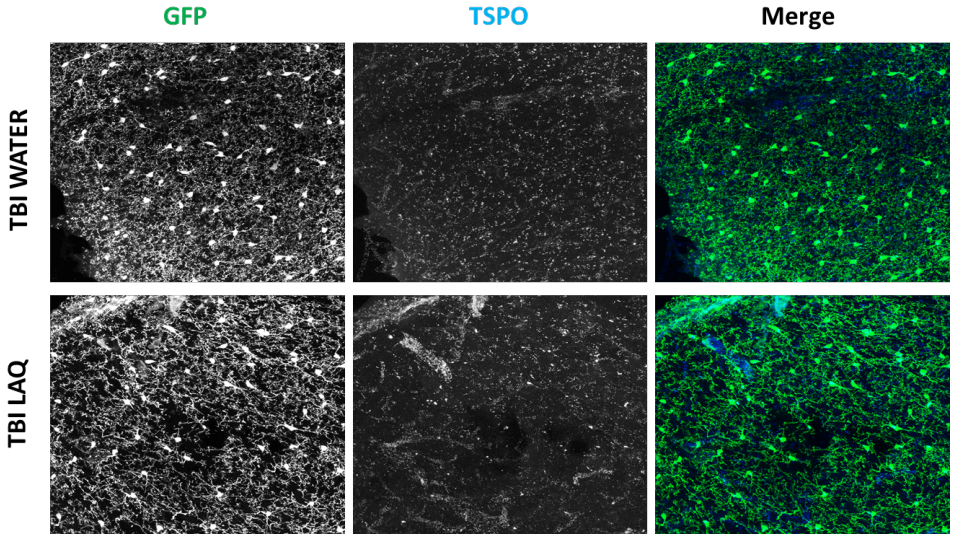

Figura 11: Representação da ativação microglial (expressão de TSPO) avaliada por meio de microscopia confocal no córtex cerebral ipsilateral (região da lesão) de camundongos *red-green* tratados com *Laquinimod* (LAQ- n=3) ou veículo (*Water*- n=3), 120 dias após indução do TCE/TBI por percussão fluídica unilateral. GFP (Green Fluorescent Protein), TSPO (Proteína translocadora 18-kDa). Magnitude 40X.

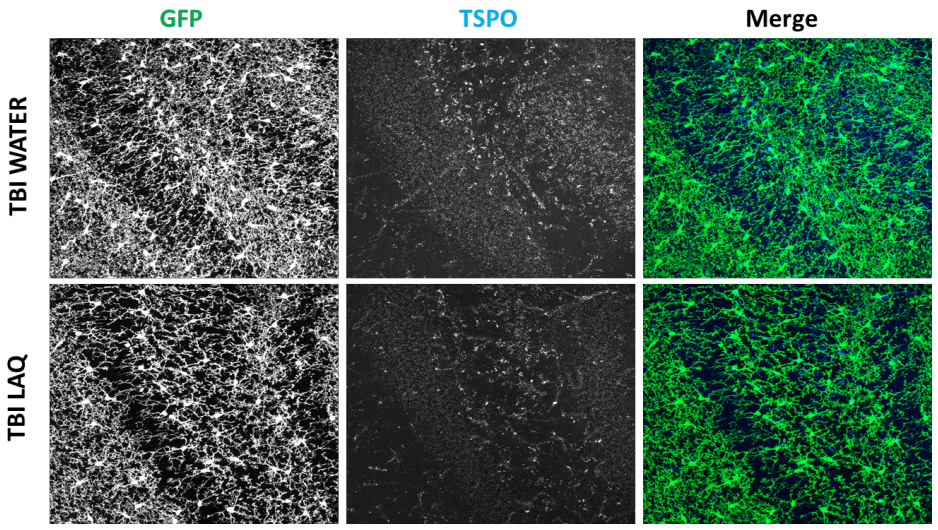

Figura 12: Representação da ativação microglial (expressão de TSPO) avaliada por meio de microscopia confocal no hipocampo ipsilateral (giro denteado) de camundongos *red-green* tratados com *Laquinimod* (LAQ- n=3) ou veículo (*Water*- n=3), 120 dias após a indução do TCE/TBI por percussão fluídica unilateral. GFP (Green Fluorescent Protein), TSPO (Proteína translocadora 18-kDa). Magnitude 40X.

Tabela 1: Efeito do traumatismo craniano (TCE/TBI) na expressão gênica em micróglia isoladas do tecido cerebral de camundongos *red-green*, submetidos ao traumatismo craniano (TBI) e não submetidos ao traumatismo craniano (Naive), tratados com água do tipo *Mili-q* (Water). Genes que aumentaram significativamente (<1.5 *fold*) após a indução do traumatismo craniano.

GENE NAME	NAIVE WATER	TBI WATER	FOLD CHANGE
Spp1	11	98	8,63
Tlr1	8	53	6,93
Sparc	15	102	6,69
HIST1H2AB	19	111	5,82
B930046C15Rik	137	768	5,61
Rgmb	34	139	4,07
Npnt	27	108	4,07
Apoe	167	667	3,99
ENSMUSG00000079376	72	279	3,86
Fcgrt	15	57	3,78
1700001E04Rik	19	72	3,76
Pik3r4	11	40	3,47
Olfr110	15	51	3,34
Sema4d	65	208	3,22
Tspan3	19	59	3,12
Csf3r	91	283	3,10
Bach2	15	45	2,94
Slc12a2	30	87	2,85
Cysltr1	19	52	2,75
LOC100038847	247	665	2,69
Fcgr1	145	359	2,49
Pon3	53	131	2,47
B4galt4	57	136	2,38
Bhlhe41	68	162	2,37
Hist1h2ac	15	36	2,35
Cadm1	42	98	2,35
Cd83	53	125	2,35
Cux1	114	266	2,33
Tlr3	15	35	2,32
Scarb2	87	203	2,32
Itga6	194	449	2,32
Ptprm	23	52	2,29
Cttnbp2nl	167	381	2,28
Fads1	42	95	2,26
Pros1	110	243	2,20
Gal3st4	46	100	2,19
Spint1	38	83	2,18
Tspan7	57	124	2,17
Inpp4b	126	268	2,14

GENE NAME	NAIVE WATER	TBI WATER	FOLD CHANGE
Eya4	27	57	2,12
Abca1	42	88	2,09
Ctsd	4868	10025	2,06
Sall1	384	788	2,05
Serpinf1	30	62	2,03
Tlr4	53	108	2,03
Atp8a2	148	299	2,02
Sesn1	65	130	2,01
Csf1r	1685	3385	2,01
Bco2	76	152	2,00
Tanc2	87	172	1,97
Il10rb	91	178	1,95
Asph	297	576	1,94
D18Ertd653e	152	294	1,93
Gbgt1	46	88	1,93
Sox4	30	58	1,92
Syngr1	65	124	1,92
Lair1	213	407	1,91
Ang	46	86	1,88
Extl3	87	163	1,87
Slc1a3	80	149	1,86
Arhgap5	262	486	1,85
Rab3il1	95	175	1,84
Smad7	30	56	1,84
Sall3	23	42	1,84
Kcnd1	19	35	1,83
Serpine2	247	451	1,83
Capn3	95	173	1,82
Rbbp9	23	41	1,82
Cd34	202	366	1,82
Ctss	2088	3777	1,81
Garnl3	23	41	1,77
Tubgcp5	118	209	1,77
Npl	103	181	1,76
Map2k1	76	134	1,76
Ctsf	110	192	1,74
Tm9sf4	38	66	1,73
Stab1	95	164	1,73
Olfml3	707	1222	1,73
Abhd12	434	746	1,72
Rab6b	27	46	1,72

GENE NAME	NAIVE WATER	TBI WATER	FOLD CHANGE
A430107D22Rik	53	91	1,72
Abcc3	76	130	1,71
Pla2g15	190	325	1,71
Mef2a	156	265	1,70
Tgfbr2	316	534	1,69
C1qc	2035	3438	1,69
Snn	42	71	1,69
Clstn1	30	51	1,69
Pmp22	65	108	1,68
Hexb	5690	9444	1,66
Abi3	236	384	1,63
C1qa	1297	2102	1,62
P2rx7	65	105	1,62
Bin1	240	386	1,61
Liph	34	55	1,61
Gpr34	1137	1824	1,60
Adora3	110	176	1,60
Mafb	350	558	1,59
C1qb	1871	2980	1,59
Tnfrsf17	27	42	1,59
P2ry6	76	120	1,58
Qdpr	27	42	1,57
Adap2	171	268	1,57
Ctsl	137	214	1,57
Tgfbr1	1118	1745	1,56
Scoc	358	556	1,56
Bach1	91	141	1,55
Ppap2a	34	53	1,54
Siglech	274	418	1,53
Fgd2	84	128	1,53
Ptgs1	434	660	1,52
Ppp1r9a	42	64	1,52
Ppfia4	57	87	1,52
Kctd12	407	615	1,51
Lgmn	620	933	1,51
Lmo2	209	314	1,50

Tabela 2: Efeito do traumatismo craniano (TCE/TBI) na expressão gênica em micróglia isoladas do tecido cerebral de camundongos *red-green*, submetidos ao traumatismo craniano (TBI) e não submetidos ao traumatismo craniano (Naive), tratados com água do tipo *Mili-q* (Water). Genes que reduziram significativamente (<1.5 *fold*) após a indução do traumatismo craniano.

GENE NAME	NAIVE WATER	TBI WATER	FOLD CHANGE
Atf3	2868	30	95,15
Egr1	9683	125	77,59
Adamts1	1396	31	45,60
Fos	2830	77	36,63
Fosb	669	21	32,31
Junb	7641	473	16,14
Dnajb4	285	24	12,11
Jun	3503	298	11,77
Ccl4	205	18	11,18
Egr2	68	8	9,08
TNF	91	11	8,43
Ccl3	806	107	7,51
Nos2	65	9	6,86
Ppp1r15a	601	95	6,35
Trim47	217	35	6,22
Igfbp5	23	4	6,05
Enpp2	190	35	5,46
Abhd6	23	4	5,38
Clu	30	6	4,97
Socs3	103	22	4,74
Rgs1	160	36	4,46
Olfr920	34	9	3,82
Ttr	34	9	3,82
Il1a	232	69	3,37
Ccrl2	76	23	3,36
Tspan18	30	10	3,08
Rhob	2419	816	2,97
Zmynd8	46	18	2,55
Myc	61	25	2,39
CXCL10	27	12	2,17
Carhsp1	30	14	2,15
Tmem204	30	16	1,96
CEBPB	152	82	1,86
Mef2d	49	28	1,78
Tmc7	49	28	1,78
Rasgef1b	42	24	1,74
Hvcn1	38	22	1,72
Il1rl2	61	37	1,66
Bend6	30	18	1,66

Tabela 3: Efeito do tratamento com o *Laquinimod* (25mg/kg/dia) na expressão gênica em micróglia isoladas do tecido cerebral de camundongos *red-green*, submetidos ao traumatismo craniano (TBI) tratados com água do tipo *Mili-q* (Water) ou com o *Laquinimod* (LAQ). Genes que aumentaram significativamente (<1.5 fold) após o tratamento com o imunomodulador *Laquinimod*.

GENE NAME	TBI WATER	TBI LAQ	FOLD CHANGE
Chi3l3	14	62	4,36
Atf3	30	99	3,27
Adamts1	31	100	3,25
Fosb	21	54	2,59
Cybb	11	26	2,40
Ccl3	107	253	2,35
Ccl4	18	42	2,30
Ccl2	45	97	2,18
Fos	77	156	2,02
TNF	11	22	1,99
CXCL10	12	23	1,87
Egr1	125	225	1,81
C4a	22	39	1,75
Jun	298	485	1,63
Lilrb4	25	41	1,62

Tabela 4: Efeito do tratamento com o *Laquinimod* (25mg/kg/dia) na expressão gênica em micróglia isoladas do tecido cerebral de camundongos *red-green*, submetidos ao traumatismo craniano (TBI) tratados com água do tipo *Mili-q* (Water) ou com o *Laquinimod* (LAQ). Genes que reduziram significativamente (<1.5 fold) após o tratamento com o imunomodulador *Laquinimod*.

GENE NAME	TBI WATER	TBI LAQ	FOLD CHANGE
Hspa1a	128	33	3,83
Nedd1	25	11	2,18
Epn2	27	13	2,10
Bach2	45	22	2,07
Tnfrsf17	42	21	2,01
Sult1a1	29	15	1,98
Errfi1	33	17	1,92
Sesn1	130	68	1,92
Ppap2a	53	28	1,87
Tlr5	20	11	1,87
Upk1b	98	53	1,86
Csmd3	66	36	1,84
Jarid2	23	13	1,81
Smad	52	29	1,78
ENSMUSG00000079376	279	158	1,76
Rtn1	43	25	1,73
Snn	71	41	1,71
Havcr2	122	72	1,71
Large	46	27	1,70
Tmcc3	90	53	1,70
Inpp4b	268	159	1,68
Garnl3	41	24	1,67
Cryl1	58	35	1,67
Pgrmc1	23	14	1,66
Slc46a1	60	36	1,66
Lrp12	47	28	1,65
Rgmb	139	85	1,63
Jam2	86	53	1,63
Gpr165	57	35	1,62
Klf12	26	16	1,62
Tspan4	25	16	1,60
Rgl1	21	13	1,60
Zfp691	94	59	1,60
Lrrc3	175	110	1,60
Slc4a2	36	22	1,59
Adamts16	22	14	1,59
Tspan3	59	37	1,59
Eng	90	57	1,58
Chn2	46	29	1,57
Adrb1	111	71	1,57
Pmepa1	190	121	1,57
Icam1	69	44	1,56
Rbbp9	41	27	1,54

1.3 – Conclusões

Durante o período do doutorado sanduíche, demonstramos, por meio de técnicas de microscopia, citometria de fluxo, *sorting* e expressão gênica, alterações nas respostas mediadas por células do sistema imune residentes no SNC (micróglia) e provindas da periferia (monócitos) após a indução do TCE/TBI por percussão fluídica unilateral. Aumento no número de micróglia e do infiltrado de monócitos foi observado durante a fase aguda (3dpi) do TCE/TBI bem como uma redução da expressão de genes associados à manutenção da função microglial. Além disso, o pré-tratamento com o imunomodulador *Laquinimod* induziu aumento da expressão do marcador de ativação microglial TSPO e preveniu a supressão dos genes responsáveis por manter a função fisiológica dessas células. É importante ressaltar que o trabalho continuará em andamento sob a coordenação do professor Richard M Ransohoff. As análises da expressão de genes em micróglia e monócitos estão sendo realizadas em colaboração com o grupo do professor Oleg Butovsky, da Universidade de *Havard*, Boston, Massachusetts, USA. Experimentos estão sendo conduzidos para investigar o efeito do *Laquinimod* na resposta mediada por essas células com inicio do tratamento após a indução do TCE/TBI. Assim, a continuidade desse trabalho pela equipe do professor Richard M Ransohoff fortalecendo a colaboração com o nosso grupo no Brasil sob a supervisão do professor Antônio Lúcio Teixeira é essencial para uma melhor conclusão acerca dos resultados obtidos.

ANEXO 2- Artigos não relacionados diretamente a tese, mas publicados durante o período do doutorado

2.1 – Artigos em colaboração relacionados ao tema da tese, publicados durante o período do doutorado

- Brant F, ***Miranda AS***, Esper L, Rodrigues DH, Kangussu LM, Bonaventura D, Soriani FM, Pinho V, Souza DG, Rachid MA, Weiss LM, Tanowitz HB, Teixeira MM, Teixeira AL, Machado FS. Role of the aryl hydrocarbon receptor in the immune response profile and development of pathology during Plasmodium berghei Anka infection. **Infect Immun**. 2014; 82(8) :3127-3140. doi: 10.1128/IAI.01733-14.

- Campos AC, Brant F, ***Miranda AS***, Machado FS, Teixeira AL. Cannabidiol increases survival and promotes rescue of cognitive function in a murine model of Cerebral Malaria, **Neuroscience**. 2015; doi: http://dx.doi.org/10.1016/j.neuroscience.2014.12.051.

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Role of the Aryl Hydrocarbon Receptor in the Immune Response Profile and Development of Pathology during *Plasmodium berghei* Anka Infection

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Infection with *Plasmodium falciparum* may result in severe disease affecting various organs, including liver, spleen, and brain, resulting in high morbidity and mortality. *Plasmodium berghei* Anka infection of mice recapitulates many features of severe human malaria. The aryl hydrocarbon receptor (AhR) is an intracellular receptor activated by ligands important in the modulation of the inflammatory response. We found that AhR-knockout (KO) mice infected with *P. berghei* Anka displayed increased parasitemia, earlier mortality, enhanced leukocyte-endothelial cell interactions in the brain microvasculature, and increased inflammation in brain (interleukin-17 [IL-17] and IL-6) and liver (gamma interferon [IFN-γ] and tumor necrosis factor alpha [TNF-α]) compared to infected wild-type (WT) mice. Infected AhR-KO mice also displayed a reduction in cytokines required for host resistance, including TNF-α, IL-1β, and IFN-γ, in the brain and spleen. Infection of AhR-KO mice resulted in an increase in T regulatory cells and transforming growth factor β, IL-6, and IL-17 in the brain. AhR modulated the basal expression of SOCS3 in spleen and brain, and *P. berghei* Anka infection resulted in enhanced expression of SOCS3 in brain, which was absent in infected AhR-KO mice. These data suggest that AhR-mediated control of SOCS3 expression is probably involved in the phenotype seen in infected AhR-KO mice. This is, to our knowledge, the first demonstration of a role for AhR in the pathogenesis of malaria.

Plasmodium falciparum malaria is a major cause of morbidity and mortality and is responsible for millions of infections, especially in sub-Saharan Africa. Falciparum malaria may be mild or lead to severe life-threatening complications, such as altered liver function, including fulminant hepatic failure; renal failure; and cerebral malaria (CM). CM is a major life-threatening complication of *P. falciparum* infection in humans, especially in young children (1). Infection of susceptible mouse strains with *Plasmodium berghei* Anka is commonly employed as a model of CM, because this model displays many features in common with human CM (2, 3). Use of this *P. berghei* Anka model has allowed the identification of several important pathogenesis pathways in CM (2, 3). While there has been discussion regarding the utility of this model as a surrogate for human CM (3, 4), it is regarded as an important and valid animal model of human CM (5–7). Moreover, the *P. berghei* Anka infection model also allows investigations into liver damage, as described in *Plasmodium*-infected humans (8, 9). Observations from humans with CM due to *P. falciparum*, as well as those from the CM mouse model using *P. berghei* Anka, suggest that the pathogenesis of CM is multifactorial and includes sequestration of infected red blood cells (iRBCs), disruption of the blood-brain barrier (BBB), upregulation of the inflammatory pathways, and dysregulation of endothelin pathways (10–13). Previously, we have demonstrated a crucial role for platelet-activating factor in experimental CM infection (10–13). Infection with *P. berghei* Anka results in a reduction in cerebral blood flow and neuronal dysfunction (14, 15). In addition, low nitric oxide (NO) bioavailability, hypoarginemia, and elevated levels of cell-free hemoglobin (16–20) have also been demonstrated to contribute to the pathogenesis of CM.

As with other infections (21), the inflammatory response triggered by malaria parasite infection is a double-edged sword. The early inflammatory response is required to control parasite replication and promote the clearance of infected erythrocytes. However, excessive levels of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), interleukin-1β (IL-1β), and IL-6, may result in cerebral dysfunction (22–24). Therefore, the outcome of infection depends on a delicate balance between appropriate and inappropriate induction of these mediators (25).

Both $CD4^+$ and $CD8^+$ T cells contribute to the pathogenesis of CM. $CD8^+$ T cells are sequestered in the brain following *P. berghei* Anka infection at the onset of altered neurological signs and

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symptoms and are postulated to cause damage to the brain endothelium via the production of perforin (26). Sequestration of $CD8^+$ T cells as well as red blood cells is associated with the development of CM (27, 28). T regulatory (Treg) cells play a critical role in the maintenance of immunological self-tolerance, as well as the control of immune responses to pathogens (29). Treg cells mediate their effects by direct cell contact or by secretion of anti-inflammatory cytokines, such as IL-10 and transforming growth factor β (TGF-β). In CM, Treg cells may contribute to the pathogenesis of infection by suppressing antiparasitic immunity (30). The role of Treg cells in the pathogenesis of CM in *P. berghei* Anka-infected mice has not been fully elucidated (31–33).

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor and a member of the Per-Arnt-Sim (PAS) superfamily of proteins, which contains proteins involved in the detection of intracellular or environmental changes, sensing of light, oxygen and redox potential, the circadian rhythm, the response to hypoxia, and hormone signaling (34, 35). In the absence of ligands, AhR is inactive and remains in the cytoplasm complexed with accessory proteins. Upon ligand binding, the receptor shifts to the nucleus and heterodimerizes with the AhR nuclear translocator (ARNT). Once transcriptional regulation has occurred, the AhR is exported to the cytosol and degraded by the proteasome (35, 36).

AhR is activated by exogenous compounds, particularly by environmental contaminants, such as 2,3,7,8-tetrachlorobenzo-*p*-dioxin (TCDD). Activation of the AhR by this environmental pollutant can lead to several toxic changes, including hepatocellular damage, epithelial changes, cancer, birth defects, thymic involution, and immunosuppression (37, 38). There are a variety of endogenous agents capable of activating AhR, such as eicosanoids, bilirubin, the tryptophan photoproduct 6-formylindolo[3,2-*b*]carbazole (FICZ), kynurenines, and indoleamine 2,3-dioxygenase (IDO) (34, 35, 37, 39). During immune responses, AhR regulates both Treg and Th17 cell differentiation in a ligand-specific fashion (40–42).

AhR has been reported to contribute to the modulation of the inflammatory response to infections with *Toxoplasma gondii* (43), *Listeria monocytogenes* (44), and herpes simplex virus (45). During *T. gondii* infection, AhR deficiency results in a reduction of suppressor of cytokine signaling 2 (SOCS2) expression in the spleen. Suppressors of cytokine signaling (SOCS) are a family of intracellular proteins modulating the immune response, acting in several signaling pathways. Several studies have evaluated the roles of SOCS1, -2, and -3 under steady-state conditions and after infection. SOCS1-deficient mice spontaneously exhibit severe lymphopenia, fatty degeneration of the liver, and macrophage infiltration of major organs. SOCS3-deficient mice have embryonic lethality due to massive expansion of erythroid progenitors. Infection of mice with either *T. gondii* or *Trypanosoma cruzi* infection in the absence of SOCS2 leads to alterations in the central nervous and cardiovascular systems. While one study has demonstrated the participation of AhR in the modulation of SOCS2 expression induced by lipoxin in dendritic cells (46), the role of AhR in the modulation of SOCS1 and SOCS3 in dendritic or other cell types is unknown. The role of AhR in malaria has not been explored.

Since inflammation is an important factor in the pathogenesis of CM and AhR is a modulator of inflammation, we examined the role of AhR in *P. berghei* Anka-infected mice. Herein, we demonstrate that AhR modulates the development of CM and protects against liver damage. These data provide new insights into the pathogenesis of CM.

MATERIALS AND METHODS

Ethics statement. This study was carried out in strict accordance with the Brazilian guidelines on animal work and the *Guide for the Care and Use of Laboratory Animals* (47). The animal ethics committee of the Universidade Federal de Minas Gerais, CETEA/UFMG, approved all experiments and procedures, including euthanasia and fluid and organ removal (permit number 202/10). All animal experiments were planned in order to minimize mouse suffering.

Parasitology and pathology. Wild-type (WT) C57BL/6 mice (6 to 8 weeks old) were obtained from the Animal Care Facilities of the Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. AhR-knockout (KO) mice were bred on a C57BL/6 genetic background under pathogen-free conditions at the Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais. Blood stages of *P. berghei* Anka constitutively expressing green fluorescent protein (GFP) (*P. berghei* Anka-GFP; the 15cy1 clone) (48), kindly provided by Claudio Marinho (Universidade de São Paulo), were stored in liquid nitrogen. Mice were infected intraperitoneally (i.p.) with 10^5 *P. berghei* Anka-infected red cells suspended in 0.2 ml phosphate-buffered saline (PBS). The percent parasitemia was quantified by determination of the frequency of GFP in whole blood using flow cytometry. Briefly, a drop of tail blood from infected and uninfected mice was collected directly into polystyrene tubes containing 2 ml of PBS for flow cytometry analysis. Each sample was analyzed using a flow cytometer (FACSCanto II; Becton, Dickinson, San Jose, CA). GFP frequency was measured using an argon laser (488 nm), and the acquisition was processed using Diva software (Becton, Dickinson, San Jose, CA). Erythrocyte populations were identified on the basis of their morphological characteristics in a dot plot graphic (forward scatter-side scatter) and analyzed for the presence/expression of GFP. A minimum of 100,000 gated events on the erythrocyte population of each sample was acquired for analysis. Mice were observed daily for parasitemia, survival, and clinical signs of CM culminating in ataxia and paralysis, which were assessed by use of the rapid murine coma and behavior scale (RMCBS) (49). For determination of hematocrit, samples of blood were collected at 5 days postinfection (dpi) and placed into heparinized capillary tubes, and the tubes were centrifuged for 10 min in a hematocrit centrifuge (HT, São Paulo, Brazil). For determination of liver function, alanine aminotransferase (ALT) activity was measured using a commercially available kit following the manufacturer's protocol (Quibasa, Bioclin, Belo Horizonte, Brazil). Serum iron levels were determined by colorimetric assay using a commercially available kit (Gold Analisa, Belo Horizonte, Brazil) following the manufacturer's protocol.

At the time points indicated below, mice were euthanized with ketamine-xylazine, the brain and a portion of the liver were removed and immediately fixed in 4% buffered formalin, and tissue fragments were embedded in paraffin. Tissue sections (4 μm thick) were stained with hematoxylin-eosin (H&E) and examined under a light microscope. Sections were captured with a digital camera (DEI-470; Optronics, Goleta, CA) connected to a microscope (IX70; Olympus, Center Valley, PA) with a magnification of ×200. Liver pathology (degeneration and inflammation) was graded from 0 to 4, considering the severity of the lesions (none, 0; minimal, 1; mild, 2; moderate, 3; marked, 4).

Intravital microscopy. Intravital microscopy of the mouse brain microvasculature was performed at 5 dpi. Mice were anesthetized i.p. with a sterile mixture of 10 mg/kg of body weight xylazine and 150 mg/kg ketamine in PBS. The tail vein was cannulated for administration of fluorescent dyes. A craniotomy was performed with a high-speed drill (Beltec, São Paulo, Brazil), and the dura and arachnoid mater were removed to expose the underlying pia mater vasculature. During the experiment, the mice were maintained at 37°C with a heating pad (Fine Science Tools Inc., North Vancouver, BC, Canada), and the exposed brain was kept moist with artificial cerebrospinal fluid buffer (pH 7.4, 132 mmol/liter NaCl,

2.95 mmol/liter KCl, 1.71 mmol/liter $CaCl_2$, 0.64 mmol/liter $MgCl_2$, 24.6 mmol/liter $NaHCO_3$, 3.71 mmol/liter dextrose, 6.7 mmol/liter urea) at 37°C. To observe leukocyte-endothelium interactions, leukocytes were fluorescently labeled by intravenous administration of rhodamine 6G (0.3/0.5 mg/kg body weight; Sigma-Aldrich, St. Louis, MO). An intravital microscope (Nikon CSRS H550L; Nikon, Japan) with a ×20 objective lens outfitted with a fluorescent light source (epi-illumination at 510 to 560 nm, using a 590-nm emission filter) was used to examine the brain microcirculation. A digital camera (Nikon DS-QiMc) was used to project the images onto a computer monitor, and images were recorded for playback analysis using Imaging software (NIS-Elements; Nikon). The number of rolling and adherent leukocytes was determined offline during video playback analysis. Leukocytes were considered adherent to the venular endothelium if they remained stationary for a minimum of 30 s, and adherent leukocytes were expressed as the number of cells per 100 μm. Rolling leukocytes were defined as white blood cells moving at a velocity lower than that of erythrocytes within a given vessel. The *P. berghei* Anka-GFP-infected red blood cells were observed in the pia mater vessel using a green fluorescent light source (epi-illumination at 510 to 560 nm, using a 560-nm emission filter).

Assessment of BBB integrity. The integrity of the blood-brain barrier (BBB) was investigated using Evans blue dye as described previously (11). Briefly, Evans blue solution (20 mg/kg; Sigma-Aldrich) was administered (1 ml/kg) via an eye vein 30 min prior to sacrifice at 6 dpi. One hour later, the mice were sacrificed and the heart was perfused with 5 ml of PBS. Brain samples were removed and weighed, and Evans blue extravasation was evaluated using 1 ml of formamide (24 h at room temperature). The amount of Evans blue in the tissue was obtained by comparing the extracted absorbance with that of a standard curve of Evans blue read at 620 nm in an enzyme-linked immunosorbent assay (ELISA) plate reader as a measurement of capillary permeability. Results are presented as the amount of Evans blue per 100 mg of tissue

Hemodynamic measurements. Systolic blood pressure (SBP) and heart rate were measured in uninfected and infected mice at 0, 3, 4, and 5 dpi. All mice were acclimated to the blood pressure measurement device for 5 days. SBP and heart rate were determined by the tail-cuff plethysmography method in nonanesthetized mice. The tail-cuff measurements were made in trained mice that had not undergone any invasive procedures, and the intra-arterial measurements were made without the stress of heating and restraints required during the tail-cuff procedure. All data are expressed as the mean ± standard error of the mean (SEM). Changes in SBP and heart rate from the baseline are expressed as absolute values as well as areas under the curves.

Quantification of nitric oxide in brain tissue. The brain was harvested and homogenized in a PBS buffer containing a protease inhibitor cocktail. Nitric oxide (NO) was quantified as nitrite. Nitrates were reduced to nitrites by enzymatic conversion by nitrate reductase (Sigma). The level of nitrites was determined by the Griess method. The NO_2^- concentration (μM) was determined by reference to an $NaNO_2$ standard curve (absorbance at 550 nm).

CBA. Serum cytokine levels were determined using a mouse Th1/Th2/Th17 cytometric bead array (CBA) kit (BD Biosciences, San Diego, CA) and analyzed on a FACSCanto II flow cytometer. Standard curves for each cytokine were determined over a range of 20 to 5,000 pg/ml. The lower limit of detection for the CBA, according to the manufacturer, is 0.1 to 16.8 pg/ml, depending on the analyte. The levels of the cytokines IL-2, IL-6, IL-10, IL-17A, IFN-γ, and TNF-α were measured and analyzed by FCAP Array software (BD Biosciences).

Quantification of tissue cytokines (ELISA). Brain and spleen tissues were homogenized in a PBS buffer containing a protease inhibitor cocktail to determine the concentrations of the cytokines IL-1β, IL-6, IL-12p70, IL-10, IL-17A, IFN-γ, TGF-β, and TNF-α by ELISA (DuoSet kits; R&D Systems), in accordance with the manufacturer's instructions.

Real-time PCR (qPCR). Brains and spleen were removed for analysis of the transcript levels of AhR, SOCS1, SOCS2, SOCS3, and FOXP3. Total RNA was obtained using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the procedure supplied by the manufacturer. Total RNA was reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) as described by the manufacturer, and quantitative PCR (qPCR) was performed on an ABI Prism Step One sequence detection system (Applied Biosystems, Carlsbad, CA), using SYBR green PCR master mix (Applied Biosystems), for the AhR, FOXP3, SOCS1, SOCS2, and SOCS3 genes. The 18S rRNA gene was used as an endogenous control for normalization, according to the manufacturer's protocol, and was tested in a Step One thermal cycler and a 7500 Fast real-time PCR system (Applied Biosystems) with specific primer pairs. The relative expression level of the genes was determined by the $2^{-\Delta\Delta CT}$ threshold cycle (C_T) method, and the data were normalized by the 18S ribosome subunit expression levels. All reactions were replicated. Test samples were expressed as the fold change of transcript abundance in uninfected WT or AhR-KO mice compared with that in infected WT or AhR-KO mice, respectively (mean expression from at least three uninfected mice). The sequences of the primers used in this study were as follows: AhR Forward, GGAGCGCTGCTTCCTCCAC; AhR Reverse, GCTGCCCTTTGGCATCACAACC; FOXP3 Forward, CCCAGGAAAGACAGCAACCTT; FOXP3 Reverse, TCCTCACAACCAGGCCACTTG; SOCS1 Forward, ACACTCACTTCCGCACCTTC; SOCS1 Reverse, GAAGCCATCTCCACGCTG; SOCS2 Forward, CGGCGGTGGAGGCGATCTG; SOCS2 Reverse, CCGAAATGGTGGCGGAGGGG; SOCS3 Forward, GTTGAGCGTCAAGACCCAGT; SOCS3 Reverse, GGGTGGCAAAGAAAAGGAG; 18S Forward, CGTTCCACCAACTAAGAACG; and 18S Reverse, CTCAACACGGGAAACCTCAC.

Flow cytometry of brain lymphocytes. *P. berghei* Anka-infected mice were sacrificed at 6 dpi and perfused by intracardiac catheter with PBS to remove circulating RBCs and leukocytes from the brain. The brains were then removed, and lymphocytes were isolated as described previously (27). Briefly, the brains were collected and gently homogenized with a sterile glass tissue grinder in RPMI 1640 medium containing 5% fetal calf serum. Homogenates were passed through a nylon cell strainer (pore size, 70 μm; Becton, Dickinson, San Jose, CA) and then centrifuged at 400 × *g* for 10 min. The pellet was resuspended in 35% Percoll gradient (Sigma-Aldrich), and this was deposited on a 70% Percoll gradient. After centrifugation (1,100 × *g*), myelin was aspirated off the top of the 35% Percoll layer and leukocytes were collected at the boundary layer, between the 70% and 35% gradients. Leukocytes were then resuspended in fluorescence-activated cell sorting buffer (PBS containing 1% fetal calf serum and 0.01% NaN_3) and counted. At 4 h posttreatment with brefeldin A (10 μg/ml), the cells were fixed and stained with labeled mouse-specific antibodies with the combinations of biotin-CD3 followed by Strep-Cy5, CD4 (phycoerythrin [PE]-Cy7), CD8 (allophycocyanin [APC]), CD25 (APC), FOXP3 (PE), IFN-γ (PE), IL-10 (APC), IL-17A (PE), TNF-α (Alexa Fluor 647), and isotype controls (all from BD Pharmingen). For each sample, 20,000 cells from the lymphocyte population were scored. Data were acquired on a FACSCanto II flow cytometer (Becton, Dickinson) and analyzed by FlowJo (version 7.6) software (Tree Star, Inc.). The gating strategy used is illustrated in Fig. S1A in the supplemental material.

Flow cytometry of liver lymphocytes. *P. berghei* Anka-infected mice were sacrificed at 6 dpi and perfused with PBS by an intraportal catheter to remove circulating RBCs and leukocytes from the liver. Liver-derived lymphocytes were isolated using a modified method previously described (50). Briefly, livers were excised and finely minced in digestive medium containing 0.05% collagenase (Life Technologies) and 0.002% DNase I (Sigma-Aldrich) in Hanks' balanced salt solution (Life Technologies). After gentle agitation at 37°C for 30 min, the concentrate was passed through a nylon cell strainer (pore size, 70 μm; Becton, Dickinson, San Jose, CA), washed twice with ice-cold PBS (pH 7.4), and centrifuged at 300 × *g* for 10 min. Lymphocytes were purified by layering the cell suspension on fluorescence-activated cell sorting buffer (PBS containing 1% fetal calf serum and 0.01% NaN_3) and centrifuged at 800 × *g* for 20 min at room temperature. Lymphocytes were washed in PBS and

counted. Liver cells were incubated with brefeldin A (10 μg/ml) for 4 h, followed by fixation and staining with specific antibody combinations: biotin-CD3 followed by Strep-Cy5, CD4 (PE-Cy7), CD8 (APC), CD25 (APC), FOXP3 (PE), IFN-γ (PE), IL-10 (APC), IL-17A (PE), TNF-α (Alexa 647), and isotype controls (all from BD Pharmingen). Data were acquired on a FACSCanto II flow cytometer (Becton, Dickinson), and viable cells were analyzed by flow cytometry using FlowJo software (Tree Star, Inc.). The gating strategy used is illustrated in Fig. S1B in the supplemental material.

Flow cytometry of spleen and thymus lymphocytes. Spleen and thymus cells were evaluated *ex vivo* for extracellular molecular expression patterns and for intracellular cytokine expression patterns. Purified splenocytes and thymus cells were removed from infected mice at 6 dpi. Spleen cells were incubated with brefeldin A (10 μg/ml) for 4 h, followed by fixation and staining with specific antibody combinations: biotin-CD3 followed by Strep-Cy5, CD4 (PE-Cy7), CD8 (APC), CD25 (APC), FOXP3 (PE), IFN-γ (PE), IL-10 (APC), IL-17A (PE), TNF-α (Alexa 647), and isotype controls (all from BD Pharmingen). Thymus cells were removed and stained directly with CD3, CD8, CD4, CD25, and FOXP3. Data were acquired on a FACSCanto II flow cytometer (Becton, Dickinson), and viable cells were analyzed by flow cytometry using FlowJo software (Tree Star, Inc.). The gating strategy used is illustrated in Fig. S1C in the supplemental material.

Statistical analysis. Results are shown as means ± SEMs. Differences were compared by using two-tailed Student's *t* tests with 95% confidence intervals, analysis of variance (ANOVA), or two-way ANOVA followed by the Bonferroni correction, as needed, for multiple comparisons when parametric assumptions were met. Otherwise, the Mann-Whitney U test was applied. Differences between lethality curves were calculated using the log rank test (GraphPad Prism software, version 5.0). Results with a P value of <0.05 were considered significant. All data are representative of those from at least 2 experiments ($n = 5$ to 7 mice).

RESULTS

AhR deficiency results in increased parasitemia and mortality in *Plasmodium berghei* Anka infection. To investigate whether AhR expression was modulated during *P. berghei* Anka infection, qPCR was performed on brain, liver, and spleen. After infection, there was a significant reduction in AhR expression in the brain compared with that in the brain of uninfected mice (Fig. 1 A), and there was a markedly increased AhR expression in the liver and spleen after infection (Fig. 1B and C). These results suggest that during infection, AhR expression is modulated in an organ-specific fashion. To determine the role of AhR in the pathogenesis of *P. berghei* Anka infection, AhR-deficient mice were infected and parasitemia (Fig. 1D) and mortality (Fig. 1E) were monitored. The absence of AhR resulted in a significant increase in parasitemia (Fig. 1D) and more rapid mortality (statistical analysis by the log rank test, $P < 0.05$; Fig. 1E) during infection. Severe neurological signs, including ataxia, paralysis, seizure, rollover, and coma, appeared earlier in infected AhR-KO mice than infected WT mice. The hematocrit at day 5 dpi was significantly lower in infected AhR-KO mice than infected WT mice (Fig. 1F). In agreement with the findings of a previous study, uninfected AhR-KO mice were found to have a slight decrease in hematocrit compared with WT mice (51).

Alterations in leukocyte-endothelial cell interaction and brain vascular permeability in AhR-KO mice during *P. berghei* Anka infection. CM is associated with inflammation in the brain, which is characterized by leukocyte adherence in the brain microvasculature, damage to the endothelium, and eventual disruption of the BBB and hemorrhage. The role of AhR in the interaction of leukocytes with the pia mater vasculature was evaluated using intravital microscopy. There was a significant increase in the number of leukocytes rolling and adhering in the vasculature of the pia mater of *P. berghei* Anka-infected AhR-KO compared with the number of such leukocytes in infected WT mice (Fig. 2A to D). Moreover, the intravital images and histological analyses demonstrated that AhR deficiency increased the hemorrhagic foci in the pia mater and neuropil (Fig. 2E to H).

Severe malaria is usually accompanied by vascular sequestration of leukocytes in the microvasculature in a variety of vital organs. In the brain, it may result in functional alterations in the endothelium and in the BBB. Experiments using Evans blue revealed that at 6 dpi AhR-KO mice had an increase in vascular permeability compared to infected WT mice (Fig. 2I), suggesting that *P. berghei* Anka infection in the absence of AhR is associated with increased vasculature permeability.

NO is an important regulator of vascular tone. It is believed that alterations in cerebral NO levels may contribute to altered neurological signs and symptoms, including coma, during *P. berghei* Anka infection. We found that AhR-KO-infected mice had increased levels of NO compared to infected WT mice (Fig. 2J). The systolic blood pressure and heart rate in WT and AhR-KO mice, however, were similar during the period of observation (see Fig. S1D and E in the supplemental material).

The absence of AhR results in severe inflammation and hepatic injury during *P. berghei* Anka infection. Examination of liver sections revealed increased liver inflammation and necrosis in infected AhR-KO mice compared to infected WT mice (Fig. 3A to H). In addition, *P. berghei* Anka-infected AhR-KO mice had a significant increase in serum levels of ALT at 5 dpi (Fig. 3I). Hemolysis often accompanies malaria parasite infection, resulting in released free heme catalyzed in iron, an important metabolite for the parasite life cycle. Both infected WT and AhR-KO mice had a significant increase in the levels of iron at 5 dpi. By 6 dpi, WT mice had a reduction in iron to basal levels; however, iron levels remained elevated in AhR-KO mice (Fig. 3J).

Since immune mediators such as T cells and cytokines have been shown to be crucial for the development of severe malaria, we investigated the phenotype of infiltrating cells found in the liver. Greater numbers of $CD4^+$ IFN-γ-positive (IFN-γ^+), $CD4^+$ TNF-α-positive (TNF-α^+), and $CD4^+$ IL-10-positive (IL-10^+) cells, but not $CD8^+$ IFN-γ^+ cells, were observed in the livers of infected AhR-KO mice than in those of infected WT mice (Fig. 3K to N). On the other hand, there were no differences detected in the number of $CD4^+$ IL-17^+ and $CD4^+$ $CD25^+$ FOXP3-positive ($FOXP3^+$) cells among all uninfected and infected groups analyzed (Fig. 3O and P). Figures 3Q and R show the proportion of Th1, Th17, and Treg cells in the liver of *P. berghei* Anka-infected WT and AhR-KO mice.

Inflammatory response to *P. berghei* Anka infection in the brain of AhR-KO mice. Several parameters of the immune response were evaluated in the brain. At 5 dpi, increased levels of IL-17 and IL-6 were observed in the brain of AhR-KO mice compared with the levels in the brain of WT mice (Fig. 4A and B), while the levels of IL-12p70, IFN-γ, TNF-α, and IL-1β at 3 and 5 dpi were either reduced or unchanged (Fig. 4C to F). The profile of infiltrating cells in the brain was next determined. The absence of AhR did not alter the numbers of infiltrating $CD4^+$ IFN-γ^+ and $CD8^+$ IFN-γ^+ cells in the brain of infected mice (Fig. 5A and B); however, there was an increase in the number of $CD4^+$ IL-17^+ T cells (Fig. 5C).

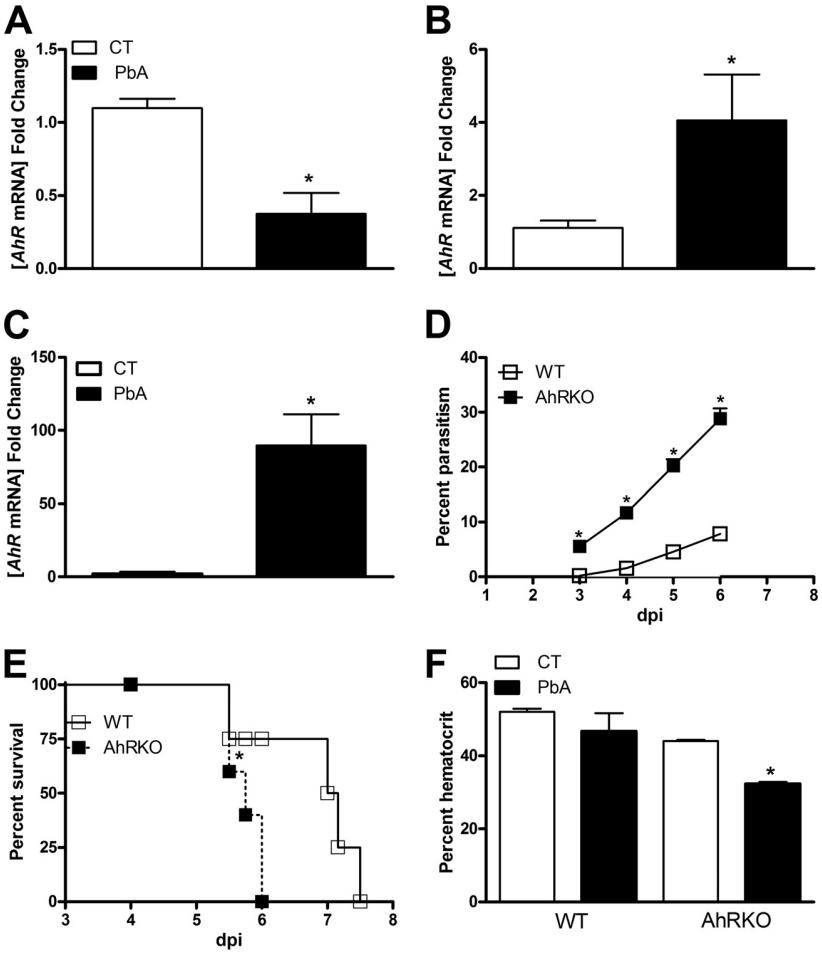

FIG 1 AhR-KO mice are highly susceptible to *Plasmodium berghei* Anka (PbA) infection. C57BL/6 (WT) mice were infected i.p. with 10^5 parasitized erythrocytes. (A to C) Brain (A), liver (B), and spleen (C) were harvested from control and infected WT mice at 6 dpi, followed by RNA extraction, and quantitative reverse transcription-PCR using primers specific for AhR in brain, liver, and spleen was performed. The data are expressed as the fold change in transcript abundance in uninfected WT mice compared with the abundance in infected WT mice. (D to F) WT and AhR-KO mice were infected i.p. with 10^5 parasitized erythrocytes, and at the indicated time points, the following parameters were assessed: the natural course of infection by flow cytometric analysis (D), the survival curves of *P. berghei* Anka-infected WT and AhR-KO mice (expressed as the number of days after infection) (E), and hematocrit at 5 dpi (F). The data are representative of those from at least two independent experiments (5 mice/group) and are shown as the means ± SEMs. *, $P < 0.05$ for the comparison of infected versus uninfected WT mice (A to C) or the comparison of infected WT versus AhR-KO mice (D to F). CT, control.

Treg cells play an important role in the control of immune responses to several pathogens. At 6 dpi, there were increased levels of TGF-β (Fig. 5D), but not IL-10 (Fig. 5E), and significantly increased numbers of $CD4^+$ $CD25^+$ $FOXP3^+$ cells (Fig. 5F) in the brain of infected AhR-KO mice compared with infected WT mice. The suppressor of cytokine signaling (SOCS) family plays an important role in immune responses by modulating cytokine signaling pathways, and AhR has been shown to modulate SOCS2 expression in the mouse model of *T. gondii* infection (46). SOCS2 is also induced during *T. cruzi* infection (52) and was shown to be modulated as a result of aspirin treatment (53). Therefore, we examined SOCS1, -2, and -3 expression in the brain tissue of infected mice. In the brain of infected AhR-KO mice, the expression of SOCS3 was significantly decreased, whereas the expression of SOCS1 was only minimally reduced and the expression of SOCS2 was unchanged compared with that in the brain of infected WT mice (Fig. 5G to I). Figures 5J and K show the proportion of Th1, Th17, and Treg cells in the brain of *P. berghei* Anka-infected WT and AhR-KO mice.

Systemic and splenic cytokine responses. At 5 dpi, infected AhR-KO mice displayed a significant increase in serum levels of IL-2, IL-17, IL-6, TNF-α, and IL-10, but not IFN-γ, compared with those in infected WT mice (Fig. 6A). However, no significant differences in the levels of IL-12p70, IFN-γ, IL-10, and IL-β in the spleen (Fig. 6B) were observed between *P. berghei* Anka-infected WT and AhR-KO mice. Infection of AhR-deficient mice resulted in an absence of TNF-α induction (Fig. 6B), reduced FOXP3 expression (Fig. 7A), and decreased TGF-β production (Fig. 7B) in the spleen compared with the results for infected WT mice. We also observed a significant increase in the number of $CD4^+$ $CD25^+$ $FOXP3^+$ cells in the spleen of infected WT mice but not in the spleen of infected AhR-KO mice (Fig. 7C).

Alterations in the generation and expansion of $CD4^+$ $CD25^+$ $FOXP3^+$ cells were demonstrated in the thymus of AhR-KO mice

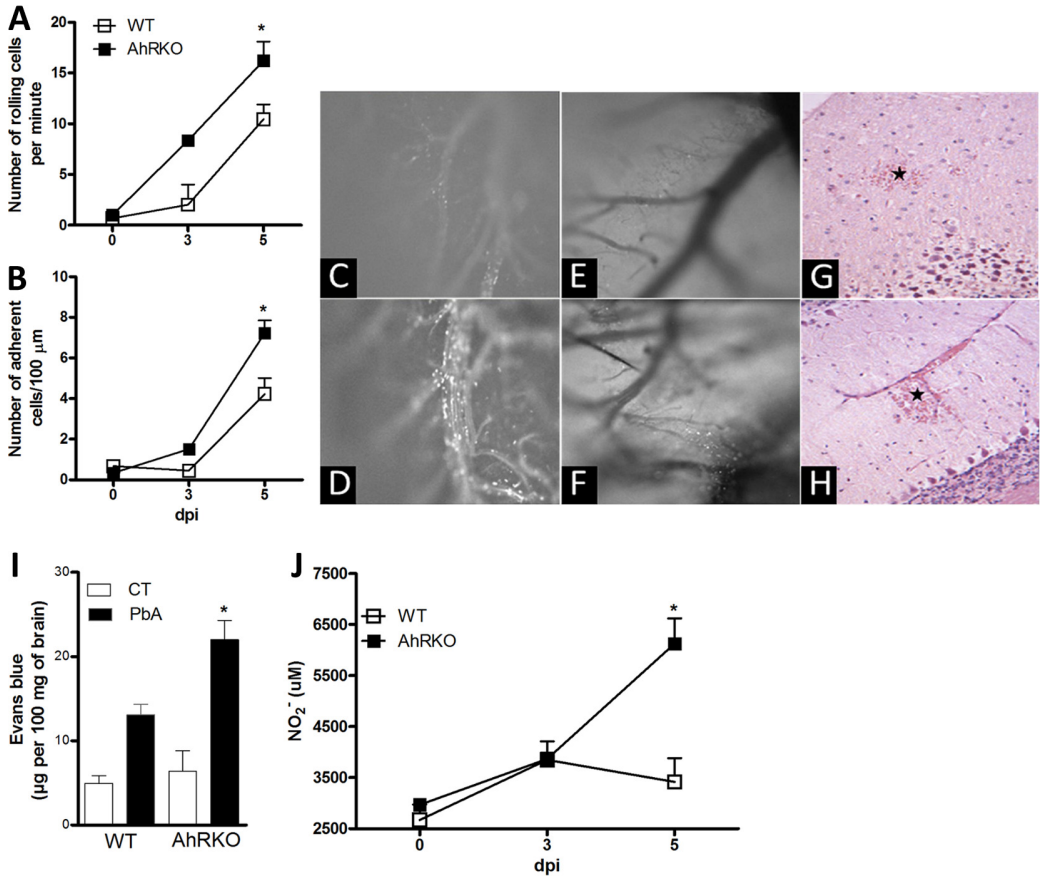

FIG 2 Leukocyte-endothelium interaction and vascular leakage in *P. berghei* Anka-infected AhR-KO brain. WT and AhR-KO mice were infected i.p. with 10^5 parasitized erythrocytes. (A, B) Intravital microscopy was used to assess the rolling (A) and adhesion (B) of leukocytes on the brain microvasculature in uninfected and infected mice at 3 and 5 dpi. The data are presented as the mean number of rolling cells per minute ± SEM (A) or the mean number of adherent cells per 100 μm ± SEM (B). (C to F) Time-lapse images reveal rolling and adherent cells in WT (C) and AhR-KO (D) mice and the presence of *P. berghei* Anka-GFP red blood cells in the pia mater of infected WT (E) and AhR-KO (F) mice. (G, H) Representative photomicrographs of H&E-stained cerebellar sections from infected WT (G) and AhR-KO (H) mice at 6 dpi. Infected WT mice displayed mild focal hemorrhage at the molecular layer (asterisk) (G). Infected AhR-KO mouse displayed extensive hemorrhagic areas (asterisk) (H). Magnifications, ×200. Quantitative assessment of brain vascular permeability using Evans blue extravasation in formamide was made by measurement of the absorbance at 650 nm in brain extract on day 6 dpi. (I) Infected AhR-KO mice showed increased vascular permeability compared with that of *P. berghei* Anka-infected WT mice. (J) The brain was harvested and homogenized, and nitrite levels were assayed by the Griess method. The data are representative of those from one of three independent experiments (5 mice/group) and are shown as the mean ± SEM. *, $P < 0.05$ for the comparison of infected WT versus AhR-KO mice.

(see Fig. S2A in the supplemental material). *P. berghei* Anka infection resulted in a minimal reduction of $CD4^+$ $CD25^+$ $FOXP3^+$ cells in the thymus of WT mice (see Fig. S2A in the supplemental material). Infection of AhR-KO mice resulted in a significant reduction of Treg cells in the thymus and impaired their generation/expansion in the spleen compared with the findings for infected WT mice (Fig. 7C; see Fig. S2A in the supplemental material). Moreover, the generation of T cells in the thymus was altered by the absence of AhR, including an increase in the number of double-positive thymocytes (see Fig. S2B in the supplemental material) and $CD4^+$ $CD8^{low}$ cells (see Fig. S2D in the supplemental material) and a decrease in the numbers of $CD8^+$ $CD4^-$ cells (see Fig. S2G in the supplemental material) compared with the results for WT mice. Upon infection, AhR deficiency resulted in decreased numbers of double-positive thymocytes (see Fig. S2B in the supplemental material), $CD4^+$ $CD8^-$ cells (see Fig. S2E in the supplemental material), $CD8^+$ $CD4^{low}$ cells (see Fig. S2F in the supplemental material), and $CD8^+$ $CD4^-$ cells (see Fig. S2G in the supplemental material) at 6 dpi. In the spleen of infected AhR-KO mice, SOCS3 expression was significantly lower than that in the spleen of WT infected mice (Fig. 7D). Figures 7E and F demonstrate the proportion of Th1, Th17, and Treg cells in the spleen of *P. berghei* Anka-infected WT and AhR-KO mice.

DISCUSSION

AhR, a highly conserved receptor found in members of the animal kingdom from invertebrates to mammals, participates in a variety of physiological functions (35, 36, 54, 55). Recent studies have identified several endogenous ligands for AhR, suggesting that AhR not only is a master regulator of drug metabolism but also participates in many physiological functions, including cell growth, differentiation, vascular and hematopoietic development, and regulation of immune responses. To our knowledge, this is the first report demonstrating a role for AhR in the pathogenesis of severe malaria caused by *P. berghei* Anka infection. AhR-KO mice

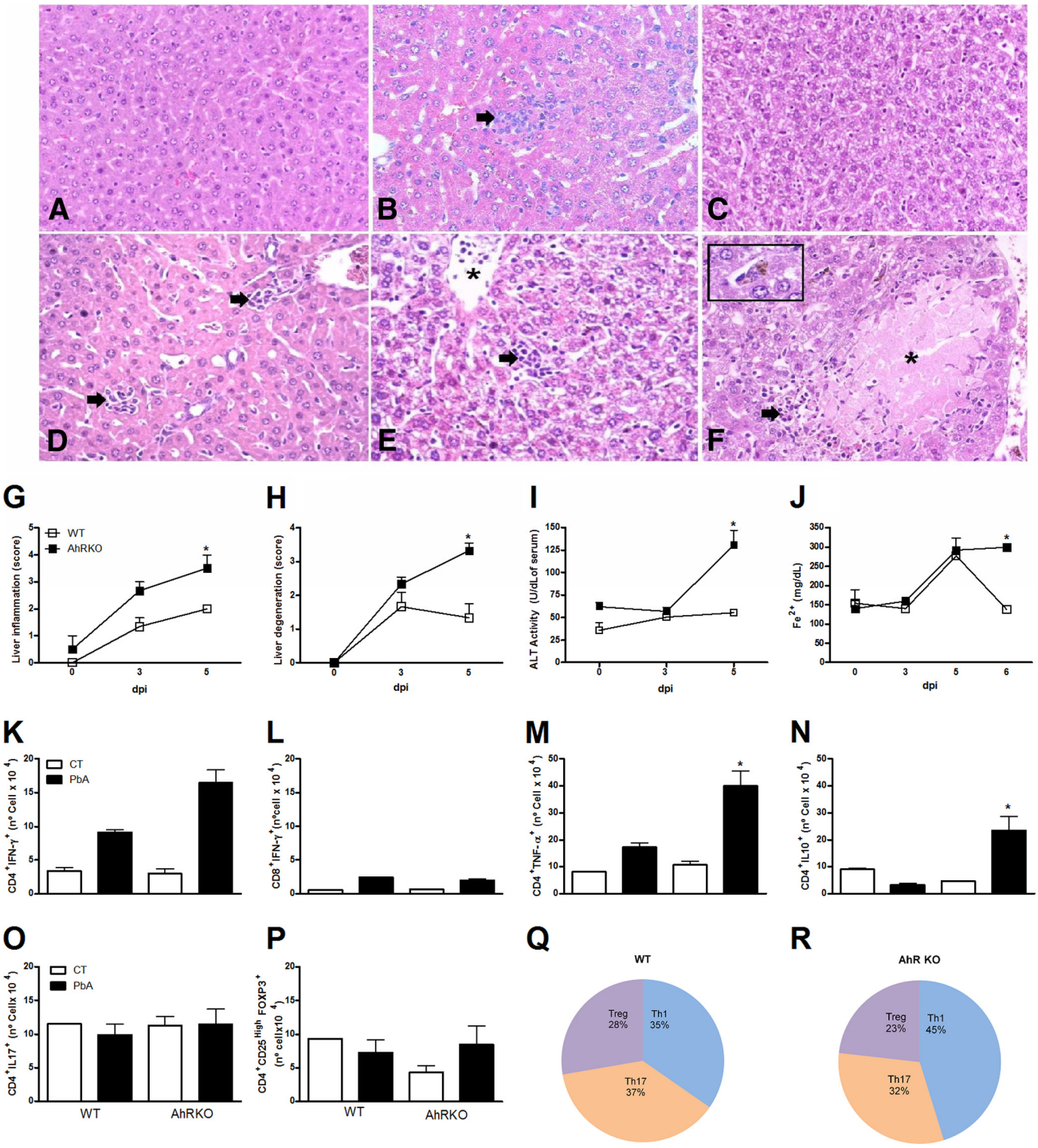

FIG 3 Increased liver injury in *P. berghei* Anka-infected AhR-KO mice. WT and AhR-KO animals were infected i.p. with 10^5 parasitized erythrocytes. (A to F) Representative photomicrographs of H&E-stained liver sections from uninfected WT (A) and AhR-KO (D) mice or infected WT (B, C) and AhR-KO (E, F) mice on days 3 (B, E) and 6 (C, F) after infection. (A) Normal histological appearance of liver. (D) Inflammatory foci (arrows) are seen to be scattered in the hepatic parenchyma. (B) focal inflammation revealed in an infected WT mouse (arrow). (E) An AhR-KO mouse displayed moderate degeneration of hepatocytes, accumulation of leukocytes in the lumen of the blood vessel (asterisk), and inflammatory infiltrates (arrow). (C) Infected WT mice exhibited moderate and diffuse hepatocellular degeneration. (F) Focal necrosis (asterisk) partially surrounded by mononuclear cells (arrow) in an AhR-KO mouse. Note the mild Kupffer cell hyperplasia and deposition of malarial pigment (inset). Magnifications, ×200 (A to F) and ×400 (inset). (G to J) At the indicated time points, the following parameters were assessed: liver inflammation (G) and injury (H), ALT levels in serum (I), and the iron concentration in serum by a ferrozine-based colorimetric assay (J). (K to P) Leukocytes were harvested from WT and AhR-KO mouse livers at 0 and 6 dpi, followed by detection of $CD4^+$ IFN-γ^+ (K), $CD8^+$ IFN-γ^+ (L), $CD4^+$ TNF-α^+ (M), $CD4^+$ IL-10^+ (N), $CD4^+$ IL-17^+ (O), and $CD4^+$ $CD25^+$ $FOXP3^+$ (P) cells by flow cytometry. (Q, R) The $CD4^+$ T cell numbers (infected WT and AhR-KO mice) found by flow cytometry are presented as pie graphs and were used for comparison of the profile/proportion of Th cell responses: Th1 ($CD4^+$ IFN^+), Th17 ($CD4^+$ IL-17^+), and Treg ($CD4^+$ $CD25^{high}$ $FOXP3^+$) cells. The data are representative of those from two independent experiments (5 mice/group) and are shown as the mean ± SEM. *, $P < 0.05$ for the comparison of *P. berghei* Anka-infected WT mice versus AhR-KO mice.

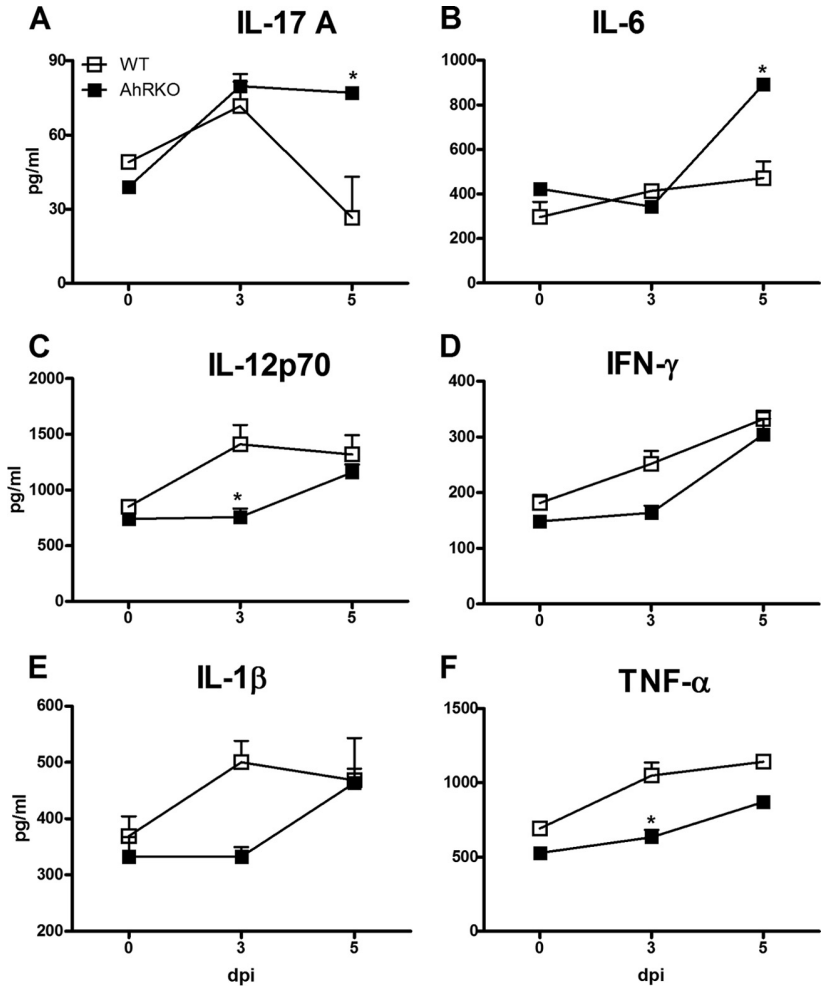

FIG 4 Increased production of IL-17A and IL-6 in the brain of *P. berghei* Anka-infected AhR-KO mice. WT and AhR-KO animals were infected i.p. with 10^5 parasitized erythrocytes. Brain was harvested and homogenized, and the kinetics of expression of the following cytokines were measured by ELISA: IL-17A (A), IL-6 (B), IL-12p70 (C), IFN-γ (D), IL-1β (E), and TNF-α (F). The data are representative of those from two independent experiments (5 mice/group) and are shown as the mean ± SEM. *, $P < 0.05$ for the comparison of infected WT versus AhR-KO mice.

are more susceptible to *P. berghei* Anka infection, displaying early neurological signs such as ataxia, paralysis, seizure, rollover, and coma. These mice also display anemia, increased parasitemia, mortality, and significant pathological changes in the brain and liver compared with infected WT mice. *P. berghei* Anka-infected AhR-KO mice displayed increases in serum levels of both pro- and anti-inflammatory mediators, including IL-2, IL-17, IL-6, TNF-α, and IL-10, which are likely associated with the defects in the generation/expansion of T and Treg cells found in the thymus and spleen. These observations underscore a role for the AhR in the control of infection.

A successful response to malaria requires a timely and proportional release of proinflammatory cytokines to minimize parasitemia, while anti-inflammatory cytokines counteract the high level of inflammatory cytokines to control the inflammatory process and minimize the pathology (56). *Plasmodium* infection is associated with increased levels of cytokines in the blood and brain tissue (10, 56–58). The high levels of proinflammatory cytokines observed in *P. berghei* Anka-infected AhR-KO mice could induce vascular cell adhesion molecule expression, promoting recruitment of leukocytes to the brain microvasculature and thus contributing to neurovascular endothelial damage and BBB disruption (4, 9, 11, 17, 27, 28, 59).

Intravital microscopy demonstrated that a deficiency of AhR resulted in significant increases in infection-induced rolling and adherent cells in the pia mater vasculature, hemorrhagic foci, and the levels of NO in brain tissue. These may contribute to the disruption of the BBB observed at 6 dpi. These alterations in the BBB during CM could allow cytokines and malaria parasite antigens to enter the brain, from which they are normally excluded, leading to activation of microglia and/or damage to astrocytes (17). Basal levels of some inflammatory cytokines were detected in the brain of uninfected WT and AhR-KO mice, but the levels were markedly enhanced after disease induction. Low levels of cytokines in the brain under basal conditions have also been found in other studies and may reflect normal brain physiology. Indeed, cytokines such as TNF-α, IL-1β, and IL-6 not only are involved in the immune response to infection and/or stress but also have critical roles in normal brain functioning (60). For example, TNF-α is produced by the resident immune cells of the central nervous system,

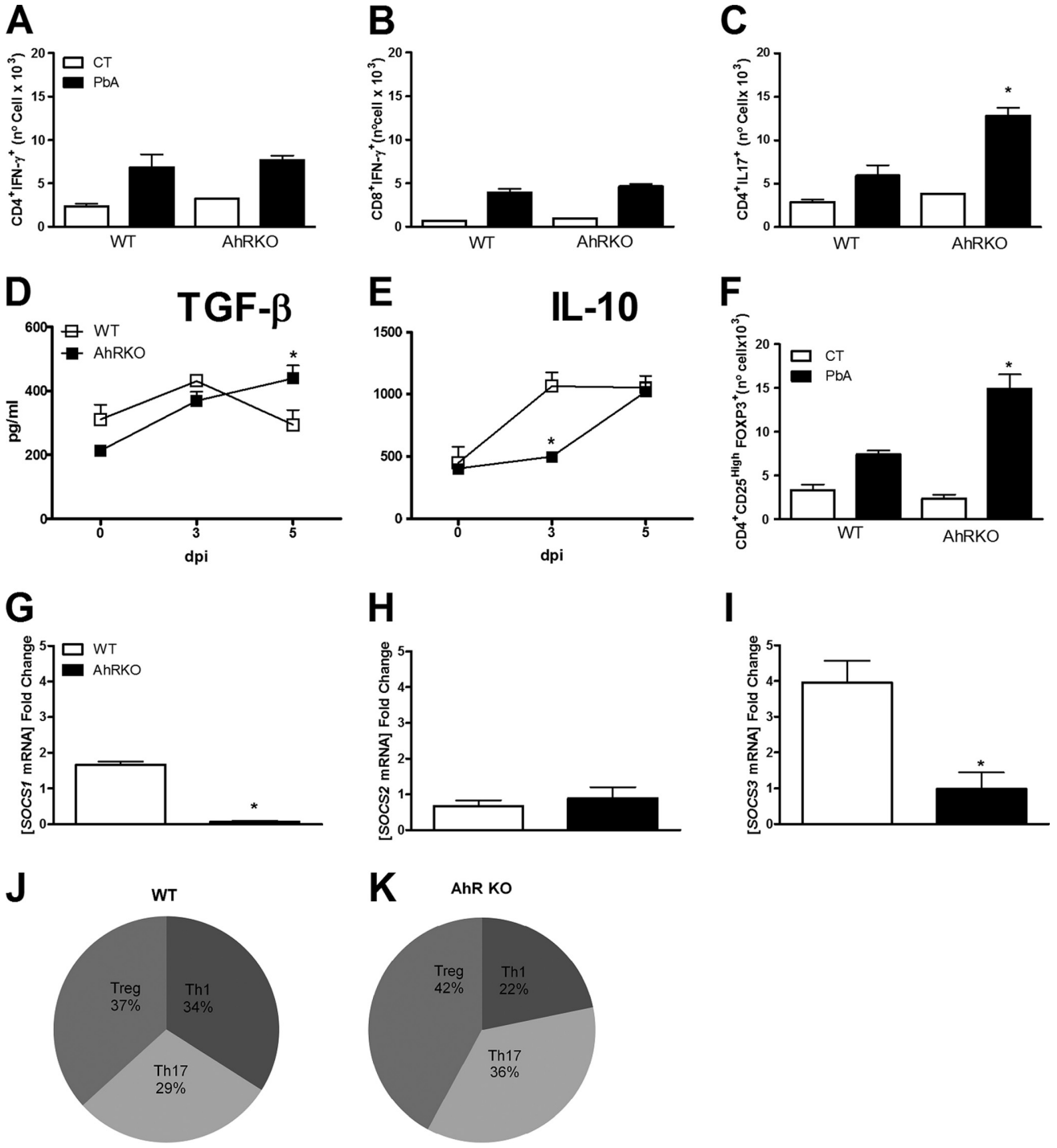

FIG 5 AhR modulates the production of anti-inflammatory mediators in the brain during *P. berghei* Anka infection. WT and AhR-KO mice were infected by the i.p. route with 10^5 parasitized erythrocytes. (A to C) Leukocytes were harvested from WT and AhR-KO mouse brain at 0 and 6 dpi, followed by detection of $CD4^+$ IFN-γ^+ (A), $CD8^+$ IFN-γ^+ (B), and $CD4^+$ IL-17^+ (C) cells by flow cytometry. (D, E) Brains were harvested and homogenized, and the kinetics of the expression of TGF-β (D) and IL-10 (E) were measured by ELISA. (F) Leukocytes were harvested from WT and AhR-KO mouse brains at 0 and 6 dpi, followed by detection of $CD4^+$ $CD25^+$ $FOXP3^+$ cells by flow cytometry. (G to I) Brains were harvested from control and infected (6 dpi) WT and KO mice, followed by RNA extraction and quantitative reverse transcription-PCR using primers specific for SOCS1 (G), SOCS2 (H), and SOCS3 (I). Data were normalized to those for uninfected WT or AhR-KO mice. (J, K) The $CD4^+$ T cell numbers (infected WT and AhR-KO mice) were recorded by flow cytometry and are represented as pie graphs used for comparison of the profile/proportion of Th cell responses: Th1 ($CD4^+$ IFN^+), Th17 ($CD4^+$ IL-17^+), and Treg ($CD4^+$ $CD25^{high}$ $FOXP3^+$) cells. The results are presented as the mean fold change compared to the level of expression in infected WT versus AhR-KO mice. *, $P < 0.05$ for the comparison of infected WT mice versus infected AhR-KO mice. Data are shown as the mean ± SEM and are representative of those from two independent experiments with at least five animals per group.

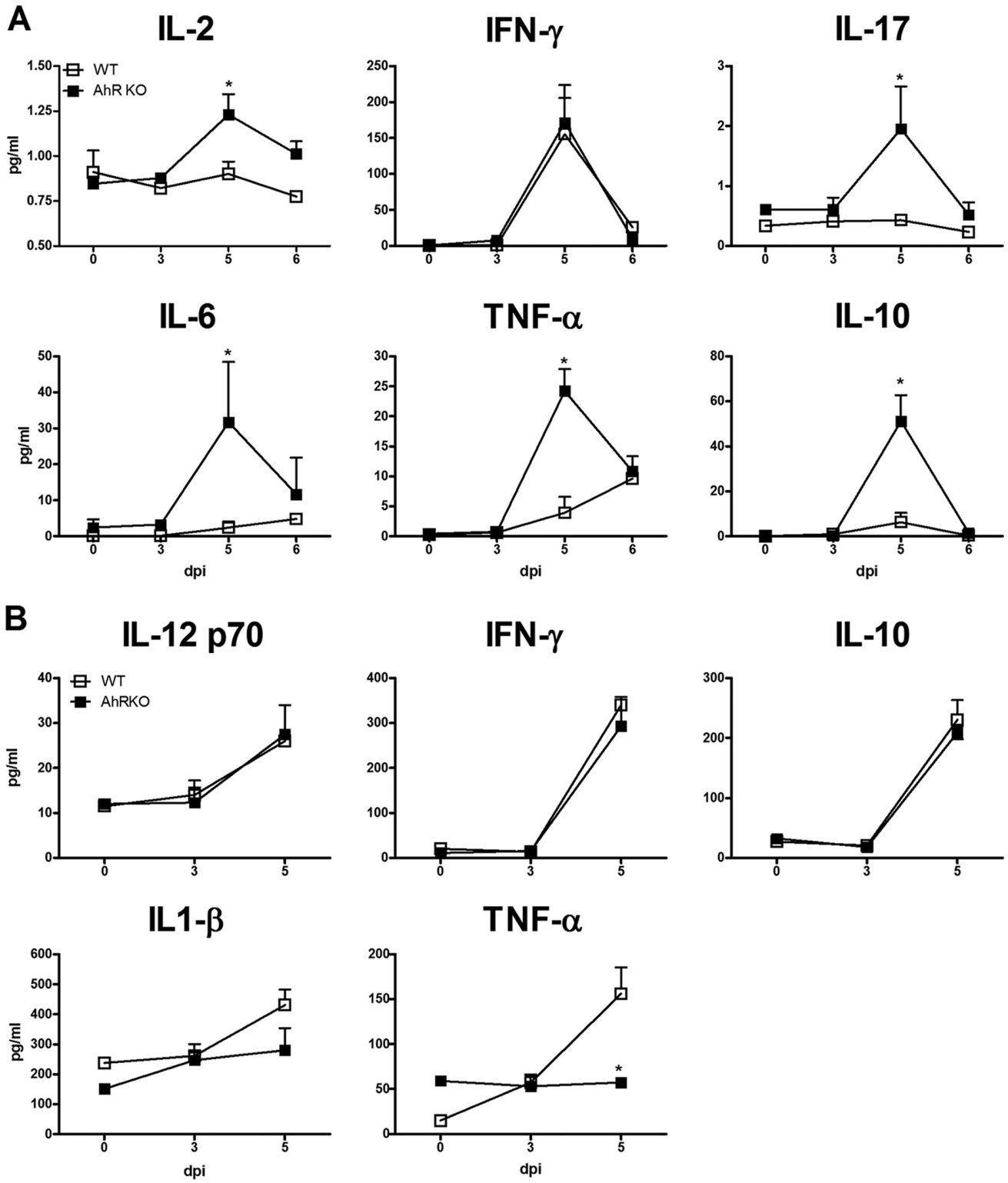

FIG 6 Systemic and splenic modulation of the inflammatory response in *P. berghei* Anka-infected AhR-KO mice. WT and AhR-KO animals were infected i.p. with 10^5 parasitized erythrocytes. (A) Serum cytokine levels were determined by CBA at the indicated time point. (B) Spleens were harvested and homogenized, and the kinetics of IL-12p70, IFN-γ, IL-10, IL-1β, and TNF-α cytokine expression were measured by ELISA. Data are shown as the mean ± SEM and are representative of those from two independent experiments with at least five animals per group. *, $P < 0.05$ for the comparison of *P. berghei* Anka-infected WT versus AhR-KO mice.

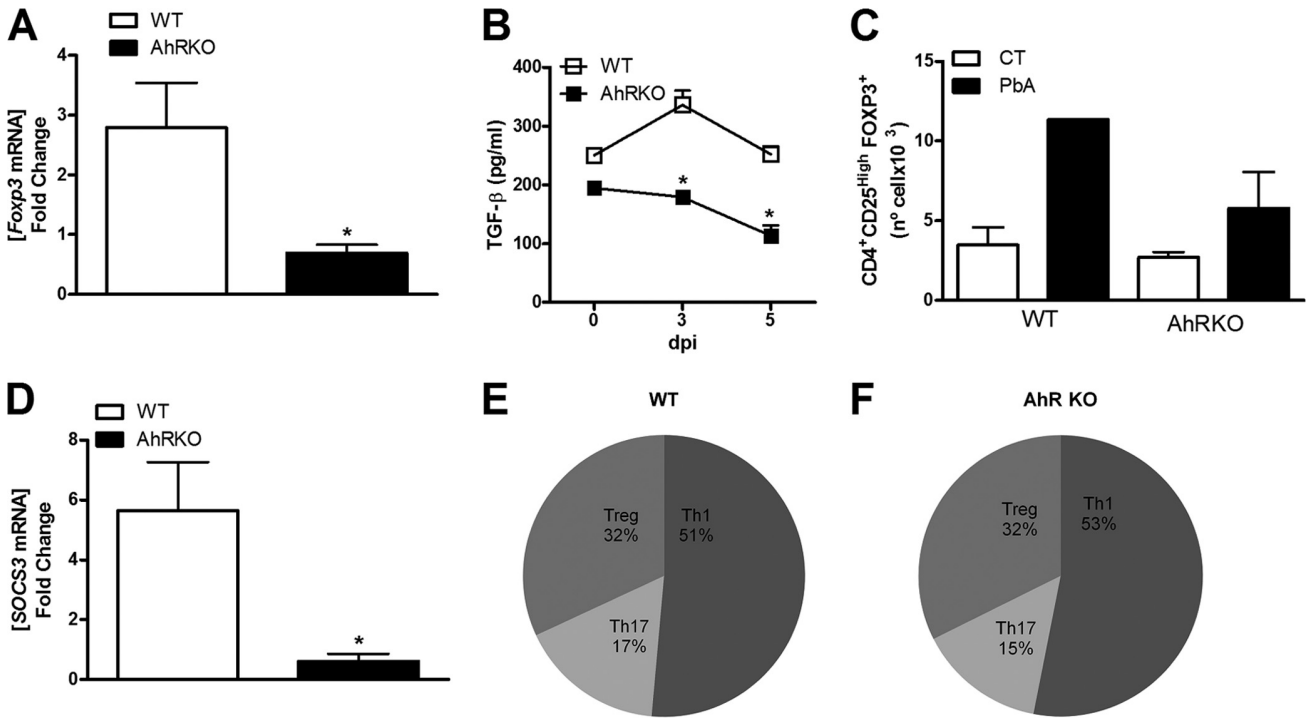

FIG 7 Modulation of TGF-β and SOCS3 in the spleen by AhR during *P. berghei* Anka infection. WT and AhR-KO mice were infected i.p. with 10^5 parasitized erythrocytes. (A) Splenocytes were harvested from control and infected WT and AhR-KO mice at 6 dpi, mRNA was extracted, and quantitative reverse transcription-PCR was performed using primers specific for FOXP3. Data were normalized to those for uninfected WT or AhR-KO mice. (B) Spleens were harvested and homogenized, and the kinetics of TGF-β expression were measured by ELISA. (C) Splenocytes were harvested from control and infected WT and AhR-KO mice at 6 dpi, followed by detection of $CD4^+$ $CD25^+$ $FOXP3^+$ cells by flow cytometry. (D) Splenocytes were harvested from control and infected WT and AhR-KO mice at 6 dpi, RNA was extracted, and quantitative reverse transcription-PCR was performed using primers specific for SOCS3. Data were normalized to those for uninfected WT and AhR-KO mice. (E, F) The $CD4^+$ T cell numbers for infected WT and AhR-KO mice found by flow cytometry are represented as pie graphs used for comparison of the profile/proportion of Th cell responses: Th1 ($CD4^+$ IFN^+), Th17 ($CD4^+$ $IL\text{-}17^+$), and Treg ($CD4^+$ $CD25^{high}$ $FOXP3^+$) cells. The results are presented as the mean fold change compared to the level of expression for infected WT versus AhR-KO mice. Data are shown as the mean ± SEM and are representative of those from two independent experiments. *, $P < 0.05$, for the comparison of infected WT versus infected AhR-KO mice.

microglia and astrocytes (61), and is essential to maintenance of synaptic scaling, and therefore, it has a role in learning and memory formation (62). Whether cytokines exert physiological or pathological effects depends on their amount and on the spatial and temporal pattern of their production (63).

P. berghei Anka-infected AhR-KO mice displayed marked anemia and increased levels of iron in serum, whereas the infected WT mice were able to reestablish the physiological levels of iron in serum. Iron is required for the proliferation of *Plasmodium*, which then utilizes diverse mechanisms to acquire iron from hosts. Nevertheless, the precocious higher level of parasitemia found in the AhR-deficient mice could not only be attributed to increased iron, since it was significantly higher than that in WT mice only at 6 dpi. However, the liver damage observed in *P. berghei* Anka-infected AhR-KO mice could be responsible, in part, for the severe hemolysis observed in these mice, which could then generate free hemoglobin that, in the presence of reactive oxygen species (ROS), would readily be oxidized, releasing free heme, a molecule cytotoxic to the host and parasite (9, 18, 64, 65). Likewise, previous studies have shown that AhR has an endogenous role in the physiology and homeostasis of the liver, preventing the development of fibrosis. Mice lacking AhR had a variable degree of hepatic fibrosis (minimal to mild portal fibrosis) without an inflammatory stimulus. The development of severe hepatic fibrosis is common only in older AhR-KO mice (9 to 11 months of age) (66–68). Infected AhR-KO mice displayed increased serum levels of ALT as well as hemozoin deposits and inflammation and necrosis in the liver, indicating hepatic damage more severe than that in WT mice. The liver injury induced by *P. berghei* Anka appears to be caused by the inflammatory response driven by the accumulation of parasites in this organ (69). In fact, we observed a greater number of infiltrating $CD4^+$ $IFN\text{-}\gamma^+$ and $CD4^+$ $IL\text{-}10^+$ cells and a markedly enhanced number of $CD4^+$ $TNF\text{-}\alpha^+$ cells in the liver of infected AhR-KO, suggesting the profile of the inflammatory response which contributed to the liver damage observed in these mice.

A balance between protective immunity and immune-mediated pathology is observed in CM. Recent studies have investigated the role of Treg cells in the regulation of the immune response against *Plasmodium*. Treg cells suppress the Th1 response, preventing *Plasmodium* elimination both in humans and in experimental models (31, 70). In *P. berghei* Anka infection, Treg cells contribute to pathogenesis by modulating the balance of pro- and anti-inflammatory responses (33). The combined induction of Treg and Th1 cells appears to follow a carefully orchestrated immune response, where insufficient or excessive induction of Treg

cells at different time points during infection may disrupt this balance and contribute to severe disease (33, 71). *P. berghei* Anka-infected AhR-KO mice displayed increased levels of TGF-β in the brain (30, 71). In AhR-KO mice, there was a decrease in $CD4^+$ $CD25^{high}$ $FOXP3^+$ cells in the thymus and a higher frequency of $CD4^+$ $CD25^{high}$ $FOXP3^+$ cells in the brain compared with the findings for WT mice. While Treg cells have been involved in the regulation of the immune response to malaria, the precise mechanism by which Treg cells modulate cerebral pathology is still unclear. In different models of infection, Treg cells have been described to be important in the modulation of T cell responses (52, 54, 72). During CM, Treg cells participate in the modulation of T cell recruitment to the brain. $CD8^+$ as well as $CD4^+$ T cells have been shown to contribute to CM pathogenesis (30). $CD8^+$ T cells accumulated in the brain just prior to the onset of signs and are believed to be the primary effectors of experimental CM (73). However, brain sequestration of $CD8^+$ T cells is not sufficient for the development of CM in C57BL/6 mice, and the concomitant presence of parasitized red blood cells appears to play an important role in the onset of neuropathology (27, 28). Our results indicate that a deficiency of AhR does not alter the number of $CD8^+$ T cells and $CD4^+$ T cells accumulated in the brain but results in a higher frequency of Th17 and Treg cells.

The balance between Treg and Th17 cells is essential for immune homeostasis. IL-6, together with TGF-β, has an important role in Th17 cell generation (74), and our results indicate that AhR could participate in this process as a regulator of Th17 as well in Treg cell differentiation (37, 40, 54). TGF-β is necessary for the induction of both Th17 and Treg cells in naive $CD4^+$ T cells. The choice of this response is controlled, in part, by IL-6, which prevents the expression of FOXP3 and initiates the Th17 profile (75). A deficiency of AhR in brain resulted in the increased levels of IL-6 and TGF-β. This change was associated with higher numbers of $CD4^+$ $IL\text{-}17^+$ cells in the brain and an induction of Th17 cell differentiation/IL-17 production during *P. berghei* Anka infection. However, one study using mice that failed to generate an IL-17 profile suggested no role for IL-17 in the development of CM (76). Our results demonstrate that in the absence of AhR, one can still find a generation/stimulation Th17 environment with a greater number of $CD4^+$ $IL\text{-}17^+$ cells infiltrated into brain and the production of higher levels of IL-17. In the liver of infected mice, the absence of AhR resulted in an increased Th1 response compared with that in WT mice. Therefore, the absence or reduction of AhR expression/activity during *P. berghei* Anka infection could be one reason for the inappropriate proportion of Th1/Th17/Treg cell responses. This suggests that the activation of AhR has an important role in the pathogenesis of the immune response.

Infected AhR-KO mice also displayed decreased levels of SOCS1 and SOCS3. These data suggest that during *P. berghei* Anka infection, IL-17 and IL-6 production in the brain could activate astrocytes, contributing to Th17 differentiation, a process modulated by SOCS3. To this end, a recent study demonstrated that astrocytes can serve as a target of Th17 cells and IL-17 in the central nervous system and that SOCS3 participates as a negative regulator of IL-17 functions in this tissue, reinforcing our hypothesis (77). Further experiments are under way in our laboratory to confirm this hypothesis in the setting of malaria.

Our data demonstrate that AhR activation modulates cytokine expression during murine *P. berghei* Anka infection. AhR has been regarded as a potential regulator of the immune response (35–37, 39, 54, 78, 79), playing various roles in the differentiation of Th cells, which are differently regulated, depending on their activating ligands (39, 40, 42, 55). For example, the tryptophan photoproduct 6-formylindolo[3,2-*b*]carbazole and kynurenine, endogenous AhR ligands, increase only the Th17 population (37, 40), while 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a high-affinity exogenous AhR ligand, appears to expand only the Treg cell population (preventing experimental autoimmune encephalomyelitis) (42). Additionally, we demonstrated that AhR expression is associated with modulation of the expression of SOCS, a well-known class of molecules contributing to the regulation of cytokine receptor signaling pathways important in the modulation of the immune response against infection (46, 52). In this regard, SOCS1 and SOCS3 expression counteracts the actions of IFN-γ and IL-6 (46, 52, 77), which play important roles in the pathogenesis of CM. Since there was a reduction in the expression of SOCS1 and SOCS3 in infected AhR-KO mice, one would expect that the inflammatory milieu found in the infected brain would have a greater ability to induce CM. However, the precise intracellular role of SOCS as a modulator of CM development still needs to be identified. Currently, we are conducting studies to clarify the role of AhR in the modulation of SOCS3 expression and its participation in the generation/differentiation of Th17 and Treg cells and the pathology resulting from *P. berghei* Anka infection.

In summary, the current report demonstrates the importance of AhR in the modulation of immune responses and development of disease during *P. berghei* Anka infection. AhR appears to contribute significantly to the control of parasite replication and the modulation of inflammatory responses, likely via controlling the expression of crucial pathways, such as the SOCS1 and SOCS3 pathways.

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2.2 – Artigos não relacionados diretamente ao tema da tese, mas publicados durante o período do doutorado

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UNIVERSIDADE FEDERAL DE MINAS GERAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE - INFECTOLOGIA E MEDICINA TROPICAL

UFMG

ATA DA DEFESA DE TESE DA ALUNA
ALINE SILVA DE MIRANDA

Realizou-se, no dia 27 de fevereiro de 2015, às 08:00 horas, Sala 029 - andar térreo da Faculdade de Medicina, da Universidade Federal de Minas Gerais, a 128ª defesa de tese, intitulada *"Estudo dos mecanismos associados à disfunção cognitiva em modelo murino de malária cerebral."*, apresentada por ALINE SILVA DE MIRANDA, número de registro 2011673067, graduada no curso de FISIOTERAPIA, como requisito parcial para a obtenção do grau de Doutor em Ciências da Saúde, pelo Programa de Pós-Graduação em CIÊNCIAS DA SAÚDE - INFECTOLOGIA E MEDICINA TROPICAL, à seguinte Comissão Examinadora: Prof. Antonio Lucio Teixeira Junior - Orientador (UFMG), Profa. Milene Alvarenga Rachid (UFMG), Prof. Eduardo Antonio Ferraz Coelho (UFMG), Prof. Antonio Carlos Pinheiro de Oliveira (UFMG), Prof. Leonardo José de Moura Carvalho (Fiocruz) e Prof. Fabio Trindade Maranhão Costa (UNICAMP).

A Comissão considerou a tese:

(X) Aprovada

( ) Reprovada

Finalizados os trabalhos, foi lavrada a presente ata que, lida e aprovada, vai assinada pelos membros da Comissão.

Belo Horizonte, 27 de fevereiro de 2015.

Prof. Antonio Lucio Teixeira Junior

Profa. Milene Alvarenga Rachid

Prof. Eduardo Antonio Ferraz Coelho

Prof. Antonio Carlos Pinheiro de Oliveira

Prof. Leonardo José de Moura Carvalho

Prof. Fabio Trindade Maranhão Costa