Journal of Neuroimmunology 330 (2019) 5–11

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Short communication

T-lymphocytes response persists following *Plasmodium berghei* strain Anka infection resolution and may contribute to later experimental cerebral malaria outcomes

Aline Silva de Miranda[a,b,*], Rodrigo Novaes Ferreira[a,b], Érica Leandro Marciano Vieira[b], Larissa Katharina Sabino Abreu[a], Fátima Brant[c], Luciene Bruno Vieira[d], Fabíola Mara Ribeiro[c], Fabiana Simão Machado[c], Milene Alvarenga Rachid[e], Antônio Lúcio Teixeira[f]

[a] *Laboratory of Neurobiology, Department of Morphology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil*
[b] *Interdisciplinary Laboratory of Medical Investigation, School of Medicine, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil*
[c] *Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil*
[d] *Department of Pharmacology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil*
[e] *Department of Pathology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil*
[f] *Neuropsychiatry Program, Department of Psychiatry and Behavioral Sciences, McGovern Medical School, University of Texas Health Science Center at Houston, USA*

ARTICLE INFO

Keywords:
Malaria
Cerebral malaria
Chloroquine
MK801
T lymphocytes
Glutamate

ABSTRACT

Several studies have proposed cerebral malaria (CM) as a CD4+ and CD8+ T lymphocyte-mediated disease. However, there are no data regarding the recruitment and/or persistence of these cells in the CNS following the phase of infection resolution. Glutamate-mediate excitotoxicity has also been implicated in CM. Blockade of glutamate NMDA receptors by its noncompetitive antagonist MK801 modulates cytokine and neurotrophic factors expression preventing cognitive and depressive-like behavior in experimental CM. Herein, we aim to investigate the role of T lymphocytes in later outcomes in CM, and whether the protective role of MK801 is associated with T lymphocytes response.

1. Introduction

Malaria is a life-threatening infectious disease and a major socioeconomic burden in endemic areas in Africa, Asia and Central and South America (Phillips et al., 2017). *Plasmodium falciparum* infection has been associated with severe complications of the disease, which includes an acute brain form known as cerebral malaria (CM). Even with antimalarial treatment, CM may lead to severe consequences, such as coma and ultimately, death in approximately 20% of cases (Bartoloni and Zammarchi, 2012; Hunt et al., 2014). CM has also been associated with short and long term cognitive and behavioral impairments (Dugbartey et al., 1998; Idro et al., 2010a,b).

The cellular and molecular mechanisms underlying CM pathogenesis remain to be fully understood. In this regard, two main hypotheses have been postulated: (1) neural injury secondary to the obstruction of capillary blood flow by parasitized red blood cells, and (2) neural damage induced by the inflammatory response against the parasite in the central nervous system (CNS) (Hunt et al., 2014; Martins and Daniel-Ribeiro, 2013). This neuroinflammatory response is characterized mainly by increased cytokine expression, brain endothelial cell dysfunction, leukocytes accumulation in the microcirculation, and blood brain barrier leakage (Hunt et al., 2014; Martins and Daniel-Ribeiro, 2013).

Immune responses mediated by brain sequestered T lymphocytes have been shown to contribute to CM development (Belnoue et al., 2002; Nie et al., 2007). The understanding of the mechanisms by which $CD4^+$ subsets and $CD8^+$ T cells mediate CM is also incomplete but a large body of experimental evidence suggests the involvement of the TH_1 cytokine interferon gamma (IFN-γ) as responsible for disease exacerbation (Nie et al., 2007). As a consequence of IFN-γ production, $CD8^+$ T cells adhere to activated brain endothelium and appear to participate in CM pathogenesis via a perforin-dependent manner (Craig et al., 2012; Hansen, 2012; Nitcheu et al., 2003). The roles of thes T lymphocyte have been described during the acute phase of CM, but to the best of our knowledge, there are no data regarding the recruitment and/or persistence of these cells in the CNS following the infection resolution phase.

Glutamate is the main excitatory neurotransmitter in the CNS and, under physiological concentrations, plays pivotal roles in cognitive and behavioral processes. However, under pathological conditions, high concentrations of glutamate lead to neuronal damage through an event known as excitotoxicity, which is mainly mediated by the activation of the *N*-methyl-D-

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[*] Corresponding author at: Departamento de Morfologia, ICB, UFMG, Av. Antônio Carlos, 6627, Pampulha, Belo Horizonte 31270-901, Brazil.
E-mail addresses: mirandas.aline@gmail.com, mirandaas@icb.ufmg.br (A.S. de Miranda).

https://doi.org/10.1016/j.jneuroim.2019.02.002
Received 11 January 2019; Received in revised form 4 February 2019; Accepted 7 February 2019

aspartate (NMDA) receptors (Newcomer and Krystal, 2001). We have previously shown a protective role of MK801 (dizocilpine maleate), a non-competitive NMDA receptor antagonist, against experimental CM (ECM)-associated cognitive decline and depressive-like behavior. The MK801 neuroprotection was associated with the up-regulation of neurotrophic factors and increased percentage of microglia cells expressing IL-10, supporting the crosstalk between glutamate and the immune system in ECM pathogenesis (de Miranda, 2016).

The current study aims to investigate the role of T lymphocytes in ECM later outcomes, and whether the protective role of MK801 is associated with T lymphocyte response.

2. Material and methods

2.1. 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 Ethics Committee for Animal Research, Universidade Federal de Minas Gerais (CETEA/UFMG, Permit Protocol Number 105/09). All tissue collection was performed under ketamine/xylazine anesthesia and all efforts were made to minimize animal suffering.

2.2. Animals

Female C57BL/6 mice (20–25 g), 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 six mice per cage in a room-controlled temperature (25 °C) with food and water ad libitum.

2.3. Study design

The *Plasmodium berghei* strain ANKA (PbA) infection, behavior and treatment protocols were carried out as previously described (for a complete description see: de Miranda, 2016). Mice were randomly assigned into five groups (control (CT) + saline (SAL); control (CT) + chloroquine (CQ); control (CT) + CQ + MK801; PbA + CQ, and PbA + CQ + MK801).

2.4. Central nervous system leukocytes analysis by flow cytometry

After 10 days of cessation of CQ treatment, brain tissues were collected and processed for immune cells profile analysis by flow cytometry. Brain tissues of controls and treated PbA-infected mice ($n = 5$ per group) were carefully removed after intracardiac perfusion with PBS to remove all circulating red blood cells (RBCs) and leukocytes from the brain. Leukocytes in the CNS were isolated and quantified as described by Brant et al., 2014. Briefly, 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 $\times g$ for 10 min. The pellet was resuspended in 35% Percoll gradient (Sigma-Aldrich), and this was deposited on a 70% Percoll gradient. After centrifugation (1100 $\times g$), 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 post-treatment with brefeldin A (10 μg/mL), the cells were fixed and stained with labeled mouse-specific anti-bodies against CD3 (PE-Cy7), CD4 (Pacific Blue, PB), CD8 (PerCP), IL-10 (APC), IFN-γ (PE), TNF-α (APC-CY7) and isotype controls (all from BD Pharmingen, San Diego, CA). For each sample, 50,000 events from the leukocytes population were scored. The frequency of positive cells was analyzed using a gate that included myeloid cells. Limits for the quadrant markers were always set based on negative populations and isotype controls. Data were acquired on a FACSCanto II flow cytometer (Becton, Dickinson) and analyzed by FlowJo (version 7.6) software (Tree Star, Inc., Ashland, OR, USA). Analysis in FlowJo software took into account size (forward light scatter) and granularity (side light scatter) of populations. Frequency of an analyzed population in front of total acquired events was used in the construction of graphs. Representative dot plot and histogram of analysis strategy are shown in Fig. 1A–D.

2.5. Data analysis

Results obtained were presented as mean ± standard deviation (SD). All data were tested for normal distribution by employing the Shapiro-Wilk test. For normally distributed variables, differences were compared by analysis of variance (ANOVA). Bonferroni post-test was used as needed for multiple comparisons. In case of non-normally distributed variables, differences were analyzed by the Kruskal-Wallis nonparametric test. The significance level was set at $p < .05$. Statistical analyses were performed using Prism 5 software (GraphPad, La Jolla, CA, USA).

3. Results

We investigated, by employing flow cytometry analysis, a potential role for lymphocytes in ECM later outcomes following infection resolution by CQ and whether the protective effect of MK801 is associated with T lymphocyte response. We have previously shown that PbA-infected mice or infected animals treated only with MK801 died between days 5 and 6 post- infection. Importantly, survival rate was similar in both MK801 + CQ and CQ- treated mice following PbA infection. Nevertheless, MK801 + CQ treatment was able to prevent impairment in the object recognition test seen in the CQ-treated mice following PbA infection resolution. No significant differences were found between control groups, indicating that MK801 and CQ per se did not influence cognitive performance (CT + SAL = 66.40% ± 11.83;CT + CQ = 65.17% ± 20.68;CT + CQ + MK801 = 67.17% ± 11.18;PbA + CQ = 20.57% ± 12.62;PbA + MK801 + CQ = 60.63% ± 19.96; where $p < .05$ when comparing PbA + CQ with controls and Pba-mice receiving the combination of MK801 + CQ). In the forced swimming test, PbA-mice treated only with CQ also presented depressive-like symptoms compared with CT + CQ and PbA + CQ + MK801 groups, as indicated by a higher immobility time (seconds) during the task (CT + SAL = 88.17 ± 32.88;CT + CQ = 76.71 ± 21.34;CT + CQ + MK801 = 73.38 ± 13.32;PbA + CQ = 114.3 ± 49.81;PbA + MK801 + CQ = 70.88 ± 40.39, where $p < .05$). Altogether these results support glutamate-based strategies as promising in ECM (de Miranda, 2016).

Flow cytometry analysis showed that PbA-infected mice treated with CQ or MK801 + CQ presented a significant decrease in the percentage of total lymphocytes cells ($CD3^+$) compared with their respective uninfected controls (Fig. 1E).

PbA-infected mice treated with CQ showed a significant decrease in the percentage of $CD4^+$ T lymphocyte cells expressing the cytokine IL-10 compared with non-infected animals. Infected mice treated with MK801 + CQ presented a significant increase in the percentage of $CD4^+IL\text{-}10^+$ T cells compared with infected animals treated with CQ (Fig. 2A). Moreover, infected mice receiving CQ presented a decrease in the percentage of $CD4^+$ T lymphocyte cells expressing the cytokine IFN-γ compared with non-infected animals (Fig. 2B). A significant decrease in the percentage of $CD4^+TNF^+$ T cells in infected mice treated with MK801 + CQ was found compared to non-infected controls (Fig. 2C).

Regarding $CD8^+$ T lymphocyte cells, a significant decrease was observed in the percentage of these cells expressing IL-10, IFN-γ and TNF in the brain of PbA-infected mice treated with MK801 + CQ compared with non-infected animals (Fig. 3A–C). Infected animals receiving only CQ presented a significant decrease in the percentage of $CD8^+$ T cells expressing TNF compared with their respective controls.

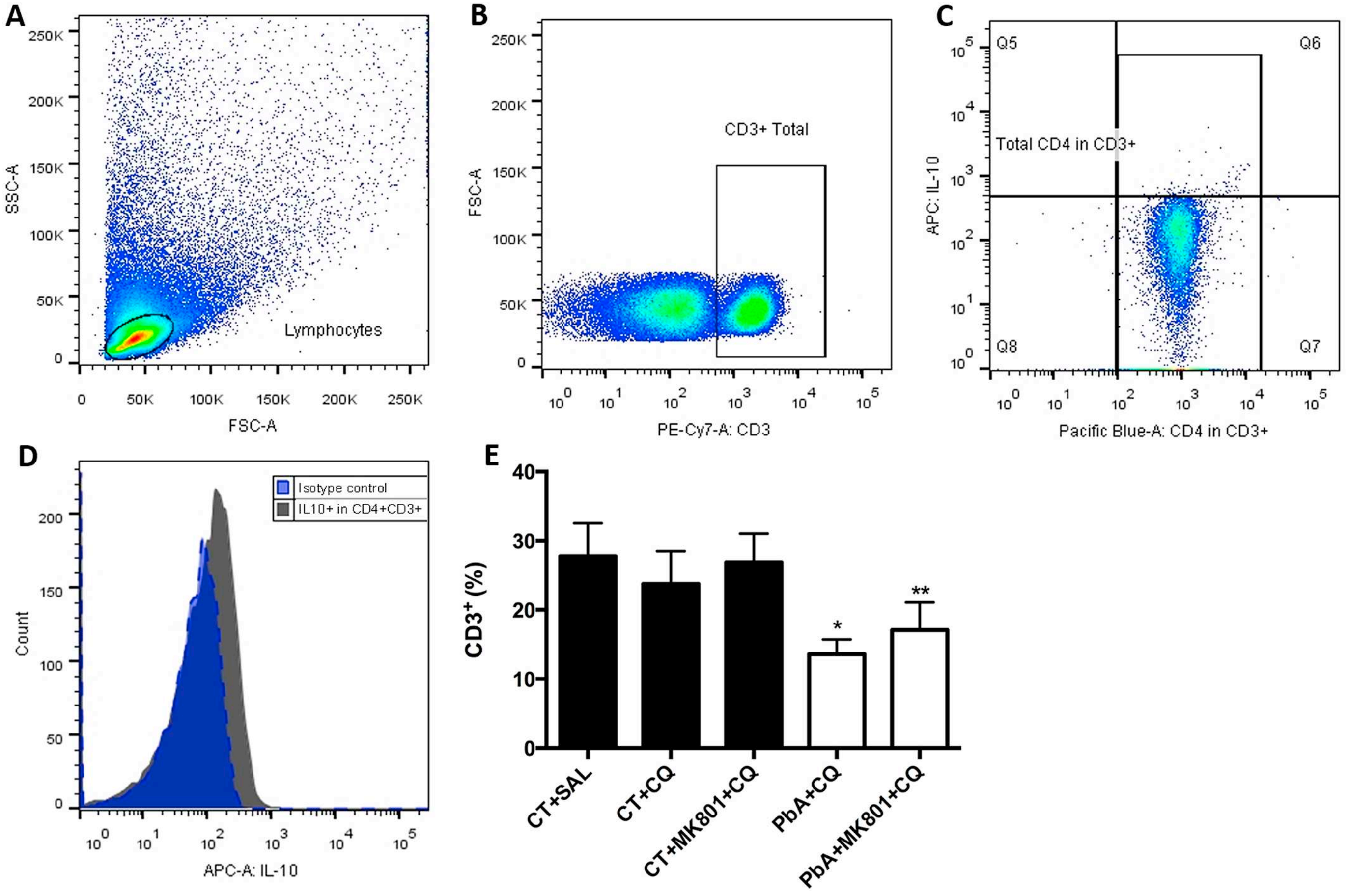

Fig. 1. Representative flow cytometry analysis strategy. After 10 days of cessation of CQ treatment, the brains were collected and processed for immune cells profile analysis by flow cytometry. Mice were randomly assigned into five groups: control + saline (CT + SAL); control + chloroquine (CT + CQ); control that received chloroquine and MK801 (CT + MK801 + CQ); PbA-infected mice treated with chloroquine (PbA + CQ), and PbA- infected mice treated with both chloroquine and MK801 (PbA + MK801 + CQ). (A) Total lymphocytes were gated and (B) fluorescent dot-plots were selected for T-lymphocytes ($CD3^+$). (C) Double-positive gates were used to verify $CD4^+$ T cells subset expressing, for example IL-10. (D) Staining for IL-10 was analyzed through histogram (blue/left: isotype control; gray/right: positive cells for IL-10) based on the $CD3^+CD4^+$ cells gate. (E) MK801 effects in the percentage of $CD3^+$ T lymphocytes following Plasmodium berghei ANKA (PbA) infection resolution by chloroquine (CQ) therapy. After 10 days of cessation of CQ treatment, the brain tissues were collected and processed for immune cells profile analysis by flow cytometry of $CD3^+$ T cells (E). Results are expressed as mean ± SD and are representative of at least two independent experiments ($n = 5$ *per group*). * indicate statistical differences regarding respective controls, where *$p < .05$, **$p < .01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The percentage of $CD8^+$ T cells expressing TNF was higher in the brain of PbA-infected mice treated with CQ compared with infected animals receiving MK801 + CQ (Fig. 3C).

4. Discussion

Over the past decades, several studies have proposed CM as a $CD4^+$ and $CD8^+$ T lymphocyte-mediated disease (Grau et al., 1986; Hermsen et al., 1997; Yanez et al., 1996). In general, inflammatory mediators like the cytokines IFN-γ and TNF-α have been implicated in ECM pathogenesis by promoting the accumulation of infected red blood cells as well as of immune cells like $CD8^+$ T lymphocytes in the brain microvasculature (Claser et al., 2011; Villegas-Mendez et al., 2012). On the other hand, the release of the anti-inflammatory cytokine IL-10 seems to counteract the excessive immune response, suppressing immune cells proliferation and migration, and promoting survival (Freitas do Rosario and Langhorne, 2012; Sarfo et al., 2011).

Apart from inflammatory mechanisms, glutamate-mediated excitotoxicity has also been implicated in ECM development and poor outcome (Dobbie et al., 2000; Medana et al., 2002; Miranda et al., 2010). We have previously shown that the blockade of the glutamate NMDA receptors by MK801 modulates cytokine and neurotrophic factors expression, preventing ECM-related cognitive impairment and depressive-like behavior after infection resolution with CQ therapy. Importantly, MK801 mediated-protection was associated with increased expression of IL-10 by microglia (de Miranda, 2016). Herein, we investigated a potential role of T lymphocytes in ECM later outcomes, and whether the protective role of MK801 is associated with T lymphocytes response.

PbA-infected mice treated with CQ or CQ + MK801 showed a significant decrease in total percentage of $CD3^+$ T cells compared with non-infected groups. There is evidence that CQ inhibit T lymphocyte proliferation through the induction of the cyclin-dependent kinase inhibitor p21. The effect of CQ in T lymphocytes seems to occur in a dose and antigen dependent manner (Bygbjerg and Flachs, 1986; Oh et al., 2016). Corroborating our findings, NMDA receptor antagonists like MK801 and memantine seem to be potent immune suppressors, reducing the proliferation and migration of T lymphocytes to inflammatory sites(Kahlfuss et al., 2014). Interestingly, non-infected mice treated with CQ or CQ + MK801 did not show the same suppression on total $CD3^+$ T cells, indicating that the CQ and MK801-associated immune effects might be dependent of a stimulus like the PbA infection.

We also found that the treatment with CQ alone suppressed the percentage of $CD4^+$ T cells expressing IL-10 in PbA-infected mice,

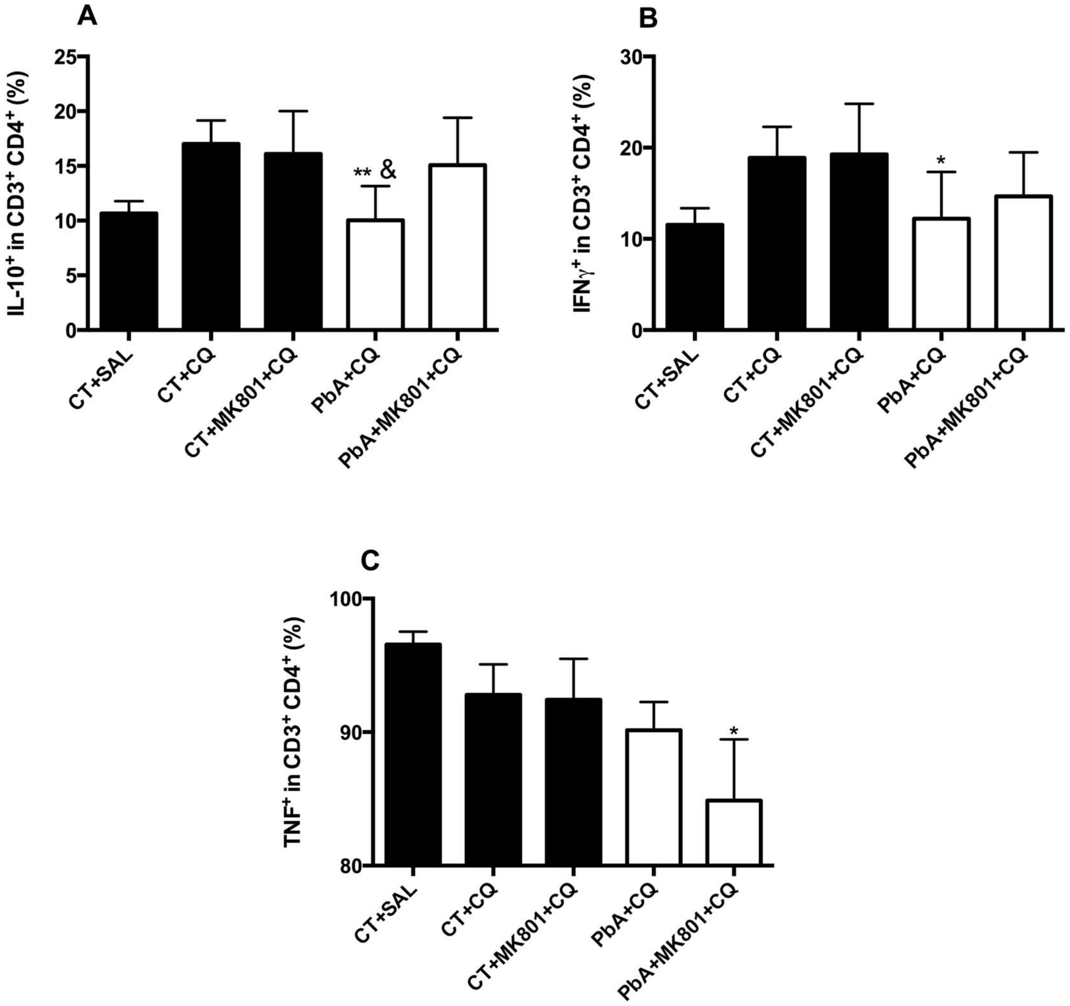

Fig. 2. MK801 effects in the percentage of $CD4^+$ T lymphocytes expressing the cytokines IL-10, IFN-γ and TNF following *Plasmodium berghei* ANKA (PbA) infection resolution by chloroquine (CQ) therapy. After 10 days of cessation of CQ treatment, the brain tissues were collected and processed for immune cells profile analysis by flow cytometry of (A) $CD3^+$, $CD4^+$, IL-10^+; (B) $CD3^+$, $CD4^+$, IFN-γ^+; and (C) $CD3^+$, $CD4^+$, TNF^+ cells. Mice were randomly assigned into five groups: control + saline (CT + SAL); control + chloroquine (CT + CQ); control that received chloroquine and MK801 (CT + MK801 + CQ); PbA-infected mice treated with chloroquine (PbA + CQ), and PbA- infected mice treated with both chloroquine and MK801 (PbA + MK801 + CQ). Results are expressed as mean ± SD and are representative of at least two independent experiments (n = 5 *per group*). * indicate statistical differences regarding respective controls, where $^*p < .05$, $^{**}p < .01$, and $^{***}p < .001$. & $p < .05$ indicate statistical differences regarding PbA-infected mice treated with CQ and PbA-infected treated with CQ + MK801.

which was restored by the MK801 adjuvant therapy. In line with this finding, MK801 enhanced the expression of IL-10 by $CD4^+$ T cells isolated from mice pooled lymph nodes (Kahlfuss et al., 2014). Specifically in ECM, the protective role of IL-10 is well known, especially during the acute phase of the disease (Amante et al., 2007; de Miranda et al., 2015; Kossodo et al., 1997; Specht et al., 2010). For instance, the production of IL-10 by $CD4^+$ T lymphocytes protected against ECM development in a co-infection model of retrovirus LP-BM5 murine leukemia virus – capable of inducing immunodeficiency – and PbA infection (Eckwalanga et al., 1994). We previously showed that the MK801 beneficial effect in ECM cognitive and behavior impairments was, at least in part, associated with an increase in the percentage of microglial cells expressing IL-10 (de Miranda, 2016). Herein, we provided further evidence that MK801 might also exert its protective effects in ECM by stimulating the expression of IL-10 by $CD4^+$ T lymphocytes. This statement is also supported by accumulating evidence showing that T-cell derived IL-10, especially $CD4^+$ T cells, plays a crucial role in down-regulating inflammatory pathology during blood-stage malaria infections (Freitas do Rosario and Langhorne, 2012).

It has been accepted that an imbalance between pro-inflammatory (IFN-γ and TNFα) and anti-inflammatory (IL-10) mediators are involved in ECM pathogenesis (Hunt et al., 2006; Hunt and Grau, 2003; Schofield and Grau, 2005). In the current study, apart from restoring the percentage of $CD4^+$ T lymphocytes expressing IL-10 in the brain of PbA-infected animals, MK801 also decreased the expression of TNFα by these same cells. The capacity of MK801 to prevent the production of TNFα was previously shown in an in vitro study with rat lymphocytes incubated with homocysteine or homocysteic acid (Vladychenskaya et al., 2011). Based on the evidence supporting the role of TNFα in ECM development and outcome (Amante et al., 2010; de Miranda et al., 2015; de Miranda et al., 2011), it is reasonable to hypothesize that the neuroprotection of MK801 also relies on its suppressive effect in the production of TNFα by $CD4^+$ T cells. The decrease in the percentage of $CD4^+$ T lymphocytes expressing TNFα might also be an indirect effect of MK801 through the stimulation of IL-10, since IL-10 can act directly on $CD4^+$ T cells, inhibiting their proliferation and production of inflammatory mediators like TNFα (Joss et al., 2000; Moore et al., 2001).

The CQ therapy alone significantly decreased the percentage of $CD4^+$ T cells expressing IFN-γ in the brain of PbA-infected mice. The inhibitory effect of CQ in $CD4^+$ T lymphocytes producing IFN-γ was reported before during the acute phase of ECM, corroborating, at least in part, our findings (Zhu et al., 2012). In an in vitro study, sustained

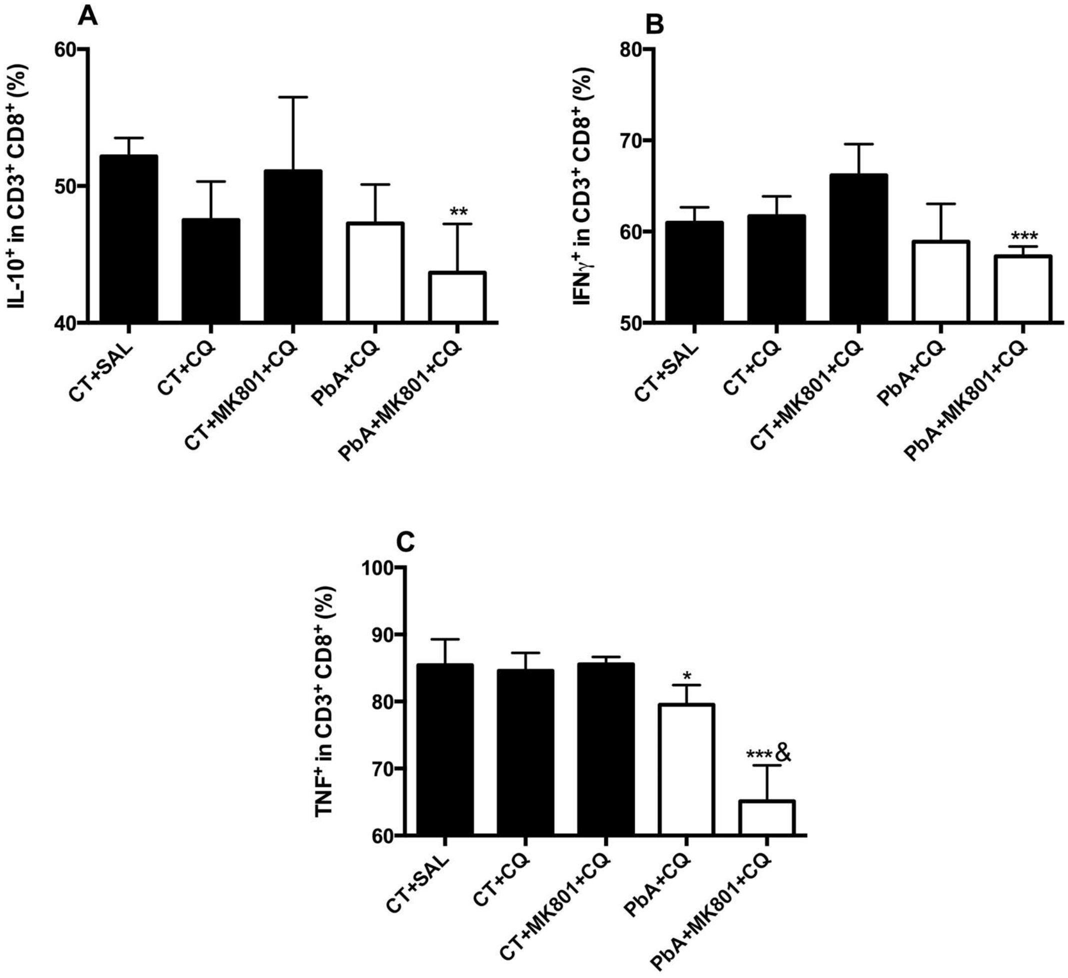

Fig. 3. MK801 effects in the percentage of $CD8^+$ T lymphocytes expressing the cytokines IL-10, IFN-γ and TNF following Plasmodium berghei ANKA (PbA) infection resolution by chloroquine (CQ) therapy. After 10 days of cessation of CQ treatment, the brain tissues were collected and processed for immune cells profile analysis by flow cytometry of (A) $CD3^+$, $CD8^+$, IL-10^+; (B) $CD3^+$, $CD8^+$, IFN-γ^+; and (C) CD3+, CD8+, TNF^+ cells. Mice were randomly assigned into five groups: control + saline (CT + SAL); control + chloroquine (CT + CQ); control that received chloroquine and MK801 (CT + MK801 + CQ); PbA-infected mice treated with chloroquine (PbA + CQ), and PbA- infected mice treated with both chloroquine and MK801 (PbA + MK801 + CQ). Results are expressed as mean ± SD and are representative of at least two independent experiments (n = 5 *per group*). * indicate statistical differences regarding respective controls, where $*p < .05$, $**p < .01$, and $***p < .001$. & $p < .05$ indicate statistical differences regarding PbA-infected mice treated with CQ and PbA-infected treated with CQ + MK801.

CQ treatment of developing T cells inhibited T cell proliferation and the differentiation of IFN-γ-producing Th1 cells (Oh et al., 2016). Although, IFN-γ has been implicated in ECM pathophysiology (Claser et al., 2011; Hunt et al., 2006), herein a decrease in IFN-γ expression by $CD4^+$ T lymphocytes following CQ treatment seems not to be sufficient to protect mice from ECM-associated later outcomes. Indeed, we have shown that CQ alone, despite improving survival, was not able to prevent object recognition memory impairment and depressive-like behavior in PbA-infected animals (de Miranda, 2016).

Emerging evidence has supported a crucial role of $CD8^+$ T-cells in ECM pathogenesis (Belnoue et al., 2008; Hermsen et al., 1997; Howland et al., 2015; Lundie et al., 2008; Miyakoda et al., 2008). $CD8^+$ T-cells might damage cerebral microvascular endothelial cells in perforin and granzyme-B dependent manners (Nitcheu et al., 2003; Potter et al., 2006). In the current study, the treatment with MK801 decreased the expression of IL-10, IFN-γ and TNF-α by $CD8^+$ T lymphocytes in the brain of PbA-infected mice after infection resolution with CQ. An in vitro study showed thatMK801 prevented granule-mediated killing of mature neurons, opening new strategies for cytotoxic T lymphocytes-mediated diseases such as CM (Malipiero et al., 1999). Further protective effects of NMDA receptors antagonists, including the MK801, on regulating $CD8^+$ T cells proliferation and migration functions were also reported (Kahlfuss et al., 2014).

In summary, we provided the first evidence that $CD4^+$ and $CD8^+$ T cells might be involved in ECM later outcomes after infection resolution with antimalarial treatment. The selective NMDA receptor antagonist, MK801, might also exert its protective effect in ECM by modulating T lymphocyte response toward an anti-inflammatory profile. Accordingly, our results reinforce the therapeutic use of NMDA antagonists as an adjunctive strategy in anti-malarial therapy to improve CM-associated cognitive and behavioral symptoms.

Author's contribution

ASM participated in the experimental design, carried out immunological assays, data analysis and drafted the manuscript. RNF carried out behavioral and immunological assays, data analysis and drafted the manuscript. ELMV participated in the experimental design, carried out behavioral and immunological assays, data analysis and drafted the manuscript. LKSA and FB carried out immunological assays and revised the manuscript. LBV and FMR carried out data analysis and revised 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.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors would like to thank FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Brazil), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for financial support. ASM is a 2016 NARSAD Young Investigator Grant Awardee from the Brain and Behavior Research Foundation.

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