

Universidade Estadual de Campinas

Rodolfo Thomé

Efeito da infecção por *Plasmodium berghei* sobre a Encefalomielite

Autoimmune Experimental

Effect of *Plasmodium berghei* infection on Experimental Autoimmune

Encephalomyelitis

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UNIVERSIDADE ESTADUAL DE CAMPINAS
Instituto de Biologia

RODOLFO THOMÉ

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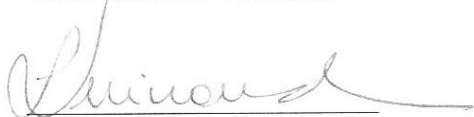
Effect of Plasmodium berghei infection on Experimental Autoimmune Encephalomyelitis

Tese apresentada ao Instituto de Biologia da
Universidade Estadual de Campinas como
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do título de Doutor em Genética e Biologia
Molecular, na área de Imunologia.

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the University of Campinas in partial
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of Doctor, in the area of Immunology.

Orientadora: Profa. Dra. Liana Maria Cardoso Verinaud

Este exemplar corresponde à versão final
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Profa. Dra. Liana Verinaud

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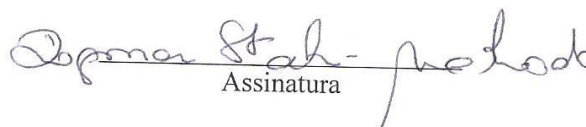
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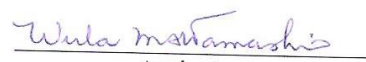
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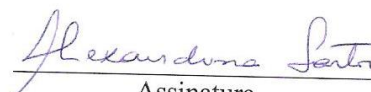
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***Digno é o Cordeiro, que
foi morto, de receber o poder,
e riquezas, e sabedoria, e
força, e honra, e glória, e
ações de graças.***

Apocalipse 5:12

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Resumo

A malária permanece como a maior doença infecciosa do mundo, sendo que metade da população mundial se encontra em risco de ser infectada. A destruição do agente causador, protozoários do gênero *Plasmodium*, é essencial para a resolução da moléstia e é mediada pela atuação coordenada entre linfócitos T e B. Foi noticiado a presença de auto-anticorpos em pacientes portadores de malária, e em alguns casos o agravamento de doenças autoimunes como o lúpus e, possivelmente, a esclerose múltipla. Estudos conduzidos em nosso laboratório demonstraram que a infecção por *P. berghei* NK65 em camundongos promoveu atrofia do timo e subsequente migração de células T CD4⁺CD8⁺ (DP) para órgãos linfoides secundários. Tendo em vista que o timo é o órgão linfóide primário responsável pela maturação e desenvolvimento de linfócitos T, desempenhando um papel primordial em gerar a tolerância central, o presente estudo teve por objetivo investigar o efeito da infecção por *P. berghei* sobre a evolução clínica da Encefalomielite Autoimune Experimental (EAE) e também sobre o perfil de ativação de células dendríticas. Os resultados obtidos mostraram que camundongos curados da malária apresentaram quadro clínico exacerbado de EAE quando comparados com animais que não entraram em contato com o plasmódio. A piora no quadro clínico de EAE se associou com a migração precoce de células T-DP para o Sistema Nervoso Central (SNC) e pela produção de citocinas inflamatórias por tais células. Camundongos resistentes a EAE desenvolveram a doença após a infecção por plasmódio, indicando que a atrofia tímica induzida pela infecção é capaz de alterar a susceptibilidade ao desenvolvimento de doenças autoimunes. Por outro lado, o tratamento de células dendríticas (DCs) com extratos derivados do plasmódio modificou seu estado de maturação/ativação para um perfil tolerogênico. A transferência adotiva de células dendríticas moduladas com extratos de plasmódio foi capaz de reduzir a EAE, bem como a neuro-inflamação do SNC pela supressão da resposta imune celular a neuro-antígenos. Tomados em conjunto, os resultados obtidos neste estudo mostram que a infecção por *Plasmodium berghei* NK65 promove alterações significativas no sistema imune que auxiliam na exacerbção da neuro-inflamação autoimune. Por outro lado, a utilização de extratos do plasmódio pode se tornar uma alternativa inovadora na modulação da inflamação por meio da transferência adotiva de células dendríticas tornadas tolerogênicas com tais extratos.

Abstract

Malaria remains as the most frequent infectious disease in the world, where half of the world population is at risk. Destruction of the causative agent, protozoan of the *Plasmodium* genus, is essential for the resolution of the disease and this is mediated by the coordinated action of both T and B lymphocytes. It has been demonstrated that malaria-bearing patients possess auto-antibodies, and in some cases, the worsening of autoimmune diseases such as lupus and multiple sclerosis. Studies, conducted by our group, have shown that *P. berghei* NK65 infection promoted thymic atrophy and the subsequent migration of CD4⁺CD8⁺ (DP) T cells towards the peripheral immune system. Since the thymus is the primary lymphoid organ responsible for the generation and maturation of T cells, playing a major role in the shaping of T cells repertoire, the present study aimed to investigate the influence of *P. berghei* infection in the clinical course of Experimental Autoimmune Encephalomyelitis (EAE), the mouse model for multiple sclerosis, and also over the maturation/activation status of dendritic cells. Results showed that malaria-cured mice developed a more severe EAE clinical course compared with control mice. The worsening in EAE score was related to the migration of DP-T cells towards the Central Nervous System (CNS), where these cells produced high amounts of inflammatory cytokines. Interestingly, EAE-resistant BALB/c mice developed the disease after plasmodia infection, indicating that the thymic atrophy induced by the infection is able to alter the susceptibility to autoimmune diseases. On the other hand, treatment of dendritic cells (DCs) with *P.berghei* extracts (PbX) modified their activation/maturation status towards a tolerogenic profile. The adoptive transfer of DC-PbX was able to suppress the development of EAE, as well as neuro-inflammation, through the reduction in cellular immune responses towards neuro-antigens. Taken together, the results collected in this study show that *Plasmodium berghei* NK65 infection promotes significant alterations in the immune system that aid the development of autoimmune neuro-inflammation. On the other hand, the use of plasmodia extracts may become an interesting approach to modulate inflammation through the adoptive transfer of tolerogenic dendritic cells.

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Dedicatória

Dedico este trabalho ao Único e Incomparável Deus.

Obrigado Senhor!

“Incomparável és pra mim

Minha história de amor com você não tem fim

Se estou triste, doente ou cansado

Sua mão toca meu rosto e eu fico curado

Amor da minha vida meu bem inigualável

Você pra mim é tudo único incomparável

Por sua causa eu atravessei atmosfera

Por sua causa eu descì até o centro da terra

Peguei de volta a chave da vida e te devolvi

E ninguém vai apagar a chama que eu próprio acendi

Por amor, foi por amor somente por amor

Pra mim você é único incomparável

É porque você existe que eu não me acabo

A sua companhia é boa e agradável

Sem você a minha vida seria insuportável”

Único Incomparável

Compositor: Pregador Luo

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Introdução

Parte das características do sistema imune é o reconhecimento e diferenciação de estruturas próprias e de não próprias dentro de um organismo. Tal reconhecimento ocorre através de receptores presentes na superfície celular. Sua interação com ligantes cognatos leva a ativação, proliferação e diferenciação celular culminando no estabelecimento da inflamação. A inflamação é importante para a eliminação de organismos invasores e de antígenos exógenos e na manutenção da homeostase. Sendo mediada por células da resposta imune inata e adaptativa, é necessário que exista um fino controle das respostas imunes inflamatórias, visto que a exacerbação da resposta imune pode levar ao surgimento de respostas contra tecidos e proteínas próprias. Tal controle é dado tanto no desenvolvimento quanto na ativação periférica dos leucócitos, as células participantes do sistema imunológico. A resposta imune específica a antígenos é precedida da seleção e estimulação de linfócitos T portando receptores capazes de reconhecer fragmentos lineares apresentados por células apresentadoras de antígenos.

Por outro lado, a invasão do organismo por agentes patogênicos, como o plasmódio (agente causador da malária), induz uma série de sinalizações que promovem a resposta imune. Contudo, evidências têm demonstrado que a ativação estimulada por plasmódio é, por muitas vezes, mal direcionada e insuficiente para conter a infecção. Existe um acúmulo de dados a respeito da exacerbação de respostas imunes indesejadas em indivíduos portadores de malária. Essas observações sugerem que existe um comprometimento tanto na geração de repertório de linfócitos T quanto na modulação da ativação celular periférica.

O desenvolvimento de linfócitos T e a geração de repertório

O timo é um órgão linfoide localizado no mediastino da cavidade torácica. Anatomicamente, o órgão é bilobado, sendo posteriormente dividido por septos dando origem a vários lóbulos. Análises histológicas revelam que cada lóbulo é formado por um córtex e uma medula. O córtex é a região mais densamente povoada, sendo composta principalmente por timócitos e células precursoras de linfócitos T, enquanto que a medula apresenta-se menos povoada e constituída principalmente por células epiteliais tímicas, células dendríticas e linfócitos B (Miller, 2011). O timo é o órgão linfoide responsável pela diferenciação, seleção e maturação de linfócitos T (Sprent, 1995; Hogquist *et al.*, 2005; Kyewski e Klein, 2006).

A geração de células T é dependente da entrada constante de precursores linfoides que adentram o órgão por meio de vênulas de endotélio alto (HEV, do inglês *High Endothelial Venules*) localizadas na região córtico-medular (Takahama, 2006). Por meio do reconhecimento de quimiocina específicas, os precursores linfoides migram para a região subcapsular do timo, área mais externa do córtex, onde a expressão de pré-receptores de linfócitos T (pré-TCR) garantem a sobrevivência da célula (Kuchroo *et al.*, 2002). As células incapazes de projetar para a superfície o pré-TCR, morrem por ausência de estímulo, conhecida por morte por negligência (Von Boehmer, 2009). Neste momento, os timócitos (as células T enquanto no timo) não apresentam TCR e seus co-receptores: as moléculas CD4 e CD8, sendo denominados timócitos duplo-negativos (DN). Em uma segunda etapa, os timócitos DN, no córtex, passam a expressar ambos os co-receptores juntamente com o TCR, sendo denominados timócitos CD4⁺CD8⁺ (duplo-positivos, DP). O timo de camundongos adultos e jovens é composto de 70-85% de timócitos DP.

A migração cortical de timócitos DP facilita a interação destas células com o microambiente tímico, especialmente células epiteliais corticais expressando peptídeos próprios associados a moléculas do Complexo Principal de Histocompatibilidade (MHC, do inglês, *Major Histocompatibility Complex*) de classe I e II. Apenas as células capazes de reconhecer moléculas de MHC próprio recebem citocinas e fatores de crescimento que permitem sua sobrevivência. Este processo é denominado de seleção positiva e é responsável pela maturação de timócitos DP a timócitos CD4⁺ (simples positivo, SP) e CD8⁺ SP (Hogquist *et al.*, 1994).

Os timócitos SP, agora na região medular, são expostos, novamente, a moléculas de MHC-I e MHC-II, contudo, células que interajam com elevada afinidade com tais moléculas serão eliminadas. De forma semelhante, os timócitos SP expressando TCRs proibitivos, como o TCR V β 5.1 e V β 5.2 e TCR V β 12.1 e V β 12.2, são eliminados devido a seu potencial auto-reativo. Além disso, o reconhecimento com alta afinidade garante a eliminação de células auto-reativas. Este processo é denominado de seleção negativa (Starr *et al.*, 2003).

Os três mecanismos constituem os processos de seleção de repertório de linfócitos que ocorrem no timo, e uma vez que os timócitos passam por tais processos, as células T deixam este órgão em direção aos órgãos linfoides secundários como células maduras e aptas a ativação por ligação de TCR com MHC conjugado a peptídeo cognato. Dentro deste contexto, é necessário que a arquitetura tímica mantenha sua organização estrutural e funcional para a correta formação de repertório e manutenção dos números de linfócitos T em órgãos linfoides periféricos.

Interessantemente, a despeito de seu papel-chave na geração das células responsáveis pela resposta imune adaptativa, o timo involui em decorrência do

envelhecimento do organismo. A atrofia tímica, caracterizada por perda da massa relativa do órgão ao corpo, observada no envelhecimento é conhecida por atrofia fisiológica do timo (Hirokawa *et al.*, 1994). A produção de hormônios sexuais e adrenais está relacionada com a perda gradativa de massa tímica e também da produção e maturação de novos linfócitos T (Savino *et al.*, 2007; Elmas *et al.*, 2008; Gruver e Sempowski, 2008). Por outro lado, indivíduos adultos e idosos apresentam quantidades similares de linfócitos T periféricos (Ligthart *et al.*, 1986; Kilpatrick *et al.*, 2008; Lee *et al.*, 2012), indicando que ou a timopoiese é mantida mesmo na redução de celularidade tecidual ou existem mecanismos de proliferação homeostática que garantem a manutenção de uma quantidade mínima necessária de linfócitos T periféricos. Ambas as hipóteses foram confirmadas. Pacientes adultos irradiados e transplantados com medula óssea apresentaram reconstituição de linfócitos T periféricos, apesar da redução do tamanho tímico (Thomas *et al.*, 1986). Em outros estudos, foi verificado que a transferência de células T a camundongos linfopênicos promoveu a proliferação celular na periferia de forma dependente da produção de IL-7 (Schluns *et al.*, 2000; Laurent *et al.*, 2004). Experimentos *in vitro* demonstraram que, possivelmente, a proliferação homeostática necessita da interação entre células dendríticas (DCs) e linfócitos T e que citocinas envolvidas na diferenciação de células T desempenham um importante papel neste fenômeno (Watanabe *et al.*, 2004). Essas e outras evidências sugerem que o pool de células T na periferia do sistema imune é mantido por meio de proliferação homeostática e da chegada de novas células provenientes do timo (Bell *et al.*, 1987; Rocha *et al.*, 1989; Mackall *et al.*, 1993; Mackall *et al.*, 1997; Mackall *et al.*, 2001).

Apesar da existência de mecanismos compensatórios para a manutenção do pool de linfócitos T, o timo é essencial para a geração de repertório e sua retirada cirúrgica acarreta consequências graves. Camundongos neonatos timectomizados são deficientes em montar

resposta imune contra agentes patogênicos (Jamieson *et al.*, 1999), porém a cirurgia em adultos não causa esse problema (Miller, 1962; McIntire *et al.*, 1964; Jamieson *et al.*, 1999). As diferenças observadas entre os camundongos tornados atímicos em idades distintas indicam que o timo é necessário nos primeiros momentos de vida, contudo sua função e importância em adultos são pouco conhecidas (Miller, 2011). Ainda assim, foi descrito um papel importante desempenhado por este órgão no estabelecimento da tolerância induzida por via oral em camundongos mais velhos (Song *et al.*, 2006).

Mesmo apresentando involução fisiológica, o timo pode sofrer influências externas ao sistema, especialmente em situações de estresse, desnutrição e infecções, que promovem sua atrofia. Por sua importância na manutenção do sistema imune, o comprometimento tímico é danoso nas infecções.

O comprometimento tímico nas infecções

Fisiologicamente o timo sofre involução por conta do envelhecimento (Hirokawa *et al.*, 1994). Contudo, alguns fatores extrínsecos como, por exemplo, má-nutrição (Savino *et al.*, 2007), estresse (Gruver e Sempowski, 2008) e doenças metabólicas (Elmas *et al.*, 2008) são capazes de induzir sua atrofia. Foi constatado que alguns agentes patogênicos podem levar à atrofia tímica tanto em camundongos quanto em humanos infectados por, entre outros, *Trypanosoma cruzi* (Savino *et al.*, 1989), HIV (Savino *et al.*, 1986) e em modelos experimentais de malária (*Plasmodium chabaudi*) (Seixas e Ostler, 2005). A interação parasita/patógeno-hospedeiro promove alterações tímicas caracterizadas por: (i) atrofia

tímica, (ii) menor liberação de células T maduras, (iii) saída de timócitos imaturos, (iv) alterações na estrutura tímica e (v) geração de células T tolerogênicas ao antígeno.

De forma geral, as infecções podem atuar sobre o timo de duas maneiras: local ou sistêmica. Localmente, a presença do patógeno promove alterações na arquitetura tímica e nas células em processo de maturação. De modo sistêmico, o timo está sob o efeito de hormônios liberados na circulação, como glicocorticoides e mediadores inflamatórios. Foi verificado que a secreção elevada de cortisol promoveu atrofia tímica em modelos de infecção aguda por *Francisella tularensis*, *T.cruzi*, *Salmonella* e *Mycobacterium avium* (Chen *et al.*, 2005; Perez *et al.*, 2007; Borges *et al.*, 2012; Deobagkar-Lele *et al.*, 2013). A atrofia tímica induzida por infecção se correlaciona com a virulência das cepas dos patógenos, indicando que fatores produzidos por bactérias, fungos, vírus e helmintos exercem efeitos tóxicos sobre os timócitos. De fato, se verificou que a administração de lipopolissacarídeo (LPS) induz a atrofia tímica de camundongos (Baroni *et al.*, 1976). Semelhantemente, a administração de enterotoxina de *Escherichia coli* e toxinas fúngicas gliotoxina (produzida por *Aspergillus fumigatus*) e T-2 (toxina causadora de leucopenia fatal em humanos) também promoveram a atrofia (Sutton *et al.*, 1994; Islam *et al.*, 1998; Tsuji *et al.*, 2000).

Além da perda de massa relativa do timo, as infecções podem incutir em mudanças na arquitetura tímica. Foi verificado que o parasita *T.cruzi* é capaz de invadir o timo e provocar atrofia tímica agressiva, com alterações em componentes não linfoides, como a matriz extra-celular, com aumento na deposição de fibronectina e laminina e aumento da produção das quimiocinas CXCL12 e CCL4 e morte das células epiteliais tímicas (Savino *et al.*, 1989; Cotta-De-Almeida *et al.*, 2003; Mendes-Da-Cruz *et al.*, 2003). Os timócitos de camundongos infectados por *T.cruzi* apresentaram-se alterados, com maior expressão de

receptores de quimiocinas e capacidade migratória *ex vivo* (Cotta-De-Almeida *et al.*, 2003; Mendes-Da-Cruz *et al.*, 2006). Consequentemente, o repertório de linfócitos T nos órgãos linfoides secundários é alterado, com maior frequência de células T-DP imaturas. É proposto que a maior frequência de resposta autoimune cardíaca observada na doença de Chagas seja resultado da presença de células T-DP que não passaram pelos mecanismos de seleção tímica (Cotta-De-Almeida *et al.*, 2003).

Além de infecções parasitárias, o timo é alvo de infecções virais. Por exemplo, o vírus da imunodeficiência humana (HIV) é capaz de infectar células epiteliais tímicas (TECs) e leva-las a morte por apoptose e necrose (Stanley *et al.*, 1993). A infecção de TECs por alguns vírus não induz morte celular, mas altera sua funcionalidade. Por exemplo, a infecção por vírus coxsackie induz a produção de citocinas GM-CSF e LIF, importantes na maturação de timócitos (Brilot *et al.*, 2002). A observação de que infecções podem não apenas induzir a morte de células epiteliais tímicas, mas também alterar sua função é importante, dado a participação de TECs na geração de células T.

O surgimento de células T-DP na periferia do sistema imune foi observado em infecções causadas por *T.cruzi*, HIV, vírus da hepatite B e C (Savino *et al.*, 2007). Embora a origem de células T-DP não possa ser totalmente esclarecida, a maior migração de timócitos observadas na vigência da infecção, sugere que tais células sejam elicitadas em decorrência da inflamação infecciosa. Por outro lado, foi verificado que a infecção por HIV é capaz de induzir a expressão de CD8 em células T CD4 (SP), tornando-as células T-DP geradas extratimicamente (Nascimbeni *et al.*, 2004).

Não por acaso, a presença de patógenos no timo sugere que tais agentes adquiram tropismo a este órgão com o intuito de gerar tolerância central. Conforme anteriormente discutido, a interação com alta afinidade entre TCR e moléculas de MHC resulta na

eliminação dos timócitos, o que configura a seleção negativa. Neste contexto, possivelmente, a interação de timócitos antígeno-específicos com moléculas de MHC portando peptídeos cognatos pode causar a eliminação de células imunocompetentes que seriam responsáveis pela resistência à infecção. Este seria um caso de tolerância central patógeno-específica. Tal probabilidade foi investigada com resultados interessantes. O grupo liderado por Correia-Neves demonstrou anteriormente que a infecção por cepa fracamente virulenta de *M.avium*, embora presente na região medular do timo, não causou sua atrofia, mas induziu a geração de clones tolerantes à micobactéria (Nobrega *et al.*, 2007; Nobrega *et al.*, 2010). Neste modelo de infecção, as células T geradas a partir de timos infectados foram incapazes de produzir quantidades detectáveis de IFN- γ frente estímulo com *M.avium* ou BCG (Nobrega *et al.*, 2010). A tolerância a patógenos também foi descrita em infecções virais, como na infecção pelo vírus de leucemia murina (MLV) e pelo vírus da coriomeningite murina (LCMV) (Jamieson e Ahmed, 1988; Korostoff *et al.*, 1990).

Um dos papéis desempenhados pelo timo é o de gerar a tolerância imune por meio da seleção positiva de linfócitos T imuno-competentes e a eliminação de clones que reconhecem peptídeos próprios (Miller, 2011). Alterações que resultem na falha de deleção dos clones auto-reativos e sua consequente migração para a periferia pode levar ao desenvolvimento de respostas autoimunes (Asano *et al.*, 1996). Interessantemente, camundongos infectados por *T.cruzi* apresentam autoimunidade a cardiomiócitos, de forma semelhante ao observado em pacientes portadores de doença de Chagas. Células T-DP apresentando TCR proibitivos (V β 5 e V β 12) foram encontradas na circulação de camundongos infectados, sugerindo que tais células não passaram pelo processo de seleção negativa (Mendes-Da-Cruz *et al.*, 2003). Adicionalmente, foi detectada a presença de

anticorpos anti-timo e células responsivas a cardiomiócitos em camundongos infectados (Savino *et al.*, 1989). Esses achados mostram que as alterações tímicas observadas na infecção por *T.cruzi* podem gerar um repertório de células T auto-reativas no sistema imune.

Tendo em vista que o timo é um órgão-alvo em infecções, é necessário manter a arquitetura de seu microambiente, contribuindo para a manutenção de repertório periférico eficiente de células T maduras selecionadas apropriadamente.

A infecção por plasmódio e as alterações tímicas na malária

A infecção por protozoários pertencentes ao gênero *Plasmodium* provoca a doença infecciosa com maior número de infectados no mundo, a malária. São reportados cerca de 300 milhões de novos casos por ano, principalmente em países subdesenvolvidos e em desenvolvimento (Guyatt *et al.*, 1999; Snow *et al.*, 1999). Aproximadamente, um milhão de crianças africanas morrem anualmente em decorrência dessa enfermidade (Miller *et al.*, 2002). No Brasil foram reportados mais de 330 mil casos novos em 2010 (Brasil, 2010). Devido a esses fatos, destaca-se a sua importância no cenário de saúde pública mundial. Até o momento quatro espécies de plasmódio conhecidamente possuem a capacidade de infectar o homem: *P. falciparum*, *P. vivax*, *P. ovale* e *P. malariae*. Dentre elas, o *P. falciparum* é o mais virulento, sendo responsável por, aproximadamente, 90% das mortes. Em camundongos, as espécies usualmente infectantes são: *P. berghei*, *P. chabaudi* e *P. yoelli*. O parasita apresenta ciclo de vida em dois hospedeiros: o mosquito *Anopheles* e o

homem. Durante a picada do mosquito são inoculadas formas esporozoítas do plasmódio, que chegam a corrente sanguínea e invadem hepatócitos (Tuteja, 2007; Chang *et al.*, 2013).

Nos hepatócitos, os protozoários se diferenciam em formas esquizontes para gerar merozoítos. É conhecido que o ciclo hepático tem duração de até quinze dias. A liberação de merozoítos na circulação possibilita o contato destes com eritrócitos, onde se diferenciam em trofozoítos para, novamente, originarem formas esquizontes. A proliferação intra-eritrocítica de esquizontes culmina no rompimento das hemácias e liberação de formas infectantes de plasmódio. Todo o ciclo sanguíneo tem duração de 48h. Nestes intervalos são observados os sinais e sintomas clínicos da malária: febre intermitente, mal-estar e eritema. Parte dos merozoítos pode se diferenciar em gametócitos, que são as formas sexuais, e contaminar mosquitos *Anopheles* durante a picada.

Enquanto na circulação, os protozoários são reconhecidos pelo sistema imune através de receptores de padrões moleculares associados a patógenos (PRRs, do inglês *Pattern Recognition Receptors*) presentes em células do sistema imune inato, como macrófagos, células dendríticas e neutrófilos (Stevenson e Riley, 2004; Zhu *et al.*, 2011; Zhu *et al.*, 2012). O plasmódio é reconhecido por apresentar em sua superfície fosfatidilinositol glicosilados (GPIs), que são reconhecidos através de receptores do tipo Toll (TLRs, do inglês *Toll-Like Receptors*) 1 e 2 (Nebl *et al.*, 2005; Zhu *et al.*, 2011). Durante seu ciclo de vida, as espécies de plasmódio produzem hemozoina, um pigmento gerado pela degradação de hemoglobina, sendo este composto reconhecido por TLR-4 e 9 (Jide *et al.*, 2009). A inflamação causada pelo reconhecimento do parasita, via TLRs, pode acarretar em danos ao organismo. Foi verificado que a ativação por TLR-4 na malária infantil predispõe a malária cerebral (Khor *et al.*, 2007; Loharungsikul *et al.*, 2008). Polimorfismos no gene de *tlr-4* estão associados com complicações da infecção por

plasmódio (Mockenhaupt *et al.*, 2006). Ainda neste contexto, camundongos deficientes na produção de CXCL-10 (em que células inflamatórias apresentam sua capacidade migratória debilitada) experimentalmente infectados por *P.berghei* ANKA apresentam elevada frequência de células T reguladoras (Tregs) produtoras de Fator de Crescimento e Transformação (TGF)- β e não sucumbem a infecção, oposto ao observado em animais selvagens, que morrem portando malária cerebral (Sarfo *et al.*, 2011).

Por outro lado, é necessária a presença de uma resposta imune protetora. Experimentalmente, para estudos de resposta imune a malária, camundongos C57BL/6 são infectados por *P.chabaudi chabaudi* AS, uma cepa não letal de plasmódio, ou por *P.berghei* ANKA e NK65, cepas letais capazes ou incapazes de induzir malária cerebral, respectivamente. Experimentos realizados em camundongos mostraram que o controle da infecção por *P.chabaudi* é dependente tanto de células T CD4⁺ quanto da produção de Interferon (IFN)- γ e Interleucina (IL)-12 (Van Der Heyde *et al.*, 1997; Su e Stevenson, 2002). Células T $\gamma\delta$ participam da resposta, mas não são essenciais para o controle da infecção, visto que camundongos Knock-out para tais células são resistentes ao inoculo do patógeno (Langhorne *et al.*, 2002). A ausência de IL-10, uma citocina anti-inflamatória, promove morte dos animais infectados por *P.chabaudi* devido à exacerbação da resposta imune (Li *et al.*, 1999; Li *et al.*, 2003). Embora a ausência de linfócitos B não provoque mortalidade dos camundongos infectados, a parasitemia é persistente nos camundongos C57BL/6, indicando um possível papel auxiliador de anticorpos no controle da infecção (Good e Doolan, 1999; Bergmann-Leitner *et al.*, 2011). Adicionalmente, foi evidenciado que citocinas como o Fator de Necrose Tumoral (TNF)- α e IFN- γ e fatores como o óxido nítrico (NO) secretados nos tecidos durante a resposta protetora contra o *Plasmodium* são importantes (Stevenson *et al.*, 1995; Jacobs *et al.*, 1996). Por outro lado, recentemente se

discute que a resposta imune celular exacerbada favoreça a persistência do parasita no hospedeiro (Miller *et al.*, 2002). Essas e outras observações mostram que é necessário existir um fino controle nas respostas imunes contra o plasmódio. Devido à sua importância, recentemente estudos têm buscado compreender a interação entre plasmódio e timo.

Foi verificado que camundongos BALB/c infectados com *P.berghei* NK65 morrem aos 14 dias após infecção apresentando elevada parasitemia e severa atrofia tímica (Andrade *et al.*, 2008). Análises estruturais mostraram o parasita dentro do timo. Posteriormente, foi demonstrado que, além das modificações morfológicas, no timo foram encontradas alterações na deposição de elementos de matriz extra-celular e aumento na expressão de moléculas relacionadas com a migração de timócitos para a periferia (Gameiro, Nagib, Andrade, *et al.*, 2010). A presença do parasita dentro do timo se correlacionou com um aumento na apoptose celular e também na migração prematura de células T CD4⁺CD8⁺ (duplo-positivos, DP) para órgãos linfoides secundários (Francelin *et al.*, 2011). É pouco conhecido o efeito que a migração prematura de timócitos à periferia pode exercer sobre o sistema imune.

Padrão semelhante de atrofia e migração de timócitos duplo-positivos foi descrito em infecção experimental com *T.cruzi* onde a cronicidade da resposta imune é acompanhada do desenvolvimento de autoimunidade a tecidos cardíacos (Cotta-De-Almeida *et al.*, 2003). A atrofia tímica seguida da migração de células T que não passaram pelo processo de seleção negativa pode estar relacionada à autoimunidade em humanos e em modelos animais com doença de Chagas (Mendes-Da-Cruz *et al.*, 2003). Nesse sentido, é possível supor que a migração precoce de timócitos à periferia na atrofia tímica induzida pela infecção por *Plasmodium* pode alterar a predisposição de camundongos e pacientes ao

aparecimento de doenças auto-imunes, dentre as quais diabetes, lúpus e esclerose múltipla, entre outras.

A influência mútua entre malária e doenças autoimunes

A relação entre malária e doenças autoimunes é alvo de publicações conflitantes. Foi descrito que pacientes maláricos apresentam níveis elevados de anticorpos anti-núcleo e proteínas nucleares, semelhante ao observado em pacientes portadores de lúpus (Bonfa *et al.*, 1987). Neste estudo, até 75% dos pacientes apresentaram quantidades detectáveis de auto-anticorpos. Pontualmente, são descritos casos de agravamento do lúpus em pacientes infectados por plasmódio (Wallace *et al.*, 1993; Datta *et al.*, 2013). Por outro lado, uma fração dos indivíduos portadores de lúpus é resistente à infecção por plasmódio (Floto *et al.*, 2005; Clatworthy *et al.*, 2007; Francis e Perl, 2010). Análises subsequentes mostraram que a susceptibilidade ao desenvolvimento de lúpus é maior em pessoas com deficiência na expressão do receptor inibitório de porção Fc γ (Fc γ RIIB), curiosamente, a redução na expressão de Fc γ RIIB confere resistência à malária e o desenvolvimento de malária cerebral e placentária (Butcher, 1996; Butcher, 2008; Willcocks *et al.*, 2010). Foi demonstrado também que o soro de pacientes portadores de lúpus reduz o crescimento de *P. falciparum* em culturas (Singh *et al.*, 2001). O efeito protetor foi dependente da produção de anticorpos anti-fosfoproteína P0 de ribossomo de *P. falciparum* (PfP0) que é estruturalmente semelhante à P0 humana (Singh *et al.*, 2001).

A constituição genética predispõe a redução da infecção por *P. berghei* ANKA bem como a severidade da malária cerebral e placentária (Floto *et al.*, 2005; Clatworthy *et al.*,

2007; Waisberg *et al.*, 2011; Waisberg *et al.*, 2013). Em camundongos, a infecção experimental por *P.berghei* ANKA também melhorou o quadro clínico de lúpus (Al-Quraishy *et al.*, 2013). Esses resultados indicam que existe uma relação excludente entre infecção por plasmódio e lúpus.

Ainda, a literatura apresenta dados acerca da possível relação entre malária e esclerose múltipla (MS, do inglês *Multiple Sclerosis*). Foi observado que regiões que erradicaram plasmódio, como a Sardenha e Sicília, apresentam elevada incidência de MS (Nicoletti *et al.*, 2001; Ragonese *et al.*, 2004). Dentre as mudanças socioambientais sofridas pela Sardenha, a mais rápida foi o controle da epidemia malárica. Interessantemente, a frequência de MS é reduzida na África, polo endêmico de malária ao passo que se verificou um aumento na frequência de MS em indivíduos africanos que se mudaram para países sem a infecção por plasmódio, como a França (Delasnerie-Laupretre e Alperovitch, 1990).

Ainda neste contexto, foi observada uma maior frequência na mutação com ganho de função do gene de chitotriosidase (Chit) entre indivíduos africanos e afrodescendentes provenientes de zonas endêmicas de malária, conferindo resistência à infecção por plasmódio (Boot *et al.*, 1995; Barone *et al.*, 2003; Malaguarnera *et al.*, 2003; Sotgiu *et al.*, 2006). A enzima Chit é produzida por macrófagos e células dendríticas em resposta a estímulos de patógenos produtores de quitina. Curiosamente, Chit é encontrada em elevados níveis no soro e líquido cefalorraquidiano de pacientes portadores de esclerose múltipla (Sotgiu *et al.*, 2006). Tais evidências sugerem que indivíduos afrodescendentes portadores de mutações em Chit apresentam vantagem imunológica frente à infecção por plasmódio, contudo, na ausência de um agente infeccioso a resposta imune exacerbada favorece o surgimento da neuro-inflamação autoimune observada na MS (Sotgiu *et al.*, 2008).

A esclerose múltipla é uma doença caracterizada por distúrbios sensoriais, neurite ótica unilateral e fraqueza progressiva dos grupos musculares por conta da ativação do sistema imune, principalmente linfócitos T (Ben-Nun *et al.*, 1981), contra os componentes da bainha de mielina que recobrem os axônios (Noseworthy *et al.*, 2000). A evolução clínica da MS é observada frequentemente no início da vida adulta e os sintomas progridem cronicamente levando a incapacitação motora, em grande parte dos casos (Weinshenker *et al.*, 1989). Pouco se conhece acerca dos eventos desencadeadores das reações autoimunes que acometem os pacientes, algumas evidências indicam que infecção por plasmódio pode induzir a resposta autoimune na MS. Uma série de estudos conduzidos nos Estados Unidos e Europa apontaram que a infecção por plasmódio na infância confere resistência ao desenvolvimento de MS ao passo que a infecção silenciosa na adolescência e idade adulta pode ser a causa da doença, embora os mecanismos não foram descritos ou sugeridos (Kissler, 2001a; b).

Recentemente, se verificou um aumento expressivo em casos novos de MS em áreas de desenvolvimento urbano (Kotzamani *et al.*, 2012). No Brasil, a MS tem início aos 30-40 anos de vida em indivíduos caucasianos, embora aproximadamente um terço dos acometidos é formado por afrodescendentes (Alves-Leon *et al.*, 2008; Pereira *et al.*, 2012). Esses dados mostram que novos estudos devem ser conduzidos com a finalidade de investigar as possíveis interações entre malária e esclerose múltipla. Para tanto, abordagens são realizadas empregando o modelo experimental mais bem caracterizado da doença humana, a Encefalomielite Autoimune Experimental (EAE), induzida em camundongos geneticamente susceptíveis após imunização com antígenos derivados do Sistema Nervoso Central (SNC) (Bernard, 1976; Krishnamoorthy e Wekerle, 2009; Chastain *et al.*, 2011).

Apesar de constatada uma importante influência genética no desenvolvimento de síndromes autoimunes, o ambiente pode desempenhar um papel decisivo nesta questão. Os fatores ambientais mais prováveis capazes de induzir uma resposta ao próprio são as infecções. Essa relação é alvo de debates conflitantes atualmente (Sfriso *et al.*, 2010). Em experimento de co-infecção por *P.chabaudi chabaudi* os camundongos tiveram redução do quadro clínico de EAE por conta da expansão de células T regulatórias (Tregs) estimuladas pelo parasito (Farias *et al.*, 2011). Por outro lado, camundongos criados em situações germ-free são mais resistentes ao desenvolvimento de EAE (Lee *et al.*, 2011). Anteriormente, foi descrito que pacientes com malária desenvolveram respostas de origem autoimune, como a formação de anticorpos anti-células da musculatura lisa e anti-núcleo (Phanuphak *et al.*, 1983). Achados similares foram encontrados em camundongos infectados com *P.berghei* (Poels *et al.*, 1980). Nesse estudo, os autores demonstraram ainda que a formação de auto-anticorpos é dependente da participação de linfócitos T, visto que camundongos atímicos não desenvolveram auto-resposta (Poels *et al.*, 1980).

As evidências apresentadas até o momento mostram que a malária pode tanto exacerbar quanto modular a inflamação. Ainda não estão totalmente esclarecidos os mecanismos que determinam a estimulação ou inibição da inflamação na vigência da malária. Tendo em vista que células dendríticas são células apresentadoras de antígenos com a capacidade exclusiva de direcionar a resposta imune no momento da ativação de linfócitos T “naïve”, é de se supor que a infecção por plasmódio pode influenciar a ativação/maturação dessas células.

Células dendríticas: elementos-chave na malária e sua modulação para fins terapêuticos

Respostas imunes adequadas exigem um balanço entre ativação e supressão das células T pelas células apresentadoras de antígenos. Vários tipos celulares são capazes de realizar apresentação de antígenos: monócitos, macrófagos, células dendríticas, linfócitos B e neutrófilos (Henry *et al.*, 1999; Guernonprez *et al.*, 2002; Chastain *et al.*, 2011). Entretanto, apenas as células dendríticas são capazes de ativar linfócitos T “naïve” e direcionar o perfil de resposta para T helper (Th) 1, Th2, Th17 e Th9 (Levin *et al.*, 1993; Banchereau *et al.*, 2000; Guernonprez *et al.*, 2002; Shortman e Liu, 2002; Emmer *et al.*, 2006; Heath e Carbone, 2009; Huang *et al.*, 2010; Lu *et al.*, 2012). A apresentação de antígenos mediada por DCs ocorre através da liberação de três sinais, principalmente: moléculas de MHC-I ou II complexadas com peptídeo antigênico interagem com TCR; moléculas co-estimuladoras CD-80 e CD-86 presentes em DCs se ligam a receptores específicos presentes nos linfócitos T como CD-28 e CTLA-4; e as citocinas secretadas pelas DCs influenciam na ativação, diferenciação e proliferação dos linfócitos T Ag-específicos. O estágio de maturação/ativação de células dendríticas influencia no tipo de resposta a ser gerada. Por exemplo, DCs imaturas são caracterizadas pela baixa expressão de MHC-II e moléculas co-estimulatórias e quando em contato com linfócitos T “naïve” estimulam sua diferenciação em Tregs (Lutz e Schuler, 2002; Lutz, 2012). DCs maduras, por sua vez, expressam elevados níveis de MHC-II e CD-80 e CD-86 e induzem a geração de resposta T helper (Banchereau *et al.*, 2000).

Essa habilidade única coloca as DCs como células-alvo na infecção por plasmódio. Recentemente foi descrito que indivíduos infectados por *P.vivax* apresentam uma proporção maior de DCs imaturas com perfil imunossupressor na circulação quando

comparados com indivíduos saudáveis (Goncalves *et al.*, 2010). Também foi demonstrado que algumas cepas de *Plasmodium* são capazes de invadir e se multiplicar dentro de células dendríticas em modelo experimental de infecção, dificultando o desenvolvimento de vacinas e a erradicação do parasita (Wykes *et al.*, 2011).

O bloqueio das funções das células dendríticas reduz a eficácia do sistema imune e dificulta a eliminação de patógenos. Tal fenômeno pode ser observado em células dendríticas de pacientes portadores de malária, que se apresentam moduladas para um fenótipo anti-inflamatório: baixa expressão de moléculas co-estimuladoras, secreção de IL-10 e estimulação de células Tregs (Urban *et al.*, 1999; Giusti *et al.*, 2011). Os mecanismos pelos quais algumas cepas de plasmódio suprimem DCs ainda não estão esclarecidos. Entretanto, foi demonstrado que hemozoina, o pigmento de plasmódio, é capaz de reduzir a maturação de DCs humanas e murinas (Millington *et al.*, 2006; Urban e Todryk, 2006; Giusti *et al.*, 2011). DCs tratadas *in vitro* com hemozoina adquirem habilidades imunossupressoras como a redução na expressão das moléculas co-estimuladoras CD80 e CD86, secreção de IL-10 e indução de células Tregs (Skorokhod *et al.*, 2004; Giusti *et al.*, 2011)

A modulação de células dendríticas tem se mostrado uma abordagem promissora no tratamento de doenças inflamatórias. Por exemplo, foi demonstrado que DCs tratadas com formulações sintéticas ou extratos derivados de patógenos adquirem um fenótipo imaturo e, após transferidas adotivamente, suprimem a severidade de artrite experimental induzida por colágeno, transplantações renais e Encefalomielite Autoimune Experimental (Moser *et al.*, 1995; Rea *et al.*, 2000; Roelen *et al.*, 2003; Tarbell *et al.*, 2004; Van Halteren *et al.*, 2004; Emmer *et al.*, 2006; Terness *et al.*, 2008; Unger *et al.*, 2009; Ko *et al.*, 2010; Sofronic-Milosavljevic *et al.*, 2013).

De fato, células dendríticas moduladas com dexametasona (DC-dexa), um composto sintético análogo ao cortisol, adquirem um fenótipo supressor e induzem a expansão de células T reguladoras (Moser *et al.*, 1995; Rea *et al.*, 2000; Emmer *et al.*, 2006; Unger *et al.*, 2009). Quando transferidas adotivamente, as DC-dexa reduziram significativamente a severidade do quadro clínico de Encefalomielite Autoimune Experimental (EAE), um modelo experimental da esclerose múltipla em humanos (Pena *et al.*, 2013). Outros compostos como dihidroartemisinina, vitamina D e CTLA-4-Ig (este um conjugado de porção Fc de anticorpo com molécula de CTLA-4) também se mostraram capazes de interferir na maturação de DCs, e quando transferidas para camundongos foram capazes de suprimir a inflamação crônica em diversos modelos experimentais (Mellor *et al.*, 2004; Bahri *et al.*, 2009; Ko *et al.*, 2010; Zhao *et al.*, 2012; Farias *et al.*, 2013). Apesar de sua plasticidade fenotípica e funcional em decorrência dos estímulos presentes no microambiente, a identificação de novos compostos com capacidade imunomoduladora é complexa. Neste contexto, este trabalho trabalhou com a hipótese de que a migração precoce de células T-DP para a periferia do sistema imune pode alterar o curso e a susceptibilidade ao desenvolvimento de doenças auto-imunes. Tendo em vista que moléculas derivadas de *P. berghei* modulam células dendríticas para um perfil tolerogênico, trabalhamos com a hipótese de que tais células tratadas com extratos de plasmódio podem ser utilizadas no controle da neuro-inflamação autoimune.

Objetivo

Objetivo

O presente trabalho teve por objetivo investigar o efeito da infecção por *Plasmodium berghei* NK65 sobre a resposta autoimune, utilizando como modelo a Encefalomielite Autoimune Experimental, e sobre a modulação de células dendríticas geradas a partir de precursores de medula óssea visando sua utilização terapêutica na autoimunidade.

Objetivos específicos

Tendo em vista que o objetivo principal do projeto é investigar o efeito da infecção por *P.berghei* sobre a resposta imune, os seguintes objetivos específicos foram almejados:

1. Desenvolvimento de modelo não letal de infecção por *Plasmodium berghei* NK65.
2. Investigação do efeito da infecção por *P.berghei* sobre o curso da Encefalomielite Autoimune Experimental.
3. Modulação de células dendríticas por extratos de plasmódio e seu emprego terapêutico na Encefalomielite Autoimune Experimental.

A presente tese de doutorado se apresenta na forma de três capítulos que abordam, individualmente, cada objetivo específico.

Material e Métodos

1. Animais. Camundongos BALB/c e C57BL/6 fêmeas, de seis semanas procedentes do Centro Multidisciplinar para Investigação Biológica na Área da Ciência em Animais de Laboratório (CEMIB/UNICAMP), foram mantidos em condições S.P.F. (*specific-pathogen free*) com água e ração autoclavados *ad libitum*, tendo a temperatura e o fotoperíodo em ciclos de 12 horas controlados durante todo o experimento. Cada grupo experimental foi constituído por seis camundongos. Os protocolos envolvendo animais de laboratório foram executados de acordo com as normas da Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL/COBEA) e aprovados pela Comissão de Ética no Uso de Animais (CEUA/UNICAMP) sob protocolo número 2687-1.

2. Infecção por *Plasmodium berghei*, índice tímico e indução de encefalomielite autoimune experimental.

2.1. Infecção por *P.berghei* NK65. Foi utilizado o isolado virulento de *Plasmodium berghei* cepa NK65, cedido pelo Prof^o. Dr^o. Fábio T. M. Costa do Instituto de Biologia da Universidade de Campinas (UNICAMP). Antes de cada infecção, amostras congeladas do parasita foram inoculadas por via intra-peritoneal (i.p.) em animal repique. Após 14 dias, estes animais foram sacrificados e foi realizada contagem das hemácias infectadas e determinação de parasitemia. Foram injetadas via i.p. 1×10^6 hemácias parasitadas por animal. A evolução da malária foi acompanhada pela determinação da parasitemia em dias alternados até o final do experimento.

2.2. Índice tímico. Animais infectados e não infectados foram pesados no dia do sacrifício, o qual foi realizado em anestesia. Em seguida, os timos foram coletados, lavados em PBS e pesados. O índice tímico foi calculado de acordo com a seguinte equação: peso

do órgão (g) / peso do animal (g) x 100. Os timos foram dissecados e fixados, cortados em micrótomos (cortes de 5µm) e corados em Hematoxilina e Eosina (HE), para análise histopatológica.

2.3. Indução e avaliação de EAE. A indução e avaliação de EAE seguiram protocolos previamente descritos (Farias *et al.*, 2011). Brevemente, camundongos foram imunizados em dois flancos por via sub-cutânea (s.c.) com 100µg total do peptídeo de neuro-antígeno MOG₃₅₋₅₅ (MEVGWYRSPFSRVVHLYRNGK) emulsionado em igual volume de adjuvante completo de Freund (CFA, Sigma, USA). Os animais foram também imunizados com duas doses via i.p. de 200ng de toxina de *Pertuxis* em 0h e 48h após a aplicação dos antígenos de MOG. A evolução e severidade da EAE foram avaliadas diariamente a partir do nono dia após imunização de acordo com sistema de escore clínico na escala de 0-5, onde: 0, não doente; 1, perda de tônus da cauda; 2, fraqueza dos membros posteriores; 3, paralisia dos membros posteriores; 4, fraqueza ou paralisia dos membros anteriores juntamente com os posteriores; 5, agonizando ou morto. Ao término do período de acompanhamento, os animais dos diferentes grupos foram anestesiados e decapitados. O cérebro e cordão espinhal foram removidos e fixados, cortados em micrótomo (cortes de 5µm) e corados em H/E, para análise histológica.

3. Tratamento dos animais infectados com a cepa letal de *P.berghei* NK65. Para o estabelecimento do modelo de atrofia tímica em infecção não letal por *P.berghei* NK65, camundongos BALB/c e C57BL/6 foram infectados com o parasita como descrito acima. O tratamento com cloroquina (Sigma-Aldrich, USA) seguiu protocolo descrito anteriormente (Nurul Aiezzah *et al.*, 2010) com modificações. Brevemente, decorridos dez dias após a inoculação do parasita, camundongos dos grupos previstos no item 1 receberam cloroquina

(5mg/kg/dia) por via i.p. durante cinco dias consecutivos. A parasitemia foi acompanhada por quinze dias após o início da administração da droga. A parasitemia em 0% foi considerada como sucesso do tratamento. A existência/inexistência de efeitos colaterais ao uso de cloroquina, como alterações no sistema imune e mortalidade dos animais, foi acompanhada em grupos controle.

4. Preparo de antígenos derivados de *P.berghei*. O preparo de antígenos totais de *P.berghei* seguiu indicações previamente descritas (Sarfo *et al.*, 2011). Brevemente, as hemácias parasitadas foram lavadas duas vezes em solução HBSS (Sigma-Aldrich) por 10 minutos a 1.100g. Células provenientes de animais não infectados foram usadas como controle. As células foram diluídas para a concentração de 10^9 /mL e sofreram ruptura de sua integridade celular por meio de vinte ciclos de congelamento e descongelamento. Posteriormente foram centrifugadas a 15.000g por 1h. Os sobrenadantes foram estocados a -20°C até o uso. A concentração de proteínas foi determinada utilizando o micrométodo de Bradford, seguindo recomendações do fabricante (Sigma-Aldrich).

5. Avaliação da proliferação específica frente a MOG₃₅₋₅₅. Para avaliar a proliferação específica frente a MOG₃₅₋₅₅, os animais dos grupos controles e experimentais foram sacrificados em câmara de CO₂. O baço de cada animal foi removido, os esplenócitos ressuspensos em PBS estéril contendo a sonda CFSE (do inglês, *Carboxyfluorescein succinimidyl ester*, 2,5 μM) na densidade de 1×10^6 células /mL e deixados em temperatura ambiente por cinco minutos. Em seguida, foi adicionado o meio RPMI com 10% de soro bovino fetal (meio RPMI completo) e as suspensões foram lavadas por centrifugação a 300g, por 10 minutos, ressuspensas no volume original com RPMI

completo contendo 50µg/mL de MOG₃₅₋₅₅ e distribuídas em placas de cultura de 96 poços. Antes da adição dos antígenos, foi efetuada a análise de uma alíquota das células marcadas com CFSE por citometria de fluxo, para definir o valor máximo de incorporação da sonda. As placas de cultura foram incubadas em estufa de CO₂ 5% a 37°C por 5 dias. Em seguida, as células em cada poço foram fixadas com PBS contendo 1% de formaldeído, transferidas para tubos apropriados, e analisadas em citômetro de fluxo (Gallios, Becman-Coulter) localizado no Departamento de Genética, Evolução e Bioagentes, do Instituto de Biologia da UNICAMP. A proliferação foi analisada pelo decaimento da fluorescência da sonda nas subpopulações de linfócitos T pertinentes a cada experimento.

6. Geração, modulação e transferência adotiva de células dendríticas.

6.1. Diferenciação de células dendríticas a partir de precursores de medula óssea. Células dendríticas foram diferenciadas da medula óssea (DCs) de camundongos C57BL/6 seguindo recomendações previamente descritas (Lutz *et al.*, 1999) com modificações. Brevemente, os precursores foram coletados dos fêmures de camundongos. A suspensão de células da medula foi ajustada para a concentração de $1,5 \times 10^6$ células/mL de meio de cultura RPMI contendo 10% SFB e 50µg/mL de gentamicina, suplementado com mrGM-CSF (20ng/mL). As suspensões foram incubadas por oito dias à 37°C, em estufa com 5% de CO₂. Metade do meio de cultura foi descartado e substituído por meio recém-preparado a cada três dias. Paralelamente, em alguns experimentos, os precursores celulares foram supridos com meio RPMI completo suplementado com 10% de sobrenadante de células L929 produtoras de GM-CSF. Nestas culturas, todo o meio foi trocado a cada três dias. O processo de diferenciação foi acompanhado por citometria de

fluxo analisando-se a presença de células CD11c⁺MHC-II⁺. Não houve diferenças significativas na eficiência de geração de células dendríticas entre os dois métodos (dados não mostrados).

6.2. Modulação de DCs *in vitro*. Para modulação de sua atividade funcional, as DCs diferenciadas *in vitro* foram retiradas das placas de cultura no oitavo dia de cultivo e semeadas em novas placas de seis poços, na presença de 50µg/mL de antígenos de *P.berghei* como preparados acima ou ainda na ausência de qualquer estímulo. As células foram estimuladas com 1ng/mL de lipopolisacarídeo (LPS) de *E.coli* (cepa 0111:B4, Sigma-Aldrich), e incubadas a 37°C, por 24 horas, em estufa contendo 5% de CO₂. Após a incubação, alíquotas dessas células foram coletadas das culturas para avaliação de seus marcadores de superfície por citometria de fluxo. As células restantes foram utilizadas em ensaios de proliferação antígeno-específica de células T e de transferência adotiva para camundongos C57BL/6, conforme descrito adiante.

6.3. Transferência adotiva de células dendríticas. 1,5x10⁶ DCs moduladas com antígenos de *P.berghei* foram transferidas adotivamente 3 dias antes (na avaliação de administração profilática) e 10 dias depois (na avaliação da administração terapêutica) da imunização com MOG₃₅₋₅₅, por via endovenosa pelo plexo retro-orbital.

6.4. Enriquecimento de linfócitos T esplênicos e ensaio de co-cultura. Após a morte dos animais dos grupos previstos no item 1 em câmara de CO₂, seus baços foram removidos com assepsia, transferidos em placas de Petri contendo meio de cultura sem soro, divulsionados com o auxílio de peneira e sedimentados por centrifugação. Os linfócitos foram separados em populações TCD4⁺ por passagem em esferas magnéticas recobertas com anticorpos anti-CD4, de acordo com recomendações do fabricante (Miltenyi Biotec, Alemanha). As células foram marcadas com a sonda CFSE conforme descrito

anteriormente e semeadas em placas de 96 poços contendo células dendríticas diferenciadas e moduladas como acima na proporção 1:1 (DC:T, respectivamente). O potencial estimulador das DCs foi avaliado pelo decaimento da intensidade da sonda e dosagem das citocinas secretadas nos sobrenadantes após 96h de cultura a 37°C em estufa de CO₂.

7. Detecção das citocinas secretadas nos sobrenadantes de culturas. Os sobrenadantes das culturas foram coletados ao final do período de incubação para dosagem de citocinas por citometria de fluxo, empregando o Cytometric Bead Array (BD Bioscience). Foram avaliados os níveis das citocinas interleucina (IL)-2, IL-4, IL-6, IL-10, IL-12, IL-17 e Interferon gama (IFN- γ) (todas do kit Th1/Th2/Th17 mouse cytokines, BD Biosciences).

8. Citometria de fluxo. A avaliação das proteínas de superfície celular e intracelular foi realizada por marcação com anticorpos monoclonais diretamente conjugados com fluorocromos. As células marcadas foram adquiridas em citômetro de fluxo (Gallios, Becton-Coulter) localizado no Departamento de Genética, Evolução e Bioagentes do Instituto de Biologia da UNICAMP e analisadas no programa FlowJo V10 (Tristar Inc.).

9. Detecção da expressão gênica por ensaio da reação de Real Time PCR.

9.1. Extração de RNA. A extração do RNA total foi realizada com o reagente Trizol, seguindo-se o protocolo recomendado pelo fabricante (Gibco Inc, USA). Brevemente, para cada amostra, em um tubo de fundo cônico, foi adicionado o reagente Trizol (1 ml para 1 mg de tecido), sendo agitado por 30 segundos e deixadas a temperatura ambiente por 5 minutos. Para cada 1 mL da suspensão foi adicionado 0,2 mL de

clorofórmio (Sigma) e centrifugadas a 12000g por 15 minutos a 4°C. A fase aquosa foi transferida para um tubo novo, ao qual foi adicionado o mesmo volume de isopropanol, sofrendo agitação e incubado por 20 minutos a -20° C para precipitar o RNA da fase aquosa. Novamente os tubos foram centrifugados a 12000g por 15 minutos a 4° C. O precipitado foi lavado em etanol 100%, sendo então seco em temperatura ambiente, com o tubo invertido sobre um papel de filtro. As amostras de RNA foram suspensas em 20µL de água deionizada e livre de RNase, sendo então armazenadas a -70° C. Uma alíquota de 1µL foi utilizada para a obtenção da concentração de RNA (Thermo Scientific, Waltham, MA, EUA).

9.2. Reações de Real Time PCR. A expressão quantitativa de genes de IL-10 (Mm00439614_m1), IL-17 (Mm00439618_m1), IFN γ (Mm01168134_m1), ROR γ t (Mm01261022_m1) e FOXP3 (Mm00475162_m1) nos tecidos foi analisada através de reações de Real Time PCR, utilizando-se os sistemas Taqman em um aparelho ABI Prism 7500 (Applied Biosystems, Foster City, CA, EUA). O DNA complementar sintetizado a partir do RNA mensageiro foi utilizado juntamente com reagente TaqMan Gene Expression Master Mix™ (Applied Biosystems, Foster City, CA, EUA). Diferentes oligonucleotídeos e sondas para a amplificação do controle endógeno GAPDH (Mm99999915_g1) e dos genes de interesse foram empregados. Os resultados foram analisados com base no valor de CT (*cycle threshold* – ou ciclo limiar), sendo este o ponto correspondente ao número de ciclos aonde a amplificação atinge um dado limiar, que permite a análise quantitativa da expressão do fator avaliado. Os gráficos estão expressos como expressão relativa tendo o grupo “naïve” como padrão (método de $2^{-\Delta\Delta CT}$).

10. Imunofluorescência. Para a análise da presença de células T duplo-positivas em SNC, cortes histológicos preparados como descrito anteriormente tiveram seus sítios reativos bloqueados com solução de PBS-BSA 1% (Sigma-Aldrich, USA). Após lavagens com salina tamponada (PBS), os cortes receberam anticorpos monoclonais anti-CD4/FITC (clone GK1.5) e anti-CD8 α /PE (clone 53-6.7) diluídos 1/100 em PBS. Os cortes foram analisados em microscópio de fluorescência (Leika). Os anticorpos foram adquiridos da eBioscience, USA.

11. Análise estatística. Os resultados foram analisados pelo teste t de “*Student*” e ANOVA (do inglês, *Analyses of Variance*), determinando-se como estatisticamente significativas as diferenças com valores de $p \leq 0,05$. Para a elaboração dos gráficos e das análises, foi utilizado o Software GraphPad Prism V5 (GraphPad Inc.).

Capítulo 1

Capítulo 1: Desenvolvimento de modelo não letal de infecção por *Plasmodium berghei* NK65.

Introdução

A malária permanece como a maior doença infecciosa do mundo, com aproximadamente 50% da população mundial sob o risco de contrair a doença (Battle *et al.*, 2014). A doença é causada pela infecção com protozoários do gênero *Plasmodium*, sendo que em humanos são descritas quatro espécies de plasmódio infectantes: *P.falciparum*, *P.vivax*, *P.ovale* e *P.malariae*. Em camundongos, as espécies usualmente infectantes são: *P.berghei*, *P.chabaudi* e *P.yoelli*. Para o estudo das respostas imunes contra o plasmódio, normalmente são usados camundongos infectados por *P.chabaudi* e *P.berghei*.

Em camundongos BALB/c foi demonstrado que a infecção por *Plasmodium berghei* NK65, uma cepa incapaz de causar malária cerebral, é caracterizada por elevada parasitemia e morte dos animais aos quatorze dias de infecção (Andrade *et al.*, 2008). Foi descrito anteriormente que a infecção também promove atrofia do timo, por meio da estimulação da morte de timócitos por apoptose e da migração precipitada de células T-DP para os órgãos linfoides secundários (Francelin *et al.*, 2011). Análises posteriores demonstraram ainda que, em camundongos infectados por *P.berghei* NK65 há alteração na deposição de elementos de matriz extracelular, bem como na expressão de quimiocinas e seus receptores em timócitos (Gameiro, Nagib, Andrade, *et al.*, 2010; Lima *et al.*, 2012). A migração de células T-DP do timo está relacionada com o desenvolvimento de respostas contra cardiomiócitos em camundongos infectados por *T.cruzi* (Mendes-Da-Cruz *et al.*, 2003). Para avaliar se as alterações no timo e, consequentemente, no repertório de linfócitos

T, provocadas pela infecção por *Plasmodium berghei* NK65 interferem no desenvolvimento de doenças autoimunes, o desenvolvimento de um modelo não letal de infecção foi necessário.

Dentre os fármacos empregados no controle da infecção por plasmódio, a cloroquina (CQ) se constitui numa alternativa interessante, visto que apresenta baixo custo e pouca exigência de armazenamento. De fato, por conta destas características a CQ foi utilizada como droga de escolha no tratamento de malária por várias décadas (Loeb *et al.*, 1946). Contudo, o uso sem controle médico e a descontinuidade do tratamento de pacientes facilitou o aparecimento de cepas resistentes de plasmódio (Harinasuta *et al.*, 1965; Sidhu *et al.*, 2002). Atualmente, o tratamento com cloroquina é indicado apenas em quimioterapia combinada com outros fármacos como a artemisinina e a mefloquina, que se constitui a terapia combinada de artemisinina (ACT) (World Health Organization., 2011).

Neste contexto, a presente etapa do projeto teve por objetivo o desenvolvimento de modelo não letal de infecção por *P.berghei* NK65. Os resultados obtidos mostraram que a administração de cloroquina foi capaz de eliminar o parasita da circulação enquanto que os camundongos permaneceram viáveis por até quarenta dias. Interessantemente, a administração de CQ mostrou-se capaz de alterar as frequências de subpopulações de linfócitos T, com especial atenção ao aumento no número de células T reguladoras.

Resultados

Tratamento com cloroquina reduz a letalidade da infecção pelo protozoário Plasmodium berghei NK65

O modelo de infecção por *Plasmodium berghei* NK65 em camundongos BALB/c foi caracterizado previamente (Andrade *et al.*, 2008), contudo carecem dados acerca da infecção em animais da linhagem C57BL/6. Os experimentos expostos nas figuras 1 e 2 foram desenvolvidos com o objetivo de avaliar a cinética da infecção por *P.berghei* NK65 em camundongos C57BL/6 fêmeas.

Para estabelecer o modelo não letal de infecção por *Plasmodium berghei* NK65 camundongos C57BL/6 infectados foram tratados com cloroquina (CQ), um agente antimalárico empregado no tratamento da malária humana (Thome, Lopes, *et al.*, 2013). Foi utilizada a dose de 5mg/kg, aplicada diariamente por cinco dias consecutivos a partir do décimo dia de infecção. Como controles, os animais foram tratados com a solução de diluição do fármaco: solução salina tamponada (PBS 0,02M pH7,2). Os resultados obtidos mostram que camundongos infectados por *P.berghei* morrem aos 14 dias de infecção. Por outro lado, camundongos que foram tratados com cloroquina permaneceram viáveis por até 40 dias (Figura 1).

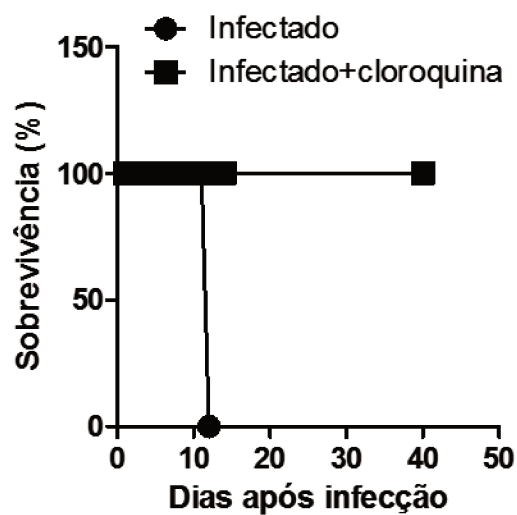


Figura 1 – Efeito da administração de cloroquina sobre a sobrevivência de camundongos infectados por *Plasmodium berghei*. Camundongos C57BL/6 fêmeas (n=10) foram infectados com 1×10^6 hemácias parasitadas por *P.berghei* NK65. Após dez dias da infecção, metade dos animais foi tratada com cloroquina (5mg/kg) por via intraperitoneal (i.p.) uma vez ao dia durante cinco dias consecutivos. A mortalidade foi acompanhada diariamente. Como controles, o restante dos animais recebeu a solução veículo (PBS 0,02M pH7,2). Os resultados mostram que a administração de cloroquina reduziu a morte dos animais. Dados representativos de cinco experimentos independentes.

Administração de cloroquina elimina a forma sanguínea de Plasmodium berghei, mas não impede a atrofia tímica

Conforme demonstrado na figura 2A, tal linhagem de camundongos desenvolve um modelo de infecção onde a malária é caracterizada por parasitemia intermediária (~40%, figura 2A), em contraponto ao observado em camundongos BALB/c onde a parasitemia se estende por até 20% (Andrade *et al.*, 2008). Devido à redução da letalidade da infecção após tratamento, a presença de formas hemoparasitárias de *P.berghei* foi investigada na vigência da administração de CQ. Os resultados obtidos mostram que a aplicação de cloroquina reduziu rapidamente a presença de *P.berghei* no sangue de camundongos infectados (Figura 2A). Mesmo com o término da administração, não foi detectado o parasita em hemácias por até quarenta dias (*dados não mostrados*).

Camundongos C57BL/6 infectados por *Plasmodium berghei* foram mortos em 0, 3, 7 e 14 dpi. O peso relativo do timo foi aferido para determinação do índice. Conforme demonstrado na figura 2B, foi observada uma redução significativa no índice tímico a partir do terceiro dia de infecção e que se agrava aos sete dias. Paralelamente, camundongos que foram tratados com cloroquina aos dez dias de infecção, não apresentaram melhora do índice tímico em relação aos animais sem tratamento (Figura 2B). Além disso, camundongos sem infecção tratados com cloroquina apresentaram índice tímico semelhante aos animais controle sem tratamento (*dados não mostrados*), indicando que a cloroquina não induz alterações no timo.

Em suma, os dados obtidos demonstram que: 1) a infecção por *Plasmodium berghei* NK65 pode ser controlada pela administração por via i.p. de cloroquina (5mg/kg) por cinco

dias consecutivos. 2) A eliminação do parasita não reverte a atrofia tímica estabelecida, sendo que o índice tímico aos quarenta dpi permaneceu semelhante aos dos animais com quatorze dpi (*dados não mostrados*).

A elevada taxa de sobrevivência dos animais por até quarenta dpi em associação com ausência de parasitos sanguíneos asseguram que o modelo de infecção não letal foi estabelecido com sucesso.

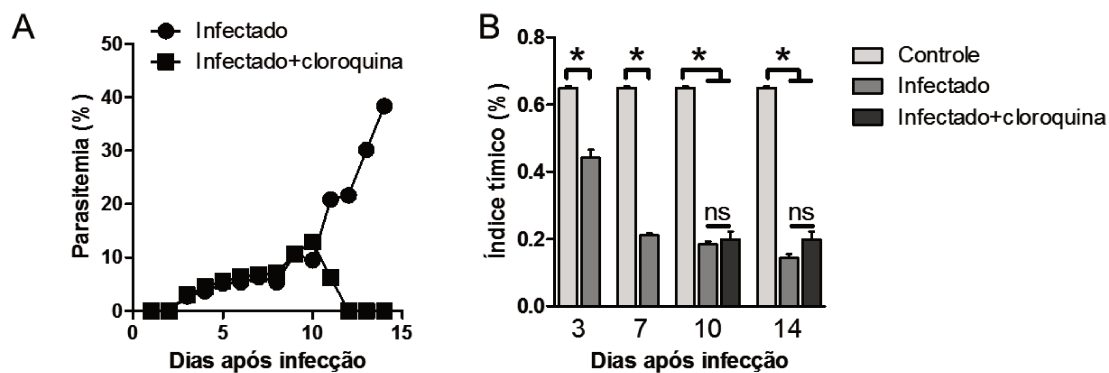


Figura 2 – Efeito da administração de cloroquina sobre a infecção por *Plasmodium berghei* e sobre o índice tímico. Camundongos C57BL/6 fêmeas (n=10) foram infectados com 1×10^6 hemácias parasitadas por *P.berghei* NK65. Após dez dias da infecção, metade dos animais foi tratada com cloroquina (5mg/kg) por via intraperitoneal (i.p.) uma vez ao dia durante cinco dias consecutivos. A) A parasitemia foi acompanhada diariamente e observa-se um rápido declínio na presença do parasita no sangue após a aplicação de cloroquina. B) Animais foram mortos em diferentes tempos de infecção, e o peso do timo em relação ao peso corpóreo (índice tímico) foi aferido. Os resultados mostram que a infecção por *P.berghei* induz perda gradual da massa tímica. Dados representativos de cinco experimentos independentes. *: $p < 0,05$.

Camundongos infectados por *P.berghei* apresentam alterações no repertório de linfócitos em órgãos linfoides primários e secundários

Tendo em vista que a infecção por *Plasmodium berghei* reduz o peso relativo do timo, importante órgão linfóide primário, as frequências das subpopulações de timócitos e linfócitos maduros foram avaliadas, respectivamente, em timos e baços de camundongos infectados. Como controles, tais análises foram conduzidas em animais saudáveis de mesma idade. Os resultados obtidos mostram que existe redução significativa na frequência de células T CD4⁺CD8⁺ (duplo-positivos, DP) em timos de camundongos infectados quando comparados com animais controle (Figura 3A). Por outro lado, verificou-se um aumento na frequência de timócitos CD4⁺CD8⁻ (duplo-negativos, DN) CD4⁺CD8⁻ (Simples-positivo, SP) e CD4⁻CD8⁺ (SP) (Figura 3A). Em menor grau, as subpopulações de linfócitos T em baços sofreram alterações em decorrência da infecção. Os baços de camundongos infectados apresentaram maior frequência de células T CD8 e DP quando comparadas com baços de camundongos saudáveis (Figura 3A).

A frequência de células T-DP foi acompanhada durante a infecção. Para tanto, camundongos C57BL/6 fêmeas foram mortas aos 3, 7, 10 e 14 dias após infecção e os baços retirados para o preparo de suspensões celulares as quais foram imunomarcadas com anticorpos fluorescentes anti-CD3ε, anti-CD4 e anti-CD8α. Os resultados obtidos mostraram uma maior frequência de células T-DP nos baços de camundongos infectados por *P.berghei*. O aumento na frequência desta população tem início discreto a partir do sétimo dia, com aumento significativo aos dez e quatorze dias após infecção (Figura 3B). Neste experimento, parte dos camundongos foi tratada com cloroquina (5mg/kg, nas mesmas condições descritas anteriormente) aos dez dias após infecção para eliminação do

parasita e a frequência de células T-DP foi avaliada também. Conforme exposto na figura 3B, o tratamento com cloroquina não impediu o aumento na frequência de células T-DP em baços de camundongos infectados. Ainda assim, com quatorze dias após infecção, houve diferença estatística na frequência de células T-DP entre os grupos infectado e infectado tratado com CQ.

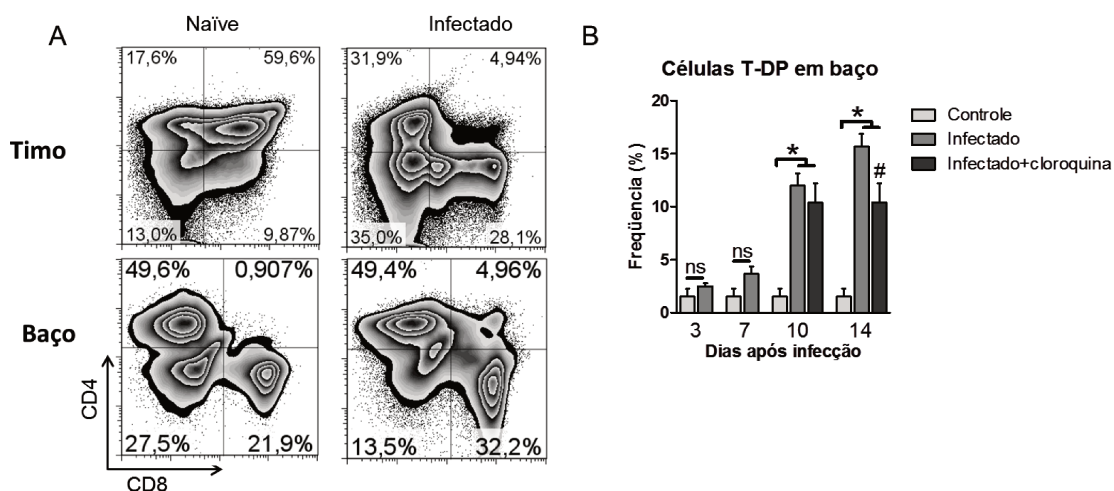


Figura 3 – Infecção por *Plasmodium berghei* promove alterações nas subpopulações de linfócitos T em órgãos linfoides primários e secundários. Camundongos C57BL/6 fêmeas (n=10) foram infectados com 1×10^6 hemácias parasitadas por *P.berghei* NK65. A) Aos dez dias de infecção, os animais foram mortos e os timos e baços coletados para análise das subpopulações de linfócitos T. Os resultados mostram que na vigência da infecção ocorrem alterações nas frequências de subpopulações de linfócitos em órgãos linfoides primários e secundários. B) A presença de células T CD4⁺CD8⁺ (duplo positivas, T-DP) em baço foi acompanhada em vários tempos de infecção e mostram um aumento gradual na frequência de células T-DP. Dados representativos de cinco experimentos independentes. *: $p < 0,05$ quando comparado ao controle, #: $p < 0,05$ quando comparado ao infectado, ns: não significativo.

Administração de cloroquina altera a frequência de células dendríticas e células T reguladoras

A cloroquina tem sido usada no controle da infecção plasmodial desde 1934, contudo, verificou-se recentemente que esta formulação possui propriedades anti-inflamatórias (revisto por Thome *et al.* 2013). Para verificar se a administração de cloroquina influencia na frequência de subpopulações celulares, camundongos C57BL/6 foram tratados com cloroquina (CQ, nas doses de 3, 5 e 10 mg/kg) por cinco dias consecutivos. Três dias após a aplicação da última dose de CQ, os animais foram mortos e os baços retirados para o preparo de suspensões celulares. As frequências de linfócitos T CD4⁺ e CD8⁺, células T reguladoras (CD4⁺CD25⁺FOXP3⁺) e células dendríticas (CD11c⁺MHC-II⁺) foram avaliadas.

Os resultados obtidos demonstram que a administração de cloroquina não promoveu alterações na frequência de células CD4⁺ e CD8⁺ (Figura 4A). Por outro lado, camundongos tratados com CQ apresentaram aumento na frequência de células T reguladoras quando comparados com camundongos controle (Figura 4B). O aumento foi proporcional à concentração da dose de cloroquina administrada (Figura 4B). Em contrapartida, houve redução no número de células dendríticas esplênicas com o tratamento com cloroquina (Figura 4C).

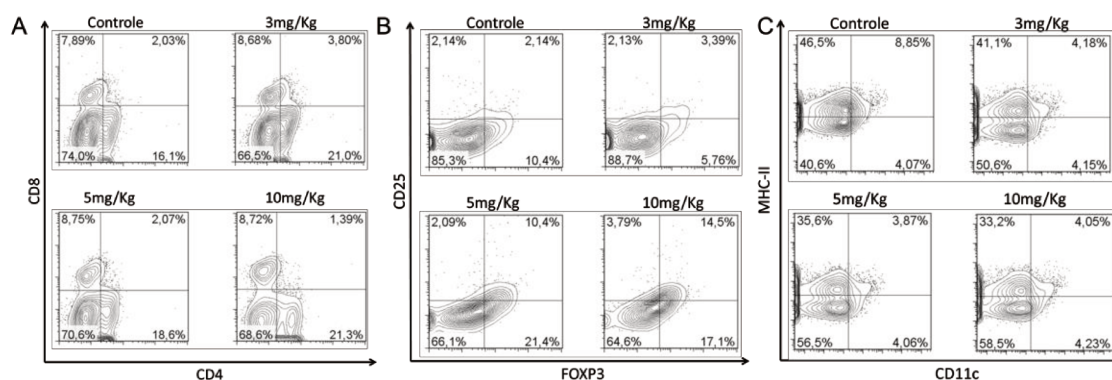


Figura 4 – Efeito do tratamento com cloroquina sobre a frequência de subtipos de linfócitos T e de células dendríticas. Camundongos C57BL/6 (n=6 camundongos/grupo) foram tratados com cloroquina (5mg/kg) por via intraperitoneal (i.p.) uma vez ao dia durante cinco dias consecutivos. Três dias após a administração da última dose de cloroquina, os animais foram mortos e os baços coletados, divulsionados e as suspensões celulares foram incubadas com anticorpos monoclonais conjugados a fluorocromos para análise da frequência de células T CD4⁺ e CD8⁺, células T CD4⁺CD25⁺FOXP3⁺ e células dendríticas. Dados representativos de dois experimentos independentes.

Discussão

Os resultados aqui apresentados mostram que a administração de cloroquina foi capaz de eliminar o parasita *Plasmodium berghei* NK65 da circulação de camundongos C57BL/6 inoculados com hemácias infectadas. A cloroquina foi um fármaco empregado por muito tempo como droga de entrada no tratamento da malária, contudo o uso desordenado e mal acompanhado por parte dos pacientes promoveu o surgimento de cepas de plasmódio resistentes (Loeb *et al.*, 1946).

De forma geral, o modelo não letal de infecção por *Plasmodium berghei* NK65 foi estabelecido com sucesso. Camundongos tratados com cloroquina apresentaram redução da parasitemia, bem como permaneceram com o timo atrófico durante todo o período experimental. Interessantemente, a eliminação do protozoário não promoveu recuperação do índice tímico, mas uma estabilização de sua massa relativa.

Mesmo em plasmódio a ação da cloroquina não está bem descrita. CQ age exclusivamente na fase eritrocítica da malária. Algumas teorias quanto ao seu mecanismo de ação ditam que CQ interage com moléculas de DNA e inibição de enzimas e transporte de nutrientes (Slater, 1993; Sullivan *et al.*, 1996; Bray *et al.*, 1998; Sullivan *et al.*, 1998; Bray *et al.*, 1999). A hipótese mais aceita afirma que cloroquina interfere com a digestão de hemoglobina pelo protozoário (Fitch, 1969; Fitch *et al.*, 1982; Krogstad *et al.*, 1985; Krogstad e Schlesinger, 1986; Sullivan *et al.*, 1996; Sullivan *et al.*, 1998). Em sua fase eritrocítica, o plasmódio invade hemácias e se desenvolve pela ingestão de hemoglobina do citoplasma celular e depositando o resíduo em seu vacúolo digestivo (VD). O VD é um compartimento acidificado sendo, portanto, alvo da ação da cloroquina. Acredita-se que CQ

se ligue a hematina, um produto tóxico oriundo da degradação de hemoglobina, impedindo sua incorporação ao cristal de hemozoina. A hematina livre, aparentemente, interfere com o processo de desintoxicação do plasmódio provocando danos às membranas do protozoário por peroxidação de lipídios.

Interessantemente, a aplicação de CQ além de eliminar o plasmódio foi capaz de alterar componentes do sistema imune em camundongos saudáveis. Com especial atenção, houve um aumento significativo no número absoluto de células T reguladoras de forma dependente da dose de CQ. Por outro lado, a frequência de células dendríticas foi reduzida. Esses achados indicam que a cloroquina pode apresentar efeitos imuno-moduladores.

De fato, a literatura apresenta estudos onde a administração de cloroquina apresentou efeitos benéficos no controle da inflamação. Foi demonstrado que pacientes portadores de artrite reumatóide apresentaram significativa melhora no edema e dor nas articulações após tratamento com CQ (Canadian Consensus Conference on hydroxychloroquine, 2000). Semelhantemente, a administração de CQ promoveu melhora nos parâmetros clínico de pacientes portadores de lúpus (Gladman *et al.*, 1998; Tam *et al.*, 2000). Ainda não está completamente esclarecido o mecanismo pelo qual CQ exerce seu efeito, porém é conhecido que este fármaco se comporta como uma base fraca apresentando tropismo a organelas ácidas, como o lisossomo (Thome, Lopes, *et al.*, 2013). Por este motivo, a administração de CQ mostrou-se capaz de impedir o processamento de antígenos por células dendríticas, visto que este mecanismo é dependente da atividade de lisossomos (Garulli *et al.*, 2008; Amaravadi *et al.*, 2011).

Dentre as funções imunomoduladoras de cloroquina, foi demonstrado que a administração de CQ suprimiu a ativação celular dependente de TLRs (Macfarlane e Manzel, 1998; Savarino *et al.*, 2003; Loehberg *et al.*, 2012). CQ não apenas reduziu a

produção de TNF- α como também suprimiu a expressão de seu receptor em células U-937 humanas (Jeong *et al.*, 2002). Esses efeitos são dependentes e independentes de pH, uma vez que CQ interfere com o metabolismo do ferro em macrófagos (Jeong e Jue, 1997). CQ também se mostrou capaz de reduzir seletivamente a secreção de IL-1 e IL-6 por linfócitos T e macrófagos (Ertel *et al.*, 1992; Sperber, 1993; Karres, 1998).

Tomados em conjunto os resultados mostram que o modelo não letal de infecção por *Plasmodium berghei* foi desenvolvido com sucesso por meio da administração de cloroquina, um fármaco com potencial imunossupressor pouco explorado. Novos estudos deverão ser conduzidos com o objetivo de determinar quais os mecanismos de ação da cloroquina sobre o sistema imune.

Conclusões

Tendo em vista os resultados apresentados neste capítulo, pode-se concluir que:

- a. Camundongos infectados tratados com cloroquina apresentaram redução da parasitemia, manutenção do baixo do índice tímico, e aumento da frequência de células T-DP em órgão linfóide secundário.
- b. O tratamento de camundongos saudáveis com cloroquina promoveu um aumento significativo na frequência de células T reguladoras e redução em células dendríticas.

Os resultados obtidos permitiram a confecção do artigo: “Chloroquine: Modes of Action of an Undervalued Drug” (Página 210).

Capítulo 2

Capítulo 2 – Efeito da infecção por *Plasmodium berghei* NK65 sobre o curso da Encefalomielite Autoimune Experimental.

Introdução

O timo desempenha um papel crucial no desenvolvimento e maturação de linfócitos T. Dentro do órgão, timócitos (os linfócitos T no timo) são expostos a um microambiente rico em citocinas, quimiocinas e interações célula-célula culminando em sua proliferação, rearranjo de receptores de antígenos (TCR) e a diferenciação de timócitos em linfócitos T maduros (Gameiro, Nagib e Verinaud, 2010). Entretanto, o timo apresenta perda de massa e celularidade por conta do envelhecimento e distúrbios ambientais, como infecções e desnutrição (Savino *et al.*, 2007). Trabalhos anteriores de nosso laboratório demonstraram que a infecção por *Plasmodium berghei* NK65, agente causador de malária não-cerebral, torna o timo atrófico por meio da indução de morte por apoptose e a migração precoce de timócitos CD4⁺CD8⁺ (duplo-positivos, DP) para a periferia (Andrade *et al.*, 2008; Gameiro, Nagib, Andrade, *et al.*, 2010; Francelin *et al.*, 2011).

Conhecidamente, infecções virais e bacterianas podem promover o desenvolvimento de autoimunidade por meio da (i) quebra da tolerância periférica de células T, (ii) pelo desenvolvimento de linfócitos T efetores reativos a auto-antígenos ou (iii) pelo fenômeno conhecido por mimetismo molecular, onde um antígeno exógeno compartilha sequências ou estruturas semelhantes a proteínas próprias (Gilden, 2005; Herrmann *et al.*, 2006). Neste último caso, a febre reumática aguda, onde anticorpos atacam cardiomiócitos, pode se desenvolver após a geração de resposta imune contra *Streptococcus* β-hemolítico do grupo

A (Read *et al.*, 1974; Kawakita *et al.*, 1976). Adicionalmente, foi proposto que células T-DP elicitadas na vigência da infecção por *Trypanosoma cruzi* desempenham um papel importante na inflamação cardíaca autoimune observada em camundongos com doença de Chagas (Mendes-Da-Cruz *et al.*, 2003).

Neste contexto, a presente etapa da tese teve por objetivo avaliar se a infecção prévia por *Plasmodium berghei* NK65 interfere no curso clínico e na susceptibilidade ao desenvolvimento de Encefalomielite Autoimune Experimental, o modelo murino de Esclerose Múltipla humana. Para tanto, camundongos geneticamente susceptíveis (C57BL/6) e resistentes (BALB/c) ao modelo de EAE foram infectados com a cepa NK65 de *P.berghei* e curados da malária pela administração de cloroquina. Posteriormente, os animais foram imunizados com peptídeo encefalitogênico MOG₃₅₋₅₅ e acompanhados diariamente para avaliação dos sinais clínicos de EAE. Foi observado que camundongos C57BL/6 desenvolveram quadro clínico agravado de EAE, com intenso infiltrado de células T-DP no Sistema Nervoso Central. Análises subsequentes mostraram que células T-DP recém-saídas do timo desempenharam papel importante na severidade da doença. Interessantemente, camundongos geneticamente resistentes, após infecção com *P.berghei*, desenvolveram parte dos sinais clínicos de EAE. Este é o primeiro estudo a demonstrar uma possível integração entre malária e EAE por meio da contribuição do timo.

Resultados

Agravamento da Encefalomielite Autoimune Experimental em camundongos previamente infectados por Plasmodium berghei NK65

O presente estudo teve por objetivo verificar se camundongos que previamente tiveram malária apresentam alteração no curso clínico de Encefalomielite Autoimune Experimental (EAE). Para tanto, camundongos C57BL/6 fêmeas foram infectados com *P.berghei* por meio da injeção por via intraperitoneal (i.p.) de hemácias parasitadas. Dez dias após a infecção, os animais foram tratados diariamente com cloroquina, na dose de 5mg/kg, por cinco dias consecutivos. Após três dias da aplicação da última dose do fármaco, os animais foram imunizados com emulsão contendo peptídeo MOG₃₅₋₅₅ e Adjuvante Completo de Freund por via subcutânea (NK+CQ+EAE). Além disso, para induzir o quadro de EAE, os animais também receberam, por via i.p., toxina de *Bordetella pertussis* em 0h e 48h após aplicação de MOG. Como controles, parte dos camundongos foi tratada com cloroquina (CQ+EAE) e outra parte com PBS (EAE).

a. Alteração no curso da doença

Os animais foram acompanhados diariamente para avaliação do surgimento dos sinais clínicos de EAE. Conforme exposto na figura 1, animais imunizados com peptídeo MOG tem o aparecimento dos sinais clínicos de EAE a partir do sétimo dia após imunização, com piora progressiva (Grupo EAE). Os animais previamente infectados por *P.berghei*, por sua vez, apresentaram quadro clínico significativamente mais severo do que

animais não infectados e imunizados com MOG (Figura 1). Interessantemente, os animais pertencentes ao grupo CQ+EAE apresentaram quadro clínico significativamente reduzido quando comparados com os animais pertencentes aos grupos EAE e NK+CQ+EAE, o que indica a possibilidade de um efeito protetor da cloroquina sobre a neuroinflamação autoimune.

b. Modificação na resposta proliferativa de linfócitos T

Decorridos dez dias da imunização com MOG, quando os primeiros sinais clínicos se manifestaram, os animais foram mortos e seus baços coletados para o preparo de suspensões celulares, as quais foram marcadas com a sonda CFSE e postos em cultura na presença dos estímulos: peptídeo MOG (para avaliar proliferação antígeno-específica) e Concanavalina-A (para avaliar proliferação inespecífica). Os resultados obtidos mostram que células provenientes de camundongos “naïve”, quando cultivadas na presença de MOG, não proliferaram significativamente quando comparadas com células de camundongos pertencentes ao grupo EAE (Figura 2). As células provenientes de camundongos pertencentes ao grupo NK+CQ+EAE, quando cultivadas com MOG, proliferaram em níveis similares às culturas de células do grupo EAE (Figura 2). Por outro lado, houve redução significativa na proliferação celular do grupo CQ+EAE em comparação ao grupo EAE e NK+CQ+EAE (Figura 2). Quando foram comparadas as culturas conduzidas na presença de concanavalina-A, verificou-se que células pertencentes aos grupos “naïve”, EAE e NK+CQ+EAE proliferaram em níveis próximos, sem a existência de diferença estatística. Contudo, as células pertencentes ao grupo CQ+EAE proliferaram significativamente menos quando comparadas aos demais grupos (Figura 2).

c. Mudança no perfil de citocinas secretadas por linfócitos T após cultivo com peptídeo MOG₃₅₋₅₅

Os sobrenadantes das culturas contendo células provenientes de camundongos dos grupos EAE, CQ+EAE e NK+CQ+EAE foram coletados ao término da incubação, e ensaiados para a dosagem de citocinas produzidas. Conforme exposto na figura 3, as culturas com células provenientes de camundongos portadores de EAE sem tratamento com cloroquina ou infectados apresentaram elevados níveis de IL-4, IL-6, IL-10, IL-17 e Fator de Necrose Tumoral (TNF)- α . Contudo, as células de animais pertencentes ao grupo NK+CQ+EAE apresentaram níveis significativamente mais elevados de IL-6, IL-17, TNF- α e IFN- γ quando comparados com as culturas do grupo EAE. Interessantemente, as culturas de células esplênicas provenientes do grupo CQ+EAE apresentaram níveis significativamente maiores de IL-4 e IL-10, ao passo que os níveis de IL-6, IL-17, TNF- α e IFN- γ se encontraram significativamente mais reduzidos quando comparados com os grupos EAE e NK+CQ+EAE (Figura 3).

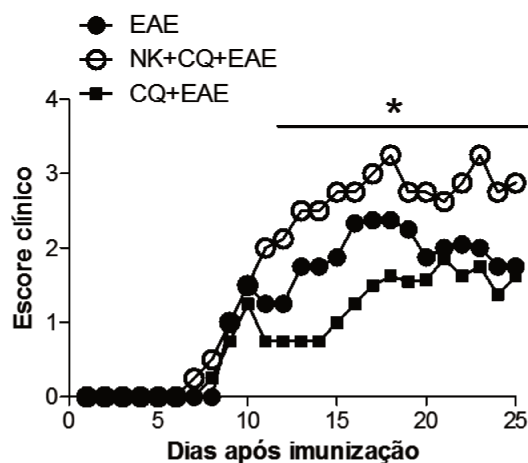


Figura 1 – Efeito da infecção por *Plasmodium berghei* sobre a severidade da Encefalomielite Autoimune Experimental. Camundongos C57BL/6 (n=6 camundongos/grupo) foram infectados com 1×10^6 hemácias parasitadas por *P.berghei* e depois de dez dias foram tratados com cloroquina (5mg/kg) por via i.p. uma vez ao dia durante cinco dias consecutivos. Três dias após a administração da última dose de cloroquina, os animais foram imunizados com 100µg de peptídeo MOG₃₅₋₅₅ e acompanhados diariamente para o surgimento dos sinais clínicos de EAE. Como controles parte dos camundongos recebeu apenas cloroquina (CQ+EAE) e outra parte foi tratada com veículo (EAE). Os resultados mostram que animais previamente infectados apresentam piora do quadro clínico de EAE em comparação dos animais dos grupos controles. Dados representativos de quatro experimentos independentes. *:p<0,05.

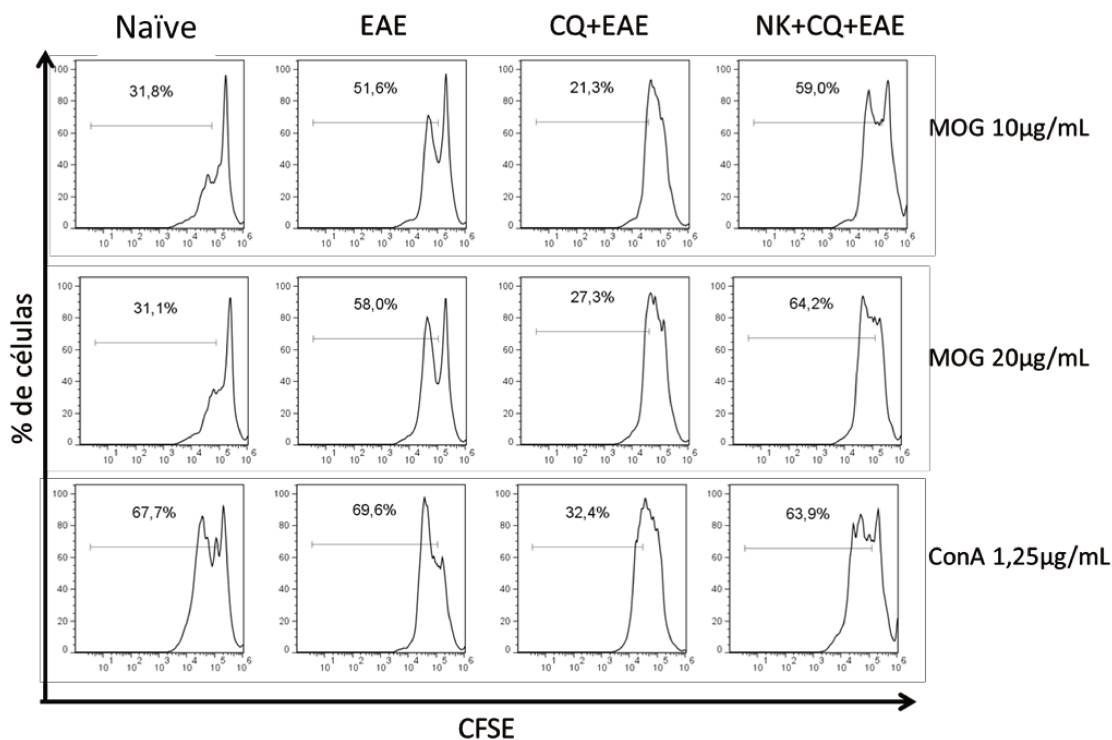


Figura 2 – Alterações na responsividade celular de células T esplênicas de camundongos portadores de EAE. Camundongos C57BL/6 (n=6 camundongos/grupo) foram infectados com 1×10^6 hemácias parasitadas por *P.berghei* e depois de dez dias foram tratados com cloroquina (5mg/kg) por via i.p. uma vez ao dia durante cinco dias consecutivos. Três dias após a administração da última dose de cloroquina, os animais foram imunizados com 100µg de peptídeo MOG₃₅₋₅₅ e acompanhados diariamente para o surgimento dos sinais clínicos de EAE. Como controles parte dos camundongos recebeu apenas cloroquina (CQ+EAE) e outra parte foi tratada com veículo (EAE). Decorridos dez dias da indução de EAE, os animais foram mortos e os baços foram recolhidos assepticamente para o preparo de suspensões celulares. As células esplênicas ($2,5 \times 10^5$ /well) foram marcadas com a sonda CFSE (1,25µM) e incubadas na presença de peptídeo MOG₃₅₋₅₅ ou de Concanavalina A por 96h em estufa de CO₂. Como controles as células foram cultivadas na ausência de estímulos. A proliferação foi analisada pelo decaimento da fluorescência da sonda dentro da “gate” de células T CD3⁺. Dados representativos de três experimentos independentes.

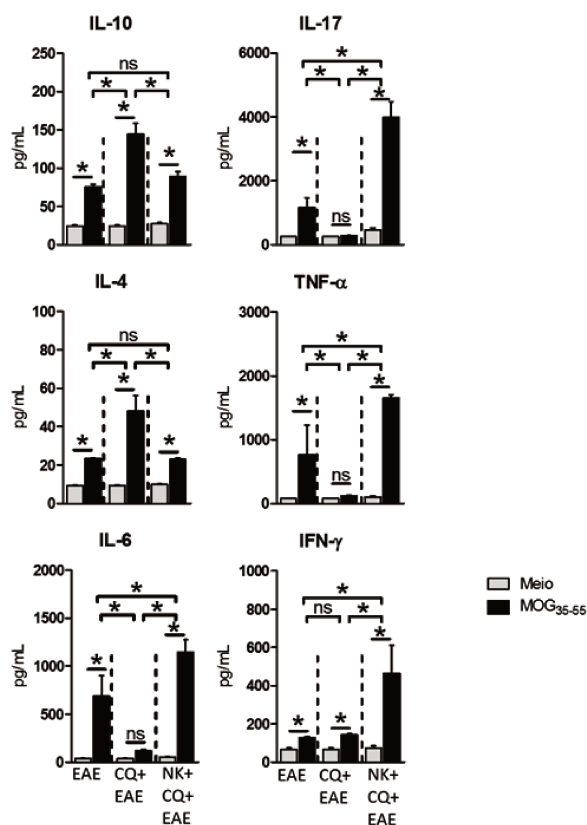


Figura 3 – Dosagem das citocinas produzidas nas culturas de células esplênicas. Camundongos C57BL/6 (n=6 camundongos/grupo) foram infectados com 1×10^6 hemácias parasitadas por *P.berghei* e depois de dez dias foram tratados com cloroquina (5mg/kg) por via i.p. uma vez ao dia durante cinco dias consecutivos. Três dias após a administração da última dose de cloroquina, os animais foram imunizados com 100µg de peptídeo MOG₃₅₋₅₅ e acompanhados diariamente para o surgimento dos sinais clínicos de EAE. Como controles parte dos camundongos recebeu apenas cloroquina (CQ+EAE) e outra parte foi tratada com veículo (EAE). Decorridos dez dias da indução de EAE, os animais foram mortos e os baços foram recolhidos assepticamente para o preparo de suspensões celulares. As células esplênicas ($2,5 \times 10^5$ /well) foram marcadas com a sonda CFSE (1,25µM) e incubadas na presença de peptídeo MOG₃₅₋₅₅ ou de Concanavalina A por 96h em estufa de CO₂. Como controles as células foram cultivadas na ausência de estímulos. Ao término do período de incubação os sobrenadantes foram coletados e analisados quanto a presença de citocinas por CBA (BD Biosciences). Dados representativos de três experimentos independentes. *:p<0,05, ns: não significativo.

Alterações histológicas no Sistema Nervoso Central (SNC) de camundongos portadores de EAE previamente infectados por Plasmodium berghei NK65

A infiltração de células do sistema imune em tecidos do Sistema Nervoso Central é um dos principais sinais observados em pacientes portadores de esclerose múltipla. De forma similar, camundongos portadores de EAE apresentam extensos infiltrados inflamatórios no SNC. A medula lombar é um tecido representativo do SNC para avaliação do infiltrado inflamatório em condições inflamatório-infecciosas (Bombeiro *et al.*, 2012; Thome, Moraes, *et al.*, 2013). Assim, tendo em vista o agravamento do quadro clínico de EAE em camundongos curados de malária, as medulas espinhais foram avaliadas quanto à inflamação tecidual.

a. Aumento do infiltrado inflamatório em camundongos previamente curados de malária

Camundongos C57BL/6 previamente infectados por *P.berghei* foram tratados com cloroquina por cinco dias consecutivos. A EAE foi induzida três dias após o término do tratamento. Como controle, foram utilizados camundongos apenas tratados com cloroquina (grupo CQ+EAE) e animais não infectados e não tratados (EAE). Aos quatorze dias após a imunização com neuro-antígenos, os camundongos foram mortos, e a porção lombar da medula espinhal foi preparada para análise histopatológica conforme descrito anteriormente. Conforme exposto na Figura 4 e corroborando com os dados de literatura, camundongos portadores de EAE apresentam infiltrado celular no SNC. Interessantemente,

camundongos do grupo NK+CQ+EAE apresentaram maior infiltrado celular do que os animais EAE. Por outro lado, não foram encontrados sítios de infiltração celular em tecidos provenientes de animais do grupo CQ+EAE (Figura 4).

b. Inflamação neural é aumentada nos camundongos EAE previamente infectados por Plasmodium berghei NK65

i. Astroglíose

Amostras de tecido neural foram analisadas quanto a expressão de GFAP, um marcador de astroglíose, que é resultado da ativação de células da glia: astrócitos, oligodendrócitos e micróglia. Conforme exposto na Figura 5, células da glia pertencentes a animais portadores de EAE apresentam elevada produção de GFAP. Contudo, não se observou diferença na expressão deste marcador quando as amostras do grupo EAE e NK+CQ+EAE foram comparadas. As amostras pertencentes aos animais do grupo CQ+EAE apresentaram redução na expressão de GFAP quando comparadas com as amostras dos animais dos grupos EAE e NK+CQ+EAE (Figura 5).

ii. IL-6

Cortes histológicos da região lombar de medulas espinhais foram imunomarcados com anticorpos anti-IL-6 para análise da produção deste fator inflamatório no SNC de camundongos portadores de EAE. Conforme exposto na Figura 6, amostras de camundongos portadores de EAE apresentaram produção de IL-6. Os animais pertencentes ao grupo NK+CQ+EAE produziram maior quantidade de IL-6 *in locu* do que os camundongos do grupo EAE. Adicionalmente, os camundongos CQ+EAE produziram menos IL-6 do que os animais dos grupos EAE e NK+CQ+EAE (Figura 6).

iii. *Enzima inducible Nitric Oxide Synthase(iNOS)*

O óxido nítrico é um importante sinalizador da resposta imune, bem como de impulsos nervosos. Durante a resposta imune, e em resposta a estímulos oriundos do microambiente, a enzima sintase de óxido nítrico pode ser induzida (isoforma iNOS) com a finalidade de auxiliar na eliminação de patógenos ou na liberação de sinais na comunicação intercelular. Nesta etapa do estudo, a expressão de iNOS foi avaliada no sítio inflamatório da EAE, i.e. SNC. Conforme exposto na Figura 7, a produção de iNOS mostrou-se maior em camundongos pertencentes ao grupo NK+CQ+EAE quando comparados com camundongos dos grupos EAE e CQ+EAE. Interessantemente, e em concordância com os marcadores previamente mostrados, verificou-se uma menor produção de iNOS nos animais pertencentes ao grupo CQ+EAE quando comparados com os demais grupos (Figura 7).

c. *As subpopulações celulares que infiltraram o SNC de camundongos previamente curados de malária são compostas principalmente de células T CD4⁺CD8⁺*

Para avaliar se as células T-DP (CD4⁺CD8⁺) – elicitadas pela infecção por *Plasmodium berghei* NK65 – exercem algum papel na inflamação *in locu*, amostras de região lombar de medula espinhal foram coletadas de camundongos pertencentes aos grupos EAE, CQ+EAE e NK+CQ+EAE e analisadas quanto à presença de células CD4⁺CD8⁺ por imunofluorescência com dupla marcação. Os resultados obtidos estão representados na Figura 8 e mostram que animais do grupo EAE apresentam infiltrado formado principalmente por células T CD4⁺ e com poucas células T CD8⁺. No entanto, os

animais pertencentes ao grupo NK+CQ+EAE apresentaram extenso infiltrado inflamatório, sendo este constituído principalmente por células $CD4^+CD8^+$. Já animais pertencentes ao grupo CQ+EAE não apresentaram infiltrado inflamatório (Figura 8).

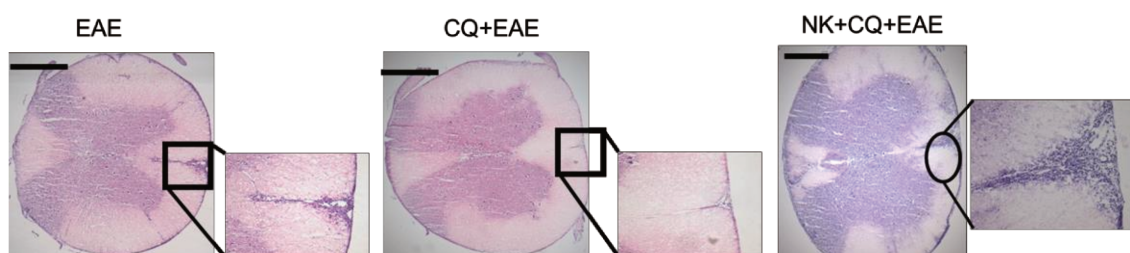


Figura 4 – Análise da frequência de células que infiltraram o Sistema Nervoso Central de camundongos portadores de EAE. Camundongos C57BL/6 (n=6 camundongos/grupo) foram infectados com 1×10^6 hemácias parasitadas por *P.berghei* e depois de dez dias foram tratados com cloroquina (5mg/kg) por via i.p. uma vez ao dia durante cinco dias consecutivos. Três dias após a administração da última dose de cloroquina, os animais foram imunizados com 100µg de peptídeo MOG₃₅₋₅₅ e acompanhados diariamente para o surgimento dos sinais clínicos de EAE. Como controles parte dos camundongos recebeu apenas cloroquina (CQ+EAE) e outra parte foi tratada com veículo (EAE). Decorridos quatorze dias da indução de EAE, os animais foram mortos e as medulas lombares foram recolhidas cuidadosamente. Na sequência as amostras foram congeladas em meio de sustentação. Cortes de 14µm foram feitos em criostato e corados com Hematoxilina e Eosina. Os resultados mostram que os animais previamente infectados apresentaram maior infiltrado de células do que os animais controle. Dados representativos de três experimentos independentes.

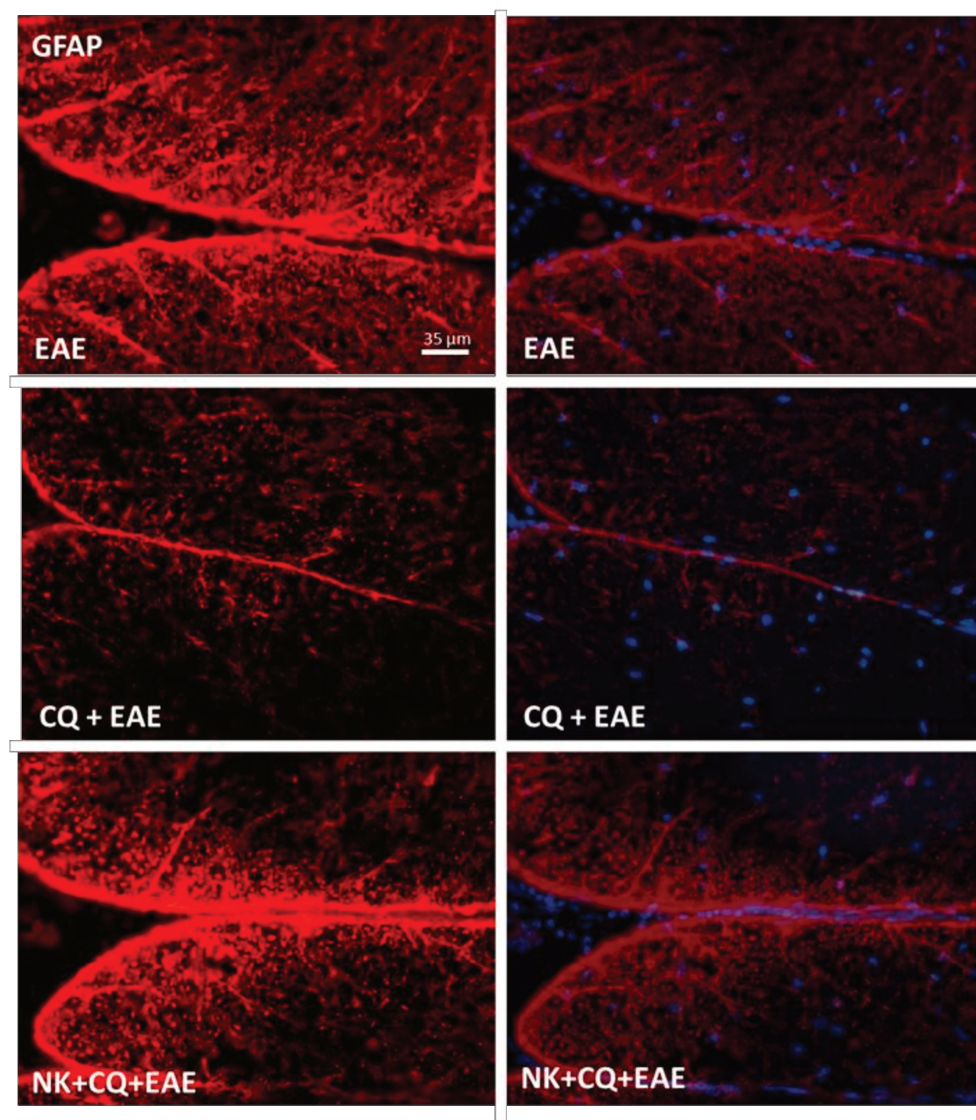


Figura 5 – Análise da produção de GFAP por imunofluorescência em medula espinhal de camundongos portadores de EAE. Camundongos C57BL/6 (n=6 camundongos/grupo) foram infectados com 1×10^6 hemácias parasitadas por *P.berghei* e depois de dez dias foram tratados com cloroquina (5mg/kg) por via i.p. uma vez ao dia durante cinco dias consecutivos. Três dias após a administração da última dose de cloroquina, os animais foram imunizados com 100μg de peptídeo MOG₃₅₋₅₅ e acompanhados diariamente para o surgimento dos sinais clínicos de EAE. Como controles parte dos camundongos recebeu apenas cloroquina (CQ+EAE) e outra parte foi tratada com veículo (EAE). Decorridos quatorze dias da indução de EAE, os animais foram mortos e as medulas lombares foram recolhidas cuidadosamente. Na sequência, as amostras foram congeladas em meio de sustentação. Cortes de 14μm foram feitos em criostato e imunomarcados com anticorpos anti-GFAP/Cy3. Os resultados mostram que os animais portadores de EAE saudáveis ou previamente infectados apresentaram maior produção de GFAP do que os animais tratados com cloroquina. Dados representativos de dois experimentos independentes.

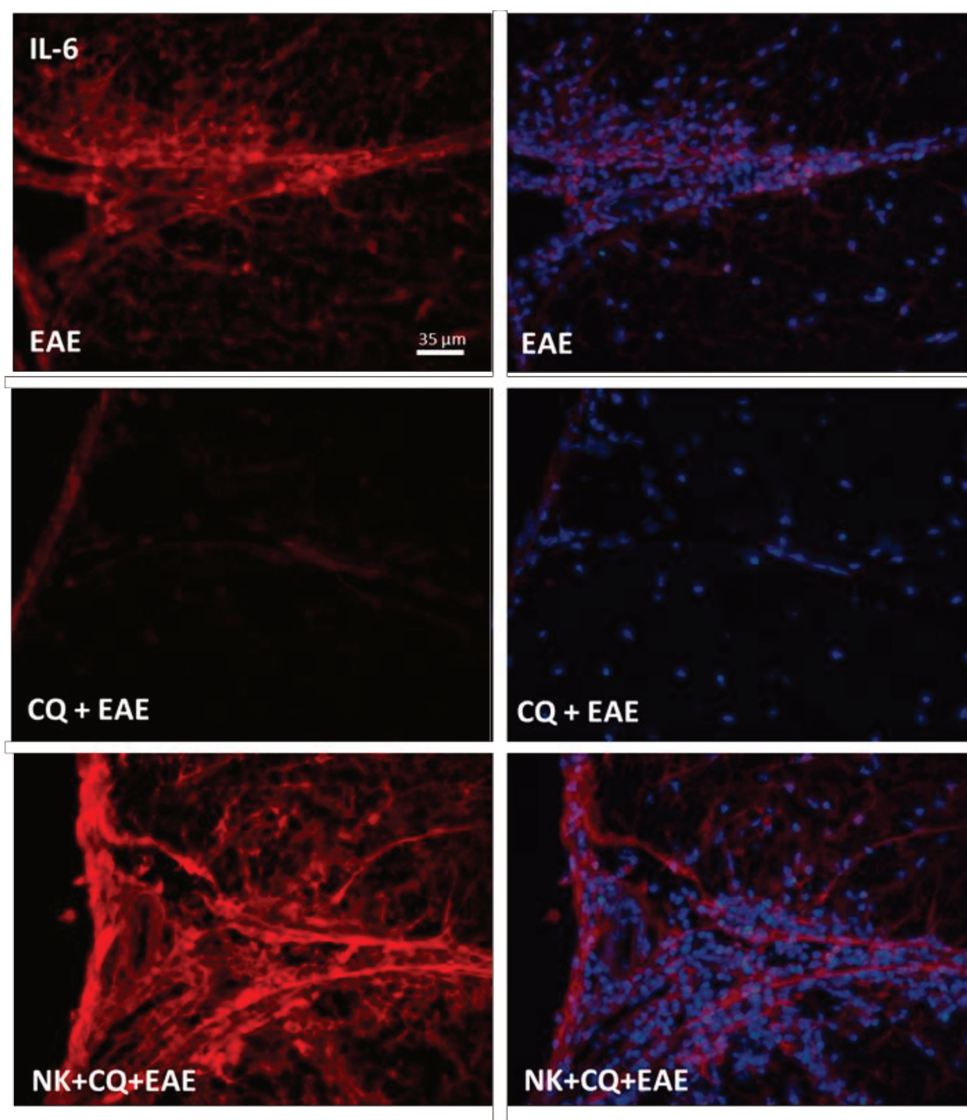


Figura 6 – Análise da produção de IL-6 por imunofluorescência em medula espinhal de camundongos portadores de EAE. Camundongos C57BL/6 (n=6 camundongos/grupo) foram infectados com 1×10^6 hemácias parasitadas por *P.berghei* e depois de dez dias foram tratados com cloroquina (5mg/kg) por via i.p. uma vez ao dia durante cinco dias consecutivos. Três dias após a administração da última dose de cloroquina, os animais foram imunizados com 100μg de peptídeo MOG₃₅₋₅₅ e acompanhados diariamente para o surgimento dos sinais clínicos de EAE. Como controles parte dos camundongos recebeu apenas cloroquina (CQ+EAE) e outra parte foi tratada com veículo (EAE). Decorridos quatorze dias da indução de EAE, os animais foram mortos e as medulas lombares foram recolhidas cuidadosamente. Na sequência, as amostras foram congeladas em meio de sustentação. Cortes de 14μm foram feitos em criostato e imunomarcados com anticorpos anti-IL-6/Cy3. Os resultados mostram que os animais portadores de EAE previamente infectados apresentaram maior produção de IL-6 do que os animais EAE não tratados ou tratados com cloroquina. Dados representativos de dois experimentos independentes.

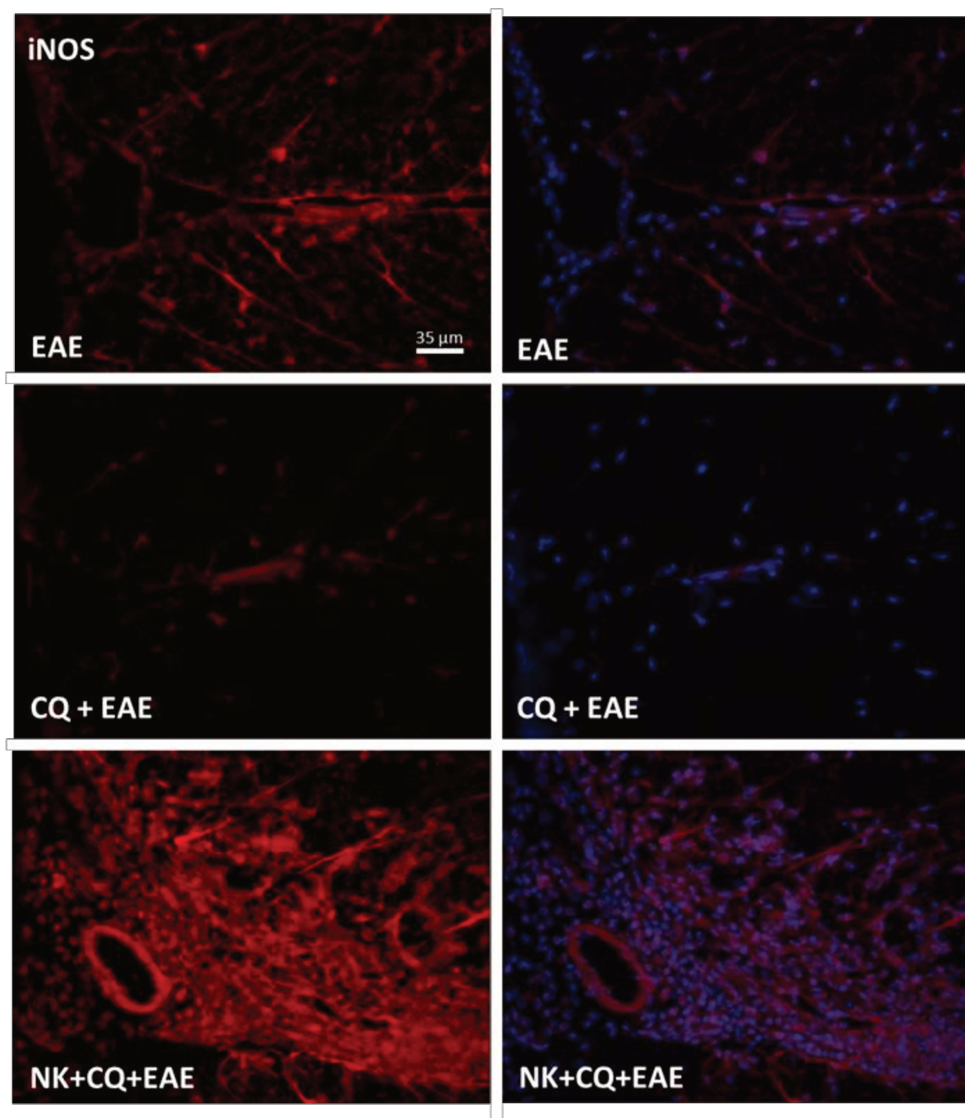


Figura 7 – Análise da produção de iNOS por imunofluorescência em medula espinhal de camundongos portadores de EAE. Camundongos C57BL/6 (n=6 camundongos/grupo) foram infectados com 1×10^6 hemácias parasitadas por *P.berghei* e depois de dez dias foram tratados com cloroquina (5mg/kg) por via i.p. uma vez ao dia durante cinco dias consecutivos. Três dias após a administração da última dose de cloroquina, os animais foram imunizados com 100μg de peptídeo MOG₃₅₋₅₅ e acompanhados diariamente para o surgimento dos sinais clínicos de EAE. Como controles parte dos camundongos recebeu apenas cloroquina (CQ+EAE) e outra parte foi tratada com veículo (EAE). Decorridos quatorze dias da indução de EAE, os animais foram mortos e as medulas lombares foram recolhidas cuidadosamente. Na sequência, as amostras foram congeladas em meio de sustentação. Cortes de 14μm foram feitos em criostato e imunomarcados com anticorpos anti-iNOS/Cy3. Os resultados mostram que os animais portadores de EAE previamente infectados apresentaram maior produção de iNOS do que os animais EAE não tratados ou tratados com cloroquina. Dados representativos de dois experimentos independentes.

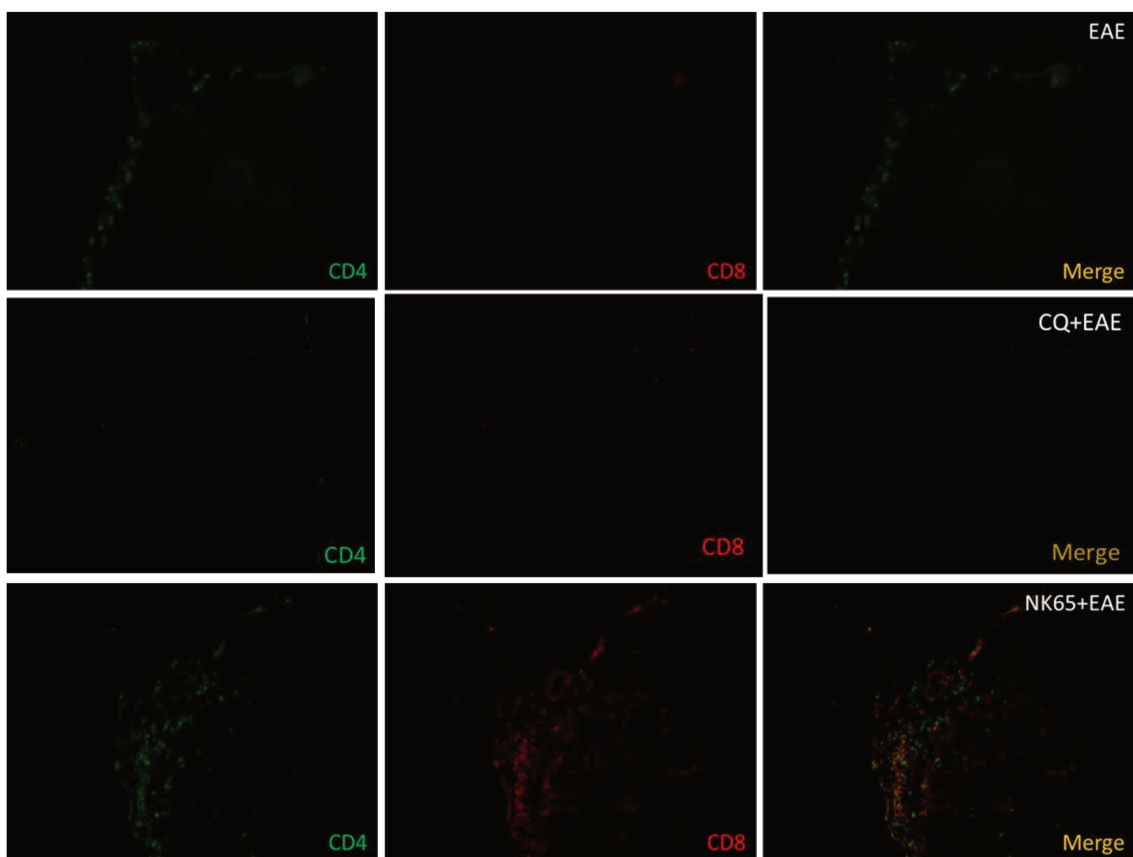


Figura 8 – Análise da frequência de células CD4⁺CD8⁺ em medula espinhal de camundongos portadores de EAE. Camundongos C57BL/6 (n=6 camundongos/grupo) foram infectados com 1×10^6 hemácias parasitadas por *P.berghei* e depois de dez dias foram tratados com cloroquina (5mg/kg) por via i.p. uma vez ao dia durante cinco dias consecutivos. Três dias após a administração da última dose de cloroquina, os animais foram imunizados com 100µg de peptídeo MOG₃₅₋₅₅ e acompanhados diariamente para o surgimento dos sinais clínicos de EAE. Como controles parte dos camundongos recebeu apenas cloroquina (CQ+EAE) e outra parte foi tratada com veículo (EAE). Decorridos quatorze dias da indução de EAE, os animais foram mortos e as medulas lombares foram recolhidas cuidadosamente. Na sequência, as amostras foram congeladas em meio de sustentação. Cortes de 14µm foram feitos em criostato e imunomarcados com anticorpos anti-CD4/FITC e anti-CD8/PE. Os resultados mostram que os animais portadores de EAE previamente infectados apresentaram maior frequência de células infiltrantes, sendo a maioria células CD4⁺CD8⁺, quando comparados com camundongos EAE. Dados representativos de quatro experimentos independentes.

Neuro-inflamação é exacerbada em camundongos portadores de EAE que previamente foram infectados por Plasmodium berghei NK65

A inflamação neuronal é responsável pelo surgimento dos sinais clínicos de EAE. Os resultados apresentados até o momento indicam que em camundongos pertencentes ao grupo NK+CQ+EAE a neuro-inflamação é exacerbada quando comparados com os animais pertencentes aos grupos EAE e CQ+EAE. Tendo em vista que células T-DP foram encontradas em elevada quantidade no SNC de camundongos que previamente tiveram malária, o passo seguinte foi investigar se tais células são capazes de produzir citocinas inflamatórias, bem como avaliar se a expressão de mediadores inflamatórios encontra-se alterada no SNC dos animais portadores de EAE.

a. Produção de citocinas inflamatórias por células T-DP que infiltraram o SNC de camundongos que previamente tiveram malária.

A fim de investigar a participação de células T-DP na neuro-inflamação de camundongos pertencentes ao grupo NK+CQ+EAE, os animais foram infectados com a cepa NK65 de *P.berghei* e, aos dez dias da infecção, o parasita foi eliminado pelo tratamento com cloroquina. Decorridos quatorze dias da imunização com peptídeo MOG, os camundongos foram mortos e as medulas lombares foram retiradas, maceradas e submetidas a centrifugação em gradiente de densidade para o enriquecimento de linfócitos T. As suspensões celulares enriquecidas em células T foram imuno-marcadas e analisadas quanto a presença de citocinas IFN- γ e IL-17 dentro da “gate” de células T-DP (CD4⁺CD8⁺). Os camundongos pertencentes ao grupo CQ+EAE não apresentaram números detectáveis de células infiltrantes no SNC, e por este motivo, as análises nesse grupo

ficaram comprometidas (dados não mostrados). Os resultados obtidos mostram que as células T-DP presentes nos tecidos de camundongos pertencentes ao grupo NK+CQ+EAE apresentam níveis maiores de IL-17 quando comparados com camundongos pertencentes ao grupo EAE. A detecção de IFN- γ apresentou-se semelhante entre os dois grupos (Figura 9).

b. Alterações no perfil de expressão gênica em tecido nervoso de camundongos portadores de EAE previamente infectados por *Plasmodium berghei*.

A expressão gênica de mediadores inflamatórios no SNC foi avaliada. Os resultados obtidos mostram que tecidos provenientes de camundongos pertencentes ao grupo NK+CQ+EAE apresentaram expressão significativamente maior de IFN- γ e IL-17 quando comparados com os animais pertencentes ao grupo EAE e CQ+EAE. Por outro lado, a expressão de IL-10 e Foxp3 foi significativamente maior em SNC de camundongos pertencentes ao grupo CQ+EAE em comparação com os demais grupos e não tendo diferença entre os grupos EAE e NK+CQ+EAE (Figura 10).

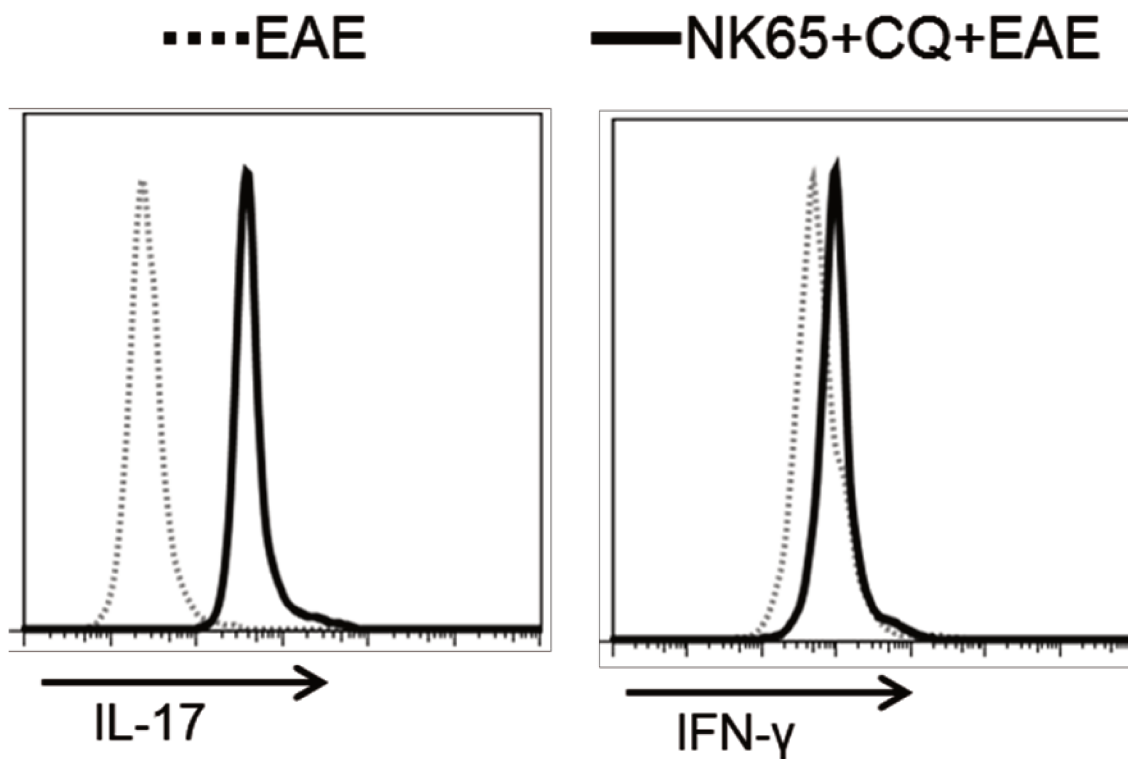


Figura 9 – Análise da presença intracelular de citocinas inflamatórias em células T-DP enriquecidas de SNC de camundongos portadores de EAE. Camundongos C57BL/6 (n=6 camundongos/grupo) foram infectados com 1×10^6 hemácias parasitadas por *P.berghei* e depois de dez dias foram tratados com cloroquina (5mg/kg) por via i.p. uma vez ao dia durante cinco dias consecutivos. Três dias após a administração da última dose de cloroquina, os animais foram imunizados com 100μg de peptídeo MOG₃₅₋₅₅ e acompanhados diariamente para o surgimento dos sinais clínicos de EAE. Como controles parte dos camundongos recebeu apenas cloroquina (CQ+EAE) e outra parte foi tratada com veículo (EAE). Decorridos quatorze dias da indução de EAE, os animais foram mortos e as medulas lombares foram recolhidas e maceradas em meio RPMI completo. Na sequência, as suspensões celulares foram centrifugadas em gradiente de densidade de Percol 30/70. As células da interface foram coletadas e imunomarcadas com anticorpos conjugados a moléculas fluorescentes. A presença de IFN-γ e IL-17 dentro da “gate” de células T-DP foi analisada por citometria de fluxo. Camundongos pertencentes ao grupo CQ+EAE não apresentaram quantidade detectável de células infiltrantes no SNC. Os resultados mostram que as células dos animais do grupo NK+CQ+EAE apresentaram maior produção de IFN-γ do que as células dos animais pertencentes ao grupo EAE. Dados representativos de três experimentos independentes.

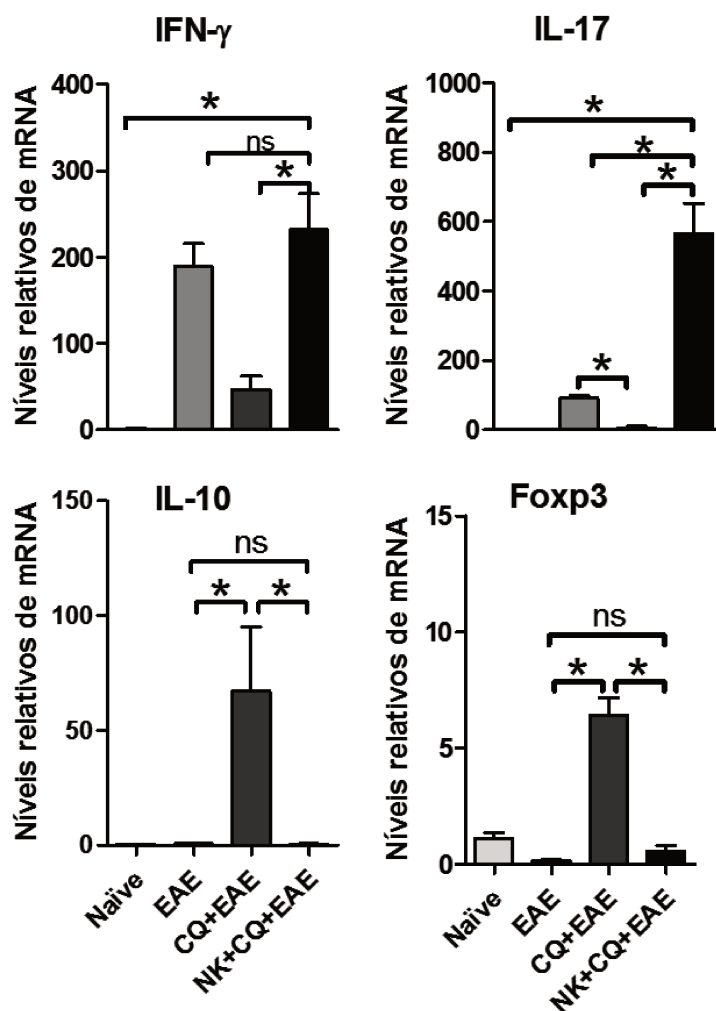


Figura 10 – Análise da expressão gênica de mediadores inflamatórios em Sistema Nervoso Central de camundongos portadores de EAE. Camundongos C57BL/6 (n=6 camundongos/grupo) foram infectados com 1×10^6 hemácias parasitadas por *P.berghei* e depois de dez dias foram tratados com cloroquina (5mg/kg) por via i.p. uma vez ao dia durante cinco dias consecutivos. Três dias após a administração da última dose de cloroquina, os animais foram imunizados com 100 μ g de peptídeo MOG₃₅₋₅₅ e acompanhados diariamente para o surgimento dos sinais clínicos de EAE. Como controles parte dos camundongos recebeu apenas cloroquina (CQ+EAE) e outra parte foi tratada com veículo (EAE). Decorridos quatorze dias da indução de EAE, os animais foram mortos e as medulas lombares foram recolhidas e congeladas imediatamente. Após extração de RNA e conversão em cDNA, reações de RT-PCR foram conduzidas a fim de analisar a expressão gênica de IFN- γ , IL-17, IL-10 e Foxp3 nos diferentes grupos experimentais. Os resultados mostram que camundongos pertencentes ao grupo NK+CQ+EAE apresentam alterações na expressão dessas moléculas quando comparados com os demais grupos. Dados representativos de três experimentos independentes. *:p<0,05, ns: não significativo.

Agravamento da Encefalomielite Autoimune Experimental é devido a estimulação recíproca entre células T encefalitogênicas e elicitadas na vigência da malária

Os resultados obtidos até o momento mostram a associação entre o agravamento da EAE com o surgimento de células T-DP no Sistema Nervoso Central de camundongos pertencentes ao grupo NK+CQ+EAE. Com a finalidade de caracterizar a participação de tais células na piora da EAE, o seguinte conjunto de experimentos foi planejado. Primeiramente, linfócitos T totais foram isolados de baço de camundongos infectados por *Plasmodium berghei* NK65 aos dez dias de infecção e co-cultivados com linfócitos T esplênicos isolados de camundongos portadores de EAE na presença de peptídeo MOG. As células encefalitogênicas foram previamente marcadas com a sonda CFSE. Como controles, os linfócitos T encefalitogênicos foram cultivados na ausência das demais células.

Numa segunda etapa, linfócitos T obtidos de camundongos infectados foram transferidos adotivamente (1×10^6 células/animal) a camundongos previamente imunizados com peptídeo MOG, e a evolução clínica da EAE foi acompanhada.

Por fim, células T $CD4^+CD8^+$ esplênicas provenientes de camundongos infectados foram isoladas por *sorting* e transferidas adotivamente ($1,5 \times 10^5$ células/animal) a camundongos portadores de EAE.

a. Análise da proliferação celular em co-culturas de células T.

As co-culturas conduzidas na presença de células T provenientes de camundongos infectados por *P.berghei* apresentaram proliferação significativamente maior do que as culturas conduzidas na presença de células T “naïve” ou na ausência de células T externas (Figura 11). Esses resultados indicam que linfócitos T provenientes de camundongos

portadores de malária estimulam a proliferação de células T encefalitogênicas por mecanismos ainda desconhecidos.

b. Agravamento da EAE em decorrência da transferência adotiva de células T provenientes de camundongos com malária.

Interessantemente, quando linfócitos T isolados de camundongos maláricos foram transferidos adotivamente a camundongos em início de quadro clínico de EAE, o desenvolvimento do modelo foi acelerado e agravado em comparação com os animais recipientes de células T obtidas de camundongos “naïve” (Figura 12).

c. Agravamento da EAE como resultado da transferência adotiva de células T-DP originadas na infecção por *Plasmodium berghei*.

Para confirmar a participação de células T-DP elicitadas na infecção no agravamento da EAE, camundongos foram infectados e dez dias depois foram mortos. Os linfócitos T-DP foram isolados por citometria de fluxo (*sorting*) e transferidos adotivamente a camundongos portadores de EAE. Como controles, células T totais foram isoladas de camundongos “naïve”. Conforme exposto na Figura 13, os animais recipientes em células T-DP apresentaram quadro clínico mais severo do que os animais recipientes de células T “naïve” (Figura 13).

Tomados em conjunto, os resultados obtidos corroboram a hipótese de que a maior severidade de EAE em camundongos previamente infectados por *Plasmodium berghei* é dependente da ativação de células T-DP.

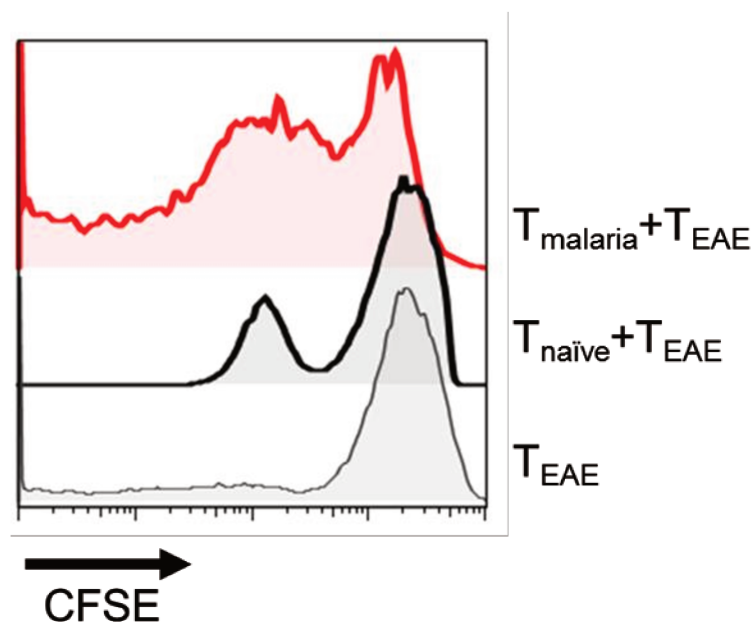


Figura 11 – Alteração na proliferação de células T encefalitogênicas co-cultivadas com linfócitos T provenientes de camundongos infectados por *Plasmodium berghei*. Camundongos C57BL/6 (n=6 animais) foram infectados com *P.berghei* NK65. Aos dez dias de infecção, esplenócitos foram coletados e células T totais foram isoladas usando esferas magnéticas. Células obtidas de camundongos “naïve” foram empregadas também. Camundongos portadores de EAE, aos dez dias após imunização com neuro-antígeno, foram mortos e seus esplenócitos foram coletados e os linfócitos T isolados e marcados com a sonda CFSE (2,5μM). As células foram co-cultivadas na proporção 1:1 por 96 na presença de peptídeo MOG (10μg/mL). Como controles, as células T encefalitogênicas foram cultivadas isoladamente. O decaimento da fluorescência foi acompanhado dentro da população de células CD3⁺CFSE⁺. Dados representativos de três experimentos independentes.

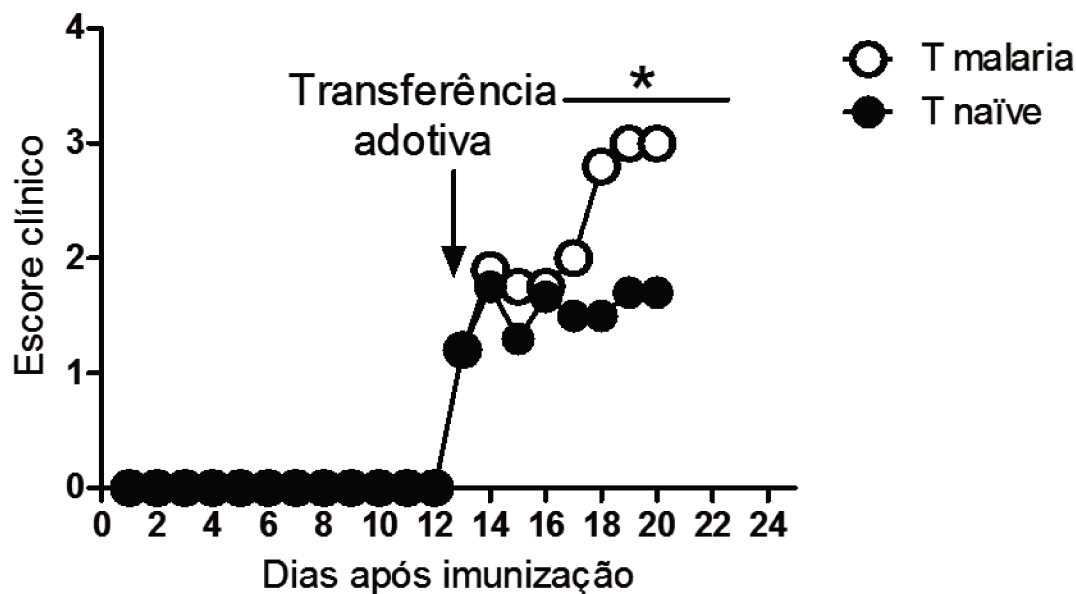


Figura 12 – Agravamento da EAE em camundongos recipientes de células T totais obtidas de animais portadores de malária. Camundongos C57BL/6 (n=6 animais) foram infectados com *P.berghei* NK65. Aos dez dias de infecção, esplenócitos foram coletados e células T totais foram isoladas usando esferas magnéticas. As células foram transferidas adotivamente a camundongos portadores de EAE (n=6 camundongos/grupo) aos dez dias da imunização com peptídeo MOG. Como controles, linfócitos T totais foram isolados a partir de camundongos “naíve”. O quadro clínico foi acompanhado diariamente e mostra que os camundongos recipientes de células T de malária desenvolveram um modelo significativamente mais agravado de EAE em comparação com o grupo controle. Dados representativos de três experimentos independentes. *: $p < 0,05$.

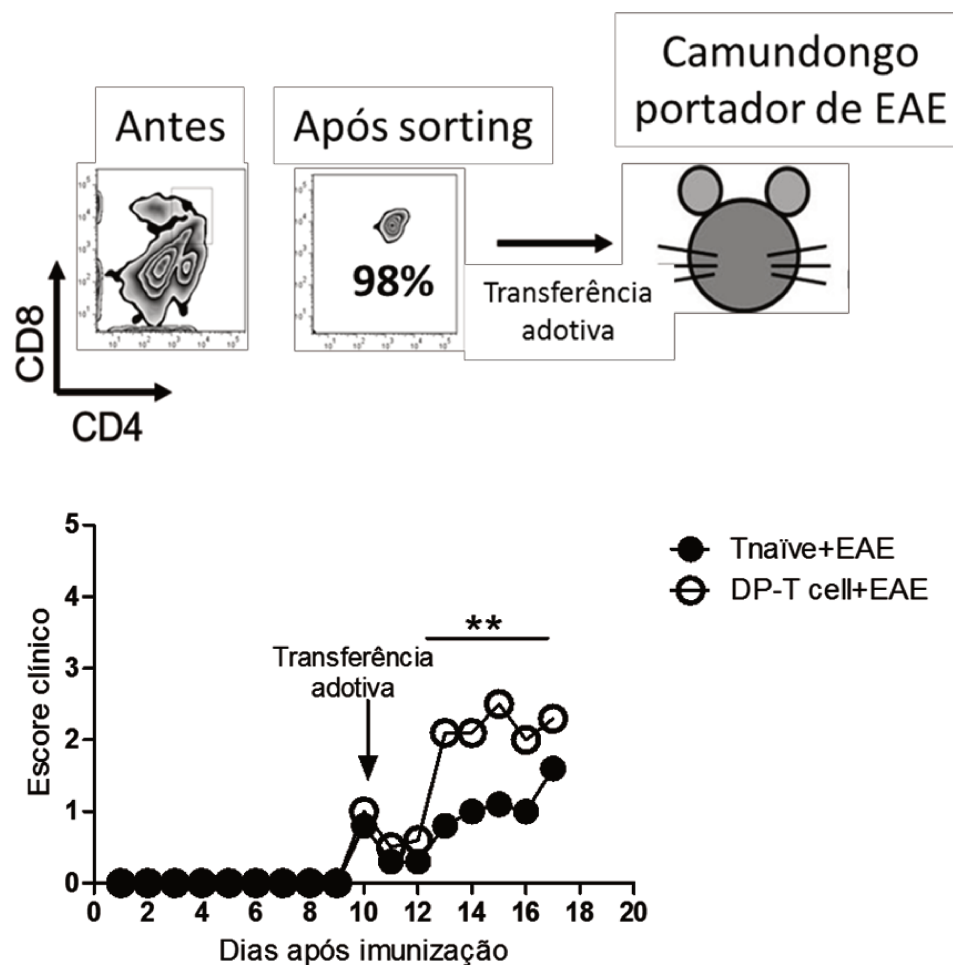


Figura 13 – Agravamento da EAE em camundongos que receberam células T-DP elicítadas na vigência da infecção por *Plasmodium berghei* NK65. Camundongos C57BL/6 (n=6 animais) foram infectados com *P.berghei* NK65. Aos dez dias de infecção, esplenócitos foram coletados e células T totais foram isoladas usando esferas magnéticas. As células foram transferidas adotivamente a camundongos portadores de EAE (n=6 camundongos/grupo) aos dez dias da imunização com peptídeo MOG. Como controles, linfócitos T totais foram isolados a partir de camundongos “naïve”. O quadro clínico foi acompanhado diariamente e mostra que os camundongos recipientes de células T de malária desenvolveram um modelo significativamente mais agravado de EAE em comparação com o grupo controle. Dados representativos de três experimentos independentes. **: p<0,01.

Encefalomielite Autoimune Experimental em camundongos geneticamente resistentes

Tendo em vista que camundongos C57BL/6 curados de malária desenvolveram quadro clínico mais agravado de EAE e que a piora na neuro-inflamação está relacionada com a migração para o SNC e secreção de citocinas inflamatórias por células T-DP, que prematuramente saíram do timo; a etapa seguinte do presente estudo teve por objetivo investigar se o aumento na frequência de células T-DP, elicitadas na vigência da malária, pode alterar a susceptibilidade ao desenvolvimento de EAE. Neste contexto, camundongos BALB/c, que são geneticamente resistentes a EAE, foram infectados por *Plasmodium berghei* NK65 e tratados com cloroquina. Nesta linhagem, a infecção por *P.berghei* é mais severa. Os camundongos morrem com 13-14 dias a partir da infecção (dpi). Em experimentos iniciais, verificou-se que o tratamento com cloroquina iniciado aos 10 dpi, resultou em sobrevida de 50% dos animais. Por outro lado, quando o tratamento teve início aos 7 dpi, a sobrevida foi de 100% (*dados não mostrados*). Portanto, nos experimentos utilizando camundongos BALB/c o tratamento com cloroquina teve início aos 7dpi. Três dias após a última dose de cloroquina, os animais foram imunizados com peptídeo MOG e acompanhados rotineiramente para avaliação dos sinais clínicos de EAE.

a. Camundongos BALB/c previamente infectados por Plasmodium berghei desenvolvem Encefalomielite Autoimune Experimental

Camundongos BALB/c imunizados com peptídeo MOG não apresentaram os sinais clínicos de EAE. Por outro lado, quando os animais foram previamente infectados por *P.berghei* e tratados com cloroquina, a imunização com neuro-peptídeo provocou o aparecimento dos primeiros sinais clínicos da doença (perda de tônus da cauda e fraqueza

dos membros posteriores) em 50% dos animais imunizados (Figura 14). Os animais tratados com cloroquina e imunizados (grupo CQ+EAE) não desenvolveram a doença (*dados não mostrados*).

b. Perfil de células infiltrantes no SNC de camundongos BALB/c portadores de EAE é inflamatório

Camundongos foram mortos quatorze dias após imunização com peptídeo MOG, a medula espinhal foi coletada e macerada imediatamente. Para o enriquecimento das células infiltrantes, as suspensões celulares obtidas das medulas foram sobrepostas ao gradiente de Percol 30/70 e submetidas a centrifugação. As células presentes na interface dos gradientes foram coletadas e imunomarcadas. Os resultados obtidos mostraram que camundongos pertencentes ao grupo NK+CQ+EAE tiveram número significativamente maior de células infiltrantes no SNC do que camundongos pertencentes ao grupo EAE (Figura 15). Interessantemente, análises de citocinas intracelulares mostraram que as células de camundongos NK+CQ+EAE apresentaram quantidade significativamente maiores de IL-17 quando comparadas com células de camundongos pertencentes ao grupo EAE, ao passo que os níveis de IL-10 foram estatisticamente reduzidos (Figura 15). A produção de IFN- γ não se apresentou estatisticamente diferente.

c. Parte das células que infiltrou o SNC de camundongos imunizados com peptídeo MOG é formado por células T-DP

Para avaliar a presença de células T-DP entre o infiltrado no SNC de camundongos imunizados com MOG, as medulas espinhais foram coletadas e maceradas quatorze dias após imunização. Após enriquecimento dos leucócitos, parte das células foi imunomarcada

e analisada por citometria de fluxo. A outra parte foi marcada com a sonda CFSE e cultivada na presença de peptídeo MOG por 96h. Os resultados obtidos mostraram que camundongos pertencentes ao grupo NK+CQ+EAE apresentam maior quantidade de células T-DP do que os camundongos do grupo EAE. Análises mostraram que as células T-DP presentes no SNC dos camundongos EAE e NK+CQ+EAE são responsivas a estímulo antigênico. As células de camundongos NK+CQ+EAE apresentaram proliferação maior do que células de camundongos EAE (Figura 16).

Tomados em conjunto, os resultados mostram que camundongos geneticamente resistentes a EAE são tornados susceptíveis após a infecção prévia por *Plasmodium berghei* NK65. No entanto, estudos deverão ser conduzidos a fim de avaliar se a susceptibilidade se deve a alterações nas regiões de reconhecimento presentes nos TCRs das células T-DP bem como se outros modelos de inflamação são alterados nos camundongos curados da malária. Este é o primeiro trabalho a mostrar o agravamento de uma doença autoimune em camundongos curados de malária.

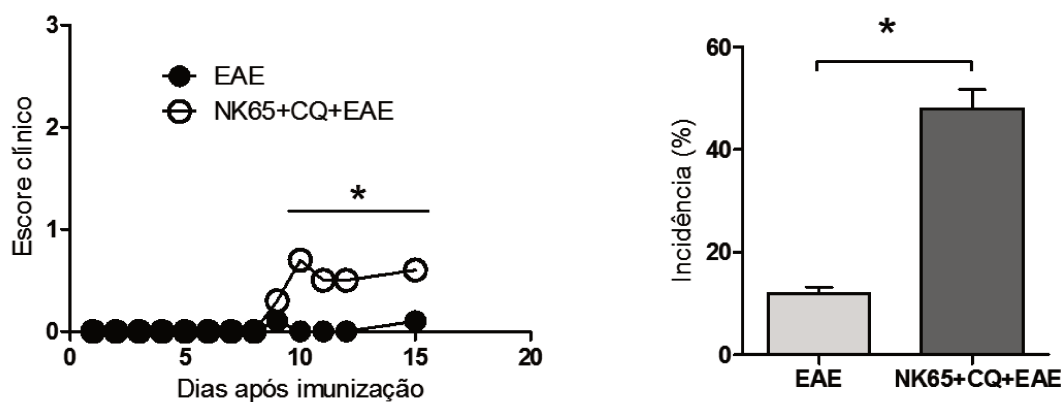


Figura 14 – Desenvolvimento de EAE em camundongos naturalmente resistentes após a infecção por *Plasmodium berghei*. Camundongos BALB/c (n=6 camundongos/grupo) foram infectados com *P.berghei* e tratados com cloroquina (5mg/kg) por cinco dias consecutivos iniciando-se sete dias após infecção. Três dias após a última dose de CQ, os camundongos foram imunizados com 100µg de MOG₃₅₋₅₅ para a indução de EAE. O quadro clínico foi acompanhado diariamente e revelou-se maior nos camundongos pertencentes ao grupo NK+CQ+EAE do que nos demais grupos, bem como a incidência da doença. Resultados representativos de três experimentos independentes. * p<0,05.

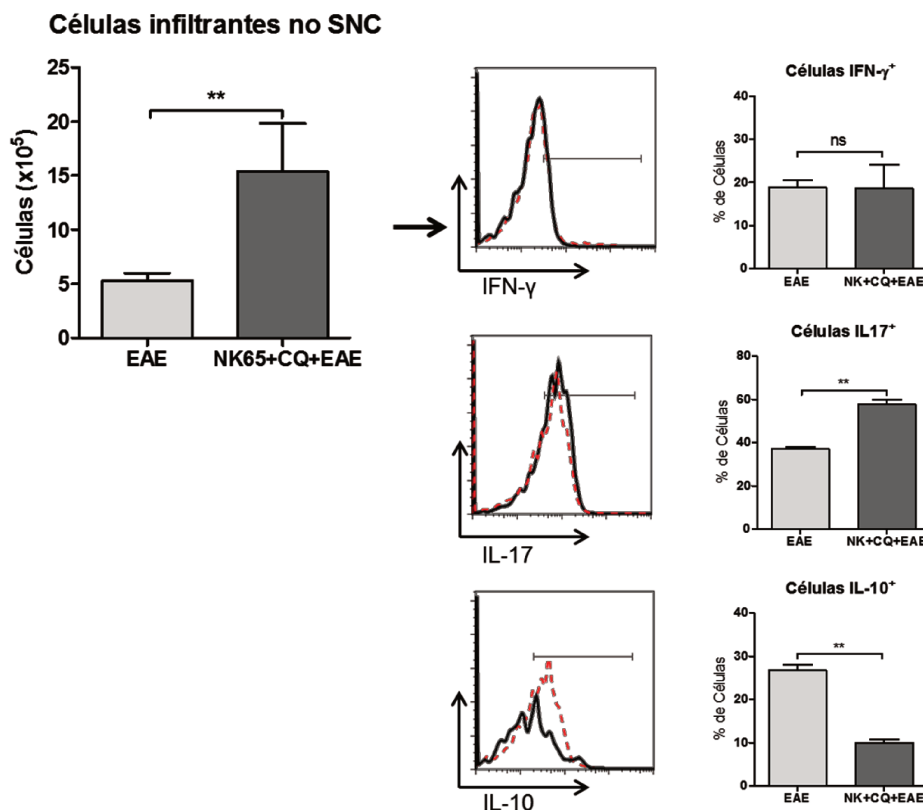


Figura 15 – Desenvolvimento de EAE em camundongos BALB/c está relacionado com a infiltração de leucócitos e células produtoras de citocinas inflamatórias. Camundongos BALB/c (n=6 camundongos/grupo) foram infectados com *P.berghei* e tratados com cloroquina (5mg/kg) por cinco dias consecutivos iniciando-se sete dias após infecção. Três dias após a última dose de CQ, os camundongos foram imunizados com 100 μ g de MOG₃₅₋₅₅ para a indução de EAE. Decorridos quatorze dias da indução de EAE, os animais foram mortos e as medulas lombares foram recolhidas e maceradas em meio RPMI completo. Na sequência, as suspensões celulares foram centrifugadas em gradiente de densidade de Percol 30/70. As células da interface foram coletadas e imunomarcadas com anticorpos conjugados a moléculas fluorescentes. A presença de IL-10, IFN- γ e IL-17 dentro da “gate” de células T (CD3⁺) foi analisada por citometria de fluxo. Resultados representativos de três experimentos independentes. ** p<0,01, ns: não significativo.

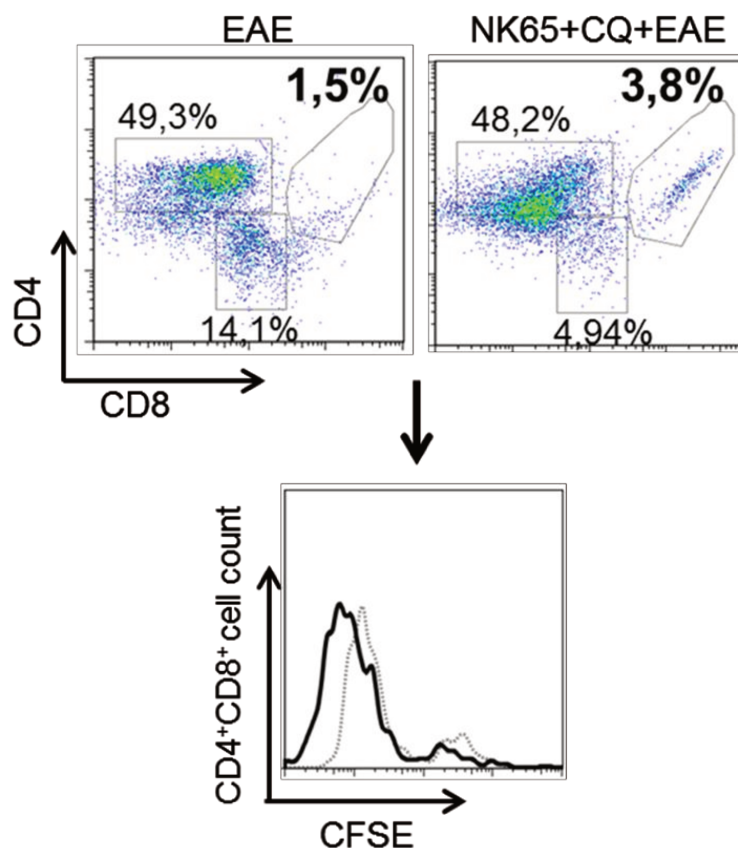


Figura 16 – Responsividade de células T-DP encontradas no SNC de camundongos BALB/c portadores de EAE. Camundongos BALB/c (n=6 camundongos/grupo) foram infectados com *P.berghei* e tratados com cloroquina (5mg/kg) por cinco dias consecutivos iniciando-se sete dias após infecção. Três dias após a última dose de CQ, os camundongos foram imunizados com 100µg de MOG₃₅₋₅₅ para a indução de EAE. Decorridos quatorze dias da indução de EAE, os animais foram mortos e as medulas lombares foram recolhidas e maceradas em meio RPMI completo. Na sequência, as suspensões celulares foram centrifugadas em gradiente de densidade de Percol 30/70. As células da interface foram coletadas e imunomarcadas com anticorpos conjugados a moléculas fluorescentes. A presença de células T-DP (CD3⁺CD4⁺CD8⁺) foi analisada por citometria de fluxo. Uma parte das células foi marcada com a sonda CFSE e cultivada na presença de peptídeo MOG (10µg/mL) por 96h. A proliferação foi determinada pela redução na fluorescência da sonda dentro da “gate” de células T-DP. Resultados representativos de três experimentos independentes.

Discussão

Nesta etapa do estudo foi demonstrado que após infecção com *Plasmodium berghei* NK65, camundongos imunizados com MOG₃₅₋₅₅ desenvolvem quadro clínico agravado de Encefalomielite Autoimune Experimental. Análises subsequentes demonstraram que timócitos prematuramente saídos do timo desempenharam um papel importante neste fenômeno.

O timo é um órgão-chave para o desenvolvimento de linfócitos T e possui um papel importante na eliminação de células T auto-reativas (Asano *et al.*, 1996; Starr *et al.*, 2003). Precusores de linfócitos T adentram o timo por meio das vênulas de endotélio alto encontradas nas regiões córtico-medulares e são desprovidos da expressão das moléculas de TCR e seus co-receptores CD4 e CD8 (Lind *et al.*, 2001). O processo de maturação consiste tanto na migração dos timócitos pelo córtex em direção à medula quanto da expressão dos receptores e co-receptores de linfócitos T (Anderson e Jenkinson, 2001). Durante este processo, células T imaturas interagem com uma miríade de citocinas, quimiocinas e peptídeos próprios apresentados em associação com moléculas do Complexo Principal de Histocompatibilidade (MHC) expressos por células epiteliais tímicas (TECs) culminando na morte das células que reconhecem com alta afinidade as moléculas próprias (Savino *et al.*, 2002). Nosso grupo demonstrou anteriormente que a infecção letal por *Plasmodium berghei* NK65 promove atrofia tímica dependente da morte timocitária e da migração prematura de tais células para a periferia do sistema imune (Andrade *et al.*, 2008; Gameiro, Nagib, Andrade, *et al.*, 2010; Francelin *et al.*, 2011). No presente estudo foi verificado um aumento na expressão de AIRE, IL-6 e IL-7 em timos de camundongos infectados, o que

indica que a seleção de repertório pode estar comprometida. Futuras análises serão conduzidas para avaliar a presença de células portadoras de TCR proibitivos (TCR V β 5 e V β 12) em órgãos linfoides secundários. A maturação e seleção inadequadas de linfócitos T pode levar à liberação de células T auto-reativas para os órgãos linfoides secundários e o consequente desenvolvimento de desordens de origem autoimune (Hogquist *et al.*, 1994; Itoh *et al.*, 1999; Capalbo *et al.*, 2012).

De fato, foi observado um agravamento na severidade da EAE em camundongos previamente infectados por *P.berghei* em comparação com animais não infectados. A análise do infiltrado inflamatório no SNC dos camundongos pertencentes ao grupo NK+CQ+EAE revelou uma elevada frequência de células T-DP. Tais células são funcionais, uma vez que proliferaram frente estímulo antígeno e apresentaram níveis intracelulares detectáveis de citocinas inflamatórias, como IL-17, responsável pelo desenvolvimento de quadro severo de EAE (Willenborg *et al.*, 1999; Chu *et al.*, 2000; O'connor *et al.*, 2008). Como esperado, quando células T totais ou duplo-positivas, provenientes de camundongos com malária, foram transferidas adotivamente a camundongos portadores de EAE, o quadro clínico aumentou significativamente. Coletivamente, este conjunto de experimentos demonstrou que as células T-DP elicitadas em camundongos infectados por *P.berghei* são inflamatórias e exacerbam a neuro-inflamação autoimune. Interessantemente, os experimentos com co-culturas mostraram que células T provenientes de camundongos infectados estimularam a proliferação de células T de camundongos imunizados com MOG. Esses dados sugerem que células T-DP, originadas no curso da malária, agravam a EAE por meio de, pelo menos, dois mecanismos diferentes: (i) pela reação e proliferação em resposta a neuro-antígenos próprios e (ii) pela estimulação da proliferação de células T encefalitogênicas.

Os dados apresentados nesta tese são preocupantes visto que a malária permanece como a principal doença infecciosa do mundo, com mais de 250 milhões de pessoas infectadas (Bhaumik, 2013). Vários estudos demonstraram a incidência de autoimunidade em países que erradicaram a infecção por plasmódio (Sotgiu *et al.*, 2008). Embora a hipótese da higiene pressuponha a resistência à autoimunidade em decorrência de infecções, tal teoria pode não ser aplicada à malária. Existe um acúmulo de evidências que corroboram ou refutam o papel da malária em doenças autoimunes, tanto de origem humana quanto animal (Cox, 1964; Loose e Di Luzio, 1973; Adu *et al.*, 1982; Jarra, 1983; Phanuphak *et al.*, 1983; Fawcett *et al.*, 1989; Lloyd *et al.*, 1994; Butcher, 1996; Daniel-Ribeiro, 2000; Daniel-Ribeiro e Zanini, 2000; Butcher, 2008). Alternativamente, foi proposto anteriormente que durante a infecção por *T.cruzi*, o perfil inflamatório das células T-DP prematuramente egressas do timo pode ter associação com o processo autoimune observado na doença de Chagas (Mendes-Da-Cruz *et al.*, 2003).

Foi demonstrado que a infecção por *Plasmodium chabaudi* promove um aumento na frequência de células T reguladoras no baço dos animais infectados, e que tais células suprimem o desenvolvimento de EAE de forma não específica (*by-stander suppression*) (Farias *et al.*, 2011). Entretanto, a infecção por *P.berghei* induz a produção e secreção de anticorpos anti-DNA e anti-núcleo de maneira dependente de linfócitos T (Poels *et al.*, 1980). Tais observações indicam que diferentes espécies de plasmódio induzem tipos de resposta imune distintos. De fato, neste estudo foi observado que a expressão genica de AIRE e de citocinas inflamatórias nos timos de camundongos infectados apresentou variações dependendo da espécie de *Plasmodium* empregada. Não obstante, hemácias infectadas por *P.falciparum*, *P.yoelli*, *P.berghei* e *P.vivax* induzem diferentes perfis de ativação em células dendríticas (Urban *et al.*, 1999; Skorokhod *et al.*, 2004; Urban e

Todryk, 2006; Bettiol *et al.*, 2010; Cockburn *et al.*, 2010; Goncalves *et al.*, 2010; Clemente *et al.*, 2011; Giusti *et al.*, 2011; Wykes *et al.*, 2011; Mauduit *et al.*, 2012; Clemente *et al.*, 2013; Thome *et al.*, 2014).

Baseado nestas observações, a presente tese propõe a hipótese de que, durante a malária por *P.berghei*, timócitos DP saem prematuramente do timo em direção ao sistema imune periférico. Nos órgãos linfoides secundários, devido a um evento inflamatório (disposição genética, infecção, radiação, entre tantos outros) as células T-DP proliferam e migram para o tecido-alvo onde produzem elevados níveis de citocinas inflamatórias levando ao surgimento de inflamação exacerbada (Figura 17).

Ainda assim, novos estudos deverão ser conduzidos com a finalidade de determinar quais os fatores de ativação de células T-DP e com relação à sua especificidade antigênica. Enquanto algumas evidências mostram a existência de células T-DP no controle da inflamação (Bang *et al.*, 2001; Das *et al.*, 2003; Tutaj e Szczepanik, 2007), os dados apresentados neste estudo indicam que as células T-DP, elicitadas na malária, são inflamatórias.

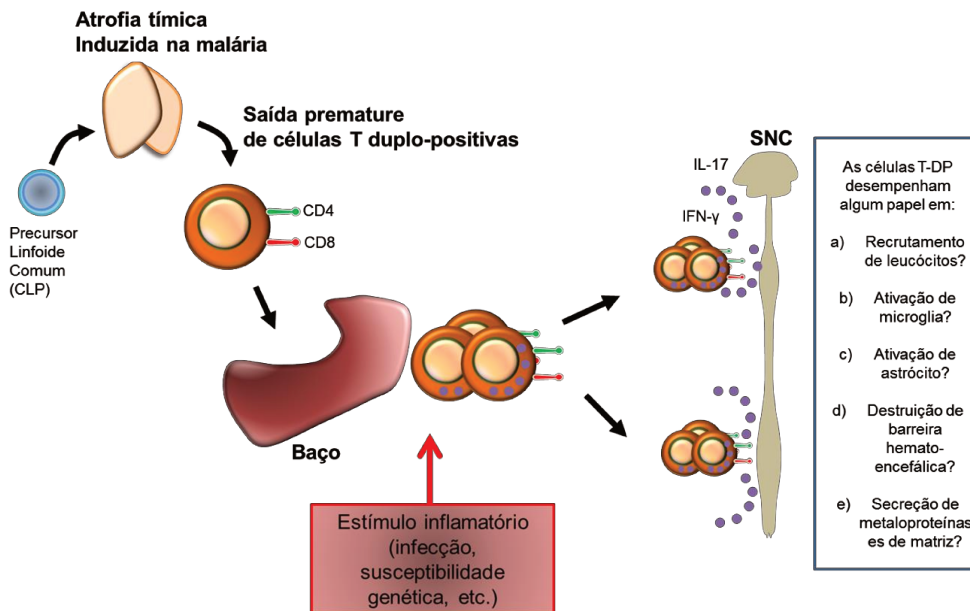


Figura 17 – Modelo hipotético para a exacerbação da Encefalomielite Autoimune Experimental em camundongos previamente infectados por *Plasmodium berghei*. Baseando-se nas observações deste estudo, a seguinte hipótese foi formulada: a infecção por malária promove atrofia tímica e a saída prematura de células T-DP, que não passaram pelos processos de seleção de repertório, para os órgãos linfóides secundários. Após um evento inflamatório, como infecções, susceptibilidade genética ou inflamação crônica, as células T-DP proliferam e migram para o tecido-alvo onde estimulam a inflamação pela secreção de citocinas inflamatórias. Entretanto, existem alguns aspectos que necessitam de melhor esclarecimento, por exemplo, se as células T-DP induzem o recrutamento de leucócitos, ativação de microglia e astrócitos, e permeabilização da barreira hemato-encefálica.

Conclusões

Tendo em vista os resultados apresentados neste capítulo, pode-se concluir que:

- a. A presença de células T DP no CNS de camundongos que previamente tiveram malária indica que a emigração precoce de timócitos em decorrência da infecção pode contribuir para o agravamento de doenças autoimunes em camundongos geneticamente susceptíveis.
- b. A elevada frequência de células T DP, estimulada pela infecção por *P.berghei* NK65, pode desempenhar um papel importante no agravamento da EAE em camundongos C57BL/6 e BALB/c.
- c. Células T DP esplênicas migram para o sistema nervoso central após a administração de neuroantígenos. No sítio inflamatório, tais células produzem elevados níveis de citocinas inflamatórias que podem contribuir para a destruição da bainha de mielina tanto diretamente quanto pela estimulação de células efectoras, contudo novas análises são necessárias para confirmar esses achados.
- d. Camundongos resistentes à EAE induzida por peptídeo MOG₃₅₋₅₅ desenvolveram a doença. Embora os sinais sejam reduzidos em comparação com animais susceptíveis, a presença de células T DP no CNS desses camundongos foi verificada.

Os resultados obtidos permitiram a confecção dos artigos científicos “Chloroquine Treatment Enhances Regulatory T Cells and Reduces the Severity of Experimental Autoimmune Encephalomyelitis”; “Reduced Severity of Experimental Autoimmune Encephalomyelitis After Violacein Treatment is a Result of Regulatory T Cells Induction and Inhibition of the Central Nervous System Inflammation” e “Exacerbation of Autoimmune Neuro-inflammation in Mice Cured from Blood-Stage *Plasmodium berghei* Infection” (Páginas 199, 264 e 285, respectivamente).

Capítulo 3

Capítulo 3 – Modulação de células dendríticas por extratos de plasmódio e terapia de Encefalomielite Autoimune Experimental.

Introdução

Células dendríticas (DCs) são células apresentadoras de antígenos profissionais que ativam linfócitos T “naïve” e ditam o curso da resposta imune adaptativa através da estimulação de células Th1, Th2, Th17 e células T reguladoras (Treg) (Banchereau e Steinman, 1998; Banchereau *et al.*, 2000; Leung *et al.*, 2010; Baxter e Jordan, 2013). Por conta deste potencial, as DCs são alvos frequentes em infecções. Por exemplo, foi demonstrado que DCs abrigam bactérias e parasitas nas infecções por *Salmonella* e *Plasmodium*, respectivamente (Niedergang *et al.*, 2000; Van Der Velden *et al.*, 2003; Bueno *et al.*, 2008; Wykes *et al.*, 2011). O bloqueio das funções das células dendríticas reduz a eficácia do sistema imune e reduz a eliminação de patógenos. Tal fenômeno pode ser observado em células dendríticas de pacientes portadores de malária, que se apresentam moduladas para um fenótipo anti-inflamatório: baixa expressão de moléculas co-estimuladoras, secreção de IL-10 e estimulação de células Tregs (Urban *et al.*, 1999; Giusti *et al.*, 2011). Os mecanismos pelos quais algumas cepas de plasmódio suprimem DCs ainda não estão esclarecidos. Entretanto, foi demonstrado que hemozoina, o pigmento de plasmódio, é capaz de reduzir a maturação de DCs humanas e murinas (Millington *et al.*, 2006; Urban e Todryk, 2006; Giusti *et al.*, 2011).

A modulação de células dendríticas tem se mostrado uma abordagem promissora no tratamento de doenças inflamatórias. Por exemplo, foi demonstrado que DCs tratadas com

formulações sintéticas ou extratos derivados de patógenos adquirem um fenótipo imaturo e, depois de transferidas adotivamente, suprimem a severidade de artrite experimental induzida por colágeno, transplantações renais e Encefalomielite Autoimune Experimental (Moser *et al.*, 1995; Rea *et al.*, 2000; Roelen *et al.*, 2003; Tarbell *et al.*, 2004; Van Halteren *et al.*, 2004; Emmer *et al.*, 2006; Terness *et al.*, 2008; Unger *et al.*, 2009; Ko *et al.*, 2010; Sofronic-Milosavljevic *et al.*, 2013).

A Encefalomielite Autoimune Experimental (EAE) é uma neuroinflamação mediada por células T que, em muitos aspectos, mimetiza a doença humana Esclerose Múltipla. Os linfócitos T CD4⁺ desempenham um papel central no desenvolvimento deste modelo de doença por meio da estimulação de células acessórias e produção de mediadores inflamatórios, que geram inflamação e danos neuronais (Chu *et al.*, 2000; Domingues *et al.*, 2010). Portanto, é desejável a supressão da resposta imune para o controle da EAE.

A interação entre células dendríticas e plasmódio precisa ser mais bem caracterizada e, quando possível, direcionada para novas abordagens terapêuticas em condições inflamatórias. Resultados obtidos em nosso laboratório mostraram que a infecção por *Plasmodium berghei* NK65 induz alterações distintas sobre o perfil fenotípico e funcional de células dendríticas tímicas e esplênicas. Camundongos BALB/c e C57BL/6 foram infectados com *P.berghei* NK65, selvagem ou GFP-*Knock in*, e aos quatorze dias de infecção as células dendríticas esplênicas e tímicas foram coletadas e analisadas quanto a expressão de moléculas envolvidas na apresentação de antígenos. O perfil funcional de DCs de camundongos infectados foi avaliado por meio de ensaios de co-cultura com linfócitos T esplênicos. Os resultados mostram que células dendríticas tímicas de camundongos infectados apresentaram-se imaturas, ao passo que DCs esplênicas apresentaram um

fenótipo inflamatório. Neste contexto, a modulação de DCs por extratos de *P.berghei* também foi investigada.

Interessantemente, quando células dendríticas geradas a partir de precursores de medula óssea foram tratadas com extratos de *P.berghei* e estimuladas com lipopolissacarídeo, a expressão de moléculas de apresentação de antígenos foi reduzida. Ensaio de funcionalidade mostraram que as DCs moduladas foram capazes de estimular a geração de células Tregs e, quando transferidas adotivamente, suprimiram o curso da EAE tanto de forma profilática quanto terapêutica de maneira antígeno-específica. Este é o primeiro estudo a mostrar ao contato com o plasmódio por diferentes vias gera alterações distintas em células dendríticas. Ao mesmo tempo, a modulação de DCs com extratos de plasmódio objetivando seu emprego no controle de neuroinflamação autoimune é inédita até o momento.

Resultados

Células dendríticas são moduladas para um perfil imaturo quando tratadas com extrato de *Plasmodium berghei* NK65.

a. Análise fenotípica de células dendríticas moduladas com extratos de Plasmodium berghei

Estudos recentes têm demonstrado o efeito de parasitas do gênero *Plasmodium* sobre o perfil de ativação de células dendríticas humanas e murinas. Foi observado que células dendríticas podem abrigar parasitas durante o curso da infecção malárica (Niedergang *et al.*, 2000; Van Der Velden *et al.*, 2003; Bueno *et al.*, 2008; Wykes *et al.*, 2011). A interferência na maturação de DCs também promove a supressão do sistema imune, o que atrasa ou até impede a eliminação do patógeno. Células dendríticas de pacientes com malária apresentam fenótipo imaturo, com reduzida expressão de moléculas envolvidas na apresentação de antígenos (MHC-II, CD-80 e CD-86) bem como numa acentuada predisposição a geração de células T reguladoras (Urban *et al.*, 1999; Giusti *et al.*, 2011). Os mecanismos pelos quais as células dendríticas tornam-se tolerogênicas ainda não estão totalmente elucidados. Contudo, foi verificado que hemozoina, o pigmento de plasmódio, é capaz de impedir a maturação de células dendríticas humanas e de camundongos (Millington *et al.*, 2006; Urban e Todryk, 2006; Giusti *et al.*, 2011). Neste sentido, o efeito do tratamento com extratos de *Plasmodium berghei* (PbX) sobre a maturação de células dendríticas foi avaliado. Para tanto, células dendríticas foram diferenciadas a partir de precursores de medula óssea e tratadas com PbX (100µg/mL) na presença de um estímulo de maturação – lipopolissacarídeo (LPS, 1ng/mL) por 18h. Como controles as células

foram tratadas com extrato de hemácias provenientes de camundongos saudáveis (RBC). Ao término do período de incubação, as células foram coletadas e imuno-marcadas para análise por citometria de fluxo de moléculas envolvidas na apresentação de antígenos. Os resultados obtidos mostram que células dendríticas tratadas com RBC apresentaram aumento na expressão de MHC-II, CD-80 e CD-86 quando comparadas com células não tratadas com LPS (*dados não mostrados*). Por outro lado, a exposição de DCs a PbX promoveu significativa redução na expressão de moléculas co-estimuladoras (CD-80 e CD-86) mas não de MHC-II quando comparadas com DC-RBC (Figura 1). Esses resultados indicam que o tratamento de DCs com extratos de *P.berghei* direcionam tais células para um estágio imaturo de ativação.

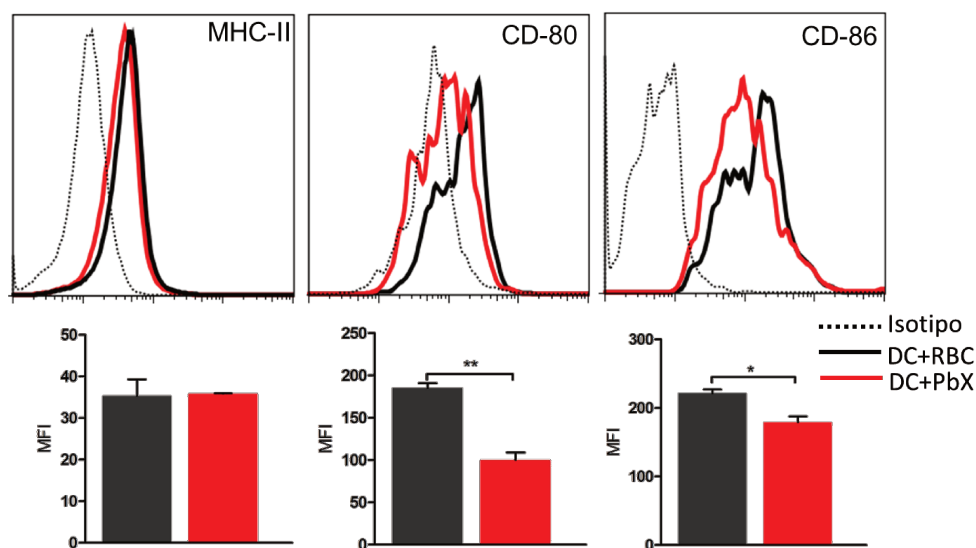


Figura 1 – Células dendríticas tratadas com extratos de *Plasmodium berghei* apresentam fenótipo imaturo. Células dendríticas geradas a partir de precursores de medula óssea foram estimuladas com Lipopolissacarídeo (LPS, 1ng/mL) por 18h na presença de PbX (100μg/mL). Como controles, as células foram estimuladas na presença de extratos de hemácias normais (RBC, 100μg/mL). A) As células foram coletadas e marcadas com anticorpos monoclonais conjugados a fluorocromos anti-MHC-II/PECy7, anti-CD-80/FITC e anti-CD-86/PE. A intensidade média de fluorescência (MFI) foi calculada a partir de dados adquiridos de 500.000 eventos em citômetro de fluxo. Resultados mostram a modulação de DCs por extratos de plasmódio. Dados representativos de três experimentos independentes realizados em sextuplicatas. * $p < 0,05$ e ** $p < 0,01$.

b. Análise funcional de células dendríticas moduladas com extrato de Plasmodium berghei.

Células dendríticas apresentando elevada expressão de MHC-II, mas com menor expressão de CD-80 e CD-86 são consideradas imaturas, ou semi-maduras (Lutz e Schuler, 2002). Outra característica de DCs imaturas é a capacidade de induzir a expressão do fator de transcrição Foxp3 em linfócitos T “naïve”, o que confere a essas células uma capacidade reguladora. Neste sentido, os experimentos realizados na sequência tiveram por objetivo avaliar a frequência de células T Foxp3⁺ e a produção de citocinas em co-culturas conduzidas na presença de células dendríticas tratadas com PbX e RBC. Os resultados mostram que os linfócitos T cultivados na presença de DC-RBC apresentaram maior produção de IL-17, IFN- γ e IL-10 quando comparados com células T cultivadas na ausência de DCs (Figura 2A, B e C, respectivamente). Por outro lado, as culturas conduzidas na presença de DC-PbX apresentaram frequência significativamente menor de linfócitos T produtores de IL-17 e significativamente maior produção de IFN- γ e IL-10 quando comparadas com culturas conduzidas na presença de DC-RBC (Figura 2A, B e C, respectivamente). Interessantemente, as culturas conduzidas na presença de DC-PbX apresentaram significativamente maior frequência de células CD4⁺Foxp3⁺ do que as demais culturas (Figura 2D).

Os níveis de citocinas presentes nos sobrenadantes das culturas foram determinados por citometria de fluxo. Resultados mostram que as culturas conduzidas na presença de DC-RBC apresentam produção significativamente maior de IL-6, TNF- α e IFN- γ quando comparadas com culturas sem DCs (Figura 2E). Em contrapartida, culturas conduzidas na presença de DC-PbX apresentaram níveis significativamente reduzidos de IL-6 e TNF- α do

que culturas com DC-RBC. Por outro lado, os níveis de IL-10 e IFN- γ foram encontrados aumentados em culturas com DC-PbX quando comparados com as demais culturas (Figura 2E). Esses achados confirmam que a administração de extrato de *P.berghei* promove a geração de células dendríticas com perfil tolerogênico. Tais células são eficazes na indução de células T reguladoras. Curiosamente, DC-PbX induzem tanto a produção de IFN- γ quanto de IL-10 por linfócitos T.

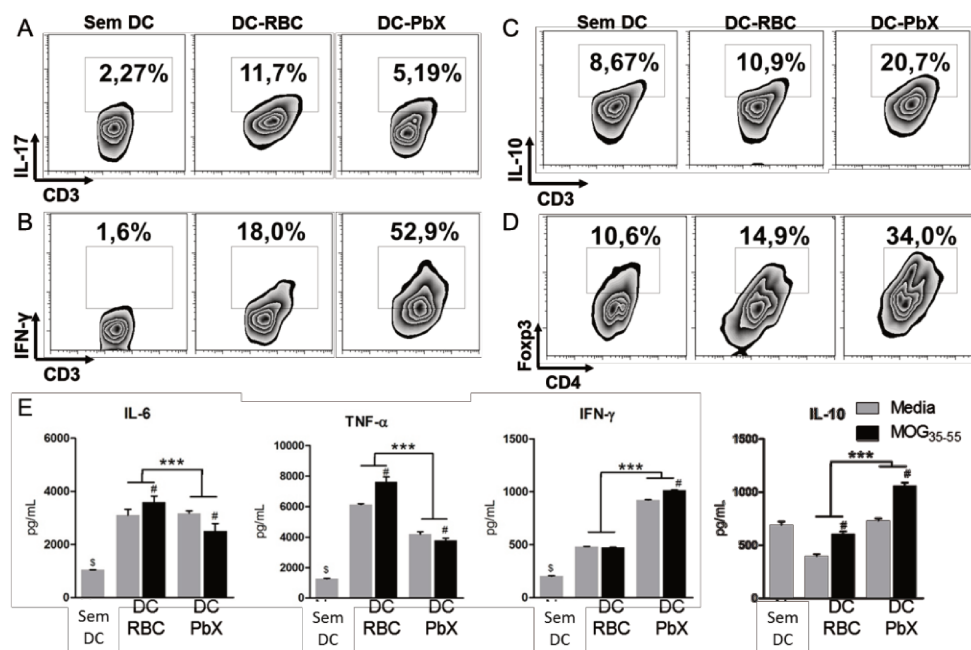


Figura 2 – Células dendríticas moduladas com extratos de *Plasmodium berghei* adquirem fenótipo tolerogênico. Células dendríticas foram tratadas com PbX ou RBC (100µg/mL), estimuladas com LPS (1ng/mL) e pulsadas com peptídeo MOG (10µg/mL) por 18h. Posteriormente, as DCs foram semeadas em placas de cultura de 96 cavidades com fundo em U (5x10⁵ células/cavidade). Linfócitos T foram isolados a partir de baços de camundongos “naïve” empregando-se beads magnéticas (Dynabeads, Life Technologies). As células T foram semeadas em conjunto com as DCs na mesma proporção (1DC:1T). As co-culturas foram incubadas por 96h na presença ou ausência de peptídeo MOG (10µg/mL). Como controles, células foram cultivadas na ausência de DCs. No final do período de incubação, as células foram tratadas com Acetato Miristato de Phorbol (PMA) e Ionomicina na presença de Brefeldina-A por 4h. A, B e C: Níveis intracelulares de IL-17, IFN-γ e IL-10 em células CD3⁺ foram determinados, respectivamente. D: A geração de células T reguladoras (CD4⁺Foxp3⁺) foi avaliada também. E: Os níveis de citocinas presentes nos sobrenadantes foram determinados por citometria de fluxo empregando-se o kit CBA (BD Biosciences). Dados representativos de três experimentos independentes com resultados similares. *** p<0,005. # p<0,05 em comparação com as respectivas culturas conduzidas na ausência de antígeno. \$ p<0,05 em relação às culturas conduzidas na presença de DCs.

Células dendríticas moduladas com extratos de *P.berghei* suprimem a Encefalomielite Autoimune Experimental

a) Transferência adotiva de DC-PbX reduz o quadro clínico de EAE.

A geração de células T reguladoras e células produtoras de IL-10 é uma característica importante em células dendríticas tolerogênicas. Tendo em vista que o desenvolvimento de inflamações crônicas de origem autoimune resulta de falhas nos mecanismos reguladores da resposta celular (Chu *et al.*, 2000), o potencial terapêutico de células dendríticas moduladas com extratos de *Plasmodium berghei* foi avaliado. O modelo de EAE se desenvolve após a migração de células T encefalitogênicas para o Sistema Nervoso Central (SNC). Fato que ocorre, normalmente, dez dias após a imunização com peptídeo MOG (Domingues *et al.*, 2010).

Neste sentido, visando a utilização profilática de células dendríticas imaturas geradas com extratos de plasmódio, DC-PbX foram transferidas adotivamente para camundongos C57BL/6 três dias antes da imunização com peptídeo MOG. O desenvolvimento da EAE foi acompanhado diariamente. Os resultados obtidos mostram que camundongos recipientes de DC-RBC desenvolveram quadro clínico de EAE similar ao observado em camundongos não tratados com células dendríticas (Figura 3). Ao passo que, camundongos pertencentes ao grupo DC-PbX+EAE tiveram sinais clínicos de EAE significativamente reduzidos quando comparados com animais dos demais grupos (Figura 3).

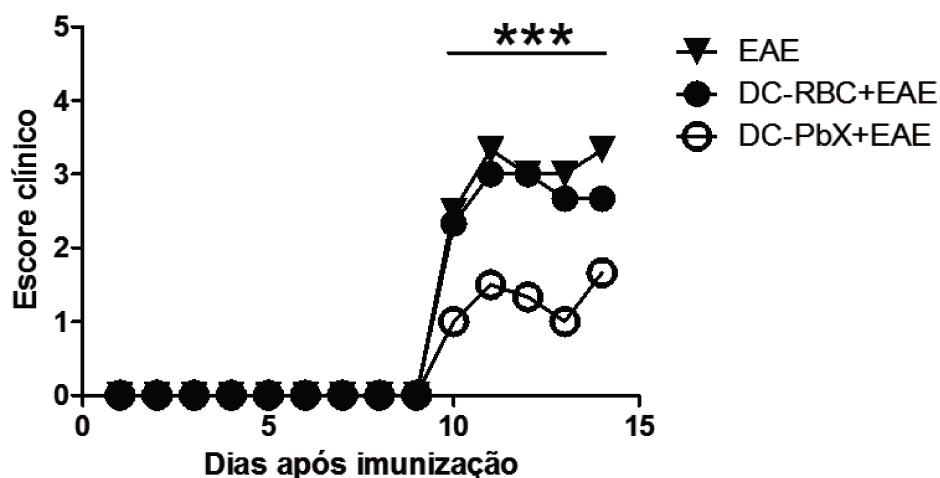


Figura 3 – Desenvolvimento de EAE em camundongos recipientes de células dendríticas moduladas com extratos de plasmódio. Células dendríticas foram estimuladas com LPS (1 η g/mL), tratadas com PbX (100 μ g/mL) e pulsadas com peptídeo MOG (10 μ g/mL) por 18h. Como controles, células dendríticas foram tratadas com extrato de hemácias normais (RBC). Após o término do período de incubação, as células foram transferidas adotivamente (1,5 $\times$ 10⁶ células/camundongo) por via endovenosa a camundongos C57BL/6 (n=6 camundongos/grupo). Decorridos três dias da transferência adotiva, os camundongos foram imunizados com peptídeo MOG (100 μ g/camundongo) emulsificado em Adjuvante Completo de Freund. Toxina de Pertussis (200 η g/camundongo) foi aplicada intraperitonealmente à 0h e às 48h da imunização. Os sinais clínicos da doença foram acompanhados diariamente e mostram que a transferência de DC-PbX promoveu supressão da severidade da EAE. Dados representativos de três experimentos independentes. *** p<0,005.

b) Análise do infiltrado inflamatório no SNC de camundongos portadores de EAE

A infiltração de células inflamatórias no SNC é um dos marcos da EAE. Neste sentido, camundongos controles e recipientes de células dendríticas moduladas, ambos portadores de EAE, foram mortos. A região lombar das medulas espinhais foi coletada e incluída em meio crioprotetor. Cortes de 14µm foram realizados e corados em hematoxilina e eosina (H&E). Os resultados mostram que a transferência adotiva de células dendríticas moduladas com PbX foi capaz de reduzir a infiltração celular no SNC de camundongos imunizados com peptídeo MOG (Figura 4).

Adicionalmente, o perfil de linfócitos T que infiltraram o SNC foi investigado. Os dados obtidos mostram que tanto a frequência quanto os números absolutos de células T produtoras de IL-17 e IFN- γ são significativamente menores em camundongos que receberam DC-PbX quando comparados com os demais grupos (Figura 5A e B, respectivamente). A frequência de células T produtoras de IL-10 estava significativamente maior em medulas lombares de camundongos recipientes de DC-PbX quando comparados com camundongos dos demais grupos, no entanto, devido à menor infiltração de células no SNC (Figura 4), os números absolutos de células produtoras de IL-10 foram significativamente reduzidos em comparação com o grupo EAE (Figura 5C).

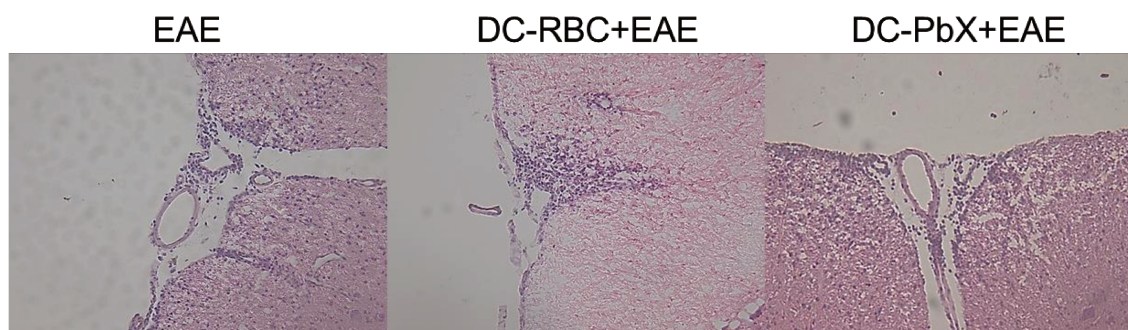


Figura 4 – Alterações histológicas no Sistema Nervoso Central de camundongos recipientes de células dendríticas. DCs foram estimuladas com LPS (1 μ g/mL), tratadas com PbX (100 μ g/mL) e pulsadas com peptídeo MOG (10 μ g/mL) por 18h. Como controles as células foram tratadas com extrato de hemácias normais. As DCs foram transferidas adotivamente (1,5x10⁶ células/camundongo) via endovenosa a camundongos C57BL/6 (n=6 camundongos/grupo) três dias antes da indução de EAE. Decorridos quatorze dias da imunização com peptídeo MOG, a região lombar das medulas espinhais foram retiradas e processadas para análise histopatológicas por coloração de HE. Aumento de 200x. Dados de dois experimentos independentes.

c) Responsividade celular em camundongos portadores de EAE

A resposta imune celular contra neuro-antígenos na Encefalomielite Autoimune Experimental foi caracterizada anteriormente (Dittel *et al.*, 1999; El-Behi *et al.*, 2011). Neste contexto, a responsividade celular frente ao peptídeo MOG, empregado na imunização, e contra proteína básica de mielina (MBP, do inglês *Myelin Basic Protein*) foi comparada entre os grupos experimentais. Os resultados obtidos mostraram que células de camundongos recipientes de DC-PbX tiveram proliferação significativamente menor do que células de camundongos pertencentes aos outros grupos (Figura 6A e B). Parte das culturas celulares foi conduzida na presença de ovalbumina (OVA), um antígeno irrelevante ao modelo de EAE. Nestas culturas, as células dos camundongos proliferaram em níveis similares entre si (Figura 6C). Esses achados indicam que a supressão imune observada nos camundongos recipientes de DC-PbX é antígeno/tecido-específica, visto que as células mantiveram seu potencial proliferativo quando cultivadas na presença de antígeno irrelevante.

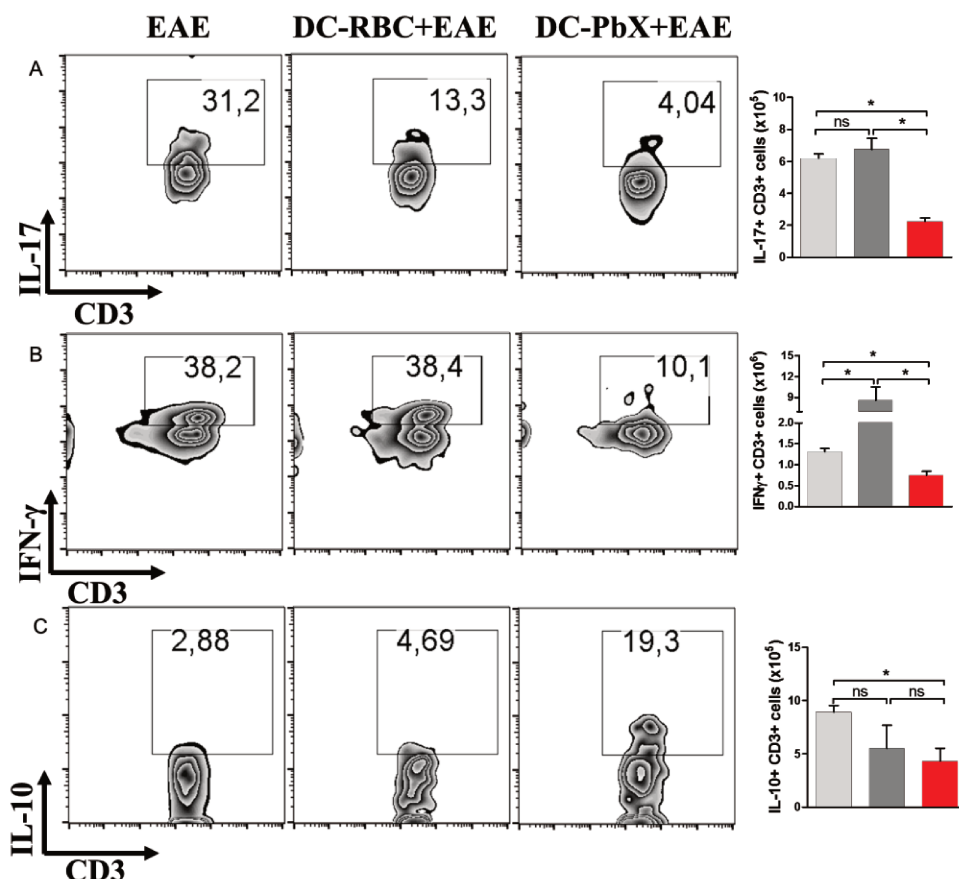


Figura 5 – Alteração no perfil de células infiltrantes no SNC de camundongos portadores de EAE. DCs foram estimuladas com LPS (1ng/mL), tratadas com PbX (100μg/mL) e pulsadas com peptídeo MOG (10μg/mL) por 18h. Como controles as células foram tratadas com extrato de hemácias normais. As DCs foram transferidas adotivamente ($1,5 \times 10^6$ células/camundongo) via endovenosa a camundongos C57BL/6 (n=6 camundongos/grupo) três dias antes da indução de EAE. Decorridos quatorze dias da imunização com peptídeo MOG, os animais foram mortos e o tecido nervoso foi coletado cuidadosamente e os leucócitos infiltrantes foram enriquecidos por meio de centrifugação em gradiente de Percol 30/70. As suspensões celulares enriquecidas em linfócitos foram incubadas por 4h na presença de PMA e Ionomicina+Brefeldina. Posteriormente as células foram marcadas com anticorpos conjugados a fluorocromos para análise por citometria de fluxo. As frequências de células produtoras de IL-17 (em A), IFN-γ (em B) e IL-10 (em C) foram determinadas. Dados de três experimentos independentes. *p<0,05, ns: não significativo.

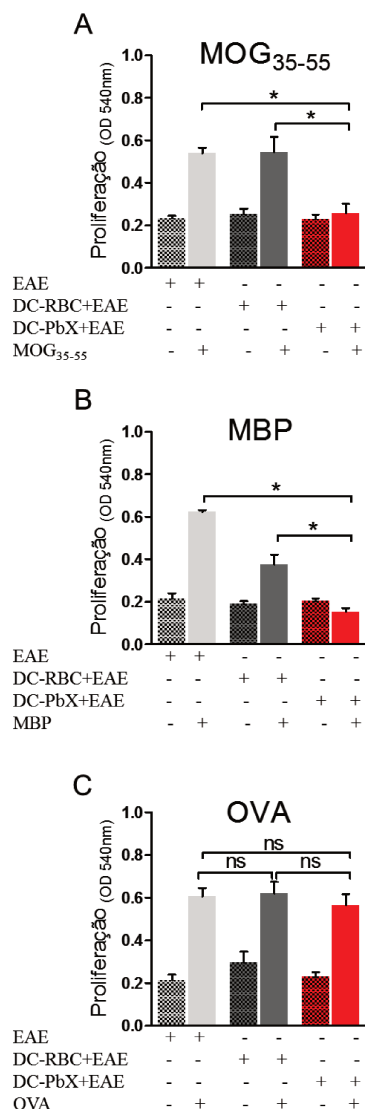


Figura 6 – Redução da responsividade celular de células proveniente de camundongos recipientes de células dendríticas moduladas com extratos de plasmódio. DCs foram estimuladas com LPS (1ng/mL), tratadas com PbX (100µg/mL) e pulsadas com peptídeo MOG (10µg/mL) por 18h. Como controles as células foram tratadas com extrato de hemácias normais. As DCs foram transferidas adotivamente (1,5x10⁶ células/camundongo) via endovenosa a camundongos C57BL/6 três dias antes da indução de EAE. Decorridos quatorze dias da imunização com peptídeo MOG, os animais foram mortos e os baços foram retirados assepticamente para o preparo de suspensões celulares. As células esplênicas foram cultivadas em placas de cultura de 96 cavidades (5x10⁵ células/cavidade) na presença de peptídeo MOG (em A), Proteína Básica de Mielina (MBP, em B) e Ovalbumina (OVA, em C) (10 µg/mL, 50µg/mL e 50µg/mL, respectivamente). Como controles, as células foram cultivadas na ausência de antígenos. A proliferação foi analisada por MTT. Resultados obtidos a partir de três experimentos independentes. * p<0,05. ns: não significativo.

Células dendríticas moduladas com extratos de plasmódio podem ser utilizadas no controle da neuro-inflamação autoimune estabelecida previamente.

- c. Células dendríticas tratadas com PbX suprimem a resposta de células T encefalitogênicas.*

A severidade da EAE foi significativamente menor em camundongos que receberam células dendríticas moduladas com extratos de plasmódio. Tendo em vista que a administração de células dendríticas foi realizada de forma profilática (três dias antes da imunização com neuro-antígeno), o potencial terapêutico da transferência adotiva foi investigado. Para tanto, camundongos com EAE estabelecida (após dez dias, quando surgem as primeiras manifestações clínicas) receberam células dendríticas, tratadas com extrato de plasmódio ou extrato de hemácias normais. Os resultados obtidos mostraram que a transferência adotiva de DC-PbX foi capaz de reduzir significativamente o quadro clínico de EAE quando comparado com os demais grupos (Figura 7).

- d. Transferência adotiva de células dendríticas moduladas reduz a severidade de EAE em curso.*

Conforme demonstrado anteriormente, DC-PbX induziu a diferenciação de células T reguladoras quando co-cultivadas com linfócitos T “naïve” (Figura 2). Essa característica, juntamente com a reduzida expressão de moléculas envolvidas na apresentação de antígenos, classifica as células dendríticas moduladas com extratos de plasmódio como células dendríticas imaturas (ou tolerogênicas). Entretanto, neste conjunto

de experimentos, DC-PbX foram transferidas a camundongos previamente imunizados com peptídeo MOG. Para investigar se DC-PbX exerce efeito sobre a funcionalidade de células T encefalitogênicas, linfócitos T esplênicos foram enriquecidos a partir de baços de camundongos portadores de EAE e co-cultivados na presença de células dendríticas tratadas com extratos. Como controles, as células foram cultivadas na ausência de DCs. A proliferação celular e a produção de citocinas foram avaliadas.

As análises das co-culturas revelaram que células T cultivadas sem DCs não apresentaram proliferação celular. Quando cultivadas na presença de DC-RBC, células T encefalitogênicas apresentaram elevada taxa de proliferação. Por outro lado, culturas conduzidas na presença de células dendríticas moduladas com extratos de plasmódio apresentaram redução na taxa de proliferação quando comparadas com as culturas de DC-RBC (Figura 8A). A determinação dos níveis de citocinas presentes nos sobrenadantes revelou que culturas conduzidas na presença de DC-PbX apresentaram níveis significativamente reduzidos de IL-6 e TNF- α , ao passo que, os níveis de INF- γ e IL-10 foram encontrados significativamente aumentados quando comparadas com culturas de DC-RBC (Figura 8B).

Tomados em conjunto, esses dados indicam que células dendríticas podem ser direcionadas a um estado tolerogênico quando tratadas com extratos de plasmódio. DC-PbX induzem a geração de células T reguladoras e produtoras de INF- γ e IL-10 quando cultivadas na presença de linfócitos T “naïve”. Quando cultivadas com linfócitos T encefalitogênicos, DC-PbX suprimem a proliferação celular e induzem a produção de INF- γ e IL-10, ao passo que reduzem a produção de IL-6 e TNF- α . Esses achados indicam que células dendríticas tratadas com extratos de plasmódio modulam a resposta imune por mecanismos distintos: (i) indução de células T reguladoras a partir de linfócitos T “naïve”,

(ii) criação de microambiente supressor por meio da estimulação da secreção de IL-10 e IFN- γ por linfócitos T e (iii) redução da responsividade celular quando cultivadas com células T ativadas.

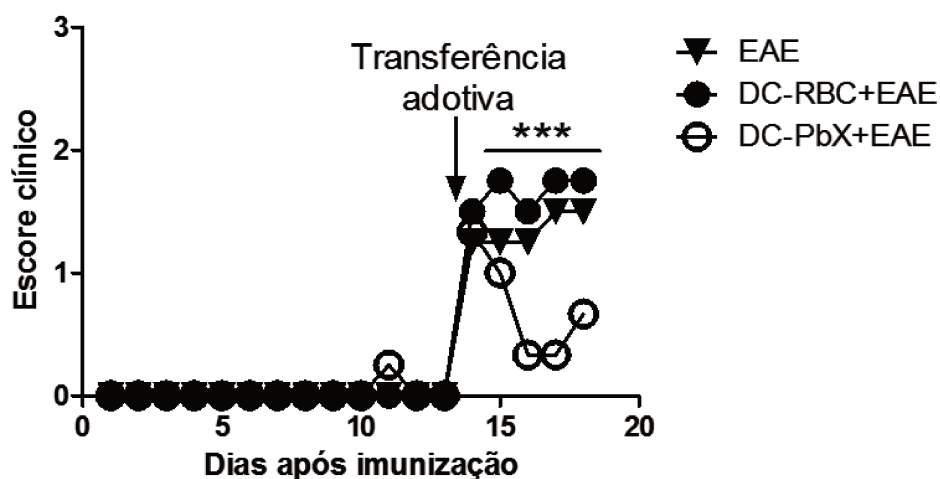


Figura 7 – Efeito terapêutico da administração de células dendríticas tolerogênicas no curso da Encefalomielite Autoimune Experimental. Células dendríticas foram estimuladas com LPS (1ng/mL), tratadas com PbX (100µg/mL) e pulsadas com peptídeo MOG (10µg/mL) por 18h. Como controles, células dendríticas foram tratadas com extrato de hemácias normais (RBC). Após o término do período de incubação, as células foram transferidas adotivamente ($1,5 \times 10^6$ células/camundongo) por via endovenosa a camundongos C57BL/6 (n=6 camundongos/grupo). A transferência adotiva ocorreu aos dez dias da aplicação de neuro-antígeno de indução de EAE, quando as primeiras manifestações clínicas são observadas. Os sinais clínicos da doença foram acompanhados diariamente e mostram que a transferência de DC-PbX promoveu supressão da severidade da EAE. Dados representativos de dois experimentos independentes. *** p<0,005.

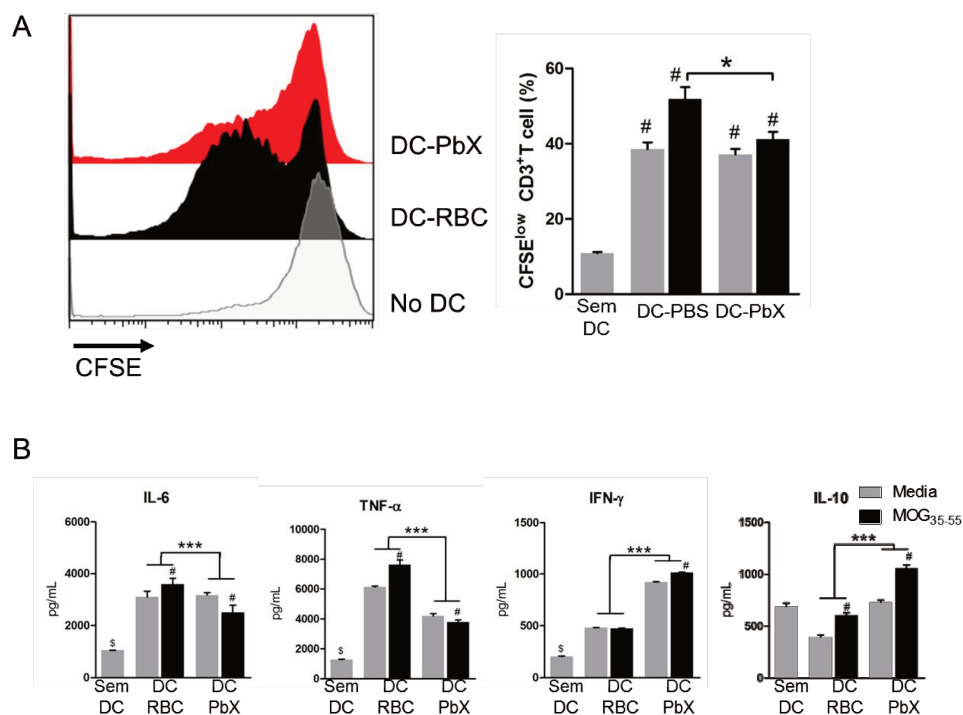


Figura 8 – Células dendríticas moduladas com extratos de *Plasmodium berghei* suprimem a proliferação de células T encefalitogênicas. Células dendríticas foram tratadas com PbX ou RBC (100µg/mL), estimuladas com LPS (1ng/mL) e pulsadas com peptídeo MOG (10µg/mL) por 18h. Posteriormente, as DCs foram semeadas em placas de cultura de 96 cavidades com fundo em U (5x10⁵ células/cavidade). Linfócitos T foram isolados a partir de baços de camundongos portadores de EAE empregando-se *beads* magnéticas (Dynabeads, Life Technologies) e, em seguida, marcadas com a sonda fluorescente CFSE (2,5µM, Sigma-Aldrich). As células T foram semeadas em conjunto com as DCs na mesma proporção (1DC:1T). As co-culturas foram incubadas por 96h na presença ou ausência de peptídeo MOG (10µg/mL). Como controles, células foram cultivadas na ausência de DCs. A: A proliferação celular foi analisada pelo decaimento da fluorescência da sonda na população de células CD3⁺. B: Os níveis de citocinas presentes nos sobrenadantes foram determinados por citometria de fluxo empregando-se o kit CBA (BD Biosciences). Dados representativos de três experimentos independentes com resultados similares. *** p<0,005. # p<0,05 em comparação com as respectivas culturas conduzidas na ausência de antígeno. \$ p<0,05 em relação às culturas conduzidas na presença de DCs.

Discussão

Foi demonstrado que células dendríticas em pacientes e roedores com malária são imaturas e não funcionais (Skorokhod *et al.*, 2004; Millington *et al.*, 2006; Urban e Todryk, 2006; Giusti *et al.*, 2011). Embora na malária a inatividade de DCs seja um problema, na inflamação autoimune, este é um fenômeno almejado. Neste estudo, é demonstrado que células dendríticas moduladas com extratos de hemácias infectadas por *Plasmodium berghei* suprimem o desenvolvimento de Encefalomielite Autoimune Experimental.

Na vigência da infecção por plasmódio, o parasita desenvolve tropismo por células dendríticas, que fagocitam o eritrócito infectado (Cockburn *et al.*, 2011; Mauduit *et al.*, 2012). Dentro das DCs, o parasita produz hemozoina, um produto gerado a partir da degradação da hemoglobina, culminando no bloqueio da maturação de células dendríticas (Urban e Todryk, 2006). DCs tratadas *in vitro* com hemozoina adquirem habilidades imunossupressoras como a redução na expressão das moléculas co-estimuladoras CD80 e CD86, secreção de IL-10 e indução de células Tregs (Skorokhod *et al.*, 2004; Giusti *et al.*, 2011). Os resultados obtidos neste estudo mostram que extratos de eritrócitos infectados por *P.berghei* (PbX) alteram a maturação de DCs, com baixa expressão de moléculas apresentadoras de antígenos. As DCs moduladas estimularam a produção de citocinas anti-inflamatórias por linfócitos T e foram capazes de induzir a conversão de linfócito T “naïve” em células T reguladoras. DC-PbX estimularam a produção de IFN- γ por linfócitos T “naïve”, sugerindo que o perfil anti-inflamatório induzido por PbX é dependente da geração de microambiente rico em IFN- γ . Um mecanismo similar de modulação por DCs foi descrito recentemente, onde DCs moduladas com proteína Sj16, derivada de *Schistosoma*

japonicum, induzem células Treg produtoras de IFN- γ e IL-10 (Sun *et al.*, 2012). Também foi demonstrado que extratos de *P.falciparum* modulam DCs humanas, induzindo-lhes um perfil imaturo e estimulando a geração de células Tregs a partir de linfócitos T autólogos (Clemente *et al.*, 2013). Por outro lado, verificou-se que extratos de *Plasmodium yoelli* induzem a maturação de DCs e sua ativação inflamatória (Bettioli *et al.*, 2010). Esses dados em conjunto com os obtidos nesta tese indicam que diferentes espécies de plasmódio são capazes de induzir alterações distintas em células dendríticas.

Os resultados obtidos neste estudo mostram que a transferência adotiva de células dendríticas moduladas com PbX reduziu o quadro clínico de EAE. A resposta imune contra neuro-antígenos foi reduzida ao passo que células T de camundongos recipientes de DC-PbX proliferaram na presença de um antígeno não relacionado, ovalbumina (OVA). Estes achados sugerem que a transferência de DC-PbX não apenas reduziu a neuroinflamação, mas também estimulou a geração de supressão antígeno/tecido-específica.

Adicionalmente, quando DCs foram co-cultivadas com linfócitos T encefalitogênicos a proliferação específica foi reduzida bem como os níveis de citocinas inflamatórias nos sobrenadantes. Essas observações podem se correlacionar com a melhora no quadro clínico da doença. Embora o mecanismo para essas observações seja desconhecido, evidências sugerem que moléculas derivadas de plasmódio modulam o sistema imune. Foi observado que extratos solúveis de *P.falciparum* potencializam a indução e a função de células T reguladoras humanas por um mecanismo dependente de TGF- β (Clemente *et al.*, 2011). Na infecção murina por *Plasmodium chabaudi*, existe um aumento na frequência de células T reguladoras, responsáveis pela redução do quadro clínico de EAE (Farias *et al.*, 2011).

Interessantemente, co-culturas de células dendríticas moduladas com PbX conduzidas na presença de linfócitos T “naïve” ou encefalitogênicos apresentaram diferenças com relação a secreção de IFN- γ . Ainda é incerto como as células que produzem ambas as citocinas (IFN- γ /IL-10) modulam a EAE (na abordagem profilática), embora células T reguladoras duplo-produtoras de IFN- γ /IL-10 foram descritas como fundamentais na modulação de resposta imune através da administração de produtos derivados de helmintos (Sun *et al.*, 2012). Tais observações sugerem que células dendríticas modulam linfócitos T “naïve” e ativados por mecanismos diferenciados, o que carece de maior aprofundamento.

É notória a modulação de células dendríticas com moléculas derivadas de patógenos. Por exemplo, foi demonstrado que ovos de *Schistosoma mansoni* bloqueiam a maturação de DCs induzindo-lhes um perfil imaturo (Osada *et al.*, 2009; Everts *et al.*, 2010). Adicionalmente, antígenos solúveis de ovos de *S.mansoni* (SEA) são enriquecidos em ômega-1, produto com a capacidade de modular DCs e induzir a expressão de Foxp3 em células T *in vitro* (Everts *et al.*, 2009; Steinfelder *et al.*, 2009). Embora a retenção da resposta imune seja um problema nas infecções por *Schistosoma* e *Plasmodium*, o bloqueio da maturação de DCs pode ser útil no controle de inflamações crônicas de origem autoimune. Neste sentido, DCs expostas a SEA e ômega-1 adquirem propriedades moduladoras e induzem células Tregs a partir de linfócitos T “naïve” provenientes de camundongos NOD, reduzindo a incidência de diabetes espontânea (Zacone *et al.*, 2011). Outros grupos também reportaram que a administração de produtos derivados de helmintos, como *Trichuris suis*, reduziu a manifestação de EAE, provavelmente pela modulação de células apresentadoras de antígenos (Kuijk *et al.*, 2012).

Tendo em vista que células dendríticas ditam o curso da resposta imune, é razoável assumir que tais células são alvos durante infecções, resultando na mudança de seu perfil de ativação/maturação para um contexto anti-inflamatório. Tomados em conjunto, os resultados apresentados nesta etapa da tese mostram que células dendríticas moduladas com extratos de *P.berghei* são supressoras e controlam a EAE. Novos estudos serão conduzidos com a finalidade de determinar o mecanismo responsável pela modulação de células dendríticas.

Conclusões

Tendo em vista os resultados apresentados neste capítulo, pode-se concluir que:

- a. Células dendríticas diferenciadas a partir de precursores de medula óssea adquirem um fenótipo imaturo quando tratadas com extratos de *Plasmodium berghei* NK65 (DC-PbX).
- b. Quando co-cultivadas com linfócitos T “naïve”, as DC-PbX induzem a geração de células T reguladoras, o que as classifica como células dendríticas tolerogênicas.
- c. A transferência adotiva de células dendríticas moduladas com extratos de plasmódio suprimiu o quadro clínico de EAE tanto de forma profilática quanto terapêutica.
- d. Camundongos recipientes de DC-PbX apresentaram alterações na expressão gênica de mediadores inflamatórios no Sistema Nervoso Central quando comparados com camundongos pertencentes aos grupos controles.
- e. Os mecanismos de atuação de DC-PbX investigados, apesar de poderem não se restringir apenas a eles, incluem: (i) indução de células T reguladoras, (ii) criação de microambiente anti-inflamatório e (iii) supressão de células T auto-reativas.

Os resultados obtidos permitiram a confecção dos artigos científicos “Dendritic cells treated with chloroquine modulate experimental autoimmune encephalomyelitis”; “Dendritic cells treated with crude *Plasmodium berghei* extracts acquire immune-modulatory properties and suppress the development of autoimmune neuroinflammation” e “Nitric oxide plays a key role in the suppressive activity of chloroquine-modulated dendritic cells” (páginas 218, 228 e 252).

Discussão

Neste trabalho mostramos que a infecção prévia por *Plasmodium berghei* promove a exacerbação de respostas neuro-inflamatórias de origem autoimune por mecanismos ainda não totalmente esclarecidos, mas que envolvem a participação de células T-DP, precocemente saídas do timo, e sua posterior migração para o Sistema Nervoso Central. Embora a ocorrência de doenças autoimunes seja reduzida em zonas endêmicas de malária, estudos têm demonstrado a presença de elevados níveis de auto-anticorpos na circulação de pacientes infectados pelo plasmódio (Wallace *et al.*, 1993; Datta *et al.*, 2013). Interessantemente, mutações de ganho de função da enzima Chit, importante na resposta contra o plasmódio, aparentemente desempenham papel fundamental na resposta autoimune observada em pacientes com esclerose múltipla (Boot *et al.*, 1995; Barone *et al.*, 2003; Malaguarnera *et al.*, 2003; Sotgiu *et al.*, 2006; Sotgiu *et al.*, 2008). Não obstante, foi proposto que a infecção silenciosa por plasmódio em adolescentes e adultos pode ser uma das causas de MS (Kissler, 2001a; b). Experimentalmente foi demonstrado que camundongos infectados por *Plasmodium berghei* NK65 apresentam níveis séricos elevados de anticorpos anti-núcleo e contra células de musculatura lisa e que tal auto-resposta é dependente da atuação de células T, uma vez que a infecção em camundongos timectomizados não provocou o surgimento de auto-anticorpos (Poels *et al.*, 1979; Poels *et al.*, 1980). Esses resultados sugerem que a infecção por *P.berghei*, de alguma forma, altera a resposta imune facilitando o surgimento de auto-resposta, possivelmente, por meio da modulação de linfócitos T.

Tendo em vista que a seleção de repertório e a exclusão de células T auto-reativas acontece no timo, nosso grupo tem por objetivo investigar as possíveis alterações tímicas que ocorrem na vigência da infecção por plasmódio. Os resultados anteriormente obtidos mostraram a presença de *P.berghei* no timo após quatorze dias da infecção (Andrade *et al.*,

2008). Análises subsequentes conduzidas com camundongos C57BL/6 infectados por *P.berghei*-GFP^{knock-in}, que é fluorescente, mostraram que o parasita invade o timo a partir do terceiro dia de infecção e se aloja na região medular tímica, em contato com células dendríticas (*dados não mostrados e artigo em preparação*). Essa observação é intrigante visto que DCs tímicas (DCts) estão envolvidas nos processos de seleção de repertório, com especial participação na seleção negativa (Watanabe *et al.*, 2004; Capalbo *et al.*, 2012; Hadeiba *et al.*, 2012). Adicionalmente, foi verificado que timócitos de camundongos infectados por *Plasmodium berghei* apresentaram elevada taxa de morte celular por apoptose, principalmente por células DN e SP. Por outro lado, observou-se uma maior frequência de linfócitos T-DP em órgãos linfoides secundários após dez dias de infecção (Francelin *et al.*, 2011). Para investigar as possíveis consequências da atrofia tímica e presença de células T-DP em órgãos linfoides secundários, os camundongos foram tratados com cloroquina a partir de dez dias de infecção a fim de eliminar o parasita do sistema. Decorridos três dias após a última aplicação de cloroquina, os animais foram imunizados com peptídeo encefalitogênico e acompanhados para o desenvolvimento de Encefalomielite Autoimune Experimental.

Conforme visto anteriormente, os animais curados de malária apresentaram uma exacerbação da EAE e as análises que se seguiram mostraram que células T-DP presentes no baço, não apenas migraram para o SNC, como produziram quantidades significativas de citocinas inflamatórias. Esses resultados são interessantes, uma vez que tais células não passaram pelos mecanismos de seleção e, em teoria, seriam pouco responsivas a estímulo antigênico (Hirokawa *et al.*, 1994; Sprent, 1995; Asano *et al.*, 1996; Anderson *et al.*, 2002; Starr *et al.*, 2003; Hogquist *et al.*, 2005; Cheng *et al.*, 2007; Gameiro, Nagib e Verinaud, 2010; Capalbo *et al.*, 2012). Corroborando a hipótese de que as células T-DP não apenas

migram para o tecido-alvo, mas são capazes de responder a estímulos, quando esplenócitos foram desafiados *in vitro* com peptídeo MOG a análise da proliferação celular mostrou que o subtipo celular que mais ativamente proliferou consistiu de células T-DP.

Esses resultados mostraram que as células T-DP elicítadas na malária são capazes de responder a peptídeo encefalitogênico, mas não confirmam se essas células participam da piora do quadro clínico na EAE. Para investigar se o mecanismo de exacerbação da resposta imune deve-se a um efeito direto ou indireto de células T na EAE, foram conduzidos experimentos de cultivo conjunto entre células T provenientes de camundongos infectados com células T provenientes de camundongos portadores de EAE. Os resultados mostraram que as células T de camundongos com malária estimularam a proliferação de linfócitos T encefalitogênicos. A transferência adotiva de linfócitos T totais ou DP, oriundos de camundongos com malária, também foi capaz de agravar a EAE. Em conjunto, tais observações indicam que a piora na inflamação neural e no quadro clínico de EAE se deve a uma participação direta e indireta de células T-DP elicítadas na malária.

Interessantemente, a literatura descreve a presença de células T-DP originadas extratimicamente e envolvidas na supressão da resposta antitumoral em melanomas, cânceres de mama e cólon, por meio da secreção das citocinas IL-4, IL-5 e IL-13 e baixo potencial proliferativo (Desfrancois *et al.*, 2009; Freeman *et al.*, 2011; Sarabayrouse *et al.*, 2011). Oposto ao observado neste estudo, onde as células T-DP produziram elevadas quantidades de IL-17 e apresentaram alta taxa de proliferação após estimulação antigênica. Não obstante, a presença de células T-DP em camundongos BALB/c se relacionou com o desenvolvimento de EAE nesta linhagem, que é geneticamente resistente.

Os resultados obtidos indicam que a maturação e seleção de repertório de linfócitos T em camundongos infectados por plasmódio estão comprometidas. A possível modulação

de células dendríticas tímicas por plasmódio pode ter efeito na maturação de linfócitos T. Estudos subsequentes conduzidos em nosso laboratório mostraram que DCts de camundongos infectados por *P.berghei* apresentam-se alteradas tanto em seu fenótipo quanto em sua função (*dados não mostrados e artigo em submissão*). A quantidade absoluta de DCts é significativamente menor quando comparadas com camundongos saudáveis ($15.10^5 \times 5.10^5$ DCts/timo) assim como a expressão de moléculas co-estimuladoras CD80 e CD86. Por outro lado, a expressão de moléculas de MHC-II apresentou-se significativamente maior em DCts de camundongos infectados quando comparadas com células de camundongos saudáveis. Quando cultivadas em conjunto com timócitos totais, as DCts inibiram a proliferação de celular e promoveram a sobrevivência de células T-DP (*dados não mostrados e artigo em submissão*).

Entretanto, a avaliação de células dendríticas esplênicas de camundongos infectados por plasmódio apresentou resultados distintos dos observados em DCts. As DCs de camundongos infectados por *P.berghei* NK65 apresentaram expressão significativamente maior de MHC-II, CD80 e CD86 quando comparadas com DCs “naïve” (*dados não mostrados e artigo em redação*). Quando cultivadas em conjunto com linfócitos T esplênicos provenientes de camundongos “naïve”, as DCs estimularam sua proliferação frente ovalbumina e extrato de plasmódio. Interessantemente, a análise de DCs esplênicas de camundongos infectados por outras espécies de plasmódio (*P.chabaudi*, *P.yoelli* XL e *P.yoelli* 17X) apresentou resultados distintos. DCs de camundongos infectados por *P.chabaudi* apresentaram expressão semelhante de moléculas CD80 e CD86 em comparação com DCs “naïve”, ao passo que em MHC-II foi aumentada. Por outro lado, DCs de camundongos infectados por *P.yoelli* apresentaram níveis elevados de expressão de MHC-II, CD80 e CD86 quando comparadas com DCs “naïve” e infectadas por *P.chabaudi*

e *P.berghei*. Esses resultados estão em concordância com a literatura, em que diferentes espécies de plasmódio induzem respostas distintas em células dendríticas periféricas (Skorokhod *et al.*, 2004; Urban e Todryk, 2006; Bettiol *et al.*, 2010; Cockburn *et al.*, 2010; Giusti *et al.*, 2011; Mauduit *et al.*, 2012; Clemente *et al.*, 2013).

Os resultados permitem supor que, em camundongos infectados por *P.berghei* NK65, timócitos DP entram em contato com DCts que estimulam sua sobrevivência. Quando saem prematuramente do timo, o encontro de células T-DP com DCs ativadas expressando fortemente moléculas envolvidas na apresentação de antígenos pode estimular a exacerbação da resposta autoimune observada nos camundongos previamente infectados e imunizados com peptídeo MOG. No entanto, novos estudos deverão ser conduzidos para avaliar tal hipótese.

Interessantemente, células dendríticas diferenciadas a partir de precursores de medula óssea quando tratadas com extratos derivados de *P.berghei* apresentaram um fenótipo imaturo e estimularam a geração de células T reguladoras. A transferência adotiva de DC-PbX mostrou-se eficaz no controle da EAE por meio da redução da resposta celular específica a neuro-antígenos. Esses achados são intrigantes, uma vez que se observou uma diferença na ativação entre DCts e DCs isoladas de camundongos infectados. O contato de DCs com extratos de patógeno promoveu alterações distintas das observadas quando a interação ocorreu com o parasita intacto. A literatura nos mostra que a forma como DCs entram em contato com o plasmódio influencia na resposta orquestrada por células dendríticas (Urban *et al.*, 1999; Skorokhod *et al.*, 2004; Wykes *et al.*, 2011; Thome *et al.*, 2014). Novos estudos serão realizados com a finalidade de determinar a possível utilização de células dendríticas moduladas com extratos de patógeno no controle de inflamações crônicas.

Tomados em conjunto, os resultados obtidos neste estudo indicam que a infecção por *Plasmodium berghei* NK65 promove alterações profundas no sistema imune, que não são normalizadas após a eliminação do parasita. A emigração precoce de células T-DP do timo, em decorrência da malária, predispõe camundongos ao desenvolvimento de inflamações exacerbadas. Por outro lado, a modulação de células dendríticas com extratos de plasmódio pode ser uma alternativa interessante para o controle de inflamações crônicas de origem autoimune.

Conclusão

Tomados em conjunto, os resultados obtidos mostram que a infecção por *Plasmodium berghei* NK65 promove alterações significativas no sistema imune que auxiliam na exacerbação da neuro-inflamação autoimune. Por outro lado, a utilização de extratos do plasmódio pode se tornar uma alternativa inovadora na modulação da inflamação por meio da transferência adotiva de células dendríticas tornadas tolerogênicas com tais extratos.

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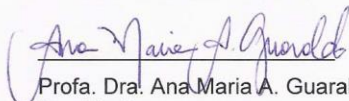
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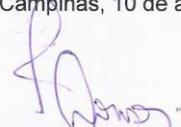
CERTIFICADO,

Certificamos que o projeto "Efeito da atrofia tímica induzida por infecção por *Plasmodium berghei* NK65 sobre a indução e desenvolvimento de EAE experimental em camundongos" (protocolo nº 2687-1), sob a responsabilidade de Profa. Dra. Liana Maria Cardoso Verinaud / Rodolfo Thomé, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em 10 de abril de 2012.

Campinas, 10 de abril de 2012.


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Artigos publicados

Chloroquine Treatment Enhances Regulatory T Cells and Reduces the Severity of Experimental Autoimmune Encephalomyelitis

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Abstract

Background: The modulation of inflammatory processes is a necessary step, mostly orchestrated by regulatory T (Treg) cells and suppressive Dendritic Cells (DCs), to prevent the development of deleterious responses and autoimmune diseases. Therapies that focused on adoptive transfer of Treg cells or their expansion *in vivo* achieved great success in controlling inflammation in several experimental models. Chloroquine (CQ), an anti-malarial drug, was shown to reduce inflammation, although the mechanisms are still obscure. In this context, we aimed to access whether chloroquine treatment alters the frequency of Treg cells and DCs in normal mice. In addition, the effects of the prophylactic and therapeutic treatment with CQ on Experimental Autoimmune Encephalomyelitis (EAE), an experimental model for human Multiple Sclerosis, was investigated as well.

Methodology/Principal Findings: EAE was induced in C57BL/6 mice by immunization with myelin oligodendrocyte glycoprotein (MOG₃₅₋₅₅) peptide. C57BL/6 mice were intraperitoneally treated with chloroquine. Results show that the CQ treatment provoked an increase in Treg cells frequency as well as a decrease in DCs. We next evaluated whether prophylactic CQ administration is capable of reducing the clinical and histopathological signs of EAE. Our results demonstrated that CQ-treated mice developed mild EAE compared to controls that was associated with lower infiltration of inflammatory cells in the central nervous system (CNS) and increased frequency of Treg cells. Also, proliferation of MOG₃₅₋₅₅-reactive T cells was significantly inhibited by chloroquine treatment. Similar results were observed when chloroquine was administrated after disease onset.

Conclusion: We show for the first time that CQ treatment promotes the expansion of Treg cells, corroborating previous reports indicating that chloroquine has immunomodulatory properties. Our results also show that CQ treatment suppress the inflammation in the CNS of EAE-inflicted mice, both in prophylactic and therapeutic approaches. We hypothesized that the increased number of regulatory T cells induced by the CQ treatment is involved in the reduction of the clinical signs of EAE.

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Introduction

The modulation of the immune system is a necessary process to prevent the development of deleterious immune response and autoimmune diseases. Several mechanisms were developed to restrain exacerbated activation of the immune system against self-antigens which includes the central and peripheral tolerance [1–3]. Thymocytes, the lymphocytes inside the thymus, are “tamed” to recognize auto-antigens and respond to non-self-antigens within the thymic environment, in a network of soluble molecules, cell-cell and cell-extracellular matrix interactions [4–6]. In periphery,

natural arising regulatory T (Treg) cells act inhibiting the activation of self-reactive lymphocytes through cell contact, secretion of anti-inflammatory cytokines and modulation of professional antigen presenting cells, like dendritic cells (DCs) [3,7,8]. It was previously shown that a reduction in number and function of Treg cells is associated with autoimmune diseases [9–11], and failure to express the nuclear transcriptional factor Foxp3 results in human X-linked IPEX (Immunodysregulation Polyendocrinopathy and Enteropathy) and mouse *scurfy*, both severe poly-autoimmune disease syndromes [12,13].

Adoptive transfer of Treg cells has proven to be a useful tool to reduce inflammatory diseases, such as human graft versus host disease [14], experimental diabetes [15], experimental autoimmune hepatitis [16], experimental arthritis [17] and experimental autoimmune encephalomyelitis [18]. Therefore, therapies that promote the expansion of regulatory T cells are desirable in order to reduce the overall chronic inflammation observed in most autoimmune diseases. Chloroquine (CQ), an anti-malarial drug, has proven to exert some anti-inflammatory effects through the down-regulation of Tumor Necrosis Factor- α (TNF- α) production and signaling in macrophages [19,20], as well as the cytokine pattern production [21]. Yet, the administration of chloroquine prevented the onset of graft-versus-host disease in a mouse model [22]. Treatment with chloroquine together with other immunosuppressive drugs resulted in amelioration of the clinical manifestations in rheumatoid arthritis patients [23]. It is not clear the precise mechanism triggered by chloroquine, but several evidences suggest that chloroquine acts as a weak base by both pH-dependent and -independent mechanisms [24–26].

Experimental Autoimmune Encephalomyelitis (EAE) is the most studied experimental model for Multiple Sclerosis, which is originated after immunization of susceptible mice with myelin-associated proteins in an inflammatory context. Activated T cells migrate into the Central Nervous System (CNS) and initiate a robust inflammatory response [27–29]. Thus far, the treatment for MS is based on high cost medicine and more recently on the administration of monoclonal antibodies [30–32]. So, the search for adjunctive therapies is of great value in the field of autoimmunity treatment, especially those that increase the frequency or function of regulatory T cells. In this sense, chloroquine is a cheap and well-tolerated drug, with some described effects on inflammatory conditions. However, the mechanisms used by chloroquine and whether regulatory T cells are involved in the immunomodulation as well as whether this drug can reduce the clinical signs of EAE, remain obscure.

In this context, we aimed to investigate if the administration of chloroquine alters the frequency of regulatory T cells and dendritic cells in the periphery of the immune system and if the treatment with CQ could ameliorate the clinical signs of EAE. We found that CQ treatment provoked an increase in the frequency of Treg cells and reduced DCs numbers in the spleen. When CQ was administered both prophylactic and therapeutically mice developed mild clinical score of EAE and this was accompanied by a reduced infiltration of inflammatory cells to the CNS. An increase in Treg cells number and in secretion of immunomodulatory cytokines was observed as well. The data obtained here strongly suggest that chloroquine may become a useful adjunct in the treatment of multiple sclerosis.

Materials and Methods

Mice

Six-to-eight week-old female C57BL/6 mice from the Multi-disciplinary Center for Biological Research, University of Campinas, were used in this study. Mice were kept in *specific-pathogen free* conditions, in a controlled temperature and photoperiod environment, with free access to autoclaved food and water throughout the experiment. All protocols involving laboratory animals were approved and performed in accordance with the guidelines of the State University of Campinas Committee on the Use and Care of Animals (Comissão de Ética no Uso de Animais – CEUA, # 2687-1).

Chloroquine Treatment

Groups of mice ($n = 7$) were created aiming the test for ideal, non-toxic chloroquine (Chloroquine diphosphate salt, Sigma-Aldrich, Brazil) concentration. The concentrations tested were 3, 5 and 10 mg/kg⁻¹. The 100 mg/kg dose was found to be lethal. Animals of each group received chloroquine via i.p. (200 μ L/mice) for five consecutive days. Control mice were injected with diluent solution (Phosphate-Buffered Saline 0,02 M pH 7,2). Three days after the last dose, mice were killed and splenic cells were collected and assayed for cellular population analysis in the presence of concanavalin-A (2,5 μ g/mL). Mice survival and spleen cellularity were evaluated as well.

EAE Induction, Evaluation and Chloroquine Treatment

EAE was induced and evaluated in mice according to a previous published paper [33]. Briefly, each mouse was injected with 100 μ g MOG_{35–55} (MEVGWYRSPFSRVVHLYRNGK, Rhea-Biotech, Brazil) emulsified with Complete Freund's Adjuvant (CFA, Sigma-Aldrich, USA). 200 ng *Pertussis* toxin (Ptx, Sigma-Aldrich, USA) was administered via i.p. at 0 and 48 h after MOG_{35–55} inoculation. Weight changes and clinical signs were followed and graded daily according to a score method, where 0: no sign, 1: flaccid tail, 2: hind limbs weakness, 3: hind limbs paralysis, 4: hind paralysis and fore limbs weakness, 5: full paralysis/dead. An intermediate non-toxic concentration (5 mg/kg/day) of chloroquine was used for EAE treatment (five consecutive days, via i.p.). For prophylactic approach, EAE was induced three days after the last dose of CQ (5 mg/kg⁻¹), and for therapeutic approach, mice received the CQ treatment after the onset of EAE (day 10th after immunization with neuro-antigens). Fourteen (prophylactic approach) and thirty (therapeutic approach) days after antigen challenge mice were killed spinal cords were removed and snap frozen; 12 μ m thin slices were made in cryostat and stained with haematoxylin and eosin (H&E).

Isolation of Treg Cells (CD4⁺CD25⁺) and Transfer Experiments

Naïve C57BL/6 mice were treated with chloroquine as described above and three days after the last dose spleen cells were collected and CD4⁺CD25⁺ cells were isolated by magnetic beads following manufacturers recommendations (CD4⁺CD25⁺ Regulatory T Cell Isolation Kit; Miltenyi Biotec, USA). 5×10^5 Treg cells per mouse were adoptively transferred (via i.v.) to EAE mice at the onset of disease (10 days after immunization). As control, EAE mice received equal numbers of CD4⁺CD25⁺ cells at the same time point. EAE induction and evaluation was performed as described above.

Lymphoproliferative Response and Cytokine Dosage

Splenic cells were aseptically collected from mice after 10 and 30 days of antigen challenge for prophylactic and therapeutic approaches, respectively, and after 16 days for Treg cells transfer experiments. Single cell suspensions were stained with Carboxy-fluorescein succinimidyl ester (CFSE, Sigma-Aldrich, USA) following the manufacturers instructions. Cells (5×10^5 /well) were diluted in RPMI 1640 media supplemented with Fetal Calf Serum (FCS; 10% vol/vol), guaranine (50 μ g/mL), 2-Mercaptoethanol (2 mM) and myelin oligodendrocyte glycoprotein peptide (MOG_{35–55}; 20 μ g/mL), plated in flat-bottom plates and incubated at 5% CO₂ and 37°C for 96 h. After the incubation period, cells were stained with PerCPy5-conjugated anti-CD3 antibodies and fixed in 1% paraformaldehyde prior to flow cytometer analysis. CFSE^{low}CD3⁺ cells were considered proliferating T cells. Culture

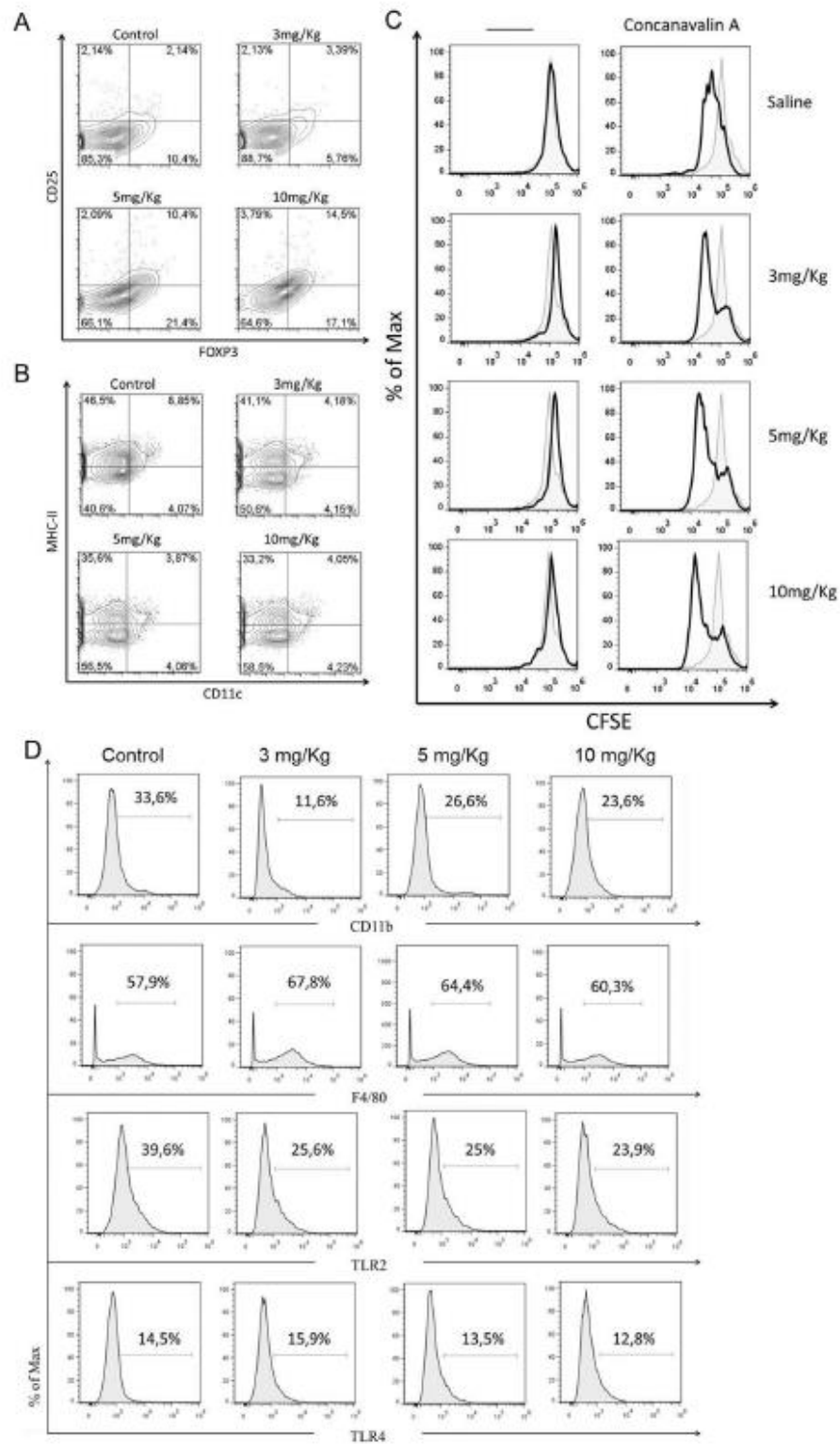


Figure 1. Chloroquine administration alters the frequency of regulatory T (Treg) cells and dendritic cells (DCs), but not the proliferative capability of T cells. Briefly, mice were treated with chloroquine *via i.p.* for five consecutive days. Three days after the last dose mice were killed and splenic cells were analyzed by flow cytometry. Increased numbers of Treg cells (A) and reduced frequency of DCs (B) was found in mice treated with chloroquine when compared to the control group. In addition, splenic T cells proliferative response was not altered in the presence of concanavalin-A (C). Subpopulations of leukocytes showed slight changes when compared to control subjects (D). Results are representative of three independent experiments.
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supernatants were collected and assayed for cytokines (IL-4, IL-6, IL-10, IL-17, IFN- γ and TNF- α) secretion using the Cytometric Bead Array (CBA, BD Biosciences, USA) according to manufacturers instructions.

Analysis of Cellular Infiltration in the CNS

Fourteen days after EAE induction (in the prophylactic approach) and thirty days after EAE induction (in the therapeutic approach), mice were anesthetized, perfused with ice cold PBS and half of the spinal cords and brains were removed and stored at -80°C until use for RT-PCR assays; the remaining tissue was prepared for the enrichment of infiltrating leukocytes according to a previously described methodology and analyzed by flow cytometry [34].

Flow Cytometry

Fluorochrome-conjugated monoclonal antibodies were used to stain leukocytes. Cells were surface stained with anti-CD4/PE-Cy7, anti-CD8/APC, anti-CD3/PerCPy5, CD11c/APC, CD11b/PE, F4-80/APC, TLR-2/PE, TLR-4/PE. For intracellular staining, cells were fixed/permeabilized (fixation/permeabilization buffers) according to manufacturers recommendations, later monoclonal anti-Foxp3/APC, IL-10/PE, IFN- γ /PE and IL-17/APC were added to cells. Isotype controls were used as well. All antibodies were purchased from eBioscience (USA). Preparations were acquired with a Gallios flow cytometer (Becton Coulter, USA) and data analyzed using FlowJo 7.6 (Tree Star Inc., USA).

RT-PCR Assays

Frozen tissues were used for RNA extraction using Trizol (Invitrogen, USA) and cDNA synthesis according to the manufacturers recommendations (Applied Biosystems, USA). Expression of IL-10 (Mm00439614_m1), IL-17 (Mm00439618_m1), IFN γ (Mm01168134_m1), FOXP3 (Mm00475162_m1) and RAR-related orphan receptor C (RORc) (Mm01261022_m1) were analyzed in comparison to GAPDH (Mm99999915_g1, housekeeping gene) levels. RT-PCR reactions were performed using Taqman reagents according to manufacturers recommendations (Applied Biosystems, USA).

Statistical Analysis

Clinical score comparisons between control and experimental groups were done by Two-Way ANOVA and post-tested with Bonferroni. Other analyses among two and three (or more) groups were carried out with Students *t* test and One-Way ANOVA, respectively. Results are expressed as mean $\pm$ standard error mean (SEM) and $p < 0.05$ value were defined as significant.

Results

Increased Frequency of Regulatory T cells and Reduced Percentage of Dendritic Cells after Chloroquine Treatment

Naïve mice were treated with CQ at different dosages for five consecutive days and the cellular subsets were evaluated three days

after the last dose of drug administration. Along with reduction in the total splenic cells number at higher doses (data not shown), our data showed that CQ treatment increased the numbers of regulatory T cells whereas the frequency of dendritic cells was reduced (Figure 1A and 1B, respectively). In order to evaluate whether CQ treatment promoted functional alterations in T cells, splenic lymphocytes from CQ treated-mice were cultured in the presence of concanavalin-A (Con-A) for 72 h. As depicted in figure 1C, the CQ treatment did not alter the proliferation capacity of T cells. Other subpopulations of leukocytes were also analyzed but only a slight change in the frequency of these cells was noticed compared to the control group (Figure 1D).

Chloroquine Treatment Reduces the Clinical Evolution and Infiltration of the CNS in EAE Mice

An increase in regulatory T cells pool is associated with mild inflammation, whereas reduced dendritic cell numbers may impair proper antigen presentation to T cells, thus dampening adaptive immune response. In this context, the next goal was to determine whether prophylactic CQ administration was capable of modulating the course and severity of EAE. Hence, mice were subjected to CQ treatment (5 mg/kg/day) for five consecutive days, and three days after the last dose EAE was induced (Figure 2A) and the development of the disease accompanied daily.

Mice that received CQ prior to EAE induction showed a significant reduction in weight loss compared with PBS-treated animals. Accordingly, the treatment was also capable to delay disease severity course (Figures 2B and 2C). As leukocytes infiltration in the CNS is directly associated with the severity of disease, we aimed to investigate whether the CQ treatment had altered brain inflammation. PBS- and CQ-treated EAE mice were killed and spinal cords were removed and stained with H/E. Corroborating results mentioned above, CQ treated-mice presented lower leukocytes infiltration in the CNS (Figure 2D). Overall, CQ administration was able to ameliorate the clinical course of EAE, most probably, because of the reduced cellular infiltration in the CNS.

We next examined the profile of leukocytes that infiltrated the CNS of CQ treated-EAE mice. For that purpose brains and spinal cords were collected, minced and cellular suspensions were prepared and analyzed as described in M&M section. Our results show that lymphocytes managed to overcome the blood-brain barrier and infiltrated the CNS of EAE mice, both of PBS- and CQ-treated groups. However, the number of infiltrating lymphocytes was significant reduced in CQ treated-mice compared to the control subjects (Figure 3A). Interestingly, the pattern of infiltrating cells in CQ-treated group was quite different from control EAE group. CQ treated-mice showed significant reduction in interleukin (IL)-17A- and interferon-gamma (IFN- γ)-producing cells and a significant increase of IL-10-producing cells in the CNS (Figure 3B). Also, the relative gene expression analyses have showed decreased pattern for IL-17 and IFN- γ and up-regulated pattern for IL-10 (Figure 3C, 3D and 3E, respectively).

As Treg cells number were increased in normal mice upon CQ treatment and an augmented frequency of this population is correlated with suppression of EAE, we aimed to assess the

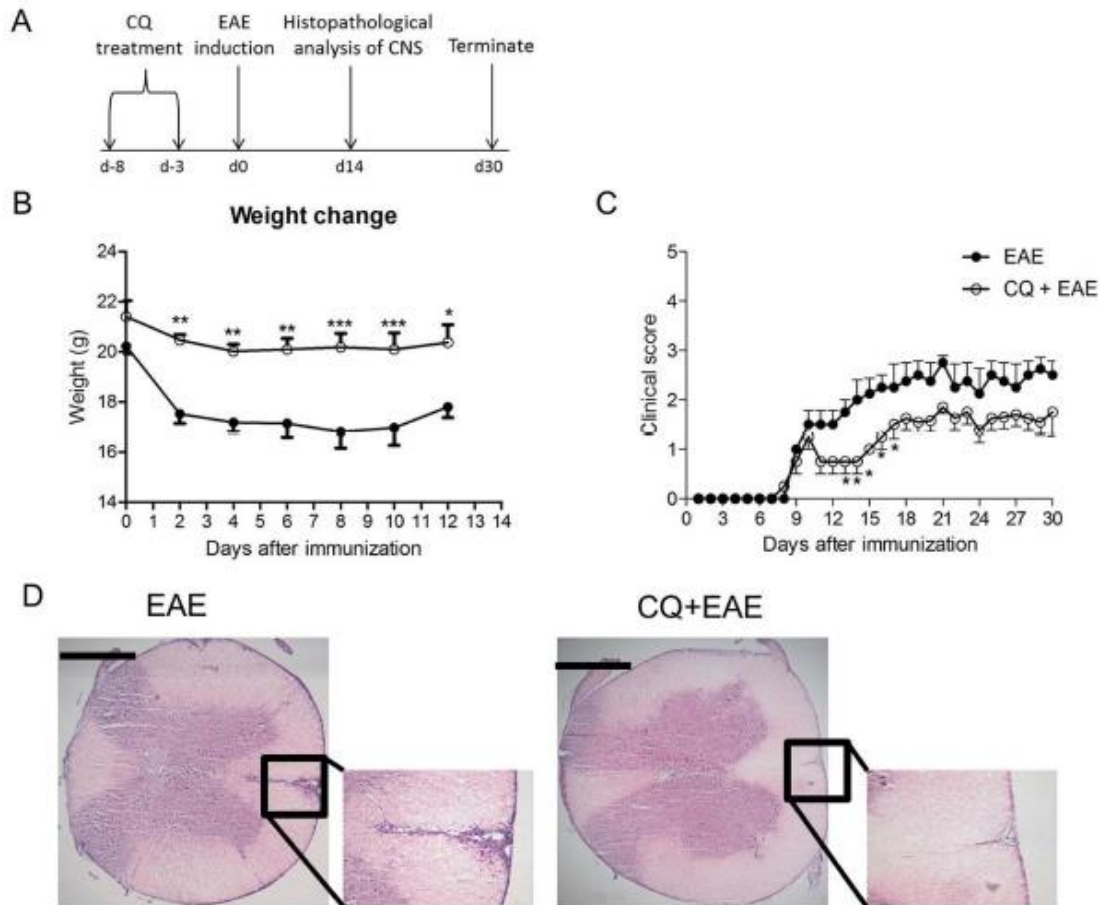


Figure 2. CQ treatment reduces the severity of EAE. (A) Briefly, mice were treated with chloroquine for five consecutive days and three days after the last dose of the drug EAE was induced through the administration of MOG₃₅₋₅₅ peptide emulsified in CFA and two injections of Ptx (200 ng/animal) at 0 and 48 h post antigen inoculum. (B and C) The weight change and clinical score were checked routinely. Results are expressed as mean $\pm$ SEM for at least five animals. $p < 0.01$ (**) and $p < 0.005$ (***). (D) At 14 days, mice were killed and spinal cords were prepared for histological analysis (H&E stained) to evaluate cellular infiltration in the CNS. The figures are representative of at least three independent experiments performed in different days. Bar: 500 μ m.
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incidence of Treg cells in spleen and the Foxp3 gene expression in the spinal cords of EAE mice fourteen days after induction of the disease. Corroborating our results, the expression of Foxp3 was found significantly augmented in CQ treated-mice (Figure 3F). In the periphery of the immune system, it was observed that EAE mice that received CQ had increased Treg cell numbers compared with the PBS treated-group (Figure 3G). These data indicate that the reduction in EAE severity observed in CQ-treated mice correlates with the increase in Treg cells number both in the CNS and the periphery.

Administration of Chloroquine Suppresses the Ag-specific Proliferation and Changes the Cytokine Production Pattern

Considering that an increase in Treg and IL-10-producing cells may correlate with the reduced clinical signs of EAE, and that the

antigen-specific cellular immune response is the cause of the disease in mice, we next evaluated whether peripheral encephalitogenic lymphocytes from CQ treated-mice proliferate in the presence of MOG₃₅₋₅₅. For that purpose, splenic leukocytes derived from mice after ten days of immunization with neuro-antigen were collected and put in culture in the presence of MOG₃₅₋₅₅ for 96 h. Our data show that lymphocytes from CQ-treated mice proliferated significantly less than cells from PBS-treated group (Figure 4A). In the culture supernatants there was also a significant reduction in IL-17 levels, whereas the concentration of IL-10, IL-6, IFN- γ , and IL-4 were found significantly up regulated from CQ-treated mice cells compared to PBS-treated ones. No difference could be observed in the levels of tumor necrosis factor- α (TNF- α) between cultures of both groups (Figure 5B).

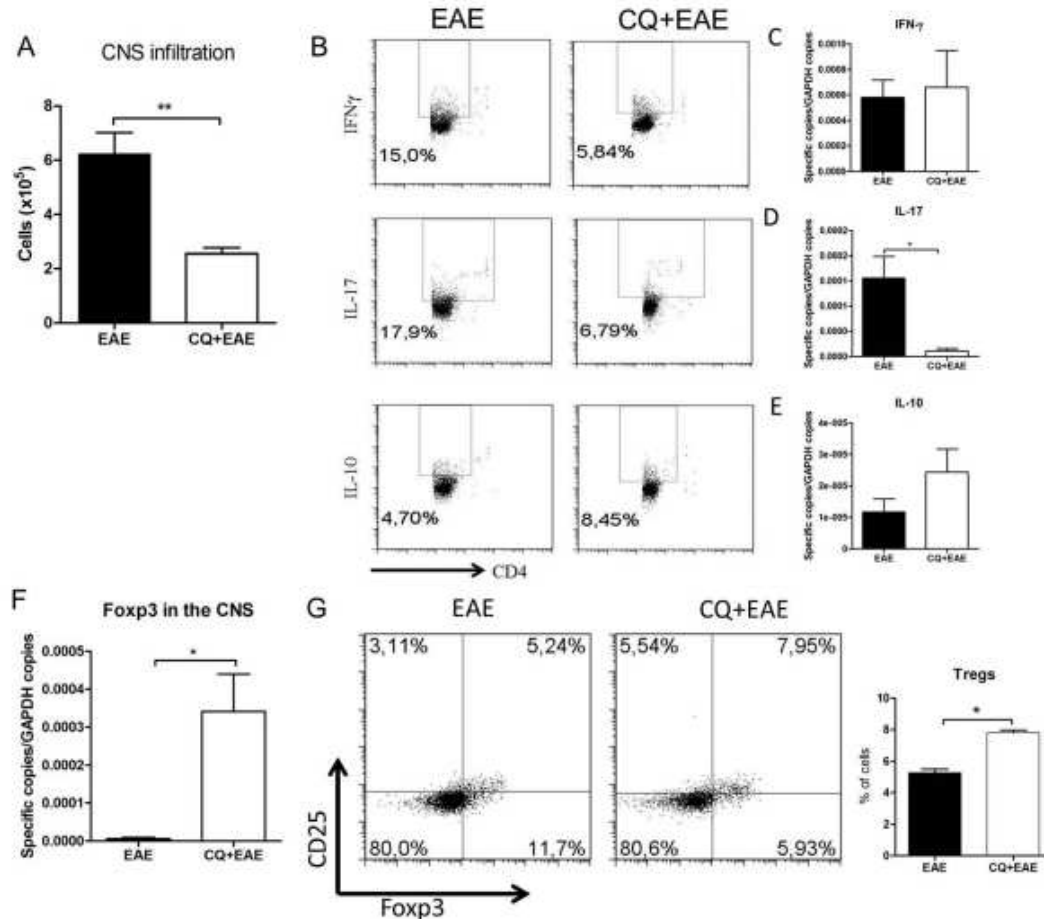


Figure 3. Analysis of the cellular infiltration of the CNS show reduced IFN- γ and IL-17 producing cells in CQ treated EAE mice. (A) CQ treated-mice presented reduced infiltration of inflammatory cells. (B) The percentage of IFN- γ - and IL-17-producing cells infiltrating the brain was reduced while the frequency of IL-10-producing cells was found augmented in brain of mice treated with CQ. (C, D and E) Gene expression of IFN- γ , IL-17 and IL-10 in the CNS followed the same pattern, respectively. (F) The expression of FOXP3 was evaluated in the CNS by RT-PCR. (G) The frequency of CD25⁺Foxp3⁺ cells was evaluated in spleens of mice. Results are representative of two independent experiments and are expressed as mean $\pm$ SEM for at least five animals. $p < 0.05$ (*) and $p < 0.01$ (**). doi:10.1371/journal.pone.0065913.g003

Chloroquine Treatment may also be Used after the Onset of EAE with Similar Results

Although CQ prophylactic approach was able to reduce the clinical evolution of EAE, the results might differ when the drug is administrated after disease onset, which corresponds to a more realistic picture for disease treatment. In order to solve this issue, mice were immunized with MOG₃₅₋₅₅ and 10 days later, after the onset of EAE, CQ treatment was initiated (Figure 5A). Results showed that CQ-treated EAE mice presented a reduction in the weight loss and amelioration of the clinical course of the disease (Figure 5B and 5C, respectively).

EAE develops after the migration of inflammatory cells to the CNS, where they produce pro-inflammatory cytokines and secrete a myriad of enzymes and soluble factors damaging the nervous

system. As the treatment started after disease onset, we next evaluated whether the cellular infiltration in the spinal cords of mice was altered. Thirty days after EAE induction spinal cords were collected and analyzed for the presence of leukocytes. We found that CQ treatment provoked a slight reduction in the infiltration of cells to the spinal cords compared with the PBS-treated group (Figure 5D). Although CQ treatment was not able to reduce leukocytes infiltration in the CNS, a significant up-regulation of Foxp3 cells in the spinal cords was observed. The expression of IFN- γ was found significantly down-regulated in the treated group as well.

The expression of IL-17 and Th17 related transcriptional factor RAR-related orphan receptor C (RORC) was not statistically different between the two groups (Figure 5E).

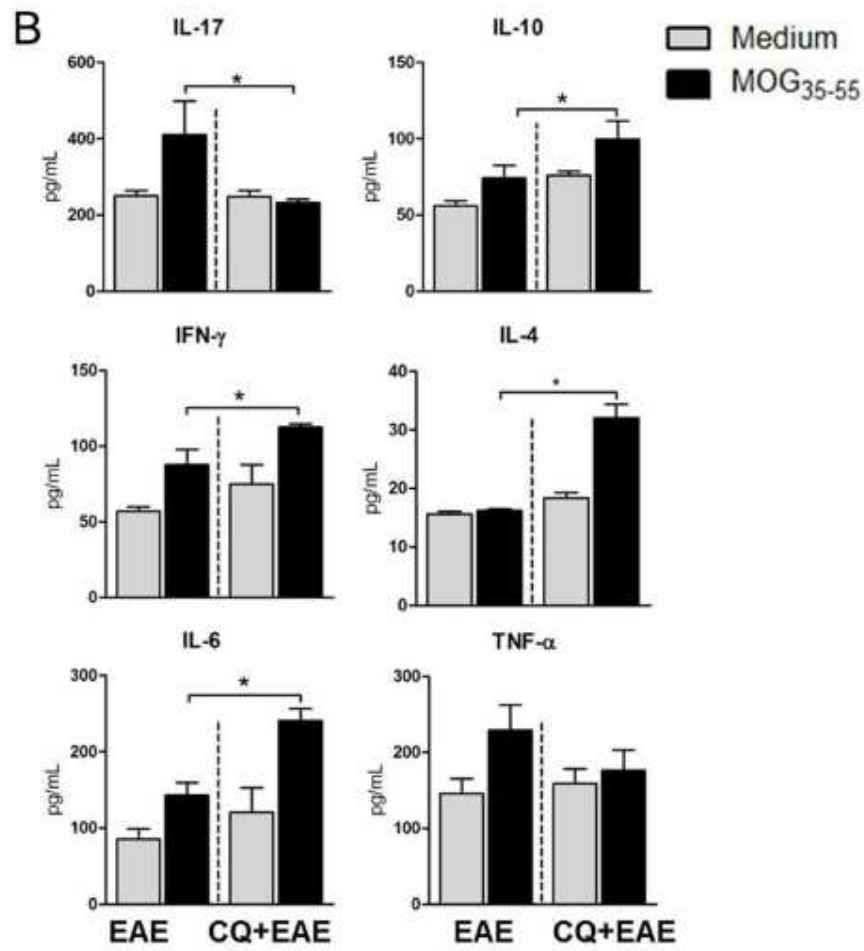
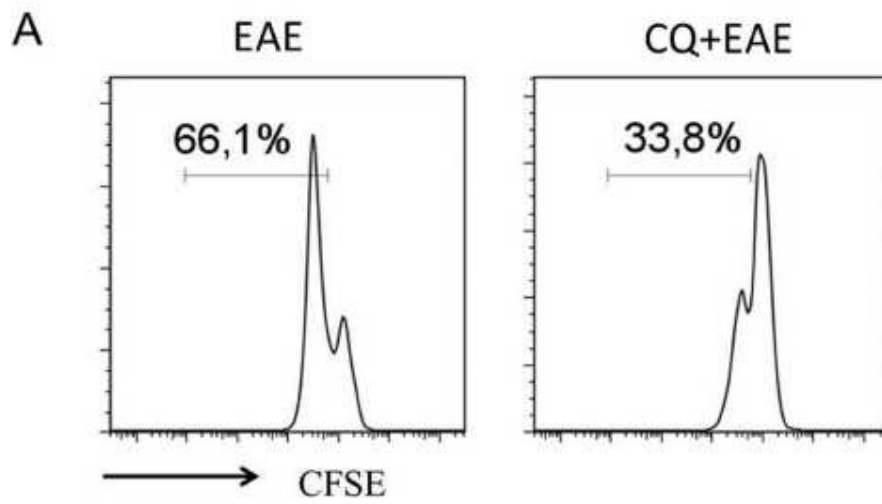


Figure 4. Chloroquine treatment reduces the Ag-specific proliferation of T cells in the spleen and the cytokine production profile. After 14 days of immunization with MOG₃₅₋₅₅ peptide, mice were killed and CFSE-stained splenocytes were cultivated in the presence of MOG₃₅₋₅₅ for 96 h. (A) The proliferation was calculated in the CFSE^{low}CD3⁺ cells. Figures presented are representative of three independent experiments. (B) At the end of the culture period the supernatants were collected and assayed for the detection of cytokines using cytometric beads assay. Results are expressed as mean $\pm$ SEM for at least five animals, $p < 0.05$ (*). doi:10.1371/journal.pone.0065913.g004

Accordingly, the profile of inflammatory cells in the CNS was altered as the frequency of IL-10-producing cells was augmented while the frequency of IFN- γ - and IL-17-producing cells was reduced in the CQ-treated group (Figure 5F). There was also reduction in MOG₃₅₋₅₅-specific proliferation of splenocytes from CQ-treated mice compared to control group and IL-17, IL-6, IFN- γ secretion. In contrast, IL-10 and IL-4 production was augmented when cells were cultured in the presence of MOG₃₅₋₅₅ peptide (Figure 5G).

Transfer of Chloroquine-elicited Regulatory T cells Reduces EAE

As we have observed that CQ in homeostatic conditions is able to promote an increase in Treg cells, we decided to investigate whether Treg cells elicited by CQ treatment played a role in the modulation of EAE severity. Then naïve C57BL/6 mice were treated with CQ for five consecutive days (5 mg/kg/day) and their isolated CD4⁺CD25⁺ (Treg) cells were transferred into EAE mice at the disease onset (day 10 after MOG₃₅₋₅₅ inoculation) (Figure 6A). Results showed that transfer of Treg cells reduced the clinical course of EAE (Figure 6B) compared to

CD4⁺CD25⁻ recipient EAE mice (Figure 6B). There was also reduction in the leukocytes infiltration in the CNS (Figure 6C). We next characterized the cytokine profile of the infiltrating cells. Mice that received Treg cells at EAE onset had lower frequency of IL-17- and IFN- γ -producing cells in the CNS compared to the control group. The frequency of IL-10 producing cells remained unchanged (Figure 6C).

The MOG₃₅₋₅₅-specific cellular response in the periphery was evaluated as well. It was observed that splenic cells from EAE mice that received CD25⁺-transferred cells proliferated significantly less than cells from CD25⁻-transferred-EAE mice (Figure 6D). We aimed to assess whether the pattern of cytokine production in the presence of MOG₃₅₋₅₅ peptide was altered. Our data showed that there was no statistical difference in the production of IL-17 and TNF- α between the two groups. However, levels of IFN- γ and IL-6 were reduced while an increase in IL-10 and IL-4 secretion was observed in cell cultures from CD25⁺ transferred- mice when compared to control group (Figure 6D).

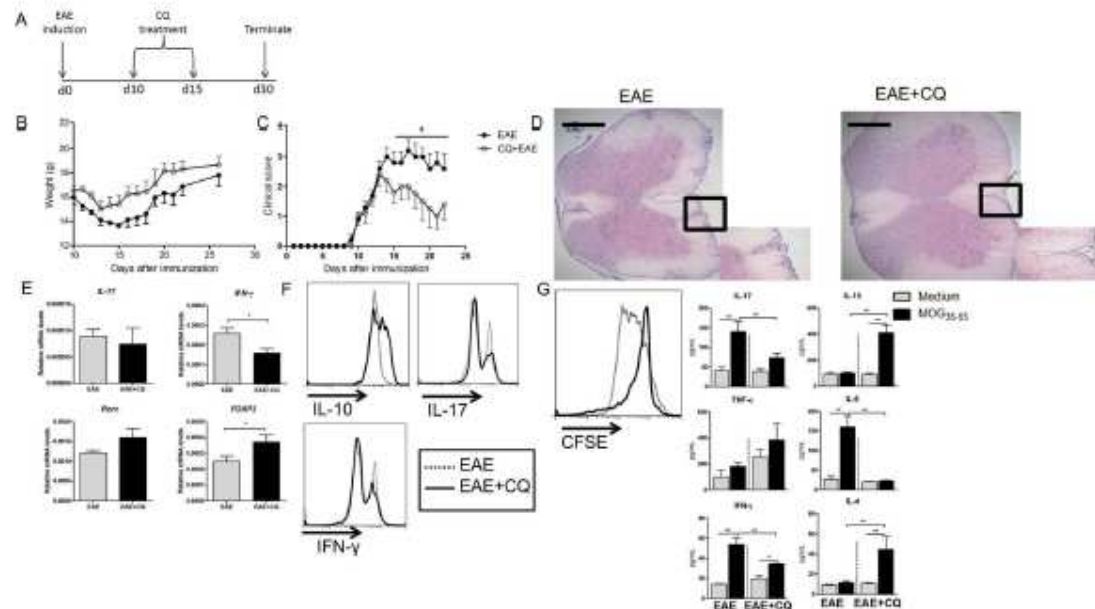


Figure 5. Chloroquine administration after the onset of EAE reduces the clinical signs of the disease. (A) CQ was administrated ten days after immunization with MOG₃₅₋₅₅. (B) and (C) Animals were accompanied for weight changes and clinical score. (D) The spinal cords were removed at day 30 and 10 μ m slices were stained with HE for detection of infiltrating cells in the CNS. Figures are representative of at least six mice. Bar: 500 μ m. (E) Gene expression of IL-17, IFN- γ , Foxp3 and ROR γ t in the CNS was evaluated. (F) The infiltrating cells in the CNS were collected and stained for flow cytometric characterization of IL-10, IL-17 and IFN- γ production. (G) Splenic cells from EAE mice, CQ- and PBS-treated mice, were removed and CFSE-stained cells were cultivated (5×10^5 /well) in the presence of MOG₃₅₋₅₅ peptide (20 μ g/mL) for 96 h. The dye decay and cytokine production were measured by flow cytometry. Results are representative of three independent experiments and are expressed as mean $\pm$ SEM for at least five animals. * $p < 0.05$. doi:10.1371/journal.pone.0065913.g005

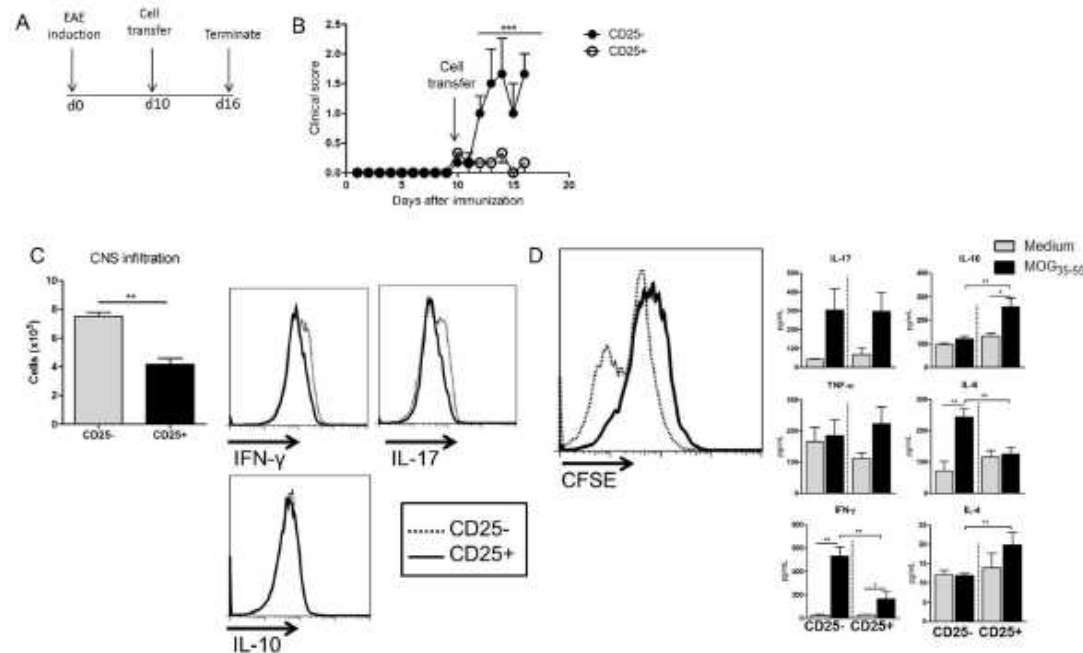


Figure 6. Transfer of CQ-elicited Treg cells reduces the severity of ongoing EAE. (A) Naïve C57BL/6 mice were treated with chloroquine (5 mg/kg/day) for five consecutive days. Three days after the last dose of the treatment, splenic CD4⁺CD25⁺ cells were isolated using magnetic beads and cells (5×10^5 cells per mouse) were transferred into mice with ongoing EAE (10 days after immunization). As controls, mice received the same number of CD4⁺CD25⁻ cells. (B) The clinical course of the disease was evaluated routinely. (C) The brains and spinal cords were collected and the enriched infiltrating cells were counted. The frequency of IL-17-, IL-10- and IFN- γ -producing cells was analyzed by flow cytometry as well. (D) The spleens were collected and CFSE-stained cells were cultivated in the presence of MOG₃₅₋₅₅ peptide for 96 h. Dye decay and cytokine production were analyzed by flow cytometry. Results are expressed as mean $\pm$ SEM for at least five animals. $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). doi:10.1371/journal.pone.0065913.g006

Discussion

Autoimmune diseases develop in deregulated immune systems that fail to control chronic inflammation. Although the events that trigger disease development are unknown, multiple sclerosis is an immune mediated syndrome with characteristics of acute and chronic inflammation [35,36]. Therapies that focus on reestablishing homeostasis and immunomodulation are of great value. Regulatory T cells play an important role in the control of inflammation and suppression of auto-reactive cells [3,8,37]. In this context, we found that chloroquine administration provokes an increase in Treg cells frequency in the spleen of normal mice. When administrated, both prophylactic and therapeutically, CQ modulated the course of EAE, an animal model for multiple sclerosis. The transfer of CQ-elicited Treg cells into mice with ongoing EAE promoted a reduction in disease severity as well.

Chloroquine, an anti-malarial agent, was shown to have anti-inflammatory properties. The administration of the drug resulted in impaired iron metabolism and TNF- α production by macrophages [20,38], as well as altered cytokine secretion profile [19,39,40]. It was also shown that chloroquine affects T cell priming to minor MHC complexes and may be used to modulate graft-versus-host disease (GVHD) [41]. The mechanisms underlying these effects are not fully understood, but may involve the changes in pH of several intracellular organelles. CQ is a weak base that has tropism for acidic organelles, such as lysosomes [42]. Although

it was already shown that CQ raises NKT cell pool [22], to our knowledge, this is the first study to show that chloroquine treatment leads to an increase in regulatory T cell numbers in the periphery as well as a decrease in DC's.

Therapies that lead to induction of regulatory T cells have provided interesting results in the amelioration of EAE. The ingestion of the lactic acid producing bacteria *Peptostreptococcus acidilactici* led to expansion of Treg cells in the mesenteric lymph nodes of mice resulting in decreased specific cellular response and consequently in EAE score [43]. Oral administration of MOG₃₅₋₅₅ also resulted in reduced EAE severity through the stimulation of antigen-specific Treg cells [44]. Therefore, we aimed to access whether prior expansion of Treg cells, due to chloroquine administration, could suppress the development of EAE. Mice treated with CQ developed a mild form of the disease, and Treg cells population was found augmented both in spleen and in the CNS. Although these Treg cells emerged before MOG₃₅₋₅₅ immunization, the MOG₃₅₋₅₅-specific cellular proliferation was reduced, suggesting that the Treg-mediated immune-suppression is antigen-unspecific. Similarly, Ovalbumin-specific regulatory T cells were able to reduce the anti-Type II Collagen responses, promoting reduced clinical signs of collagen-induced arthritis in a by-stander fashion [45,46]. In cultures of spleen cells in the presence of MOG₃₅₋₅₅ peptide we observed a change in the pattern of cytokine secretion. The increased IFN- γ , IL-4 and IL-6 production indicates that CQ treatment altered the

T cell subsets responsive to the neuro-antigen. These cytokines may be involved in the deviation of the immune response towards neuro-antigens *in vivo* after CQ administration.

Th1 and Th17 cells are important for EAE development. Both cells act synergistically to induce the lesions in the CNS [47,48], although IFN- γ -producing cells seems to suppress exacerbated disease [49,50]. Neutralization of IL-17 by antibodies leads to mild disease severity [51]. Thus, suppressing inflammatory cytokines may result in down-modulation of EAE. The treatment with chloroquine also changed the pattern of cytokine secretion of the infiltrating cells in the CNS; the reduction in the IFN- γ and IL-17-producing cells was correlated with mild disease. It was previously published that administration of MOG antigen, by the oral route, resulted in a change of the inflammatory cells in the CNS, and this promoted low disease severity [34]. The same pattern of suppression was recently observed when DNA vaccine was administered together with Tacrolimus [52]. Also, MOG-DNA vaccination promoted expansion of regulatory T cells in the periphery and Foxp3 expression in the spinal cords of EAE mice, as well as augmented the expression of neuroprotective genes in the CNS [53].

It is of recent concern that regulatory T cells may turn into effector inflammatory cells. It was found that natural arising and periphery induced Treg cells may become Th1 and Th17 cells *in vivo* and *in vitro* [54–57]. The events that lead to this conversion are based on the stimulation of the mTOR cascade, which induces the differentiation of Th1 and Th17 cells in inflammatory and lymphopenic conditions [56]. We did not observe this effect in the treatment of ongoing EAE. In fact, our results show that regulatory T cells raised by the CQ treatment were not converted into effector T cells, even at the 30th day after disease onset, as seen by the augmentation of Foxp3 expression and the reduction in IFN- γ production. So, the treatment with chloroquine of established EAE resulted in reduction of EAE suggesting that a long-lasting immunomodulation can be achieved with this therapy. When CQ-elicited Treg cells are transferred to mice with ongoing EAE, the disease severity was reduced. The cellular response towards neuro-antigens in the periphery was contained and the pattern secretion of cytokines was altered as well. Transfer of CQ-Treg cells also reduced the infiltration of cell into the CNS, although the frequency of IL-10-producing cells was unaltered, which is distinct from the data observed with CQ treatment. The reduced dendritic cells number after CQ therapy may favor the higher suppression profile of CQ treatment over CQ-Treg cells transfer experiments. These data indicate that the amelioration of EAE after CQ treatment is a result of Treg-dependent and -independent mechanisms.

Other anti-malarial drugs are being tested in experimental models of inflammation. Recently, it was published that

artemisinin derivative, dihydroartemisinin (DHA), promoted suppression on EAE course [58]. The effects observed were dependent on Treg stimulation in the periphery. The authors showed that the mammalian target of rapamycin (mTOR) signaling cascade was attenuated in T cells [58], which also inhibits Th1 and Th17 differentiation [56]. The hypothesis that CQ treatment interferes with mTOR cascade was not investigated here. However, recent reports demonstrated that chloroquine targets the mTOR pathway in cancer cells inducing cell death [59]. We do not discard the possibility that chloroquine may influence the mTOR pathway promoting regulatory T cells and decreasing the frequency of Th1 and Th17 cells. Other studies must be conducted in order to define the precise mechanism by which chloroquine stimulates Treg cells expansion or differentiation.

It was noticed that chronic abuse of chloroquine (cumulative ingestion of over 1000 g) in rheumatoid patients results in ocular toxic effects that may lead to blindness [60–62]. However, proper control of CQ consumption (3–5 mg/kg/day) reduced the incidence of retinopathy to 0.3–2% [63–65]. The dose used in this study is acceptable within the dose range for human treatment to produce less collateral effects. But, we highlight that the treatment period in this study was brief. So, other studies must be conducted to assess the efficacy of this treatment in human multiple sclerosis.

The results presented herein indicate that chloroquine reduces the clinical course of EAE. We propose that this effect is due to the expansion of regulatory T cells in the spleen, which reduces the specific cellular response in the periphery. CQ may also affect other T cell subtypes that contribute to the reduced EAE severity. Interestingly, Treg cells also migrate to the CNS to reduce local inflammation and promote protection of the nervous system. Taken together, our data suggest that chloroquine may be a potential drug to be used as an adjunctive therapy in the treatment of multiple sclerosis.

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Author Contributions

Conceived and designed the experiments: RT LV. Performed the experiments: RT CF TAdC RDG AdSF ALB. Analyzed the data: RT ASM ALB AdSF LV. Contributed reagents/materials/analysis tools: LV LMBdS ALRdO. Wrote the paper: RT AdSF LMBdS ALRdO LV.

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Review

Chloroquine: Modes of action of an undervalued drug

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ABSTRACT

For more than two decades, chloroquine (CQ) was largely and deliberately used as first choice drug for malaria treatment. However, worldwide increasing cases of resistant strains of *Plasmodium* have hampered its use. Nevertheless, CQ has recently been tested as adjunct therapy in several inflammatory situations, such as rheumatoid arthritis and transplantation procedures, presenting intriguing and promising results. In this review, we discuss recent findings and CQ mechanisms of action vis-à-vis its use as a broad adjunct therapy.

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1. Introduction

Malaria remains the most infectious disease in the world, with one million deaths per year and over 200 million infected individuals [1]. Still, vaccine development is damped by the partial and temporary nature of naturally acquired protective immunity to malaria [2]. The search for new drugs that fight malaria parasites is of great interest since multi-resistant strains of *Plasmodium* are already spread worldwide [3]. Another approach that may result in successful treatments is the creation of drug analogs that restores susceptibility to chemotherapy [4].

Notwithstanding, over the years, it was found that some antimalarial drugs are able to modulate the immune system. In this context, chloroquine (CQ) has been used in the treatment of rheumatic diseases and in the control of inflammation [5–7]. Although the precise mechanisms are not fully understood, the administration of CQ raises the lysosomal pH and interferes with several processes within the cells [8]. CQ has also been used in the control of viral infections, although the data of antiviral actions exerted by chloroquine are contradictory [9,10]. The abuse of CQ usage promoted the development of several side effects, including sight loss [11,12].

In this review, we discuss the increasing amount of evidence suggesting that chloroquine exerts several modulatory effects on the immune system. Although malaria treatment with chloroquine is undervalued in some countries recent data points out that this drug can be modified in order to restore its antimalarial activity. The same line of investigation may be used in the therapy of rheumatic and inflammatory conditions with reduced collateral effects.

2. Chloroquine in malaria

Chloroquine was first synthesized, in 1934, by Hans Andersag while working for the German chemical and pharmaceutical company Bayer AG [13]. At that time, huge efforts were made by chemical companies to develop new antimalarial drugs such as quinine, a natural product isolated from the bark of the Chinchona tree, was not fully available [14]. Although the antimalarial properties of chloroquine were identified, CQ was still considered extremely toxic for scaling up development. Consequently, other synthetic compounds such as quinacrine (also known as atabrine), were used to treat malaria [14]. Then, in the early 40s an American Company (Winthrop Company) resynthesized chloroquine, which has shown to be more active than quinacrine and surprisingly less toxic than CQ previous formulation [13]. After pharmacokinetics trials, to establish treatment dosage [13], CQ was finally produced in large scale and recommended for the prophylaxis and treatment of all blood stage human malaria infections [15].

Indeed, CQ was for several decades the antimalarial of choice because it was effective, well tolerable, safe and low-cost [16]. Nevertheless, due its deliberative use, CQ failed to treat malaria

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10–20 years after its introduction [16]. *P. falciparum* chloroquine resistance (CQ-resistance) was first noticed at the Thai-Cambodia border in the late 1950s [16] and has already spread to the vast majority of malaria-endemic countries [17]. Today, the use of chloroquine to treat falciparum malaria is restricted to few countries in Caribbean and Central America and the World Health Organization (WHO) strongly recommends artemisinin based combination therapy (ACT) for all *P. falciparum* confirmed cases [1]. Moreover, some studies have described an increase in CQ susceptibility after this drug withdrawn from first-line treatment [18–24], indicating that in the absence of the drug-pressure it is possible to restore CQ efficacy percentage.

Chloroquine is still widely used as antimalarial in vivax malaria infections [1,25]. Its use in combination, or not, with primaquine, the unique antimalarial drug that acts in the *Plasmodium* liver stage preventing relapses, is the first choice in the majority endemic areas of *P. vivax* [1]. *P. vivax* CQ-resistance was first noticed at the Papua New Guinea in 1989 [26], almost thirty years after the emergence of *P. falciparum* resistance [16]. To date, there are also evidences of CQ-resistant *P. vivax* in many other countries, including South America [27–29]. The high prevalence of chloroquine-resistant *P. vivax* in some areas [30,31] has been hypothesized as an important contributor to the high risk of severe vivax disease [32] and contributes to the overall morbidity. Accordingly, WHO still recommends the treatment with chloroquine where this drug is effective, or an appropriate ACT in areas where *P. vivax* is resistant to chloroquine [1].

2.1. Antimalarial mode of action and resistance

Chloroquine acts exclusively in the erythrocyte stage of malaria parasites. The antimalarial mechanism of action from chloroquine has not been fully elucidated. Many theories have been developed to elucidate the chloroquine mechanism of action, including DNA binding and inhibition of various enzymes and/or transporters [13]. The most explored and accepted theory is that chloroquine interferes with the hemoglobin digestion by the parasite [33]. At the erythrocyte stage, *Plasmodium* invades the red blood cells and the parasite grows by ingesting hemoglobin from the host cell cytosol and depositing it in the digestive vacuole (DV). DV is a lysosomal isolated acidic compartment where hemoglobin is digested by the parasite. During this process, the protein moiety is degraded to related peptides and heme is transformed into an inert crystalline polymer named hemozoin (HZ) [34]. Chloroquine accumulates in the DV, where it is believed to bind hemozoin, a toxic product of hemoglobin proteolysis [35,36]; thus preventing its incorporation into the hemozoin crystal [13,37–39]. The free hemozoin seems to interfere with the parasite detoxification processes and thereby damage the *Plasmodium* membranes by lipid peroxidation mechanism [40].

There are three major hypotheses to explain chloroquine accumulation in the DV, however, the exact mechanism of action still is a matter of debate. By nature, chloroquine is a diprotic weak base and, at physiological pH, can be found in unprotonated (CQ), mono-protonated (CQ⁺) and di-protonated (CQ²⁺) forms. According to the weak base model, CQ is the only form permeable in the membrane and able to diffuse into the erythrocyte up to DV. Inside this compartment, chloroquine molecules become protonated and trapped, as membranes are not permeable to the charged forms of this drug [41,42]. The second hypothesis is based on carrier-mediated mechanism, in which a Na⁺/H⁺ exchanger (NHE) on parasite plasma membrane seemed to be involved in chloroquine uptake [43,44]. This hypothesis suggests that the plasmodial NHE mediates chloroquine uptake together with sodium in exchange for protons and have been supported by the fact that chloroquine uptake were inhibited by five specific inhibitors of eukaryotic NHE [43,44]. The

third one is known as "ferriprotoporphyrin IX receptor hypothesis". According to this hypothesis the free hemozoin acts as an intravacuolar receptor for chloroquine, thus CQ entry into DV is regulated by the amount of free hemozoin [35,36].

Chloroquine was known to exert its activity by accumulating in parasite DV and so compromising hemoglobin digestion by the parasite leading to the accumulation of toxic compounds that eventually kill the parasite. In CQ-resistant *P. falciparum* strains, chloroquine accumulates in a much-reduced degree than in sensitive ones [45,46]. Therefore, drug transport is believed to be of crucial importance for understanding the mechanism(s) of chloroquine resistance. The genetic basis of this phenomenon involves mutation in the gene named *pfcr* (*Plasmodium falciparum* chloroquine resistance transporter). The *pfcr* gene, located on chromosome 7, encodes a 49 kDa protein (PfCRT) localized in parasite's food vacuole [47] with 10 predicted transmembrane domains [48]. An amino acid substitution at position 76 from lysine to threonine (K76T), located on the first transmembrane domain, has been reported to be directly associated with CQ-resistance in 40 laboratory-adapted *P. falciparum* clones from wide geographic areas [47]. Removal of the K76T mutation, which is ubiquitous in CQ-resistant lines, results in parasites becoming fully chloroquine sensitive [49]. As well as chloroquine mode of action, how this *pfcr* mutation confers the resistant phenotype has also been subject of intense debate. Recently, Martin et al. showed that the resistant form of PfCRT expressed in *Xenopus laevis* oocytes surface are able to transport CQ, whereas the oocytes expressing PfCRT wild-type protein are not able to transport the drug. Then, these data suggested that CQ resistance is due to direct transport of the drug via mutant PfCRT [50].

Another candidate gene for CQ resistance is *pfmdr1*. This gene, located on chromosome 5, encodes a homologue of the mammalian multidrug resistance gene in *P. falciparum*, termed P-glycoprotein homolog 1 (Pgh-1) [51,52]. Several studies have demonstrated partial associations between *P. falciparum* resistance to chloroquine and mutation of *pfmdr1* gene in both laboratory and field isolates [53–56]. Although it was demonstrated by allelic exchange experiments that *pfmdr1* mutations themselves are insufficient to confer chloroquine resistance in *P. falciparum*, these mutations still play a role in modulating levels of resistance [53,54,57]. Therefore, it was proposed that the increased frequency of *pfmdr1* polymorphisms in CQ-resistant parasites could reflect physiological compensation for the altered function of mutant *pfcr* [58].

2.2. The current scenario in antimalarial treatment

Although ACT are the most effective therapy to treat malaria falciparum to this point, delays in parasite clearance and isolated cases of treatment failure has been recently reported in parts of Thailand, Cambodia, Myanmar and Vietnam [59]. Therefore, it is still important to seek new strategies for the treatment of malaria and accordingly reversal of resistance to old drugs remains one acceptable strategy to deal with this problem.

Verapamil was found to reverse chloroquine resistance in 1987 [60], since these observation more than 40 compounds have been identified as CQ-resistance reversers [61,62]. Verapamil seems to interfere with the chloroquine efflux through the mutant PfCRT, then maintaining sufficient CQ levels inside the food vacuole to kill the parasite. The antimalarial primaquine and other compounds such as xanthene derivatives, an isomer of chlorpheniramine, the natural product vocamine, (diphenylmethyl)piperazines and fluoxetine were reported to reverse chloroquine resistance *in vitro* [4]. Interestingly, some antiretroviral protease inhibitors such as saquinavir [63] are also able to revert chloroquine resistance and saquinavir activity in reversing the resistance is probably due to the inhibition of chloroquine resistance transporter (mutant PfCRT)

[63]. Indeed, the fact that antiretroviral agents are able to revert CQ resistance may have significant implications for the treatment of HIV- and malaria parasite co-infected patients.

However, none of CQ-resistance reversers have already been investigated in clinical trials. Another approach to deal with antimalarial resistance might be the re-designing of available drugs. Some chloroquine analogs have been developed to restore the antimalarial activity. These include compounds with shortened side chains [64,65] or with substantially altered side chains [66], but none of these compounds has been clinically used so far.

3. Chloroquine in non-malaria conditions

Although chloroquine-resistant parasites were found, and the use of this drug is not indicated for malaria treatment in several countries, over the years CQ was found to have immunomodulating properties that are being tested both in experimental models and in humans. Indeed, there is an increase body of information pointing to chloroquine anti-inflammatory activity by mechanisms pH-dependent or independent.

3.1. Chloroquine effects in lysosomes and endosomes, and on cell death

As a weak base, chloroquine lipophilic unprotonated form (CQ) diffuses passively toward the lysosomes, where it becomes protonated and is retained by ionic trapping [67–69]. Upon chloroquine treatment lysosome pH of murine peritoneal macrophages is augmented [70] as unprotonated CQ accumulates inside lysosomes. However, due to the action of ATPase proton pumps, the drug becomes protonated and unable to leave this vesicle. Inside the lysosome, CQ sequesters protons leading to increase in pH, which interferes with most of the enzymes inside this vacuole [8,71,72]. CQ is also able to inhibit lysosomal proteases, such as Cathepsin B [70].

Moreover, it was found that chloroquine may interact with the FAD-linked enzyme Quinone Reductase 2 (NQO2) [73], which is ubiquitously expressed in human cells, and is related to the stabilization of p53 tumor suppressor [74]. It is believed that chloroquine competes with the quinone substrate of this enzyme [75]. The physiological function of NQO2 is still unclear, but it has been implicated in apoptosis and cancer [76,77]. Accumulation of chloroquine inside lysosomes interferes with several mechanisms, especially those related to antigen processing. In fact, lysosome dysfunction leads to cell debris accumulation and toxicity [78]. Lysosomes of CQ-treated cells eventually are found "leaking" substances into the cytosol, which might be facilitated by ATPases proton pumps [79]. The leaking proteins may become ubiquitinated in the cytosol and then, enhancing major histocompatibility complex (MHC) class I cross-presentation by antigen presenting cells [80]. Therefore, administration of chloroquine promotes an increase in ubiquitinated proteins in cytosol that will be degraded in the proteasomes. However, ubiquitinated proteins in excess exert cytotoxic effects [81]. Chloroquine is also capable of inducing apoptosis on jurkat cells through the acidification of the cytoplasm [82]. The mechanism of cell death induced by chloroquine is still obscure, however, it seems that accumulation of ubiquitinated proteins or augmented lysosomal permeability may be related to cell death rather than apoptosis, autophagy or oxidative stress [83].

3.2. Chloroquine, autophagy and immune response

Autophagy is an important cellular mechanism developed to degrade aged and dysfunctional proteins and organelles, as well as a mean to overcome lack of nutrients during starvation [84] and

it is also involved in eliminating some microorganisms [85,86]. This process occurs when autophagy vacuole (AV) fuses with lysosomes, resulting in protein and organelles destruction [87]. Disruption of proteins inside of lysosomes favors the creation of peptides that bind to class II MHC molecules in antigen presenting cells (APC) that will further activate antigen-specific CD4+ T cell. Autophagy also leads to peptides capable of binding to class I MHC present in all nucleated cells enhancing CD8+ T cell-mediated cytotoxic response against viruses and tumors [88]. Generation of small peptides that fits into MHC-I groove is dependent on protein ubiquitination and its degradation inside proteasome [89]. As previously discussed, chloroquine treatment increases the levels of ubiquitinated protein [80], resulting in increased protein degradation by immunoproteasomes, which favors the production of tumor-specific peptides that enhance anti-tumor immune responses.

Tumors, in turn, may use autophagy to retrieve antigens presented in the MHC-I, to escape from host immune responses [87]. A recent study demonstrates that autophagy creates epitopes that are recognized by cytotoxic T cells [88], which is highly inhibited by the addition of CQ. This process indicates that CQ interferes with the generation of CD8+ T cell mediated anti-tumor responses, by impairing MHC-I epitope production by APC [88]. Indeed, THP-1 macrophages treated with CQ also showed impaired capacity to eliminate the intracellular bacteria *Francisella tularensis* through autophagosomes [86].

Despite the studies demonstrating that chloroquine interferes with the elimination of intracellular pathogens and autophagy-dependent generation of MHC-I peptides, it was previously shown that the administration of CQ co-administered with phorbol 12-myristate 13-acetate (PMA) facilitates cross-presentation of exogenous proteins by dendritic cells (DCs) to CD8+ T cells [80], indicating that PMA acts synergistically with CQ in DCs. Moreover, whereas CQ promotes leaking of proteins from the lysosomes and reduction of protein degradation in the endosomes, PMA stimulates the engulfment of antigens inwards the cells. In fact, the use of hydroxychloroquine (HCQ), an autophagy inhibitor, together with an immune stimulating interleukin (IL-2) increases eradication efficiency of tumors in mice [87].

Among the immune-modulatory actions of chloroquine, it has been described that CQ inhibits toll-like receptor signaling cascade by reducing secretion and production of inflammatory cytokines [78,90]. CQ is able to suppress immune activation induced by CpG treatment [91] and interferes in the production of IL-6, IL-1 and tumor necrosis factor- α (TNF- α) by macrophages [92–94]. Important, CQ not only reduces the secretion of TNF- α but also suppresses the expression of the TNF- α receptor in human histiocytic U-937 cells [95]. These effects are both pH-dependent and pH-independent, since CQ administration alters the iron metabolism in macrophages and calcium signals in T cells [96–99]. Also, CQ was found to selectively regulate IL-1 and IL-6 secretion by macrophages and T cells [100]. Thus, CQ capacity to reduce secretion of pro-inflammatory cytokines to whole blood [101] predisposes its use in the prevention of sepsis [102]. Nevertheless, although chloroquine possesses recognized anti-inflammatory properties little is known about the molecular mechanisms of immune-modulation exerted by the drug.

Recently, it was found that CQ administration provokes a G1-arrest in cell cycle *in vitro* in human mammary cancer cell line MCF10A [103]. It was observed that upon CQ treatment, the levels of p53 and p51 were up regulated and the phosphorylation of Akt, also known as protein kinase B (PKB), was increased. These results suggest that CQ exerts an anti-cancer effect partially due to the alterations in the IGF-1/PI3K/Akt/mTOR and p53 signaling pathway, the major signals that regulate postnatal growth of skeletal muscles and promote protein synthesis [103].

3.3. Chloroquine and the control of viral infections

Although chloroquine was developed as an antimalarial drug, several other processes are altered by CQ administration, including the suppression of rheumatic diseases and interference with viral infections. For instance, chloroquine was shown to inhibit the infection by influenza type A and type B and adenovirus in target cells [104–107]. It has been demonstrated that CQ inhibits the replication of the human viruses H1N1 and H3N2 [107] as well as the avian virus H5N9 [108]. As the infection by the pH-resistant virus H7N3 could not be abolished after chloroquine administration, these findings demonstrate that CQ inhibits viral replication by pH-dependent mechanisms. Moreover, CQ reduces monocyte infection by the single-stranded DNA non-enveloped virus PCV2 (porcine virus 2), however, infectivity on epithelial cells was enhanced [109]. Indeed, CQ is capable of inhibiting infection process, but in previously infected cells the viral replication is unaffected [108]. However, chloroquine may present other mechanisms of antiviral action that are pH-independent, such as the inhibition of polynucleotidyl transferases like HIV-1 integrase [110].

In HIV infection, chloroquine has gained special attention due to exciting results achieved when the drug is used both in mono or combined therapy. In clinical trials, HIV-infected patients treated with chloroquine monotherapy presented reduced IL-6 and total viral load in sera, corresponding to a better prognostic [9,10,111]. Treatment with CQ reduced the numbers of circulating memory CD8⁺ T cells, which are correlated with disease progression in HIV patients [112] and do not alter immune response in these individuals since the levels of TNF- α , IFN- γ and IL-2 remained unchanged compared to the placebo-treated group [112]. Combined with HCQ treatment (400 mg/day for six months), CQ provoked an increase in plasmacytoid dendritic cells (pDCs), important cells in the control of viral infections in HIV-infected patients [113]. The number of circulating pDCs is reduced in HIV patients [114], resulting in a decreased number of activated pDCs and reduction in type I interferons secretion. CQ also blocked the expression and action of immune-modulatory molecules secreted by pDCs such as Indoleamine 2,3 dioxygenase [115] and inhibits HIV-1 replication inside monocytes and T lymphocytes [104]. Furthermore, CQ reduces the Tat-mediated HIV-1 LTR transactivation *in vitro*, reducing the production of new viruses [116]. On the other hand, the alkalization of the lysosomes of 293T and HeLa Magi cells increased the infectivity by the virus [117].

3.4. Chloroquine in the treatment of autoimmune diseases and inflammation

CQ has been used as adjunct therapy in rheumatic diseases, and recently has been indicated for the treatment of systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) [118,119].

Systemic lupus erythematosus (SLE) is an autoimmune disease that affects multiple organs in the body [120]. The manifestation of clinical symptoms varies among affected individuals avoiding a proper classification. SLE is characterized by the production of IgG autoantibodies that target nuclear constituents [121,122]. Clinical manifestation often includes skin rash, joint pain and severe and progressive kidney involvement (glomerulonephritis) and treatment usually resides with the use of cytotoxic drugs like cyclophosphamide, although experimental trials using monoclonal antibodies has showed promising results [120]. However, several reports have showed that chloroquine and hydroxychloroquine prevented clinical flare-ups and organ damages, increases survival rates and reduces vascular damages [123–127]. Hydroxychloroquine can be administered during pregnancy [128]. It was previously demonstrated that the administration of CQ inhibited angiogenesis and reduced blood vessels in the dermis of patients

with discoid lupus erythematosus [129]. HCQ was revealed to be a good therapeutic approach for the treatment of cutaneous lupus, improving skin lesions of the patients [130,131]. Chloroquine is able to reduce the signs of lupus in epidermal lesions and joints [132]. A recently retrospective study conducted in Japan revealed that HCQ treatment was able to reduce the clinical symptoms of SLE without the development of side effects [133].

Rheumatoid arthritis (RA) is an autoimmune disease that affects the joints and articular cartilage, with overall incidence of about 1% of the general population [134–136]. RA differs from SLE in many characteristics, but mostly in damage caused to the joints is orchestrated by the cellular immune responses, mainly T cells and macrophages-osteoclasts [137–141]. Chloroquine has also been employed with relative success in the treatment of RA as a second-line drug and provoked less toxicity than methotrexate, one of the most used drugs in therapy [142]. The efficacy of HCQ treatment on RA patients was assessed previously in a 36-week randomized double-blind study [5]. The authors found that HCQ treatment (400 mg/day) reduced the infiltration in the joints as well as the general pain and increased the physical function of the patients. No side effects were noticed after 36 weeks of treatment, allowing the use of CQ and HCQ as anti-rheumatic drugs [143–146].

In our laboratory we aimed to assess whether chloroquine could be used to treat experimental autoimmune encephalomyelitis (EAE), a disease in mice that mimics human multiple sclerosis. We found that CQ administration provoked a decrease in EAE severity, and this was accompanied by reduced infiltration of inflammatory cells in the central nervous systems. The treatment can be used both in prophylactic and therapeutic approach [147].

3.5. Chloroquine improving transplantation

Albeit being used in rheumatic diseases, chloroquine has proven to be a great asset in the manipulation of the immune response observed in graft-versus-host disease (GVHD), the most frequent cause of morbidity and mortality after transplantation [7,148]. CQ treatment improves GVHD, by inhibiting IL-2 and IL-4 secretion by CD8 T cells, interferes in allo-reactivity and affects the T cell priming to minor MHC complexes [149–152].

4. Ocular toxicity related to chloroquine (Ab)use

Since 60s, the ocular toxicity effect of chloroquine is known [12,153]. Together with ocular toxicity, the prolonged ingestion of chloroquine (500 mg/day for up to ten years) results in the development of undesirable side effects like dry mouth, fatigue, and loss of appetite and weight. Elder individuals (~67.4 years) under chronic chloroquine therapy (~8.4 years) are at risk for retinal toxicity [154]. A follow up study on 1207 patients with retinal toxicity caused by chloroquine treatment indicated that daily doses significantly contribute to the ocular damage [155], thus considered as a key factor for retinal toxicity [156]. Ingestion of doses higher than 3 mg/kg/day of CQ for over five years predisposes to ocular damage [157]. In late 80s, about 20% of the chloroquine users developed ocular damage due to high doses and treatment frequency [158]. In Japan, chloroquine-associated retinopathy became a social problem and the manufacture and sale of CQ in this country ended in 1974 [133].

Ocular toxicity observed in chloroquine treatment is associated with disruption of the blood retinal barrier (BRB) [159] and the continuous use of antimalarials resulted in an incidence of retinopathy of 0.3–2% [160–162]. So, the ocular fundus examination or color vision test of patients under chloroquine therapy is indispensable to detect retinal toxicity at a stage in which it is still reversible after treatment interruption [156,157]. In this context, the use of

hydroxychloroquine seems to be less toxic to the retina. Rheumatoid arthritis patients under CQ treatment (~11 years therapy and total consumption of 1000 g) developed retinopathy, whereas those under HCQ treatment (~5 years therapy and total consumption of 450 g) did not [159]. The rise in lysosome pH from cells of the retinal pigment epithelium (RPE) favors the accumulation of lipofuscin, which may lead to the retinal toxicity [67]. Lipofuscin is a complex polymer made of peroxidized lipids and protein residues. The age-related macular degeneration (AMD) is the main cause of progressive sight loss in elderly. It is believed that overload of lysosomal function in retinal pigment epithelium might be related to the development of AMD. ARPE-19 cells, an established line of retinal pigment epithelium cells, treated with chloroquine presented vacuoles formation, cellular death, cytosolic lipid accumulation and reduction in phagocytosis [83,163]. CQ administration promoted a drastic increase in LAMP1 expression in ARPE-19 cells. The raise in LAMP-1 expression is correlated with initiation of apoptosis [164]. As chloroquine has a tropism toward lysosomes, the use of Bafilomycin A1, an H⁺-ATPase pump blocker inhibits lysosomes acidification, reducing vacuole formation and death of ARPE-19 cells [83].

5. Take-home messages

Chloroquine has a natural tropism for lysosome, leading to increased pH inside it and to malfunction of vesicle function. Although chloroquine has an anti-inflammatory activity, when used in association with immune stimulators, the drug may enhance immune responses. The adoptive transfer of CQ-treated cells may become a useful tool to modulate undesired immune responses without the side effects observed in CQ treatment.

6. Conclusion

Chloroquine, an antimalarial drug, can also be used in the manipulation of the immune system. In this review, we collected data that demonstrate the multitude of actions displayed by CQ, mostly dependent on lysosome dysfunction promoted by pH increase, although pH-independent mechanisms also exist. The administration of the drug was shown to reduce overall inflammation in several conditions with little toxicity. However, new studies are needed to precisely determine chloroquine mechanism of action and to develop strategies to be used in the clinic.

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ORIGINAL ARTICLE

Dendritic cells treated with chloroquine modulate experimental autoimmune encephalomyelitis

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Chloroquine (CQ), an antimalarial drug, has been shown to modulate the immune system and reduce the severity of experimental autoimmune encephalomyelitis (EAE). The mechanisms of disease suppression are dependent on regulatory T cell induction, although Tregs-independent mechanisms exist. We aimed to evaluate whether CQ is capable to modulate bone marrow-derived dendritic cells (DCs) both phenotypically and functionally as well as whether transfer of CQ-modulated DCs reduces EAE course. Our results show that CQ-treated DCs presented altered ultrastructure morphology and lower expression of molecules involved in antigen presentation. Consequently, T cell proliferation was diminished in coculture experiments. When transferred into EAE mice, DC-CQ was able to reduce the clinical manifestation of the disease through the modulation of the immune response against neuroantigens. The data presented herein indicate that chloroquine-mediated modulation of the immune system is achieved by a direct effect on DCs and that DC-CQ adoptive transfer may be a promising approach for avoiding drug toxicity.

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Dendritic cells (DCs) are antigen-presenting cells that have an important role in activation and modulation of naïve T lymphocytes.^{1–3} The modulation of T cell functions occurs during antigen presentation, where DCs provide three signals: first, epitope-major histocompatibility complexes are recognized by the T cell receptor; second, co-stimulatory signals sent by the interaction of DCs' CD80 and CD86 with CD-28 expressed by T cells; third, the cytokine milieu created by DCs and other cells in the microenvironment.^{4,5} The combination of these signals leads to T cell differentiation into Th1, Th2, Th17 and Th9 effector CD4⁺ T cells.^{4,6–8} The functional and phenotypical plasticity of DCs is dependent on the activation/maturation status of these cells turning them an interesting target for therapy studies.^{9–11} Indeed, the adoptive transfer of *in vitro*-modulated DCs was able to reduce the severity of collagen-induced arthritis, diabetes and experimental autoimmune encephalomyelitis (EAE).^{12–16}

Chloroquine (CQ), an antimalarial drug, has been used as an anti-inflammatory agent in the control of rheumatic conditions.^{17–19} Although the mechanisms are not fully elucidated, CQ acts as a weak base and passively diffuses through the cytoplasm toward the lysosomes where it is retained by ionic trapping.²⁰ It was previously demonstrated that treatment of DCs with CQ inhibits antigen processing and presentation *in vitro* in a pH-dependent manner.^{21–23} An increasing amount of evidence suggests that CQ also exerts its

effects through pH-independent mechanisms.^{24–27} Recently, we showed that CQ administration ameliorates the clinical and immunological parameters of EAE, a model for the human multiple sclerosis.²⁸

EAE is established after neuroantigen-specific effector T cells breaks in the central nervous system (CNS) of mice.²⁹ In the CNS, effector T cells secrete a myriad of molecules that activates resident microglia and recruits inflammatory leukocytes ultimately leading to demyelination.³⁰ The clinical sign of the EAE start as paw weakness and progressively aggravate as the damage to the myelin sheath increases, resulting in death.³¹ Therefore, the cellular immune response in EAE must be restrained. In this context, DCs may be modulated toward an anti-inflammatory profile. Our previous results with CQ administration in EAE have indicated that both Treg-dependent and -independent mechanisms are responsible for disease suppression.²⁸ We hypothesize that CQ also interferes with dendritic cell maturation thus reducing the cellular response against neuroantigens.

In this study, we evaluated the effect of CQ administration to DCs. Our results showed that CQ treatment promotes alterations in the expression of molecules related to antigen presentation as well as morphological and functional changes after *in vitro* antigen stimulation. We also observed that adoptive transfer of CQ-modulated DCs ameliorated the clinical course of EAE by the downregulation

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of the immune response toward neuroantigens and reduction in the glial reactivity and local inflammation. Collectively, our data suggest that CQ exerts its suppressive effects by interfering with the function of DCs and that adoptive transfer of CQ-treated DCs may be an interesting approach for the modulation of inflammatory conditions, such as EAE.

RESULTS

CQ treatment modulates bone marrow-derived DCs *in vitro*

The expression of major histocompatibility complex class II molecules was significantly reduced in cultures of DCs treated with CQ (Figure 1a). Similarly, the expression of CD80 and CD86 molecules on DCs was reduced after the treatment with the drug at higher concentrations—ranging from 25 to 100 μM (Figure 1a). The morphology of DCs after microbial challenge was altered in cells treated with the drug compared with PBS-treated controls, such as reduction on absolute size and dendropoiesis (Figure 1b). High CQ concentrations exert toxic effects *in vivo*; in order to evaluate the possible cytotoxic action of the drug, DCs were cultivated in the presence of CQ at several doses. As shown in Figure 1c, the treatment with CQ did not reduce the viability of DCs.

For functional analysis, DCs were treated with CQ, dexamethasone or PBS (DC-CQ, DC-Dexa and DC-PBS, respectively) and cultivated with CD4⁺ T cells in the presence of MOG₃₅₋₅₅ peptide. The results

showed that T lymphocytes cultivated in the presence of DC-CQ proliferated significantly less compared with cells cultivated with other DCs (Figure 2a). It was previously observed that CQ administration promotes an increase in the expression of nitric oxide synthase (NOS) in several cell types, interfering with renal function and other NO-dependent mechanisms.^{32–34} However, in macrophages, CQ treatment reduced the NO synthesis.^{35,36} There is lack of evidences about NO production by CQ-treated DCs. In order to solve this question, DCs were cultivated in the presence of increasing doses of CQ and NO secretion was measured later. Surprisingly, the results showed that there was a dose-dependent augmentation in NO production by DCs (Figure 2b). The next goal was to determine whether the higher production in NO was directly influencing the T cell proliferation. Thus, DCs were treated with CQ and cultivated with CD4⁺ T cells in the presence of a NOS inhibitor, L-NAME. The data obtained show that T cells cultured with DC-CQ proliferated significantly less than cells cultivated with DC-PBS; however, the treatment with L-NAME restored the proliferative response of T cells from DC-CQ mixed cultures (Figure 2c).

Transfer of CQ-modulated DCs ameliorates EAE

The data presented here show that DCs are modulated by CQ *in vitro*. The therapeutic usage of CQ-modulated DCs was investigated as well. For that purpose, PBS- and CQ-treated DCs were adoptively

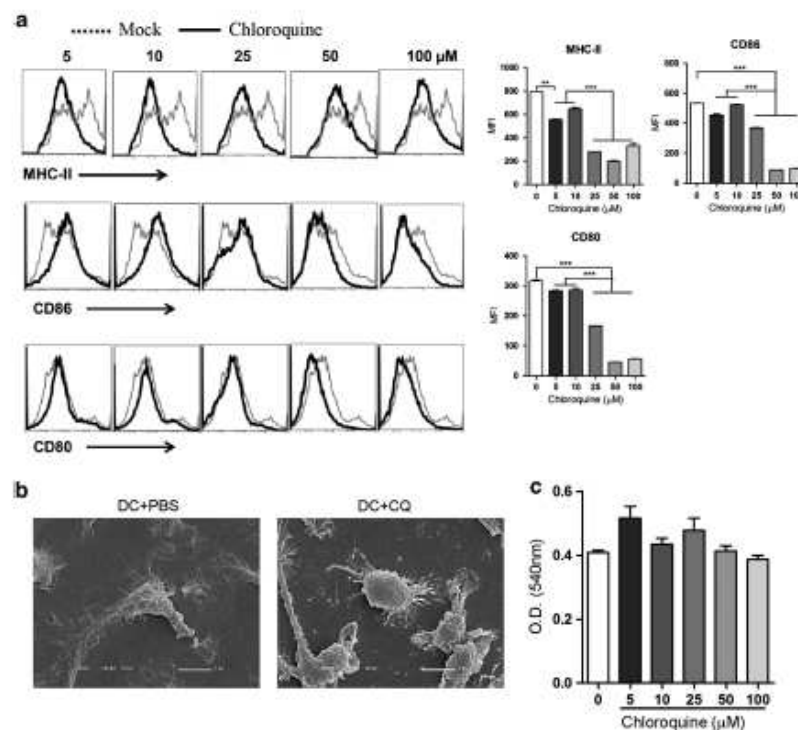


Figure 1 CQ treatment provokes phenotypical alterations in DCs. Bone marrow-derived DCs were treated with distinct CQ doses and stimulated with LPS ($1 \mu\text{gml}^{-1}$) for 18 h. (a) The mean fluorescence index (MFI) of MHC-II, CD86 and CD80 was reduced in CQ-treated cells (solid line) compared with the PBS-treated ones (dotted line); ** $P < 0.01$ and *** $P < 0.001$. (b) Scanning electron microscopy analysis showed that the treatment with CQ promoted alterations in the structure of DCs. The figures are representative of three independent experiments. (c) The toxicity of CQ was also evaluated by MTT assay.

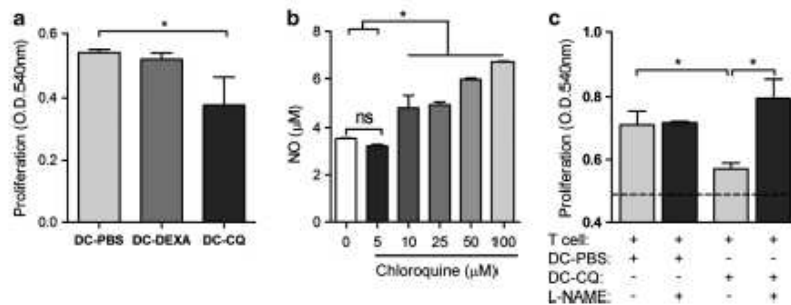


Figure 2 CQ treatment provokes functional alterations in DCs. Bone marrow-derived DCs were treated with CQ and stimulated with LPS (1 ng ml^{-1}) for 18 h. (a) DCs were treated either with dexamethasone (DC-Dexa, $50 \mu\text{M}$) or CQ (DC-CQ, $50 \mu\text{M}$) and stimulated with LPS (1 ng ml^{-1}) and then pulsed with MOG₃₅₋₅₅ peptide ($10 \mu\text{g ml}^{-1}$) for 18 h. CD4⁺ T lymphocytes were isolated from spleens of naïve mice using magnetic beads and cocultured with DCs (1:1) for 96 h in the presence of the antigen. The proliferation was evaluated by MTT. (b) The production of nitric oxide was determined by adding Griess reagent on culture supernatants from DCs treated with CQ. (c) DCs were treated with CQ (DC-CQ, $50 \mu\text{M}$) and stimulated with LPS (1 ng ml^{-1}) and then pulsed with MOG₃₅₋₅₅ peptide ($10 \mu\text{g ml}^{-1}$) for 18 h. CD4⁺ T lymphocytes were isolated from spleens of naïve mice using magnetic beads and cocultured with BMDCs (1:1) for 96 h in the presence of the antigen and an NOS inhibitor (L-NAME, 1 mM). The proliferation was evaluated by MTT. Data are representative of three independent experiments. * $P < 0.05$.

transferred to naïve mice and the development of EAE was monitored. EAE was induced by immunization of mice with MOG₃₅₋₅₅ peptide in an inflammatory context. Mice recipient of DC-PBS presented reduction of body weight and development of severe EAE, whereas the transfer of DC-CQ was capable to reduce the clinical signs of the disease (Figures 3a and b, respectively). In all experiments, DC-CQ-recipient mice presented lower cellular infiltration in the CNS in comparison with DC-PBS transferred and untreated EAE-inflicted mice (Figure 3c). To assess the presence of MOG-reactive cells in the periphery, EAE mice were killed and spleen cells were cultivated in the presence of MOG₃₅₋₅₅ peptide and whole myelin basic protein. The cells were cultivated in the presence of ovalbumin as a control of an unrelated antigen. The results showed that spleen cells from DC-CQ-recipient EAE mice proliferated significantly less compared with the DC-PBS transferred and untreated EAE-inflicted mice in the presence of neuroantigens; however, there were no statistical differences between groups regarding the proliferation against ovalbumin (Figure 3d).

Transfer of CQ-modulated DCs reduces the glial reactivity in the CNS

The transfer of CQ-modulated DCs reduced the clinical course of EAE and was correlated with the lower T cell responsiveness in the spleen. Inflammation in the CNS results in neurodegeneration. The infiltration of inflammatory cells in the CNS of DC-CQ + EAE mice was found reduced compared with the control groups, DC-PBS transferred and untreated EAE mice (Figure 3c). Gliosis is the result of the activation of the glial cells, comprising astrocytes, oligodendrocytes and microglia (Iba-1⁺ cells) after microenvironmental disturbances. The activation of astrocytes during inflammatory conditions favors the tissue microglial response, which ultimately leads to neuronal death.³⁷ Astrocytes express the glial fibrillary acidic protein (GFAP) upon stress. Due to its importance as a marker for glial reactivity, the presence of GFAP⁺ cells was analyzed in the CNS of EAE mice. The results showed that untreated and DC-PBS-transferred EAE-inflicted mice had significantly higher frequency of GFAP⁺ cells compared with the naïve group, whereas the transfer of DC-CQ was able to reduce the overall activation of astrocytes (Figure 4). Interestingly, the frequency of Iba-1⁺ cells was found significantly upregulated in both treatment groups compared with the

naïve and untreated EAE, although the Iba-1 expression in the CNS from DC-CQ-treated mice was significantly reduced compared with the DC-PBS group.

Transfer of CQ-modulated DCs alters the gene expression in the CNS of EAE mice

As adoptive transfer of CQ-modulated DCs was capable of reducing the clinical manifestation of EAE and as mice recipient of DC-CQ presented lower cellular infiltration in the CNS as well as reduced activation of glial cells, the gene expression of inflammatory mediators was investigated. The results indicated that although the expression of TGF- β and IFN- γ was not altered between groups, the levels of IL-6 and IL-17 were found significantly reduced in CNS tissue from mice recipient of CQ-modulated DCs compared with EAE groups, both untreated and DC-PBS transferred. There was also an increase in the expression of IL-10 in the spinal cords of DC-CQ-recipient mice compared with all other groups (Figure 5). The expression of FOXP3 in CNS from DC-CQ-transferred EAE mice was found significantly elevated compared with healthy mice. Collectively, the data presented here show that the reduction in clinical outcome of EAE correlated with the downregulation of inflammatory mediators in the CNS and in the periphery of the immune system.

DISCUSSION

The development of new approaches in the treatment of autoimmune diseases is a common feature in today's research. It is known that autoimmune diseases arise in uncontrolled inflammatory responses, and that loss of self-tolerance is a key step in this process. Therefore, therapies that restore either the control of inflammation or the tolerance to self are desired. In this context, DCs are an interesting target, because they can stimulate the generation of Tregs and control inflammation.^{3,38} The present study shows that transfer of CQ-modulated DCs reduces the clinical signs of EAE.

CQ, an antimalarial drug, has been used in the control of rheumatic diseases as well.^{39,40} The mechanism of action resides in the fact that CQ is a weak base that passively diffuses toward acidic organelles, like the lysosomes.^{41,42} The rise of pH interferes with the function of several enzymes and metabolic processes within the cell.^{43,44} However, pH-independent mechanisms also occur, as

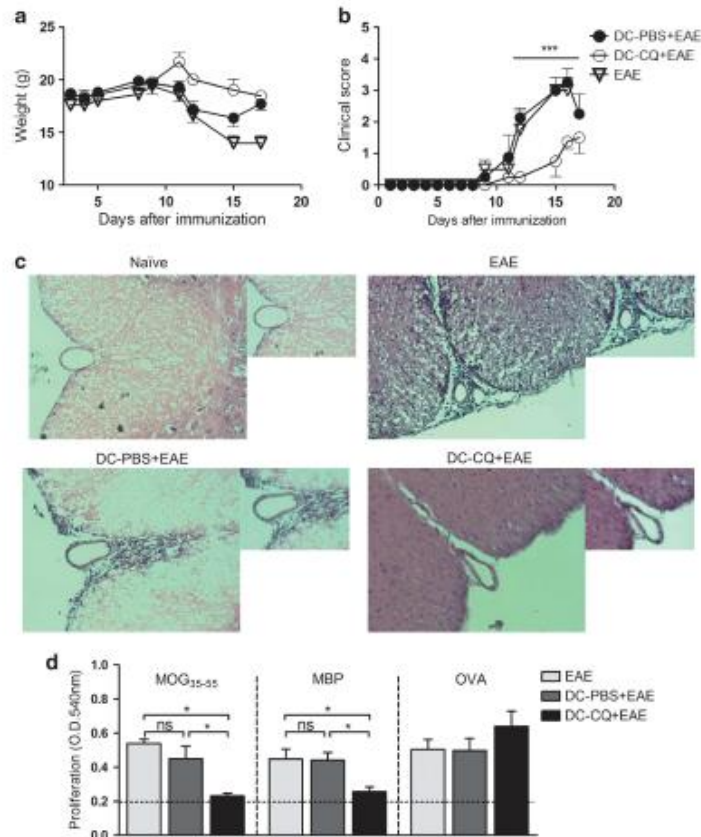


Figure 3 Adoptive transfer of CQ-treated DCs ameliorates EAE. DCs were treated with CQ ($50 \mu\text{M}$) and LPS (1 ng ml^{-1}), and then pulsed with MOG₃₅₋₅₅ peptide ($10 \mu\text{g ml}^{-1}$) for 18 h and adoptively transferred (1.5×10^6 cells per mouse) into C57BL/6 mice ($n=6$ per group) before the induction of EAE. (a) The weight variations were evaluated daily. (b) The clinical score of DCs-transferred and untreated EAE-inflicted mice was evaluated as well; *** $P<0.001$. (c) The histological analysis of the spinal cords from DC-CQ-recipient mice showed mild infiltration of inflammatory cells than in DC-PBS recipient and untreated EAE mice. (d) The spleens of naive, EAE and DC-transferred EAE (both DC-PBS and DC-CQ) mice were collected and total leukocytes (5×10^5 per well) were cultured in the presence of MOG₃₅₋₅₅ peptide, Myelin basic protein and Ovalbumin ($10 \mu\text{g ml}^{-1}$, $50 \mu\text{g ml}^{-1}$ and $50 \mu\text{g ml}^{-1}$, respectively) for 96 h. The proliferation was analyzed using the MTT method; * $P<0.05$. Data are representative of three independent experiments. Magnification of histological sections: $\times 200$ (main) and $\times 400$ (details).

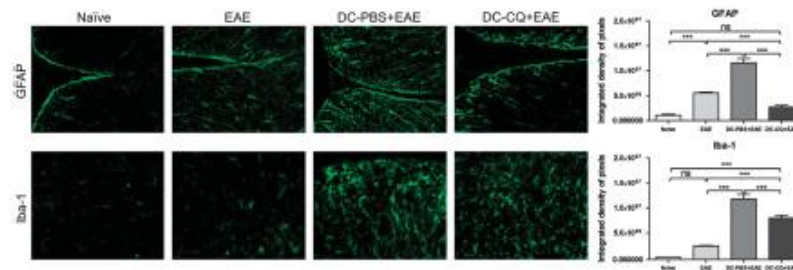


Figure 4 Microglia and astroglial response in EAE mice after DC transfer. DCs were treated with CQ ($50 \mu\text{M}$) and LPS (1 ng ml^{-1}), and then pulsed with MOG peptide ($10 \mu\text{g ml}^{-1}$) for 18 h and adoptively transferred (1.5×10^6 cells per mouse) into C57BL/6 mice ($n=6$ per group) before the induction of EAE. The spinal cords of EAE mice were collected 14 days after immunization. Frozen thin sections ($12 \mu\text{m}$) were made, fixed in formalin and the reactivity sites were blocked with PBS-BSA 3%. GFAP and Iba-1 primary antibodies were added and treated with secondary antibodies conjugated with Cy2. The sections were analyzed in epifluorescence microscope. The images were analyzed by ImageJ software and the integrated density of pixels was determined. *** $P<0.001$. Figures are representative of three independent experiments. Magnification: $\times 200$.

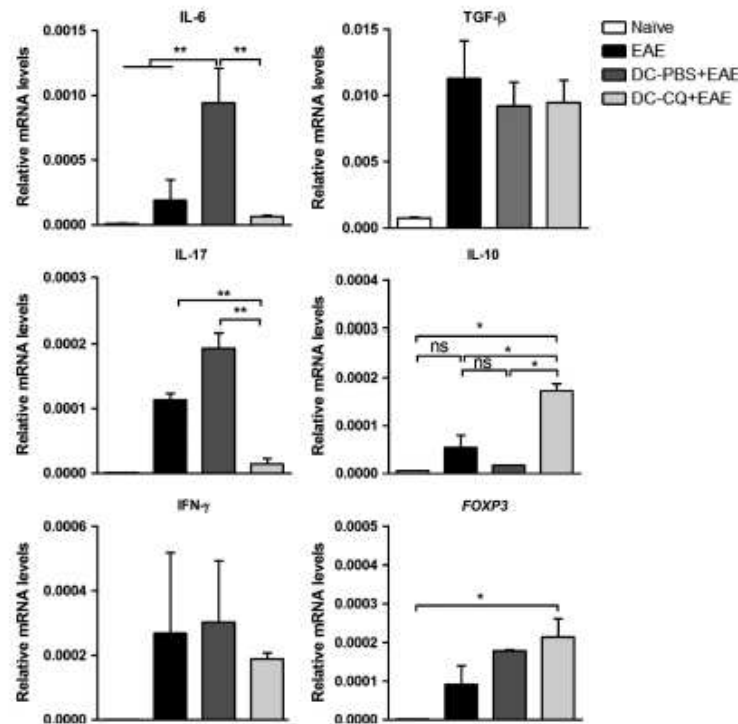


Figure 5 Altered gene expression of inflammatory mediators in CNS tissue of DC-transferred mice. Mice received PBS- and CQ-treated DCs (1.5×10^6 cells per mouse) 3 days before EAE induction. The CNS tissue (brain and spinal cord) was removed at the peak of the disease. RNA was extracted, converted to cDNA and the relative messenger RNA expression of IL-6, TGF- β , IL-17, IL-10, IFN- γ and FOXP3 was determined by reverse transcriptase-PCR reactions in naive, EAE and DC-transferred EAE (both DC-PBS and DC-CQ) mice. * $P < 0.05$ and ** $P < 0.01$. Results are expressed as mean $\pm$ standard error mean (s.e.m.) for at least four animals from each group from two independent experiments.

the treatment with CQ interferes with the iron metabolism in macrophages.²⁷ The treatment of DCs with CQ was shown to inhibit processing of antigens, which is pH-dependent.⁴⁵ Interestingly, CQ administration favors the cross-presentation by DCs and improves the development of adaptive immune responses toward ovalbumin.²² We observed that the treatment of DCs with CQ reduced the expression of molecules related to antigen presentation. Likely, the treatment promoted ultrastructural changes in the morphology of the cells. These observations suggest that the drug has an anti-inflammatory effect. The lysosome dysfunction promoted by CQ facilitates accumulation of cell debris and leads to cell death, especially cells of the retinal pigment epithelium.^{41,46} We found that CQ, in the doses tested in this study, has no toxic effect on DCs.

The maturation status of DCs defines the profile of activation of T cells.⁴⁷ Mature DCs express higher levels of class II major histocompatibility complex and co-stimulatory B7 molecules, whereas the immature/semi-mature phenotype produces intermediate expression of these molecules.¹¹ CQ does change the profile of DC maturation phenotypically. Functionally, mature DCs stimulate the differentiation of Th1, Th17 and Th2 T cells.⁴⁸ Semi-mature DCs induce the differentiation of naive T cells in regulatory T cells by differentiated co-stimulation.⁴⁷ Failure in antigen presentation leads to improper activation of T cells. We observed that CQ-modulated DCs failed to induce proliferation of CD4⁺ T cells *in vitro*. One could

argue that DCs, under the effect of CQ, do not process antigens. However, in these set of experiments, DCs were pulsed with protein peptide, which overcomes the need for lysosome-dependent antigen degradation.

Previous reports showed that CQ stimulates the expression of NOS isoforms.³²⁻³⁴ Although NO is believed to aggravate neuroinflammation,⁴⁹ its role as an immunomodulator has gained attention. It was previously demonstrated that NO blockade favors the development of antigen-specific response toward *Listeria monocytogenes* and mycobacterial infection.^{30,51} It was also shown that EAE is exacerbated in the absence of NO.⁵² Conversely, nitric oxide stimulates the generation of Tregs while suppressing IL-17-producing cells.^{52,53} In this context, the existence of TNF/iNOS-producing 'Tip'-DCs has been demonstrated.⁵⁴ These DCs are responsible for determinant spread in EAE, and are believed to have an important role in neuroinflammation.⁵⁵ Nevertheless, Tip-DCs seem to limit the expansion of autoreactive T cells in experimental autoimmune myocarditis.⁵⁶ Thus, the dual role of Tip-DCs must be better characterized in autoimmune conditions. Our results showed that CQ stimulated NO production by DCs in a dose-dependent manner and produced high amounts of TNF- α after lipopolysaccharide stimulation (data not shown), which may classify these cells as Tip-DCs. NO production, in our analysis, seemed to have a role in the stimulation of T cell proliferation. We

observed that the suppressive effect of CQ-modulated DCs was abolished after L-NAME, an NOS inhibitor, treatment. These observations suggest that the suppressive effects exerted by CQ-modulated DCs are NO dependent.

The transfer of tolerogenic DCs is an interesting approach for the control of chronic inflammation.^{33,57,58} However, it is unclear which signals turn DCs into fully mature and which turn them immunosuppressive.¹¹ For instance, it was previously demonstrated that vitamin D is an important regulator of DC function.⁵⁹ Transfer of vitamin D-modulated DCs was shown to reduce the clinical signs of EAE through the expansion of regulatory T cells.¹⁶ Also, treatment with apoptotic cells drives these cells into a permissive state, and that transfer of apoptotic cell-induced tolerogenic DCs ameliorates the course of EAE through the downmodulation of inflammatory T cells.⁶⁰ Several studies also showed that pathogen-derived molecules interfere with DC maturation status, prompting them for the use in the control of inflammatory conditions.^{58,61,62} We observed that CQ reduces the expression of antigen-presentation molecules and the functional profile of DCs *in vitro*. Thus, CQ administration induces a semi-mature phenotype in DCs. In addition, the transfer of CQ-modulated DCs reduced the clinical signs of EAE, a T cell-mediated disease. The peripheral cellular response toward neuroantigens (both MOG and myelin basic protein) was diminished as well as the infiltration of inflammatory cells in the CNS. Interestingly, the immune response toward ovalbumin, an unspecific antigen, was maintained, which strongly suggests that the reduced response generated after DC-CQ transfer is antigen/tissue specific.

The infiltration of leukocytes in the CNS promotes local inflammation and may lead to neurodegeneration, in which the glial reactivity is an important hallmark.⁶³ Astrocytes, upon environmental disturbances, express higher levels of the intermediary filament GFAP in sites of nerve injury.⁶⁴ Uncontrolled astrogliosis leads to the formation of glia scars that are irreversible, also called anisomorphic gliosis.^{65–67} We found an upregulation in the expression of GFAP in EAE-inflicted mice both untreated and treated with DC-PBS. Interestingly, mice that received CQ-modulated DCs showed lower glial reactivity. The numbers of Iba-1⁺ cells were similar between the treatment groups, which may indicate: (1) the frequency of microglia does not dictate the course of EAE, but their activation profile does; (2) the transferred DCs (which may express Iba-1⁶⁸) migrated to the CNS; (3) both these phenomena occur concurrently. In this context, we observed that the expression of cytokines within the CNS was found altered in EAE-inflicted mice, with an increase in regulatory IL-10 and reduced inflammatory IL-17 and IL-6 after transfer of DC-CQ. Our data support the hypothesis that a change in the inflammatory status of the CNS toward an anti-inflammatory profile is responsible for the amelioration observed in the clinical course of DC-CQ-treated mice.

It is not yet clear the mechanism by which CQ exerts its immunomodulatory effects on DCs. Although pH-dependent interference exists, recently it has been demonstrated that CQ interacts with the mTOR pathway in MCF7 cancer cells.⁶⁹ In DCs, it was shown that manipulation of the mTOR signaling cascade facilitates the antigen presentation in a Treg-inducing milieu while inhibiting Th1/Th17 differentiation.⁷⁰ In this context, more studies must be conducted in order to solve whether CQ and mTOR pathway are related in DCs modulation.

The chronic CQ usage has been shown to provoke collateral side effects, including retinal toxicity.^{71–73} We demonstrated that low-dose CQ administration is capable of reducing the clinical sign of EAE through the stimulation of regulatory T cells.²⁸ Although the

treatment period was brief and that the dose used was low, the chances of retinal toxicity still exists in a chronic treatment regimen. We propose that an alternative for the chronic consumption of the drug is the modulation of DCs *in vitro* and its adoptive transfer. This approach not only prevented toxicity of the drug but also reduced the EAE severity in an antigen-specific manner. Other treatments currently in use such as those with anti-inflammatory drugs and monoclonal antibodies, although still important and necessary, act promoting a general nonspecific suppression. In this sense, the administration of CQ or CQ-modulated DCs together with classical FDA-approved anti-inflammatory drugs/monoclonal antibodies may reduce the doses of the medicines consumed by the patients, thus decreasing the elevated costs of the multiple sclerosis treatment. In addition, transfer of mitomycin C-treated DCs was able to reduce the cellular response toward neuroantigens *in vivo*.¹² In this study, the authors showed that the *in vitro* modulation of DCs with a clinically approved drug and its posterior adoptive transfer is an interesting approach for the treatment of multiple sclerosis and EAE. Indeed, in coculture experiments with patient's mitomycin C-treated DCs and effector T cells, the proliferation of the latter was inhibited, whereas the dendritic cell immature phenotype was not reverted.

Collectively, our data indicate that CQ modulates DCs both phenotypically and functionally. The adoptive transfer of CQ-modulated DCs was able to reduce the clinical signs of EAE by the downregulation of cellular immune response toward neuroantigens. Consequently, the glial reactivity was diminished in the CNS of mouse that received CQ-treated DCs and may have contributed to the amelioration of disease course. Taken together, our data indicate that adoptive transfer of CQ-modulated DCs may become an alternative for the chronic consumption of the drug, thus avoiding toxicity.

METHODS

Mice

Six-to-eight week-old female C57BL/6 mice from the Multidisciplinary Center for Biological Research, University of Campinas, were used in this study. Mice were kept in specific-pathogen free condition, in a controlled temperature and photoperiod environment, with autoclaved food and water *ad libitum* throughout the experiment. The Institutional Committee (Comissão de Ética no Uso de Animais-CEUA/UNICAMP; Protocol number 2687-1) approved the protocols involving laboratory animals.

Generation of DCs, treatment and adoptive transfer

Bone marrow-derived precursors were used in the generation of DCs, according to a previous report.⁷⁴ Briefly, femurs were collected and the bone marrow cells were flushed out with RPMI medium supplemented with 2-mercaptoethanol (2 mM), fetal bovine serum (10% v-v) and gentamicin (50 µg ml⁻¹), referred to as complete medium. 5×10^6 cells were seeded in 24-well culture plate containing complete medium supplemented with GM-CSF (10 ng ml⁻¹). Fresh medium was added at days 3 and 6 of culture. This culture method results in DC generation of 85–95% purity, assessed by flow cytometry. DCs were used in transfer or coculture experiments. For adoptive transfer experiments, DCs were treated with CQ (50 µM, Sigma-Aldrich, St Louis, MO, USA) or dexamethasone (50 µM) for 18 h in the presence of lipopolysaccharide from *Escherichia coli* O111:B4 (1 ng ml⁻¹, lipopolysaccharide, Sigma-Aldrich) and pulsed overnight with 10 µg ml⁻¹ of MOG_{35–55} peptide (Rheabiotec, Campinas, Brazil). PBS was added as a control reagent. 1.5×10^6 cells were adoptive transferred via intravenously by the retroorbital route 3 days before EAE induction.

Coculture experiments

For the coculture experiments, 5×10^5 per well DCs treated as above were seeded in flat-bottom 96-well plates. Splenocytes were enriched in lymphocytes by centrifugation in Percoll gradient, following a previously published

protocol.⁷⁵ CD4⁺ T lymphocytes were isolated using Dynabeads following manufacturer's instructions (CD4⁺ T cell isolation kit, Applied Biosystems, Life Technologies, Austin, TX, USA). This protocol resulted in a purity of 90–95% CD4⁺ T cell, confirmed through analysis by flow cytometry. 5×10^5 per well CD4⁺ T cells were seeded together with DCs at a ratio of 1:1 (DC:T) in 200 μ l of complete RPMI medium containing MOG_{35–55} (MEVG-WYRSPFSRVVHLYRNGK, 10 μ g ml⁻¹, R&B Biotech). As controls, CD4⁺ T cells were grown in the absence of DCs. The plates were incubated at 37 °C for 96 h. For the evaluation of NO blockade, some cultures were conducted in the presence of the NOS inhibitor, N (G)-nitro-L-arginine methyl ester (L-NAME, 1 mM, Sigma-Aldrich). After the incubation period, the cells were treated with 10 μ l of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg ml⁻¹, Sigma-Aldrich) for 4 h and the formazan crystals dissolved with 100 μ l of isopropanol. Absorbances were read at 540 nm by spectrophotometer (VersaMax, Molecular Devices, Sunnyvale, CA, USA).

NO dosage

Nitrite production was determined using the Griess reagent following a well-established protocol.^{76,77}

EAE induction and evaluation

EAE was induced and evaluated in mice according to a previously published paper.²⁸ Briefly, each mouse was injected with 100 μ g MOG_{35–55} (R&B Biotech) emulsified with complete Freund's adjuvant (CFA, Sigma). Pertussis toxin (Ptx, Sigma) 200 ng was administered via i.p. at 0 and 48 h after antigen challenge. Clinical signs were followed and graded daily according to a score method, were 0: no sign, 1: flaccid tail, 2: hind limbs weakness, 3: hind limbs paralysis, 4: hind paralysis and fore limbs weakness, 5: full paralysis/dead. At the indicated time points after antigen challenge, mice were killed, spinal cords were removed and snap frozen in OCT Tissue Tek; 12- μ m thin slices were made and stained with haematoxylin and eosin.

Immunofluorescence

Fourteen days after EAE induction, mice were killed and the spinal cords were collected, snap frozen as mentioned above and 12- μ m thin slices were made. The reactive sites were blocked with phosphate buffer (0.1 M, pH 7.2)–BSA 3%. The cells were stained with purified rabbit anti-glial fibrillary acid protein (GFAP, at a 1:1200 dilution, Dako, Glostrup, Denmark) and anti-Iba-1 antibodies (at a 1:1200 dilution, Dako). The reaction was treated with Cy2-conjugated goat anti-rabbit immunoglobulin G (diluted 1:250, Vector) and analyzed under epifluorescence microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA).

Scanning electron microscopy

1×10^6 DCs treated both with CQ or PBS were stimulated with lipopolysaccharide (1 ng ml⁻¹) and cultivated over glass coverslips for 18 h at 37 °C. Cells were fixed (1 ml of a solution consisting of glutaraldehyde 2% and tannic acid 1%) for 1 h. The post fixation was made in aqueous 1% osmium tetroxide for 2 h. The material was dehydrated with series of ethanol and then brought to the critical point with liquid CO₂ (Balzers CPD 030) and covered with gold bath (Sputter Coater Balzers SCD 050, Capovani Brothers Inc., Scotia, NY, USA). Cells were viewed in a JEOL 5800LV scanning electron microscope.

Lymphoproliferative analysis

For the evaluation of antigen-specific proliferation, leukocytes were collected from spleens of control and EAE-inflicted mice. Briefly, spleen were collected aseptically and macerated with the aid of piston and sieve to prepare cellular suspensions. Cells were treated with ACK lysis buffer (ammonium-chloride-potassium) for 3 min and washed with complete RPMI medium. 5×10^5 cells per well were seeded in 96 flat-well plates with 200 μ l of complete RPMI medium containing 10 μ g ml⁻¹ of MOG_{35–55} (R&B Biotech) and incubated at 37 °C for 96 h. As control, cells were grown in the absence of antigen. After the incubation period, the cultures were treated with 10 μ l of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg ml⁻¹, Sigma-Aldrich)

for 4 h and the formazan crystals dissolved with 100 μ l isopropanol. Absorbances were read at 540 nm by spectrophotometer.

Reverse transcriptase PCR

The RNA was extracted from the samples with TRIzol reagent (Life Technologies, Austin, TX, USA). RNA concentration was calculated by reading the absorbance at 280 nm and the purity guaranteed by the ratio of 280/260 nm (ratios ranging from 1.8 to 2.0 were used). One microgram of RNA was converted to cDNA using High Capacity cDNA Reverse Transcription kit following the manufacturer's recommendations (Life Technologies). Expression of IL-6 (Mm00446190_m1), IL-10 (Mm00439614_m1), IL-17 (Mm00439618_m1), IFN- γ (Mm01168134_m1), TGF- β (Mm01178820_m1) and FOXP3 (Mm00475162_m1) was analyzed in comparison with GAPDH (Mm99999915_g1, housekeeping gene). Reverse transcriptase PCR reactions were performed using Taqman reagents according to manufacturer's recommendations (Applied Biosystems).

Flow cytometry

For flow cytometry analysis, 1×10^6 cells were labeled with fluorochrome-conjugated monoclonal antibodies. Cells were incubated with antibody cocktail for 30 min at 4 °C and fixed with 1% formaldehyde. The antibodies used were as follows: anti-CD11c/APC (clone N418), anti-major histocompatibility complex-II/PECy7 (clone M5/114.15.2), anti-CD80/FTTC (clone 16-10A1), anti-CD86/PE (clone GL-1), all from eBioscience (San Diego, CA, USA). 50,000 events were acquired from each sample in flow cytometer (FACSCanto, BD Biosciences, Franklin Lakes, NJ, USA) and analyzed in FlowJo 7.5 software (Tree Star Inc., Ashland, OR, USA).

Statistical analysis

For clinical score and weight comparisons, collected data were analyzed by two-way analysis of variance with Bonferroni *post hoc* test. Analyses between two groups were performed by Student's *t*-test. All analyses were performed using GraphPad prism version 5.0 (GraphPad software, San Diego, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Dendritic cells treated with crude *Plasmodium berghei* extracts acquire
immune-modulatory properties and suppress the development of
autoimmune neuroinflammation

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Summary

Dendritic cells (DCs) are professional antigen-presenting cells specifically targeted during *Plasmodium* infection. Upon infection, DCs show impaired antigen-presentation and T cell activation abilities. In this study, we aimed to evaluate whether cellular extracts obtained from *P.berghei*-infected erythrocytes (PbX) modulate DCs phenotypically and functionally and the potential therapeutic usage of PbX-modulated DCs in the control of Experimental Autoimmune Encephalomyelitis (EAE, the mouse model for human Multiple Sclerosis). We found that PbX-treated DCs have impaired maturation and stimulated the generation of regulatory T cells when cultured with naïve T lymphocytes *in vitro*. When adoptively transferred to C57BL/6 mice the EAE severity was reduced. Disease amelioration correlated with a diminished infiltration of cytokine-producing T cells in the Central Nervous System (CNS) as well as the suppression of encephalitogenic T cells. Our study shows that extracts obtained from *Plasmodium berghei*-infected erythrocytes modulate DCs towards an immunosuppressive phenotype. In addition, the adoptive transfer of PbX-modulated DCs was able to ameliorate EAE development through the suppression of specific cellular immune responses towards neuro-antigens. To our knowledge, this is the first study to present evidence that DCs treated with *Plasmodium berghei* extracts are able to control autoimmune neuroinflammation.

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Introduction

Dendritic cells are professional antigen-presenting cells that activate naïve T cells and help to dictate the course of the adaptive immune response through the stimulation of T helper (Th) 1, Th2, Th17 or regulatory T cells¹⁻⁴. Because of this potential, DCs are frequent targets during infection. For instance, it was shown that DCs harbor bacteria and parasites in *Salmonella* and *Plasmodium* infections, respectively⁵⁻⁸. The impairment of DC function also dampens the immune system and slows down or even abrogates the elimination of the pathogen. This can be observed in dendritic cells from malaria patients, which are modulated towards an anti-inflammatory phenotype: lower expression of co-stimulatory molecules, secretion of IL-10 and stimulation of regulatory T cells (Tregs)^{9, 10}. The mechanisms by which some strains of *Plasmodium* suppress DCs are not yet fully understood. However, it was previously demonstrated that hemozoin, the *Plasmodium* pigment, is able to arrest human and mouse DC maturation¹⁰⁻¹².

The modulation of DCs has proven to be a promising field for the treatment of inflammatory diseases. For instance, it was previously demonstrated that DCs treated with synthetic drugs or pathogen-derived extracts acquire immature phenotype and upon adoptive transfer suppress the severity of collagen-induced arthritis, mouse kidney grafts and experimental autoimmune encephalomyelitis (EAE)¹³⁻²². The mechanisms of DC induced suppression are mostly dependent on the generation of Tregs *in vivo*¹⁷. EAE is a T cell-dependent neuroinflammation that, in many aspects, mimics human Multiple Sclerosis. CD4⁺ T cells play a pivotal role in disease development with the stimulation of accessory cells and production of inflammatory mediators, which leads to

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inflammation and neuronal damage^{23, 24}. Thus, suppression of the cellular response is desirable for the control of EAE.

The interplay between DCs and *Plasmodium* parasites must be better characterized and, when possible, directed towards to new approaches in inflammatory conditions. In this study, we aimed to evaluate whether extracts from *Plasmodium berghei*, the causative agent of malaria, modulate dendritic cells towards a regulatory phenotype aiming its use for the control of inflammatory conditions. We found that DCs treated with *Plasmodium berghei* extracts stimulated the generation of Tregs *in vitro*. Upon adoptive transfer, modulated DCs reduced the severity of EAE through the reduction of the inflammation in the CNS and suppressed the proliferation of auto-reactive T lymphocytes.

Materials and Methods

Mice. Six-to-eight week-old female C57BL/6 mice from the Multidisciplinary Center for Biological Research, University of Campinas, were used in this study. Mice were kept in specific-pathogen free conditions, in a controlled temperature and photoperiod environment, with free access to autoclaved food and water throughout the experiment. All protocols involving laboratory animals were approved and performed in accordance with the guidelines of the State University of Campinas' Committee on the Use and Care of Animals (Comissão de Ética no Uso de Animais – CEUA, # 2687-1).

***Plasmodium berghei* NK65 infection, enrichment of infected erythrocytes and PbX preparation.** 1×10^6 malaria infected red blood cells (iRBC) were obtained from a source mice and injected via i.p. into naïve C57BL/6 mice. At the 14th day of infection, mice were euthanized and blood samples were collected in heparinized tubes. Mice with

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parasitemia higher than 30% were used for the enrichment of iRBC, according to a previously describe method ²⁵. Briefly, blood samples were centrifuged over a Percoll 65% gradient (300g, 20 minutes, 4°C with no brakes) and the cells from the interface were collected and washed with PBS 0,02M pH 7,2. This suspension consists mainly in 95% of iRBC as confirmed by Giemsa staining in blood smears. For the preparation of *P. berghei* extracts (PbX), the iRBC-enriched suspension was submitted to 20 cycles of freeze/thawing in liquid nitrogen and warm bath (37°C), as previously described ²⁶. As controls, normal RBC underwent the same freeze/thaw process. The protein concentration was determined using the Bradford Protein Assay following the manufacturer's instructions (Sigma-Aldrich, St Louis, MO, USA).

Generation of dendritic cells, *in vitro* modulation and adoptive transfer. Bone marrow-derived precursors were used in the generation of dendritic cells, according to a previously report ²⁷. Briefly, femurs were collected and the bone-marrow cells were flushed out with RPMI medium supplemented with 2-Mercaptoethanol (2mM), Fetal Bovine Serum (10% v:v) and Gentamycin (50µg/mL) – referred to as complete medium. 5×10^6 cells were seeded in 24-wells culture plate containing complete medium supplemented with GM-CSF (10ng/mL). Fresh medium was added at days 3 and 6 of culture. This culture method results in DC generation of 85-95% purity, assessed by flow cytometry. DCs were used in transfer or co-culture experiments. For DC modulation, the cells were treated with PbX (100µg/mL) for 18h in the presence of an activating factor – lipopolysaccharide (LPS) from *E.coli* O111:B4 (1ng/mL, LPS, Sigma-Aldrich) and pulsed overnight with 10 µg/mL of MOG₃₅₋₅₅ peptide (Genemed Syn, CA, USA) or ovalbumin (50µg/mL, OVA, Sigma-Aldrich, St Louis, MO, USA). RBC extracts were used as a control reagent. $1,5 \times 10^6$ cells were adoptive transferred via

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i.v. by the retro-orbital route three days before (prophylactic) and fourteen days after (therapeutic approach) EAE induction.

Antigen-presentation assays and cytokine dosages. The following assay was performed as previously described²⁸. Briefly, 5×10^5 /well DCs treated as above were seeded in U-bottom 96 wells plates. Splenocytes were enriched in lymphocytes by centrifugation in Percoll gradient, following a previously published protocol²⁹. Total T lymphocytes were isolated using dynabeads following manufacturer's instructions (Mouse Pan T cell isolation kit, Life Technologies, Austin, TX, USA). Responder T cells from EAE-inflicted mice were stained with carboxyfluorescein succinimidyl ester (CFSE, $2.5 \mu\text{M}$, Sigma-Aldrich) following the manufacturer's instructions. T cells were seeded together with DCs at a ratio of 1:1 (DC:T) in complete RMPI medium containing MOG₃₅₋₅₅ ($10 \mu\text{g/mL}$, Genemed Syn). As controls, T cells were grown in absence of DCs. The plates were incubated at 37°C for 96 hours. The proliferation in these set of experiments was measured by the decay of the dye in flow cytometer. Culture supernatants were collected and assayed for cytokines (IL-10, IL-17, IFN- γ and TNF- α) secretion using the Cytometric Bead Array (CBA, BD Biosciences, Franklin Lakes, NJ, USA, USA) according to manufacturer's instructions.

EAE induction and evaluation. EAE was induced and evaluated in mice according to a previous published paper³⁰. Briefly, each mouse was injected with $200 \mu\text{g}$ MOG₃₅₋₅₅ (Genemed Syn, CA) emulsified with Complete Freund's Adjuvant (CFA, Sigma-Aldrich). 300 ng Pertussis toxin (Ptx, Sigma-Aldrich) was administrated via i.p. at 0 and 48h after antigen challenge. Clinical signs were followed and graded daily according to a score method, were 0: no sign, 1: flaccid tail, 2: hind limbs weakness, 3: hind limbs

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paralysis, 4: hind paralysis and fore limbs weakness, 5: full paralysis/dead. At the indicated time-points after antigen challenge mice were killed spinal cords were removed, dehydrated in serial alcohol and xylene solutions and paraffin embedded; 10µm thin slices were made and stained with haematoxylin and eosin (H&E).

Lymphoproliferative response and cytokine dosage. Splenic cells from EAE-inflicted mice were aseptically collected after 10 days of antigen administration. Cells (5×10^5 /well) were diluted in RPMI 1640 media supplemented with Fetal Calf Serum (FCS; 10% vol/vol), guaramicine (50µg/mL) and 2-Mercaptoethanol (2mM). Media was supplemented with myelin oligodendrocyte glycoprotein peptide (MOG₃₅₋₅₅; 10µg/mL), Myelin Basic Protein (MBP, 50µg/mL) or Ovalbumin (OVA, 50µg/mL). The cells were plated in flat-bottom plates and incubated at 5% CO₂ and 37°C for 96h. As controls, cells were grown in the absence of antigen. After the incubation period, the cells were treated with 10µL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5mg/mL, Sigma-Aldrich) for 4 hours and the formazan crystals dissolved with 100µL of isopropanol. Absorbances were read at 540nm by spectrophotometer (VersaMax, Molecular Devices, Sunnyvale, CA, USA).

Analysis of cellular infiltration in the Central Nervous System (CNS). Fourteen days after EAE induction, mice were anesthetized, perfused with ice cold PBS and CNS tissue was prepared for the enrichment of infiltrating leukocytes according to a previously described methodology and analyzed by flow cytometry ³¹.

Flow cytometry. Fluorochrome-conjugated monoclonal antibodies were used to stain leukocytes. Cells were surface stained with anti-CD4/PE-Cy5 (clone GK1.5), anti-

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CD8/APC (clone 53-6.7), anti-CD3/APC-Cy7 (clone 145-2C11), CD11c/APC (clone N418), MHC-II/PE-Cy7 (clone M5/114.15.2), CD-80/FITC (clone 16-10A1) and CD-86/PE (clone GL-1). For intracellular staining, cells were cultured for 4h in the presence of Phorbol 12-myristate 13-acetate (PMA, 50ng/mL, Sigma) and Ionomycin (100ng/mL). Brefeldin A (10μg/mL) was added as well. Later, the cells were fixed/permeabilized (fixation/permeabilization buffers) according to manufacturer's recommendations, and monoclonal anti-Foxp3/APC (clone FJK-16s), IL-10/PE (clone JES5-16E3), IFN-γ/PE (clone XMG1.2) and IL-17/APC (clone eBIO17B7) were added to cells. Isotype controls were used as well. All antibodies were purchased from BD Biosciences (USA). Preparations (50.000 events/sample, unless otherwise stated) were acquired with a Gallios flow cytometer (Becman Coulter, USA) and data analyzed using FlowJo 7.6 (Tree Star Inc., Ashland, OR, USA). The analysis of cytokine-producing cells was performed using the Fluorescence Minus One (FMO) methodology to help identify gating boundaries, in which cells are stained with all surface antibodies but the ones for intracellular staining³².

Statistical analysis. Clinical score comparisons between control and experimental groups were done by Two-Way ANOVA and post-tested with Bonferroni. Other analyses among two and three (or more) groups were carried out with Student's t test and One-Way ANOVA, respectively. The statistical analyses were performed in GraphPad Prism Software (v.5, GraphPad Software, Inc., La Jolla, CA, USA). Results are expressed as mean ± standard error mean (SEM) and p<0,05 value were defined as significant.

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Results

Dendritic cells acquire an immature phenotype after contact with Plasmodium berghei extracts stimulating the generation of regulatory T cells and changing the profile of T-cell derived cytokines in vitro

It has been previously shown that splenic DCs from *Plasmodium*-infected patients and mice present impaired antigen presentation^{9, 26}. In addition, treatment of DCs with *P. falciparum* extracts drives DCs towards a permissive state⁹. In this context, we aimed to evaluate whether the contact of dendritic cells with crude extracts from *P. berghei*-infected erythrocytes (PbX) would promote changes in the maturation phenotype of DCs. Our results showed that after stimulation with LPS, DCs treated with PbX presented a significant reduction in the expression of CD-86 and CD-80, but not in MHC-II, when compared to cells treated with non-infected Red Blood Cell extracts (RBC) (Figure 1A).

Our next goal was to investigate whether the PbX-treated DCs (DC-PbX) were modulated towards a suppressive phenotype. For that purpose, T cells were isolated from naïve mice and co-cultured with MOG-pulsed DC-PbX or MOG-pulsed non-infected RBC-treated DCs (DC-RBC) as control. The data obtained show that DC-RBC, but not DC-PbX, stimulated the generation of IL-17-producing T cells (Figure 1B). Also, DC-RBC were able to induce the production of IFN- γ in naïve T cells, but not as efficiently as DC-PbX (Figure 2C). DC-PbX were able to stimulate T cells to produce IL-10 (Figure 2D). Interestingly, further analysis showed that DC-PbX induced IFN- γ /IL-10 double-producing T cells (*data not shown*). As the differentiation of naïve T cells into regulatory T cells is a desirable ability in the immunosuppressive phenotype

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of DCs, we then investigated whether DC-PbX were able to induce Tregs. Co-cultures conducted in the presence of DC-PbX had higher frequency of Foxp3⁺Tregs than cultures conducted in the presence of DC-RBC (Figure 2E).

Transfer of DCs treated with Plasmodium berghei extracts (DC-PbX) reduces EAE through the suppression of antigen-specific cellular responses

In order to evaluate whether DCs treated with *Plasmodium berghei* extracts show a therapeutic potential, we transferred DC-PbX to naïve C57BL/6 mice three days before the immunization with MOG₃₅₋₅₅ peptide as a prophylactic approach. As shown in figure 2, mice recipient of DC-RBC (controls) showed an aggravated disease course whereas the transfer of DC-PbX significantly reduced the clinical signs of EAE (Figure 2A). It is well known that the MOG-induced EAE is dependent on the cellular immune response towards the CNS tissue³³. In this context, we compared the reactivity of splenic leukocytes from EAE-inflicted mice towards MOG₃₅₋₅₅ peptide and whole Myelin Basic Protein (MBP) with the reactivity of splenic leukocytes from mice adoptively transferred with DC-PbX or DC-RBC. Our data showed that splenocytes from EAE mice that received DC-PbX proliferated significantly less than cells derived from DC-RBC or untreated EAE-inflicted mice when cultured in the presence of neuro-antigens (Figure 2B and 2C). Interestingly, splenocytes from MOG₃₅₋₅₅-immunized mice cultivated in the presence of ovalbumin (OVA) proliferated at the same level as the cells cultivated in the presence of MOG₃₅₋₅₅ and MBP. This indicates that some antigen presentation occurs *in vitro* due to the presence of antigen-presenting cells in the culture, but it also shows that the cellular immune response towards an unrelated antigen was unaffected (Figure 2D).

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Next, we investigated whether the infiltration of inflammatory cells in the CNS was changed. Our data showed that DC-RBC recipient and untreated EAE-inflicted mice presented high infiltration of leukocytes, while the transfer of DC-PbX reduced the cellular entry to the CNS (Figure 3A). In addition, the profile of the invading T cells was altered. We observed that both the frequencies and the absolute numbers of IL-17- and IFN- γ -producing T cells were reduced in mice that received DC-PbX compared to DC-RBC and untreated EAE group (Figure 3B and 3C, respectively). The frequency of IL-10-producing T cells was significantly augmented in DC-PbX transferred group compared with the DC-RBC and un-transferred EAE-inflicted mice, however, due to the lower infiltration of T cells in the CNS from DC-RbX transferred mice (Figure 3A), the absolute numbers of infiltrating IL-10-producing cells was significant reduced in comparison to the EAE group (Figure 3D).

Dendritic cells treated with extracts from Plasmodium berghei suppress ongoing EAE and encephalitogenic T cells

In this study, we show that the adoptive transfer of DC-PbX reduced the clinical course of EAE. Our data demonstrated, in Figures 2 and 3, that the cellular immune response towards neuro-antigens as well as the frequency of inflammatory T cells in the CNS was reduced in EAE-inflicted mice that received DC-PbX (DC-PbX+EAE) in comparison with EAE-inflicted mice that received DC-RBC (DC-RBC+EAE). However, in these set of experiments, the DCs encountered naïve T cells by the time of adoptive transfer, since cells were transferred before EAE induction. To investigate whether DC-PbX would suppress the clinical course of ongoing EAE, we adoptively transferred these cells at disease onset. Results showed that the transfer of DC-PbX significantly reduced the clinical course of EAE (Figure 4A). In addition, the

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suppressive effect of DC-PbX over encephalitogenic T cells was evaluated. For that purpose, we co-cultured DC-PbX with splenic T cells from MOG₃₅₋₅₅-immunized mice. Our results showed that the proliferation of MOG-specific T cells was significantly suppressed in the presence of DC-PbX, but not when cells were co-cultured with DC-RBC (Figure 4B). Besides, in the culture supernatants, levels of TNF- α , IL-6 and IFN- γ were reduced in DC-PbX co-cultures in comparison with the cultures conducted with DC-RBC. There were no statistical differences in IL-10 levels between the groups (Figure 4B).

Discussion

It has been demonstrated that DCs in malaria patients and mouse are immature and non-functional^{10-12, 34}. Although in malaria infection, the paralyzed dendritic cell is a problem, in the autoimmune inflammation, this is a desired phenomenon. In this study, we show, for the first time to our knowledge, that dendritic cells modulated by extracts of *Plasmodium berghei*-infected erythrocytes protect mice from the development of EAE.

During the *Plasmodium* infection, the parasite develops tropism towards DCs, which phagocytize the infected Red Blood Cells^{35, 36}. Inside dendritic cells, the parasite produces hemozoin, a product of hemoglobin degradation, ultimately leading to impaired DC maturation¹². *In vitro* hemozoin-treated DCs acquire immunosuppressive abilities such as reduced expression of co-stimulatory molecules CD80 and CD-86, secretion of IL-10 and induction of regulatory T cells^{10, 34}. Semi-mature dendritic cells are so called because they express low-to-intermediate levels of co-stimulatory molecules and have the ability to induce the differentiation of regulatory T cells when

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co-cultured with naïve T cells (reviewed in ^{37, 38}). Our data showed that extracts from *P.berghei*-infected erythrocytes (PbX) changed the maturation status of DCs, with lower expression of antigen-presenting molecules, which may characterize these cells as semi-mature DCs. Indeed, we observed that these modulated DCs also stimulated the production of anti-inflammatory cytokines by T cells and were able to induce the conversion of naïve T cells into regulatory T cells. DCs treated with PbX stimulated the production of IFN- γ by naïve T cells, suggesting that the induced anti-inflammatory profile of DC-PbX is dependent on the stimulation of an IFN- γ -biased milieu. A similar mechanism of DC modulation can be observed with *Schistosoma japonicum*-derived Sj16 protein that induces IL-10- and IFN- γ -producing Tregs ³⁹. Also, it was recently shown that *P.falciparum* schizont extracts also modulate human dendritic cells, that acquire the ability to induce Tregs from naïve autologous T cells ²⁵. On the other hand, it was demonstrated that extracts of *P.yoelli*-infected erythrocytes induced DC maturation and inflammatory activation ⁴⁰. These data in conjunction with our results indicate that diverse *Plasmodium* species are able to promote distinct effects on DCs.

Here we demonstrated that transfer of DC-PbX into EAE-inflicted mice reduced the clinical signs of the disease. The peripheral immune response towards neuro-antigens was also suppressed while splenic T cells were able to proliferate against an unrelated antigen, ovalbumin (OVA). This finding suggests that the transfer of modulated DCs not only reduced the neuroinflammation but also stimulated the generation of an antigen-specific immunosuppression.

Moreover, when DCs treated with PbX were co-cultured with previously activated encephalitogenic T cells the specific proliferation was reduced as well as the

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supernatant's inflammatory cytokine levels. These observations might correlate with the amelioration of the disease. Although the precise mechanism for this observation is unknown, an increasing amount of evidence suggest that *Plasmodium*-derived molecules modulate the immune system. It was previously demonstrated that *P.falciparum* soluble extracts potentiate the induction and function of human regulatory T cells through a TGF- β -dependent mechanism⁴¹. In mouse *Plasmodium chabaudi* infection, there is an increase in the frequency of peripheral regulatory T cells, which modifies the course of EAE⁴².

Interestingly, dendritic cells modulated with PbX presented different results when cultured with naïve and encephalitogenic T cells regarding the secretion of IFN- γ . It is uncertain how these IFN- γ /IL-10- double-producing T cells generated from naïve cells suppress the establishment of EAE (in the prophylactic approach), although the IFN- γ /IL-10 double-producing regulatory T cells have been previously reported when helminth-derived products were used to modulate T cell activation³⁹. These observations suggest that PbX-modulated DCs possess a dual role: (i) to stimulate the differentiation of regulatory T cells from naïve T cells and (ii) to suppress the proliferation of inflammatory T cells.

Modulation of dendritic cells with pathogen-derived molecules is notorious. For instance, it was previously demonstrated that eggs from *Schistosoma mansoni* arrests DCs maturation towards and immature phenotype^{43, 44}. In addition, soluble egg antigens (SEA) from *S.mansoni* is enriched in omega-1, which modulates DCs and induces the expression of Foxp3 in T cells *in vitro*^{45, 46}. Although the restrained immune response in malaria and schistosomiasis is a problem, the impairment in DC function may

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become useful for the control of chronic inflammatory conditions. Likewise, DCs exposed to SEA and omega-1 acquire modulatory properties and induces Tregs in CD4⁺T cells from non-obese diabetic (NOD) mice, reducing the incidence of spontaneous diabetes⁴⁷. Other groups have also reported that the administration of helminth products, such as those from *Trichuris suis*, reduced the clinical manifestation of EAE, likely through the modulation of antigen presenting cells⁴⁸.

As DCs dictate the course of the immune response, it is reasonable to assume that they are targets during infections, which change the maturation/activation profile of these cells towards an immunosuppressive phenotype. Taken together the results presented herein show that dendritic cells modulated with *P.berghei*-derived extracts are suppressive and control EAE. Further elucidations are necessary in order to define the mechanisms that control the modulation of dendritic cells.

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Authors' contributions: Conceived and designed the experiments: RT, FTMC & LV. Performed the experiments: RT, LKI, TAdC, RDG, ITF, CR & SCPL. Analyzed the data: RT, MACH, FTMC & LV. Contributed reagents/materials/analysis tools: LV FTMC & MACH. Wrote the paper: RT & LV.

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Conflicts of interest

The authors declare no competing interest.

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Figure legends

Figure 1 – Dendritic cells treated with *Plasmodium berghei* extracts acquire an immature phenotype and stimulate the generation of regulatory T cells. Bone marrow-derived dendritic cells were stimulated with LPS (1 η g/mL) for 18h in the presence of PbX (100 μ g/mL). As control, cells were stimulated in the presence of non-infected Red Blood Cell extracts (RBC, 100 μ g/mL). A: The cells were collected and stained with fluorochrome-conjugated antibodies for MHC-II, CD80 and CD86. The mean fluorescence intensity (MFI) was calculated from 500.000 acquired events. Dendritic cells were treated with PbX (100 μ g/mL) or non-infected Red Blood Cells extracts (100 μ g/mL), stimulated with LPS (1 η g/mL) and pulsed with MOG₃₅₋₅₅ peptide (10 μ g/mL) for 18h. Then cells were seeded in 96 U-bottom wells (5x10⁵ cells/well). T lymphocytes were isolated from spleens of naïve mice using Dynabeads and seeded with DCs (1 DC:1 T). The cells were allowed to grow for 96h in the presence or absence of MOG peptide (10 μ g/mL). As controls, T cells were cultivated without DCs. At the end of culture period, the supernatant was collected and the cells were treated with PMA and Ionomycin together with Brefeldin-A for 4h. B, C and D: Intracellular IL-17, IFN- γ and IL-10 in CD3⁺ cells was determined, respectively. E: The generation of Foxp3⁺ Regulatory T cells was determined as well. Data from three separate experiments with similar results. *p<0,05 and **p<0,01.

Figure 2 – Reduced EAE severity in mice that received PbX-modulated dendritic cells correlates with decreased cellular response towards neuro-antigens. DCs were stimulated with LPS (1 η g/mL) then treated with PbX (100 μ g/mL) and pulsed with MOG₃₅₋₅₅ peptide (10 μ g/mL) for 18h and adoptively transferred (1,5x10⁶ cells/mouse) into C57BL/6 mice (n=6/group) before the induction of EAE. A: The clinical score of

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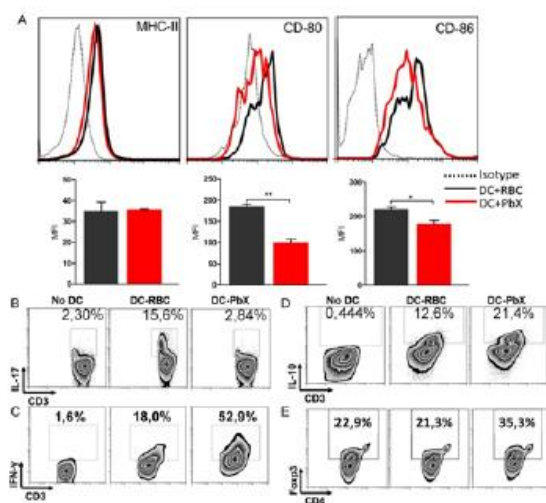
DCs-transferred and untreated EAE-inflicted mice was monitored daily; *** $p < 0.001$. The spleens of EAE and DC-transferred EAE (both DC-RBC and DC-PbX) mice were collected and total leukocytes (5×10^5 /well) were cultured in the presence of MOG₃₅₋₅₅ peptide (in B), Myelin basic protein (in C) and Ovalbumin (in D) ($10 \mu\text{g/mL}$, $50 \mu\text{g/mL}$ and $50 \mu\text{g/mL}$, respectively) for 96h. The proliferation was analyzed using the MTT method; * $p < 0.05$. Representative data of three independent experiments.

Figure 3 – Changes of infiltrating cells in the CNS from DC-transferred mice. DCs were stimulated with LPS (1 ng/mL) then treated with PbX ($100 \mu\text{g/mL}$) and pulsed with MOG₃₅₋₅₅ peptide ($10 \mu\text{g/mL}$) for 18h and adoptively transferred (1.5×10^6 cells/mouse) into C57BL/6 mice ($n=6$ /group) before the induction of EAE. A: At the 14th day after immunization, the CNS tissue of EAE-inflicted mice was removed and processed for histopathology analysis by H&E staining. Magnification: 200x. In addition, the CNS tissue of EAE-inflicted mice (both DC transferred and untreated) was removed at the 14th day post-immunization and the infiltrating leukocytes were enriched through gradient centrifugation using Percol reagent. Cells were stimulated with PMA + ionomycin in the presence of Brefeldin-A for four hours before staining with fluorochrome-conjugated antibodies. The frequencies of T cells producing IL-17 (in B), IFN- γ (in C) and IL-10 (in D) were evaluated by flow cytometry. Data from three independent experiments. * $p < 0.05$.

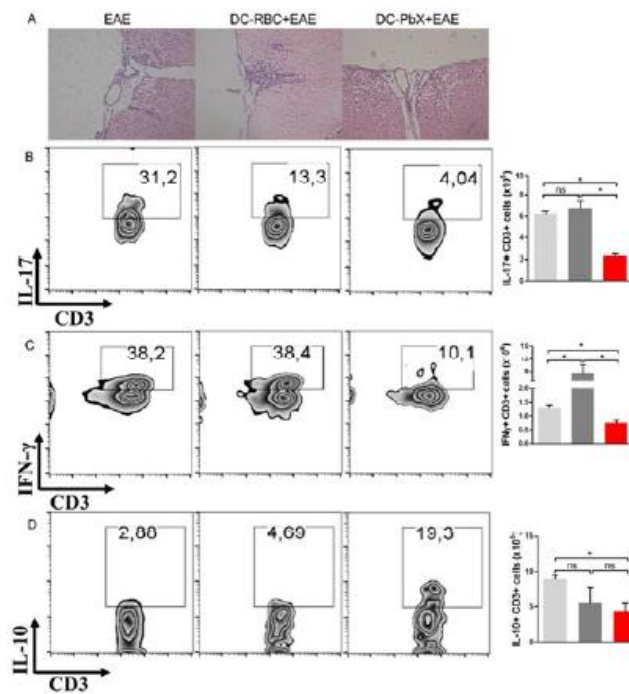
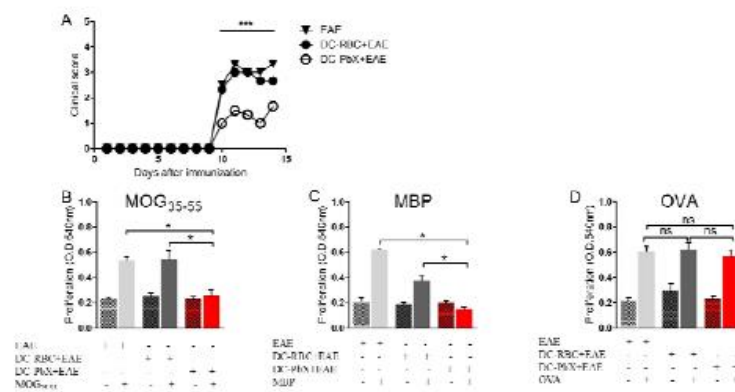
Figure 4 – Dendritic cells treated with *Plasmodium berghei* extracts suppress ongoing EAE and encephalitogenic T cells. DCs were stimulated with LPS (1 ng/mL) then treated with PbX ($100 \mu\text{g/mL}$) and pulsed with MOG₃₅₋₅₅ peptide ($10 \mu\text{g/mL}$) for 18h and adoptively transferred (1.5×10^6 cells/mouse) into C57BL/6 mice ($n=6$ /group) at

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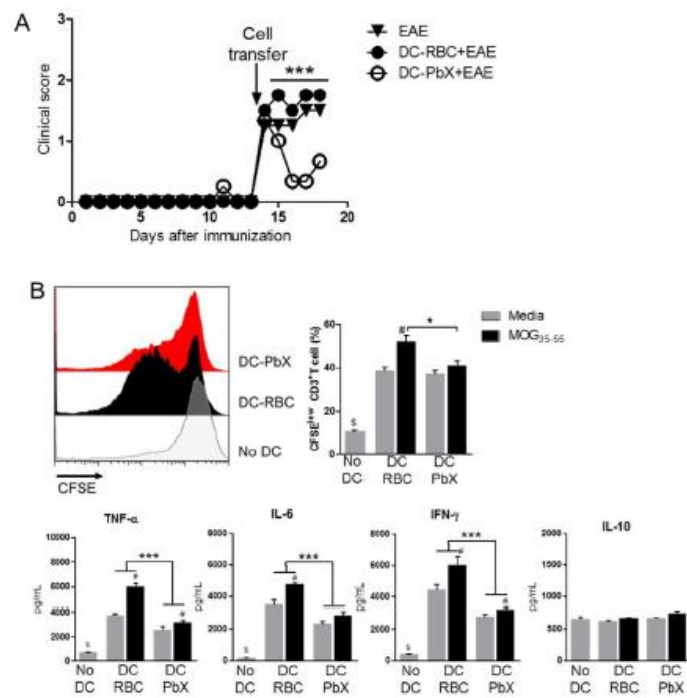
the onset of EAE (day 14th after immunization). A: The clinical score of DCs-transferred and untreated EAE-inflicted mice was monitored daily; *** $p < 0,001$. DCs were modulated with PbX (100 μ g/mL) stimulated with LPS (1 η g/mL) and pulsed with MOG peptide (10 μ g/mL) for 18h. Splenic T cells from MOG₃₅₋₅₅-immunized mice were isolated at the 10th day post-immunization, CFSE-stained and co-cultured with DC-PbX (1:1, DC:T) in the presence or absence of MOG₃₅₋₅₅ (10 μ g/mL) for 96h. As controls, cells were grown with DC-RBC or alone. B: The proliferation of T cells was measured by the decay of the dye at the end of the culture period by flow cytometry. Cytokine levels in culture supernatants was determined by Cytometric Bead Assay. *** $p < 0,001$; * $p < 0,05$ (in comparison to the DC-RBC co-culture); # $p < 0,05$ (in comparison to co-cultures without antigen in the same DC regimen); \$ $p < 0,05$ (in comparison to all DC co-cultures). Representative data from two independent experiments with similar results.



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Manuscritos submetidos

**NITRIC OXIDE PLAYS A KEY ROLE IN THE SUPPRESSIVE ACTIVITY OF
CHLOROQUINE-MODULATED DENDRITIC CELLS**

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Running title: Dendritic cell-derived NO reduces EAE

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Abstract

We have previously shown that chloroquine (CQ), an antimalarial drug, modulates the immune system through the stimulation of regulatory T (Treg) cells and suppression of dendritic cells (DCs). Later, we showed that CQ-modulated DCs (CQ-DC) reduced the severity of Experimental Autoimmune Encephalomyelitis (EAE) and that these cells produce high amounts of Nitric Oxide (NO). In this study, we aimed to evaluate whether the tolerogenic activity of CQ-treated DCs lies on NO production and secretion. For that purpose, wild type (WT) and *inducible Nitric Oxide Synthase* (iNOS)-Knockout DCs were treated with chloroquine and phenotypical, functional and therapeutical analyses were carried out. Results showed that in the absence of NO, DCs modulatory function was diminished. Upon adoptive transfer, CQ-DC_{iNOS^{-/-}} were unable to control the development of EAE, but CQ-DC_{WT} were. Taken together, our data shows that the suppressive effect of chloroquine-modulated dendritic cells is due to both NO-dependent and -independent mechanisms.

Introduction

Dendritic cells are antigen-presenting cells with the ability to dictate the course of the immune response through the modulation of naïve T cells by the time of their activation. It is known that DC modulation constitute a fine approach to deal with the immune-suppression caused by tumors^{1, 2} and the exacerbated immune response observed in autoimmune diseases^{3, 4}. Therefore, identification of novel compounds with the ability to modulate dendritic cells can be extremely useful.

We have previously described that chloroquine, an antimalarial drug, has anti-inflammatory activity that may be directed towards the modulation of Experimental Autoimmune Encephalomyelitis, the mouse model of human Multiple Sclerosis⁵. It is believed that the main mechanism of action exerted by chloroquine is the alkalization of acid organelles, like the lysosomes⁶. We reported that chloroquine administration provokes an increase in the frequency of regulatory T cells in the periphery and a decrease in dendritic cells numbers⁵. Conversely, chronic consumption of chloroquine leads to the development of toxic effects, such as blindness⁶. Still, it was shown that drug-modulated DCs are an interesting approach to bypass toxicity⁴. Indeed, we showed that DCs treated with chloroquine acquire immune-modulatory abilities and suppress the development of EAE by mechanisms not yet fully understood⁷. Interestingly, chloroquine-modulated DCs produce high amounts of nitric oxide (NO), an important mediator of inflammation. However, its pro-inflammatory activity has been questioned.

Lack of nitric oxide prompts mice to develop a more severe form of EAE⁸. In addition, nitric oxide inhibits the differentiation of naïve T cells into Th17 cells favoring the generation of Treg cells^{8, 9}. Thus, the production of NO by CQ-modulated DCs is striking. In this context, we aimed to evaluate whether nitric oxide plays a role in the modulatory activity of chloroquine-treated dendritic cells.

Results and Discussion

Lack of Nitric Oxide dampens dendritic cell-stimulated generation of Treg cells

To verify whether nitric oxide is involved in the modulation of dendritic cells by chloroquine, we treated DCs obtained from wild type mice (DC_{WT}) with CQ (CQ-DC_{WT}), or vehicle (PBS-DC_{WT}), and measured NO levels. We found that CQ-DC_{WT} produced high amounts of NO (Figure 1A). It has been demonstrated that chloroquine

administration results in NO production by the endothelium as well as expression of endothelial Nitric Oxide Synthase (eNOS) in some cell types leading to renal failure and oxidative distress¹⁰⁻¹². We aimed to investigate whether the NO production was iNOS-dependent. To solve this question, DCs generated from iNOS^{-/-} mice (DC_{iNOS^{-/-}}) were submitted to the same treatment as wild-type DCs. The results showed that CQ-treated DC_{iNOS^{-/-}} (CQ- DC_{iNOS^{-/-}}) produced significantly less NO than controls (CQ-DC_{WT}), indicating that the immune isoform of Nitric Oxide Synthase (iNOS) is responsible for the nitric oxide production elicited by chloroquine (Figure 1A). As so far there is no evidence that CQ stimulate iNOS expression, we decided to evaluate this possibility. Results showed that DC_{WT} expressed high levels of iNOS (Figure 1B). Interestingly, the expression of the Indoleamine 2,3 Dioxygenase (IDO), an immune-modulatory enzyme, was found augmented as well, and this effect was observed both in CQ-DC_{WT} and CQ-DC_{iNOS^{-/-}} (Figure 1B).

In the next set of experiments, we aimed to assess whether NO plays a role in the differentiation and activation of T cells. For that purpose, we co-cultured DCs with naïve T cells. We found that both CQ-DC_{WT} and CQ-DC_{iNOS^{-/-}} stimulated the generation of Treg cells. However, CQ-DC_{iNOS^{-/-}} were not as efficiently as CQ-DC_{WT} in this ability (Figure 1C). The production of IFN- γ and IL-10 by T cells was then investigated. The data obtained show that CQ-DC_{WT} was unable to induce IFN- γ production whereas IL-10 was significantly up-regulated (Figure 1D). On the other hand, DC_{iNOS^{-/-}}, treated or not with CQ, did not induce IFN- γ production, but was able to stimulated the production of IL-10 (Figure 1D), although not at the same levels as CQ-DC_{WT}. These data confirms that NO is important for the generation of Treg cells⁹.

DCs from iNOS-knockout suppress EAE through the modulation of the local inflammation

So far, our data indicate that nitric oxide may be involved in the modulation of the immune response by chloroquine-modulated DCs. To ascertain whether NO play a major role in DC modulatory activity, we adoptively transferred CQ-DC_{WT} and CQ-DC_{iNOS^{-/-}} towards WT EAE-bearing mice. Corroborating our previous report⁷, CQ-DC_{WT} was able to significantly reduce the clinical signs of EAE when compared to control group (PBS-DC_{WT}) (Figure 2A). However, in the absence of nitric oxide production by DCs, the modulatory effect of chloroquine was abolished as demonstrated by the exacerbation of EAE in mice recipient of PBS-DC_{iNOS^{-/-}} and CQ-DC_{iNOS^{-/-}} (Figure 2A). As NO production has been shown to suppress EAE and the generation of Th17 cells^{8,9,13}, we then aimed to evaluate whether inflammation in the CNS was affected.

Our analysis showed that the transfer of CQ-DC_{WT} into EAE mice (CQ-DC_{WT} + EAE) provoked a significant increase of FOXP3, IL-10, TGF- β , IL-4, iNOS and IDO expression in the central nervous system, whereas a decrease was observed in IFN- γ , IL-17, TNF- α and IL-6 expression when compared with control mice (PBS-DC_{WT} + EAE) (Figure 2B).

Surprisingly, the transfer of CQ-DC_{iNOS^{-/-}} into EAE mice (CQ-DC_{iNOS^{-/-}} + EAE) stimulated the expression of anti-inflammatory mediators, such as FOXP3, IL-10, TGF- β and IDO, as well as inflammatory molecules, such as IL-17, IL-6, IFN- γ and TNF- α (Figure 2C).

Collectively, these data indicate that CQ-DC_{WT} suppresses neuro-inflammation through the production of NO and that lack of this inflammatory mediator results in exacerbated autoimmune neuro-inflammation. Interestingly, the stimulation of regulatory T cells by CQ-DC_{iNOS^{-/-}} suggests that NO-independent mechanisms must

exist. Therefore, further studies must be conducted to characterize the mechanistic spectrum of chloroquine action.

Methods

Mice

All protocols involving laboratory animals were approved by the institutional committee (Protocol number 2687-1). iNOS^{-/-} and Wild-type mice, on the C57BL/6 background, were used in this study. Mice were from CEMIB, University of Campinas. In all experiments, mice were kept in *specific-pathogen free* condition, in a controlled temperature and photoperiod environment.

DC generation, treatment, co-cultures and EAE induction

Bone marrow-derived precursors were used in the generation of dendritic cells, according to our previously report⁷. The treatment with chloroquine (50μM, Sigma-Aldrich, St Louis, MO, USA) and LPS (1ng/mL, Sigma-Aldrich) pulse as well as co-cultures, adoptive transfer and induction of EAE experiments followed that paper's instructions as well.

Flow cytometry

Anti-mouse antibodies anti-CD4/PE-Cy5 (clone GK1.5), anti-CD25/FITC (PC61), anti-FOXP3/APC (FJK-16s), anti-IL-10 (JES5-16E3) and anti-IFN-γ (XMG1.2) were used. All antibodies were from BD Biosciences (Franklin Lakes, NJ, USA). Intracellular staining followed manufacturer's instructions.

Reverse Transcriptase PCR

RNA extracted from lumbar spinal cords was converted to cDNA (High Capacity cDNA Reverse Transcription kit, Life Technologies, Austin, TX, USA). Expression of IL-4 (Mm00445259_m1), IL-6 (Mm00446190_m1), IL-10 (Mm00439614_m1), IL-17 (Mm00439618_m1), IFN- γ (Mm01168134_m1), TGF- β (Mm01178820_m1), TNF- α (Mm00443258_m1), FOXP3 (Mm00475162_m1), iNOS (Mm00440502_m1) and IDO (Mm00492586_m1) was analyzed in comparison to GAPDH (Mm99999915_g1, housekeeping gene). Reactions were performed with Taqman reagents (Life Technologies). The results are shown as relative expression ($2^{-\Delta\Delta CT}$).

NO dosage

Nitrite production was determined using the Griess reagent following a well-established protocol¹⁴.

Statistical analysis

For clinical score and weight comparisons, collected data were analyzed by two-way ANOVA with Bonferroni post-test. Analyses between two groups were performed by Student's *t* test. All analyses were performed using GraphPad prism version 5.0 (GraphPad software Inc., San Diego, CA, USA).

Acknowledgements

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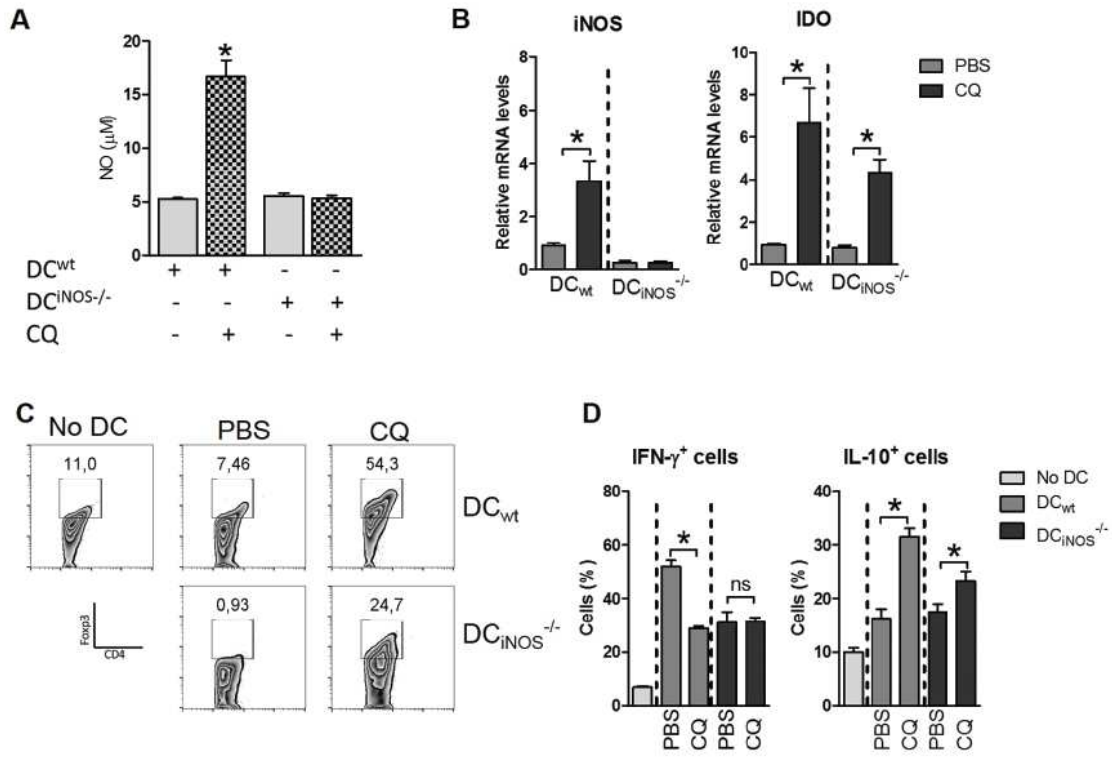
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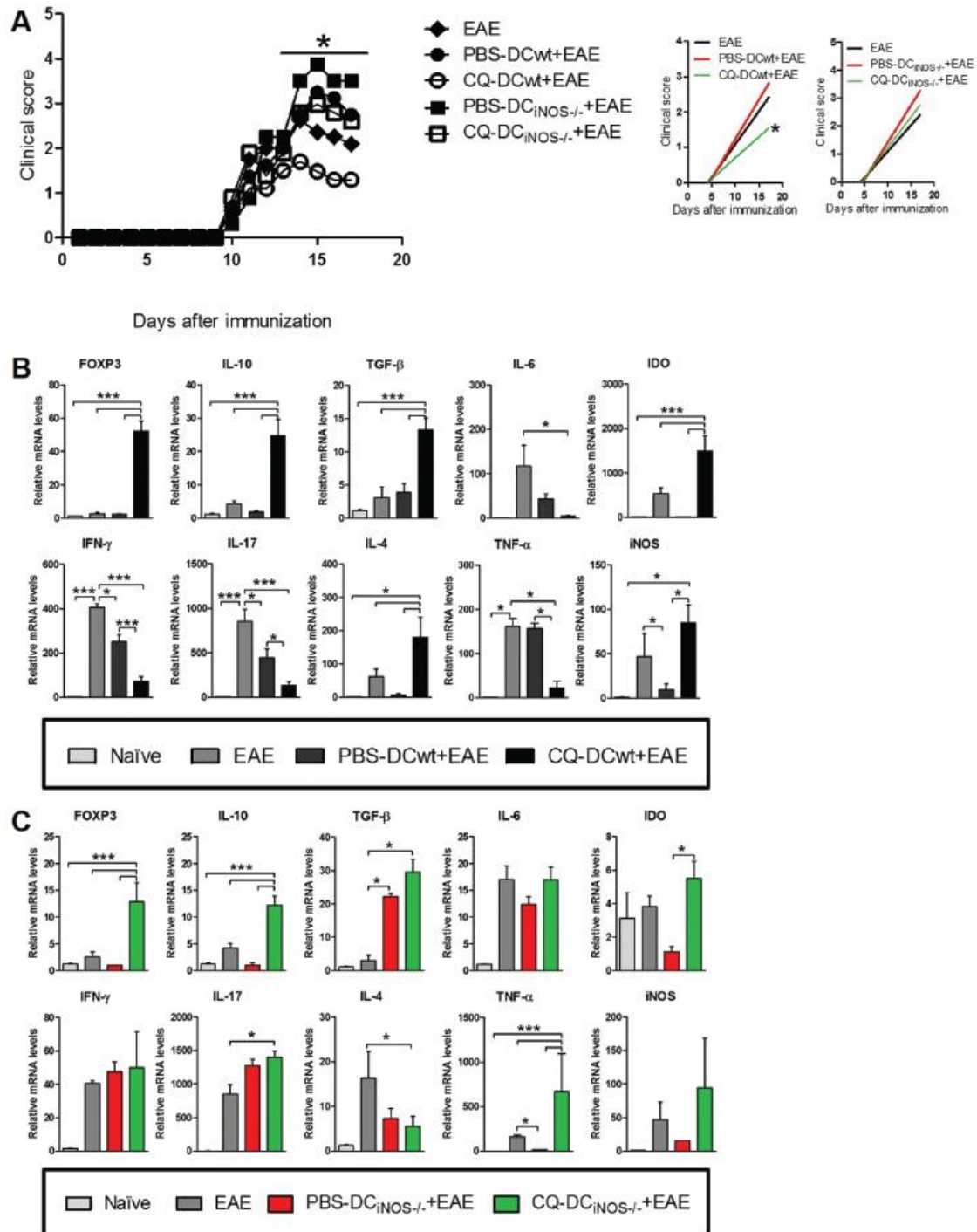
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Figure legends

Figure 1 – Nitric oxide production and dendritic cell modulation following chloroquine treatment. Dendritic cells were generated from bone marrow from WT and iNOS^{-/-} C57BL/6 mice. Bone marrow-derived DCs were treated with chloroquine (50µM) and stimulated with LPS (1ng/mL) for 18h. A) The production of nitric oxide was determined by adding Griess reagent on culture supernatants from DCs treated with chloroquine. B) The cultures were submitted to RNA extraction followed by cDNA conversion. The relative mRNA expression of iNOS and IDO was determined by RT-PCR reactions. C) DCs were treated either with chloroquine (DC-CQ, 50µM) and stimulated with LPS (1ng/mL) then pulsed with MOG₃₅₋₅₅ peptide (10µg/mL) for 18h. Total T lymphocytes were enriched from spleens of naïve mice using Percol gradient centrifugation and co-cultured with DCs (1:1) for 96h in the presence of MOG₃₅₋₅₅ peptide. The frequency of CD4⁺CD25⁺FOXP3⁺ (Treg) cells was determined at the end of the experiment. D) The co-cultures were conducted as above, and by the end of the incubation time, T cells were stained for the presence of intracellular cytokines (IFN-γ and IL-10). Data are representative of three independent experiments made in quadruplicates. * *p*<0,05.

Figure 2 – Exacerbation of Experimental Autoimmune Encephalomyelitis after adoptive transfer of iNOS^{-/-}DCs. Dendritic cells were generated from bone marrow from WT and iNOS^{-/-} C57BL/6 mice. Bone marrow-derived DCs were treated with chloroquine (50μM) and stimulated with LPS (1ng/mL) for 18h then adoptively transferred towards EAE-inflicted mice (n=6 mice/group). A) The clinical course of the disease was evaluated daily. Linear regression lines are in the side panels. B) Gene expression of inflammatory mediators in the CNS of mice recipient of DC_{WT} is shown. C) Gene expression of inflammatory mediators in the CNS of mice recipient of DC_{iNOS^{-/-}} is shown Data are representative of three independent experiments. * $p < 0,05$ and *** $p < 0,005$.





**REDUCED SEVERITY OF EXPERIMENTAL AUTOIMMUNE
ENCEPHALOMYELITIS AFTER VIOLACEIN TREATMENT IS A RESULT
OF REGULATORY T CELLS INDUCTION AND INHIBITION OF THE
CENTRAL NERVOUS SYSTEM INFLAMMATION**

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Abstract

Violacein (viola), a quorum sensing metabolite from the Gram-negative bacterium *Chromobacterium violaceum*, has been shown to protect mice from malaria and to have beneficial effects on tumors until now. However, viola mechanisms of action are still unknown. In this study, we aimed to evaluate whether viola treatment would modulate the clinical course of Experimental Autoimmune Encephalomyelitis, an animal model of human Multiple Sclerosis. For that purpose, EAE-inflicted mice were given viola intraperitoneally at disease onset. Viola-treated mice developed mild EAE disease in contrast with placebo-treated mice. Also, in viola-treated mice, the frequency of regulatory T cells (CD4+Foxp3+) were found up-regulated while the expression of inflammatory mediators were found reduced in the Central Nervous System. When viola-elicited regulatory T cells were isolated and adoptively transferred to EAE-bearing mice, clinical course was significantly reduced. These findings show, for the first time, that violacein was able to reduce the clinical signs of EAE through the stimulation of regulatory T cells, opening new perspectives for the use of this compound in inflammatory-related diseases.

Keywords: Violacein, Experimental Autoimmune Encephalomyelitis, Regulatory T cells.

1. Introduction

Experimental Autoimmune Encephalomyelitis (EAE) is a murine model to study the human disease Multiple Sclerosis. The mechanisms that trigger the development of Multiple Sclerosis are not fully understood. However, it is well-established that EAE develops after encephalitogenic T cells have been activated in the periphery of the immune system and then extensively proliferated and migrated to the Central Nervous System (CNS), where they secrete inflammatory cytokines, enzymes and chemo-attractive molecules [1]. The local inflammation in the CNS is a problem normally bypassed after treatment with anti-inflammatory drugs. As the modulation of the immune system is a necessary step to achieve efficient treatments for chronic inflammatory diseases, several new drugs are being tested in animal models of autoimmune disease aiming their future use in the clinic. Drugs such as chloroquine, dihydroartemisinin, and primaquine have already been used to modulate EAE and experimental arthritis [2-5] and some of them are currently in use in the clinic [6]. Still, other compounds must be characterized for future treatment design.

Violacein (viola) is a compound derived from bacterium *Chromobacterium violaceum* and acts as a quorum sensing metabolite that also possess anti-microbial, anti-tumor and anti-malarial activity [7-9]. It was previously shown that viola treatment results in elimination of *Plasmodium falciparum* parasites within erythrocytes [10]. In addition, administration of viola has been demonstrated to inhibit tumor growth [11-13]. It is believed that viola acts through inhibition of cell growth and induction of apoptosis [11, 12]. Although the mechanism of action has not been fully characterized, these findings suggest that viola may present a broad range of action [7, 10-15].

In this context, we aimed to evaluate whether the treatment with viola would modulate the clinical course of Experimental Autoimmune Encephalomyelitis (EAE). Our results show that upon viola administration the EAE severity was significantly reduced in comparison with the placebo-treated group. In these viola-treated mice, levels of regulatory T cells were found augmented and CNS expression of inflammatory cytokine was abolished. The sole adoptive transfer of viola-elicited Tregs was able to mimic the results observed with the full compound administration. To our knowledge, this is the first study to show that the bacterial compound viola is able to suppress EAE through the inhibition of local inflammation and induction of Treg cells.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice (n=6, 6-8 week-old) were used in this study. Mice were obtained from the University of Campinas Center for animal care (CEMIB). All mice were specific-pathogen free and acclimatized in clean cages with autoclaved food and water *ad libitum*. The photoperiod was controlled (12h/12h, light/dark) as well as the temperature (22°C). All protocols involving laboratory animals were approved and performed in accordance with the guidelines of the University of Campinas' Committee on the Use and Care of Animals (CEUA) under protocol number 2687-1.

2.2. Experimental Autoimmune Encephalomyelitis (EAE) induction.

EAE was induced and evaluated in mice according to a previous published paper [16]. Briefly, mouse was injected with MOG₃₅₋₅₅ peptide (100µg, Genescript, USA) emulsified in Complete Freund's Adjuvant (CFA, Sigma-Aldrich, USA). Pertussis toxin (200ng, Sigma-Aldrich, USA) was administrated intraperitoneally at 0 and 48h after MOG immunization. Clinical signs of the disease were followed and graded daily according to a score method, where: 0-no sign, 1-limp tail, 2-hind limbs weakness, 3-hind limb paralysis, 4-hind paralysis and fore limbs weakness, 5-full paralysis/death.

2.3. Violacein (viola) production and treatment.

Viola was extracted from *Chromobacterium violaceum* as previously described [7, 10]. Briefly, violacein (3 - (1,2 -dihydro- 5-(5 -hydroxy- 1H -indol-3 -yl) -2-oxo -3H-pyrrol - 3ylidene) -1,3- dihydro -2H -indol-2 -one) was extracted through purification with chloroform, followed by diethyl ether and then ethanol. After evaporation of ethanol, the viola was purified by crystallization (methanol/water) followed by HPLC (High

Performance Liquid Chromatography). DMSO was used to dissolve viola due to its poor solubility in water. Lipopolysaccharide residues were not detected (using the Limulus Amebocyte Lysate QCL -1000 ® kit, Cambrex).

To verify drug toxicity, mice were intraperitoneally treated with viola at the doses 1.75; 3.5 and 7 mg/Kg. The treatment regimen consisted in daily intraperitoneal (i.p.) administration of viola for three consecutive days. Mice receiving the 7mg/Kg dose died after the second dose (*data not shown*). The maximum tolerable dosage was used to treat EAE-bearing mice. The treatment started at disease onset (around the 10th day after MOG immunization) and continued for three consecutive days. As control, naïve mice were treated with viola (viola group).

2.4. Analysis of the gene expression in CNS.

At the 20th day after EAE induction, mice were killed and the lumbar spinal cords were removed and frozen immediately. The frozen tissues of CNS were used for RNA extraction using Trizol (Life Technologies, USA) and cDNA synthesis was performed according to the manufacturer's recommendations (High Capacity c DNA Converter Kit, Life Technologies, USA). FOXP3 (Mm00475162_m1), *Indoleamine 2,3-Dioxygenase* (IDO) (Mm00492586_m1), *Inducible Nitric Oxide Synthase* (iNOS) (Mm00440502_m1), IL-17 (Mm00439618_m1), IL-10 (Mm00439614_m1), TGF-β (Mm00498255_m1), IFN-γ (Mm01168134_m1) and TNF-α (Mm00443258_m1) were analyzed in comparison to GAPDH (Mm99999915_g1, housekeeping gene) levels. RT-PCR reactions were performed using Taqman reagents according to manufacturer's recommendations (Applied Biosystems, USA). The results are shown as relative expression ($2^{-\Delta\Delta CT}$).

2.5. Analysis of cellular infiltration in the CNS.

At the 20th day after EAE induction, mice were anesthetized, perfused with ice cold PBS and the lumbar spinal cords were removed and prepared for the enrichment of infiltrating leukocytes according to a previously described methodology [17]. Briefly, spinal cord-derived cells were suspended in Percoll 30%, and then overlaid in Percoll 70%. The suspension was centrifuged for 30 minutes at 300g and no breaks. The cells in the interface were collected and washed in Phosphate-Buffered Saline (PBS, 0,02M pH7,2) and counted in hemocytometer.

2.6. Analysis of the frequency of regulatory T cells after viola treatment.

Naïve C57BL/6 mice were treated with viola and after the treatment, mice were killed and splenocytes were collected. Single cell suspensions were prepared and surface stained with fluorochrome-conjugated antibodies against: anti-CD4/PE-Cy5 (clone GK1.5), anti-CD3/APC-Cy7 (clone 145-2C11) and anti-CD25/FITC (clone PC61). For intracellular staining of Foxp3, the cells were treated with "fixation/permeabilization" buffers according to the manufacturer's recommendations (eBioscience, USA). Anti-FOXP3/APC (Clone FJK-16s) antibodies were added to the cells. All antibodies were from eBiosciences. Preparations (at least 50.000 events) were acquired in a Gallios Flow Cytometer (Becman Coulter, USA) and data analyzed using FlowJo 7.6 software (Tree Star Inc., USA).

2.7. Isolation of Tregs (CD4⁺CD25⁺) and transfer experiments.

Naïve C57BL/6 mice were treated with viola as described above and at the end of the treatment period spleen cells were collected and CD4⁺CD25⁺ cells were isolated using magnetic beads following manufacturer's instruction (Mouse Regulatory T Cell

Dynabeads, Life Technologies, USA). 5×10^5 Tregs per mouse were adoptively transferred (by the venous route) to EAE-inflicted mice at the onset of the disease (10 days after MOG immunization). As controls, EAE mice received equal numbers of CD4⁺CD25⁻ cells (effector T cells, Teff) at the same time-point. EAE induction and evaluation was performed as described above.

2.8. Statistical analyses.

Clinical score comparisons between control and experimental groups were carried out by Two-Way ANOVA and post-tested with Bonferroni. Other analyses among two and three (or more) groups were done with Student's t test and One-Way ANOVA, respectively. Results are expressed as mean $\pm$ standard error mean (SEM) and values of $p < 0,05$ were defined as significant.

3. Results

3.1. *Violacein treatment reduces the clinical score of EAE.*

Mice developed the EAE first clinical sign (limp tail) at the 10th day after MOG immunization. A group of mice was treated for three days with viola (3,5mg/Kg), the EAE+Viola group. As shown in Figure 1, the treatment with viola reduced the clinical course of EAE. Although mice were treated only for three days, the suppression of the disease persisted until the 30th day after immunization. The treatment triggered a statistically significant improvement in the disease progression (Figure 1).

3.2. *Reduced CNS inflammation and cellular infiltration in EAE mice treated with viola.*

The inflammation in CNS of viola-treated mice was evaluated. At the 20th day after MOG immunization, mice were killed and lumbar spinal cords were collected and

followed by mRNA extraction. The gene expression of inflammatory mediators and transcription factors were analyzed. PBS-treated mice bearing EAE presented significant high expression levels of iNOS, IL-17, IFN- γ , TNF- α and TGF- β compared with naïve and viola mice. There was no statistical differences in IFN- γ and TGF- β expression between EAE and EAE+Viola groups. However, in CNS from viola-treated EAE-bearing mice (EAE+viola group), the expression of Foxp3, IDO and IL-10 was significantly higher compared to the EAE control group (Figure 2A). In order to investigate whether the reduction in the expression of inflammatory mediators were related to the infiltration of cells, mice were killed at the same time-point as described previously. The infiltrating cells were enriched in density gradient centrifugation and counted. The results showed that the treatment with viola reduced the number of cells infiltrating the CNS in comparison with untreated EAE mice (Figure 2B).

3.3. Increased numbers of Regulatory T cells in mice treated with viola.

The increase in the expression of Foxp3, IL-10 and TGF- β following viola treatment suggests that regulatory T (Treg) cells are involved in the suppression of EAE. To assess this possibility, naïve mice were treated with viola at the doses 1.75, 3.5 and 7 mg/Kg. Mice that received the 7mg/Kg dose died shortly after the second dose (data not shown). After the administration of the last dose of viola, mice were killed and the splenic levels of CD4⁺CD25⁺FOXP3⁺ (regulatory T cells) were assessed. The data obtained showed that the treatment with viola promotes a significant up-regulation in the frequency of Treg cells when compared with vehicle-treated mice (Figure 3). There was no statistical difference between mice that received 1.75 and 3.5 mg/Kg dose.

3.4. Adoptive transfer of violacein-elicited Regulatory T cells reduces the clinical course of EAE.

To investigate whether viola-elicited Treg cells are responsible for the suppression of EAE, naïve mice were treated with viola as described previously. At the end of the treatment, mice were killed and spleen Treg cells were isolated and adoptively transferred towards EAE-bearing mice (at the 10th day after MOG immunization). As demonstrated in Figure 4, the transfer of Treg cells significantly reduced the severity of EAE when compared with control EAE mice and effector T cell (Teff, CD25⁺)-transferred EAE mice. Interestingly, the single transfer of regulatory T cells was able to produce a persistent amelioration of the disease (Figure 4).

3.5. Reduced EAE clinical course correlates with reduction in gene expression of inflammatory cytokine within the CNS.

The gene expression levels in the CNS of EAE-bearing mice recipient of Teff and Treg cells were analyzed and compared to the levels of EAE control mice. The results show that mice recipient of Treg cells presented significant higher levels of IDO expression when compared with the other groups (Figure 5A). Levels of IFN- γ , IL-17 and TNF- α were found down regulated in comparison with CD25⁺-recipient mice and EAE control group. Interestingly, levels of FOXP3 and IL-10 were not statistically different among groups (Figure 5A). To assess if these results are related to the infiltration of cells in the CNS, mice were killed and the infiltrating cells were enriched from the spinal cords. The results show that EAE-bearing mice recipient of Treg cells had significantly lower infiltrating cells than mice from the EAE and Teff-EAE groups (Figure 5B).

4. Discussion

In this paper, we show that the quorum sensing metabolite violacein is able to suppress autoimmune neuro-inflammation. Using the Experimental Autoimmune Encephalomyelitis model of human multiple sclerosis, we found that the administration of viola reduced the inflammation through the down-modulation of inflammatory mediators in the Central Nervous System. To our knowledge, we are the first group to present evidence that viola possess anti-inflammatory activity in autoimmunity.

The modulation of autoimmune diseases is a common goal in immunology research. Although the search for new pharmacological agents is necessary, some known compounds may present a broad range of action. In this context, antimalarial drugs have proven to be excellent anti-inflammatory agents [6, 18, 19]. It was previously shown that dihydroartemisinin reduced the clinical course of EAE through the inhibition of the mammalian target of rapamycin (mTOR) signaling pathway and increase in Treg numbers [3]. Likewise, we showed previously that, chloroquine is able to reduce the severity of Experimental Autoimmune Encephalomyelitis through the elicitation of regulatory T cells [2]. Thus, drugs that rise Treg cells numbers are desired to control autoimmunity [20, 21]. In this sense, we observed that viola-treated mice presented higher frequency of Treg cells than control mice. These viola-elicited Treg cells were, somehow, involved in the suppression of EAE, as high FOXP3 expression was detected in CNS of viola-treated mice, but in a much lower degree in the other groups.

Interestingly, the gene expression profile in the CNS was significantly changed after the treatment with viola. We observed a reduction in the expression of IL-17 in mice treated with viola, but not in EAE-bearing mice control group. IL-17 was recently recognized as a key player in autoimmune diseases, especially in EAE [1, 22-26].

Although it was shown that, inflammation continues in the CNS despite local production of IL-17 [27], our data indicate that the modulation of EAE by viola is intimately related to the reduced production of IL-17. Of note, IFN- γ expression levels were not changed in the treatment regimen, which indicate that the mechanism of viola-induced suppression is independent of this cytokine. Notwithstanding, viola-treated mice presented higher expression of IDO and IL-10. Dendritic cells expression of Indoleamine 2,3 - Dioxygenase (IDO) enzyme is believed to play a significant role in the suppression of activated effector T cells and induction of regulatory T cells [28-30]. This finding indicates that one of the actions exerted by viola involves the modulation of dendritic cells as well as induction of Treg cells. Further studies will be conducted in order to investigate this possible association.

The role of regulatory T cells in the anti-inflammatory activity of viola was confirmed when we transferred viola-elicited Treg cells towards EAE-bearing mice. Thus, the adoptive transfer was able to overcome the inflammatory process and suppressed the disease even after mice developed the initial symptoms of EAE. Similarly as in chloroquine-elicited Treg cells [2], these transferred regulatory T cells were generated in naïve mice (not in MOG-immunized mice), thus we are persuaded to believe that a bystander suppression was promoted by viola-induced Treg cells.

Other mechanism by which viola may have exerted its effects is the suppression of inflammation *in locu*. It is known that following MOG-immunization, encephalitogenic T cells migrate towards the CNS after 10 days, where they promote inflammation [1, 31, 32]. As the treatment with viola and the adoptive transfer of Treg cells were initiated at this time-point, it is highly probable that part of the anti-inflammatory effect of viola was exerted locally in the CNS. The following observations corroborate this hypothesis: (i) viola-treated mice presented as much as 10×10^5 cells in

the CNS, whereas mice recipient of Treg cells presented 0.2×10^5 cells and (ii) there were differences in the gene expression between EAE-viola and EAE+CD25⁺ groups, mainly in FOXP3 and IFN- γ expression. Taken together these findings indicate that the adoptively transferred regulatory T cells inhibited the migration of encephalitogenic T cells towards the CNS, whereas the treatment with viola suppressed the inflammatory cells that had already infiltrated the CNS.

Although viola demonstrated impressive therapeutic potential, this metabolite is toxic at large concentrations. Mice treated with viola at a 7mg/Kg dose succumbed after the second dose of the treatment. In Ehrlich ascites tumors, the treatment with viola induced apoptosis of the tumor cells through oxidative stress and imbalance in the antioxidant defense of cells [11]. A similar mechanism was observed in Caco-2, human colon cancer cells, where reactive oxygen species were induced at high levels leading to cell death [13]. Chloroquine presents similar effects in tumor cells and in lymphocytes [6]. Antimalarials are also known for their toxicity. In this context, we showed that dendritic cells are an interesting approach to bypass this hindrance. When DCs were modulated by chloroquine they acquired an immature phenotype and suppressed EAE upon adoptive transfer [16]. These results suggest that although toxic, some compounds may be used to modulate dendritic cells towards an inflammatory/anti-inflammatory profile to be used as an adjunct therapy. It is possible that viola-modulated dendritic cells acquire an immature phenotype as well as chloroquine-modulated DCs. Further studies must be conducted to ascertain this possibility.

5. Conclusion

In this study, we show that violacein, a quorum sensing metabolite, has anti-inflammatory activity through the stimulation of regulatory T cells and local suppression of inflammation. Although violacein is toxic at high doses, future studies will be conducted in order to overcome this toxicity through the usage of violacein-modulated dendritic cells.

Conflict of interest.

There was no conflict of interest associated with this work for any of the authors.

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Figure files and legends

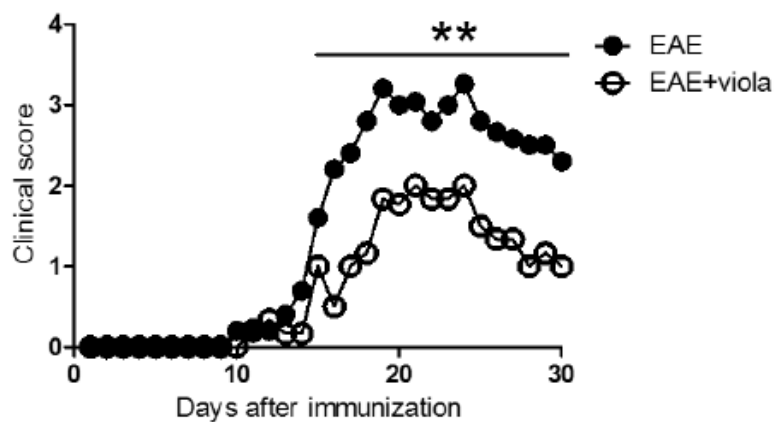


Figure 1 – Treatment with viola reduces the severity of Experimental Autoimmune Encephalomyelitis. C57BL/6 mice (n=6 mice/group) were immunized with MOG35-55 peptide emulsified in Complete Freund's Adjuvant (CFA) and intraperitoneally injected with Pertussis toxin (200ng) at 0 and 48h from immunization. Ten days after immunization, when the first clinical signs started to appear, mice were treated with viola (3.5 mg/Kg) for three consecutive days. Results show that the treatment reduced the severity of EAE. Representative data from three independent experiments. **p<0.01.

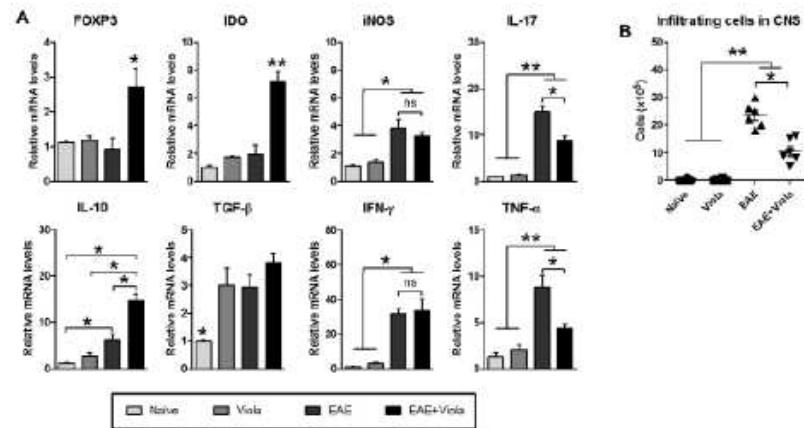


Figure 2 – Treatment with violacein reduces the severity of Experimental Autoimmune Encephalomyelitis through the modulation of CNS inflammation and infiltration of cells. C57BL/6 mice (n=6 mice/group) were immunized with MOG₃₅₋₅₅ peptide emulsified in Complete Freund's Adjuvant (CFA) and intraperitoneally injected with Pertussis toxin (200ng) at 0 and 48h from immunization. Ten days after immunization, when the first clinical signs started to appear, mice were treated with viola (3.5 mg/Kg) for three consecutive days. At the 20th day after immunization, mice were killed and lumbar spinal cords were removed and submitted to mRNA extraction protocols. A) The expression of FOXP3, IDO, iNOS, IL-17, IL-10, TGF-β, IFN-γ and TNF-α was evaluated and show that viola treatment changed the CNS gene expression profile of EAE-bearing mice. B) The infiltration of cells in the CNS was evaluated as well. For that, spinal cords were collected and enriched in T cells, which were counted in hemocytometer. Results show that the treatment reduced the infiltration of cells in the CNS of EAE-bearing mice. Representative data from two independent experiments. *p<0.05 and **p<0.01.

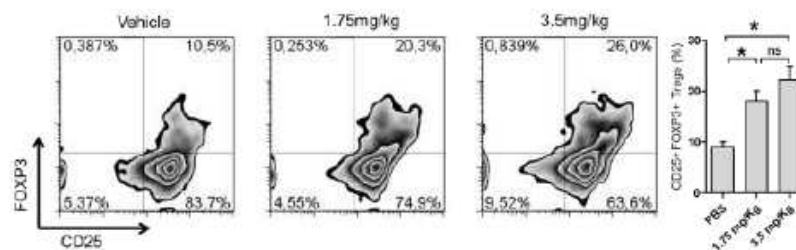


Figure 3 – Violacein treatment up-regulates the frequency of Regulatory T cells. C57BL/6 mice (n=6 mice/group) were treated with viola (1.75, 3.5 and 7 mg/Kg) for three consecutive days. Mice receiving the 7mg/Kg dose died after the second administration. After the last dose of viola, mice were killed and spleen cells were stained for flow cytometry analysis of CD4⁺CD25⁺FOXP3⁺ (Treg) cells. Results show an increase in the frequency of Treg cells after viola treatment. Representative data from three independent experiments. *p<0.05.

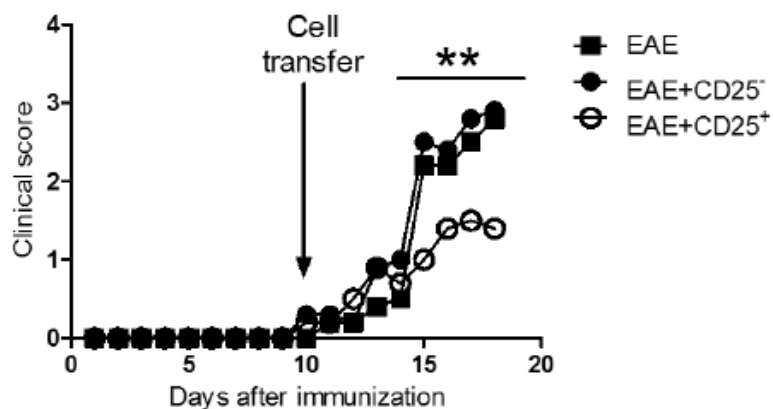


Figure 4 – Reduction in EAE severity after adoptive transfer of violacein-elicited Treg cells. C57BL/6 mice (n=6 mice/group) were treated with viola (3.5mg/Kg) for three consecutive days. After the last dose of viola, mice were killed and spleen cells were prepared for single cell suspension. CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were isolated using dynabeads, following manufacturer's instructions (Life Technologies). 5x10⁵ cells were adoptively transferred into EAE-bearing mice, at the 10th day after immunization (arrow), the clinical course was evaluated daily and showed reduction in the severity of CD4⁺CD25⁺-transferred mice compared to the other groups. Representative data from two independent experiments. **p<0.01.

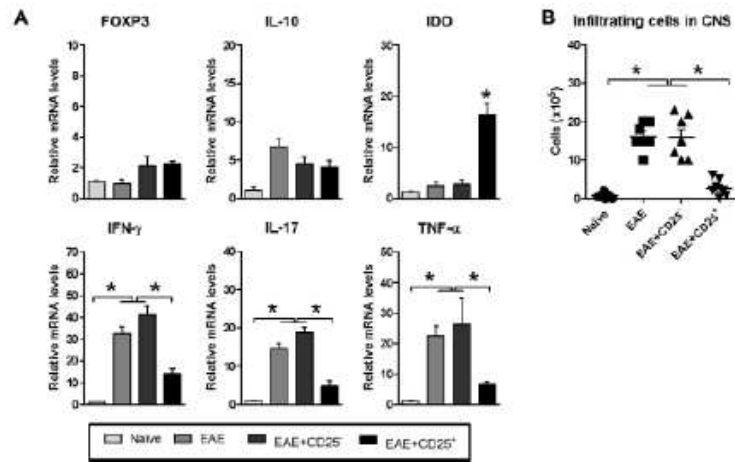


Figure 5 – Adoptive transfer of regulatory T cells elicited after violacein treatment reduces EAE through the modulation of CNS inflammation and inhibition infiltration of cells. C57BL/6 mice (n=6 mice/group) were treated with viola (3.5mg/Kg) for three consecutive days. After the last dose of viola, mice were killed and spleen cells were prepared for single cell suspension. CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were isolated using dynabeads, following manufacturer's instructions (Life Technologies). 5x10⁵ cells were adoptively transferred into EAE-bearing mice, at the 10th day after immunization, the clinical course was evaluated daily. At the 20th day after immunization, mice were killed and lumbar spinal cords were removed and submitted to mRNA extraction protocols. A) The expression of FOXP3, IDO, IL-17, IL-10, IFN- γ and TNF- α was evaluated. Results show that adoptive transfer of viola-elicited Treg cells changed the CNS gene expression profile of EAE-bearing mice. B) The infiltration of cells in the CNS was evaluated as well. For that, spinal cords were collected and enriched in T cells, which were counted in hemocytometer. Results show that the treatment inhibited the infiltration of cells in the CNS of EAE-bearing mice. Representative data from two independent experiments. *p<0.05.

1 Exacerbation of Autoimmune Neuro-inflammation in Mice Cured from Blood-
2 Stage *Plasmodium berghei* Infection

3

4

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19

20 Running title: Malaria-induced thymic atrophy aggravates EAE.

21

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24

1 **Abstract**

2

3 The thymus plays an important role shaping the T cell repertoire in the
4 periphery, partly, through the elimination of inflammatory auto-reactive cells. It has
5 been shown that, during *Plasmodium berghei* infection, the thymus is rendered atrophic
6 by the premature egress of CD4⁺CD8⁺ double-positive (DP) T cells to the periphery. To
7 investigate whether autoimmune diseases are affected after *Plasmodium berghei* NK65
8 infection, we immunized C57BL/6 mice, which was previously infected with *P.berghei*
9 and treated with chloroquine (NK65+CQ+EAE), with MOG₃₅₋₅₅ peptide and the clinical
10 course of Experimental Autoimmune Encephalomyelitis (EAE) was evaluated. Our
11 results showed that NK65+CQ+EAE mice developed a more severe disease than control
12 EAE mice. The same pattern of disease severity was observed in MOG₃₅₋₅₅-immunized
13 mice after adoptive transfer of *P.berghei*-elicited splenic DP-T cells. The higher
14 frequency of IL-17⁺- and IFN- γ ⁺-producing DP lymphocytes in the Central Nervous
15 System of these mice suggests that immature lymphocytes contribute to the aggravated
16 disease. To our knowledge, this is the first study to integrate the possible relationship
17 between malaria and multiple sclerosis through the contribution of the thymus.
18 Notwithstanding, further studies must be conducted to assert the relevance of malaria-
19 induced thymic atrophy in the susceptibility and clinical course of other inflammatory
20 autoimmune diseases.

21

22 **Keywords:** malaria, experimental autoimmune encephalomyelitis, thymus,
23 autoimmunity, double-positive T cells.

24

1 Introduction

2

3 The thymus plays a crucial role in the development and maturation of T
4 lymphocytes. Inside the organ, thymocytes (T cells in the thymus) are provided with a
5 wealthy microenvironment consisting of cytokines, chemokines and cell-cell
6 interactions that ultimately leads to proliferation, T-cell receptor gene rearrangements
7 and thymocyte differentiation into mature T cells¹. However, the thymus undergoes
8 weight and cellular loss caused by ageing and environmental disturbances, such as
9 infections and malnutrition². We have previously shown that infection with
10 *Plasmodium berghei* NK65 infection, the non-cerebral malaria pathological agent,
11 renders the thymus atrophic through the enhanced thymocyte death by apoptosis and
12 premature egress of CD4⁺CD8⁺ (Double-positive, DP) T cells to the periphery³⁻⁵.

13 It is already known that some viral and bacterial infections can promote the
14 development of autoimmunity by inducing the breakdown of T cell tolerance and
15 development of effector T cells reactive with the self-antigens or by the phenomenon
16 called molecular mimicry, where a foreign antigen shares sequence or structural
17 similarities with self-antigens^{6,7}. For instance, acute rheumatic fever, where antibodies
18 attack the heart, can occur after the body makes immune responses against Group A β -
19 hemolytic streptococci^{8,9}. In addition, it has been proposed that the prematurely
20 egressed DP-T cells observed during *Trypanosoma cruzi* infection play an important
21 role in the autoimmune cardio-inflammation¹⁰.

22 In this context, we aimed to evaluate whether the previous infection with
23 *Plasmodium berghei* NK65 would interfere with the clinical course of Experimental
24 Autoimmune Encephalomyelitis, a mouse model for human Multiple Sclerosis. We
25 observed that EAE-susceptible mice cured from malaria developed an aggravated form

1 of EAE, with increased infiltration of DP-T cells in the Central Nervous System (CNS).
 2 Further analyses showed that thymic-prematurely egressed DP-T cells were important
 3 for the enhanced clinical manifestation of the disease. To our knowledge, this is the first
 4 study to demonstrate the possible integration between malaria and EAE through the
 5 contribution of the thymus.

6

7 **Materials and Methods**

8

9 *Animals.* Six-to-eight week-old female C57BL/6 mice from the Multidisciplinary
 10 Center for Biological Research, University of Campinas, were used in this study. Mice
 11 were kept in specific-pathogen free conditions, in a controlled temperature and
 12 photoperiod environment, with free access to autoclaved food and water throughout the
 13 experiment. All protocols involving laboratory animals were approved and performed in
 14 accordance with the guidelines of the Institutional Committee on the Use and Care of
 15 Animals (CEUA, # 2687-1).

16

17 *Infection and treatment.* For these experiments, we used the NK65 strain of
 18 *Plasmodium berghei*, because this parasite does not migrate to the CNS of mice to cause
 19 cerebral malaria. Mice (n=6 mice/group) were intra-peritoneally injected with 10^6
 20 infected red blood cells (iRBC) obtained from a source mouse. The frequency of
 21 *P.berghei*-infected RBCs was assessed daily by examination with Giemsa-stained thin
 22 blood smear. Ten days after infection, animals of each group started the treatment with
 23 chloroquine (5mg.kg^{-1} , via i.p. for five consecutive days). Three days after the last dose
 24 of the drug, mice were immunized for the induction of EAE.

25

1 *EAE induction and evaluation.* EAE was induced and evaluated in mice according to a
 2 previous published paper ¹¹. Briefly, each mouse was subcutaneously injected with
 3 100µg MOG₃₅₋₅₅ (MEVGWYRSPFSRVVHLYRNGK, Genemed Syn, USA) emulsified
 4 with Complete Freund's Adjuvant (CFA, Sigma-Aldrich, USA). 200ng Pertussis toxin
 5 (Ptx, Sigma-Aldrich, USA) was administrated via i.p. at 0 and 48h after MOG₃₅₋₅₅
 6 inoculation. Clinical signs were followed and graded daily according to a score method,
 7 where 0: no sign, 1: flaccid tail, 2: hind limbs weakness, 3: hind limbs paralysis, 4: hind
 8 paralysis and fore limbs weakness, 5: full paralysis/dead. Disease severity was
 9 evaluated for thirty days.

10

11 *Cell sorting and flow cytometry.* Spleen cells were obtained from *P.berghei*-infected
 12 mice at ten days of infection. Total splenic T lymphocytes were isolated using
 13 Dynabeads following the manufacturer's instructions (Mouse Pan T cell isolation kit,
 14 Life Technologies, USA). For DP-T cell isolation, spleen-derived single cell suspension
 15 was incubated with FITC-conjugated anti-CD4 (clone H129.19) and PE-conjugated
 16 anti-CD8a (clone 53-6.7) for 20 minutes. The double-positive T lymphocytes
 17 (CD4⁺CD8⁺) were sorted in FACSaria II cell sorter (BD Biosciences, USA). The purity
 18 of both isolations was assessed and accounted for 98% pure subpopulations. 1.5×10^5
 19 DP-T cells were adoptively transferred (via i.v.) to each group of mice at the onset of
 20 EAE (around the tenth day after immunization). As controls, mice received total T cells
 21 obtained from naïve mice and EAE induction and evaluation was performed as describe
 22 above. In some set of experiments, total T cells isolated from naïve and malaria-bearing
 23 mice were transferred (1×10^6 cells/mouse) at the onset of EAE.

24

1 *Analysis of cellular infiltration in the CNS.* Fourteen days after EAE induction, mice
 2 were anesthetized, perfused with ice cold PBS and half of the spinal cords were
 3 removed and stored at -80°C until use for RT-PCR assays; the remaining tissue was
 4 prepared for the enrichment of infiltrating leukocytes according to a previously
 5 described methodology and analyzed by flow cytometry [34].

6

7 *RT-PCR assays.* Mice were killed at the indicated time-points and frozen tissues were
 8 used for RNA extraction (Trizol reagent, Life Technologies, USA) and cDNA synthesis
 9 according to the manufacturer's recommendations (High Capacity RNA-to-cDNA
 10 converter kit, Life Technologies, USA). RNA extraction from DP-T cells was carried
 11 out immediately after sorting, using an extraction kit according to the manufacturer's
 12 instruction (RNeasy Micro kit, Qiagen USA). Expression of AIRE (Mm00477461_m1),
 13 IL-7 (Mm01295803_m1), IL-6 (Mm00446190_m1), IL-17 (Mm00439618_m1), IFN γ
 14 (Mm01168134_m1), FOXP3 (Mm00475162_m1) and RAR-related orphan receptor C
 15 (RORc) (Mm01261022_m1) were analyzed in comparison to GAPDH
 16 (Mm99999915_g1, housekeeping gene) levels. RT-PCR reactions were performed
 17 using Taqman reagents according to manufacturer's recommendations (Applied
 18 Biosystems, USA).

19

20 *Histopathology and Immunofluorescence.* At the indicated time-points mice were
 21 killed, spinal cords were removed and snap frozen; 12 μ m thin slices were made in
 22 cryostat and stained with haematoxylin and eosin (H&E) for histopathological analysis.
 23 For the characterization of the infiltrating cells, the reactive sites of the slices were
 24 blocked with Phosphate-Buffer (0.1M, pH7.2)-BSA 3%. The cells were stained with
 25 FITC-conjugated rat anti-mouse CD4 (clone H129.19, at a 1:100 dilution, BD

1 Biosciences, USA) and PE-conjugated rat anti-mouse CD8a (clone 53-6.7, at a 1:100
2 dilution, BD Biosciences, USA). In some set of experiments, the lumbar spinal cords
3 were incubated with purified anti-mouse GFAP, COX-2, IL-6, NF- κ B, phosphorylated
4 iKB and iNOS. Later, secondary Cy3- or TRITC-conjugated antibodies were added.
5 The reaction was analyzed under epifluorescence microscope (Leica, GER).

6

7 *In vitro re-stimulation and cytokine dosage.* Splenic cells were aseptically collected
8 from mice after 10 days of MOG₃₅₋₅₅ immunization. Single cell suspensions were
9 stained with Carboxyfluorescein succinimidyl ester (CFSE, 2.5 μ M, Sigma-Aldrich,
10 USA) following the manufacturer's instructions. Cells (5x10⁵/well) were diluted in
11 RPMI 1640 media supplemented with Fetal Calf Serum (FCS; 10% vol/vol),
12 guaranine (50 μ g/mL), 2-Mercaptoethanol (2mM) and myelin oligodendrocyte
13 glycoprotein peptide (MOG₃₅₋₅₅; 20 μ g/mL), plated in U-bottom plates and incubated for
14 96h. After the incubation period, cells were stained with PercPCy5-conjugated anti-
15 CD3e, PE-conjugated anti-CD8a and PECy7-conjugated anti-CD4 antibodies and fixed
16 in 1% paraformaldehyde prior to flow cytometer analysis. CFSE^{low} cells inside each
17 population were considered proliferating T cells. Culture supernatants were collected
18 and assayed for cytokines (IL-4, IL-6, IL-10, IL-17, IFN- γ and TNF- α) secretion using
19 the Cytometric Bead Array (CBA, BD Biosciences, USA) according to manufacturer's
20 instructions.

21

22 *T cell co-cultures.* For the co-cultures, total spleen T cells were isolated from malaria-
23 bearing and EAE-inflicted mice after ten days of immunization. T cells derived from
24 EAE mice were stained with CFSE according to the manufacturer's instructions
25 (2.5 μ M, Sigma-Aldrich, USA) and seeded in U-bottom 96-well plates (5x10⁵

1 cells/well). T cells derived from naïve or *P.berghei*-infected mice were added
 2 afterwards at a 1:1 proportion. MOG₃₅₋₅₅ peptide was added to a final concentration of
 3 20µg/mL. The cultures were incubated for 96h at 37°C. At the end of culture time, the
 4 cells were collected and stained with PercP-conjugated anti-CD3e antibodies and fixed
 5 in 1% paraformaldehyde prior to flow cytometer analysis. CFSE^{low}CD3⁺ cells were
 6 considered proliferating responder T cells.

7

8 *Statistical analysis.* Clinical score comparisons between control and experimental
 9 groups were done by Two-Way ANOVA and post-tested with Bonferroni. Other
 10 analyses among two and three (or more) groups were carried out with Student's t test
 11 and One-Way ANOVA, respectively. Results are expressed as mean ± standard error
 12 mean (SEM) and p<0,05 value were defined as significant.

13

1 Results

2 *Exacerbation of Experimental Autoimmune Encephalomyelitis in mice cured from* 3 *P.berghei*

4 The present study aimed to evaluate whether the previous infection with
5 *P.berghei* NK65 would interfere with the clinical outcome of Experimental
6 Autoimmune Encephalomyelitis. For that purpose, C57BL/6 mice were infected with
7 parasitized erythrocytes and at the tenth day of infection, parasites were eliminated
8 following chloroquine (CQ) treatment. Three days after the last dose of CQ, mice were
9 immunized with MOG₃₅₋₅₅ peptide for the induction of EAE, the NK+CQ+EAE group.
10 As controls, naïve mice were treated with chloroquine before EAE induction, the
11 CQ+EAE group. The results showed that mice from the NK+CQ+EAE group developed
12 an aggravated disease course compared to EAE control animals with higher clinical
13 score (Figure 1A). As recently published, the CQ+EAE group developed mild clinical
14 scores (Figure 1A)¹¹. Thus, *P.berghei* infection was able to overcome the suppressive
15 effect of chloroquine.

16

17 *The aggravation of EAE correlates with DP-T lymphocytes reactivity to neuro-* 18 *peptides*

19 Since EAE model is highly dependent on the cellular response against neuro-
20 antigens, the cellular immune response against MOG peptide was evaluated. Spleen
21 cells from EAE-bearing mice were harvested ten days after neuro-peptide immunization
22 and cultivated in the presence of MOG₃₅₋₅₅. The data obtained showed that
23 NK+CQ+EAE-derived cells proliferated significantly more when compared to cells
24 from EAE control group. Surprisingly, double positive (DP)-T cells presented higher
25 proliferation levels than the other subpopulations (Figure 1B). Cells from CQ+EAE

group did not present significant proliferation nor the presence of DP-T cells in the spleens (*data not shown*). The cytokine secretion was altered as well. Cell culture supernatants from NK+CQ+EAE mice presented significant higher levels of pro-inflammatory cytokines (TNF- α , IL-17 and IFN- γ) compared with the EAE group, whereas the levels of IL-10, IL-6 and IL-4 remained similar between two groups (Figure 1C). Interestingly, cell cultures from CQ+EAE mice showed significantly higher levels of IL-10 and IL-4 whereas levels of IL-17, IL-6 and TNF- α were reduced compared with the other groups (Figure 1C).

9

Plasmodium berghei NK65 infection promotes thymic alterations and the premature egress of CD4⁺CD8⁺ double-positive lymphocytes to the periphery

The results presented so far show that the exacerbated clinical score from NK+CQ+EAE mice correlates with an enhanced cellular immune response towards neuro-antigens orchestrated, partly, by DP-T cells. The presence of DP-T cells in the spleens of NK+CQ+EAE mice is interesting. It was previously shown that during *P.berghei* infection, the thymus of BALB/c mice undergoes structural and phenotypical changes that together with the uncontrolled parasitemia culminates in death of the host fourteen days after infection ³. To investigate whether the infection with *P.berghei* would promote similar alterations in the spleens and thymuses of a different mouse strain, C57BL/6 (B6) mice were injected with infected Red Blood Cells. In this strain, the infection is controlled when chloroquine (5mg.kg⁻¹) is administrated for five consecutive days starting at day 10 after infection (Figure 2A). Similarly as in the BALB/c model, the thymus of B6 mice is reduced with loss of the thymic index, starting at the third day of infection (Figure 2B). Interestingly, the expression of the Autoimmune Regulator (AIRE) gene was found up-regulated compared with control

10

1 mice as well as the expression of IL-7 and IL-6 (Figure 2C). The gene expression within
2 the thymus of B6 mice infected with other *Plasmodium* species, such as *P.yoelli* and
3 *P.chabaudi* was also evaluated. Results showed that the gene expression of cytokines
4 and AIRE differs between groups (Supplementary Figure 1).

5 It is already clear nowadays that disarranged thymic structure associated with an
6 inflamed microenvironment result in altered maturation of thymocytes¹². Accordingly,
7 the thymocyte subpopulations were altered in the thymus of *P.berghei*-infected mice,
8 with reduced numbers of CD4⁺CD8⁺ (Double-positive, DP) thymocytes (Figure 2D). In
9 the spleen, results showed an increased frequency of DP-T lymphocytes as well as
10 CD4⁺ and CD8⁺ T cells. Naïve mice treated with chloroquine showed no alterations
11 regarding the frequency of DP-T cells in the spleen and in thymic T cell subpopulations
12 in comparison to mice without treatment (*data not shown*). Thus, it is possible that the
13 responding DP-T cells observed in the NK+CQ+EAE mice was elicited during
14 *P.berghei* NK65 infection. However, it is not clear yet whether these cells are related to
15 the exacerbated EAE outcome.

16

17 *Infiltration of inflammatory cells in the Central Nervous System is characterized by the*
18 *presence of DP-T cells and the local production of inflammatory cytokines*

19 To investigate the possible association of DP-T cells with the exacerbation of
20 EAE, the frequency of these cells in the Central Nervous System was evaluated.
21 Fourteen days after EAE induction, mice were killed and spinal cords were prepared for
22 histological analyses. The phenotype of the infiltrating T cells was determined by direct
23 immunofluorescence labeling technique using FITC-conjugated anti-CD4 and PE-
24 conjugated anti-CD8 antibodies. Results showed that in EAE group, among the
25 infiltrating cells, a high frequency of CD4⁺ T cells was observed, while no infiltration

1 was observed in the CNS of CQ+EAE mice (Figure 3). Surprisingly, an elevated
2 frequency of CD4⁺CD8⁺ (DP) T cells among the infiltrating cells in the CNS of
3 NK+CQ+EAE mice was identified (Figure 3). These data indicate that the double-
4 positive T cells that prematurely migrated from the thymus during *P.berghei* infection
5 are antigen-responsive (Figure 1B) and capable to migrate to the CNS during neuro-
6 inflammation (Figure 3). In addition, the CNS of NK+CQ+EAE mice showed an
7 increased production of IL-6, NF- κ B, phosphorylated κ B (κ B α -p), iNOS, COX-2 and
8 GFAP compared with EAE-inflicted mice (Supplementary figure 2).

9 In the CNS, an increased gene expression of IL-17 in tissue from NK+CQ+EAE
10 mice was observed in comparison with EAE mice (Figure 4A). In contrast, the
11 expression of Foxp3 and IL-10 was found down-regulated (Figure 4A). IFN- γ
12 expression was not significantly altered. To investigate whether the DP-T cells found in
13 the CNS tissue were able to produce inflammatory mediators, mice were killed at the
14 fourteenth day of EAE and the infiltrating cells were enriched from the CNS. The
15 intracellular staining results showed that DP-T cells from NK+CQ+EAE mice produced
16 high levels of IL-17 but not IFN- γ compared with cells from EAE group (Figure 4B).

17

18 *Transfer of lymphocytes from P.berghei-infected mice increases the severity of EAE*

19 In order to better characterize the relationship among malaria-elicited DP-T
20 lymphocytes and the exacerbation of EAE, a series of experiments to demonstrate that
21 the mechanism of disease aggravation is T-cell dependent was conducted. First, splenic
22 T cells isolated from *P.berghei*-infected or naïve mice were co-cultured with splenic T
23 cells from EAE-inflicted mice in the presence of MOG₃₅₋₅₅ peptide for four days.
24 Results showed that the cultures conducted in the presence of T cells from malaria-

1 infected mice proliferated significantly more than cells from co-cultures with naïve T
2 cells (Figure 5A).

3 Then, it was investigated whether the adoptive transfer of splenic T cells from
4 malaria mice would alter the course of EAE. For that purpose, B6 mice were infected
5 with *Plasmodium berghei* NK65 and the total splenic T cells were isolated ten days
6 after infection. These cells were adoptively transferred to EAE mice at the beginning of
7 the clinical signs (around the 10th day after immunization). As expected, the clinical
8 course of EAE was significantly aggravated in malaria T cells-transferred mice
9 compared to naïve T cell-recipient mice (Figure 5B).

10 Lastly, since the infiltrating T cells in the CNS of NK+CQ+EAE mice
11 comprised mostly of DP-T cells, the next goal was to evaluate whether these cells play a
12 role in the aggravation of EAE. Therefore, sorted splenic DP T cells from malaria-
13 infected mice were intravenously transferred to EAE mice as soon as the clinical signs
14 of EAE started to appear (around the 10th day after MOG₃₅₋₅₅ immunization). The
15 results showed that mice that received DP-T cells developed a more severe disease than
16 mice that received (naïve) T cells from control mice (Figure 5C). Interestingly, gene
17 expression analysis showed that the splenic double-positive T cell raised during
18 *Plasmodium* infection had higher levels of IFN- γ and ROR γ t mRNA copies compared
19 with splenic naïve T cells (Supplementary Figure 3).

20

21 Discussion

22

23 In this study, we show that, after infection with *Plasmodium berghei* NK 65,
24 MOG-immunized mice develop an aggravated form of Experimental Autoimmune
25 Encephalomyelitis. Further analysis demonstrated that the thymic prematurely egressed

1 CD4⁺CD8⁺ (double-positive) T cells play an important role in the enhanced severity of
2 the disease.

3 The thymus is a key organ for the development of T lymphocytes and plays a
4 major role in the elimination of auto-reactive T cells^{13, 14}. T cell precursors enter the
5 thymus through the high endothelial venules found in the cortico-medullary junction
6 and lack the expression of antigen receptor and co-receptors CD4 and CD8¹⁵. The
7 maturation process consists of both migration of thymocytes through the cortex towards
8 the medulla and expression of T cell receptor and co-receptors¹⁶. During this process,
9 immature T cells interact with a myriad of cytokines, chemokines and self-peptides
10 presented in association with MHC molecules on thymic epithelial cells (TECs) and
11 cells that recognize antigens with high avidity are deleted¹². We have previously shown
12 that the lethal *Plasmodium berghei* NK65 infection promotes thymic atrophy and that
13 this phenomenon is dependent on thymocyte death and its premature egress to the
14 periphery³⁻⁵. The increased expression of AIRE concurrently with IL6 and IL-7 mRNA
15 in *P.berghei*-infected mouse thymuses indicates that an altered repertoire selection
16 might be occurring. An impaired T cell maturation and selection in the thymus may lead
17 to the release of self-reactive T cells to the periphery and the sub-sequential
18 development of autoimmune disorders¹⁷⁻¹⁹.

19 Indeed, we observed that mice that were cured from *P.berghei* infection
20 developed a more severe form of EAE compared with uninfected animals. When we
21 analyzed the inflammatory infiltration in the CNS, we found that most of the infiltrating
22 cells were double-positive T cells. These DP-T cells were functional as they produced
23 high levels of IL-17, an inflammatory cytokine related to EAE worsening²⁰⁻²². As
24 expected, when we adoptively transferred total T cells or sorted DP-T cells derived from
25 malaria-bearing mice towards EAE-inflicted mice, the clinical score arose significantly.

1 Taken together, these set of experiments demonstrated that the DP-T cells elicited in
 2 *P.berghei*-infected mice are inflammatory and exacerbate autoimmune neuro-
 3 inflammation. Of note, co-cultures showed that malaria-elicited T cells stimulated the
 4 proliferation of T cells from MOG-immunized mice. Together with the proliferative
 5 response observed in DP-T cells from NK+CQ+EAE, these data suggest that DP-T
 6 cells, originated in the course of malaria, exacerbate EAE by, at least, two different
 7 mechanisms: (i) DP-T cells self-react and proliferate against neuro-antigens and (ii) DP-
 8 T cells stimulate the proliferation of encephalitogenic T cells.

9 The data presented here might be concerning as malaria remains the world's
 10 most prevalent infectious disease, with over 250 million people infected ²³. Several
 11 reports have correlated autoimmunity prevalence in countries that have eradicated the
 12 *Plasmodium* infection ²⁴. Although the hygiene hypothesis supports the idea of
 13 infection-induced autoimmune resistance, this theory may not fully apply to malaria.
 14 There is an increasing amount of evidence supporting or rejecting the role of malaria in
 15 autoimmune disease both in human and animal models ²⁵⁻³⁵. Similarly, it was previously
 16 shown that in *Trypanosoma cruzi* infection, the inflammatory profile of prematurely
 17 egressed DP-T cells from the thymus may be involved in the autoimmune process
 18 observed in murine and human Chagas' disease ¹⁰.

19 It was demonstrated that *Plasmodium chabaudi* infection increases the frequency
 20 of regulatory T cells in spleens, and these cells suppressed the development of EAE in a
 21 by-stander fashion ³⁶. However, *P.berghei* infection was shown to induce the secretion
 22 of anti-DNA and anti-nuclear antibodies in a T cell-dependent manner ³⁷. These
 23 observations indicate that different *Plasmodium* species induce distinct types of immune
 24 responses. Indeed, we observed that the gene expression of inflammatory cytokines and
 25 AIRE within the thymus vary depending on the type of *Plasmodium* species.

1 Nonetheless, infected Red Blood Cells from *P.falciparum*, *P.yoelli*, *P.berghei* and
2 *P.vivax* induce distinct maturation status of dendritic cells ³⁸⁻⁴⁸.

3 Based on our observations, we hypothesized that during malaria infection,
4 thymocytes prematurely egress from the thymus towards the peripheral immune system.
5 In the periphery, due to a trigger event (which can be genetic susceptibility, infection, or
6 radiation, for instance) these cells proliferate and migrate to the target organ, where they
7 produce high amounts of inflammatory cytokines leading to exacerbated inflammation
8 (Figure 6).

9 Further studies must be conducted in order to evaluate how these DP-T cells are
10 activated and whether they are antigen-specific or not. Although an increasing amount
11 of evidences show the presence of extra-thymic DP-T cells in the control of
12 inflammation ⁴⁹⁻⁵¹, our data clearly shows that in malaria infection, the thymic-derived
13 DP-T cells are inflammatory. To our knowledge, this is the first study that shows the
14 interplay between malaria infection and autoimmune neuro-inflammation through the
15 contribution of the thymus-derived double-positive T cells.

16

17

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25

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18

1 **Figure legends**

2 **Figure 1 – Aggravation of EAE in mice cured from malaria correlates with**
3 **increased cellular immune response towards myelin.** C57BL/6 mice (n=6
4 mice/group) were intraperitoneally (i.p.) infected with 1×10^6 *P. berghei*-infected Red
5 Blood Cells and treated with chloroquine (CQ, 5mg/Kg) for five consecutive days
6 starting at the 10th day after infection. Three days after the last dose of CQ, mice were
7 immunized with 100µg of MOG₃₅₋₅₅ peptide and Pertussis toxin was administrated (via
8 i.p.) at 0 and 48h after peptide immunization for EAE induction. A) The clinical course
9 of EAE was then monitored. Linear regression analyses are exposed in the side panels.
10 B) At the 10th day after MOG-immunization, the spleens of mice were collected and
11 dissociated. Total leukocytes (5×10^5 /well) were CFSE-stained (2,5 µM) and cultured in
12 the presence of MOG₃₅₋₅₅ (10µg/mL) peptide for 96h. At the end of culture period, the
13 cells were surface stained with anti-CD3/CD4/CD8 antibody cocktail and events were
14 acquired in a flow cytometer. The proliferation was analyzed inside CD4⁺CD8⁺ T cell
15 population. C) The culture supernatants were assayed for the secreted cytokines IL-10,
16 IL-4, IL-6, IL-17, TNF-α and IFN-γ. *: p<0,05, **:p<0,01 and ***: p<0,005.
17 Representative data of three independent experiments.

18
19 **Figure 2 – *P. berghei* infection provokes thymic alterations and the**
20 **premature egress of double-positive T lymphocytes in the spleens.** C57BL/6 mice
21 (n=6 mice/group) were intraperitoneally (i.p.) infected with 1×10^6 *P. berghei*-infected
22 Red Blood Cells and treated with chloroquine (CQ, 5mg/Kg) for five consecutive days
23 starting at the 10th day after infection. A) Mice treated with CQ showed decrease in the
24 parasite burden. B) The thymuses were collected at different time-points and the relative
25 body weight was determined. C) The gene expression of AIRE, IL-7 and IL-6 was

assessed. D) Flow cytometry analysis of the T lymphocytes subpopulations (CD4⁺ and CD8⁺) in the thymuses and spleens. *: p<0,05 and **:p<0,01. Representative data of four independent experiments.

Figure 3 – Central Nervous System of malaria-cured EAE mice show increased cellular infiltration of DP-T cells. C57BL/6 mice (n=6 mice/group) were intraperitoneally (i.p.) infected with 1x10⁶ *P.berghei*-infected Red Blood Cells and treated with chloroquine (CQ, 5mg/Kg) for five consecutive days starting at the 10th day after infection. Three days after the last dose of CQ, EAE was induced. As controls, naïve mice were treated with CQ or vehicle before EAE induction. The spinal cords of EAE-inflicted mice were collected fourteen days after MOG-immunization. Frozen thin sections (12µm) were made and fixed in formalin. Cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 and analyzed in epifluorescence microscope. Figures are representative of three independent experiments. Magnification: 200X.

Figure 4 – Inflammation in the CNS of NK+CQ+EAE mice correlates with an increased production of inflammatory cytokines by DP-T cells. Groups of mice (n=6 mice/group) subjected to infection and EAE induction. A) At the 10th day after MOG-immunization, mice were killed and spinal cords were removed to analyze the gene expression of IL-17, IFN-γ, Foxp3 and IL-10 in the lumbar spinal cords of mice. B) The infiltrating cells of the CNS were enriched and stimulated by Phorbol Myristate Acetate and Ionomycin in the presence of Brefeldin A for 4h. The frequency of IFN-γ-

1 and IL-17-producing cells inside CD4⁺CD8⁺ T cell gate was analyzed. *: p<0,05.

2 Representative data of three independent experiments.

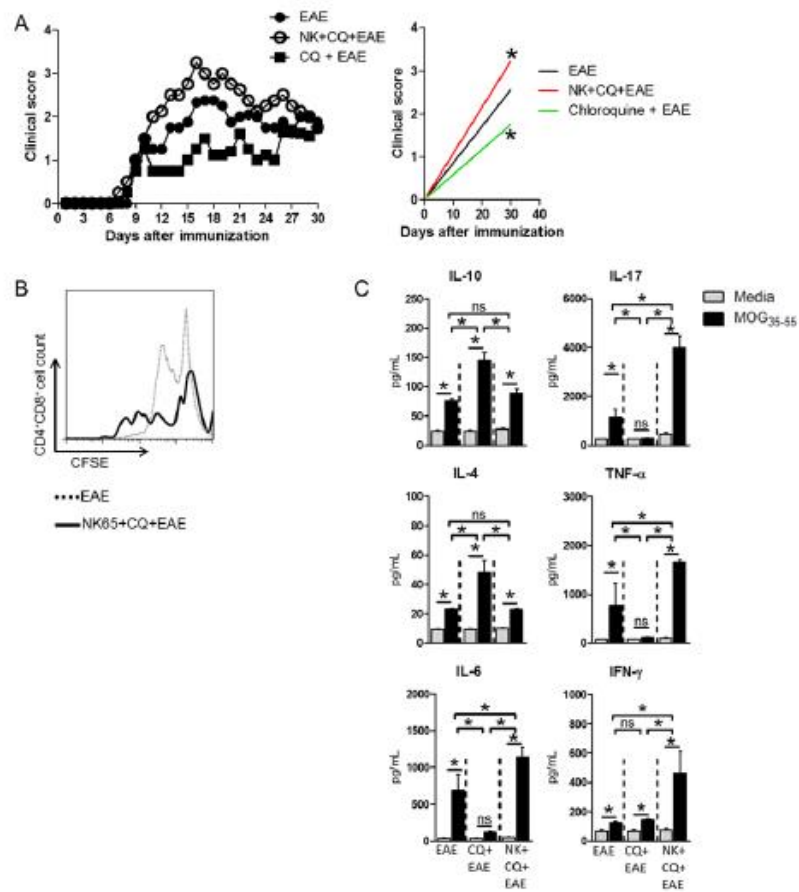
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4 **Figure 5 – Exacerbation of EAE in mice transferred with total T from naïve**
5 **mouse or DP-T cells from malaria-cured mouse.** A) C57BL/6 mice (n=6) were
6 intraperitoneally (i.p.) infected with 1x10⁶ *P.berghei*-infected Red Blood Cells. At the
7 10th day of infection, splenocytes were collected and total T cells were isolated using
8 dynabeads (Pan T cell isolation kit). Naïve-derived splenic T cells were used as well.
9 EAE-inflicted mice (n=6) were killed at the 10th day after immunization and the total
10 splenic T cell were isolated with the same methodology and CFSE-stained (2,5 µM). As
11 controls, T cells from EAE-inflicted mice were cultured without the presence of other
12 cells. B) Total T cells isolated from naïve and malaria-bearing mice were transferred
13 (1x10⁶ cells/mouse) at the onset of EAE (n=6 mice/group), and clinical course of the
14 disease was evaluated daily. C) Splenic CD4⁺CD8⁺ T cells from malaria-bearing mice,
15 or total splenic T cells from naïve mice, were sorted and adoptively transferred (1,5x10⁵
16 cells/mouse) to EAE-inflicted mice (n=6 mice/group) at disease onset. The clinical
17 score of the disease was evaluated daily. *: p<0,05. **: p<0,01. Representative data of
18 two independent experiments with similar results.

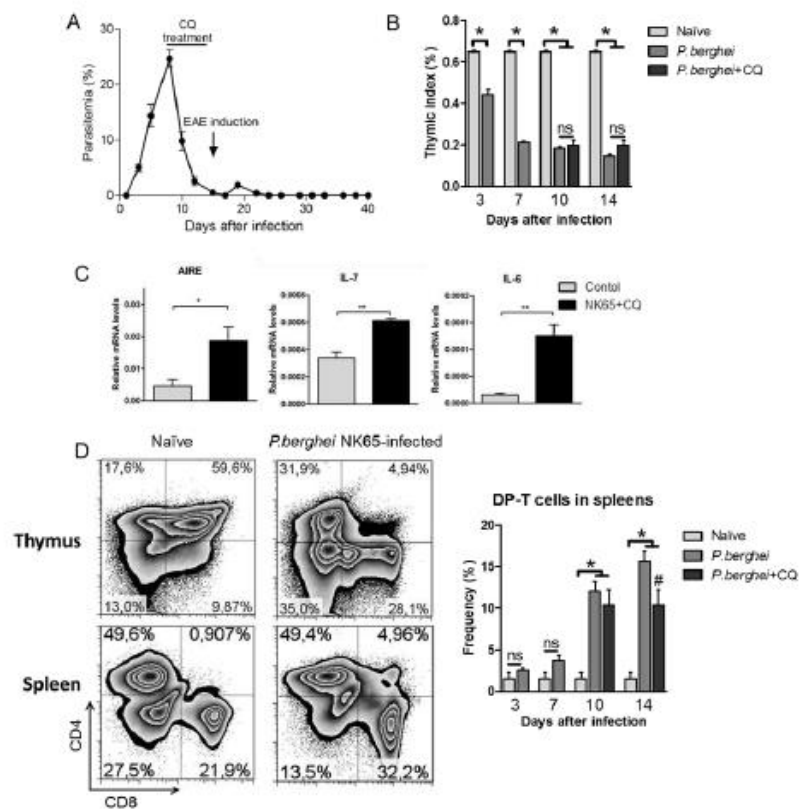
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20 **Figure 6 – Hypothesis model for the exacerbation of autoimmune neuro-**
21 **inflammation.** Based in our observations, we propose a model for EAE exacerbation.
22 Malaria infection promotes thymic atrophy and the premature egress of DP-T cells to
23 the peripheral immune system. After an inflammatory trigger, which can be infection,
24 genetic susceptibility or chronic inflammation, these cells proliferate and migrate to the
25 target organ where they stimulate CNS inflammation by secreting cytokines. However,

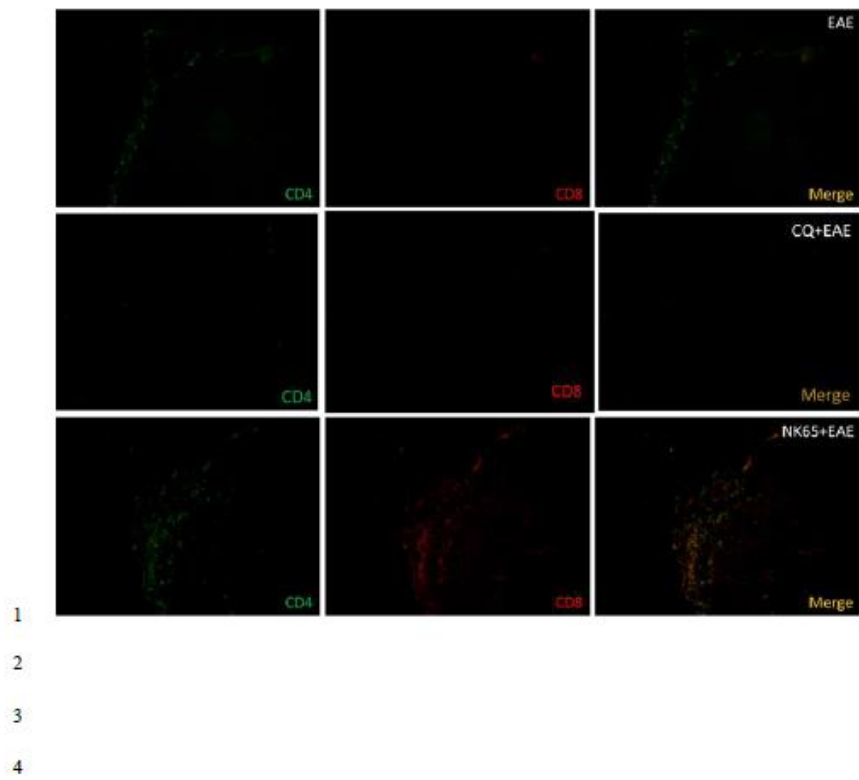
1 there is still much to be explored, as for example, whether these DP-T cells are able to
2 induce leukocyte recruitment, microglia and astrocyte activation, and, Blood-Brain
3 Barrier (BBB) destruction.
4

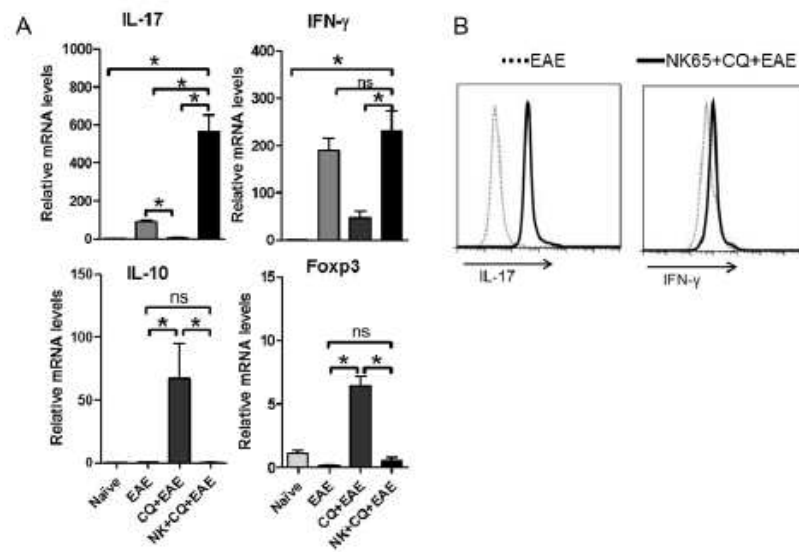


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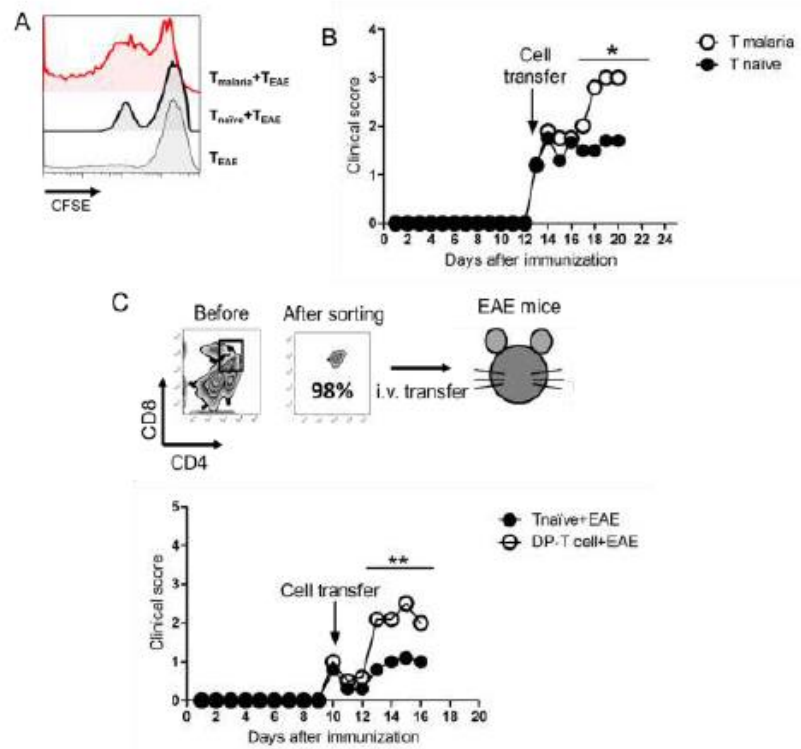


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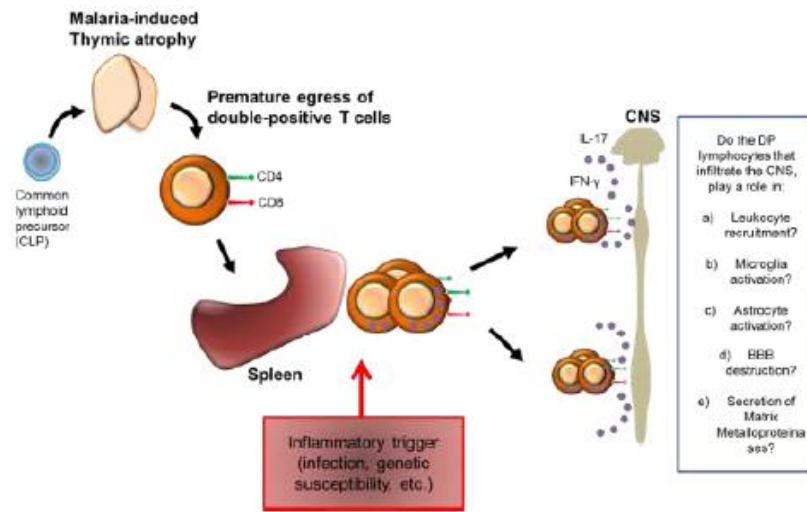


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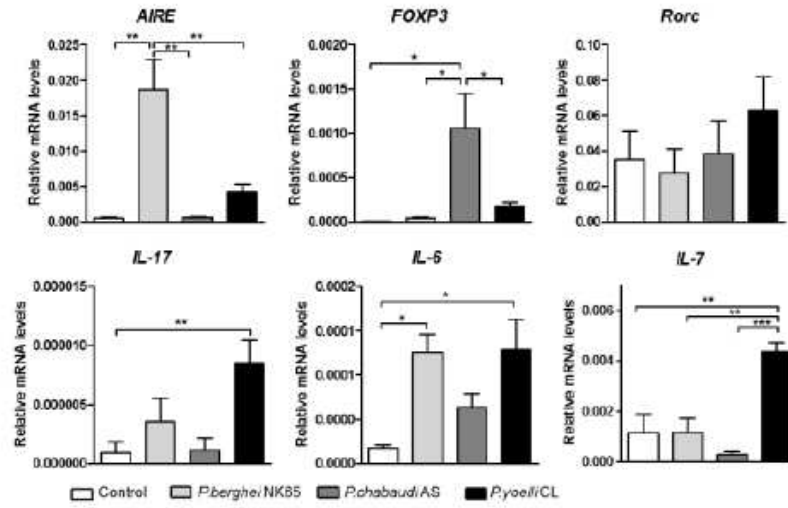
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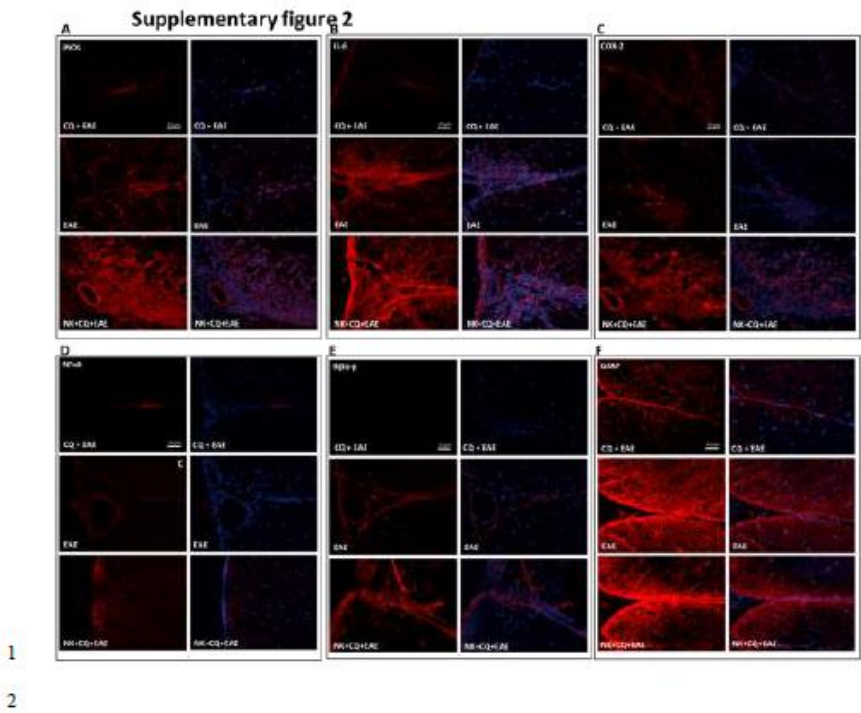
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Supplementary figure 1

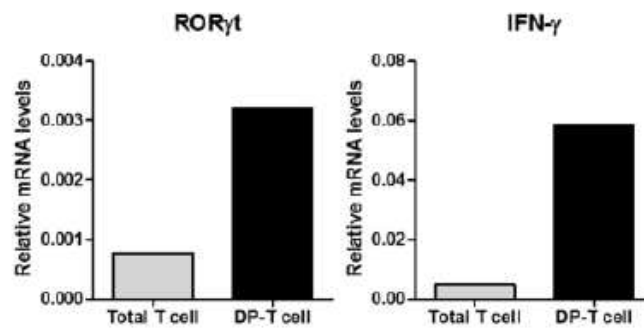


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Supplementary figure 3



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Outros artigos científicos

Effect of Helium-Neon (HeNe) laser irradiation on lesions in experimental paracoccidioidomycosis

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ABSTRACT

Cutaneous lesions caused by the fungus *Paracoccidioides brasiliensis* lead to ulcerative wounds, which are difficult to be cured by conventional treatment with anti-fungal drugs due to their adverse effects and the multidrug resistance that some fungal isolates present. So, the laser therapy emerges as an alternative treatment since its anti-inflammatory effects and wound healing properties are already known. In this work, we showed that lesions caused by the inoculation of yeast cells into the back foot-pad of BALB/c mice and treated with HeNe laser present greater histologic organization, milder inflammatory infiltrate, higher organization of the granulation tissue around the lesion, and enhanced immunolabeling for iNOS. In addition, an increased SOD activity and NO concentration, higher percentage of macrophages, and lower neutrophil numbers were observed. The percentage of NK and NKT cells were also slightly higher after the first laser session. Altogether these results point towards a dual effect of the laser treatment, decreasing the inflammatory response and accelerating the wound healing of the lesions. In this sense, the HeNe laser can be considered as an effective adjunctive treatment to be combined with pharmacologic therapies for

improving the treatment of painful non-healing paracoccidioidomycotic wounds.

KEYWORDS: low-level laser therapy, HeNe laser, skin lesions, inflammatory response, paracoccidioidomycosis, *Paracoccidioides brasiliensis*

INTRODUCTION

The thermodynamically human pathogenic fungus *Paracoccidioides brasiliensis* (Pb) is the etiological agent of the most important systemic mycosis in Latin America, the paracoccidioidomycosis (PCM) [1]. The infection starts in the lung, due to the inhalation of infecting propagules, but has a tendency to disseminate to other tissues or organs via lymphatic and/or blood vessels [2-5]. In the skin and mucous membranes the infection leads to painful and sensitive lesions where the fungi remain fully pathogenic and active for a long period even after the beginning of pharmacologic therapy. Furthermore, it is not uncommon that mucocutaneous lesions progress to ulcerative wounds that predispose the host to secondary microbial infection [6, 7].

Cutaneous lesions in PCM are conventionally treated with systemic antifungal agents, which include sulfonamides, amphotericin B, or imidazoles, but very few patients achieve full remission from treatment with these medications either when

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given alone or in combination. Besides, such therapies induce systemic adverse effects to the patients, are long-term treatments, and have their efficacy frequently questioned due to the multidrug resistance that some *Pb* isolates present [8]. So, the search for non-pharmacologic therapies, such as the low-level laser therapy (LLLT), which may also be effective alternative and/or adjunctive treatments for maximizing patient healing with avoidance of toxic effects [9], is necessary, desirable, and justified.

The helium-neon (HeNe) laser has emerged as an alternative treatment since its microbicidal activity and its ability to heal wounds without changing cell function are already well known [10, 11]. Although the exact mechanism of LLLT remains to be established, some reports document anti-inflammatory effects, improvement in tensile strength of wounds, and reduction of painful symptoms through the biostimulation of cellular events [12, 13].

Using an experimental model of paracoccidioidomycotic lesions caused by the inoculation of yeast cells into the back foot-pad of BALB/c mice, our group has explored the biological mechanisms underlying the inflammatory process after three sessions of HeNe laser treatment.

Our results demonstrated that HeNe laser-treated lesions presented reduction in footpad edema, faster cutaneous wound healing, confluent granulomas, diffuse and more loosely-distributed immunolabeling for Tumor Necrosis Factor- α (TNF- α), and enhanced labeling of Interferon- γ (IFN- γ) [14]. In another study, we reported alterations in lesion microenvironment after laser treatment such as inhibition of the CXCL10 chemokine secretion and hypoxia-inducible factor 1 (HIF-1) expression, increase of vascular endothelial growth factor (VEGF) expression, and decrease of reticulin fiber deposition [15]. Finally, in a more recent study, we have also observed decreased levels of pro- and anti-inflammatory cytokines and of chemokine ligand 3 (CCL3), as well as decreased density of fibronectin and laminin fibers in lesions exposed to HeNe laser irradiation [16].

The purpose of this work was to analyze some immune modulatory effects occurring early on the next day after the action of HeNe laser on the healing of paracoccidioidomycotic injuries to support and corroborate the data obtained previously.

MATERIALS AND METHODS

Animals

Specific pathogen free BALB/c male mice, 6-8 week-old, were purchased from Centro Multi-Institucional de Bioterismo (CEMIB) and maintained in transparent acrylic plastic isolators under aseptic conditions throughout the study, with sterile water and feed provided *ad libitum*. All procedures were carried out in accordance with the guidelines proposed by the Brazilian Society on Animal Care and approved by the animal care and use committee of the University of Campinas (protocol 1944-1).

Fungal strain

The virulent isolate Pb18 of *P. brasiliensis* was used throughout the experiments. Fungal cells were grown at 37°C in Fava Netto's medium and used at the 7th day of cell culture growth. Viability was determined by Lactophenol cotton blue staining [17] and was higher than 90%.

Infection of mice

Thirty mice received 5×10^6 yeast forms of *P. brasiliensis* contained in 50 μ l buffer into the left back footpad, followed immediately by 50 μ l sterile saline into the opposite one. Animals were monitored daily for the progression of lesions, which peaked at 7 days after inoculation.

Laser treatment

Seven days after *P. brasiliensis* infection, the animals were separated into twelve sets of five mice each (six sets in the experimental group and six sets in the control group). On days 7, 8 and 9 following infection, the lesions of mice from the experimental group were irradiated with laser (referred as T1, T2 and T3, respectively). The control group underwent the same handling, but was sham-irradiated (referred as non-treated animals). The laser used in this study was a helium-neon

laser with a measured output of 3.5 mW, which emits light in a collimated beam (diameter 4 mm) with a wavelength of 632.8 nm. The irradiating flux for each treatment (previously determined by dose response studies) was 3.0 J/cm² with a stimulation time of 110 seconds. The laser probe was applied punctually, in contact with the wound, without anesthesia or sedation. Twenty-four hours after each treatment, five animals from each group were killed by CO₂ inhalation and the footpads removed for analysis.

Histopathological analysis

The footpads were collected and fixed in 4% paraformaldehyde solution for 16 hours at 4°C. The specimens were submitted to diafanization with xylene, dehydrated by graded ethanol, embedded in paraffin and cut in 5-µm-thick sections. Histologic changes were evaluated on sections stained with hematoxylin and eosin (H&E).

Flow cytometry analysis of cell populations in paracoccidioidomycotic lesions

After sacrifice, the footpads from laser-treated and non-treated animals were removed, soaked and mashed in phosphate-buffered saline (PBS) containing 5% fetal calf serum. The solution obtained passed by a cell strainer with mesh pore size of 100 µm (BD, San Diego, California, USA) and was then centrifuged at 1000 g for 15 minutes at 4°C for subsequent cellularity evaluation. The red cells were removed by using ammonium chloride lysis buffer (0.16 M NH₄Cl, 0.01 M KHCO₃, 0.001 M EDTA, pH 7.4). Multi-color flow cytometric phenotypic analysis was performed with a Gallios device (Beckman Coulter) using fluorescent isothiocyanate (FITC), phycoerythrin (PE) and allophycocyanin (APC) as fluorescent dyes. Directly conjugated anti-mouse monoclonal antibodies were used: CD3, CD11b, Ly6G/6C (BD Pharmingen, San Jose, CA, USA), CD11b and NK1.1 (eBioscience, San Diego, CA, USA). For macrophages detection anti-mouse F4/80-like receptor was used with Mouse BD Fc Block™ purified anti-mouse CD16/CD32 mAb 2.4G2, the staining was amplified by using a biotinylated anti-rat IgG2a mAb RG7/1.30 second-step antibody followed by a "bright" third-step reagent, Streptavidin-APC (BD Pharmingen, San Jose,

CA, USA). Fluorochrome-labelled isotype-matched negative controls for the specific monoclonal antibodies were also obtained from Pharmingen. The assessment of neutrophil population was performed using the subtraction of macrophages population from CD11b and Ly6G and Ly6c positive cells. Cells were stained for 20 min and then washed with PBS, fixed with paraformaldehyde 4% and analyzed by flow cytometry in a Gallios device (Beckman Coulter). Analyses were performed after recording 10.000 events for each sample using Flow Jo 7.6.5 (Ashland, OR, USA).

Immunohistochemical analysis

The production of inducible isoform of Nitric Oxide Synthases (iNOS) by macrophages in laser-treated and non-treated lesions was done by using immunohistochemical method. The footpad sections were deparaffinized with xylene, hydrated in an ethanol gradient and water before stain reaction. Directly after, incubation was performed with 3% H₂O₂ in absolute methanol (v/v) to block endogenous peroxidase activity (10 min). From here all procedures were done according with ImmunoCruz™ rabbit ABC Staining System (sc-2018) datasheet. Briefly, all sections were incubated with goat serum to avoid secondary antibody nonspecific binding for one hour at 4°C and then incubated with specific primary antibodies to mouse iNOS both from AbCam (Cambridge, Cambridgeshire, UK) for 16 hours at 4°C. After washing, sections were overlaid for 1 h with the secondary antibody biotin-conjugated. This was followed by incubation with AB enzyme reagent to amplification of signal reaction. Bound antibodies were detected by reactivity with 3,3'-diaminobenzidine plus H₂O₂. After tap washing, the slides were counterstained by Harris Haematoxylin and mounted with Permount. For immunohistochemical controls primary antibodies were omitted from the staining procedure and were negative for any reactivity. Quantification of the immunostaining (integrated density of pixels/area) was done by using the Image J software [18].

Assessment of tissue nitric oxide (NO) concentration and superoxide dismutase (SOD) activity

After sacrifice, the footpads from laser-treated and non-treated animals were removed, soaked and

mashed in phosphate-buffered saline (PBS). The solution obtained was filtered with a syringe filter of 0.22 μm and then centrifuged at 1000 g for 15 minutes at 4°C. The SOD activity in the supernatant was measured using the SOD Assay Kit-WST (Sigma-Aldrich, USA) and standardized with SOD from bovine erythrocytes (Sigma Chemical Co., St. Louis, MO, USA). Nitrate (NO_3^-) + nitrite (NO_2^-) were measured in the supernatant using the Cayman Chemical Nitrate/Nitrite Colorimetric Assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). This assay converts NO_3^- to NO_2^- with nitrate reductase, and total NO is measured as total nitrite using the Griess reagent.

Statistical analysis

Mann Whitney test was used for statistical evaluation of the results comparing laser treated and non-treated lesions. Results are expressed as mean values $\pm$ SEM, and *p* values lower than 0.05 were considered to be statistically significant.

RESULTS

Histopathological analysis

The paracoccidioidomycotic lesions obtained from non-treated animals had the same pattern at

the 8, 9 and 10 days post infection, showing a disorganized epidermis and superficial dermis with an intensive mixed inflammatory infiltrate where some fungi can be seen (Figure 1A). At the center of the lesion, a substantial necrotic center with draining of fibrinous exudate, cellular debris and viable fungi was visible (Figure 1B). The animals that were treated with one session of laser irradiation (T1) showed the same morphological pattern observed with the untreated specimens, but with hyalinization of the collagen fibers on the connective tissue and lack of vacuolated macrophages against a larger area of granulomatous inflammation (data not shown). In mice treated with two sessions of laser irradiation (T2) the epidermis and superficial dermis showed greater histologic organization, milder mononuclear infiltrate and a light increase in collagen deposition (Figure 1C). At the center of the lesion, there was still a substantial necrotic center, but less viable fungi and a mixed inflammatory infiltrate are seen (Figure 1D). After three laser sessions (T3), the dermis presented a normal histological appearance with an intense collagen deposition (Figure 1E). At this time, the lesions also presented a higher organization of the granulation tissue pointing to the encapsulation of the lesion (Figure 1F).

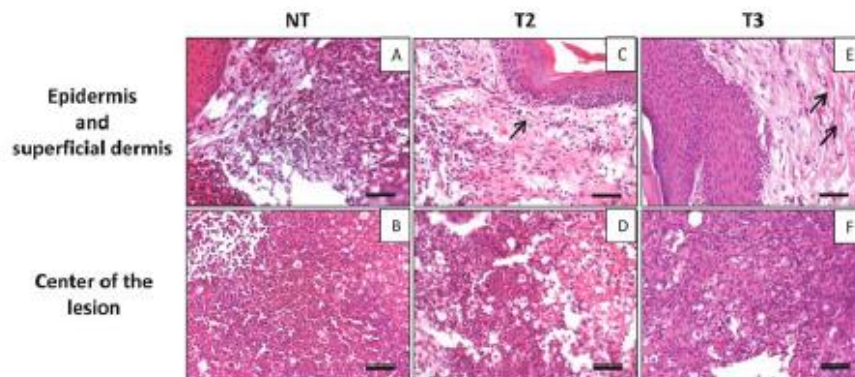


Figure 1. Histopathological features of PCM lesions treated or not with HeNe laser. Animals were inoculated with yeast forms of *P. brasiliensis* into the left back footpad, and 7, 8 and 9 days following infection the lesions were irradiated with laser. (A and B: non laser-treated animal (NT); C and D: animals treated with 2 laser sessions (T2); E and F: animals treated with 3 laser sessions (T3). Note that laser-treated lesions show greater histologic organization, milder mononuclear infiltrate and an increase in collagen deposition (arrows). The figures are representative of at least three experiments performed on different experimental days. Bar: 50 μm .

Flow cytometry

Cell populations present in the experimental PCM footpad lesions were analyzed by flow cytometry (Figure 2).

The analysis of lymphocyte subsets revealed a significant increase in the percentage of cells expressing NK and NKT cells associated markers soon after the first day of treatment (T1). After two (T2) and three laser sessions (T3), a gradual

reduction of these subpopulations was observed in the lesions and the same cell percentage in laser treated and non-treated animals at days 9 and 10 post infection was found (Figure 2A/D).

The analysis of the total phagocytes population showed that these cells correspond to more than 80% of the total cells present in the lesion, and no significant differences between laser treated and non-treated groups could be demonstrated (Figure 2B/D).

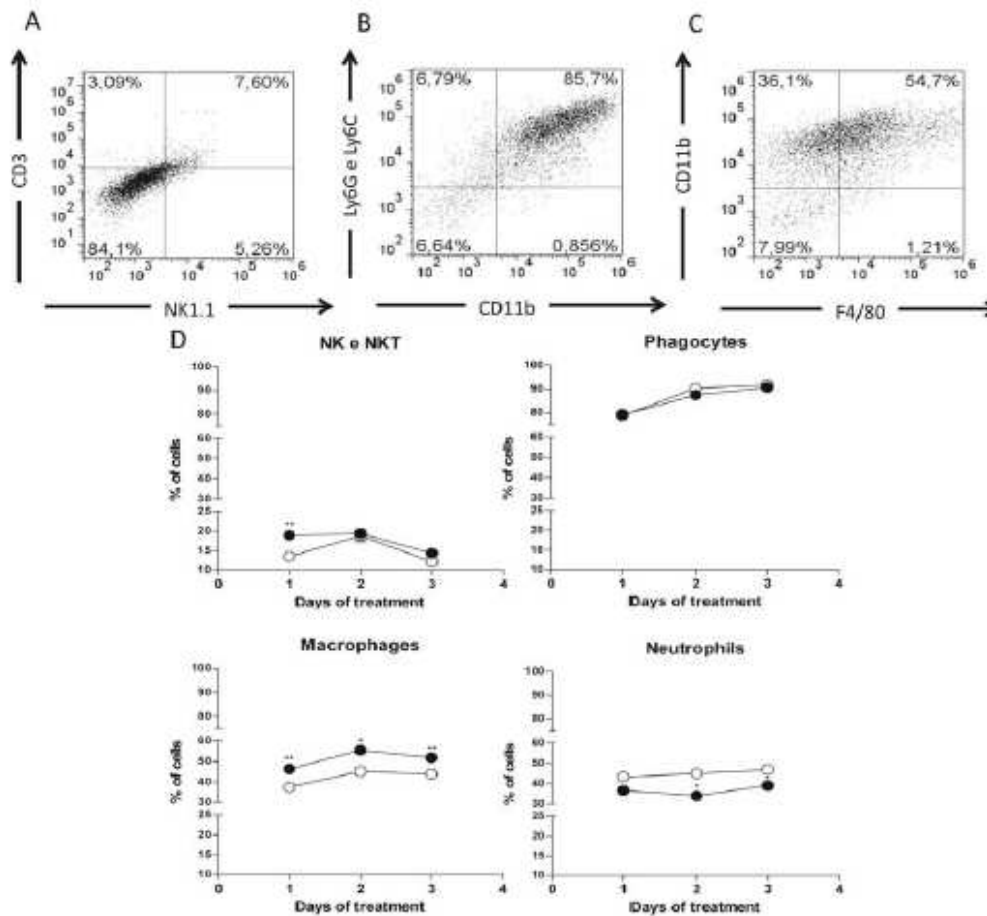


Figure 2. Analysis of cell populations at the experimental PCM lesion. A, B and C are representative dot-plots for: NK and NKT cell subsets, total phagocytes, and macrophage population, respectively. D: percentage of cells in lesions from non-treated (○) and laser treated animals (●). A higher percentage of macrophages and NK and NKT cells can be observed on the lesions after HeNe laser irradiation. Results are expressed as mean ± standard error (SEM) for at least five animals. * $p < 0.05$; ** $p < 0.01$.

However, the percentage of macrophages was higher (Figure 2C/D), while the percentage of neutrophils was significantly lower, in laser-treated animals than in the non-treated controls (Figure 2D).

Immunohistochemistry

The analysis of iNOS production, by the immunohistochemistry technique, revealed an

increased expression in laser treated animals when compared to non-treated ones (Figures 3A to 3D). While non-treated animals showed weak labeling for iNOS (Figure 3A), laser treated animals presented strong labeling for this molecule after one (T1), two (T2) or three treatments (T3) (Figures 3B, 3C and 3D, respectively). These results were quantitatively confirmed by histometric computer-based analyses (Figure 3E).

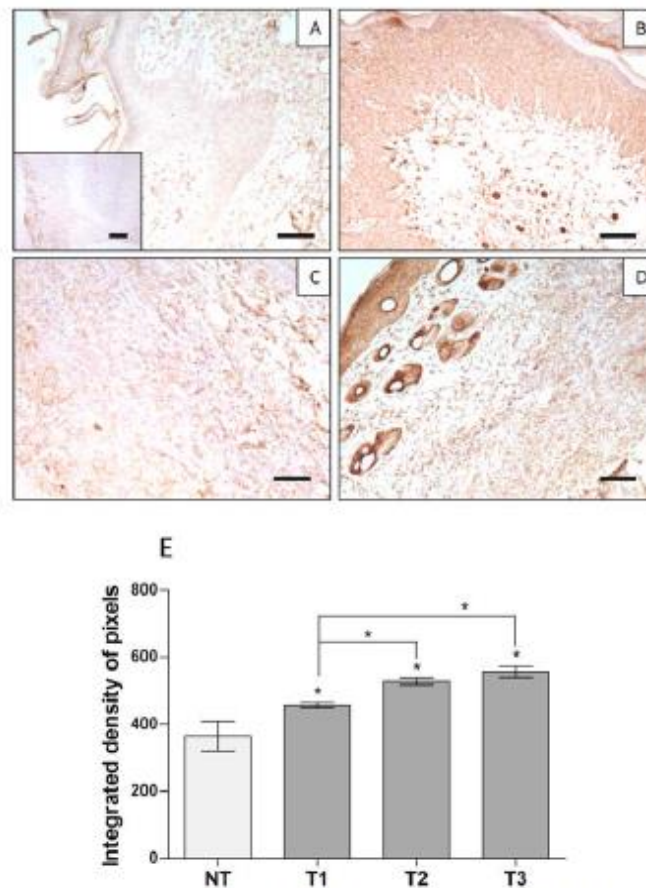


Figure 3. Immunohistochemistry staining for iNOS. A: Non-treated animals (NT); B to D: animals treated with one (T1), two (T2) or three (T3) laser irradiation session, respectively. Inset panel depicts negative control; no primary antibody. Note that laser-treated lesions present strong labeling for iNOS after treatment. The figures are representative of at least three experiments performed on different experimental days. Bars: 250 μ m (inset) and 100 μ m. E: The graphic corresponds to quantitative analysis of selected microscopic fields of lesions from laser-treated and non-treated lesions in terms of iNOS. Results are expressed in total pixels and represent the mean $\pm$ SEM for at least five animals. * $p < 0.05$.

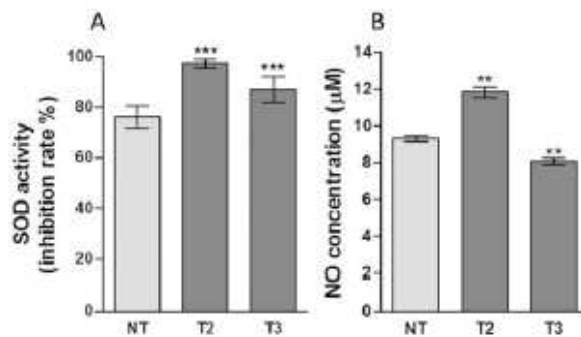


Figure 4. Superoxide dismutase (SOD) activity and tissue nitric oxide (NO) concentration in lesions from non-treated (NT) mice and after two (T2) or three (T3) laser treatment. Both the SOD activity and the NO concentration show a significant increase after two laser irradiation sessions and a reduction after three sessions of laser treatment. Results are expressed as mean $\pm$ standard error (SEM) for at least five animals. ** $p<0.01$; *** $p<0.0001$.

Superoxide dismutase (SOD) activity and nitric oxide (NO) concentration

The laser treatment of the PCM lesions leads to dramatic changes in the superoxide dismutase activity and nitric oxide production when compared to the non-treated group.

The SOD activity showed a significant increase after two laser irradiation sessions (T2). After three laser irradiation sessions (T3) the enzyme activity was reduced, but it remained significantly higher than the non-treated animals (Figure 4A). The nitric oxide concentration also significantly increased after two sessions of laser irradiation (T2). After the third day of treatment (T3), however, tissue NO concentration was diminished, remaining considerably lower than the non-treated group (Figure 4B).

DISCUSSION

The study of skin and soft-tissue lesions in PCM is important not only because of their frequency, difficulty of treatment and diagnostic value for the disease but also because they predispose patients to secondary bacterial infections [6, 7, 19].

Because of the discomfort evoked by PCM lesions and collateral drugs effects, the HeNe laser irradiation can represent an adjunctive, or even an alternative, therapy since its microbicidal effect, wound healing properties and non-invasive characteristics are already well known [9, 10, 20-23]. Although the exact mechanism of laser therapy

remains obscure, it is known that it reduces the time and improves the quality and tensile strength of tissue repair, has anti-inflammatory effects, and provides reduction of painful symptoms at injured tissue areas [12, 13, 24, 25].

In leukocytes, the therapeutic effect of HeNe laser begins with photo-chemical reactions that are able to enhance the phagocytic activity and to increase the synthesis of bactericidal substances, like nitric oxide (NO) and peroxide anion, by a mechanism that involves the increased permeability of cell membranes to ions including Ca^{2+} [23-25]. Moreover, under the action of HeNe laser the enzyme superoxide dismutase (SOD), which is responsible for catalyzing the reaction that converts superoxide radicals into oxygen and hydrogen peroxide (a potent killer of microbes), is reactivated [24, 26].

Histopathological results presented here showed that laser treatment of lesions was efficient in accelerating tissue repair since epidermis and dermis are better organized with increased collagen deposition and granulation tissue formation, which represent typical processes of fibrogenesis. Evidence that laser photostimulation increases collagen synthesis has already been obtained from different studies [14, 27-29]. Beyond these effects, lesions that were exposed to three sessions of HeNe laser irradiation present less viable fungi and this fact certainly minimizes fungal infection dermal effects.

NK and NKT lymphocyte subpopulations are critical in protection against pathogens [30-32]. In experimental PCM, NK cells are very effective during the precocious phase of the disease and are involved in natural host resistance against the fungus since they would be potential sources of interferon-gamma (IFN- γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) at an early stage of infection [30-34]. Data presented here show an increase in the percentage of NK and NKT cells in laser-treated animals. Previously, we have demonstrated increased levels of IFN- γ in animals treated with the laser, which may be, at least partially, produced by NK cells [14]. The increase of these cells in animals treated with one session of irradiation may be the result of a major attraction of these cells to injury, helping to control and limit the spread of the pathogen by both directly and/or inducing death by producing IFN- γ that is important for phagocyte activation.

The prolonged presence of neutrophils in PCM lesions has long been reported [18, 35, 36]. However, neutrophils are less efficient in eliminating *Paracoccidioides brasiliensis* as compared with macrophages, since many of them are susceptible to infection due to deficiencies in the process of phagocytosis of pathogens [37, 38]. Here, we observed that laser-treated lesions show reduced percentage of neutrophils and substantially increased percentage of macrophages.

Macrophages play a central role in wound healing and are the main phagocytic cells in tissue repair, removing pathogens in addition to the remaining fragments (debris) from tissues, cells and matrix [39, 40]. When activated at the injury site, they secrete large amounts of growth factors and cytokines amplifying the initial signals released by degranulation of platelets and neutrophils [39]. Some growth factors such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) stimulate proliferation of endothelial cells, which are likely to have pro-angiogenic activity in tissue healing [39]. So, the greatest number of macrophages in laser-treated lesions can indicate an antifungal response more efficient as well as a healing process more advanced, which is consistent with the histopathological findings.

It is well established that activated macrophages kill phagocytosed pathogens by converting molecular oxygen into reactive oxygen intermediates (ROIs), and by producing reactive nitrogen intermediates, particularly nitric oxide (NO), through the action of an enzyme called inducible nitric oxide synthase (iNOS). Indeed, in the current experimental model of PCM, we have observed that laser-treated mice present significant increase in iNOS protein expression and NO release.

This increase in NO release observed in laser-treated animals, is certainly related to greater fungicidal activity of macrophages and increased resistance to infection PCM, as already observed by other authors [41, 42]. Our experimental model also revealed that after three laser sessions the NO released is reduced. This may be related to an accelerated healing because of a greater elimination of the pathogen, and, therefore, a milder inflammatory process. In fact, the literature has shown that an overproduction of NO, or a continuous production over a long period, can enhance host susceptibility to the fungus, making wound healing difficult [43, 44]. *In vitro* experiments to evaluate the formation of nitric oxide, peroxynitrite, and superoxide anion radical in mice peritoneal macrophages exposed to HeNe laser radiation are being conducted in our laboratory and such results certainly will improve our understanding regarding the effects of laser therapy on macrophages.

Data presented here also show significant differences between laser-treated and non-treated animals in relation to the superoxide dismutase (SOD) activity. SOD is an enzyme which catalyzes the dismutation of superoxide radicals ($O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$) and such a reaction may play an important role in PCM lesion for two reasons. The first reason is that high concentrations of superoxide radical (O_2^-) can delay the healing process because of its interaction with NO to form peroxynitrite-anion with the subsequent formation of hydroxyl radical, which has an extremely potent cytotoxic effect [24, 45]. The second reason is that increased generation of hydrogen peroxide (H_2O_2) plays an important role in the elimination of microbes [42, 46, 47]. Thus, the increase in SOD activity observed in laser-treated animals results in both the reduced concentration of

superoxide, which leads to an acceleration of wound healing, and a greater release of H_2O_2 , which is an important inflammatory mediator involved in the resistance against *P. brasiliensis* [48, 47, 49, 50].

We have also evaluated the direct action of HeNe laser on *Paracoccidioides brasiliensis* yeast cells. Our results show that HeNe laser is able to decrease viability and fungal growth, as well as to increase the expression of fungal structures that are used by the pathogen as virulence mechanisms (personal report). It is most probably that these effects also play an important role in accelerating the wound healing process.

CONCLUSION

Altogether our results indicate a dual effect of the laser treatment, decreasing the inflammatory response and accelerating the wound healing of the lesions. In this sense, we believe that the HeNe laser can be considered as an effective adjunctive treatment to be combined with conventional drug therapy for improving the treatment of painful non-healing paracoccidioidomycotic wounds.

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Effects of helium-neon (HeNe) laser treatment on *Paracoccidioides brasiliensis* yeast cells

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ABSTRACT

In this work, we evaluated the effects of laser treatment on fungal cells, aiming to provide support for the use of this therapy during Paracoccidioidomycosis (PCM). HeNe laser treatment reduced the growth capacity and the viability of fungal cells, and increased gp43 expression. Laser-treated cells also showed pronounced structural alterations, since they were collapsed and presented deep folds. Additionally, remarkable changes were observed in the nucleus that presented a very electron dense form. The pathogenicity of *P. brasiliensis* after laser treatment was also evaluated, and no differences were observed in pulmonary lesions, comparing animals infected with laser-treated and non-treated yeast cells. Possibly HeNe laser is able to modify fungal structures involved in resistance and virulence mechanisms and therefore can be considered as an adjunctive therapy in the treatment of skin lesions observed during PCM.

KEYWORDS: low level laser therapy, HeNe laser, *Paracoccidioides brasiliensis*, yeast cells

INTRODUCTION

The human pathogenic fungus *Paracoccidioides brasiliensis* (*P. brasiliensis*) is the etiological

agent of Paracoccidioidomycosis (PCM), the most prevalent systemic mycosis in several Latin American countries [1]. This pathogenic dimorphic fungus grows in a mycelial form at environmental temperatures (20-26°C), and in a yeast phase at the temperature of the mammalian host (36-37°C). The disease is acquired by inhalation of dust containing the infecting propagules produced by the fungal mycelium. The infection takes place first in the lungs and afterwards is disseminated via the circulatory and/or lymphatic system to different organs such as the liver, spleen, adrenal glands, skin and mucosae, among others, where it induces severe lesions and remains viable even with antimycotic therapy [2].

The cutaneous lesions, which are extremely painful and sensitive, can progress to ulcerative wounds and predispose the patients to secondary bacterial infections [3, 4]. The currently available treatment with antifungal agents is frequently toxic at therapeutic dosages, and some drugs affect structures that are shared by humans and yeast causing side effects to patients.

Because of the discomfort caused by lesions and collateral drug effects, new non-harmful strategies are being proposed that can be used as adjuvant and/or alternative therapy to eradicate the pathogen and accelerate the cure of lesions. In this way, low intensity laser therapy using helium-neon (HeNe) laser irradiation can represent an excellent tool

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due to its microbicidal and anti-inflammatory effects, wound healing properties, and non-invasive characteristics that are already well known [5, 6, 7].

Previous studies from our laboratory, which explored the biological mechanisms underlying the inflammatory process of paracoccidioidomycotic lesions treated by HeNe laser, demonstrated the ability of this therapy to increase the production of proinflammatory cytokines as well as the synthesis of extracellular matrix components, leading to faster and more effective wound healing. In addition, fungal cells recovered from the lesions after laser treatment showed no capability of growth when cultured *in vitro* [8, 9, 10].

The present study evaluates the effects of HeNe laser treatment on *P. brasiliensis* yeast cells, aiming to provide additional support for the use of this therapy as an adjuvant tool to be combined with anti-fungal agents in the treatment of PCM ulcerations.

MATERIAL AND METHODS

Fungus

The virulent isolate Pb 18 of *P. brasiliensis* was maintained in the yeast form at 37°C in Fava Netto's culture medium, and was used at the 7th day of cell culture growth.

Laser treatment

P. brasiliensis isolate (Pb 18) in its yeast phase was grown in Petri dishes containing semi-solid BHI medium supplemented with 4% (vol/vol) fetal calf serum and 5% (vol/vol) yeast extract of *P. brasiliensis*, and incubated at 36°C. After seven days in culture, *P. brasiliensis* colonies were individually exposed to HeNe laser (incident energy of 3 J/cm²) for 110 seconds for three consecutive days. Twenty-four hours after the last exposure, *P. brasiliensis* cells were collected from the cultures; suspensions were prepared and cells were analyzed for growth inhibition, viability, and infectivity. For ultra-structural analyses, *P. brasiliensis* yeast cells were evaluated after 2 and 24 hours of laser treatment. Non-treated cultures were maintained in parallel and used as control.

Yeast cell viability by Trypan Blue

HeNe laser-treated fungal cultures were resuspended in PBS 0.15 M pH 7.4 and the concentration was adjusted to 1 x 10⁶ cells/mL. 50 µL of the suspension were incubated with 50 µL of Trypan Blue and dead cells were tallied in a Neubauer chamber.

Growth inhibition

Individual colonies were collected and resuspended in 1 ml PBS, washed and counted in a Neubauer chamber. Inoculum concentrations ranging from 10³ to 10⁷ cells/mL were plated in Petri dishes, incubated for 7 days at 36°C, and colony forming units (CFUs) were counted.

SEM

The morphological alteration of *P. brasiliensis* yeast cells were monitored by scanning electron microscopy (SEM). Laser-treated and non-treated *P. brasiliensis* yeast cells were washed in PBS 0.1 M pH 7.4 and fixed for 12 h in PBS 0.1 M pH 7.4 solution containing 2% of paraformaldehyde and 1.5% of glutaraldehyde. The samples were then fragmented and post fixed in 1% OsO₄ in 0.1 M cacodylate buffer for 2 h. After thorough rinsing in 0.1 M phosphate buffer, samples were dehydrated in a graded series of ethanol and transferred into liquid CO₂ in a critical point dryer. The dried specimens were mounted, sputter coated with gold, and examined under the scanning electron microscope JEOL 5800LV (Jeol BV, Zaventem, Belgium).

TEM

The ultra-structural alteration of *P. brasiliensis* yeast cells were surveyed by transmission scanning electron microscopy (TEM). For chemical fixation, immediately after collection, laser-treated and non-treated *P. brasiliensis* yeast cells were washed three times with PBS 0.1 M pH 7.4. Afterwards, samples were fixed overnight in PBS 0.1 M pH 7.4 solution containing 2% of paraformaldehyde and 1.5% of glutaraldehyde. After fixation, they were rinsed in PBS 0.1 M pH 7.4 and fragmented. Postfixation was done in PBS 0.1 M pH 7.4 solution containing osmium tetroxide 1% for 90 min, followed by a series of ethanol dehydration steps. The fragments were

then washed with increasing concentrations of propylene oxide and infiltrated with propylene oxide/Epon (1:1) and pure Epon resin. The sections were contrasted with uranyl acetate and lead citrate, and examined using a LEO 906 transmission electron microscope (ZEISS, Oberkochen, Germany).

Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed to analyze and compare the expression of the secreted fungal cell wall protein, the 43,000-Da glycoprotein (gp43), as previously described [11]. The membranes were incubated with an anti-gp43 polyclonal antibody (kindly provided by Prof. Ronei Mamoni from University of Campinas) for 1 h at room temperature. The membranes were washed in phosphate-buffered saline-Tween 20 (PBS-T) and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgG secondary antibody (Dako) in PBS-T for 1 h at 37°C. The sites of antibody-antigen reactions were visualized with a solution containing 5 mg of 3,3'-diaminobenzidine (Sigma) and 10 µl of H₂O₂ in 50 ml of Tris buffer, pH 7.4.

In vivo infection

Specific pathogen free C57Bl/10 male mice, 6-8 week-old, obtained from Centro Multi-Institucional de Bioterismo (CEMIB) were used. Animals were maintained in transparent acrylic plastic isolators under aseptic conditions throughout the study with sterile water and feed provided *ad libitum*. All animals received humane care in compliance with the guidelines proposed by the Brazilian Council on Animal Care (SBCAL) and the experimental protocol was approved by the animal care and use committee of the University of Campinas (CEUA/UNICAMP). One day after the last laser treatment of *Paracoccidioides brasiliensis* cultures, groups of fifteen mice were inoculated intraperitoneally with 5×10^6 viable yeast cells/animal. Control groups were also inoculated via i.p. route with 5×10^6 non-laser-treated *P. brasiliensis* cells. At days 7, 14 and 30 post infection, five animals from both experimental and control groups were sacrificed by cervical dislocation and lungs removed for histopathological analysis.

Lung histopathology

The lungs were removed surgically and fixed in 4% paraformaldehyde solution for 16 hours at 4°C. The specimens underwent diafanization with xylene, dehydrated by graded ethanol, and embedded in paraffin. Slices 5-µm thick were cut and stained with haematoxylin-eosin (HE).

Statistical analysis

When required, the Mann Whitney test was employed to determine the significant differences between control values and experimental values. The level of significance was defined as $p \leq 0.05$.

RESULTS

Yeast cell viability by Trypan Blue

The viability of *P. brasiliensis* yeast cells following exposure to HeNe laser irradiation was measured by Trypan blue assay. Figure 1 shows that the viability of laser-treated cells decreased by approximately 30%.

Growth inhibition

To evaluate the effect of laser irradiation on fungi growth, different inoculum concentrations of non-treated and laser-treated yeast cells were plated in Petri dishes, incubated for 7 days at 36°C, and colony forming units (CFUs) were counted. An important reduction of yeast growth was observed in laser-treated cells, reaching approximately 90% when a 10^7 cells/mL inoculum was used (Figure 2).

SEM

Laser-treated yeast cells were examined by scanning and transmission electron microscopy to evaluate the fungal structure after the treatment. Non-treated *P. brasiliensis* yeast cells presented a regular morphology with oval shape and multiple sprouts (Figure 3A). Observations made 2 hours after the first treatment and 24 hours (Figures 3B and 3C, respectively) after the last laser treatment, showed that yeast cells had pronounced structural alterations. Most of the yeast cells were collapsed and presented deep folds.

TEM

The ultra-structural features of non-treated *P. brasiliensis* yeast cells by TEM analysis (Figure 4A) include typical rounded cells with

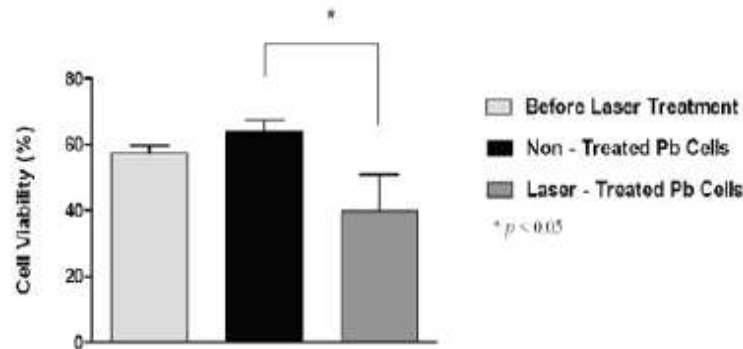


Figure 1. Effect of HeNe laser on *P. brasiliensis* cells viability. Fungus colonies were individually exposed to HeNe laser for three consecutive days, and one day after the last exposure yeast cells were collected from the cultures and analyzed for viability. There was an increase in cell death with HeNe laser exposure compared with control groups. Values are expressed as means $\pm$ SE, with $n = 6$ independent experiments.

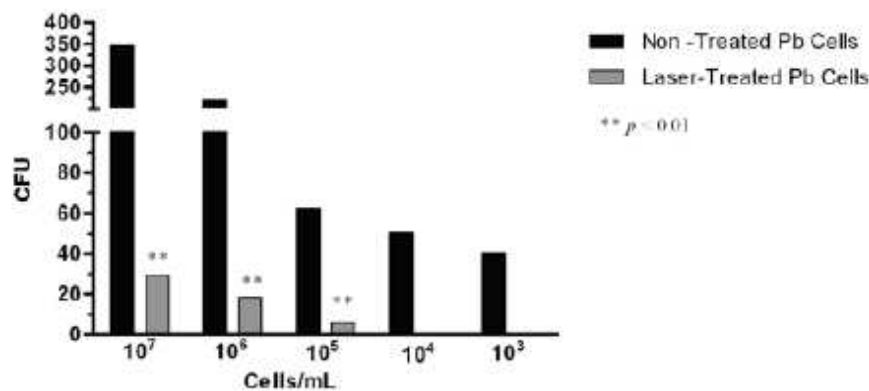


Figure 2. Effect of HeNe laser on *P. brasiliensis* cell growth. Fungus colonies were individually exposed to HeNe laser for three consecutive days, and one day after the last exposure yeast cells were collected from the cultures and analyzed for growth inhibition. A decrease in cell growth was noted after HeNe laser exposure compared with the non-treated group. Values are expressed as means $\pm$ SE, with $n = 6$ independent experiments.

thick walls of low electron density, easily visible plasma membrane, and several vacuoles and mitochondria varying in both shape and size. Laser-treated yeast cells showed both cell wall and plasma membrane intact and preserved (Figure 4B), despite structural changes observed in the SEM analysis. Also, mitochondria and vacuoles were not significantly different from non-treated yeast cells. However, laser-treated

yeast cells were more electron dense at the nuclear and cytoplasmic regions and showed less evident cytoplasmic membranes than non-treated ones.

Immunoblotting

The expression of the 43-kDa glycoprotein (gp43), the immunodominant antigen and the main antigenic component of *P. brasiliensis*, was analyzed by immunoblotting reaction obtained

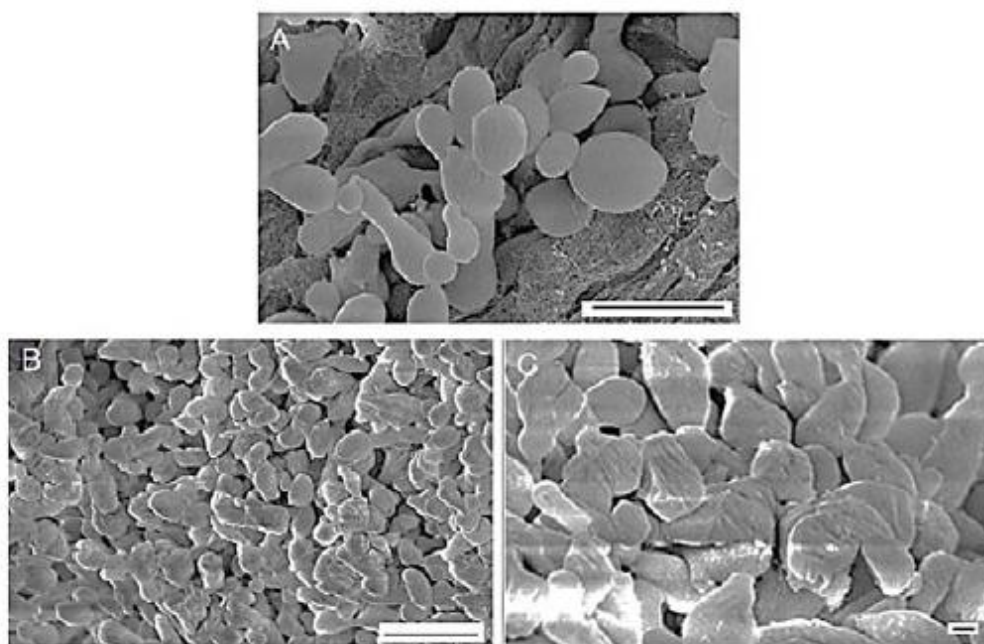


Figure 3. Representative SEM micrographs of non-treated (A) and laser-treated *P. brasiliensis* yeast cells collected two hours after the first treatment (B) and one day (C) after the last treatment. Note that laser-treated yeast cells present deep folds and are collapsed. Results are representative of 6 similar experiments. Bar: 10 μ m (A and B); 1 μ m (C).

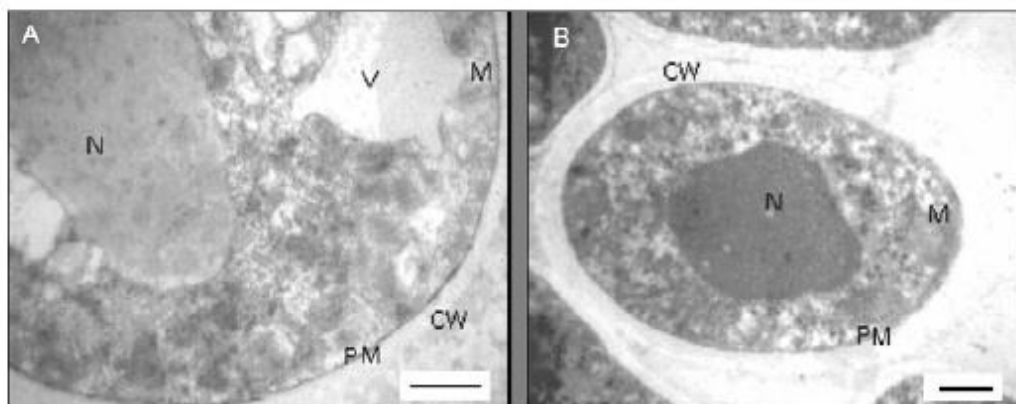


Figure 4. Representative TEM micrographs of non-treated (A) and laser-treated (B) *P. brasiliensis* yeast cells collected 24 hours after the treatment. Note that the laser-treated cell plasma membranes (PM) and cell wall (CW) are intact and preserved, while remarkable changes can be observed in the nucleus (N), which presents a very electron dense form. Mitochondria (M) and vacuoles (V) are not significantly different from non-treated cells. Results are representative of 6 similar experiments. Bar: 10 μ m.



Figure 5. Representative immunoblotting reactions obtained with non-treated (lane 1) and laser-treated *P. brasiliensis* cells (lane 2) probed with anti-gp43 polyclonal antibody. Note increase of gp43 expression in laser-treated *P. brasiliensis* yeast cells. S, standard purified gp43.

with crude exoantigen from non-treated and laser-treated yeast cells (Figure 5). Results show an increase of gp43 expression by laser-treated *P. brasiliensis* yeast cells in comparison with non-treated cells. This result was confirmed by ELISA assay (data not shown).

Lung histopathology

In order to evaluate the effects of laser irradiation on fungal pathogenicity, C57BL/10 mice were infected with non-treated and laser-treated *P. brasiliensis* yeast cells. Figure 6 shows the histology of the lesions in lungs after 7, 14 and 30 days of infection with non-treated (left panel) and laser-treated (right panel) yeast cells. At 7 days of infection (Figures 6A and 6B), the main

difference between the two groups was the presence of a more intense inflammatory process around blood vessels and newly recruited macrophages in lungs from mice infected with non-treated yeast cells. The presence of numerous yeast cells in clusters, edema, and congestion were also observed. However, at 14 (Figures 6C and 6D) and 30 days (Figures 6E and 6F) after infection no differences were observed in the characteristics of the lesions between animals receiving laser-treated and non-treated yeast cells, since both groups present a similar lung inflammation pattern and multiple granulomatous lesions with many giant cells presenting phagocytized fungal cells. Moreover, at this time of infection no difference in fungal load was observed between lungs from mice infected with non- or laser-treated yeast cells (data not shown).

DISCUSSION

A number of studies have reported positive results for the use of low-intensity laser with therapeutic purposes [12, 13, 14, 15, 16, 17, 18]. Laser therapy for skin lesions caused by *P. brasiliensis* has been studied by our group, showing inflammation reduction of the injured areas, and improvement of tissue healing [8, 9, 10].

Nevertheless, it has been argued that microorganisms might not be affected by the radiation produced by low-power lasers unless they are previously treated with a photosensitizer, such as Methylene blue [19, 20].

The use of different lasers as microbicides has been investigated previously, and the results indicate that they are effective against a variety of microorganisms [21, 22, 23]. Türkün and co-workers [24] have demonstrated antibacterial activity of Er, Cr:YSGG laser with two different power outputs on *S. mutans*. More recently, Yasuda and his group [25] have evaluated the effects of Nd:YAG and Er:YAG lasers on an *Enterococcus faecalis*-infected root canal model. The authors showed that both lasers have a bactericidal effect in infected root canals.

In this work, we investigated the effects of HeNe laser on *P. brasiliensis* yeast cells without using any photosensitizer. Our findings show that laser treatment was very effective in significantly reducing the viability and growth capacity of

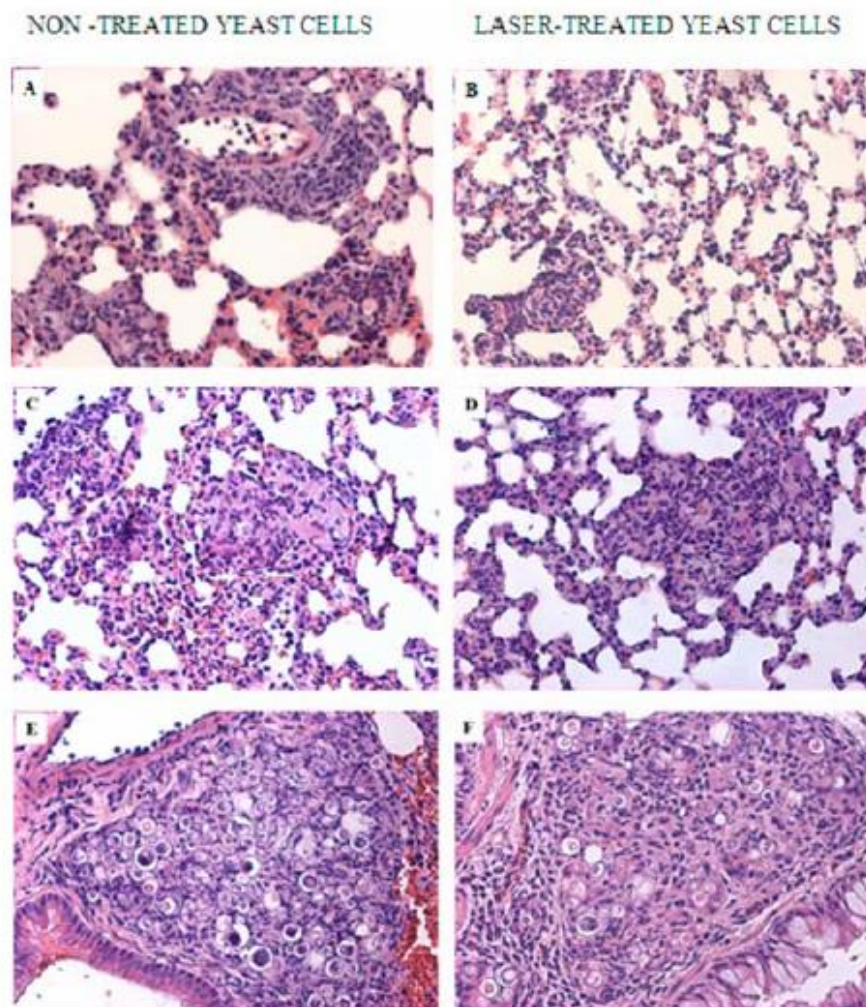


Figure 6. Representative histological findings in C57Bl/10 mouse lungs at 7 (A and B), 14 (C and D) and 30 (E and F) days post infection with laser-treated (right panel) and non-treated (left panel) *P. brasiliensis* yeast cells. At 7 days post-infection, a significant inflammatory infiltrate and the massive presence of macrophages in mice inoculated with non-treated yeast cells can be observed. At 14 and 30 days, however, histological changes are not different in the character or frequency of the pulmonary lesions in the animals that were inoculated with non- or laser-treated yeast cells. HE. Magnification: x 400.

P. brasiliensis yeast cells. These results partially disagree with those found by Andrade *et al.* [26] showing that *Pb* yeast cells lose their reproductive ability but retain the viability up to 21 days after gamma irradiation treatment.

Several cytological changes in HeNe laser-treated yeast cells of *Paracoccidioides brasiliensis* were also observed in this study. However, we could not detect any DNA damage caused by HeNe laser treatment as reported earlier by Andrade and

his group [26] that has demonstrated extensive DNA fragmentation, along with nuclear alterations, while studying the same yeast cells treated with gamma irradiation. It does seem that the HeNe laser, contrary to gamma irradiation, has no direct effect on the nucleus of fungal cells but more studies are necessary to evaluate this matter.

A notable observation in the treatment of yeast cells with HeNe laser was the increased expression of gp43, the most important antigen of *P. brasiliensis*.

One hypothesis to explain this fact is that HeNe laser alters the mechanism that regulates the expression of the gp43 molecule. Alternatively, it could represent a strategy adopted by the fungus to cope with the detrimental process caused by HeNe laser treatment.

This increased expression of gp43 certainly must explain the *in vivo* pathogenicity of *P. brasiliensis* even after laser treatment. Both hypotheses, as well as their implications for the pathogenicity of the fungus, are presently being contemplated in our laboratory.

Previously, we have reported that HeNe laser applied to the paracoccidioidomycotic lesion increases the production of proinflammatory cytokines and the synthesis of extracellular matrix components, improving wound healing [8, 9, 10]. The data reported here showing that HeNe laser has also a direct action on the fungus corroborates our previous findings, providing further evidence that HeNe laser can be an adjunctive therapy during PCM treatment.

CONCLUSION

Although the laser treatment cannot be used as a vaccine in preventing paracoccidioidomycotic infection, it can be useful in clinical practice as an additive and synergistic treatment in combination with drugs in order to accelerate the healing of cutaneous lesions caused by *Paracoccidioides brasiliensis*.

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Research report

Role of iNOS-NO-cGMP signaling in modulation of inflammatory and myelination processes

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ABSTRACT

Nitric oxide (NO) is the main activator of the soluble guanylate cyclase (sGC)-guanosine 3'/5' cyclic monophosphate (cGMP) pathway. The level of cGMP is regulated by phosphodiesterases (PDEs), which break down cGMP. It has been reported that levels of NO in the central nervous system (CNS) can greatly increase during demyelination and/or neuroinflammation. Controversially, in demyelination models, mice without iNOS may develop more severe cases of disease. Furthermore, cGMP accumulation caused by PDE inhibitors has an anti-inflammatory/neuroprotective effect in MS-models. The role of the NO-cGMP pathway in the nervous tissue is, therefore, complex and not fully understood. The aim of the present study was to contribute to existing knowledge of the role of this pathway in the CNS. Wild type (WT – C57BL/6) and iNOS^{-/-} animals were treated with sildenafil (25 mg/kg) for 8 weeks. Control animals were not treated. VCAM and ICAM (adhesion proteins), GFAP and Iba-1 (astrocyte and microglia markers, respectively), PKG (cGMP-dependent protein kinase), sGC, eNOS (constitutive endothelial NO synthase) and GSTpi (a marker of mature oligodendrocytes) were evaluated in the cerebellum using immunohistochemistry or western blotting. Myelin was assessed by luxol fast blue staining and electron transmission microscopy. Treatment with sildenafil reduced ICAM and VCAM levels (anti-inflammatory effect) and increased GFAP and Iba-1 expression (clearance phenotype) in WT animals. The expression of VCAM, ICAM, GFAP, PKG and sGC was lower in iNOS^{-/-} mice than in WT control animals. The treatment of iNOS^{-/-} animals with sildenafil resulted in an increase of all proteins (pro-inflammatory effect). There was overexpression of eNOS in untreated iNOS^{-/-} mice. The myelin structure of iNOS^{-/-} animals was damaged in comparison with WT control. Sildenafil increased GSTpi and resulted in an improved myelin structure in iNOS^{-/-} mice. In conclusion, NO-cGMP signaling plays a role in the regulation of inflammation and myelination processes. The accumulation of cGMP produced opposite effects in WT and iNOS^{-/-} mice. This can be explained by the overexpression of eNOS in iNOS^{-/-} mice, unbalancing cGMP signaling, or cGMP has a dual role in inflammation. Drugs that modulate the NO-sGC-cGMP pathway may be clinically beneficial in the treatment of neuroinflammatory/demyelinating disorders, but further studies of the regulation of this pathway are required.

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1. Introduction

Neuroinflammation involves complex signaling pathways. An increase of blood flow, cytokine interaction and the recruitment of a number of cell types can lead to a variety of events, inducing oxidative stress, excitotoxicity, cell death, angiogenesis and neurogenesis (Woodcock and Morganti-Kossmann, 2013; Kyritsis et al., 2014). In addition to peripheral inflammatory cell recruitment, resident cells (astrocytes and microglia) also

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contribute to the development and modulation of neuroinflammation (Forshammar et al., 2011, 2013). The neuroinflammatory process forms part of a number of pathological conditions, such as traumatic brain injuries (Woodcock and Morganti-Kossmann, 2013), strokes (Lartey et al., 2014), meningitis (Ramesh et al., 2013) and neurodegenerative/demyelinating diseases (Mc Guire et al., 2013). Control of this process remains challenging and the effectiveness of current techniques is limited.

Nitric oxide (NO) is an important molecule in inflammation modulation. NO is generated by three nitric oxide synthase (NOS) isoforms: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). It is also the main activator of soluble guanylate cyclase (sGC), an enzyme that synthesizes guanosine 3′5′ cyclic monophosphate (cGMP). The level of cGMP is regulated by specific phosphodiesterases (PDEs), enzymes that break down the phosphodiesteric bond of cGMP. Several PDE inhibitors have been developed and used as therapeutic agents as they increase cyclic nucleotide levels by blocking PDE function, enhancing NO-cGMP signalization (Puzzo et al., 2008).

The iNOS/NO system forms part of the pathogenesis of neuroinflammatory diseases, and NO levels are greatly increased in the CNS during inflammatory demyelination in both patient and animal models (Danilov et al., 2003; Lieb et al., 2003). Nevertheless, it has been found that mice lacking iNOS exhibit higher levels of demyelination than wild type mice, and increased levels of disease have been observed in the demyelinating model induced in iNOS^{−/−} mice (Fenyk-Melody et al., 1998; Arnett et al., 2002) and after iNOS inhibition (Ruuls et al., 1996; Kahl et al., 2003). Furthermore, studies have shown that the selective PDE5 inhibitors sildenafil (Viagra; Pfizer) and vardenafil (Levitra; Bayer), and the PDE4 inhibitor ibudilast increase levels of cGMP/cAMP in the brain and have a protective effect, including improved memory in aged rats (Prickaerts et al., 2002) and mice (Baratti and Boccia, 1999), decreased white matter damage in the event of ischemic cerebrovascular injury (Wakita et al., 2003), and protection of multiple sclerosis (MS) patients from neurodegeneration through increased gray matter perfusion in the brain (Manson et al., 2005). Recently, the authors of the present study showed that sildenafil improves myelination and decreases neuroinflammation in a cuprizone-demyelinating model (Nunes et al., 2012). Subsequently, it was shown that untreated iNOS^{−/−} animals have damaged myelin, compared to wild type mice, and that these animals suffered severe neuroinflammation following cuprizone treatment (Raposo et al., 2013). Also, although the neuroinflammatory effects of sildenafil were partially negated, myelin conditions were improved in iNOS^{−/−} mice (Raposo et al., 2013). Therefore, the NO-cGMP pathway is important for the control of neuroinflammation and myelination, although its role in these processes is complex and not yet fully understood.

The aim of the present study was to add to existing knowledge of the role of the NO-cGMP pathway in the CNS. Wild type and iNOS^{−/−} mice, either treated with sildenafil (Viagra®, Pfizer) or untreated, were used to study the role of iNOS-generated NO and of cGMP production in the modulation of inflammatory and myelination processes. The study of the role of signaling pathways in the regulation of neuroinflammation and in the control of myelination may contribute to the development of new strategies for the control of neuroinflammatory/demyelinating diseases.

2. Material and methods

2.1. Experimental design

Pubertal B6.129 P2-Nos2 (knockout iNOS) mice (obtained from Jackson Labs and maintained in the Centro de Pesquisas Aggeu Magalhães/FIOCRUZ, Recife, Brazil), and genetic background C57BL/6

wild type mice, aged 25 days and weighing 15–20 g were used in all experiments ($n = 5$ per group). The health of the mice was examined and the animals were acclimated to the laboratory environment at 25 °C and a 12 h light:12 h dark photoperiod. The animals were housed in metal cages and fed a standard laboratory diet. The experimental groups (iNOS^{−/−} and wild type) received 25 mg/kg body weight of sildenafil (Viagra®, Pfizer), for 8 weeks, administered through drinking water, according to previously standardized methodology (Zhao et al., 2003; Saraiva et al., 2009). Since the drug is soluble in water, pure water was used as a vehicle. Body weights were recorded every day and the drug concentration in the water was adjusted to maintain the dose. The control groups (iNOS^{−/−} and wild type) received only pure water. The sildenafil dosage (25 mg/kg) was based on a previous study (Nunes et al., 2012). All experiments were carried out according to ethical guidelines (L-0035/08-CEUA/FIOCRUZ, CIBIO 06/10). After the treatment period, the experimental and control animals were anaesthetized with ketamine (115 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.).

2.2. Immunohistochemistry

After anesthesia, the animals were transcardially perfused with physiological saline (20 ml), followed by 4% paraformaldehyde (Sigma-Aldrich) (40 ml) in 0.1 M phosphate (sodium phosphate monobasic and dibasic heptahydrate, Sigma-Aldrich) buffered saline (PBS), pH 7.2. The cerebellum of each animal was immediately removed and post-fixed overnight in the same fixative. The samples were dehydrated in an ethanol series (Isolar Chemical Co., RJ, Brazil), cleared in xylene and embedded in paraffin (Merck #1071642504). Sections with a thickness of 5 μ m were cut using an RM 2035 microtome (Reichert S, Leica), re-hydrated, washed in 0.05 M PBS and incubated in the buffer with 1% bovine serum albumin (BSA, fraction V) (Miles, Naperville, IL, USA) for 1 h. Endogenous peroxidase was blocked and antigen retrieval was performed, pre-treating the sections with 20 mM citrate buffer, pH 6.0, at 100 °C, for 30 min. All groups were incubated with rabbit polyclonal primary anti-PKG antibodies (Lifespan #LS-B2219), sGC (Sigma-Aldrich #G4405), GFAP (DakoCytomation, CA, USA #Z0334), ICAM or VCAM (Ebioscience #14-0542-82 and 14-1061, respectively) at a dilution of 1:100 overnight at 4 °C. After washing, the sections were overlaid for 1 h with a biotin-conjugated secondary antibody using a HRP-kit (DakoCytomation, CA, USA, Biotinylated Link Universal HRP #K0690) and visualized with 3′-3′-diaminobenzidine (DAB) as the chromogen. The slices were then weakly counter-stained with Harris' hematoxylin and mounted in entellan (Merck #1079610100). The sections were observed under an inverted microscope (Zeiss MicroImaging GmbH) equipped with a camera (Zeiss AxioCam MRM) and Release 4.7.2 image analysis software (Zeiss). Three arbitrarily chosen stained areas (0.35 mm²) were measured per animal, using GIMP 2.6.11 software (GNU Image Manipulation Program software, CNET Networks, Inc. Australia). Analysis was performed by two observers (double blind).

2.3. Immunofluorescence

After anesthesia and perfusion, the cerebella were dissected and immersed in 15% sucrose overnight, followed by immersion in 30% sucrose for a second night (36 h total). The specimens were then embedded in an OCT-Tissue Tek compound (Sakura Finetek, Torrance, CA, USA) and frozen in n-hexane (Dinamica, Sao Paulo, SP, Brazil) with liquid nitrogen. Cryo-sections measuring 8 μ m in thickness were permeabilized with 0.3% Triton X-100 and incubated for 1 h with blocking solution (3% BSA plus 0.2% Tween 20 in Tris buffered saline). Sections were then incubated with GFAP (DakoCytomation #Z0334) or Iba-1 (Wako, Osaka, Japan #019-19741) antibodies at a dilution of 1:100. Primary antibodies

were incubated overnight and then incubated with polyclonal Cy3-conjugated secondary antibodies (Jackson #705-165-147) against rabbit immunoglobulin (1:200) for 1 h. The slices were washed and mounted in fluorescent Prolong Gold Antifade medium (Life Technologies #P36930). Observation was performed using an inverted fluorescence microscope (Zeiss MicroImaging GmbH) equipped with a camera (Zeiss AxioCam MRM) and Release 4.7.2 image analysis software. Three arbitrarily chosen stained areas (0.35 mm²) were measured per animal, using GIMP 2.6.11 software (GNU Image Manipulation Program software, CNET Networks, Inc. Australia). Analysis was performed by two observers (double blind).

2.4. Western blot (WB)

Control and treated groups were anesthetized as described above and killed by decapitation. The cerebella were quickly dissected and homogenized in an extraction cocktail (10 mM EDTA, 2 mM PMSF, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM NaVO₄, 10 mg of aprotinin/ml and 100 mM Tris, pH 7.4). The samples were mixed and homogenized to create a pool from each group. Western blot was performed in accordance with Nunes et al. (2012). After electrophoresis and protein transfer, the membranes were incubated at room temperature for 4 h with rabbit polyclonal antibody against GSTpi (1:250, Abcam, Canada #ab53943) or GFAP (1:10,000, DakoCytomation, CA, USA #Z0334), and eNOS (1:1000, BD Biosciences #610299) diluted in a TBS-T buffer solution containing 3% non-fat milk. This was followed by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-goat secondary antibody (1:800 and 1:1600 dilution; Sigma-Aldrich #A9169, #A5420, respectively) diluted in TBS-T with 1% nonfat milk, for 1 h, at room temperature. An enhanced chemiluminescence reagent (Super Signal, Pierce #34080) was used to visualize the labeled protein bands and the blots were developed on X-ray film (Fuji Medical, Kodak #Z358487 – 50EA). For quantification, the density of pixels of each band was determined by the Image J 1.38 program (available at <http://rsbweb.nih.gov/ij/download.html>; developed by Wayne Rasband, NIH, Bethesda, MD). For each protein investigated the results were confirmed in three sets of experiments. Immunoblot for β -actin (1:250, Sigma-Aldrich #A2228) was performed as a control for the above protein blots. The ratio of each studied protein/ β -actin was calculated and compared between groups.

2.5. Luxol fast-blue (LFB)

Samples were dehydrated in a graded ethanol series, cleared in xylene and embedded in paraffin. Sections with a thickness of 5 μ m were cut using an RM 2035 microtome. Myelin was detected using LFB staining (Solvent Blue 38; Sigma-Aldrich) in accordance with Nunes et al. (2012). The sections were observed under an inverted microscope (Zeiss MicroImaging GmbH) equipped with a camera (Zeiss AxioCam MRM) and with Release 4.7.2 image analysis software (Zeiss).

2.6. Transmission electron microscopy (MET)

After anesthesia, the animals were sacrificed by transcardial perfusion with physiological saline (20 ml), followed by 40 ml of fixative – 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate (Sigma-Aldrich) buffer, pH 7.2. The cerebella were quickly dissected and post-fixed overnight in the same fixative. Next, cerebellum fragments were post-fixed in a solution containing 1% osmium tetroxide (Sigma-Aldrich), 2 mM calcium chloride and 0.8% potassium ferricyanide (Sigma-Aldrich) in 0.1 M sodium cacodylate buffer, pH 7.2, dehydrated in acetone series and embedded in SPIN-PON resin (Embed 812 – Electron

Microscopy Science – EMS, Washington, PA, USA). Resin polymerization was performed at 60 °C for three days. Semi-thin sections (0.5 μ m in thickness) were placed on glass slides and stained with toluidine blue. Ultrathin sections (70 nm in thickness) were placed on 300-mesh nickel grids, counterstained with 5% uranyl acetate (EMS) and lead citrate (Sigma-Aldrich) and examined using an FEI Morgani 268D transmission electron microscope.

2.7. Purified astrocyte primary cultures, nitrite and IL-2 measures

Primary culture of cerebral cortical-derived astrocytes was purified from 0 to 2 day old newborn C57BL-6 wild type and iNOS^{-/-} mice as previously described (McCarthy and de Vellis, 1980; Raposo et al., 2012). Cells were cultured in DMEM containing 10% fetal bovine serum (FBS), supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and propagated at 37 °C in a 5% CO₂ humidified atmosphere. They were then seeded onto 24-well cell culture plates (10⁴ cells/well). When wild type and iNOS^{-/-} cells reached ~95% confluence, they were treated with sildenafil (0.375 mg/ml) per 24 h (in 37 °C in a 5% CO₂ humidified atmosphere). This dose was based on an *in vivo* dose of 25 mg/kg, considering approximate total blood volume in mice. Supernatant was then used for reactions. For nitrite measure, Griess colorimetric assay was performed, in accordance with Green et al. (1982). Levels of IL-2 were accessed according to the instructions of the ELISA Quantikine Mouse IL-2 immunoassay kit (R&D #M2000). Reactions were performed in triplicate and analyzed in a Thermoplate spectrophotometer (TP-reader).

2.8. Statistic data analysis

The densitometric values of the immunoreactive bands (immunoblotting) and of immunohistochemistry quantified areas were analyzed using the GraphPad Prism software package (San Diego, CA, USA). One-way variance analysis (ANOVA), followed by the Dunnett test, was used to compare multiple groups. The *t*-student test was used to compare two groups. The results were expressed as mean $\pm$ S.E., where appropriate. A *p* < 0.05 value was adopted for statistical significance.

3. Results

3.1. VCAM (vascular cell adhesion molecule) and ICAM (endothelial intercellular adhesion molecule)

VCAM and ICAM, observed in the blood vessels of the granular, molecular and Purkinje layers and in white matter, were physiologically expressed in the cerebellum of untreated wild type animals (Fig. 1A and E). After treatment with sildenafil, VCAM and ICAM immunolabeling was significantly reduced (*p* < 0.001; Fig. 6A and B) or was completely absent from the vessels of the cerebellum layers, as shown in Fig. 1B and F.

In iNOS^{-/-} animals, VCAM and ICAM labeling was significantly reduced (*p* < 0.001; Fig. 6A and B) in the vessels of the cerebellum (Fig. 1C and G). Interestingly, after treatment with sildenafil, the vessels of animals without iNOS showed increased adhesion protein levels (Fig. 1D and H), compared to untreated iNOS^{-/-} mice (*p* < 0.001; Fig. 6A and B).

3.2. GFAP (glial fibrillary acidic protein) – reactive gliosis marker

The wild type animals presented physiologic GFAP labeling. This protein was seen in astrocytes of the granular layer, between the neurons; in the molecular layer (Bergmann glia); and in the Purkinje layer around the Purkinje cells (Fig. 2A and B). After 8 weeks of sildenafil treatment, there was a dramatic increase

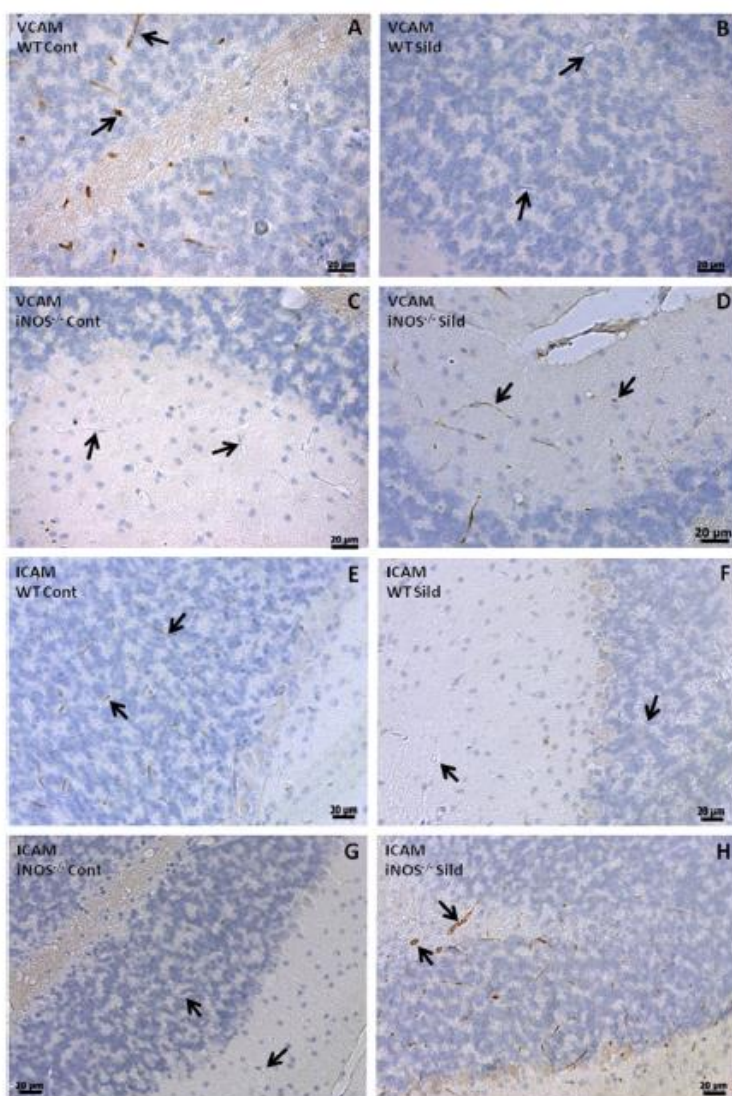


Fig. 1. Immunohistochemistry for VCAM and ICAM adhesion proteins in wild type (WT) and iNOS knockout (iNOS^{-/-}) mice with (Sild) or without (Cont) sildenafil treatment. Sildenafil reduced VCAM and ICAM labeling in WT animals. Note that the expression of adhesion proteins was eradicated in iNOS^{-/-} animals. Arrows indicate blood vessels; bars: 20 μ m.

($p < 0.001$; Fig. 6C) of GFAP in wild type animals (Fig. 2C and D). This increase was clearly visible, mainly through immunofluorescence labeling (Fig. 2, compare panels B and D). In addition to more intense immunolabeling, astrocytes processes were thicker after sildenafil treatment. Quantitative analysis of the total cerebellum by immunoblotting also showed that GFAP expression increased

after sildenafil treatment ($p < 0.05$; Fig. 6C) in wild type animals (Fig. 3).

In untreated iNOS^{-/-} animals, GFAP expression was significantly lower ($p < 0.001$; Fig. 6C) than in wild type mice (Fig. 2E and F; comparing E with A and F with B). Sildenafil increased ($p < 0.001$; Fig. 6C) the expression of GFAP in these animals (Fig. 2G and H).

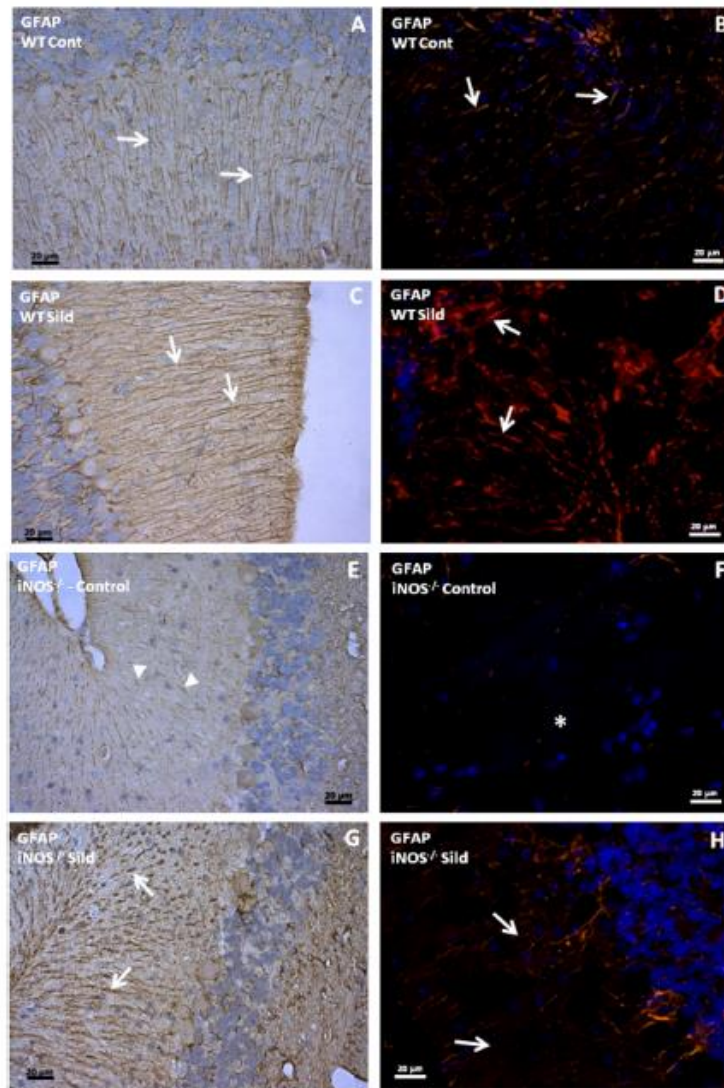


Fig. 2. Immunohistochemistry and immunofluorescence for GFAP in wild type (WT) and iNOS knockout ($iNOS^{-/-}$) mice with (Sild) or without (Cont) sildenafil treatment. Sildenafil increased the expression of GFAP in WT and $iNOS^{-/-}$ animals (arrows). $iNOS^{-/-}$ mice had a lower expression of GFAP than in WT animals (arrowheads). Arrows indicate positive astrocyte processes and the asterisk shows very low fluorescence labeling in $iNOS^{-/-}$ mice; bars = 20 μ m.

Immunoblot analysis confirmed this data, showing that mice without iNOS had a significantly lower band ($p < 0.001$) than wild type mice (see blots in Fig. 3A). Immunoblot analysis also showed that treatment with sildenafil significantly increased ($p < 0.001$) GFAP levels in $iNOS^{-/-}$ mice, compared with knockout animals without treatment (Fig. 3A and B).

3.3. Iba-1 (ionized calcium binding adaptor molecule 1) – marker of microglial activation

Microglia was labeled by immunofluorescence. In the cerebellum of untreated wild type mice, these cells had fine, ramified processes, typical of latent state phenotypes (Fig. 4A). After

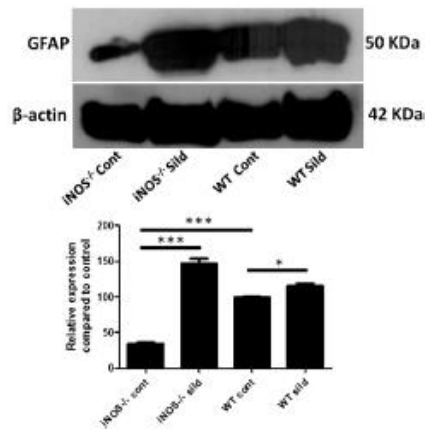


Fig. 3. GFAP expression by western blotting. Untreated Animals without iNOS (iNOS^{-/-} Cont) had a lower expression of GFAP, compared to wild type control (WT Cont). Sildenafil treatment (iNOS^{-/-} Sild and WT Sild) significantly increased levels compared with untreated animals. β -actin control of blots is shown. * $p < 0.05$, *** $p < 0.001$.

sildenafil was administered, Iba-1 was significantly increased ($p < 0.001$; Fig. 6D), compared to untreated wild type mice. Microglia cells became more numerous, assuming a phagocytic phenotype, with amoeboid appearance and thicker processes

(Fig. 4B), indicating microglial activation. The highlighted part of panel B of Fig. 4 shows an activated phagocytic microglia in detail.

Mice lacking iNOS had a similar standard of Iba-1 labeling to that observed in wild type animals (Fig. 4C). After treatment with sildenafil, Iba-1 was significantly enhanced ($p < 0.001$; Fig. 6D), compared to untreated iNOS^{-/-}, microglia were activated, revealing a greater number of cells with thicker processes and there was more intense labeling in the cell body, compared to untreated iNOS^{-/-} mice (Fig. 4D).

3.4. PKG (cGMP-dependent protein kinase) and soluble GC

Immunostaining showed that GMP dependent kinase (PKG) was expressed mainly in the Purkinje cells (Fig. 5A–D). Wild type mice expressed this enzyme physiologically (Fig. 5A), but, interestingly, after PDES inhibition by sildenafil treatment, expression of PKG decreased and the Purkinje cells had less intense labeling ($p < 0.05$; Fig. 6E) in the cell body (Fig. 5B), compared to untreated wild type animals. PKG expression was significantly reduced ($p < 0.001$; Fig. 6E) in animals lacking iNOS (Fig. 5C), compared to wild type animals (Fig. 5A and C). In iNOS^{-/-} mice, sildenafil treatment increased ($p < 0.001$; Fig. 6E) PKG labeling in the Purkinje cells (Fig. 5D).

The enzyme sGC was seen in all layers of the cerebellum (Fig. 5E–H). As with PKG, wild type mice without treatment displayed physiological expression of this enzyme (Fig. 5E). When sildenafil was administered, this enzyme decreased ($p < 0.001$; Fig. 6F) in the cerebellum (Fig. 5F). Also, iNOS^{-/-} animals (Fig. 5G) showed less intense sGC labeling ($p < 0.001$; Fig. 6F) than untreated wild type mice (Fig. 5E and G). Sildenafil subtly (not significant) increased the sGC expression of animals without iNOS (Fig. 5H).

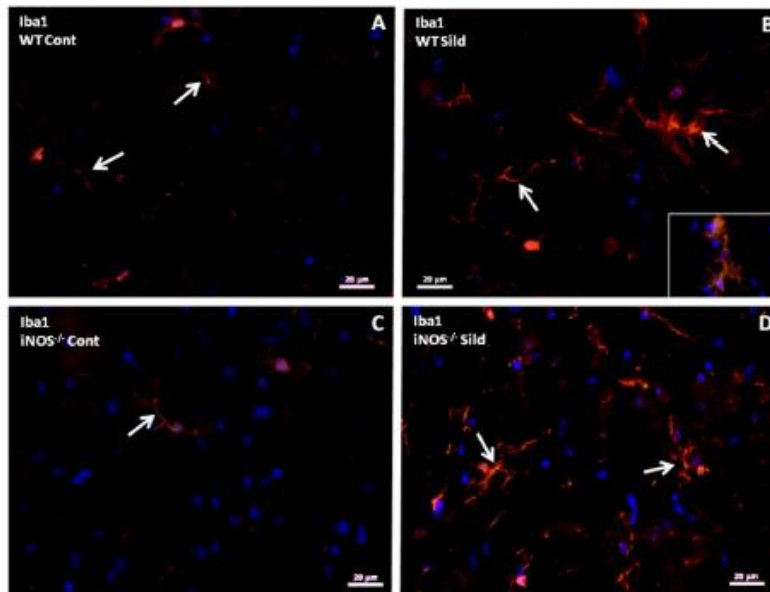


Fig. 4. Immunofluorescence for Iba-1 in wild type (WT) and iNOS knockout (iNOS^{-/-}) mice with (Sild) or without (Cont) sildenafil treatment. Sildenafil increased the expression of Iba-1, compared to untreated animals in both iNOS^{-/-} and WT mice. Also, sildenafil treated animals showed thicker processes than untreated animals. Arrows showed positive processes of microglia. Detail in B shows some strongly positive cells; bars = 20 μ m.

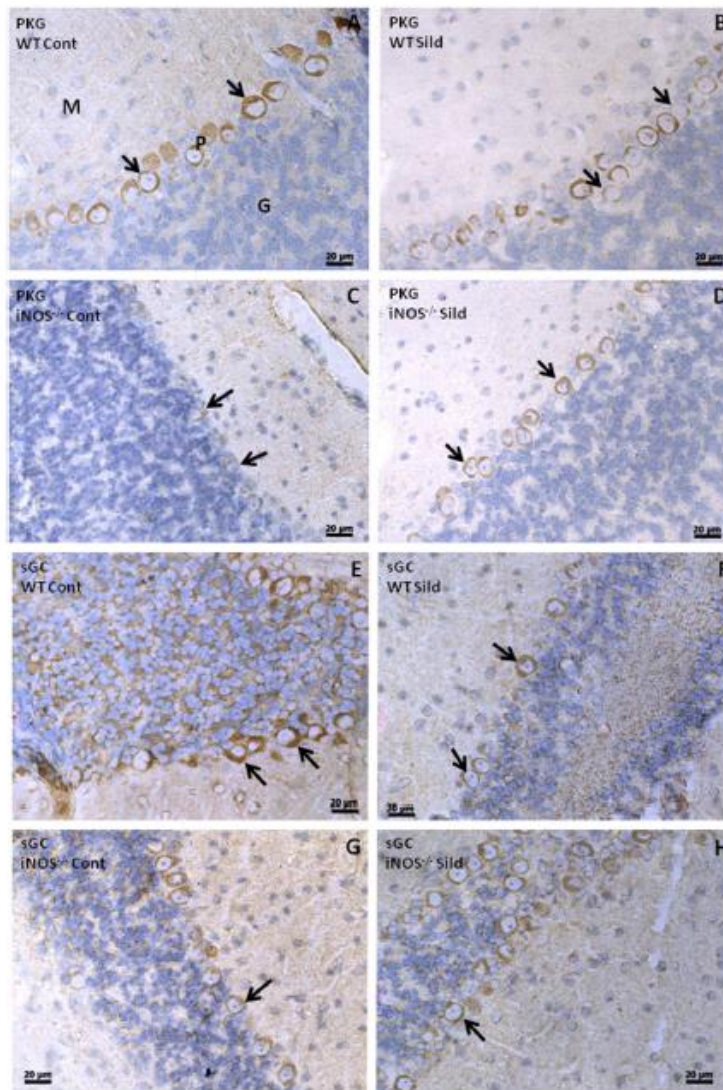


Fig. 5. Immunohistochemistry for PKG and sGC in wild type (WT) and iNOS knockout (iNOS^{-/-}) mice with (Sild) or without (Cont) sildenafil treatment. WT Sild showed decreased PKG and sGC labeling compared to WT Cont animals. The expression of both proteins was greatly reduced in iNOS^{-/-} Cont mice compared to WT Cont. Arrows show Purkinje cells; bars: 20 μ m.

3.5. eNOS (constitutive endothelial NO synthase)

The expression of eNOS was analyzed in control animals (wild type and iNOS^{-/-} without treatment) by western blotting (Fig. 7). Bands quantification showed that eNOS was overexpressed in iNOS^{-/-} mice, compared to wild type ($p < 0.01$).

3.6. Nitrite and IL-2 in isolated astrocytes

Indirect measuring of NO by nitrite (Fig. 8A) showed that, in isolated astrocytes from wild type mice, the treatment of cells with sildenafil significantly increased ($p < 0.01$) levels of nitrite, compared to untreated (control) wild type astrocytes.

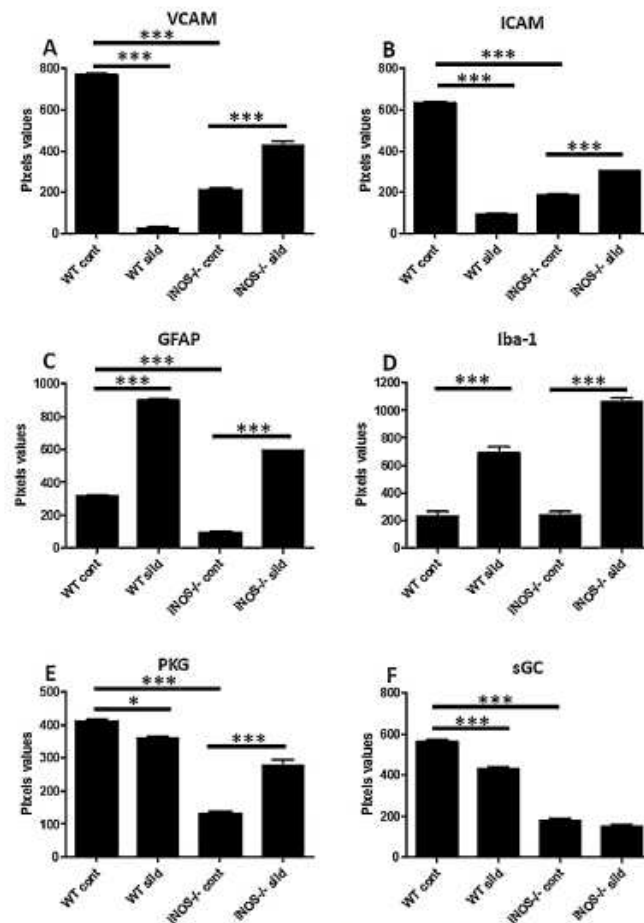


Fig. 6. Graphs representing quantitative analyses of immunohistochemistry for VCAM, ICAM, GFAP, Iba-1, PKG and sGC. WT cont = untreated wild type animals, WT sild = wild type animals treated with sildenafil, iNOS^{-/-} cont = untreated iNOS knockout animals, iNOS^{-/-} sild = iNOS knockout animals treated with sildenafil. **p* < 0.05, ****p* < 0.001.

Levels of nitrite in iNOS^{-/-} and wild type untreated cells were not significantly different. When iNOS^{-/-} astrocytes were exposed to sildenafil, there was a subtle increase in nitrite levels, but this was not significant.

Contrastingly, the level of IL-2 (Fig. 8B) was significantly (*p* < 0.001) lower in wild type astrocytes exposed to sildenafil. Astrocytes isolated from iNOS^{-/-} mice showed a lower level of IL-2 (*p* < 0.001), compared with wild type cells. Interestingly, sildenafil was not anti-inflammatory, in iNOS^{-/-} astrocytes, but significantly increased IL-2 (*p* < 0.001).

3.7. Myelin structure and ultrastructure

White matter appearance was normal in wild type mice, as seen through LFB staining (Fig. 9A). After treatment of wild

type animals with sildenafil, the white matter of the cerebellum remained normal, but was more strongly stained by LFB (Fig. 9B).

The iNOS knockout mice had damaged white matter that was strongly stained and had slots and vacuoles (Fig. 9C). Interestingly, sildenafil administration improved the appearance of white matter in iNOS^{-/-} mice, making white matter more homogeneous, without slots and vacuoles (Fig. 9D). However, the intensity of LFB staining was still higher than that of the white matter of wild type control mice.

Ultrastructural analysis (by MET) showed that myelin of wild type control was normal, with compact sheaths and preserved axons (Fig. 9E). In animals that received sildenafil, the myelin sheath remained normal and compact, and the axons were preserved (Fig. 9F).

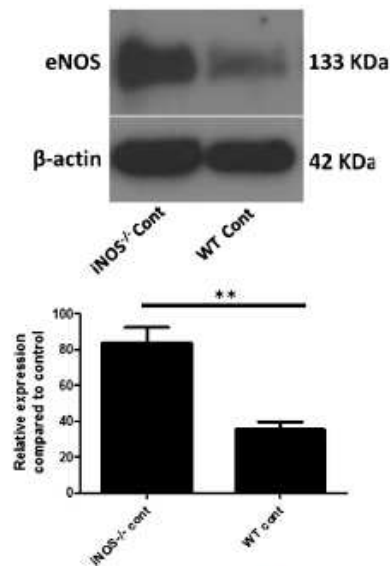


Fig. 7. eNOS expression by western blotting in iNOS^{-/-} and wild type (WT) untreated animals (iNOS^{-/-} cont and WT cont, respectively). Animals without iNOS have an overexpression of eNOS, comparing with WT cont. β-actin control of blots is shown. ***p* < 0.01.

Animals lacking iNOS had a damaged myelin ultrastructure. The sheath was pulled back, with spaces between layers of myelin (Fig. 9G). Axons were apparently normal. The iNOS^{-/-} mice which received sildenafil had a well preserved myelin sheath (Fig. 9H).

3.8. GSTpi (glutathione S-transferase pi) – marker of mature oligodendrocytes

GST-pi immunoblotting (Fig. 10) showed that wild type animals physiologically expressed this protein. Sildenafil treatment did not

induce any changes in the expression of this mature oligodendrocyte marker in wild type mice.

In untreated mice without iNOS, there was a significant decrease in GSTpi levels, compared to wild type control mice. Sildenafil treatment increased GST-pi expression in iNOS^{-/-} animals compared to untreated mice.

4. Discussion

Neuroinflammatory events involve the recruitment and/or activation of macrophage, leukocytes, microglia and astrocytes. Although inflammatory events have been comprehensively described, little is known about the signaling pathways which control these events. Several diseases with neuroinflammation and demyelination as key mechanisms remain incurable. It is important to clarify the signaling involved in these processes. Therefore, the present study investigated whether the iNOS-NO-sGC-GMPc pathway is involved in the potential recruitment of peripheral immune cells into the CNS, and if this pathway modulates the activation of neural resident cells. For this analysis, VCAM, ICAM (adhesion proteins), GFAP and Iba-1 (astrocytes and microglia markers, respectively), PKG (cGMP dependent kinase protein), sGC (the enzyme responsible for the production of cGMP), and eNOS (constitutive NO synthase) were evaluated in wild type and iNOS knockout mice. In addition, indirect measuring of NO through nitrite and IL-2 levels was performed in astrocytes isolated from iNOS^{-/-} and wild type mice brains. Moreover, it has been demonstrated that demyelination and oligodendrocyte degeneration follows inflammation (Frank-Cannon et al., 2009). On the other hand, there is considerable heterogeneity of mechanisms of myelin and oligodendrocyte destruction in active lesions of primary demyelinating diseases such as MS, in which no specific pattern of inflammation has been recognized in the same (Gay et al., 1997). Therefore the present study additionally investigated whether the iNOS-NO-sGC-GMPc pathway controls myelin integrity and mature oligodendrocyte viability. GSTpi (a marker of mature oligodendrocytes) and the structure and ultrastructure of myelin were assessed.

The role of NO in vivo has been extensively investigated in studies with NOS inhibitors and in studies of mice lacking NOS isoforms. However, the specificity of NOS inhibitors continues to be an issue of debate, while in mice NOS^{-/-} for each isoforms, a compensatory mechanism by other NOSs appears to be involved (Tsutsui et al., 2007). In spite of this compensatory mechanism, knockout animals are considered to be useful animal models for NOS deficiencies (Huang, 2000), and their phenotypes reflect the functions

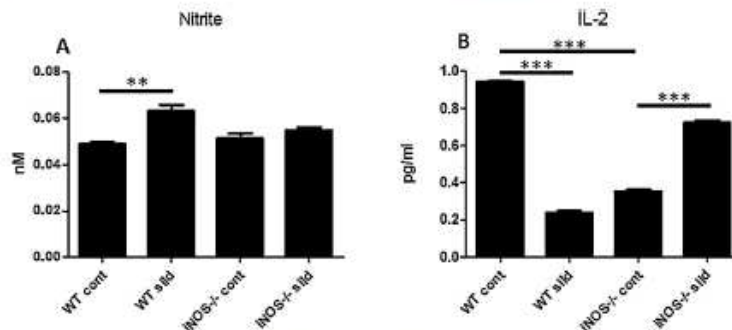


Fig. 8. Quantity of nitrite and IL-2 released in isolated astrocytes from iNOS^{-/-} and C57BL/6 wild type (WT) mice. There is no significant differences in nitrite concentration between iNOS^{-/-} and WT cells. Sildenafil increased nitrite concentration in wild type, but not in iNOS^{-/-} astrocytes. On the other hand, IL-2 level in iNOS^{-/-} was significantly less than in WT astrocytes. Sildenafil decreased IL-2 in wild type, but had an opposite effect in iNOS^{-/-} cells, increasing IL-2 levels. ***p* < 0.01, ****p* < 0.001.

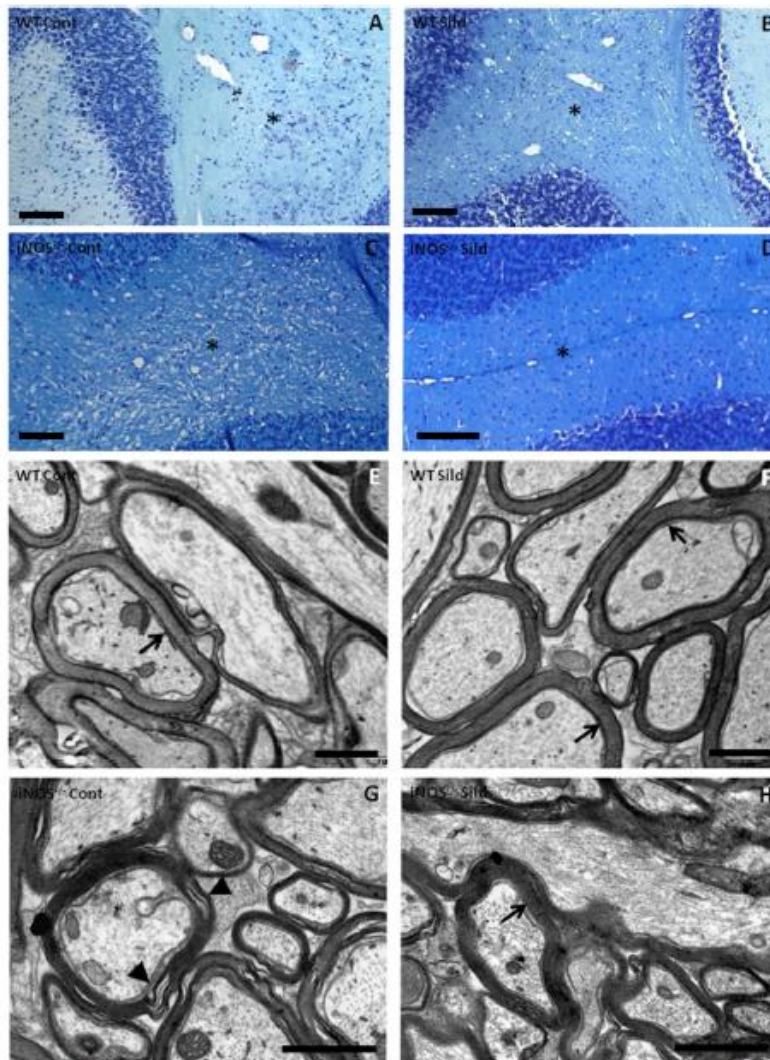


Fig. 9. Structure (Luxol Fast Blue-LFB staining) and ultrastructure (transmission electron microscopy – MET) of myelin. Wild type treated with sildenafil (WT Sild) showed more intense LFB than WT without treatment (WT cont). No difference was observed by MET between WT Cont and WT Sild. iNOS knockout animals without treatment (iNOS^{-/-} Cont) showed impaired myelin sheath, compared to WT Cont. Sildenafil improved myelin structure and ultrastructure of iNOS^{-/-} (iNOS^{-/-} Sild). Asterisks indicate white matter; arrows show normal myelin sheath; arrowheads indicate damaged myelin; bars in A–D= 100 μm; bars in E–H= 1 μm.

of each NOS isoform. As such, nNOS knockout mice exhibit distended stomachs because of abnormalities in pyloric relaxation (Huang et al., 1993); eNOS knockout mice are hypertensive (Huang et al., 1995); and iNOS knockout mice are more sensitive to certain infections (Laubach et al., 1995; MacMicking et al., 1995; Wei et al., 1995). Therefore, iNOS^{-/-} mice were chosen for this study, as this

is considered a valid investigation model for the role of iNOS in pathophysiological conditions (De Luca et al., 2006). The study was performed in the cerebellum as this region has been reported as an important region of the CNS that is affected by neurodegenerative diseases, such as MS, showing severe white matter atrophy and inflammation (Riccitelli et al., 2012; Shields et al., 2012).

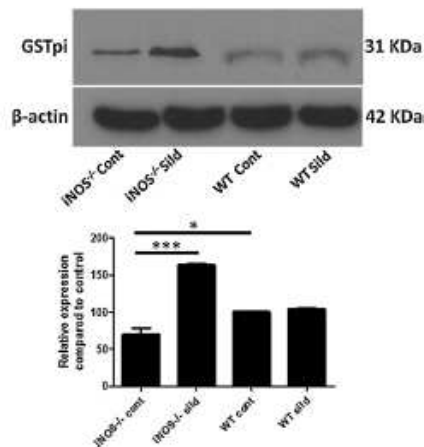


Fig. 10. Western blotting to GST-pi oligodendrocytes marker in wild type (WT) and iNOS knockout (iNOS^{-/-}) mice with (Sild) or without (Cont) sildenafil treatment. No difference in GST-pi expression was seen comparing WT Cont and WT Sild. However, iNOS^{-/-} Cont resulted in a significant reduction in the expression of protein in relation to WT Cont. iNOS^{-/-} Sild resulted in an increased expression of GST-pi compared to iNOS^{-/-} Cont. β -actin control of blots is shown. * $p < 0.05$, *** $p < 0.001$.

Previous studies have shown the anti-inflammatory effects of sildenafil in the cerebellum of mice-MS model, induced by cuprizone (Nunes et al., 2012; Raposo et al., 2013). Therefore, it is important to clarify the role of the iNOS-NO-sGC-cGMP pathway in cerebellar inflammation/myelin parameters.

Endothelium represents an important control in the recruitment of the peripheral inflammatory cells and transvascular migration (Williams and Hickey, 1995). Adhesion proteins, such as ICAM-1 and VCAM-1, are expressed in brain microvessel endothelial cells in active MS lesions. Although it has been found that glial cells express some of these adhesion molecules upon activation (astrocytes express ICAM-1, VCAM-1 and E-selectin and microglia expresses ICAM-1 and VCAM-1) (Lee and Benveniste, 1999), adhesion proteins have often been used as a marker of T cell access into the CNS in pathologic conditions (Klotz et al., 2007; Pifarre et al., 2011). ICAM-1, VCAM-1 and PECAM-1 act as ligands for leukocyte receptors and also serve as signal transducers (Greenwood et al., 2002). Besides the peripheral immune response, resident cells of the CNS, such as astrocytes and microglia, contribute to the neuroinflammatory process. In MS, the sustained inflammatory mediators released by the microglia activate additional microglia, which proliferate and result in a further release of inflammatory factors (Mrak and Griffin, 2005). Therefore, the question of whether the iNOS-NO-sGC-cGMP pathway regulates adhesion proteins and glial cell markers is of considerable importance.

In the present study, it was demonstrated that cGMP accumulated from eight weeks of sildenafil treatment reduced ICAM and VCAM expression in the cerebellar blood vessels of wild type animals, indicating possible down regulation by cGMP in the potential access of peripheral inflammatory cells to the brain tissue. This result agrees with the findings of Nossaman and Kadowitz (2013), who showed that the cGMP pathway inhibits leukocyte adhesion. In contrast to these anti-inflammatory effects, the results of the present study showed that GFAP and Iba-1 levels were increased by sildenafil treatment in wild type mice, suggesting activation of astrocytes and microglia, respectively. Cyclic

GMP-mediated pathways have been implicated in the regulation of actin cytoskeleton and cell morphology in different cell types, including macrophages (Ke et al., 2001; Borán and García, 2007). In macrophages, the atrial natriuretic peptide (ANP)-cGMP pathway was shown to increase ingestion of opsonized particles (Vollmar et al., 1997). However, ANP-cGMP signaling decreased expression of the typical proinflammatory genes NOS-2 and TNF- α (Vollmar, 2005). Borán and García (2007) demonstrated that stimulation of the PKG pathway by NO or ANP regulates cytoskeleton dynamics and motility in rat astrocytes in culture. The same research group (Borán et al., 2008) showed that the cGMP-PKG pathway stimulated by ANP (but not by NO) regulates microglial cell morphology by inducing a dramatic reorganization of the actin cytoskeleton. Both ANP and the permeable cGMP analog, dibutytyl-cGMP, promote a rapid and concentration-dependent increase in size, rounding, and lamellipodia and filopodia formation in rat brain cultured microglia. These morphological changes involve an increase in and redistribution of F-actin and result in increased phagocytic activity. However, ANP-cGMP does not induce iNOS or TNF- α expression and is able to decrease LPS-elicited induction of these inflammatory genes. These results support the hypothesis that, in microglia, the ANP-pGC-cGMP pathway promotes a response with characteristics similar to those reported for clearance of apoptotic cells by phagocytes. This microglial control can be important to treatment of chronic neuroinflammatory diseases, such as MS, as microglia could clear the environment without contributing to inflammation. These results corroborate the present results, which demonstrated that cGMP accumulated by sildenafil administration induces phagocytic phenotype of microglia and activates astrocytes. This data suggests that stimulation of the cGMP-PKG pathway in microglia and astrocytes could play a beneficial role in the resolution of neuroinflammation by removing dead cells and limiting the lesion by promoting astrocyte scars.

In addition, the present study showed that sildenafil treatment reduced sGC and PKG expression in wild type animals. Previous studies have demonstrated that the NO-cGMP pathway itself is an important regulator of GCs activity and expression (Papapetropoulos et al., 1996). The astroglial sGC can be desensitized and down-regulated by NO (Derbyshire and Marletta, 2009; Xia et al., 2012). Studies of cerebellar astrocytes showed that desensitization occurs a few seconds after the addition of NO (Bellamy and Garthwaite, 2001). Also, an increase of cAMP levels seems to be involved in the regulation of sGC (Shimouchi et al., 1993). An increase of cAMP (Shimouchi et al., 1993; Papapetropoulos et al., 1996) causes reduction in the sGC mRNA and, consequently, its product, sGC protein. A NO- and cGMP-dependent decrease in sGC mRNA has been reported in smooth muscle cells and decreased expression of the mRNA stabilizing protein HuR has been associated with this effect (Pyriochou and Papapetropoulos, 2005). It has been demonstrated that an increase in intracellular cGMP levels leads to feedback inhibition of the pathway by downregulating sGC activity (Murthy, 2001). Furthermore, PKG, but not PKA, phosphorylates soluble sGC and inhibits its activity. PKG-specific phosphorylation of soluble sGC attenuates the synthetic activity of this enzyme. The reduction of sGC mRNA (and sGC protein) or inhibition of sGC activity by PKG-phosphorylation maintain cGMP levels within the physiological range (Murthy, 2001). One or both of these mechanisms could explain the decrease in the levels of sGC protein after sildenafil treatment (cGMP accumulating) observed in the present results.

The results of the present study showed that the expression of VCAM, ICAM, GFAP, PKG and sGC was eradicated or strongly diminished in iNOS^{-/-} mice, compared to wild type control. This data indicates that the iNOS/NO system is involved in peripheral inflammatory cell recruitment and resident inflammatory cell activation (astrocytes, but not microglia). The different effects on

microglia was expected, as it has been previously observed that rat microglia did not express active sGC and did not accumulate cGMP in response to NO donors (Agullo et al., 1995), but only in response to ANP (Borán et al., 2008). Sildenafil administration in these animals increased ICAM, VCAM, GFAP, Iba-1, PKG and sGC labeling. These results corroborate with the recent study by the authors (Raposo et al., 2013), which showed that in a cuprizone-demyelination model induced in iNOS^{-/-} animals, sildenafil treatment for 4 weeks had no anti-inflammatory effects, but in contrast, increased GFAP, COX-2 and cytokine expression. Although these studies are different, with a cuprizone model used in the previous study and naïve mice used in the present study, it is interesting to note that sildenafil had no anti-inflammatory effect on iNOS^{-/-} in both conditions. This data points to the relevance of the physiologic expression of iNOS, in a normal or toxic environment, for the anti-inflammatory effects of sildenafil. In order to evaluate if there was a compensatory balance in eNOS expression due to the lack of iNOS, eNOS expression in wild type and iNOS^{-/-} animals without treatment was tested in the present study. It was shown that iNOS^{-/-} mice express high levels of eNOS compared to wild type mice. This result corroborates the findings of a previous study (Raposo et al., 2013). One possible explanation is that over signaling of NO-cGMP occurs in iNOS knockout animals after sildenafil treatment (as eNOS is overexpressed), resulting in a pro-inflammatory effect.

This overexpression of eNOS also could explain reduced sGC and PKG expression in iNOS^{-/-} knockout animals. Xia et al. (2012) questioned whether iNOS or eNOS derived NO has a greater effect on the regulation of sGC activity and expression. It was found that there was an increase in iNOS expression, accompanied by reduced sGC expression and activity, as well as reduced cGMP synthesis in hypoxic and hypercapnic rat lungs. In the present study it was found that there was reduced expression of sGC and PKG in the cerebellum of iNOS^{-/-} mice. It is possible that the overexpression of eNOS is responsible for sGC desensitization in these animals. It is therefore suggested that both iNOS and eNOS have important roles in sGC regulation.

To analyze levels of NO in animals lacking iNOS, nitrite concentration was accessed in the supernatant of astrocytes isolated from iNOS^{-/-} and wild type mice. Astrocytes were chosen because, as shown here, these cells were considerably altered in iNOS^{-/-} animals. However, in spite of eNOS overexpression in the tissue, nitrite levels in iNOS^{-/-} and wild type cells were not significantly different. Sildenafil treatment increased nitrite concentration in wild type, but not in iNOS^{-/-} astrocytes. Interestingly, IL-2 was downregulated in iNOS^{-/-} astrocytes, compared with wild type cells. Since iNOS produces NO quickly and in large amounts, in specific situations, its absence, even with eNOS overexpression, is enough to induce a typical iNOS^{-/-} profile of deficiencies in the inflammatory parameters. Corroborating with *in vivo* results, sildenafil had an anti-inflammatory effect in wild type astrocytes, decreasing IL-2 levels. However sildenafil had the opposite effect on iNOS^{-/-} cells, increasing IL-2. This data suggested that sildenafil anti-inflammatory effects are regulated by iNOS. It is possible that the cGMP accumulated by sildenafil has a feedback mechanism with iNOS, balancing the inflammatory parameters. Yoshioka et al. (2010) showed that NO attenuates its own production in LPS-stimulated BV-2 cells by reducing the induction of iNOS protein via cGMP dependent mechanisms, and that the NO-cGMP signaling pathway attenuates the activation of c-Jun N-terminal kinase (JNK), which is important for LPS-induced iNOS protein production. This balance of inflammation by iNOS-cGMP circuit needs to be explored. iNOS^{-/-} animals showed damaged myelin structure and ultrastructure and there was a significant decrease in GSTpi levels compared to wild type control animals. Interestingly, although sildenafil increased the inflammatory proteins (ICAM,

VCAM, Iba1 and GFAP), the protective effect on the myelin structure remained in these animals. Sildenafil increased GSTpi and improved myelin structure and ultrastructure in iNOS^{-/-} mice. Some studies (Yoshioka et al., 2000; Benjamins and Nedelkoska, 2007; Wang et al., 2011) have highlighted the protective role of sildenafil in the myelin structure, including in peripheral myelinated fibers. Protection against oligodendroglial apoptosis may be involved, since ibudilast inhibit excitotoxicity in cultured oligodendroglia (Wang and Robinson, 1997) and cGMP analog, 8-bromo-cyclic GMP protects differentiated oligodendrocytes from caspase-mediated death *in vitro* (Benjamins and Nedelkoska, 2007). Therefore, the results of the present study indicate that the protective role of sildenafil/cGMP in myelin/oligodendrocytes is independent of its anti-inflammatory effects.

In conclusion, iNOS-NO-cGMP signaling is involved in the regulation of inflammation and myelin parameters. iNOS knockout animals have reduced inflammatory markers and present myelin damage. The accumulation of cGMP, induced by treatment with sildenafil is anti-inflammatory in wild type animals. However, the opposite (pro-inflammatory) effect was found in animals lacking iNOS. This can be explained by the overexpression of eNOS in iNOS^{-/-} mice, unbalancing cGMP signaling. On the other hand, it is possible that a feedback mechanism between cGMP and iNOS is necessary to balance inflammatory conditions. Interestingly, despite the pro-inflammatory effect of sildenafil in iNOS^{-/-} mice, this drug protects myelin, indicating that myelin protection by cGMP is independent of its anti-inflammatory effects. Drugs that modulate the NO-sGC-cGMP pathway could be clinically beneficial in the treatment of neuroinflammatory/demyelinating disorders, but more studies of the regulation of this pathway are required. Studies that contribute to the knowledge of the effects and mechanisms of these drugs can help understand their potential usage.

Conflict of interest

The authors declare that there is no conflict of interest in the present study.

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