UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

Efeito da infecção com *Candida albicans* no desenvolvimento da Encefalomielite Autoimune Experimental

THAIS FERNANDA DE CAMPOS FRAGA DA SILVA

Tese apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração *Biologia de parasitas e micro-organismos.*

Orientadora: *Alexandrina Sartori*

BOTUCATU – SP
2016

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MARIA SUELI PARREIRA DE ARRUDA

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FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

Fraga-Silva, Thais Fernanda de Campos.
Efeito da infecção com Candida albicans no desenvolvimento da Encefalomielite Autoimune Experimental / Thais Fernanda de Campos Fraga-Silva. - Botucatu, 2016

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: Alexandrina Sartori
Coorientador: Maria Sueli Parreira de Arruda
Capes: 20100000

1. Candida albicans. 2. Doenças auto-imunes. 3. Esclerose múltipla. 4. Encefalomielite Autoimune Experimental. 5. Candidíase.

Palavras-chave: Candida albicans; Candidíase sistêmica; Encefalomielite Autoimune Experimental; Esclerose múltipla.

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Câmpus de Botucatu

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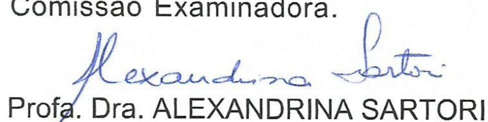
ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE THAIS FERNANDA DE CAMPOS FRAGA DA SILVA, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA GERAL E APLICADA, DO INSTITUTO DE BIOCIÊNCIAS.

Aos 25 dias do mês de fevereiro do ano de 2016, às 14:00 horas, no(a) Sala de Defesa da Pós-Graduação, reuniu-se a Comissão Examinadora da Defesa Pública, composta pelos seguintes membros: Profa. Dra. ALEXANDRINA SARTORI do(a) Departamento de Microbiologia e Imunologia - Instituto de Biociências de Botucatu / Instituto de Biociências de Botucatu, Profa. Dra. ALEXANDRA IVO DE MEDEIROS do(a) Departamento de Ciências Biológicas da Faculdade de Ciências Farmacêuticas de Araraquara - UNESP / Faculdade de Ciências Farmacêuticas de Araraquara - UNESP, Prof. Dr. JEAN PIERRE SCHATZMANN PERON do(a) Depto. de Imunologia / Instituto de Ciências Biomédicas IV de São Paulo - USP, Prof. Dr. EDUARDO BAGAGLI do(a) Departamento de Microbiologia e Imunologia do Instituto de Biociências do Campus de Botucatu. / Instituto de Biociências de Botucatu, Profa. Dra. MARIA TEREZINHA SERRÃO PERAÇOLI do(a) Depto. de Microbiologia e Imunologia / IB/Botucatu - Unesp, sob a presidência do primeiro, a fim de proceder a arguição pública da TESE DE DOUTORADO de THAIS FERNANDA DE CAMPOS FRAGA DA SILVA, intitulada **Efeito da Infecção com *Candida albicans* no desenvolvimento da Encefalomielite Autoimune Experimental**. Após a exposição, a discente foi arguida oralmente pelos membros da Comissão Examinadora, tendo recebido o conceito final:_ APROVADA _ _ _ _ . Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelos membros da Comissão Examinadora.



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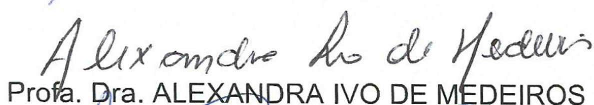

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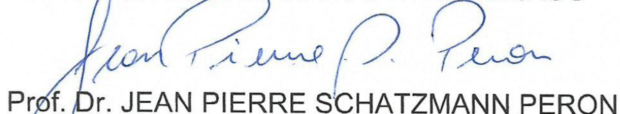

Prof. Dr. JEAN PIERRE SCHATZMANN PERON

Prof. Dr. EDUARDO BAGAGLI

Profa. Dra. MARIA TEREZINHA SERRÃO PERAÇOLI

Dedicatória

Dedico este trabalho à minha família,

meus pais, José e Izabel, e meus irmãos, Thiago e Gabriele,

por todo o amor e compreensão.

Obrigada por não medirem esforços para me ajudar em todos os momentos

e tornarem possível a realização dos meus sonhos.

Agradecimentos

Agradeço,

À Dra. Alexandrina Sartori, minha orientadora, pela confiança, orientação e dedicação. Serei sempre grata por esses anos de muito aprendizado.

À Dra. Maria Sueli Parreira de Arruda, minha co-orientadora, pelas oportunidades concedidas desde a iniciação científica, pela confiança e estímulo para buscar o meu melhor.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pela concessão da Bolsa de Doutorado Direto (#13/14353-2) e do Auxílio Regular (#12/12540-7).

Ao Dr. Robson Francisco Carvalho, à Dra. Ângela Maria V. de Campos Soares e à Dra. Sofia Fernanda G. Zorzella-Pezavento, pela contribuição durante meu exame de Qualificação.

À Dra. Liana Maria Cardoso Verinaud e o Dr. Rodolfo Thomé por me acolherem no Laboratório de Imunopatologias (UNICAMP - Campinas), pela atenção e ensinamentos.

Aos professores, alunos e funcionários do Departamento de Microbiologia e Imunologia (UNESP – Botucatu) e do Departamento de Ciências Biológicas (UNESP – Bauru) pelo suporte técnico e acadêmico. Em especial à Dra. Graziela Romagnoli, ao Luiz S. dos Santos (Lula) e ao Thiago Tozi.

À Seção de Pós-Graduação do Instituto de Biociências de Botucatu (UNESP – Botucatu) pelo suporte técnico. Em especial ao funcionário Davi Müller, sempre disposto a ajudar.

Às queridas companheiras do Laboratório de Vacinas e Imunomodulação, Sofia Zorzella-Pezavento, Thais Donegá, Larissa Lumi e Fernana Chiuso Minicucci, por me receberam com muito carinho e atenção, mas também por todo auxílio teórico e prático indispensável para a realização deste trabalho. Gostaria de agradecer também às mais novas integrantes, Larissa Ragazo, Karen Pinke e Aline Missio por todo o apoio.

Aos amigos e colaboradores do Laboratório de Imunopatologia Experimental,
James Venturini, Gisele Locachevic, Débora Almeida, Angela Finato,
Amanda Ribeiro, Bárbara Amorim, Laysla Leite e a todos que já passaram pelo LIPE,
por tudo que realizamos e pelos ensinamentos de cada dia.
Agradeço aos mais "velhos" a parceria desde a iniciação científica.

Aos biólogos, veteranos e amigos: Thiago Davanso & Andrea Mourão, Pedro Guerra,
Filipe Nathan, Ricardo Judice, Leonardo Saldanha, João Alexandre, Abner Carvalho,
Fernanda Rezende, Sarah Teodoro, Natalia Violato, André Gobatto & Maiara Gonçalves,
Mônica Fiore e Lucas Farinelli, por todos os ensinamentos, científicos e não científicos.

Às minhas amigas-irmãs Camila Marchetti, Luiza Mimura e Nara Lígia
por estarem presentes desde a graduação e tornarem o caminho mais feliz.
Muito obrigada pelo apoio nos momentos difíceis e por compartilhar tantas alegrias.

Ao meu melhor amigo, meu amor, João Alberto Pantaleão.
Por toda compreensão e incentivo. Por todas as discussões científicas!
E, por mesmo em momentos não tão bons, me fazer sorrir!
Obrigada por me proporcionar a felicidade de compartilhar sonhos e conquistas.

Aos meus avós, Antônio Fraga e Eva Zanatta,
Clodemyl Pires e Dirce Corá, por sempre me acolherem,
pelos grandes ensinamentos da vida e por todo amor e carinho.

Finalmente, agradeço aos animais de experimentação.
Que perdem a vida para que nossas conquistas sejam possíveis.
"Minha eterna gratidão, meu respeito e a certeza que fiz o máximo para amenizar
seu sofrimento, aproveitando cada dado extraído, cada experiência profissional,
e também pessoal, de meu contato com eles". Marchetti, CM.

"Tenho a impressão de ter sido uma criança brincando à beira-mar,
divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita
que as outras, enquanto o imenso oceano da verdade
continua misterioso diante de meus olhos".

Isaac Newton

Resumo

Resumo

FRAGA-SILVA, T. F. C. Efeito da infecção com *Candida albicans* no desenvolvimento da Encefalomielite Autoimune Experimental. 2016. 100 p. Tese (Doutorado) – Instituto de Biociências de Botucatu, Universidade Estadual Paulista, Botucatu, 2016.

A esclerose múltipla é uma doença autoimune ainda sem cura que acomete o sistema nervoso central (SNC). Evidências observadas em pacientes e em modelos experimentais indicam que infecções fúngicas e/ou derivados fúngicos podem contribuir com a patogênese da doença. Neste contexto, o objetivo deste projeto foi avaliar o efeito da infecção por *Candida albicans* e derivados fúngicos no desenvolvimento da encefalomielite autoimune experimental (EAE) que é o modelo murino desta patologia. Para avaliar o papel da infecção, camundongos C57BL/6 foram infectados com *C. albicans* três dias antes da indução da EAE. A infecção prévia agravou os sinais clínicos da doença. Este agravamento foi relacionado com a disseminação do fungo para o SNC e aumento da produção de citocinas encefalitogênicas (TNF-α, IL-6, IL-17 e IFN-γ) tanto na periferia (baço) quanto no SNC. Para avaliar o efeito de derivados fúngicos no desenvolvimento da EAE, camundongos foram inoculados com três doses de gliotoxina ou de leveduras mortas de *C. albicans* após a indução da doença. A inoculação de gliotoxina resultou em sintomatologia mais grave, associada com um intenso infiltrado inflamatório e maior produção de TNF-α no SNC. De forma distinta a inoculação de leveduras mortas resultou em uma doença bem mais branda, com menor porcentagem de células apoptóticas e baixa produção de citocinas encefalitogênicas no SNC. Considerando que recentemente nosso grupo de pesquisa demonstrou que a vacinação com MOG associada à vitamina D (MOG+VitD) preveniu a encefalomielite, investigamos também se a infecção por *C. albicans* é capaz de quebrar a tolerância induzida por esta vacinação. Para isto, camundongos foram vacinados com MOG+VitD, infectados com *C. albicans* e depois submetidos à indução da EAE. A vacinação determinou proteção caracterizada por melhora significativa da sintomatologia, decréscimo acentuado na produção de citocinas encefalitogênicas no baço e no SNC e expansão de células T reguladoras. No seu conjunto os resultados desta investigação indicam que derivados fúngicos, dependendo de suas características, podem ser deletérios ou protetores no desenvolvimento da EAE.

Palavras-chave: *Candida albicans*; candidíase sistêmica; encefalomielite autoimune experimental; esclerose múltipla.

Abstract

Abstract

FRAGA-SILVA, T. F. C. Effect of *Candida albicans* infection on experimental autoimmune encephalomyelitis development. 2016. 100 p. Thesis (PhD) – Institute of Biosciences of Botucatu, São Paulo State University, Botucatu, 2016.

Multiple sclerosis (MS) is an autoimmune disease that affects the central nervous system (CNS). Data from patients and experimental models suggest that fungal infection and fungal-derived antigens could contribute to the immunopathogenesis of this pathology. The main objective of this research was to investigate the effect of *C. albicans* and fungal derivatives on experimental autoimmune encephalomyelitis (EAE) that is a widely employed animal model to study MS. Initially, C57BL/6 mice were infected with *C. albicans* and three days later they were submitted to EAE induction. Infected animals developed a more severe disease associated with fungus spread to the CNS and increased production of encephalitogenic cytokines as TNF-α, IL-6, IL-17 and IFN-γ by cells from the spleen and the CNS. To test the effect of fungal derivatives mice were submitted to EAE induction and then injected with three doses of gliotoxin or dead yeasts of *C. albicans*. Gliotoxin inoculation resulted in clinical disease aggravation, higher local inflammatory infiltration and higher production of TNF-α by the CNS. Dead *C. albicans* inoculation determined a less severe disease associated with a lower production of encephalitogenic cytokines and a lower degree of apoptosis by CNS eluted cells. Lastly we demonstrated that *C. albicans* infection did not disrupt the prophylactic efficacy of MOG+VitD tolerogenic vaccination. Even in infected mice this vaccine decreased clinical signs, downmodulated the production of encephalitogenic cytokines and also increased the percentage of regulatory T cells. All together these results indicate that *C. albicans* infection and gliotoxin inoculation aggravate EAE development whereas dead *C. albicans* inoculation protected the animals.

Keywords: *Candida albicans*; candidíase sistêmica; experimental autoimmune encephalomyelitis; multiple sclerosis.

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Sumário

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Introdução

1. Introdução

1.1. Esclerose múltipla e encefalomielite autoimune experimental

A esclerose múltipla (EM) é uma doença inflamatória e desmielinizante do Sistema Nervoso Central (SNC) que é a principal causa de incapacidade neurológica em adultos jovens (Hohlfeld, 2009). A doença acomete mais mulheres do que homens, na proporção 2:1. A maior incidência ocorre na Europa (80 a cada 100.000) e a menor incidência na África (0,3 a cada 100.000); no continente Americano a incidência é de 8,3 casos a cada 100.000 habitantes (WHO, 2008). O número estimado de pessoas com EM aumentou de 2,1 milhões em 2008 para 2,3 milhões em 2013 (Browne et al., 2014). Inicialmente a maioria dos pacientes apresenta um quadro transitório de sintomas, com períodos de exacerbação e remissão da doença; a este pode se seguir a fase secundária progressiva, caracterizada por perdas irreversíveis e neurodegeneração (Imitola et al., 2005; Hohlfeld, 2009). As manifestações clínicas iniciais da EM incluem fraqueza de um ou mais membros, perda de visão, falta de coordenação motora e parestesia (Silberberg, 1992).

A causa e a patogênese da EM não são completamente conhecidas, mas os estudos indicam que esta é, fundamentalmente, uma doença autoimune mediada por células Th1/Th17 com especificidade para antígenos do SNC. Essa assertiva se baseia tanto no quadro histológico, que envolve a infiltração de macrófagos e linfócitos T e B (Lucchinetti et al., 2000) como no fato de citocinas como TNF-α, IFN-γ e IL-17 estarem envolvidas no processo inflamatório e subsequente degeneração axonal, morte dos oligodendrócitos e disfunção neuronal (Elloso et al., 2005; Sospedra & Martin, 2005; Furuzawa-Carballeda et al., 2007). As principais características histopatológicas da EM são: presença de infiltrado inflamatório composto por linfócitos T e B e macrófagos; desmielinização, ocasionada pela destruição da bainha de mielina ou pela morte dos oligodendrócitos; dano ou perda dos axônios e gliose, que se caracteriza por aumento do número de células da glia na substância branca em resposta ao dano no SNC (Constantinescu et al., 2011).

Experimentalmente a EM é estudada utilizando-se o modelo denominado encefalomielite autoimune experimental (EAE) que é induzida em ratos e camundongos por imunização com antígenos derivados de mielina (*myelin basic protein* - MBP, *proteolipid protein* - PLP, *myelin oligodendrocyte glycoprotein* - MOG ou peptídeos derivados destas proteínas) associados com o Adjuvante Completo de Freund (ACF). A transferência adotiva

de linfócitos com receptores de célula T (TCR) específicos para mielina, em animais sadios, também desencadeia a doença, indicando que a EAE é uma doença autoimune mediada pela imunidade celular (Link & Xiao, 2001). Em ratos Lewis a EAE se manifesta como doença aguda, grave e monofásica, caracterizada por infiltrado mononuclear meníngeo, perivascular e parenquimal no SNC (Link & Xiao, 2001). Nestes animais, a EAE é de recuperação espontânea e está associada ao desenvolvimento de células T supressoras (Varriale et al.,1994). Embora este modelo seja bastante utilizado, o modelo murino mimetiza melhor o decurso crônico e/ou de exacerbação-remissão visto na patologia humana (Gold et al., 2006). Exemplo disso é a paralisia e o extenso processo de desmielinização, desencadeados pela imunização de camundongos C57BL/6 com o peptídeo (35-55) derivado de MOG ($MOG_{35\text{-}55}$). Após a imunização, os animais desenvolvem uma doença crônica que perdura por, pelo menos, 45 dias (Bernard et al., 1997). De acordo com Murphy e colaboradores (2010) a partir do sétimo dia após a indução da EAE já ocorre aumento significativo de linfócitos produtores de IL-17 ($CD3^{+}CD4^{+}IFN\text{-}\gamma^{-}IL\text{-}17^{+}$) no baço, no cérebro e na medula espinhal de camundongos C57BL/6 com EAE induzida por $MOG_{35\text{-}55}$. O modelo da EAE induzida por $MOG_{35\text{-}55}$ em camundongos C57BL/6 tem sido bastante explorado, especialmente em estudos envolvendo procedimentos imunomoduladores e terapêuticos.

De modo geral, a maioria dos autores concorda que fatores ambientais, como os agentes infecciosos, em conjunto com fatores genéticos, favorecem o desencadeamento ou agravamento desta doença. Vírus, como Epstein-Barr e vírus herpes tipo 6, têm sido classicamente associados com EM (Sundqvist et al, 2011; Tait & Straus, 2008). A possibilidade de que infecções fúngicas, como as causadas pela *Candida albicans,* possam contribuir para a gravidade desta patologia tem sido sugerida mais recentemente (Benito-León et al, 2010; Pisa et al, 2011; Pisa et al, 2013).

1.2. *Candida albicans*: aspectos gerais e resposta imune

O gênero *Candida* abrange fungos pleomórficos que vivem como comensais no trato gastrointestinal e urinário de muitas espécies de mamíferos. Em indivíduos sadios raramente causam infecção persistente, mas sob certas circunstâncias, esses fungos podem agir como patógenos, com envolvimento frequente de órgãos vitais como o cérebro, fígado e rins (Van De Veerdonk et al., 2010). *Candida* spp. é o patógeno mais comum em infecções fúngicas hematogênicas (Arendrup, 2013), sendo a *C. albicans* a espécie mais frequente, seguida pela *C. tropicalis*, *C.* do complexo psilosis e *C. glabrata* (Nucci et. al., 2010).

Enquanto estão nas mucosas, isto é, em sua forma comensal, esses fungos encontram-se na forma de leveduras; ao atingir tecidos mais profundos, se transformam em hifas e/ou pseudohifas, que são formas virulentas do fungo (Brown & Gow, 1999). Este processo (morfogênese) constitui seu principal mecanismo de virulência. Segundo alguns pesquisadores, ele estaria relacionado, pelo menos em parte, ao fato das hifas impedirem a maturação de células dendríticas (DCs) e a ativação do sistema complemento (Romagnoli et al., 2004; Van Der Graaf et al., 2005).

Os mecanismos que mantêm o fungo no estado comensal e impedem a passagem para a circulação sistêmica dependem tanto da resposta imune inata como da adaptativa (Fidel & Huffnagle, 2005). Os neutrófilos são as células mais eficientes para matar e/ou inibir a formação de hifas e pseudohifas (Schuit, 1979). O reconhecimento do fungo pelas células da imunidade inata se dá por diferentes receptores de reconhecimento de padrões moleculares associados a patógenos (PAMPs) como, por exemplo, os do tipo *toll-like* (TLR), a dectina-1 e o receptor de manose (Netea et al, 2006). Destes, os TLRs são os mais estudados. Em DCs, o reconhecimento das leveduras ocorre via TLR-4 e resulta na produção de citocinas pró-inflamatórias e indução de resposta adaptativa do tipo Th1 (d'Ostiani et al., 2000). Por outro lado, a interação com as hifas ocorre via TLR-2 e resulta na produção de citocinas anti-inflamatórias e na indução de uma resposta adaptativa do tipo Th2 (Netea et al.; 2002, Kundu & Noverr; 2011). Segundo Curtis & Way (2009) a subpopulação de células Th17 confere proteção contra bactérias extracelulares e fungos, como a *C. albicans*. A possibilidade das células Th17 estarem envolvidas na modulação das candidíases é apoiada pelos achados de Saijo & Iwakura (2011). Segundo estes pesquisadores, a ligação das moléculas fúngicas β-glucanas e α-mananas aos receptores dectina-1 e dectina-2, presentes em DCs e macrófagos, levaria a indução e expansão de células Th17.

Embora sejam raros os relatos clínicos de acometimento do cérebro em pacientes com candidíase sistêmica, *Candida* spp. é frequentemente encontrada em cérebro de pacientes que morreram devido à esta infecção (Parker et al, 1981). A disseminação deste patógeno para o cérebro é mais comum em recém-nascidos (Faix & Chapman, 2003), sendo também responsáveis por grande parte dos abcessos cerebrais em pacientes imunocomprometidos (Yampolsky et al, 2010). O acometimento do SNC associado à candidíase sistêmica experimental vem sendo relatado por alguns autores. Lionakis e colaboradoes (2011) observaram expansão significativa da microglia no cérebro de camundongos C57BL/6 fêmeas após infecção. Os autores sugerem que a gliose associada ao acúmulo transitório de neutrófilos controla a infecção no cérebro. Recentemente constatamos que na infecção sistêmica utilizando a cepa FCF14, o fungo se dissemina para o cérebro e induz migração de fagócitos peritoneais para os focos de infecção (Fraga-Silva et al., 2013).

Para invadir o SNC a *C. albicans* precisa atravessar a barreira hemato-encefálica (BHE). As células endoteliais da microvasculatura do cérebro (Human Brain Microvascular Endothelial Cells – HBMECs) diferem das células endoteliais dos vasos sistêmicos. Segundo Jong e colaboradores (2001) a *C. albicans* é capaz de penetrar nestas células e de determinar a formação de pseudohifas em seu interior. Além disto, estes autores constataram que a *C. albicans* é capaz de atravessar estas células através de transcitose. A identificação de algumas moléculas que participam deste processo de migração foi recentemente descrita. Liu e colaboradores (2011) descreveram que a *C. albicans* infecta o cérebro ligando-se a gp96, uma proteína de choque térmico, expressa exclusivamente em HBMECs. Esta proteína é reconhecida pela Als3 (invasina fúngica) da *C. albicans*, o que permite a internalização do fungo pela célula endotelial e sua disseminação para o SNC.

1.3. Relação entre esclerose múltipla e fungos

A possibilidade de que toxinas fúngicas possam contribuir ou mesmo ser agentes causais da EM foi sugerida na década de 80, mas foi negligenciada durante vários anos. Recentemente, entretanto, este assunto foi alvo de revisão por Purzycki e Shain, (2010). Segundo esta revisão, certas espécies de fungos como *Aspergillus* e *Candida* conseguiriam evadir-se da resposta imunológica por se encontrarem revestidos com uma capa de manana. Esta característica lhes permitiria sobreviver no organismo humano, em um sítio não neurológico e liberar seus metabólitos continuamente para a corrente sanguínea. Entre estes produtos existem toxinas que são capazes de alterar a permeabilidade da BHE e atingir o SNC onde causariam degradação e liberação de componentes da bainha de mielina. É possível, portanto, hipotetizar que os autoantígenos assim liberados atinjam os órgãos linfoides secundários desencadeando uma resposta autoimune específica.

A possível contribuição de componentes fúngicos para o desenvolvimento da EM é sustentada tanto por achados em modelos experimentais como por algumas evidências observadas em pacientes com EM. Por exemplo, fumonisina B é uma micotoxina produzida pelo *Fusarium verticillioides*, comumente encontrado em produtos agrícolas e também em locais com excesso de umidade. Esta substância é tóxica para células da microglia e também para astrócitos primários obtidos de camundongos (Stockmann-Juvala & Savolainen, 2008). Além disto, esta toxina altera a síntese de esfingolipídeos. Coincidentemente, foi demonstrado que nos pacientes com EM ocorre uma mudança na composição lipídica dos neurônios caracterizada por aumento no conteúdo dos fosfolipídeos e diminuição na quantidade de esfingolipídeos (Wheeler et al., 2008). Do mesmo modo, experimentos realizados em ratos têm mostrado que a micotoxina penitrem A produzida pelo *Penicillium crustosum* causa tremores prolongados, ataxia, pseudoparalisis e grave disfunção neurológica (Cavanagh et al., 1998).

Outra toxina fúngica associada com alterações no SNC é a gliotoxina, um metabólito termo-estável produzido por várias espécies de *Aspergillus* e *Candida* (Kosalec & Pepeljnjak, 2005). A produção da gliotoxina por *Candida* vem sendo discutida. Kupfahl e colaboradores (2007) analisaram 100 isolados clínicos de diferentes espécies de *Candida* e não constataram a produção de gliotoxina intracelular ou extracelular *in vitro*. Kosalec e colaboradores (2008) também não detectaram a produção de gliotoxina ou metabólitos similares em diferentes cepas de *C. albicans* e *C. dubliniensis* cultivadas em meio sintético. Entretanto, alguns autores

sugerem a produção de gliotoxina ou gliotoxina-*like* por diferentes cepas de *Candida*. Shah e Larsen (1991) analisaram 50 cepas de *Candida* cultivadas durante sete dias e observaram que 32 cepas produziram uma substância semelhante à gliotoxina comercial, chamada de gliotoxina-*like*. Estes mesmos autores identificaram a gliotoxina em amostras vaginais de pacientes com candidíase vaginal e sugerem que a gliotoxina esta relacionada à virulência de *C. albicans* (Shah et al, 1995). Esta virulência ocorreria devido aos efeitos tóxicos comprovados da gliotoxina e também pelo fato da gliotoxina afetar a capacidade de invasão e disseminação do fungo, exacerbando a infecção (Kosalec & Pepeljnjak, 2004).

A inoculação intraperitoneal de gliotoxina em ratos determina a morte apoptótica de astrócitos e oligodendrócitos (Willis et al., 2004). Evidência mais direta da participação da gliotoxina na patogênese da EM foi descrita por Ménard e colaboradores (1998). Estes autores constataram, inicialmente, que o líquido cérebro-espinhal destes pacientes, mesmo quando termo-inativados, causavam morte apoptótica de astrócitos e de oligodendrócitos, mas não de outros tipos celulares, como fibroblastos e células endoteliais. O fator envolvido nesta citotoxicidade seletiva foi identificado como sendo a gliotoxina. Esta atividade gliotóxica foi inclusive identificada na urina de pacientes com EM, tendo sido sugerido que sua detecção serviria como um marcador para acompanhar recidivas ou fazer correlação com a intensidade da doença (Malcus-Vocanson et al., 1998).

Trabalhos recentes reforçam a possível participação de *Candida* spp. na EM. Benito-León *et al.* (2010), mostraram uma associação significativa entre níveis de anticorpos séricos entre *Candida* e EM. No estudo de Pisa *et al.* (2011) foi constatada a presença de macromoléculas fúngicas, de B-1,3-glucana e de anticorpos anti-*Candida* no sangue periférico de um paciente com EM. Anticorpos específicos e antígenos de *Candida* também estavam presentes no líquido cefalorraquidiano deste indivíduo. Recentemente, estes autores comprovaram a presença de antígenos de *Candida* no líquor de vários pacientes com EM (Pisa et al, 2013). Apesar da relevância deste assunto, o mesmo não tem sido estudado de forma mais detalhada. Neste contexto, nossa proposta de trabalho é, em linhas gerais, investigar a relação da *C. albicans* com a EAE que é um modelo de EM.

1.4. Relação entre esclerose múltipla e vitamina D

O tratamento para esclerose múltipla inclui medicamentos que visam reduzir a frequência e a gravidade das exacerbações, mas não ocorre cura da doença. Assim, em sua fase aguda, a EM é usualmente tratada com corticosteroides enquanto que em sua fase crônica utilizam-se drogas imunomoduladoras como o IFN-β e o acetato de glatiramer (Gold & Wolinsky, 2010). Atualmente outras drogas têm sido associadas ao tratamento da EM, como mitoxantrone, fingolimod e natalizumab (Castro-Borrero et al, 2012). Dados gerais sobre os mecanismos de ação destas drogas são descritos na tabela 1. Além disso, um grande número de ensaios clínicos tem sido conduzido para avaliar a segurança e eficácia de novas drogas, incluindo alemtuzumab, fumarato de dimetila, laquinimod, rituximab, daclizumab e cladribina (Minagar, 2013).

Estudos epidemiológicos têm demonstrado que a deficiência de vitamina D3 pode estar relacionada à etiologia da EM (Ascherio et al., 2012). Os trabalhos realizados por Munger e colaboradores mostraram que os níveis séricos de vitamina D3 (VitD) são significativamente menores em pacientes com EM em comparação com os níveis encontrados em indivíduos saudáveis. Recentemente foi demonstrado que a suplementação com VitD está associada com redução do risco de desenvolver a doença (Munger et al., 2004; Munger et al., 2006). Em um estudo clínico de fase 2 foram avaliados 49 pacientes com EM, sendo 25 tratados com altas doses de VitD durante 52 semanas (Burton et al, 2010). Neste estudo, não foram observados efeitos adversos relacionados às altas doses de VitD e os pacientes tratados apresentaram menor quantidade de recidivas da doença e redução na proliferação de linfócitos T em comparação com indivíduos controle (Burton et al, 2010).

Nos últimos anos nosso laboratório vem explorando o conceito de imunização tolerogênica profilática e terapêutica em modelos experimentais de artrite e EM. Temos seguido a linha de investigação que avalia a eficácia das imunizações diretas, ou seja, inteiramente *in vivo*, uma vez que estas estratégias poderiam ser mais facilmente traduzidas do modelo animal para o paciente. A substância de escolha é a VitD não só porque seus efeitos imunomoduladores estão bem estabelecidos mas também porque acredita-se que ela tenha um papel importante na redução do risco de desenvolver doenças autoimunes e infecciosas (Yin & Agrawal, 2014).

Tabela 1. Mecanismos de ação de drogas utilizadas no tratamento da esclerose múltipla.

Droga	Mecanismo de ação	Referência
IFN-β1a/ IFN-β1b (Avonex, Rebif, Betaseron)	*APCs* - diminui a apresentação de antígenos e a ativação de células T; *Células T* - diminui a produção de citocinas inflamatórias e determina expansão de células T reguladoras; *Células B* - diminui a expressão de MHC II e CD80, aumenta a produção de IL-10 e TGF-β.	Kasper & Reder, 2014
Acetato de glatiramer (Capoxone)	*APCs* - compete pela ligação com o MHC II bloqueando a interação de antígenos da mielina com MHC II; *Células T* - diminui a expansão de Th17 e induz expansão de Th2 e células T reguladoras; *Células B* - aumenta a produção de anticorpos e citocinas anti-inflamatórias como a IL-10; *Células do SNC* - induz proliferação, migração e diferenciação de células neuronais progenitoras e oligodendrócitos, resultando em remielinização.	Aharoni, 2014
Mitoxantrone (Novantrone)	*Células T* - inibe a proliferação de células Th e a produção de citocinas inflamatórias; *Células B* - induz apoptose de células B.	Minagar 2013
Fingolimod (FTY 720, Gilenya)	*Células T* - impede a migração de células T autorreativas para o sangue periférico e também para o SNC; *Células do SNC* - induz migração e diferenciação de células neuronais progenitoras, proliferação de astrócitos e expansão de células progenitoras de oligodendrócitos, resultando em remielinização.	Gajofatto *et al.*, 2015
Natalizumab (Tysabri)	*Células T e B* - inibe a aderência e subsequente migração de linfócitos através da BHE, atenuando a inflamação do SNC.	Salhofer-Polanyi & Leutmezer, 2014

Inicialmente a atividade biológica do hormônio 1α,25(OH)2D3 (VitD; calcitriol) foi focada na regulação da homeostasia de cálcio e fósforo. Mas atualmente já se sabe que seus efeitos biológicos se estendem a diversos tipos celulares, como por exemplo, as células do sistema imunológico e do SNC (Kongsbak et al., 2013; Eyles et al., 2005). O papel da VitD nas imunidades inata e específica é mediado por via endócrina, ou seja, através do calcitriol circulante, por via parácrina (em células adjacentes ou próximas) e também por via autócrina (dentro da própria célula) (Lang et al., 2013). Este efeito tão amplo e relevante da VitD no sistema imune está relacionado com a existência de receptores para VitD (VDR) na maioria das células deste sistema (Wöbke et al., 2014). Além disso, o sistema imune é capaz de produzir a enzima 25-hidroxivitamina D-1α- hidroxilase que converte a forma circulante da vitamina D na forma ativa. Células típicas da imunidade inata tais como monócitos, macrófagos e DCs não só expressam VDR como também produzem a hidroxilase acima citada. De forma geral, a adição de VitD em monócitos/macrófagos resulta em diminuição da produção de derivados de O_2, de NO e das citocinas pró-inflamatórias (Neve et al., 2014; Jain, 2013). Acredita-se que esta inibição, que aparentemente contrasta com a atividade microbicida da VitD, seja um efeito de feedback negativo para manter a homeostase, ou seja, evitar uma resposta inflamatória excessiva. Foi recentemente demonstrado por Chen et al., 2013, que a sinalização via VDR atenua a inflamação mediada por receptores do tipo Toll em macrófagos.

Outra atividade relevante da VitD é a modulação de DCs e a indução de células T reguladoras (Tregs). DCs humanas diferenciadas na presença de calcitriol são imaturas e se caracterizam por expressão reduzida de CD1a, MHC II, CD40, CD80 e CD86 (Adorini & Penna, 2009). Estas células também produzem menor quantidade de IL-12 e maior concentração de IL-10. Estes efeitos em conjunto determinam uma condição tolerogênica na qual estas DCs se tornam incapazes de estimular a proliferação de células T. Este fenômeno tem sido, pelo menos em parte, atribuído à geração de Tregs, pois a co-cultura de DCs pré-tratada com calcitriol e células T $CD4^{+}CD25^{+}$ leva à indução de células Tregs $CD4^{+}FoxP3^{+}$ (Penna et al., 2005). A VitD também atua sobre células T produtoras de IL-17. Hamzaoui e colaboradores (2014) demonstraram que a adição de VitD inibiu a diferenciação de células $TCD4^{+}$ em células $TCD4^{+}IL\text{-}17^{+}$ tanto em pacientes com asma quanto em controles normais. Seu efeito sobre células Th2 é menos claro, sendo que alguns relatos indicam aumento da produção de IL-4, IL-5 e IL-10 (Boonstra et al., 2001; Sloka et al., 2011), enquanto outros mostram redução no número de células produtoras de IL-4 ou até mesmo ausência de efeito

(Dimeloe et al., 2010). A dependência das concentrações utilizadas *in vitro* tem sido apontada como causa destes resultados contraditórios.

Recentemente tem sido demonstrado o papel imunomodulador benéfico da VitD na EAE. Chang e colaboradores demonstraram que camundongos C57BL/6 tratados com VitD apresentaram melhora significativa na sintomatologia acompanhada de redução na produção de citocinas pró-inflamatórias (IL-17, IFN-γ e IL-22) (Chang et al., 2010). A administração de VitD ou a transferência adotiva de DCs imaturas induzidas pela VitD foram capazes de expandir a população de células Tregs reduzindo a gravidade da EAE (Farias et al., 2013). Também foi demonstrado em camundongos C57BL/6 que a suplementação de vitamina D determinou efeito profilático e terapêutico mediado pela IL-10 (Spach et al., 2006).

Dentro deste contexto, o aspecto mais inovador desta linha de pesquisa do laboratório é investigar se a VitD funciona como um adjuvante tolerogênico quando associada com antígenos específicos. O possível papel de drogas imunossupressoras como adjuvantes tolerogênicos só foi investigado mais recentemente. Kang et al. (2008), por exemplo, demonstraram que camundongos BALB/c sensibilizados com OVA poderiam ser dessensibilizados por um tratamento concomitante com um peptídeo de OVA e dexametasona. Este processo de tolerização foi associado com bloqueio na maturação de DCs e com expansão de Tregs FoxP3+. Estes autores também constataram que a dexametasona era tolerogênica e profilática em camundongos NOD quando associada com um peptídeo derivado de insulina. Kang et al. (2009) mostraram que este conceito também era válido na EAE. A imunização de camundongos com uma vacina gênica contendo o gene da MOG na presença de FK506 preveniu o desenvolvimento da EAE.

Nesta área de pesquisa mostramos recentemente que a inoculação, em camundongos C57BL/6, de MOG na presença de VitD, preveniu o desenvolvimento da EAE. Estes estudos geraram dois trabalhos, sendo um deles relativo ao efeito profilático (Mimura et al., 2016) e o outro relacionado com o efeito terapêutico (Chiuso-Minicucci et al., 2015).

1.5. Racional do projeto

A EM é uma doença autoimune grave que acomete o SNC. Acredita-se que fatores ambientais tais como agentes infecciosos possam agravar ou até mesmo desencadear esta patologia. Evidências observadas em pacientes e em modelos experimentais indicam que fungos podem contribuir com a patogênese da EM. Neste contexto, o objetivo deste projeto é avaliar o efeito da infecção com *C. albicans* e do contato com derivados fúngicos no desenvolvimento da EAE que é o modelo utilizado para estudos desta patologia.

Nossa hipótese de trabalho é que a infecção com *C. albicans* e o contato com derivados fúngicos agrave esta doença. Este possível agravamento ocorreria, por exemplo, pela presença de toxinas fúngicas na circulação que seriam capazes de alterar a permeabilidade da BHE e, assim, penetrar no SNC, causando lesão do tecido nervoso e liberação de autoantígenos. Alternativamente a presença do fungo ou dos antígenos no SNC seria capaz de determinar uma reação inflamatória específica para o fungo que contribuiria para a expressão clínica da EAE. Alternativa ou concomitantemente, a presença do fungo e/ou derivados fúngicos no SNC poderia contribuir com a liberação de neuroantígenos.

Considerando ainda a necessidade de estratégias mais específicas e que sejam eficazes mesmo na presença de outros fatores agravantes da EM como as infecções, por exemplo, objetivamos ainda investigar se o fungo interfere na eficácia profilática da associação de MOG com VitD.

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2. Referências

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Objetivos

3. Objetivos

3.1. Objetivo geral

Avaliar o efeito da infecção com *C. albicans* e do contato com derivados fúngicos no desenvolvimento da encefalomielite autoimune experimental (EAE).

3.2. Objetivos específicos

I. Avaliar o efeito da infecção prévia com *C. albicans* no desenvolvimento da EAE;

II. Avaliar o efeito de derivados fúngicos no desenvolvimento da EAE;

III. Avaliar se a infecção por *C. albicans* interfere na eficácia da profilaxia determinada por imunização com MOG associada à vitamina D na EAE.

Resultados e discussão

4. Resultados e discussão

Os resultados e discussão dos dados obtidos encontram-se apresentados na forma de artigos científicos.

4.1. Artigo científico I

Experimental Autoimmune Encephalomyelitis Development Is Aggravated by *Candida albicans* Infection. **Journal of Immunology Research**, 2015: 635052. doi.org/10.1155/2015/635052.

4.2. Artigo científico II

Differential modulation of experimental encephalomyelitis by distinct fungal derivatives (manuscrito em preparação).

4.3. Artigo científico III

Encephalomyelitis aggravated by *C. albicans* is attenuated by tolerogenic vaccination with MOG/VitD (artigo submetido).

Artigo científico I

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 635052, 11 pages
http://dx.doi.org/10.1155/2015/635052

Hindawi

Research Article

Experimental Autoimmune Encephalomyelitis Development Is Aggravated by *Candida albicans* Infection

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Received 18 September 2014; Revised 19 January 2015; Accepted 22 January 2015

Academic Editor: Oscar Bottasso

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Multiple sclerosis (MS) is an inflammatory/autoimmune disease of the central nervous system (CNS) mainly mediated by myelin specific T cells. It is widely believed that environmental factors, including fungal infections, contribute to disease induction or evolution. Even though *Candida* infection among MS patients has been described, the participation of this fungus in this pathology is not clear. The purpose of this work was to evaluate the effect of a *Candida albicans* infection on experimental autoimmune encephalomyelitis (EAE) that is a widely accepted model to study MS. Female C57BL/6 mice were infected with *C. albicans* and 3 days later, animals were submitted to EAE induction by immunization with myelin oligodendrocyte glycoprotein. Previous infection increased the clinical score and also the body weight loss. EAE aggravation was associated with expansion of peripheral $CD4^+$ T cells and production of high levels of TNF-α, IFN-γ IL-6, and IL-17 by spleen and CNS cells. In addition to yeast and hyphae, fungus specific T cells were found in the CNS. These findings suggest that *C. albicans* infection before EAE induction aggravates EAE, and possibly MS, mainly by CNS dissemination and local induction of encephalitogenic cytokines. Peripheral production of encephalitogenic cytokines could also contribute to disease aggravation.

1. Introduction

Multiple sclerosis (MS) is an inflammatory/autoimmune and demyelinating disease of the central nervous system (CNS). It is considered one of the most common neurological disorders and causes of disability in young adults [1]. The estimated number of people with MS has increased from 2.1 million in 2008 to 2.3 million in 2013 [2]. Animal models, particularly experimental autoimmune encephalomyelitis (EAE), have been essential to decipher the pathophysiology of MS [3–6]. MS and EAE are characterized by an autoimmune response against CNS proteins, mediated mainly by T cells, that culminates in inflammatory infiltrate, gliosis, damage of myelin sheath, and neuronal death [7–9].

This disease is thought to be triggered by a complex interaction between genetic and environmental factors. Expressive data confirm that genetic variation is an important determinant for MS risk. Population, family, and molecular studies strongly support a polygenic model of inheritance, driven primarily by allelic variants relatively common in the general population. The major histocompatibility complex is believed to be the strongest MS susceptibility locus genomewide and was identified in all studied populations [10]. It has also long been recognized that infections may serve as environmental triggers for this disease. A large number of pathogens, including worldwide distributed fungi, have been proposed to be associated with MS [11]. As most of the systemic fungal pathogens have been associated with

dissemination to the CNS [12], they could contribute to local tissue destruction by their presence or, alternatively, by the induction of a local immune response.

Candida spp. is one of these pathogens that could contribute to MS development. *C. albicans* is a pleomorphic fungus that colonizes the majority of healthy human individuals. This fungus can behave as a normal component of the microbiota and also as an opportunistic pathogen that causes superficial mucosal infections as well as disseminated disease [13, 14]. As the fourth most common cause of nosocomial infections, *C. albicans* is commonly isolated from immunocompromised individuals, including those with HIV, those immunosuppressed due to cancer treatment, and premature babies [15]. A possible association between MS and *Candida* spp. has been suggested by serological evidences. A significantly higher level of *Candida* specific antibodies was detected in MS patients than in normal control individuals [16]. In addition, *Candida* spp. antigens were also demonstrated in the cerebrospinal fluid of some MS patients [17].

The possible contribution of *Candida* spp. to MS pathogenesis was initially attributed to cross-reactivity with human tissues, including brain structures [18]. More recently, it was proposed that *Candida*, sequestered in nonneuronal tissues, could release toxins that would destroy astrocytes and oligodendrocytes generating myelin debris that would then trigger a pathogenic immune response in the CNS [19]. Furthermore, the presence of yeast and hyphae in the brain recruits inflammatory cells and elicits expansion of microglia cells [20]. Considering that the possible contribution of *C. albicans* to MS needs to be investigated and that elucidation of this could affect the treatment of this disease, we evaluated the possible deleterious effect of a previous *C. albicans* infection on EAE development.

2. Methods

2.1. Animals. Female C57BL/6 mice 9–11 weeks old were purchased from University of São Paulo (USP) (Ribeirão Preto, SP, Brazil). The animals received sterilized food and water *ad libitum* and were manipulated in accordance with the local Ethics Committee for Animal Experimentation (CEEA), São Paulo State University (UNESP) (Botucatu, SP, Brazil; protocol number 351).

2.2. EAE Induction. MOG35–55 peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Genemed Synthesis Inc. (San Antonio, Texas, USA). Mice were immunized subcutaneously with 100 μg of MOG35–55 peptide emulsified in 25 μL of Complete Freund's Adjuvant (CFA) containing 4 mg/mL of *Mycobacterium tuberculosis*. Mice also received 2 intraperitoneal doses, 0 and 48 hours after immunization, of 200 ng of *Bordetella pertussis* toxin (Sigma-Aldrich Corporation, St. Louis, MO, USA). EAE clinical assessment was daily performed according to the following criteria: 0, no symptoms; 1, limp tail; 2, hind legs weakness; 3, partially paralyzed hind legs; 4, complete hind leg paralysis; and 5, complete paralysis/death. The % of weight loss and the maximum clinical score were calculated considering the highest body weight loss and the highest clinical score that each animal reached during the experiment, independently of the period, and the result was expressed as the mean per experimental group.

2.3. Fungi. C. albicans strain FCF 14 (Genbank Accession EF591020) was originally obtained from the mycology collection of the Faculdade de Odontologia de São José dos Campos, UNESP, and maintained in our mycological collection on Sabouraud-dextrose agar (Difco Laboratories, Detroit, MI, USA). For mice infection, *C. albicans* was cultured on solid media during 24 hours at 37°C. The fungal concentration was adjusted to 5.0×10^7/mL viable yeast cells in sterile saline solution (SSS). Fungus suspension was then inoculated into the lateral tail vein (0.1 mL/animal).

2.4. Fungal Load Determination. Samples from spleen, kidney, liver, brain, and spinal cord were weighted and macerated in 1.0 mL of SSS. Afterwards, 0.1 mL from each tissue homogenate was spread over culture plates containing Sabouraud-dextrose agar using a Drigalski T loop. The procedures were performed in duplicate. The plates were then sealed and incubated at 37°C for 3 days. The number of colony forming units (CFU) was normalized per gram of tissue.

2.5. CNS-Mononuclear Cells Isolation. Fourteen days after EAE induction, mice were anesthetized with ketamine/xylazine and perfused with 10 mL of SSS. Brain and spinal cord were collected, macerated, and digested with 2.5 mg/mL of collagenase D (Roche Applied Science, Indianapolis, IN, USA) in 4 mL of RPMI (Sigma) at 37°C for 45 min. Then, suspensions were washed in RPMI and centrifuged at 450 ×g at 4°C for 15 min. Cells were resuspended in Percoll (Sigma) 37% and gently laid over Percoll 70% in tubes of 15 mL. The tubes were centrifuged at 950 ×g for 20 min with centrifuge breaks turned off. After centrifugation the ring containing mononuclear cells was collected, washed in RPMI, and centrifuged at 450 ×g for 10 min. Cells were then resuspended in complete RPMI medium (RPMI supplemented with 10% of fetal bovine serum), counted, and analyzed.

2.6. Cell Culture Conditions and Cytokine Quantification. Spleen and CNS-isolated cells were collected and adjusted to 5×10^6 cells/mL and 2×10^5 cells/mL, respectively, in complete RPMI medium. Spleen and CNS-isolated cells were plated and stimulated with MOG (20 μg/mL and 50 μg/mL, resp.) and with *C. albicans* (5 yeasts/1 cell). Cytokine levels were evaluated 48 h later by enzyme-linked immunosorbent assay (ELISA) in culture supernatants using IFN-γ BD OptEIA Sets (Becton, Dickinson and Company, BD, Franklin, San Diego, CA, USA) and IL-2, IL-4, IL-6, IL-10, IL-17, and TNF-α Duosets (R&D Systems, Minneapolis, MN, USA). The assays were performed according to the manufacturer's instructions.

2.7. FACS Analysis. Spleen cells were collected; the red blood cells were lysed with buffer containing NH_4Cl, and adjusted to 10^6 cells/tube. CNS-extracted cells were plated at 5×10^5 cells/well and stimulated with MOG (125 μg/mL) and with *C. albicans* (5 yeasts/1 cell). After incubation at 37°C for

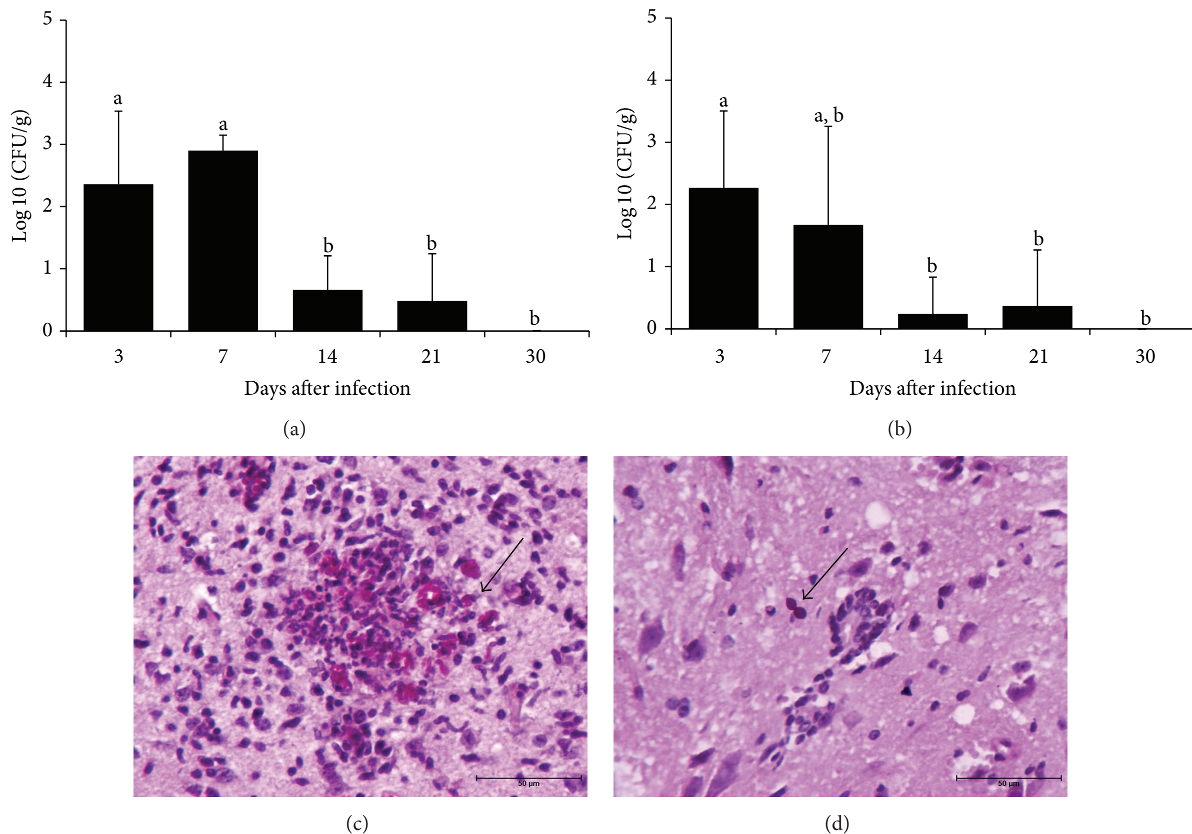


Figure 1: Dissemination of *C. albicans* to the central nervous system. C57BL/6 mice were infected with *C. albicans* and fungal load was evaluated 3, 7, 14, 21, and 30 days after in the brain (a) and in the spinal cord (b). The results are expressed as mean ± SEM (n = 5-6 mice/group) of the CFU (log 10) per gram of tissue. ANOVA, Tukey's test, $P < 0.05$. Different letters indicate statistical difference among the experimental time points. Periodic acid-Schiff revealed yeasts and hyphae in brain (c) and yeast in cervical spinal cord (d) sections.

48 h, cells were collected and stained. Spleen and CNS-extracted cells were blocked with rat serum 1% for 20 min to prevent nonspecific binding via Fc receptor. After Fc blocking, cells were stained with 0.2 μg of PerCP-conjugated anti-mouse CD3 and 0.25 μg of FITC-conjugated anti-mouse CD4 for 20 min at 4°C. Intracellular FoxP3 transcription factor analysis was performed only in spleen samples by using CD3-PercP, CD4-FITC plus 0.13 μg of APC-conjugated anti-mouse CD25 and 0.2 μg of PE-conjugated anti-mouse FoxP3 and staining set (eBiosciences, San Diego, CA, USA) according to manufacturer's instructions. After staining, the cells were washed, resuspended in FACS buffer, and fixed in paraformaldehyde 1%. Analysis was performed using a FACSCanto II (BD) from Bioscience Institute (Botucatu, SP, Brazil) and the data were analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

2.8. Histopathology of the CNS. After euthanasia, brain and lumbar spinal cord samples were removed and fixed in 10% neutral buffered formalin. Paraffin slides with 4 μm were stained with hematoxylin and eosin (H&E) to evaluate the inflammatory process. A semiquantitative analysis of CNS inflammation was performed according to the following criteria: (0) inflammatory infiltration absent; (+/++) mild/moderate inflammatory infiltration; (+++) intense inflammatory infiltration. Sections were also stained with periodic acid-Schiff to visualize fungal structures.

2.9. Statistical Analysis. Results were expressed as mean ± standard deviation or with median and interquartile (25–75%) ranges. To test for the normality of data, results were analyzed by Shapiro-Wilk's test. Comparisons between two samples were made by t-test and more than three samples were made by one way ANOVA followed by Tukey's test for parametric variables and by Kruskal-Wallis followed by Dunn's test for nonparametric variables. Fisher's test was performed to estimate the frequency of *C. albicans*-positive tissues and to compare the semiquantitative analysis of CNS tissue inflammation. The data were analyzed using SigmaPlot statistical package for Windows version 2.0 (1995, Jandel Corporation, CA, USA) and values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Candida albicans Infection Disseminates to the CNS. We initially tested the characteristics of the *C. albicans* infection in C57BL/6 mice as this is one of the strains that are susceptible to EAE induction. Experimental infection with *C. albicans* in C57BL/6 mice determined a disseminated infection that

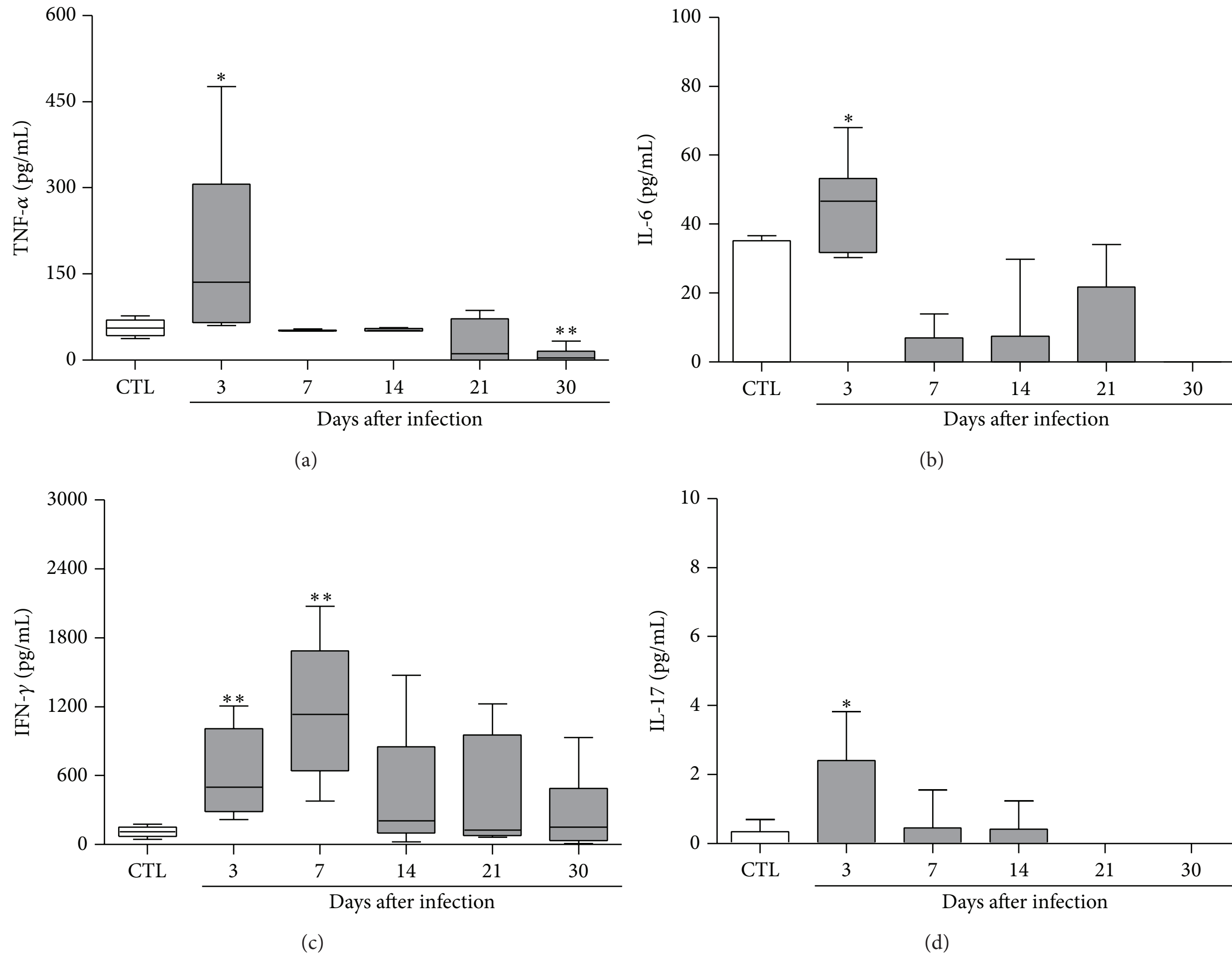


FIGURE 2: Kinetics of cytokine production by spleen cells from mice infected with *C. albicans*. C57BL/6 mice were inoculated with *C. albicans* and the spontaneous production of cytokines by spleen cells was evaluated 3, 7, 14, 21, and 30 days after fungal inoculation. The results are expressed as median, 25–75% (box), and minimum-maximum (error bars) of 5-6 mice/group. Mann-Whitney test, $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistical difference between each experimental time point and the control group (uninfected).

also reached the CNS. As observed in Table 1, the viable fungi were recovered from all evaluated organs, including the brain and the spinal cord. After 30 days all organs, except the spleen, exhibited fungal clearance. The kinetics of fungal load, during 30 days, is showed in Figures 1(a) and 1(b) for brain and spinal cord, respectively, and indicates that the fungus load is more accentuated in the first week of infection. The presence of yeasts and hyphae in the brain and yeast in the spinal cord is illustrated in Figures 1(c) and 1(d), respectively.

3.2. Production of Potentially Encephalitogenic Cytokines during C. albicans Infection. As many of the most encephalitogenic cytokines are also involved in the defense against *C. albicans* and other fungi, we tested their production during the time periods when the fungus was being detected. Spleen cell cultures from infected mice produced elevated levels of TNF-α, IL-6, IFN-γ, and IL-17 (Figure 2). Cytokine levels were especially elevated in the 3rd day after infection.

TABLE 1: Frequency of *C. albicans*-positive tissues.

Period	Tissue				
	Spleen	Kidney	Liver	Brain	Spinal cord
3 days	6/6	6/6	5/6	5/6	5/6
7 days	5/5	5/5	3/5	5/5	3/5
14 days	4/6	2/6	0/6	4/6	1/6
21 days	4/6	1/6	0/6	2/6	1/6
30 days	2/5	0/6	0/6	0/6	0/5
P value	0.0606	0.0022	0.0152	0.0152	0.0152

Data were expressed as number of *C. albicans*-positive animals/total number of animals per group.

3.3. Infection with C. albicans Aggravates EAE Development. To test the possible deleterious role of *C. albicans* on EAE development, EAE was induced in mice that had been infected three days before with the fungus. Mice previously infected, denominated EAE+Ca group, developed a more

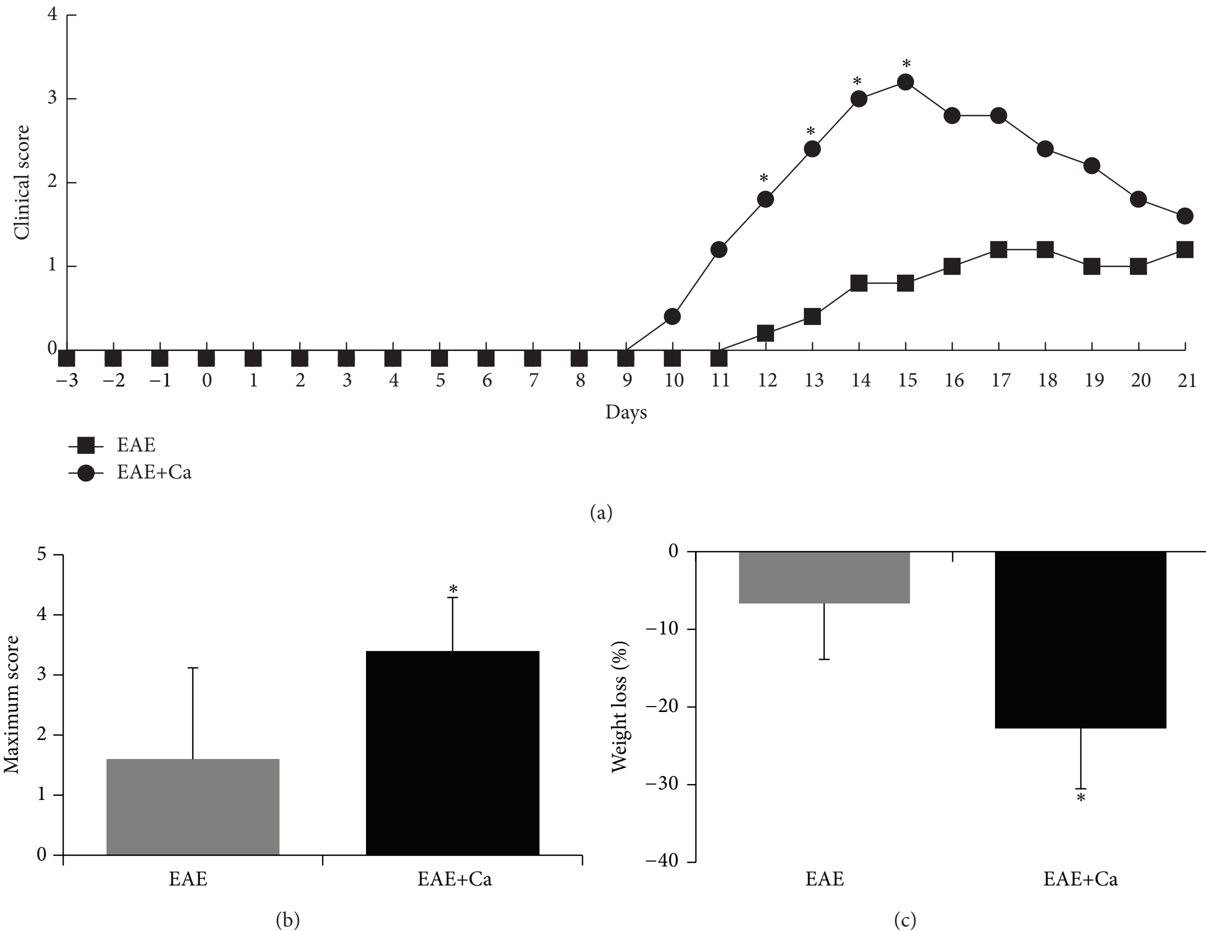
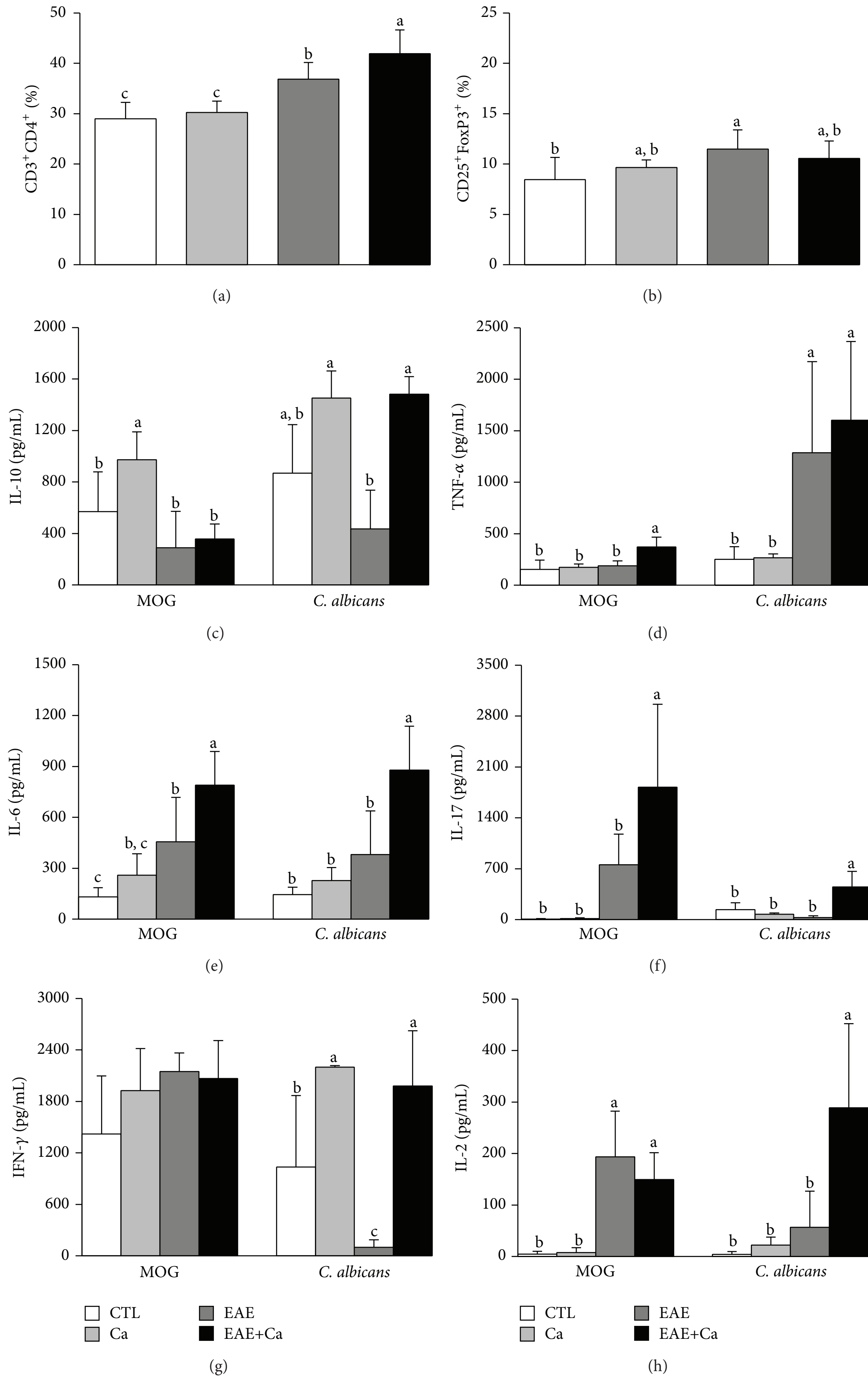


Figure 3: Effect of *C. albicans* on EAE development. C57BL/6 mice were infected with *C. albicans* 3 days before EAE induction. Disease development was followed during 21 days. Clinical scores (a) were checked every day and are expressed as mean; maximum clinical score (b) and % of body weight loss (c) were calculated as described in Methods section. The results (a and b) are expressed as mean ± SD ($n = 6$–8 mice/group). Unpaired t test, $^{*}P < 0.05$ indicates difference between EAE and EAE+Ca groups.

severe form of encephalomyelitis. As shown in Figure 3(a), these animals already showed paralysis signs at the 9th day after EAE induction whereas the EAE control group presented paralysis only 2 days later. This higher disease severity was detected during the whole acute disease phase. The average maximum clinical score, as depicted in Figure 3(b), confirmed this worst clinical evolution. Weight loss was also more accentuated in this experimental group as can be observed in Figure 3(c).

3.4. Peripheral Immunological Alterations during EAE Aggravation by C. albicans Infection. To evaluate if peripheral immunological parameters could explain this detrimental fungal effect on EAE, we tested the % of $CD3^{+}CD4^{+}$ and $CD3^{+}CD4^{+}CD25^{+}$ $FoxP3^{+}$ T-cell subsets. The cytokine production by spleen cells restimulated with MOG or with heat-killed *C. albicans* yeasts was also determined. Normal mice and mice only infected were also analyzed. A higher percentage of $CD3^{+}CD4^{+}$ T cells were found in EAE+Ca and EAE groups in comparison to normal and infected groups. In addition, the % of this T-cell subset was significantly higher in the group that was previously infected with the fungus (EAE+Ca) in comparison to the EAE group (Figure 4(a)). The % of the $FoxP3^{+}$ T cells was significantly higher in the EAE, but not in the Ca and EAE+Ca groups, in comparison to the control group, as illustrated in Figure 4(b). Concerning cytokines induced by MOG, the EAE+Ca group presented a significant production of TNF-α (Figure 4(d)), IL-6 (Figure 4(e)), and IL-17 (Figure 4(f)) in comparison to all other experimental groups. IL-2 (Figure 4(h)) and IL-4 (Figure 4(i)) were similarly elevated in EAE and EAE+Ca groups. These two groups also produced low and similar amounts of IL-10 (Figure 4(c)). Comparison of EAE+Ca and EAE cytokine production induced by heat-killed *C. albicans* clearly showed that IL-10, IL-6, IL-17, IFN-γ, IL-2, and IL-4 were significantly higher in the previously infected group.

3.5. Local Immunological Alterations during EAE Aggravation by C. albicans Infection. H&E staining clearly indicated a strong and similar inflammatory process in the brain and spinal cord of both EAE and EAE+Ca animals, as shown in Figure 5. This analogous inflammatory process was confirmed by a semiquantitative analysis done in both brain and spinal cord samples (data not shown). As expected,



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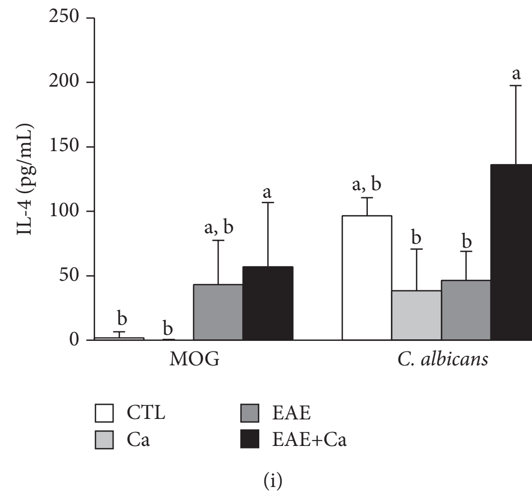

(i)

Figure 4: Modulation of MOG-induced cytokine production by previous infection with *C. albicans*. C57BL/6 mice were infected with *C. albicans* and 3 days later they were submitted to EAE induction. Fourteen days after EAE induction, some immunological parameters were evaluated in the spleen. The percentage of $CD3^+CD4^+$ (a) and $CD3^+CD4^+CD25^+FoxP3^+$ (b) was performed by cytometric analysis in 100.000 acquired events. IL-10 (c), TNF-α (d), IL-6 (e), IL-17 (f), IFN-γ (g), IL-2 (h), and IL-4 (i) levels were measured in spleen cell cultures stimulated with MOG or heat-killed *C. albicans*. The results are expressed as mean ± SD (n = 6–8 mice/group). ANOVA, Tukey's test, and $P < 0.05$. Different letters indicate statistical difference among the groups (a and b) or among the groups under the same *in vitro* stimulation (c, d, e, f, g, h, and i).

no inflammatory infiltrates were present in normal mice (Figures 5(a) and 5(d)). The amount of total leukocytes eluted from the CNS from both experimental groups was also similar as depicted in Figure 5(g). The percentage of $CD3^+CD4^+$ T cells was always higher in the EAE+Ca group, independently of their previous stimulation with MOG or heat-killed *C. albicans* yeasts (Figure 5(h)). Cells eluted from the CNS of both groups respond in a similar way to *in vitro* stimulation with MOG, that is, they produced similar amounts of TNF-α, IL-17, IFN-γ, IL-2, and IL-10 (Figure 6). However, cells eluted from mice previously infected with *C. albicans* (EAE+Ca group) produced much more TNF-α, IL-6, IL-17, IFN-γ, and IL-10 in response to *C. albicans in vitro* restimulation (Figure 6).

4. Discussion

Multiple sclerosis (MS) is one of the world's most common neurological disorders [2]. The disease develops as a result of interactions between the environment and the immune system in genetically susceptible individuals and it has long been recognized that infections may serve as environmental triggers for MS [11]. Even though viral agents have been more usually suspected as aggravating or triggering agents of this disease, fungi, especially their toxins, were recently incriminated as relevant underlying causes of MS and thus may offer an approach towards a more effective adjunct treatment [19]. *C. albicans* is the most common fungal pathogen of humans and its spreading to the brain has been described during acute infections [21, 22]. Interestingly, fifty percent of patients with disseminated candidiasis underwent CNS fungal invasion [23]. Even though *C. albicans* is usually more prevalent in immunocompromised individuals, it has also been reported to cause meningoencephalitis in healthy individuals [24]. Considering these aspects and the fact that a possible relationship between *Candida* spp. and MS patients [16, 17, 19] was recently described, we evaluated the effect of an experimental infection with this fungus on the development of EAE, which is a largely accepted model to study the pathophysiological mechanisms of MS [25].

We initially evaluated the characteristics of *C. albicans* infection in C57BL/6 mice, which is one of the strains that develop encephalomyelitis upon immunization with antigens from the CNS [26]. This strain developed a widespread infection characterized by involvement of the majority of the organs, including the brain and the spinal cord. This diffuse infection was, however, very well controlled by the immune system since almost no fungi were recovered after 30 days of infection. This dissemination of *C. albicans* to the brain was already demonstrated not only in C57BL/6 mice [20] but also in other mouse strains as BALB/c [27] and Swiss [28]. However, this is the first report that indicates spreading of this fungus to the spinal cord portion of the CNS in mice.

As expected, the infectious process triggered by *C. albicans* induced an elevated production of inflammatory cytokines as TNF-α, IL-6, IFN-γ, and mainly IL-17. This proinflammatory environment was more pronounced by the 3rd day of infection. As these cytokines have been clearly associated with MS and EAE due to their encephalitogenic properties [29–32], we choose this period of infection to induce EAE. This choice was also based on the fact that the fungus had already reached the CNS at this early time. C57BL/6 mice were then infected with *C. albicans* by intravenous route and 3 days later they were submitted to EAE induction. A very clear deleterious effect was observed in EAE development. The animals became sick earlier and,

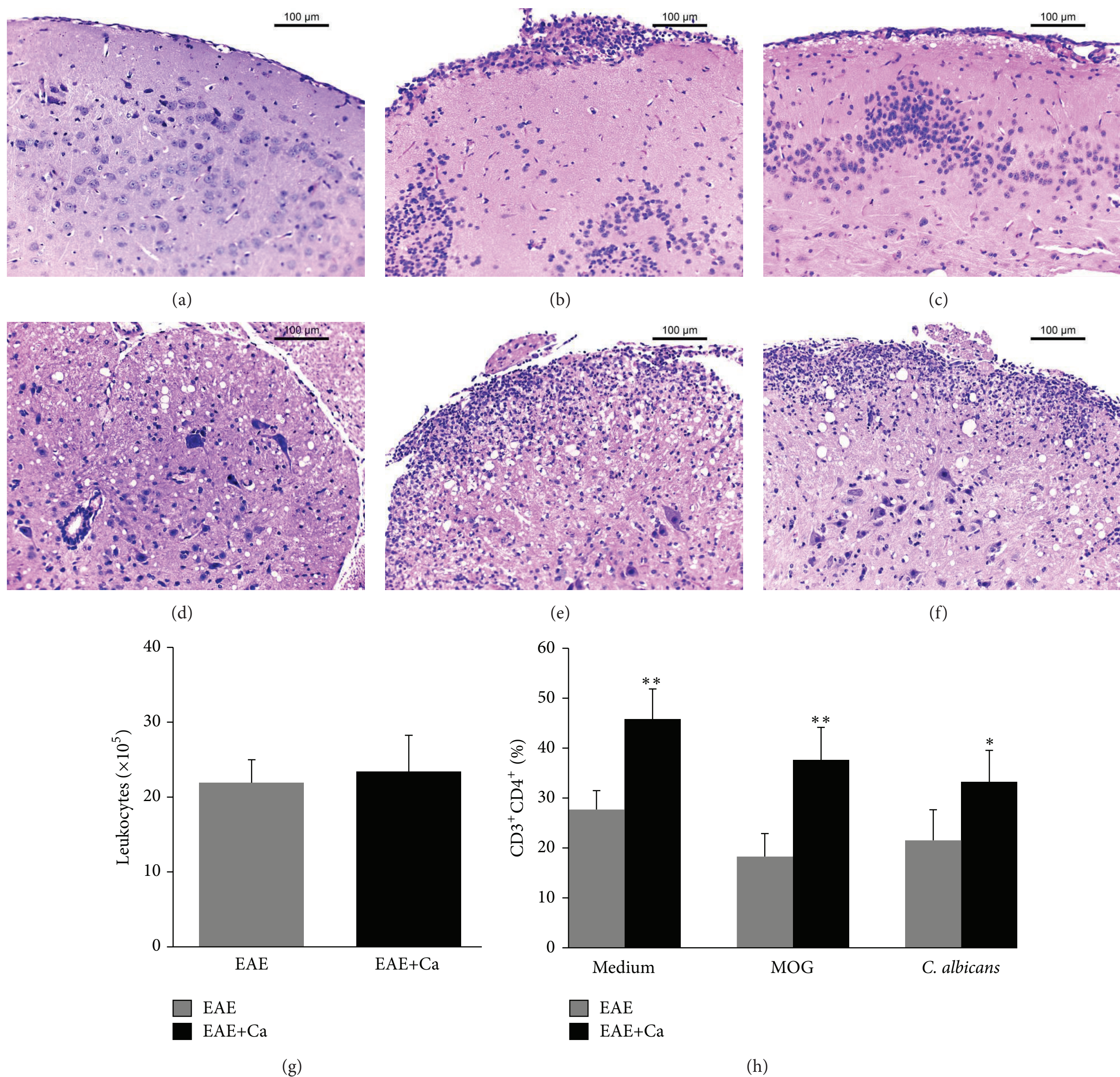

Figure 5: Previous infection with *C. albicans* increases the amount of $CD4^+$ T cells in the CNS. C57BL/6 mice were infected with *C. albicans* and 3 days later they were submitted to EAE induction. Fourteen days after EAE induction, inflammation and % of $CD4^+$ T cells were evaluated in the CNS. Inflammatory infiltrates detected by H&E staining are shown in brain samples from EAE (b) and from EAE+Ca (c) groups and in spinal cord samples from EAE (e) and from EAE+Ca (f) groups. A brain and spinal cord samples from a normal mouse is shown in (a) and (d), respectively. Total leukocyte number (g) and percentage of $CD3^+CD4^+$ T-cell subset (h) (analysis performed in 50.000 acquired events). The results are expressed as mean ± SD (n = 6-7 mice/group). Unpaired t test, $^*P < 0.05$ and $^{**}P < 0.01$ indicate difference between EAE and EAE+Ca groups under the same *in vitro* stimulation.

in addition, developed a more severe disease. Higher severity was characterized by both a higher body weight loss and a more accentuated degree of paralysis. To the best of our knowledge, this is the first demonstration that a previous experimental infection with *C. albicans* triggered EAE exacerbation. These findings are relevant because a direct contribution of *C. albicans* to this neurological disease has not been deeply investigated. However, a series of indirect and epidemiological findings supports this possibility. For example, Purzycki and Shain [19] proposed that certain pathogenic fungi could release toxins that, by destroying CNS astrocytes and oligodendrocytes, would degrade myelin triggering the onset of MS and its associated symptoms. By using immunofluorescence analysis, Benito-León et al. [16] suggested a serological evidence of a link between *Candida* infection and MS condition. By comparing the amount of anti-*Candida* antibodies in the sera of normal subjects and MS patients, these authors suggested that infections with

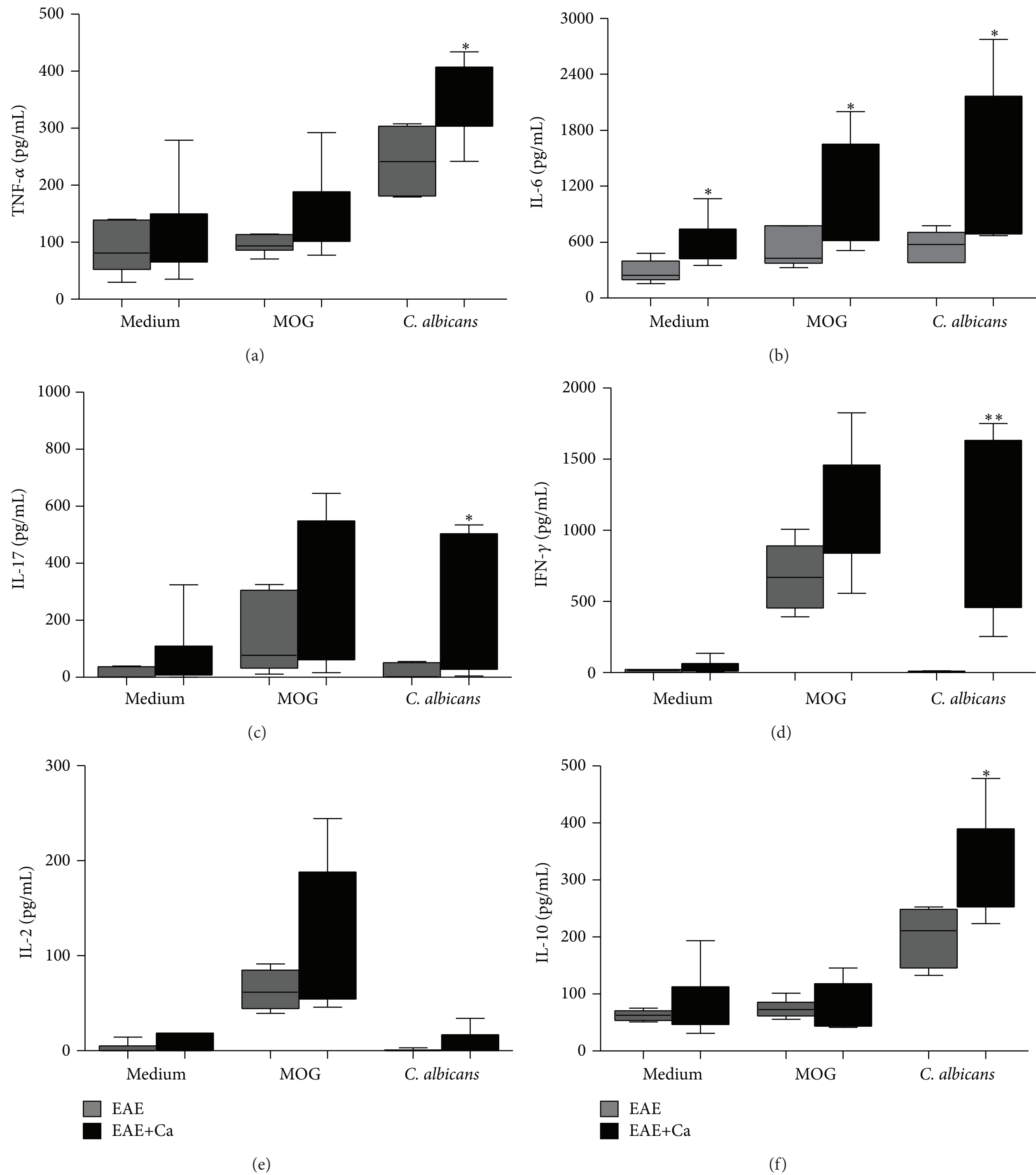

FIGURE 6: *Candida* specific T cells contribute to elevated production of encephalitogenic cytokines in the CNS. CNS eluted cells were restimulated with MOG or heat-killed *C. albicans* and TNF-α (a), IL-6 (b), IL-17 (c), IFN-γ (d), IL-2 (e), and IL-10 (f) levels were measured by ELISA. The results are expressed as median, 25–75% (box), and minimum-maximum (error bars) of 6 to 7 mice/group. Mann-Whitney test, $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistical difference between EAE and EAE+Ca groups under the same *in vitro* stimulation.

Candida spp. could be associated with increased odds of MS [16]. In addition to specific antibodies, fungal macromolecules such as proteins, polysaccharides, and DNA were also detected in blood samples from MS patients [33]. Besides these serologic evidences, antibodies against *Candida* spp. [33] and fungal DNA [17] were also detected in the cerebrospinal fluid of MS patients.

To unravel, at least partially, the immunological mechanism involved in this effect, some peripheral immunological parameters were compared among EAE, EAE+Ca,

C. albicans infected (Ca), and normal (CTL) experimental groups. Even though T regulatory (Treg) mediated responses remain poorly understood in *Candida* infection, data indicate increased proportion of this subset during candidiasis [34, 35]. As FoxP3$^+$ T cells are mostly responsible for EAE recovery in C57BL/6 mice [36, 37], we initially hypothesized that Treg expansion could theoretically downregulate EAE development. This assumption was based on the fact that Treg cells induced during infectious diseases can regulate EAE in an apparently nonspecific manner [38]. To test this possibility we evaluated the effect of the *C. albicans* infection on the percentage of this T-cell subset. The expected increase in the percentage of FoxP3$^+$ T cells was found in the spleen of the EAE group. However, the proportion of this T-cell subset was not modified in Ca and in EAE+Ca groups. This finding can be attributed, at least partially, to the complex relationship, including cell plasticity, between Treg and Th17 responses during *C. albicans* infection [35]. In addition to Treg cells we also evaluated the percentage of CD4$^+$ T cells and cytokine production. Previous fungal infection increased CD4$^+$ T-cell subset in spleen of EAE-mice (EAE+Ca group) and clearly upmodulated the production of many encephalitogenic cytokines by spleen cells stimulated with MOG or heat-killed *C. albicans*. Even though the effect of EAE on fungal load was not the focus of this investigation, fungi recovery was usually significantly lower in the infected animals that had also EAE (not shown). This finding suggests that the immune response against MOG, or maybe the presence of the CFA, is increasing fungicidal activity of the immune system. The higher production of encephalitogenic cytokines by both stimuli, MOG and *C. albicans*, was interpreted as a possible cause of EAE increased severity as cytokines can easily cross the blood-brain barrier and directly affect CNS functions [39, 40].

As the histopathology analysis from brain and spinal cord sections suggested similar degrees of inflammation, we compared the amounts of leukocytes and CD3$^+$CD4$^+$ T cells eluted from the CNS. Confirming the H&E analysis, this comparison revealed the presence of similar numbers of total cells in EAE and EAE+Ca groups, demonstrating therefore that the higher disease severity was not due to a higher degree of inflammatory infiltration. Nevertheless, the immunophenotyping analysis showed a higher proportion of CD3$^+$CD4$^+$ T-cell population in the EAE+Ca group. Culture of the cells eluted from the CNS showed, as expected, that they produced proinflammatory cytokines in the presence of MOG. Interestingly, they also produced significant amounts of proinflammatory cytokines when stimulated with *C. albicans*.

Together, these results are suggesting that both peripheral and local fungus effects are contributing to a more severe disease development. The translation of these findings to human patients certainly requires much more investigation in this area. However, we believe that these findings add more evidence that *C. albicans* is one of the fungi that can affect this type of neurological pathology.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

Special thanks are due to São Paulo Research Foundation (FAPESP), Grant no. 2013/14353-2 and Grant no. 2012/12540-7.

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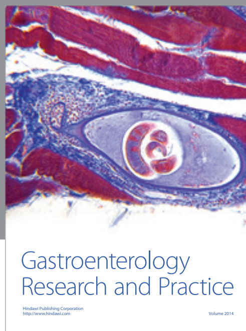

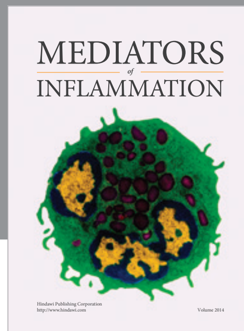

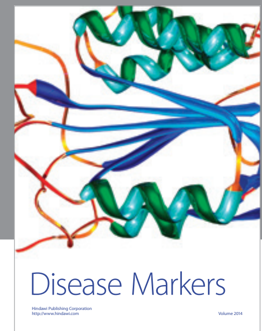

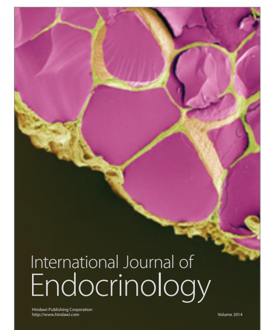

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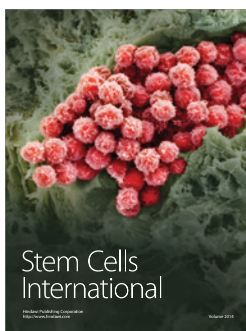

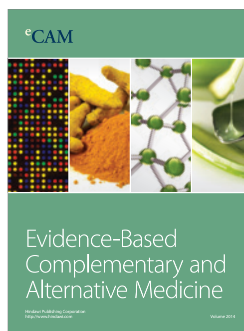

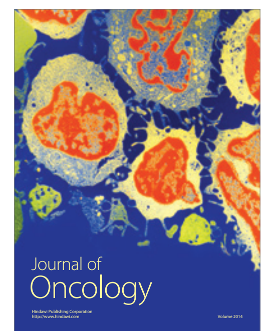

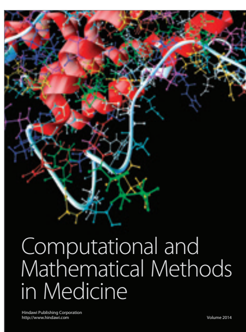

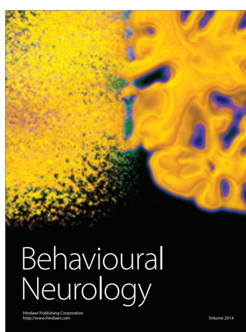

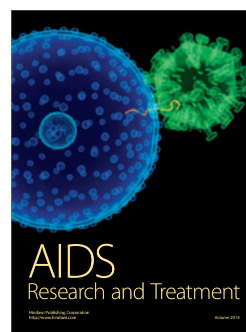

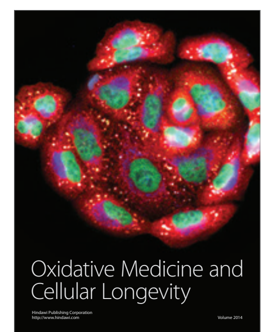

Artigo científico II

RESEARCH ARTICLE

Differential modulation of experimental encephalomyelitis by distinct fungal derivatives

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Conflict of interest. The authors declare no conflict of interest.

ABSTRACT

Background

The development of multiple sclerosis (MS), an autoimmune disease of the CNS, has been strongly associated with viral and bacterial infections. Most CNS fungal infections, that trigger considerable morbidity and mortality, are associated with *Aspergillus*, *Cryptococcus*, *Candida* and *Mucorales*. Additionally, certain fungi species sequestered in non-neuronal tissues can release toxins that destroy CNS cells. The deleterious effect of CNS fungal infections and derived toxins on the CNS has been associated with development or aggravation of MS and experimental autoimmune encephalomyelitis (EAE). In this context, we investigated the effect of gliotoxin (GTX) and dead yeasts of *C. albicans* (dead Ca) in the EAE development.

Methods

C57BL/6 mice were injected with three doses of GTX (1 mg/kg) or dead Ca ($5x10^6$ yeasts) after EAE induction. The doses were administered at the fourth, seventh and tenth days after EAE induction. Body weight and clinical score were daily evaluated. Histopathological (CNS) and immunological parameters (periphery and CNS) were evaluated during the acute disease phase (17th day).

Results

GTX aggravated disease evolution and increased the inflammatory process in the CNS. Cells eluted from the CNS of these animals produced more TNF-α and IL-10 but less IFN-γ in response to *in vitro* stimulation with myelin antigen (MOG). Otherwise, Ca reduced clinical signs but did not change the amount of cells infiltrated in the CNS. In this case, CNS cells presented less apoptosis and produced less encephalitogenic cytokines in response to MOG stimulation.

Conclusions

Fungal-derived antigens can protect or exacerbate experimental encephalomyelitis. A deeper investigation in this area can reveal the fungal components that are deleterious and that need, therefore, to be neutralized. Otherwise, protective fungal derivatives could be further explored for EAE control.

Keywords: Multiple Sclerosis; Experimental Autoimmune Encephalomyelitis (EAE); Gliotoxin; *Candida albicans*

BACKGROUND

Multiple Sclerosis (MS) is an autoimmune disease characterized by the presence of autoreactive T cells specific for CNS self-antigens. Expressive data suggest that autoimmune diseases result from the interactions of environmental and genetic risk factors. While considerable progress has been made in understanding multiple genetic risk factors for autoimmune diseases, relatively little information is available regarding the role of the environment in the development of these diseases (Miller, 2011). Environmental risk factors considered essential for MS development include infections and non-infectious factors that comprise differences in diet and other behaviors, such as cigarette smoking and sunlight exposure (Ascherio and Munger, 2007a, b). Most chronic inflammatory CNS disorders have an infectious origin and a strong association of viral and bacterial infections with MS was already demonstrated (Gilden 2005).

CNS fungal infections are associated with considerable morbidity and mortality and comprise a wide spectrum of clinical syndromes, including abscesses, meningitis/meningoencephalitis, stroke/vasculitis, and spinal pathologies such as arachnoiditis (Murthy and Sundaram, 2014). The main etiologic agents of these infections are *Aspergillus*, *Cryptococcus*, *Candida*, *Mucorales*, dematiaceous molds, and dimorphic endemic fungi. The main routes of infection are respiratory or traumatic inoculation with subsequent hematogenous or contiguous spreading (Panackal and Williamson, 2015). The association between fungal toxins and MS has been suggested. Certain fungal species isolated in non-neuronal tissues could release toxins that target and destroy CNS astrocytes and oligodendrocytes (Purzycki and Shain, 2010). Fumonisin B1, isolated from species of *Fusarium*, is cytotoxic to murine microglia and primary astrocytes and disrupts the biosynthesis of sphingolipids (Stockmann-Juvala and Savolainen, 2008), which are frequently lost from the white matter of MS patients (Wheeler et al, 2008). Also, Penitrem A from *Penicillium crustosum* causes neurological problems in rats including sustained tremors, nystagmus, ataxia, pseudoparalysis, mitochondrial swelling and severe neurologic dysfunction (Cavanagh et al, 1998).

Gliotoxin (GTX) is a mycotoxin that was initially isolated from cultures of the fungus *Gliocladium* (Weindling and Emerson, 1936). More recently it was described that it can be produced by various fungal species, e.g. *Aspergillus fumigatus*, *Eurotium chevalieri*, *Trichoderma virens*, *Neosartorya pseudofischeri* and some *Penicillium* and *Acremonium* species (Scharf et al, 2015). GTX affects fungal invasiveness and their dissemination from the primary

site throughout the organism, contributing to exacerbated and disseminated mycoses (Kosalec and Pepeljnjak, 2004). This characteristic has been associated to toxicity and immunosuppressive properties of GTX resulting in a reduced adaptive immune response against fungal infection (Stanzani et al, 2005). A gliotoxic factor for glial cells was described in monocyte cultures and in cerebrospinal fluid (CSF) from MS patients. This gliotoxic factor that is a 17 kDa glycosylated protein, called GTX or GTX-like, was present in heat-treated CSF of all ten patients with relapsing–remitting MS at relapse and caused *in vitro* apoptotic death of astrocytes and oligodendrocytes, but not fibroblasts, myoblasts, Schwann cells, endothelial cells and neurons, *in vitro* (Menard et al, 1998). More recently it was also demonstrated that only certain areas of the brain are vulnerable to GTX-like neurotoxic effect (Willis et al, 2004).

Even though the production of GTX by *Candida* species has been controversial (Shah and Larsen, 1991, Shah et al, 1995; Kosalec and Pepeljnjak, 2004; Kupfahl et al, 2007; Kosalec et al., 2008) a possible deleterious effect of *Candida* on MS patients is suggested by a few reports. Benito-Leon *et al.* (2010) by using immunofluorescence analysis detected a higher prevalence of anti-*Candida* spp antibodies in MS patients than in matched controls. This possible association is reinforced by the findings of anti-*Candida* spp antibodies also in cerebrospinal fluid of 12 MS patients (Pisa et al, 2010; Pisa et al, 2013). Our research team recently demonstrated that *C. albicans* infection before experimental autoimmune encephalomyelitis (EAE) induction aggravated encephalomyelitis clinical signs. Yeast and hyphae from this fungus were found in the CNS of these animals (Fraga-Silva et al, 2015). Considering that fungal derivatives could contribute to the cytotoxicity and/or inflammation of the CNS in MS patients (Purzycki and Shain, 2010; Pisa et al, 2013), and that we recently described that *C. albicans* increases EAE severity, we analyzed the effect of fungal derivatives in EAE development. For this, mice submitted to EAE induction were injected with three doses of GTX or dead yeasts of *C. albicans*. The fungal toxin GTX aggravated EAE whereas dead yeasts of *C. albicans* significantly protected the animals from EAE development.

METHODS

Animals

Female C57BL/6 mice with 9–11 weeks old were purchased from the University of São Paulo (USP) (Ribeirão Preto, SP, Brazil). Mice were allocated in specific-pathogen free conditions, in cages (maximum 5 mice per cage) with free food and autoclaved tap water in a controlled photoperiod (12h/12h, dark/light cycle) environment. All procedures were performed in ways that minimized animal suffering. The animals were manipulated according to the ethical principles for animal research adopted by the National Council for the Control of Animal Experimentation. This study was approved by the local Ethics Committee for Animal Experimentation, São Paulo State University (UNESP) (Botucatu, SP, Brazil; protocol number 351).

EAE Induction

Animals were subcutaneously immunized with 25 µL (100 µg) of $MOG_{35–55}$ peptide emulsified in 25 µL of Complete Freund's Adjuvant (Sigma) containing 4 mg/mL of *Mycobacterium tuberculosis*. Mice also received 2 intraperitoneal doses, 0 and 48 hours after immunization, of 200 ng of *Bordetella pertussis* toxin (Sigma). Clinical score and body weight were daily recorded until the 17th day. EAE clinical scores were monitored according to the following criteria: 0, no symptoms; 1, limp tail; 2, hind legs weakness; 3, partially paralyzed hind legs; 4, complete hind leg paralysis; and 5, complete paralysis/death. The percentage of weight loss was calculated considering the day of immunization as day 0.

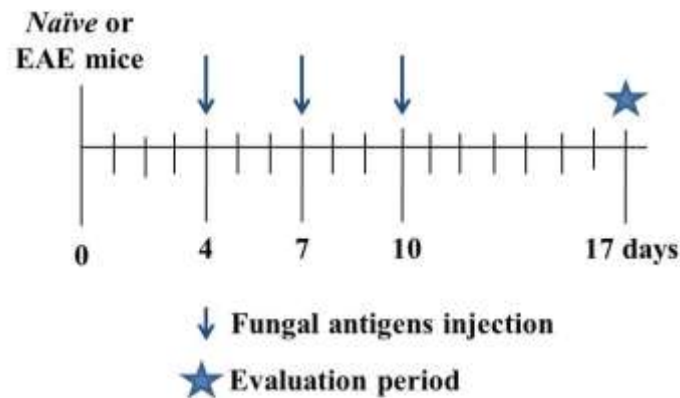
Fungal derivatives

Gliotoxin (GTX) from *Gliocladium fimbriatum* (Merck Millipore Corporation, Darmstadt, Germany) was initially diluted in DMSO (1 mg/mL) and then adjusted in sterile saline solution (SSS) to be intraperitoneally injected in mice (1 mg/kg). *C. albicans* strain FCF 14 (Genbank Accession EF591020) was originally obtained from the mycology collection from the São José dos Campos Dental School (UNESP), and maintained in our mycological collection on Sabouraud-dextrose agar (Difco Laboratories, Detroit, MI, USA). *C. albicans* was cultured on solid media during 24 hours at 37°C. The fungal concentration was adjusted to 5.0×10^7/mL viable yeast cells in SSS. Fungus suspension was then heat killed by autoclaving at 121ºC for 15

minutes. These dead yeasts of *C. albicans* were inoculated into the peritoneal cavity (0.1 mL/animal) and also used in cell culture stimulation (5 yeasts to 1 cell).

Experimental design

Mice were allocated into six groups: *NT*, injected with three doses of DMSO/SSS that was used to prepare GTX and dead Ca; *GTX*, injected with three doses of GTX; *Ca*, injected with three doses of dead yeast of *C. albicans*; *EAE/NT*, submitted to EAE induction and injected with three doses of DMSO/SSS; *EAE/GTX*, injected with three doses of GTX at the fourth, seventh and tenth days after EAE induction and, *EAE/Ca*, injected with three doses of dead yeast of *C. albicans* at the fourth, seventh and tenth days after EAE induction. Evaluations were performed seven days after last fungal antigen dose. The chronology of the experimental design is illustrated below by a timeline scheme.



CNS-mononuclear cells isolation

Seventeen days after EAE induction, mice were anesthetized with ketamine/xylazine and perfused with 10 mL of SSS. Brain and spinal cord were collected, macerated, and digested with 2.5 mg/mL of collagenase D (Roche Applied Science, Indianapolis, IN, USA) in 4 mL of RPMI 1640 medium (Sigma) at 37°C for 45 min. Then, suspensions were washed in RPMI and centrifuged at 450 ×g at 4°C for 15 min. Cells were resuspended in Percoll (GE Healthcare, Uppsala, Sweden) 37% and gently laid over Percoll 70% in tubes of 15 mL. The tubes were centrifuged at 950 ×g for 20 min with centrifuge breaks turned off. After centrifugation the ring containing mononuclear cells was collected, washed in RPMI, and centrifuged at 450 ×g for 10

min. Cells were then resuspended in supplemented RPMI medium (1% gentamicin, 2% glutamine, 1% sodium pyruvate, 1% non-essential amino acids, and 10% of FBS), counted, and cultured. Pools from 2 animals were used to get enough cell number to perform the experiments.

Cell culture conditions and cytokine and nitric oxide quantification

Spleen cells were collected and adjusted to 5×10^6 cells/mL in complete RPMI medium (1% gentamicin, 2% glutamine, and 10% of fetal calf serum). CNS-isolated cells were adjusted to 2×10^5 cells/mL in supplemented RPMI medium. Spleen and CNS-isolated cells were plated and stimulated with MOG (20 µg/mL and 50 µg/mL, respectively) and with dead yeasts of *C. albicans* (5 yeasts/1 cell). Cytokine levels were evaluated 48 h later by enzyme-linked immunosorbent assay (ELISA) in culture supernatants using IL-2, IFN-γ and IL-10 BD OptEIA Sets (Becton, Dickinson and Company, BD, Franklin, San Diego, CA, USA) and IL-6, IL-17, TNF-α, and TGF-β Duosets (R&D Systems, Minneapolis, MN, USA). The assays were performed according to the manufacturer's instructions. The production of nitrite, a stable end product of nitric oxide (NO), was measured in culture supernatants by Griess reagent containing 1% sulfanilamide (Synth, Diadema, SP, Brazil), 0.1% naphthalene diamine dihydrochloride (Sigma), and 2.5% H_3PO_4. The nitrite accumulation was quantified using a chemiluminescence microreader (ELx 800; BioTek Instruments Inc., Winooski, VE, USA). The concentration of nitrite was determined using sodium nitrite (Sigma) diluted in RPMI-1640 medium as a standard.

Flow cytometry

Lymph nodes (axillary + inguinal) were collected and the red blood cells were lysed with buffer containing NH_4Cl. For regulatory T cells analysis, lymph node cells were incubated with PerCP-conjugated anti-mouse CD3 (clone 145-2C11, 0.2 µg), FITC-conjugated anti-mouse CD4 (clone GK1.5, 0.25 µg) and APC-conjugated anti-mouse CD25 (clone PC61.5, 0.13 µg) for 20 min at 4°C. Intracellular Foxp3 transcription factor was detected using PE-conjugated anti-mouse Foxp3 (clone FJK-16s, 0.2 µg) and FoxP3 Staining Set (eBiosciences, San Diego, CA, USA) according to manufacturer's instructions. After staining, the cells were washed, resuspended in flow cytometry buffer, and fixed in paraformaldehyde 1%. Flow cytometry was performed using a FACS Canto II (BD) from Institute of Biosciences of Botucatu (UNESP, Botucatu, SP, Brazil) and the data were analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

Histopathology of the CNS

The histological analysis was performed during clinical EAE phase, that is, 17 days after disease induction. After euthanasia, lumbar spinal cord samples were removed and fixed in 10% neutral buffered formalin. Paraffin slides with 4 μm were stained with hematoxylin and eosin (H&E) and analyzed with a Nikon microscope. A semi-quantitative analysis of the CNS inflammatory infiltration was performed according to the following criteria, adapted from Soellner et al. (2013): (0) no infiltrates; (1) partial meningeal infiltration; (2) pronounced meningeal infiltration, and (3) pronounced meningeal and some parenchymal infiltration.

Statistical Analysis

Results were expressed as mean ± SEM standard deviation or with median and interquartile (25–75%) ranges. To test for the normality of data, results were analyzed by Shapiro-Wilk's test. Comparisons among three groups were made by one way ANOVA followed by Tukey's test for parametric variables and by Kruskal-Wallis followed by Dunn's test for nonparametric variables. The GraphPad Prism v5.0 Statistical Guide (2007, GraphPad Software Inc., USA) for Windows was used to analyze data and create graphs.

RESULTS

Immunodulatory effect of fungal derivatives

Three doses of gliotoxin (GTX group) or dead yeasts of *C. albicans* (Ca group) modulated cytokine production in the periphery and in the CNS of *naïve* mice. To evaluate the peripheral immune response splenic cell cultures were stimulated with dead yeasts of *C. albicans* (5 yeasts/1 cell). The *in vivo* treatment with GTX or Ca increased TNF-α, IL-6 and IFN-γ production in comparison to non-treat mice (NT group) as demonstrated in figure 1 (a, b and d, respectively). However this procedure did not modulate IL-17 (figure 1c) and no differences were observed between GTX and Ca groups. To assess cytokine production by the CNS, the cells were stimulated with MOG. Both treatments increased IL-6 and IL-17 as illustrated in figure 1 (f,g). Both treatments decreased IFN-γ production (figure 1h) but TNF-α (figure 1e) and IL-10 (not shown) were not affected by any of these two treatments.

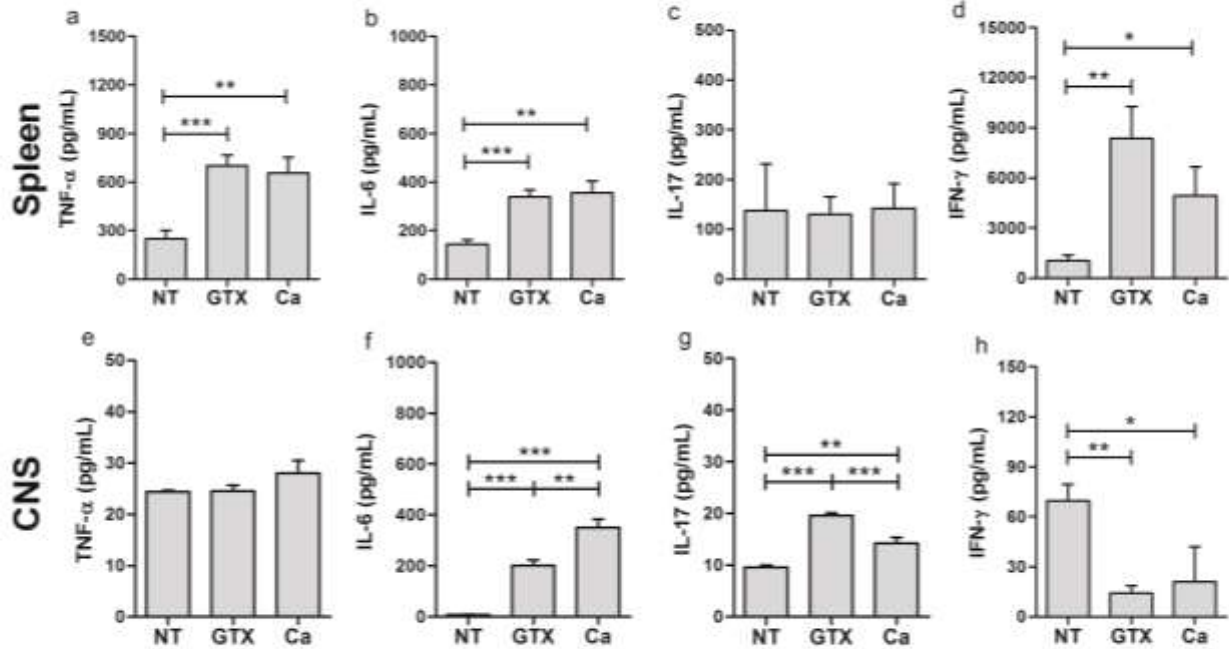


Figure 1. Immunomodulatory effect of fungal derivatives in *naïve* C57BL/6 mice. Mice were injected with three doses of gliotoxin (GTX group) or dead yeasts of *C. albicans* (Ca group) and seven days after the last dose the levels of TNF-α, IL-6, IL-17 and IFN-γ were measured in spleen cell cultures stimulated with dead yeasts of *C. albicans* (a-d) and CNS cell cultures stimulated with MOG (e-h). *p<0.05; **p<0.01 and ***p<0.001 represent comparisons among groups made by one way ANOVA followed by Tukey's test for parametric variables. Results were expressed as mean ± SEM of 5-9 samples per group. Data are representative of two independent experiments whose results were associated.

Gliotoxin aggravates EAE whereas dead *C. albicans* decreases EAE severity

To test the potential deleterious effect of fungal derivatives in EAE development, C57BL/6 mice received three doses of GTX or Ca at the fourth, seventh and tenth days after EAE induction. Disease incidence is shown in table 1. The incidence in the non-treat EAE mice (EAE group) was 100%. These animals presented the expected clinical signals as paralysis and body weight loss, as shown in figure 2. Injection of GTX maintained the incidence in 100% and also aggravated the disease. This group presented a high degree of paralysis (figure 2a,b) and also a more accentuated body weight loss (figure 2c,d) in comparison to the non-treat EAE group. Differently, injection of dead yeasts of *C. albicans* decreased EAE incidence (table 1) and significantly attenuated clinical disease manifestations (figure 2a-d).

Table 1. EAE incidence, Chi-square test (7-12 mice/group), results from two independent experiments were combined.

Groups	Incidence	P value
EAE	100.0 % (10/10)	< 0.001
EAE/GTX	100.0 % (7/7)	
EAE/Ca	16.7 % (2/12)	

The clinical findings were reinforced by the CNS histopathological analysis. A semi-quantitative analysis of lumbar spinal cord samples (figure 3a) showed an increased inflammatory infiltration in samples obtained from EAE/GTX group in comparison to both, EAE and EAE/Ca groups (figure 3b). Additionally, the number of leukocytes per gram of CNS was higher in EAE/GTX group (figure 3c). The percentage of apoptotic cells (Annexin V^+ cells) in the CNS was lower in EAE/Ca group in comparison to EAE and EAE/GTX groups (figure 3d).

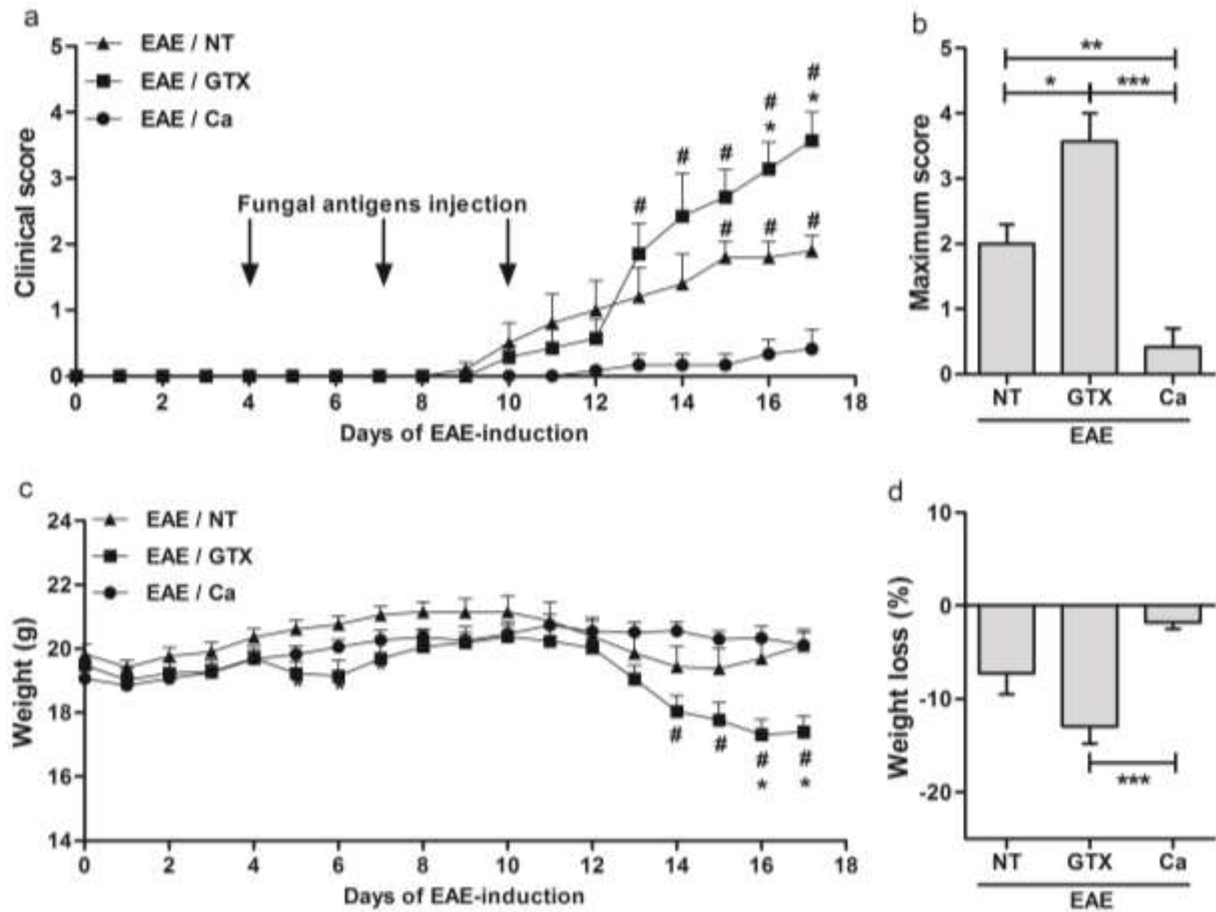


Figure 2. Gliotoxin aggravates EAE whereas dead yeasts of *C. albicans* decrease EAE severity. Mice submitted to EAE induction received three doses of gliotoxin (EAE/GTX group) or dead yeasts of *C. albicans* (EAE/Ca group). Clinical signs were analyzed daily until the 17th day when clinical signs in the EAE group were very evident. Clinical scores (a), maximum clinical score (b), body weight (c) and the weight variation was determined during EAE-development, that is, from day 0 to day 17 (d). *p<0.05 *vs* EAE group and #p<0.05 *vs* EAE/Ca group represent comparisons among groups made by one way ANOVA followed by Tukey's test for parametric variables. Results were expressed as mean ± SEM of 7-12 animals per group. Data are representative of two independent experiments whose results were associated.

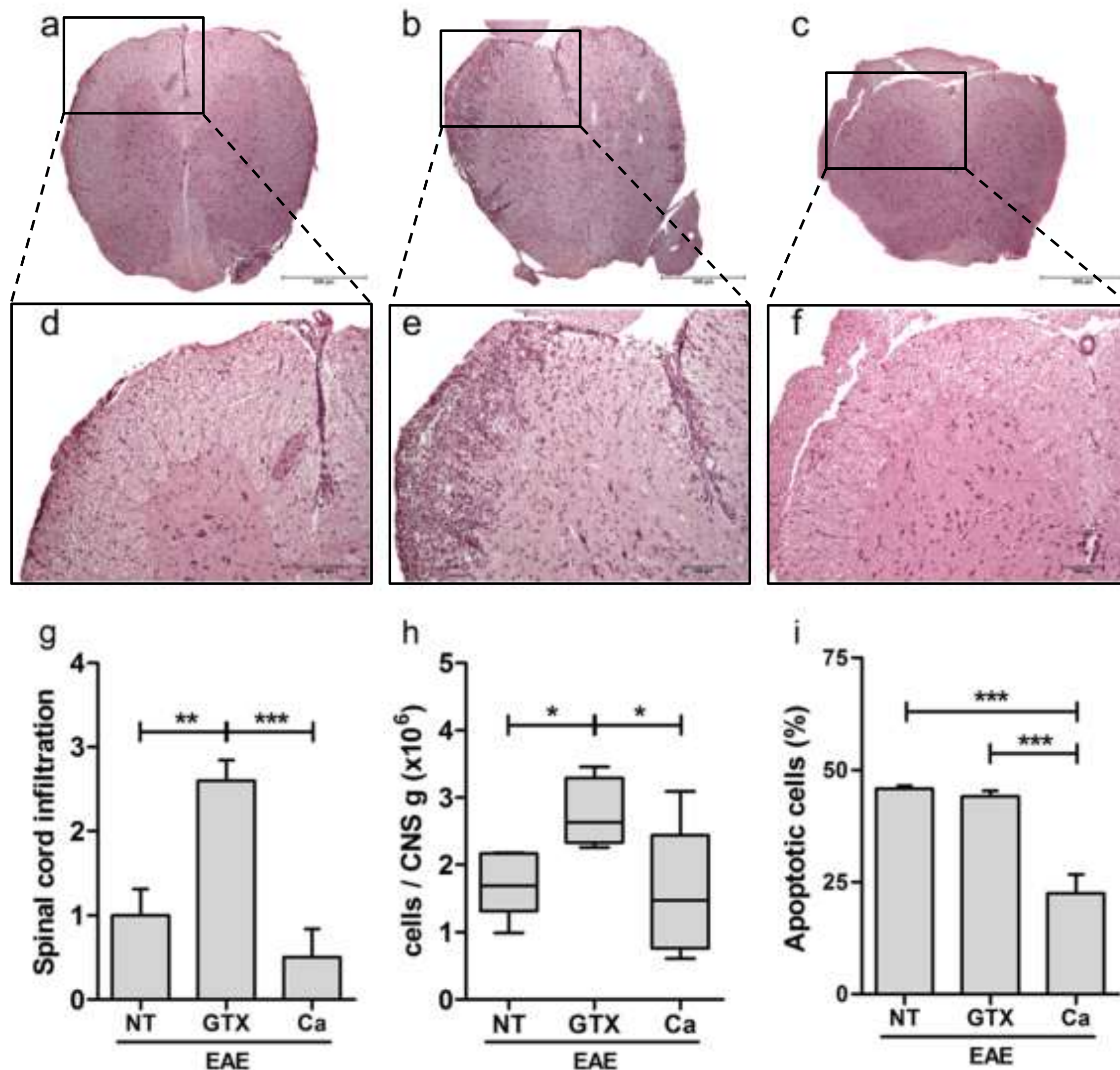


Figure 3. Inflammatory process in the CNS. Mice submitted to EAE induction received three doses of gliotoxin (EAE/GTX group) or dead yeasts of *C. albicans* (EAE/Ca group). Inflammatory infiltrates in the lumbar spinal cord samples stained with hematoxylin & eosin were evaluated at the 17th day after EAE induction. Images are representative of five samples of EAE/NT (a, d), EAE/GTX (b, e) and EAE/Ca (c, f). Scale bar = 500 µm (a-c) and 100 µm (d-f). The semi-quantitative analysis (a) was done according to the following criteria: (0) no infiltrates; (1) partial meningeal infiltration; (2) pronounced meningeal infiltration, and (3) pronounced meningeal and some parenchymal infiltration. The number of leukocytes (b) and apoptotic cells (Annexin V$^+$ cells in total in 50.000 acquired events) (c) were determined per gram of CNS tissue (in a pool of brain and spinal cord) at clinical EAE phase (17th day). *$p<0.05$ *vs* EAE group and #$p<0.05$ *vs* EAE/Ca group represent comparisons among groups made by one way ANOVA followed by Tukey's test for parametric variables and by Kruskal-Wallis followed by Dunn's test for non-parametric variables. Results were expressed as mean ± SEM or medians (25-75%, box) of 5-6 animals per group. Data are representative of one experiment.

Effect of fungal derivatives on cytokine production and T cells in EAE mice

To assess if fungal derivatives were also able to up-regulated cytokine production in EAE mice, as they did in naïve mice, spleen cells were *in vitro* stimulated with MOG or dead yeasts of *C. albicans* (5 yeasts/1 cell). Cultures stimulated with MOG produced similar amounts of cytokines (data not shown). Upon stimulation with dead *C. albicans*, cell cultures from EAE/Ca group produced higher levels of IFN-γ, IL-2 and IL-5 (figure 4d, e and f, respectively). Although the proportions of $CD3^{+}CD4^{+}$ T cells were similar in the three experimental groups (figure 4i), the percentage of $CD25^{hi}FoxP3^{+}$ regulatory T cells in the inguinal lymph nodes was lower in the EAE/Ca group in comparison to EAE/NT group (figure 4j).

Fungal derivatives modify cytokine profile in the CNS of EAE mice

To evaluate if fungal derivatives were modifying the MOG specific immune response in the CNS of EAE mice, CNS eluted cells were stimulated *in vitro* with MOG. In mice injected with GTX (EAE/GTX group) there was a higher production of TNF-α and IL-10 in comparison to non-treat EAE group (figure 5a and g, respectively). On the other hand these animals produced less IFN-γ than non-treat group (figure 5d). No differences were observed in IL-6, IL-17, IL-2, IL-5 and NO production between EAE/GTX and EAE/NT groups, as illustrate in figure 5. The cytokine profile in response to MOG was clearly distinct in the EAE/Ca group. In this case, the production of TNF-α, IL-17, IFN-γ, IL-2 and IL-10 was significantly lower in EAE/Ca group in comparison to EAE/NT group (figure 5a, c-e, g). The comparison between EAE/GTX and EAE/Ca groups indicated that the EAE/GTX group produced higher levels of TNF-α, IL-17, IL-2 and IL-10 than EAE/Ca group (figure 5a,b,e,g, respectively). To evaluate if *Candida*-specific T cells migrated to the CNS of EAE mice, CNS eluted cells were stimulated *in vitro* with dead yeasts of *C. albicans* (5 yeasts/1 cell). In EAE/GTX group there was a higher production of IL-10 in comparison to EAE group (figure 5o). In mice whose EAE was ameliorated by injection of dead yeasts of *C. albicans* (EAE/Ca group), there was a lower production of TNF-α, IL-17 and IFN-γ in comparison to EAE group, as illustrated in figure 5 (i, k and l, respectively). On the other hand, this group produced a higher level of IL-5 (figure 5n). TGF-β was not detected in the CNS cell cultures.

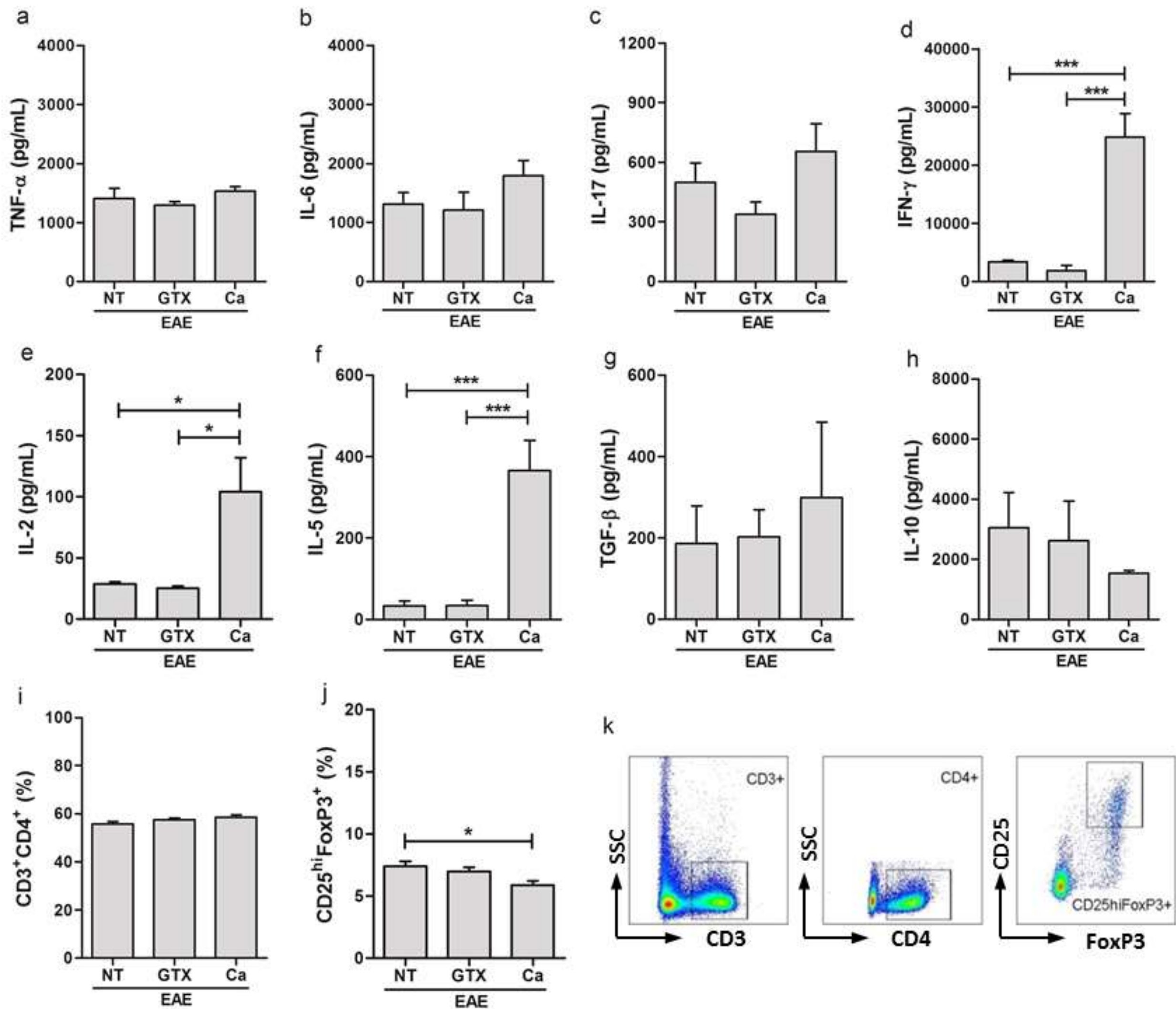


Figure 4. Peripheral immunomodulatory effect of dead *C. albicans* in EAE-mice. Mice submitted to EAE induction received three doses of gliotoxin (EAE/GTX group) or dead yeasts of *C. albicans* (EAE/Ca group). TNF-α (a), IL-6 (b), IL-17 (c), IFN-γ (d), IL-2 (e), IL-5 (f), TGF-β (g) and IL-10 (h) were measured in spleen cell cultures stimulated with Ca (5 yeasts/1 cell). The percentage of $CD4^+$ T cell (i) in total $CD3^+$ (100,000 acquired events) and $CD25^{hi}Foxp3^+$ Tregs in total $CD3^+CD4^+$ T cell (j) was assessed in a pool of inguinal lymph nodes. A typical gating scheme is illustrated (k). *p<0.05 *vs* EAE group and #p<0.05 *vs* EAE/Ca group represent comparisons among groups made by one way ANOVA followed by Tukey's test for parametric variables. Results were expressed as mean ± SEM of 5-6 animals per group. Data are representative of one experiment.

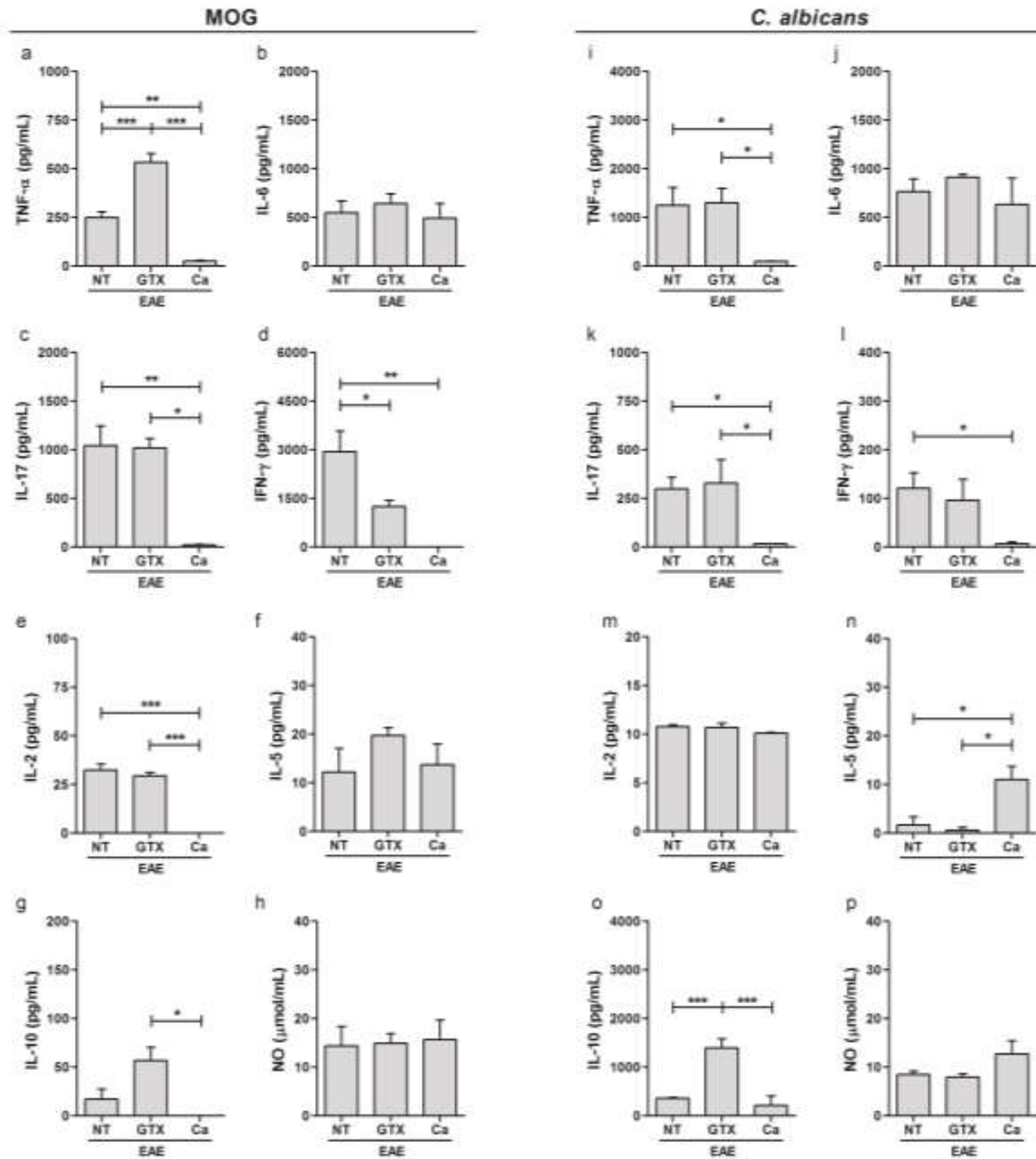


Figure 5. CNS immunomodulatory effect of fungal derivatives in EAE-mice. Mice submitted to EAE induction received three doses of gliotoxin (EAE/GTX group) or heat-dead yeasts of *C. albicans* (EAE/Ca group). TNF-α, IL-6, IL-17, IFN-γ, IL-2, IL-5, IL-10 and nitric oxide (NO) were measured in in CNS cell cultures stimulated with MOG (a-h) or dead *C. albicans* (i-p). *p<0.05 *vs* EAE group and #p<0.05 *vs* EAE/Ca group represent comparisons among groups made by one way ANOVA followed by Tukey's test for parametric variables. Results were expressed as mean ± SEM of 3-5 samples per group, each sample represent a pool of two animals. Data are representative of one experiment.

DISCUSSION

The etiology of multiple sclerosis (MS) is still unknown but there is strong evidence that genetic predisposition associated with environment factors, including fungal infections, can trigger or aggravate the disease. In the present work we evaluated and compared the effect of gliotoxin (GTX) and dead yeasts of *C. albicans* (dead-Ca) in experimental autoimmune encephalomyelitis (EAE) that is a murine model to study MS (Lavi and Constantinescu, 2005). The choice of GTX was based on the potentially deleterious effect of this mycotoxin due to its neurotoxic properties (Axelsson et al, 2006; Speth et al, 2011). Dead-Ca was also tested because we recently demonstrated that infection with *C. albicans* spread to the CNS and aggravated encephalomyelitis development (Fraga-Silva et al, 2015). In this scenario we wanted to clarify if deleterious effect was associated with fungal dissemination and colonization in the CNS or to the presence of particulate *Candida* antigens in the circulatory system.

Initially, *naïve* C57BL/6 mice were injected with three doses of these fungal derivatives and then checked for both, peripheral and CNS cytokine production. Dead-Ca was chosen to stimulate spleen cell cultures based on the previous observation that spleen cells from GTX injected mice presented a recall response to *C. albicans*. Cultures from the CNS were stimulated with myelin peptide (MOG). Inoculation of both fungal derivatives significantly up-regulated TNF-α, IL-6 and IFN-γ production by splenic cell cultures. These antigens also affected cytokine production by CNS eluted cells. In this case, they increased IL-6 and IL-17 production but they decreased IFN-γ production. This pro-inflammatory environment observed in the periphery and also in the CNS and the fact that these cytokines have been clearly associated with MS and EAE due to their encephalitogenic properties (Fletcher et al, 2010; Luchtman et al, 2014) reinforced the possibility of these fungal derivatives could exacerbate EAE development.

To evaluate the effect of these two antigens on EAE development, C57BL/6 mice were submitted to EAE induction and then injected with three doses of each fungal antigen. GTX inoculation clearly aggravated EAE development. These animals lost more body weight and reached more elevated clinical scores than non-treat EAE group. Dead-Ca inoculation had an opposite effect, that is, the animals developed a less severe disease characterized by less body weight loss and lower clinical scores than non-treat EAE group. Additionally, dead-Ca inoculation was also able to significantly decrease the incidence of the disease. These effects were supported by the histopathological CNS analysis made in comparison to non-treat EAE

mice. Spinal cord sections stained with H&E from GTX treat EAE-mice presented more inflammatory infiltration. A higher number of cells were also eluted from the CNS of these animals. As expected, fewer cells infiltrated the spinal cord of dead-Ca treat EAE-mice. We believe that these findings are new because no similar procedures were found in the literature. However, in a very recently report Takata et al. (2015) interestingly demonstrated that the oral ingestion of heat-killed *C. kefyr* ameliorated the severity of EAE. Even though our protocol was clearly distinct, the two procedures employing dead yeasts of different *Candida* species decreased EAE severity.

To check if the opposite clinical effects of GTX and dead-Ca were due to differences in the peripheral cytokine production determined by these antigens, we analyzed and compared splenic cytokine production induced by stimulation with dead-Ca. Differently from the up-regulatory effect observed in naïve mice, no changes in pro or anti-inflammatory cytokines were detected in GTX treat EAE-mice. Even though the mechanism underlying this finding was not directly evaluated, it could be related to the GTX-mediated suppression of the adaptive immunity (Kupfahl et al, 2006). Differently from this situation, the EAE/Ca group displayed up-regulation of IFN-γ, IL-2 and IL-5 and also a decreased percentage of regulatory T cells (Tregs). Up-regulation of these cytokines was interpreted as an indication that dead-Ca inoculation determined activation of both, Th1 and Th2 specific T cells. This possibility is partially supported by the fact that heat killing of *C. albicans* exposes β-glucans on cell wall surface (Gantner et al, 2005). Interaction of these β-glucans with macrophages in vitro promoted decrease in Th1 and increase in Th2 cytokines and also an increased expression of FoxP3 in lymphocytes (Chen et al, 2013). The decreased amount of regulatory T cells in the periphery could mean that part of these cells migrated to the CNS to regulate inflammation. The migration of peripheral Tregs to the CNS has been described and supports this possibility (Zozulya and Wiendl, 2008).

To analyze if the peripheral presence of fungal derivatives was affecting the immune response to MOG in the CNS or if *Candida*-specific cells had migrated to the CNS, cells eluted from brain and spinal cord were stimulated with MOG and dead-Ca. Cytokine profiles triggered by MOG and dead-Ca stimulation were clearly distinct. GTX injected EAE-mice produced more TNF-α and IL-10 and less IFN-γ than the non-treat EAE group in response to MOG stimulation. These cells also produced more IL-10 in response to dead-Ca stimulation. Considering the

findings obtained so far we were not able to explain why GTX aggravated EAE. In this scenario we would like to test if GTX is being directly neurotoxic. This possibility is supported by some reports. GTX was able to induce apoptosis in many cell types and this effect was a consequence of mitochondrial depolarization and induction of reactive oxygen species release (Pardo et al., 2006). It was also demonstrated that GTX induced a significant increase in calcium concentration in differentiated human neuroblastoma cells and that caspase activation contributed to its degenerative neurite effect (Axelsson et al, 2006). In addition, GTX reduced the viability of astrocytes, neurons and microglia by triggering apoptosis (Speth et al, 2011). In the present study we evaluated the percentage of annexin+ cells eluted from the CNS but no differences were observed between EAE/GTX and EAE/NT groups. Alternatively, GTX could provoke demyelination or BBB permeability alterations. There are no direct supports for these possibilities because the effect of fungal-derived GTX on demyelination or BBB was not tested yet. Recent studies employing chemical GTX suggest, however, that these hypotheses are worthwhile to be tested. Kalakh and Mouihate (2015) demonstrated that direct inoculation of GTX (ethidium bromide) in the rat corpus callosum determined demyelination. Also, Camire et al. (2015) described that the in vivo inoculation of GTX (3-chloropropanediol) compromised BBB integrity. Interestingly, the authors proposed that the integrity of this barrier is lost together with a cytokine unbalance towards pro-inflammatory cytokines. In addition, by using in vitro experiments, they suggested that TNF-α and IL-10 are involved in loss and restoration of BBB integrity, respectively (Camire et al., 2015). Curiously, these were the two cytokines that we detected in cells eluted from the CNS upon MOG stimulation.

The injection of dead-Ca triggered a very distinct cytokine profile. In this case, the production of encephalitogenic cytokines (TNF-α, IL-17, IFN-γ and IL-2) in response to MOG stimulation was very low in comparison to EAE/NT group. These findings are in accordance with the fact that these animals were significantly protected from the disease. A significant production of IL-5, but not TNF-α, IL-17 and IFN-γ, was detected in CNS cell cultures stimulated with dead-Ca suggesting the local presence of Th2 *Candida*-specific cells. Similarly to our findings, intraperitoneal treatment with zymosan, that is a yeast-derived β-glucan- and mannan-rich particle (Brown et al. 2002), ameliorated both chronic and relapsing EAE. This effect was associated with low MHC class II expression on microglia, decreased encephalitogenic T cell

proliferation, decreased production of IL-17 and IFN-γ but increased production of IL-4, IL-5 and IL-10 by spleen cell cultures (Li et al, 2013).

CONCLUSIONS

The results from this investigation indicate that fungal derivatives can be both, protective or deleterious, depending upon their characteristics. Considering that both aspects are relevant in the context of MS, we believe that a wide screening process needs to be done to identify deleterious molecules that need to be inactivated. Molecules with potential for protection are also worthwhile to be characterized because they can be explored as therapeutical weapons.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Conceived and designed the experiments: TFCFS, MSPA and AS. Performed the experiments: TFCFS, LCTL, LANM, LLWI, SFGZP. Analyzed the data and wrote the paper: TFCFS and AS.

ACKNOWLEDGEMENTS

Special thanks are due to São Paulo Research Foundation (FAPESP), Grant no. 2013/14353-2 and Grant no. 2012/12540-7.

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Artigo científico III

ORIGINAL ARTICLE

Tolerogenic vaccination with MOG/VitD overcomes aggravating effect of *C. albicans* in experimental encephalomyelitis

Running Title – MOG/VitD attenuates EAE aggravated by *C. albicans*

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Conflict of interest. The authors declare no conflict of interest.

ABSTRACT

Multiple sclerosis (MS) is an immune-mediated demyelinating disorder of the central nervous system (CNS). We recently described that *Candida albicans* (Ca) aggravates experimental autoimmune encephalomyelitis (EAE) that is a model to study MS. We also observed that treatment with myelin oligodendrocyte glycoprotein (MOG) in the presence of 1α,25dihydroxyvitamin D3 (VitD) protected mice against EAE development. In this work we investigated if Ca infection interferes with the efficacy of this vaccine. EAE was induced in C57BL/6 female mice previously vaccinated with MOG+VitD and then infected with Ca three days before encephalomyelitis induction. Vaccination with MOG+VitD was able to control EAE development in Ca infected mice. These animals gained weight and only a few progressed to very low clinical scores in comparison to the EAE control group. Protection was confirmed by a much lower inflammatory infiltration in the CNS and was also associated with a reduced production of encephalitogenic cytokines by both, peripheral and CNS eluted cell cultures. The presence of an elevated number of $CD25^{+}FoxP3^{+}$ regulatory T cells in the lymph nodes, at both early clinical and clinical disease stages, suggests that these cells are involved in the protective effect. Adoptive transfer of splenocytes from mice vaccinated with MOG+VitD supports the view that protection is mediated, at least part, by immunoregulatory cells. Together these experiments provide evidence demonstrating that EAE can be prevented by the inverse vaccination with MOG+VitD even in the presence of a disease aggravating infectious agent.

KEYWORDS: multiple sclerosis; tolerogenic vaccination; myelin oligodendrocyte glycoprotein; active vitamin D; fungal infection, disseminated candidiasis.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory condition of the central nervous system (CNS) determined by a presumed autoimmune process mainly directed to myelin components[1]. More than 2.5 million people have MS worldwide and the incidence continues to increase and leads to substantial disability in most patients[2]. Acute demyelination shows as clinical relapses that may fully or partially resolve, while chronic demyelination and neuroaxonal injury lead to continuous and permanent neurological symptoms that usually progress over time[3]. Experimental autoimmune encephalomyelitis (EAE) is an animal model that is very useful to understand MS pathophysiology and treatment[4]. This model is usually induced by immunization of susceptible mice strains with CNS antigens associated with strong adjuvants[5]. Inoculation of C57BL/6 mice with myelin oligodendrocyte glycoprotein-$_{35-55}$ peptide (MOG_{35-55}) triggers a chronic form of the disease that does not remit[6]. This MOG_{35-55} EAE model has supplied a growing number of evidences on the role of immune system in the pathogenesis of MS, elucidating the contribution of B, T and other cell subsets in the pathogenesis of this disease[7-9].

MS is a multifaceted condition, with a range of environmental, behavioral and genetic factors implicated in its etiology and clinical course[10]. Considering that most of the systemic fungal infections have been associated with dissemination to the CNS[11], these pathogens could contribute to local tissue destruction. Alternatively, the local immune response against them could also participate in the immune process that damages the CNS. A possible association between MS and *Candida* spp. has been suggested by serological evidences. A significantly higher level of *Candida* specific antibodies was detected in MS patients than in normal control individuals[12]. In addition, *Candida* spp. antigens were also demonstrated in the cerebrospinal fluid of some MS patients[13]. We recently used MOG_{35-55} EAE to demonstrate that *Candida albicans* infection before encephalomyelitis induction aggravated EAE clinical parameters. This higher severity was concomitant to fungus dissemination to the CNS and also to a much higher production of encephalitogenic cytokines by peripheral and local cell cultures[14].

Even though clinical studies are required to test new therapies, this EAE model is considered a first-line model in the development of novel therapeutic approaches for MS, especially for shedding light on specific mechanistic questions[5]. Myelin-specific induction of

tolerance represents a promising strategy to modify the course of autoimmune inflammatory demyelinating diseases such as MS[15]. Inverse vaccination, for example, which refers to an antigen-specific tolerogenic immunization procedure, is able to silence immunity to myelin in MS[16]. Different vaccine preparations have given encouraging results. Cytokine-neuroantigen association is a new class of tolerogenic/therapeutic vaccines for treatment of EAE[15,17,18]. This approach is based on the association between recognition of self-antigens and the tolerogenic adjuvants action. Overall, these fusion proteins are able to induce tolerance and inhibit the effector phase of autoimmune response in EAE[15]. Similarly, polymeric biodegradable lactic-glycolic acid nano/microparticles loaded with MOG_{35-55} autoantigen and recombinant IL-10 significantly ameliorated the course of EAE[18].

We recently demonstrated that the MOG_{35-55} injection in the presence of active vitamin D is highly tolerogenic being able to work as a therapy[19] and also as a prophylaxis[20]. In this context we evaluated if this kind of tolerogenic vaccination was also efficient in the presence of an aggravating EAE agent as is *C. albicans*.

METHODS

Experimental design

Mice were allocated into three groups: *EAE*, submitted to EAE induction; *Ca/EAE*, infected with *C. albicans* three days before EAE induction and, *MOG+VitD/Ca/EAE*, vaccinated with MOG+VitD, infected with *C. albicans* and then submitted to EAE induction. Evaluations were performed during the early clinical and clinical disease phases, 10th and 17th days after EAE induction, respectively. The chronology of the experimental design is clarified in figure 1(a).

Mice

Female C57BL/6 mice with 9–11 weeks old were purchased from the University of São Paulo (USP) (Ribeirão Preto, SP, Brazil). Mice were allocated in specific-pathogen free conditions, in cages (maximum 5 mice per cage) with free food and autoclaved tap water in a controlled photoperiod (12h/12h, dark/light cycle) environment. All procedures were performed in ways that minimize animal suffering. The animals were manipulated according to the ethical principles for animal research adopted by the National Council for the Control of Animal Experimentation (CONCEA). This study was approved by the local Ethics Committee for

Animal Experimentation (CEUA), São Paulo State University (UNESP) (Botucatu, SP, Brazil; protocol number 351).

Vaccination with MOG associated with vitamin D3

Active vitamin D3, 1α,25-dihydroxyvitamin D3 (VitD) (Sigma-Aldrich Corporation, St. Louis, MO, USA) was diluted in 15% ethanol and then inoculated intraperitoneally in C57BL/6 mice. Each animal received eight VitD doses (0.1 μg/dose) every other day during two weeks (on days 18, 16, 14, 12, 10, 8, 6 and 4 before EAE induction). They also were inoculated with two doses (150 μg/dose) of $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) (Genemed Synthesis Inc., San Antonio, Texas, USA) that were co-administered on days 16 and 8[19,20]. The vaccination with MOG associated with vitamin D3 was represented by MOG+VitD in the text and figures.

***Candida albicans* infection**

C. albicans strain FCF 14 (Genbank Accession EF591020) was originally obtained from the mycology collection from the São José dos Campos Dental School (UNESP), and maintained in our mycological collection on Sabouraud-dextrose agar (Difco Laboratories, Detroit, MI, USA). For mice infection, *C. albicans* was cultured on solid media during 24 hours at 37°C. The fungal concentration was adjusted to 5.0×10^7/mL viable yeast cells in sterile saline solution (SSS). Fungus suspension was then inoculated into the lateral tail vein (0.1 mL/animal). For culture stimulation *C. albicans* suspension (5.0×10^7/mL) was killed by heat and pressure during 15 min at 121ºC in autoclave.

EAE Induction

Animals were subcutaneously immunized with 25 μL (100 μg) of $MOG_{35–55}$ peptide emulsified in 25 μL of Complete Freund's Adjuvant (Sigma) containing 4 mg/mL of *Mycobacterium tuberculosis*. Mice also received 2 intraperitoneal doses, 0 and 48 hours after immunization, of 200 ηg of *Bordetella pertussis* toxin (Sigma). Clinical score and body weight were daily recorded until the 17th day. EAE clinical assessment was performed according to the following criteria: 0, no symptoms; 1, limp tail; 2, hind legs weakness; 3, partially paralyzed hind legs; 4, complete hind leg paralysis; and 5, complete paralysis/death. The percentage of weight loss was calculated considering the day of immunization as day 0.

Fungal Load Determination

Samples from brain and spinal cord were weighted and macerated in 1.0 mL of SSS. Afterwards, 0.1 mL from each tissue homogenate was spread over culture plates containing Sabouraud-dextrose agar using a Drigalski T loop. The plates were then sealed and incubated at 37°C for 3 days. The number of colony forming units (CFU) was normalized per gram of tissue and logarithmized.

CNS-Mononuclear Cells Isolation

Ten and seventeen days after EAE induction, mice were anesthetized with ketamine/xylazine and perfused with 10 mL of SSS. Brain and spinal cord were collected, macerated, and digested with 2.5 mg/mL of collagenase D (Roche Applied Science, Indianapolis, IN, USA) in 4 mL of RPMI 1640 medium (Sigma) at 37°C for 45 min. Then, suspensions were washed in RPMI and centrifuged at 450 ×g at 4°C for 15 min. Cells were resuspended in Percoll (GE Healthcare, Uppsala, Sweden) 37% and gently laid over Percoll 70% in tubes of 15 mL. The tubes were centrifuged at 950 ×g for 20 min with centrifuge breaks turned off. After centrifugation the ring containing mononuclear cells was collected, washed in RPMI, and centrifuged at 450 ×g for 10 min. Cells were then resuspended in supplemented RPMI medium (1% gentamicin, 2% glutamine, 1% sodium pyruvate, 1% non-essential amino acids, and 10% of FBS), counted, and cultured. Pools from 2 animals were used to get enough cell number to perform the experiments.

Cell Culture Conditions and Cytokine Quantification

Spleen cells were collected and adjusted to 5×10^6 cells/mL in complete RPMI medium (1% gentamicin, 2% glutamine, and 10% of fetal calf serum). CNS-isolated cells were adjusted to 2×10^5 cells/mL in supplemented RPMI medium. Spleen and CNS-isolated cells were plated and stimulated with MOG (20 μg/mL and 50 μg/mL, respectively) and with heat-killed *C. albicans* (5 yeasts/1 cell). Cytokine levels were evaluated 48 h later by enzyme-linked immunosorbent assay (ELISA) in culture supernatants using IL-2, IFN-γ and IL-10 BD OptEIA Sets (Becton, Dickinson and Company, BD, Franklin, San Diego, CA, USA) and IL-6, IL-17, TNF-α, and TGF-β Duosets (R&D Systems, Minneapolis, MN, USA). The assays were performed according to the manufacturer's instructions.

Flow cytometry

Lymph nodes (axillary + inguinal) were collected and the red blood cells were lysed with buffer containing NH_4Cl. For regulatory T cells analysis, spleen and lymph node cells were incubated with PerCP-conjugated anti-mouse CD3 (clone 145-2C11, 0.2 µg), FITC-conjugated anti-mouse CD4 (clone GK1.5, 0.25 µg) and APC-conjugated anti-mouse CD25 (clone PC61.5, 0.13 µg) for 20 min at 4°C. Intracellular Foxp3 transcription factor was detected using PE-conjugated anti-mouse Foxp3 (clone FJK-16s, 0.2 µg) and FoxP3 Staining Set (eBiosciences, San Diego, CA, USA) according to manufacturer's instructions. For myeloid dendritic cells analysis, spleen and lymph node lysed cells were incubated with PerCP-conjugated anti-mouse F4/80 (clone BM8, 0.25 µg), FITC-conjugated anti-mouse CD11c (clone N418, 0.25 µg), APC-conjugated anti-mouse MHCII (clone M5/114.15.2, 0.03 µg) and PE-conjugated anti-mouse CD86 (clone GL1, 0.13 µg), for 20 min at 4°C. After staining, the cells were washed, resuspended in flow cytometry buffer, and fixed in paraformaldehyde 1%. Flow cytometry was performed using a FACS Canto II (BD) from Institute of Biosciences of Botucatu (UNESP, Botucatu, SP, Brazil) and the data were analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

Histopathology of the CNS

The histological analysis was performed during clinical EAE phase, that is, 17 days after disease induction. After euthanasia, lumbar spinal cord samples were removed and fixed in 10% neutral buffered formalin. Paraffin slides with 5 µm were stained with hematoxylin and eosin (H&E) and analyzed with a Nikon microscope. A semiquantitative analysis of CNS inflammation was performed according to the following criteria, adapted from Soellner *et al.*[21]: (0) no infiltrates; (1) partial meningeal infiltration; (2) pronounced meningeal infiltration, and (3) pronounced meningeal and some parenchymal infiltration.

Adoptive transfer experiment

For adoptive transfer experiment mice were vaccinated with MOG+VitD as previously described. One day after the end of vaccination, 1.5 x 10^6 splenocytes from vaccinated mice were adoptively transferred via the retroorbital route to naïve mice. A control groups received splenic cells from non-vaccinated naïve mice. After 3 days mice were infected with *C. albicans* and after three additionally days they were submitted to EAE induction. Clinical

score and body weight were daily recorded until the 10th day. In this period CNS-isolated cells were used to assess cytokine production and lumbar spinal cord were used to assess gene expression.

RT-PCR assays

Frozen lumbar spinal cord samples were used for RNA extraction (Trizol reagent, Life Technologies, USA) and cDNA synthesis according to the manufacturers recommendations (High Capacity RNA-to-cDNA converter kit, Life Techcnologies, USA). Expression of IDO (Mm00477461_m1) and FoxP3 (Mm00475162_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). Expression levels of genes were represented as a relative copy numbers by using the method of delta threshold ($2^{-\Delta\Delta Ct}$).

Statistical Analysis

Results were expressed as mean ± standard deviation. To test for the normality of data, results were analyzed by Shapiro-Wilk's test. Comparisons among three groups were made by one way ANOVA followed by Tukey's test for parametric variables and values of $p < 0.05$ were considered statistically significant. The GraphPad Prism v5.0 Statistical Guide (2007, GraphPad Software Inc., USA) for Windows was used to analyze data and create graphs.

RESULTS

Tolerogenic vaccination is efficient even in the presence of *C. albicans* infection

To investigate if tolerogenic vaccination with MOG_{35-55} in the presence of active vitamin D (MOG+VitD) still efficient even in exacerbated EAE, mice were vaccinated with MOG+VitD strategy and then infected with *C. albicans* before EAE induction (figure 1a). Animals were daily monitored for disease development during 17 days. As expected *C. albicans* infection triggered a much more severe disease characterized by higher clinical scores (figure 1b) and more accentuated body weight loss (figure 1c). Vaccination significantly decreased the incidence of EAE in previously infected mice (table 1). Animals from MOG+VitD/Ca/EAE group developed a much milder disease with very low clinical scores (figure 1b) and gained body weight during disease development (figure 1c). Although vaccinated mice lost body weight during VitD administration (figure 1d), they subsequently gained weight whereas non-vaccinated mice clearly lost weight (figure 1e).

Table 1. EAE incidence, Chi-square test (7-13 mice/group), results from two independent experiments were combined.

Groups	Incidence	P value
EAE	100.0 % (7/7)	< 0.001
Ca/EAE	100.0 % (11/11)	
MOG+VitD/Ca/EAE	38.5 % (5/13)	

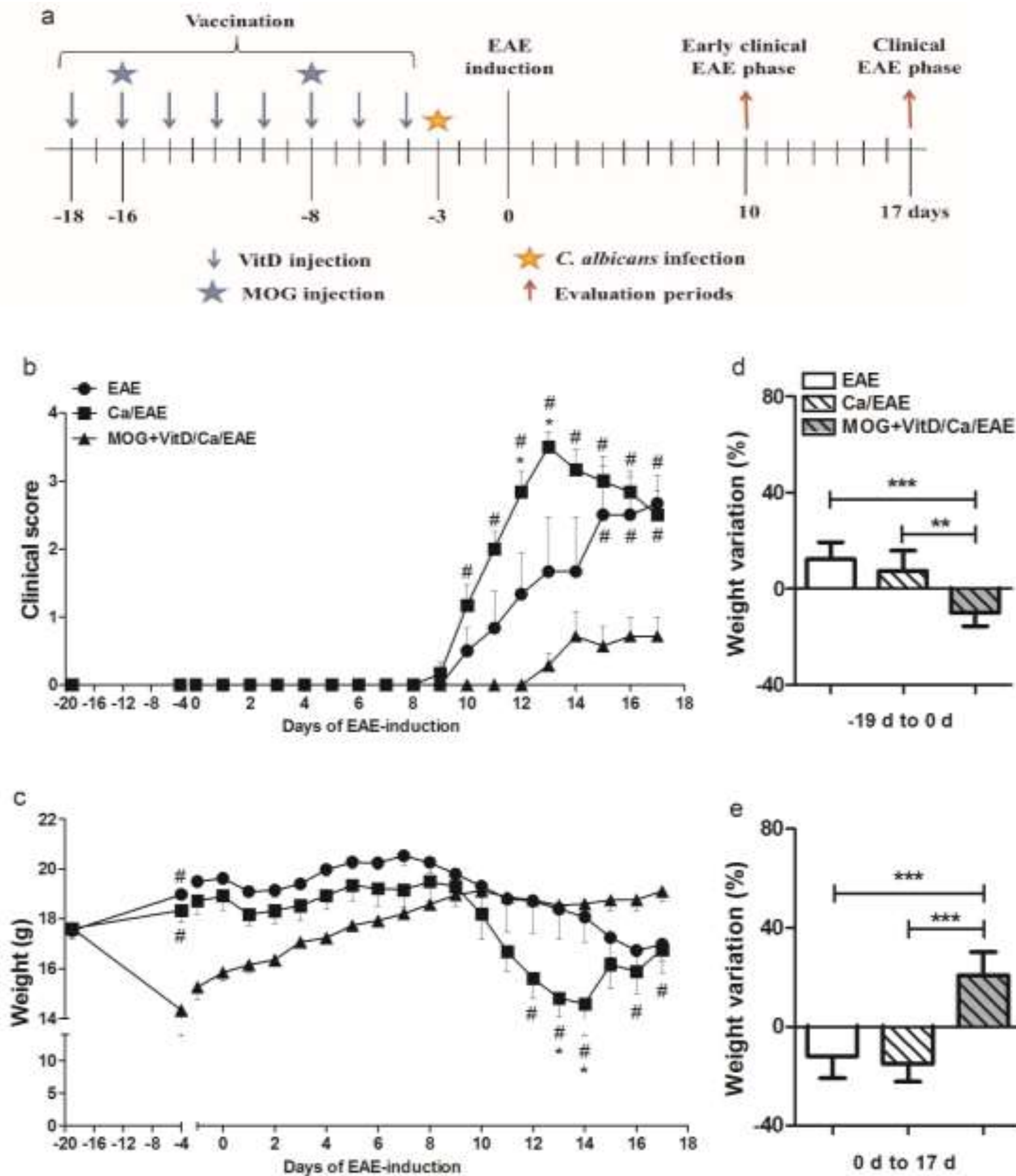

Figure 1. Efficacy of tolerogenic vaccination with MOG+VitD in EAE exacerbated by *C. albicans* infection. Experimental design (a); vaccination procedure included eight VitD doses administered every other day and two MOG doses injected at days -16 and -8. One day after last VitD dose, C57BL/6 mice were infected with *C. albicans* and three days after they were submitted to EAE induction. Peripheral and local immunological parameters were assessed in early clinical and clinical disease phases, 10th and 17th days after EAE induction, respectively. Clinical score (b) and body weight (c) were daily recorded until the 17th day. The percentage of weight variation was determined during vaccination, that is from day -19 to day 0 (d) and during EAE-development, i.e. from day 0 to day 17 (e). The results are expressed as mean ± SD (6-7 mice/group), one experiment representative of two is shown. (b,c) # indicates difference between vaccinated and non-vaccinated groups; * indicate difference between EAE and Ca/EAE groups. (d,e) **p<0.01 and ***p<0.001 indicate difference between vaccinated and non-vaccinated groups.

Vaccination downmodulated peripheral cytokine production in infected EAE-mice

As non-conventional vaccines have been associated with tolerance induction in EAE models[15,17,18], we hypothesized that cytokine production could be decreased in the peripheral lymphoid organs. Therefore, cultures of spleen cell from distinct EAE phases (early clinical and clinical phase) were stimulated with MOG. At the early clinical EAE phase there was a significant downmodulation of Interleukin (IL)-17, Tumor Necrosis Factor (TNF)-α, Interferon (IFN)-γ and IL-2 production (figure 2b-e) in the MOG+VitD/Ca/EAE group in comparison to the Ca/EAE group. In this stage no difference was observed in IL-6 and IL-10 production (figure 2a,f) and Transforming Growth Factor (TGF)-β levels were lower in the Ca/EAE group in comparison to the two other experimental groups (figure 2g). Nonetheless, a very distinct scenario was observed when cytokine levels were evaluated in cultures from the clinical EAE phase. Even though vaccination determined a significant decrease in IL-17 production (figure 2b) in the previously infected mice, the production of other proinflammatory cytokines, IL-6, TNF-α, IFN-γ and IL-2, was similar between MOG+VitD/Ca/EAE and Ca/EAE groups (figure 2a,c-e). In addition, there was no difference in the levels of IL-10 and TGF-β levels between these two groups (figure 2f,g). The comparison between the two clinical time points in the non-vaccinated EAE groups revealed a decrease in IL-6, IL-17 and IL-2 production and an increase in TGF-β production (figure 2a,b,e,g). In the vaccinated group, with exception of IL-6 and IL-10, there was no difference in the levels of cytokines between the two time points (figure 2b-e,g).

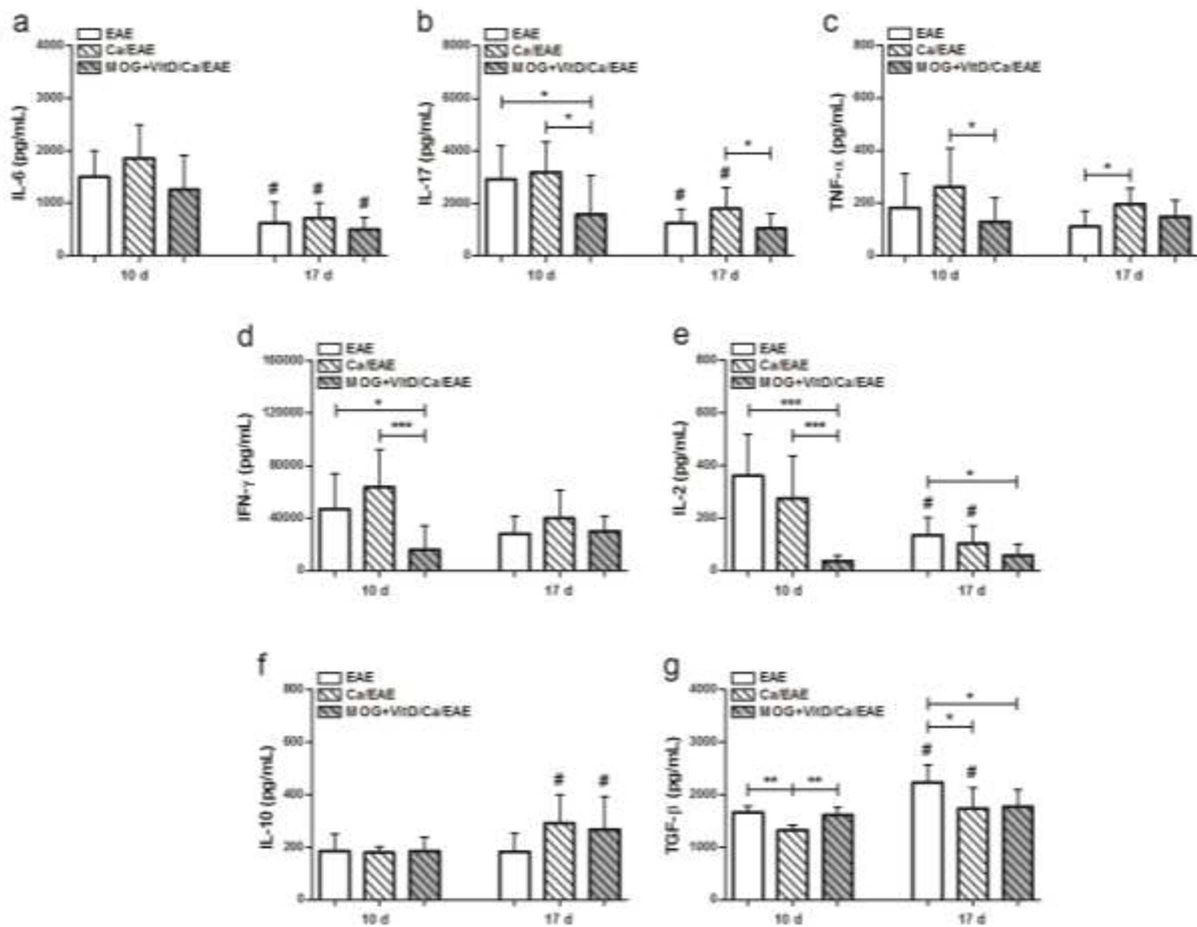

Figure 2. Vaccination downmodulated peripheral cytokine production in infected EAE-mice. C57BL/6 mice were vaccinated with MOG+VitD, infected with *C. albicans* and then submitted to EAE-induction. IL-6 (a), IL-17 (b), TNF-α (c), IFN-γ (d), IL-2 (e), IL-10 (f) and TGF-β (g) levels were measured in spleen cell cultures (5 x 106 cells/mL) stimulated with MOG (20 µg/mL) at early clinical (10th day) and clinical EAE phase (17th day). The results are expressed as mean ± SD (6-14 cultures/group), results from two independent experiments were combined. *p < 0.05, **p<0.01 and ***p<0.001 indicate difference among groups. # indicates difference between time points of analysis.

MOG+VitD vaccination and *C. albicans* infection lead to expansion of $CD25^+FoxP3^+$ T cells and myeloid dendritic cells in lymph nodes

We next tested if this peripheral cytokine downmodulation was concomitant with alterations in the proportion of T cells and myeloid Dendritic Cells (DCs). The percentage of $CD3^+CD4^+$ T cells in the lymph nodes was significantly elevated in the Ca/EAE group (figure 3a) compared to uninfected EAE group. In the vaccinated group the percentage of $CD3^+CD4^+$ T cells was lower than in the Ca/EAE group at both, early clinical and clinical EAE phase (figure 3a). Even though the three experimental groups presented elevated percentages of regulatory T cells ($CD3^+CD4^+CD25^+Foxp3^+$) at the clinical phase compared to the early clinical EAE phase, the values were significantly higher in Ca/EAE and MOG+VitD/Ca/EAE groups than in the EAE control group (figure 4b). The $CD25^+FoxP3^+/CD3^+CD4^+$ ratio was higher in the MOG+VitD/Ca/EAE group in comparison to non-vaccinated groups during the early clinical EAE phase (figure 3c). In this EAE phase, the percentage of DCs ($F4/80^-$ $CD11c^+CD86^+MHC\ II^+$) was similar in the three experimental groups (figure 3d). During clinical EAE phase, even though the percentage of DCs was higher in both infected groups (Ca/EAE and MOG+VitD/Ca/EAE) in comparison to the EAE group, vaccination decreased the percentage of myeloid DCs in MOG+VitD/Ca/EAE group compared with Ca/EAE group (figure 3d).

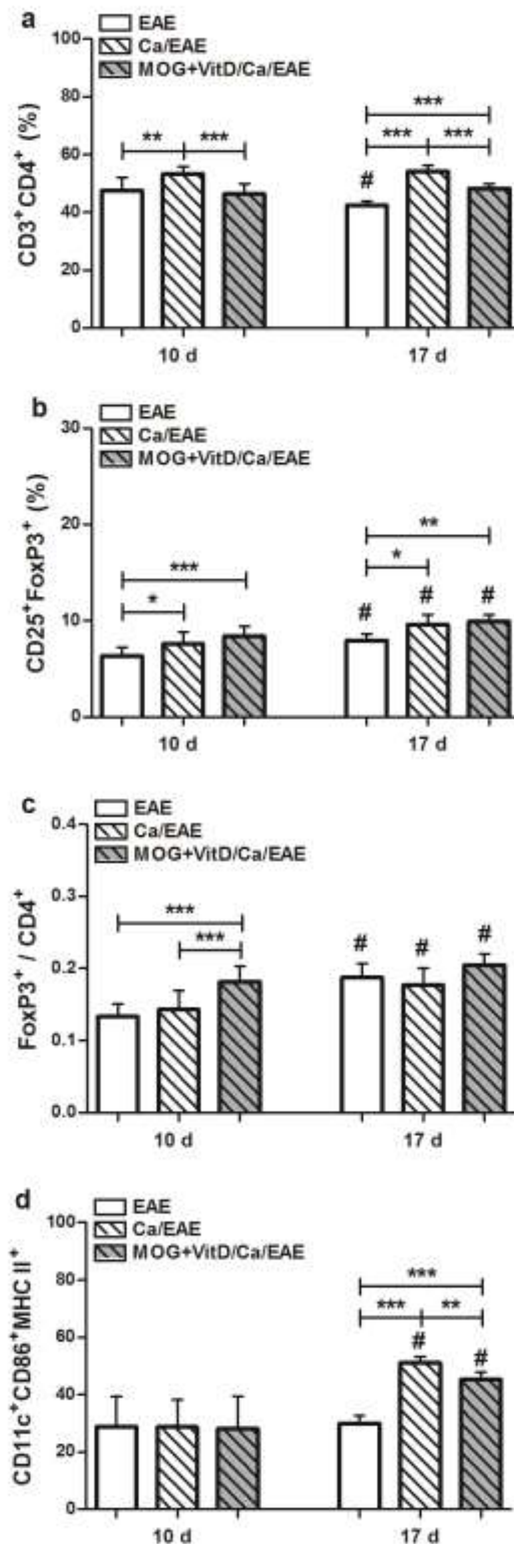

Figure 3. Expansion of regulatory FoxP3^{+} T cells in MOG+VitD vaccinated mice. C57BL/6 mice were vaccinated with MOG+VitD, infected with *C. albicans* and then submitted to EAE-induction. The percentage of T cells and dendritic cells was determined in a pool of axillary and inguinal lymph node cells at early clinical (10th day) and clinical EAE phase (17th day). Percentage of CD3^{+}CD4^{+} cells (a) in 100,000 acquired events and CD25^{+}Foxp3^{+} cells (b) in total CD3^{+}CD4^{+} cells. Ratio between percentage of FoxP3^{+} cells and CD4^{+} cells (c). Percentage of CD86^{+}MHC II^{+} (d) in F4/80^{-}CD11c^{+} dendritic cells in 500,000 acquired events. The results are expressed as mean ± SD (6-14 samples/group), results from two independent experiments were combined. *p < 0.05, **p<0.01 and ***p<0.001 indicate difference among groups, ANOVA. # indicates difference between time points of analysis.

Less pro-inflammatory activity in the CNS of vaccinated infected EAE-mice

To ascertain that the vaccination procedure was also efficient reducing the cellular infiltration in the CNS, we semi-quantitatively evaluated stained lumbar spinal cord sections concerning the degree of inflammation during the clinical EAE phase (figure 4a). Whereas the EAE and Ca/EAE groups presented a similar degree of inflammation, MOG+VitD/Ca/EAE presented a much less pronounced inflammatory process as illustrated in figure 4(b). The number of leukocytes per gram of CNS (brain plus spinal cord) was lower in vaccinated group compared to non-vaccinated groups in the early clinical EAE phase (figure 4c). This difference was not observed when this comparison was done at the clinical EAE phase. Despite the lower inflammation in the CNS in previously vaccinated mice, they presented the same amount of fungus at the CNS as non-vaccinated mice at both disease phases (figure 4d). In spite of this reduced inflammation it was possible to recover enough cells to be stimulated in vitro with MOG to assess cytokine production. During the early clinical EAE phase Ca/EAE group produced elevated levels of proinflammatory cytokines while vaccinated group showed a downmodulation of these cytokines (figure 5a-e). During the clinical EAE phase, when EAE and Ca/EAE presented similar clinical scores, comparable levels of IL-6, IL-17 and TNF-α were detected, as showed in figure 5 (a-c). On the other hand, the amount of these same mediators was significantly reduced in the MOG+VitD/Ca/EAE group in comparison to non-vaccinated groups. The production of IL-2 and IL-10 followed a similar pattern, i.e., lower levels were observed in the MOG+VitD/Ca/EAE group (figure 5e,f). No difference was observed in TGF-β levels (*data not shown*).

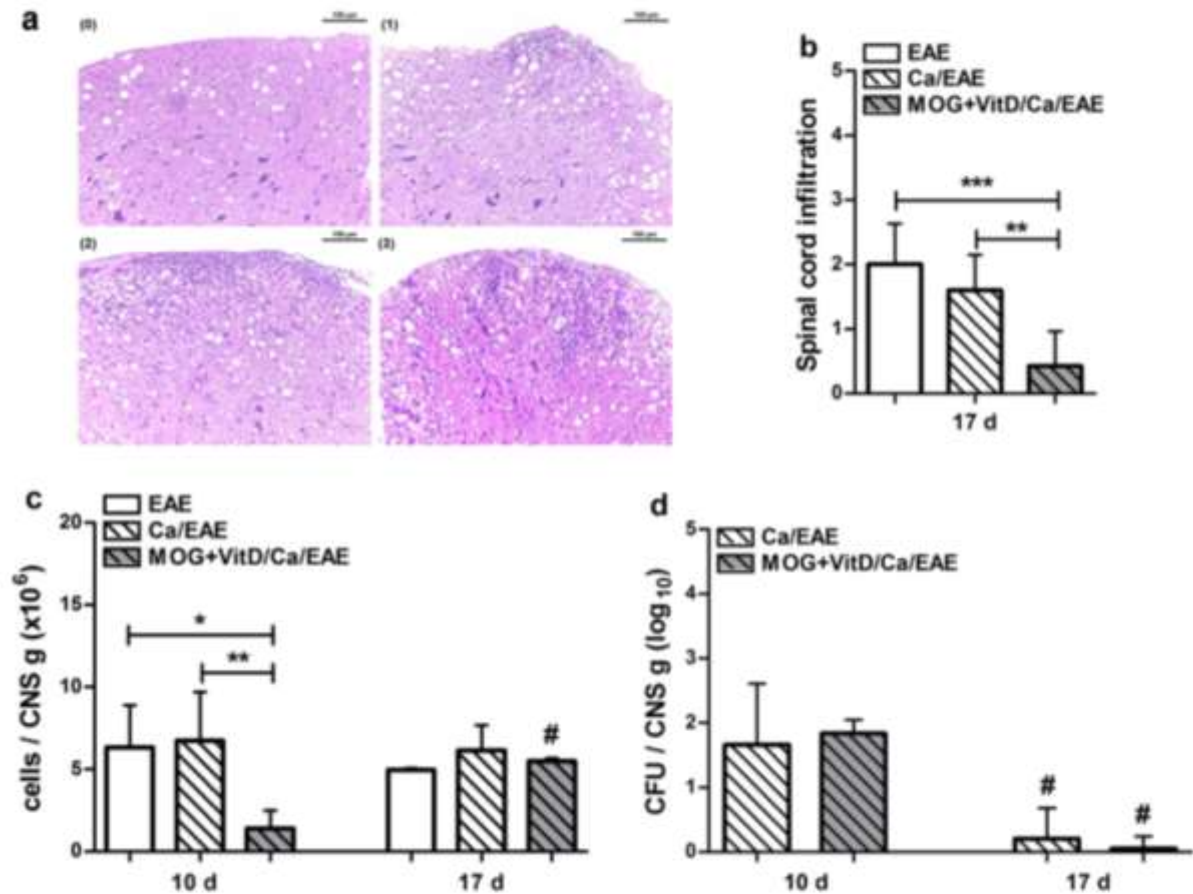

Figure 4. CNS inflammatory infiltration and fungal load. C57BL/6 mice were vaccinated with MOG+VitD, infected with *C. albicans* and then submitted to EAE-induction. Lumbar spinal cord inflammation was evaluated according to the following criteria (a): (0) no infiltrates; (1) partial meningeal infiltration; (2) pronounced meningeal infiltration, and (3) pronounced meningeal and some parenchymal infiltration. This semiquantitative analysis was used to assess the inflammatory infiltration (b) during the clinical EAE phase. The number of leukocytes (c) and fungal burden (d) was determined per gram of CNS tissue (in a pool of brain and spinal cord) at early clinical (10th day) and clinical EAE phase (17th day). The results are expressed as mean ± SD (6-14 mice/group), results from two independent experiments were combined. *p < 0.05, **p < 0.01 and ***p < 0.001 indicate difference among.

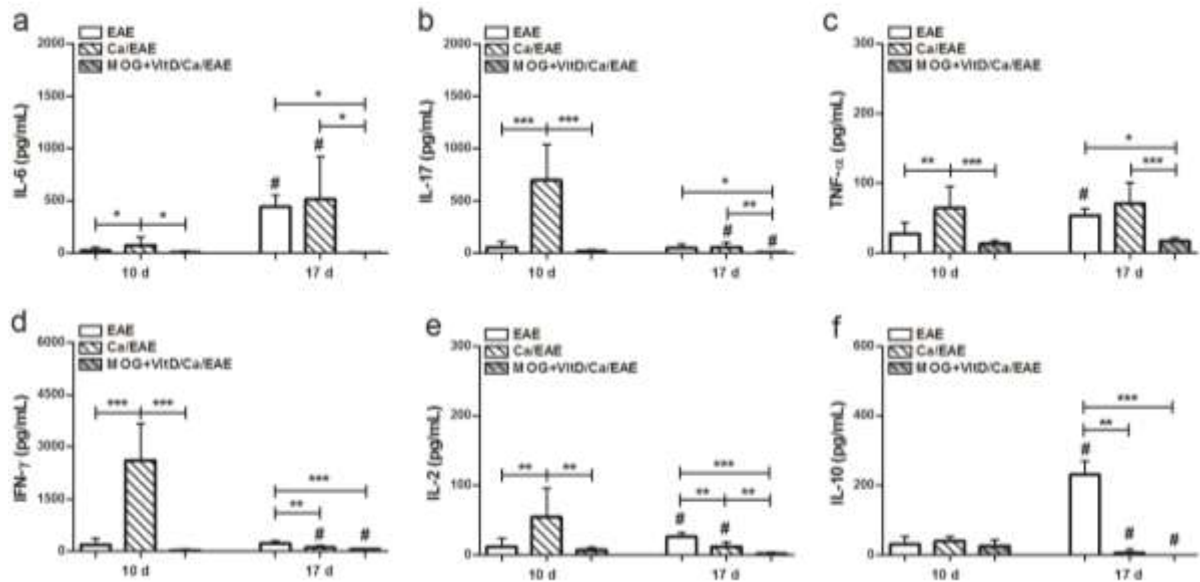

Figure 5. CNS cytokine production. C57BL/6 mice were vaccinated with MOG+VitD, infected with *C. albicans* and then submitted to EAE-induction. IL-6 (a), IL-17 (b), TNF-α (c), IFN-γ (d), IL-2 (e) and IL-10 (f) levels were measured CNS cell culture (2 x 105 cells/mL) stimulated with MOG (50 μg/mL) at early clinical (10th day) and clinical EAE phase (17th day). The results are expressed as mean ± SD (4-11 cultures/group), results from two independent experiments were combined. *$p < 0.05$, **$p<0.01$ and ***$p<0.001$ indicate difference among groups. # indicates difference between time points of analysis.

Splenocytes from vaccinated mice transfer protection against EAE development

In order to test whether the protection induced by vaccination involves the specific cellular immune response against EAE and not systemic (or nonspecific) effects of vitamin D, we evaluated the ability of splenocytes from vaccinated *naïve* mice to suppress EAE (figure 6a). Adoptive transfer of MOG+VitD-splenocytes obtained from previously vaccinated mice reduced clinical EAE manifestations in mice infected before encephalomyelitis induction. Until early clinical EAE phase, Ca/EAE-mice recipient of MOG+VitD-splenocytes showed no signs of paralysis and no body weight loss in comparison to control recipient Ca/EAE-mice (figure 6b,c). Considering that FoxP3, IDO and cytokines are factors usually involved in VitD immunomodulation[22], the relative mRNA expression for FoxP3 and Indoleamine 2, 3-Dioxygenase (IDO) and also the production of cytokines was assess in the CNS. An increased expression of FoxP3 was detected in the lumbar spinal cord from Ca/EAE-mice recipient of MOG+VitD-splenocytes in comparison with mice from control recipient group (figure 6d) and no difference was observed in IDO expression (figure 6e). Additionally, the significantly

lower production of TNF-α and IFN-γ (figure 6f,g) provide evidence that the vaccination with MOG plus with vitamin D induced tolerance to CNS-antigen that reduced EAE severity.

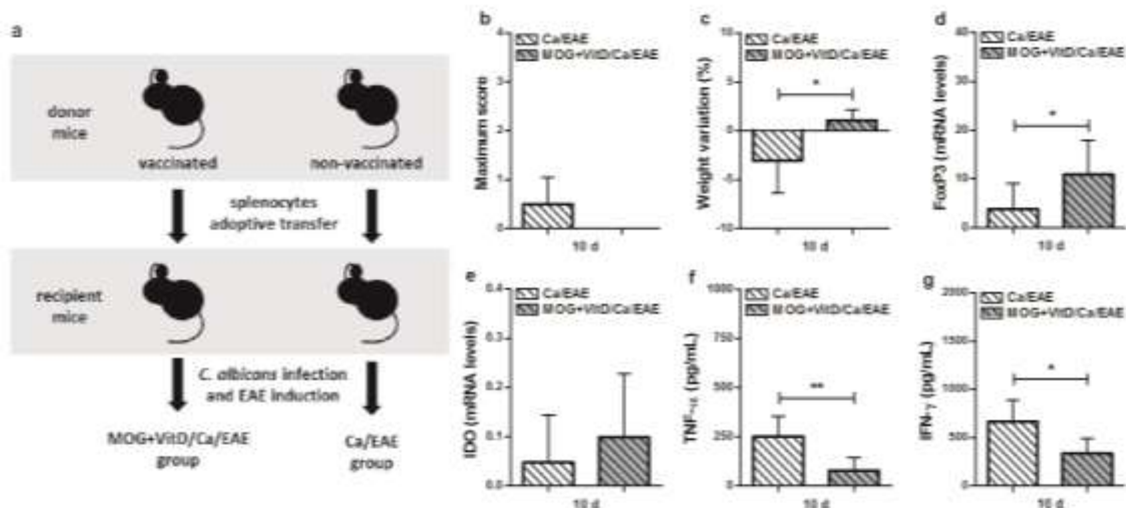

Figure 6. Splenocytes from vaccinated mice transfer protection against EAE development. Experimental design (a); vaccination procedure included eight VitD doses administered every other day and two MOG doses injected at days -16 and -8. One day after last VitD dose splenocytes were adoptively transferred (1.5 x 106 cells/mouse) into C57BL/6 naïve mice. Recipient mice were infected with *C. albicans* 3 days before EAE induction. Disease development was followed during the early clinical EAE phase to assess maximum clinical score (b) and percentage of weight variation (c). Expression of FoxP3 (d) and IDO (e) were analyzed in lumbar spinal cord. The TNF-α (f) and IFN-γ (g) levels were measured in CNS cell culture stimulated with MOG. The results are expressed as mean ± SD (6 mice/group), one experiment is shown. *p < 0.05 and **p < 0.01 indicate difference between groups.

DISCUSSION

Our research team has investigated strategies to prevent or to treat autoimmune diseases. We recently described that the association of the myelin oligodendrocyte glycoprotein (MOG_{35-55}) with active vitamin D3 (VitD) is highly efficient as a precocious treatment for experimental autoimmune encephalomyelitis (EAE)[19] and also as a prophylactic vaccination[20]. We also focus our work on the effect of infectious agents on EAE development. Whereas *Staphylococcus aureus* infection attenuated this experimental disease[23], *Candida albicans* triggered a much more accentuated encephalomyelitis[14]. In this context we questioned if

prophylaxis with MOG+VitD could be also effective to reduce EAE even in the presence of *C. albicans* infection.

Clinical, immunological and histopathological findings contributed to conclude that vaccination with MOG+VitD can decrease EAE severity even in the presence of *C. albicans* that is a disease aggravator. To get evidences of the mechanism that could be involved in this protective effect, we evaluated cytokine production, the percentage of regulatory T cells (Tregs) and presence of myeloid Dendritic Cells (DCs) during both, the early clinical (10th day) and clinical (17th day) EAE phases. Even though only more recently explored, alterations present at the EAE onset or early clinical disease phase are relevant to determine the involvement and kinetics of different cell types and their mediators in disease aggravation or disease control[24,25]. The relevance of this early analysis was very clear in our study. Furthermore, in this case, the results obtained during the clinical disease phase could be misunderstood because the acute phase in the EAE control group coincided with a recovery phase in the Ca/EAE group.

In a general way, proinflammatory cytokine production by splenocytes was significantly downmodulated in the vaccinated group during the early clinical EAE. Lower levels of IL-17 were maintained during the clinical phase. Similar alterations were triggered by subcutaneous inverse vaccination with nano/microparticles loaded with a MOG_{35-55} in EAE; this procedure also decreased the secretion of IL-17 and IFN-γ induced by MOG_{35-55} in splenic T cell cultures[18]. Additionally, during the early clinical EAE, the MOG+VitD/Ca/EAE group produced more TGF-β than Ca/EAE group. This finding seems relevant because the production of TGF-β has been implicated in the efficacy of prophylactic and therapeutic strategies in EAE[26] and also in MS[27]. Several studies have shown that the TGF-β suppressor role has been associated, at least in part, to its ability to inhibit activation and maturation of DCs[28,29] and to induce transcription of FoxP3[30]. Surprisingly we found no differences in the amount of myeloid DCs ($F4/80^{-}CD11c^{+}CD86^{+}MHC\ II^{+}$) among experimental groups during this disease phase. A reduced amount of myeloid DCs was expected based on our previous results[19] and also in the extensive literature disclosing the ability of vitamin D3 to interfere with DCs maturation[31-32]. Differently from expected we observed an expansion of myeloid DCs in infected groups in comparison to the EAE group during the clinical EAE phase. Also the MOG+VitD/Ca/EAE group showed a lower amount of myeloid DCs in comparison to Ca/EAE group. The increased in the percentage of myeloid DCs was attributed to *C. albicans*

immune response[33] because the comparison between infected groups revealed that the vaccination decreased the percentage of myeloid DCs.

Considering that the active vitamin D3 is able to generate tolerogenic DCs that promote the induction of regulatory T (Treg) cells[34], we investigated if there was an expansion of Treg cells in vaccinated group. As expected, since *Candida* infection *per se* results in a $FoxP3^+$ cells expansion[35], the infected groups showed an elevated percentage of Treg cells ($CD3^+CD4^+CD25^+FoxP3^+$) in the lymph nodes at both disease phases. Interestingly, the vaccinated group exhibited lower percentage of $CD4^+$ cells. The ratio between the number of $Foxp3^+$ and $CD4^+$ cells indicated that this relationship was significantly more elevated in the MOG+VitD/Ca/EAE group in comparison to Ca/EAE group. Together these results suggest that Treg cells expansion has contributed to the protective effect of MOG+VitD. A similar protective effect mediated by a tolerogenic vaccination was already described. Treatment with MOG-DNA vaccines reduces the clinical and histopathological signs of EAE when administered in both prophylactic and therapeutic settings through dampening of antigen (Ag)-specific proinflammatory Th1 and Th17 immune responses and expansion of Treg in the periphery[36].

This protective effect was clearly confirmed when the CNS was analyzed concerning the degree of inflammation and cytokine production. A semiquantitative evaluation showed that the inflammatory process in the lumbar spinal cord was very discrete in the vaccinated group comparing with the two other experimental groups. In spite of this, enough cells were isolated from the CNS to evaluate cytokine production. By comparing cytokine levels produced by the same number of cells, very low amounts of encephalitogenic cytokines were detected during both, early clinical and clinical disease phases. This lower production of pro-inflammatory cytokines was not due to a high local production of IL-10 or TGF-β. It could be, at least in part, associated with the downmodulation of peripheral MOG-specific immunity by the vaccination with MOG+VitD. In this context a lower amount of encephalitogenic T cells would be available to reach the CNS. Despite this, as the fungal burden recovered from the CNS was similar in vaccinated and non-vaccinated groups, we concluded that protection was not interfered with a local fungal clearance.

To ascertain that this protective effect was due to an active process mediated by peripheral cells, we transferred splenocytes from mice previously vaccinated with MOG+VitD to naïve mice before both, *C. albicans* infection and EAE induction. This

adoptive transference was able to avoid early clinical disease manifestations. In comparison to mice that received splenocytes from naïve mice and were already sick 10 days after EAE induction, the ones that received splenocytes from vaccinated mice did not lose weight neither presented paralysis at this time point. This protection mediated by these splenic cells was associated with a decreased TNF-α and IFN-γ production in the CNS. The most of the immunoregulatory ability of VitD in EAE has been linked to tolerogenic DCs that produce indoleamine 2,3-dioxygenase (IDO) which results in Treg expansion[22]. Considering that both, DCs and Treg, can be isolated from CNS of MS patients and EAE-mice[37] we initially compared the amounts of IDO and FoxP3 mRNA expression in lumbar spinal cord samples from the two groups. A similar amount of IDO mRNA expression was detected. However, a higher FoxP3 mRNA expression was detected in the CNS from protected mice, suggesting that transferred regulatory T cells are moving from the periphery to the CNS.

Together, our data indicate that *C. albicans* infection is not able to disrupt the efficacy of tolerogenic vaccination with MOG+VitD in EAE. Even in the presence of this aggravating infectious agent, the vaccination was able to reduce the clinical signs of EAE by down-regulating cellular immune response towards neuroantigens in both, periphery and CNS. The adoptive transfer of splenocytes vaccinated with MOG+VitD reinforced this cellular protective effect. Vaccination with MOG+VitD is therefore very effective. The potential efficacy as a vaccination or therapy by associations between specific autoantigens and vitamin D in other autoimmune pathologies deserves full investigation.

ACKNOWLEDGEMENTS

This work was supported by São Paulo Research Foundation (FAPESP, grant number 2012/12540-7). TFCFS received a FAPESP scholarship (grant number 2013/14353-2).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Conclusões

5. Conclusões

I. A infecção sistêmica prévia por *C. albicans* antecipou e agravou os sinais clínicos da encefalomielite autoimune experimental (EAE). Este agravamento foi associado com disseminação do fungo para o sistema nervoso central (SNC) e intensificação da resposta autoimune, tanto na periferia quanto no SNC.

II. A inoculação sistêmica de derivados fúngicos desencadeou efeito deletério ou protetor que dependeu da identidade do componente fúngico. A administração de gliotoxina resultou no agravamento da EAE que foi concomitante com aumento de infiltrado inflamatório e maior produção de TNF-α no SNC. Este agravamento também pode estar associado com permeabilização da barreira hematoencefálica e/ou efeito citotóxico direto da gliotoxina em células do SNC. Distintamente, a administração de leveduras mortas de *C. albicans* determinou um efeito protetor provavelmente associado com a migração, para o SNC, de células Th2 específicas para *C. albicans*.

III. O efeito profilático da associação de um antígeno da mielina com vitamina D foi eficaz mesmo em animais com doença agravada pela infecção por *C. albicans*, indicando que o expressivo agravamento causado pelo fungo não quebrou a tolerância determinada pela vacinação.

O presente estudo contribuiu para o esclarecimento da participação de fatores ambientais na etiologia da esclerose múltipla, mostrando que derivados fúngicos podem agravar ou proteger contra o desenvolvimento da EAE que é o modelo experimental para estudo desta doença humana. Esta abordagem sugere que o assunto é complexo e que merece uma investigação aprofundada no sentido de revelar quais derivados fúngicos agravam a doença e quais têm potencial protetor.