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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