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Efeito da infecção com *Strongyloides venezuelensis* no desenvolvimento da encefalite autoimune experimental (EAE)

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Tese apresentada ao Programa de Pós-Graduação em Doenças Tropicais da Faculdade de Medicina da Universidade Estadual Paulista – UNESP – Botucatu, para obtenção do título de Doutor em Doenças Tropicais.

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"Deus costuma usar a solidão
para nos ensinar sobre a convivência.

Às vezes, usa a raiva para que possamos
compreender o infinito valor da paz.

Outras vezes usa o tédio, quando quer
nos mostrar a importância da aventura e do abandono.

Deus costuma usar o silêncio para nos ensinar
sobre a responsabilidade do que dizemos.

Às vezes usa o cansaço, para que possamos
compreender o valor do despertar.

Outras vezes usa a doença, quando quer
nos mostrar a importância da saúde.

Deus costuma usar o fogo,
para nos ensinar a andar sobre a água.

Às vezes, usa a terra, para que possamos
compreender o valor do ar.

Outras vezes usa a morte, quando quer
nos mostrar a importância da vida."

Fernando Pessoa

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Sumário

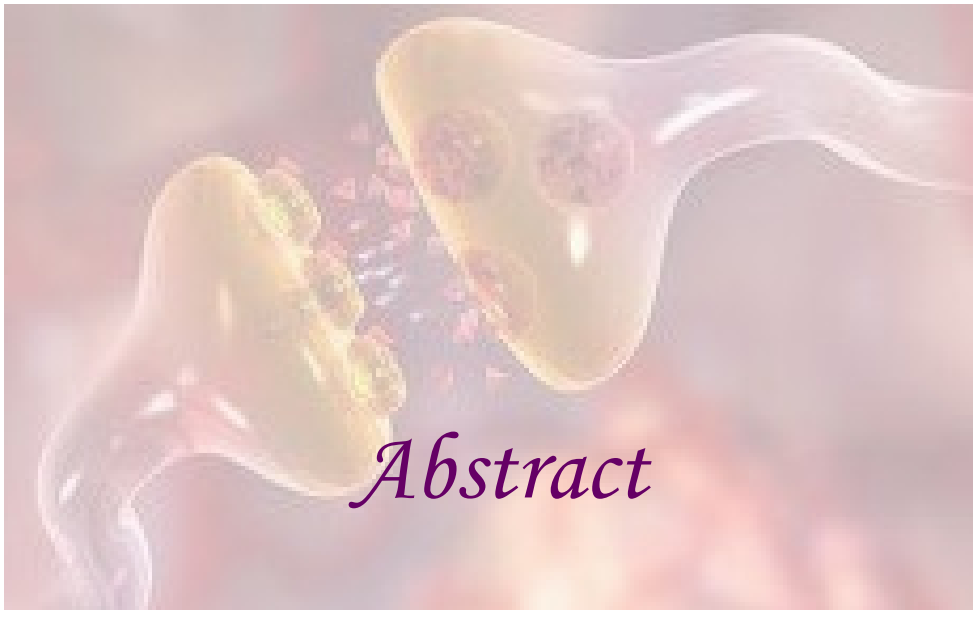
SUMÁRIO

Resumo

A esclerose múltipla (EM) é uma doença inflamatória, crônica e desmielinizante do sistema nervoso central (SNC). A caracterização de uma estratégia profilática e/ou terapêutica na EM é necessária, já que não há cura para essa doença. No contexto da hipótese da higiene, a exposição diminuída a certos agentes infecciosos como os helmintos, os lactobacilos e as micobactérias saprófitas estaria relacionada com o aumento na incidência de doenças alérgicas e autoimunes. Assim, o objetivo deste trabalho foi caracterizar a infecção por *Strongyloides venezuelensis* em ratos Lewis e avaliar se a mesma modula as características clínicas, imunológicas e histopatológicas da encefalite autoimune experimental (EAE) nestes animais. Na primeira etapa, caracterizamos as fases aguda e de recuperação da infecção e avaliamos os padrões de resposta imune nestas duas fases. Na segunda etapa, avaliamos o efeito de uma ou várias infecções com *S. venezuelensis* na evolução da EAE. Os animais foram avaliados diariamente quanto ao peso e escore clínico da doença e a eutanásia foi realizada na fase de recuperação da EAE para avaliação da resposta imune (produção de citocinas e anticorpos) e do processo inflamatório no SNC. A frequência de células T CD4+CD25+Foxp3+ no baço e nos linfonodos (inguinais e poplíteos) também foi determinada após infecção única (fase aguda e de recuperação) ou múltipla com *este* helminto. De acordo com os diversos parâmetros avaliados, os resultados demonstraram que a infecção com *S. venezuelensis* não modificou a progressão da EAE em ratos Lewis e também não alterou a frequência de células T CD4+CD25+Foxp3+ nos órgãos linfóides secundários. Considerando a hipótese da higiene, estes resultados sugerem a necessidade

de um estudo comparativo entre as diferentes espécies de helmintos para avaliar seu potencial imunorregulatório.

Palavras-chave: encefalite autoimune experimental, *Strongyloides venezuelensis*, hipótese da higiene, células T CD4+CD25+Foxp3+


Abstract

Multiple sclerosis (MS) is a chronic immune-mediated demyelinating disease of the central nervous systems (CNS). This and other immune-mediated diseases are clearly increasing in more developed countries. According to the hygiene hypothesis this is due to a decreased contact between the human population and certain organisms as helminths, mycobacteria and lactobacillus. In this context, the main objective of this investigation was to evaluate if one or multiple infections with *S. venezuelensis* was able to modify the development of experimental autoimmune encephalomyelitis (EAE) in Lewis rats. Based on the hygiene hypothesis, an at least partial protective effect was expected. Initial assays indicated that recovery from *S. venezuelensis* in Lewis rats was associated with a strong Th2 response. Rats infected one or multiple times with this helminth were then submitted to EAE induction by immunization with myelin associated with complete Freund adjuvant. Differently from what was supposed, previous infection with *S. venezuelensis* was not able to modify body weight, clinical score and inflammation at the CNS. In addition, this infection was not associated with alteration in the frequency of CD4+CD25+Foxp3+ T cells in the spleen and lymph nodes (inguinal + popliteal). Considering the hygiene hypothesis, our data and literature reports, we believe that a comparative study employing different helminth species will be necessary to elucidate this subject.

Keywords: experimental autoimmune encephalomyelitis, *Strongyloides venezuelensis*, hygiene hypothesis, CD4+CD25+Foxp3+ T cells

1. Introdução

1. INTRODUÇÃO

1.1 Esclerose múltipla

A esclerose múltipla (EM) é uma doença inflamatória e desmielinizante do Sistema Nervoso Central (SNC) que é descrita como a principal causa de incapacidade neurológica em adultos jovens (Hohlfeld, 2009). A doença afeta 2,5 milhões de pessoas em todo o mundo, tendo maior prevalência na Europa e na América do Norte. Além disso, a EM acomete mais mulheres do que homens, na razão 2:1 (WHO, 2006). A maioria dos pacientes apresenta inicialmente um quadro transitório de sintomas com períodos de exacerbação e remissão da doença, seguido por fase secundária progressiva, caracterizada por perdas irreversíveis e neurodegeneração (Imitola et al., 2005; Hohlfeld, 2009). As primeiras manifestações clínicas da EM incluem fraqueza de um ou mais membros, perda da visão, falta de coordenação motora e parestesia (Silberberg, 1992). Causa e patogênese da EM não são completamente conhecidas, mas ainda acredita-se que esta seja, fundamentalmente, uma doença autoimune mediada por células Th1 com especificidade para antígenos do SNC (Steinman, 1996; Sospedra & Martin, 2005). Além disso, evidências recentes demonstram que uma nova subpopulação de células T CD4+ produtoras de IL-17 (Th17) desempenha um papel importante na patogênese da EM (Aranami & Yamamura, 2008). Foi demonstrado, por exemplo, que pacientes com EM apresentam níveis elevados de IL-17 nas lesões do SNC (Lock et al., 2002). Além disso, foram encontradas concentrações significativamente mais elevadas de IL-17 no fluido cerebroespinhal de pacientes com EM em comparação com indivíduos saudáveis (Ishizu et al., 2005). Assim, as citocinas produzidas por células Th1 e Th17, como IFN-γ, TNF-α e IL-17 parecem

mediar o processo inflamatório e conseqüente degeneração axonal, morte dos oligodendrócitos e disfunção neuronal (Steinman, 1996; Lucchinetti et al., 2000; Elloso et al., 2005; Sospedra & Martin, 2005; Furuzawa-Carballeda et al., 2007). As principais alterações histológicas presentes no SNC de pacientes com EM incluem infiltração de células T, B e macrófagos, além de degradação da mielina presente nos axônios e astrócitos (Lucchinetti et al., 2000).

A imunopatogênese da EM também tem sido atribuída a defeitos na atividade funcional de células T reguladoras (CD4+CD25+). Essas células são importantes não apenas para a manutenção da tolerância periférica, mas também para controlar a autoimunidade órgão-específica através da supressão de células T autorreativas (Skaguchi et al., 1995; Tang et al., 2004). Apesar de alguns trabalhos mostrarem níveis normais dessas células em pacientes com EM (Viglietta et al., 2004; Haas et al., 2005; Venken et al., 2006) tem sido descrita redução na atividade funcional das mesmas *in vitro* (Haas et al., 2005; Huan et al., 2005). Um possível comprometimento da atividade das células T reguladoras na EM tem sido sugerido por estudos utilizando modelos animais. Por exemplo, a presença dessas células é observada no SNC na fase de recuperação da encefalite autoimune experimental (EAE). Por outro lado, a depleção de células T reguladoras, que expressam o fator de transcrição Foxp3 (*forkhead transcription factor* 3), exacerba as manifestações clínicas da doença (McGeachy et al., 2005). Esses achados ressaltam a importância da atividade supressora dessas células na inflamação do SNC.

O tratamento da EM é realizado com medicamentos que visam reduzir a frequência e a gravidade das exacerbações, uma vez que não há cura para a doença. Os corticosteróides, como a metilprednisolona, são utilizados na fase aguda da

doença. Já as drogas imunomoduladoras, como o IFN-β e o acetato de glatirâmer, são utilizadas em pacientes com quadros de exacerbação e remissão da doença (Rudick, 2005; Gold & Wolinsky, 2010)**.** Esses dois medicamentos impedem a ativação, a proliferação e a migração de células inflamatórias para o SNC (Gold & Wolinsky, 2010).

1.2 Encefalite autoimune experimental

A encefalite autoimune experimental (EAE) é o modelo animal utilizado para estudar a esclerose múltipla. A doença pode ser induzida em ratos e camundongos através da imunização com antígenos derivados de mielina (myelin basic protein – MBP, proteolipid protein – PLP, myelin oligodendrocyte glycoprotein – MOG ou peptídeos derivados destas proteínas) em associação com o Adjuvante Completo de Freund (ACF). A transferência adotiva de células T específicas para mielina também desencadeia a doença, indicando que a EAE é uma doença autoimune mediada pela resposta imune celular (Link & Xiao, 2001).

A EAE no rato Lewis é caracterizada como uma doença aguda, grave e monofásica com recuperação espontânea. Esta recuperação é associada com o desenvolvimento de células T com atividade supressora (Varriale et al., 1994). Neste modelo, os animais apresentam infiltração de células T no SNC, acompanhada de ativação da microglia (Namer et al., 1998). As características histopatológicas da EAE no rato Lewis incluem infiltração mononuclear meníngea, perivascular e parenquimal no SNC (Link & Xiao, 2001). A grande vantagem deste modelo é que o desenvolvimento da doença ocorre em quase 100% dos animais. (Swanborg, 2001). Por outro lado, a doença desencadeada em modelos murinos

de EAE, empregando diferentes cepas de camundongos isogênicos, mimetiza melhor o decurso crônico e/ou de exacerbação-remissão que é o mais comum na EM (Gold et al., 2006). Por exemplo, a imunização de camundongos C57BL/6 com um peptídeo (35-55) derivado de MOG desencadeia uma doença neurológica caracterizada por paralisia e também um extenso processo de desmielinização. Os animais desenvolvem doença crônica após a imunização que perdura por, pelo menos, 45 dias (Bernard et al., 1997).

A EAE tem sido considerada uma doença Th1 não só porque clones Th1 específicos para mielina transferem adotivamente a doença, mas também porque tem sido demonstrado que citocinas pró-inflamatórias, tais como IFN-γ e TNF-α danificam a bainha de mielina (Klinkert et al., 1997). De maneira similar ao que é observado na EM, as células Th17 parecem desempenhar um papel crítico na patogênese da EAE. Por exemplo, animais deficientes em IL-17 desenvolvem EAE mais tardiamente e com sintomatologia mais branda (Komiyama et al., 2006). Além disso, tem sido constatado que infiltração de células T e inflamação no SNC de animais com EAE só ocorrem se houver uma maior proporção de células Th17 do que de células Th1. A observação de que células Th17 específicas para mielina podem determinar o desenvolvimento de EAE por transferência adotiva de células comprovam o papel da Th17 na EAE (Awasthi et al., 2008).

Estes modelos experimentais murinos também são largamente empregados para avaliar o efeito neuroprotetor de substâncias imunomoduladoras e a eficácia da tolerância oral (Marques et al., 2009; Peron et al., 2010).

1.3 Gênero *Strongyloides*: ciclo biológico, infecções experimentais e resposta imune

Os nematódeos do gênero *Strongyloide*s compreendem mais de 52 espécies que acometem diversos hospedeiros (Costa-Cruz, 2005). A estrongiloidíase humana, causada pelo *Strongyloides stercoralis*, afeta entre 30-100 milhões de pessoas em 70 países diferentes (Genta, 1989; Siddiqui & Berk, 2003). A doença pode ocasionar uma infecção crônica persistente, com uma característica de auto-infecção e hiperinfecção envolvendo os pulmões e o sistema gastrintestinal, podendo ainda se disseminar para outros órgãos (Siddiqui & Berk, 2001).

Strongyloides spp. apresenta um ciclo de vida livre e um ciclo parasitário. A fase parasitária se caracteriza pela presença de fêmeas partenogenéticas no intestino delgado as quais produzem ovos larvados por partenogênese. Após a eclosão, as larvas diferenciam-se em adultos, machos e fêmeas, de vida livre, o que pode ser seguido por uma sucessão de gerações de vida livre. Entretanto, sob certas condições, provavelmente relacionadas com temperatura e umidade, ocorre a diferenciação para larvas infectantes (L3) que podem infectar o hospedeiro por penetração cutânea ou ingestão. Estas larvas migram via sistema venoso para pulmões e traquéia sendo posteriormente deglutidas e alojadas no intestino delgado onde se transformam em fêmeas partenogenéticas (Urquhart et al., 1998).

Strongyloides venezuelensis é um parasita de roedores, naturalmente encontrado em ratos (*Rattus norvegicus*). Este parasita tem sido utilizado como modelo experimental para estudos de biologia, imunologia, mecanismos de

expulsão de parasitas, características bioquímicas e atividade anti-helmíntica. Na infecção por *S. venezuelensis*, larvas infectantes de terceiro estágio penetram através da pele, migram para o pulmão, onde ocorre muda para larva de quarto estágio, e do pulmão migram para o intestino delgado onde finalmente se transformam em parasitas adultos (Tindall & Wilson, 1988).

As infecções helmínticas caracterizam-se por indução de respostas celulares tipo Th2, as quais resultam em eosinofilia, aumento do número de mastócitos na mucosa intestinal e produção de anticorpos IgG1 e IgE (Lawrence, 2003; Antony et al., 2007). A geração dessa resposta imune do tipo Th2 ocorre tanto nos linfonodos mesentéricos quanto em outros tecidos linfóides associados com o intestino. Após ativação, essas células produzem várias citocinas, como IL-4, IL-13, IL-9 e IL-5 e, além disso, estimulam as células B a secretarem IgE. Uma vez ativadas essas células migram para o local de residência dos parasitas, ou seja, a submucosa intestinal, para tentar combater a infecção (Anthony et al., 2007). Os mastócitos são importantes células efetoras na infecção por nematódeos. Eles possuem muitos grânulos contendo histamina, heparina e proteases e podem secretar citocinas como IL-4 e IL-5, bem como leucotrienos e quimiocinas. A ativação clássica resultante da degranulação de mastócitos envolve a ligação da IgE e a ligação cruzada do receptor FcεRI (de Veer et al., 2007). Essas células trabalham em conjunto com outras populações celulares, incluindo eosinófilos, neutrófilos, basófilos e macrófagos na tentativa de eliminar a infecção (Anthony et al., 2007). Os eosinófilos são as células mais comuns no local da infecção por nematódeos. Eles contêm grânulos com proteínas catiônicas

e podem liberar citocinas pró-inflamatórias, quimiocinas e mediadores lipídicos constituindo células efetoras potentes (de Veer et al., 2007).

Os helmintos podem sobreviver no organismo do hospedeiro por longos períodos causando, assim, infecções crônicas. A cronicidade dessas infecções tem sido atribuída a mecanismos imunoregulatórios, como a indução de células T reguladoras que produzem citocinas antiinflamatórias como IL-10 e TGF-β (Maizels et al., 2004). Essa resposta antiinflamatória é importante não apenas para prevenir a eliminação do verme, mas principalmente para proteger o hospedeiro contra uma resposta inflamatória excessiva (Helmby, 2009).

1.4 Hipótese da Higiene

A hipótese da higiene foi proposta na década de 80, a partir do trabalho de Strachan, (1989). O pesquisador observou que crianças oriundas de famílias mais numerosas apresentavam incidência menor de doenças alérgicas do que crianças cujas famílias tinham apenas um filho. Segundo esta hipótese, a ausência ou o menor contato com determinados agentes do meio ambiente durante a infância determina aumento na incidência de alergias e doenças autoimunes. Este menor contato ocorreria em função de melhores condições sanitárias, vacinação e uso de antibióticos (Fleming & Fabry, 2007). Foi proposto que este aumento na incidência de doenças alérgicas e autoimunes resulta da não indução de células T reguladoras. Isso ocorre devido à menor exposição a determinados microorganismos como lactobacilos, micobactérias saprófitas e helmintos, conhecidos como "*old friends*". Esses microorganismos estariam presentes durante todo o processo evolutivo dos mamíferos e seriam reconhecidos pelo

sistema imune inato como "*old friends*" e, por isto não causariam uma resposta imune destrutiva. (Rook et al., 2004; Rook et al., 2007).

Dando respaldo a esta teoria, foram publicados vários estudos mostrando a relação inversa entre as infecções e as doenças autoimunes (Cooke et al., 1999; Bach, 2002; La Flamme et al., 2003; Sewell et al., 2003; Zheng et al., 2008). No caso da EM, estudos experimentais mostraram que a infecção ou imunização de camundongos com *Schistossoma mansoni* reduz a gravidade da EAE (La Flamme et al., 2003; Sewell et al., 2003). Nesses trabalhos foi observada uma redução nos infiltrados celulares no SNC e uma melhora no quadro clínico da doença. Corroborando com esses resultados, recentemente, foi observado que a inoculação de antígeno solúvel de ovo do *S. japonicum* também diminui a gravidade e a progressão da EAE em camundongos (Zheng et al., 2008). Este potencial protetor também tem sido observado em outras espécies de helmintos. Por exemplo, a infecção com *Trichinella spirallis* determinou efeito protetor na EAE induzida em ratos Dark Agouti (DA) (Gruden-Movesijan et al., 2008). Além disso, também foi constatado por Lafaille et al. (1994), e mais recentemente por nosso grupo (Zorzella et al., 2007) que animais mantidos em condições "*germ-free*" apresentam maior incidência e quadros mais graves de EAE.

Nos últimos anos, visando uma possível aplicação clínica destes conhecimentos na profilaxia e terapia de doenças imunomediadas, aumentou o interesse de muitos pesquisadores em desvendar os mecanismos imunomoduladores exercidos pelos helmintos (Helmby & Bickle, 2006). Um dos mecanismos envolvidos nesse processo é o desvio da resposta imunológica. Por exemplo, o antígeno solúvel de ovo do *S. japonicum* induziu uma polarização Th2

tanto na periferia quanto no CNS em camundongos com EAE (Zheng et al., 2008). Resultados similares foram observados em camundongos imunizados com ovalbumina de *S. mansoni* (Sewell et al., 2003). Além disso, os helmintos também podem modular a resposta imune através da indução e/ou expansão de células T reguladoras. No caso do diabetes experimental, foi constatado que os helmintos podem induzir células T reguladoras tanto no baço (Zaccone et al., 2009) quanto no local da inflamação (pâncreas) (Hübner et al., 2008). Por outro lado, Liu et al., (2009) mostraram que apesar da infecção helmíntica induzir elevada frequência de células T reguladoras nos linfonodos (mesentéricos e pancreáticos), a proteção do diabetes ocorreu por mecanismos independentes destas células. Ainda neste estudo, os autores observaram um aumento da ativação de macrófagos pela via alternativa. O conceito de macrófagos alternativamente ativados é relativamente recente e surgiu por comparação com a via clássica de ativação destas células. Na via clássica, os macrófagos são ativados por IFN-γ o que resulta em resposta inflamatória e microbicida acentuada para patógenos intracelulares. Por outro lado, a via alternativa de ativação é induzida por IL-4 e IL-13. Este tipo de ativação é identificado pela presença de macrófagos com determinadas características, como expressão de elevados níveis de arginase-1, cadeia α do receptor para IL-4 e CD206 e pela contribuição destas células no controle de infecções parasitárias (Anthony et al., 2007; Kreider et al., 2007). Esse mecanismo foi evidenciado em um trabalho bastante recente demonstrando o efeito anti-diabético da *Taenia crassiceps* (Espinoza-Jiménez et al., 2010).

1.5 Células T reguladoras

A existência de uma subpopulação de células T com atividade supressora foi descrita inicialmente na década de 70 por Gershon et al. (1972). Entretanto, dificuldades na caracterização destas células, bem como a ausência de marcadores específicos, fizeram com que as pesquisas envolvendo as células T supressoras fossem abandonadas no final dos anos 80. Em 1995, Sakaguchi et al., descreveram uma subpopulação de células T CD4+ que expressavam também a proteína de superfície CD25 (cadeia α do receptor para a IL-2). Essas células apresentavam uma importante atividade regulatória e eram capazes de controlar as células T autorreativas *in vivo*. A partir deste período, estas células foram renomeadas e passaram a ser chamadas de células T reguladoras. As células T reguladoras $CD4^+CD25^+$ representam aproximadamente 5-10% dos linfócitos T $CD4^+$ periféricos de humanos e de camundongos e podem atuar tanto no controle da imunidade inata quanto da imunidade adaptativa (Shevach, 2002).

Embora o marcador CD25 seja muito utilizado para análise de células T $CD4^+$ reguladoras, é importante lembrar que todas as células T ativadas também expressam essa proteína de superfície. Além desse marcador, as células T reguladoras expressam constitutivamente outras moléculas como CD45RB, CTLA-4 (*Cytotoxic T Lymphocyte Antigen 4*), GITR (*Glucocorticoid-induced TNF-Receptor-related protein*), LAG-3 (*lymphocyte-activation gene 3*), CD127, OX40, receptor 4 de folato, CD39 e CD73 (Cools et al., 2007; Vignali et al., 2008; Corthay, 2009; Workman et al., 2009). Assim como o CD25, esses marcadores também não são exclusivos dessa subpopulação. As células T reguladoras CD4+CD25+ também expressam o gene regulador Foxp3, que codifica o fator de

transcrição Foxp3, que é fundamental para o desenvolvimento e função desta linhagem (Fontenot et al., 2003; Hori et al., 2003). O Foxp3 regula a transcrição de genes que codificam algumas proteínas, como por exemplo, CTLA-4 e CD39 (Ozdemir et al., 2009). A constatação de que camundongos e humanos com mutações no gene Foxp3 desenvolvem uma doença autoimune linfoproliferativa grave (Bennett et al., 2001; Brunkow et al., 2001) ressalta a importância desse fator como fundamental no desenvolvimento destas células. Apesar do Foxp3 ser considerado o melhor marcador para células T reguladoras de camundongos atualmente, ele parece não ser o marcador mais indicado para identificação dessas células em humanos. Estudos recentes indicam que em humanos o Foxp3 também pode ser expresso por linfócitos T CD4 ativados (Bacchetta et al., 2007; Roncarolo & Gregori, 2008).

As células T reguladoras são classificadas como naturais ou adaptativas. As células T reguladoras naturais (células TC4+CD25+Foxp3+) são originadas no timo, enquanto as induzidas são geradas na periferia. A conversão de células TCD4 *naïve* em células TCD4+Foxp3+ que ocorre na periferia requer contato com o antígeno (Mucida et al., 2009). As células T reguladoras induzidas são divididas em dois grupos: as Tr1, produtoras de IL-10 e as Th3, produtoras de TGF-β. Segundo Workman et al. (2009), a expressão de Foxp3 é observada nas células Th3 mas não nas células Tr1.

Os mecanismos de supressão empregados pelas células T reguladoras podem ser divididos em quatro tipos distintos (Vignali et al., 2008; Workman et al., 2009). O primeiro é a supressão mediada por citocinas inibitórias, como IL10, TGF-β e IL-35. O segundo é supressão mediada por citólise. A lise mediada por

células T reguladoras é dependente de granzima A e perforina nos humanos e de granzima B em camundongos. O terceiro mecanismo é a supressão por distúrbio metabólico. Além de sofrer apoptose pela privação de IL-2, as células T reguladoras também expressam altos níveis de CD39 e CD73, que liberam adenosina na região pericelular. Essa molécula atua nas células T efetoras por meio do receptor de adenosina 2A, determinando uma alteração metabólica que inibe a função dessas células. O último mecanismo tem como alvo de ação as células dendríticas (DC). Neste caso, as células T reguladoras induzem a produção de indoleamina 2,3-dioxigenase (IDO) pelas DC. Esta enzima causa imunossupressão por determinar degradação de triptofano e produção de metabólitos pró-apoptóticos. Esse mecanismo depende ainda da interação de CTLA-4 com CD80/CD86 presentes nas células T reguladoras e DC, respectivamente. Além disso, a maturação das DC pode ser suprimida pela interação entre LAG3 (presente nas células T reguladoras) com o MHC de classe II.

As células T reguladoras desempenham um papel fundamental na regulação da resposta imune. Elas estão envolvidas na supressão de doenças alérgicas e autoimunes, na prevenção de resposta imunopatológicas em infecções intestinais e em mecanismo de indução da tolerância materno-fetal (Maloy et al., 2005; Aluvihare & Betz, 2006; Workman et al., 2009). No entanto, a ativação dessas células nem sempre é benéfica ao hospedeiro. Isso pode ser observado, por exemplo, pela atividade supressora dessas células na resposta imune antitumoral (Sakaguchi et al., 2001).

1.6 Racional do projeto

A caracterização de uma estratégia profilática e/ou terapêutica na EM é essencial, já que não há cura para essa doença. No contexto da hipótese da higiene, vários trabalhos mostram que o contato com antígenos ambientais pode diminuir ou até impedir as manifestações clínicas da EM. Assim, a proposta desse projeto foi investigar o efeito da infecção prévia com *S. venezuelensis* nas manifestações clínicas da EAE no rato Lewis. Na primeira etapa caracterizamos a interação entre o parasita e o hospedeiro e avaliamos a resposta imunológica desencadeada por esse helminto. Na segunda etapa, avaliamos o efeito de uma ou várias infecções com *S. venezuelensis* na evolução da EAE.

Nossa hipótese inicial de trabalho previa que a infecção prévia com *S. venezuelensis* impediria o desenvolvimento parcial ou total da EAE através da modulação da resposta imune por polarização no sentido Th2 ou pela indução de células T reguladoras.

2. Referências

2. REFERÊNCIAS*

Aluvihare VR, Betz AG. The role of regulatory T cells in alloantigen tolerance. Immunol Rev. 2006;212:330-43.

Anthony RM, Rutitzky LI, Urban JF Jr, Stadecker MJ, Gause WC. Protective immune mechanisms in helminth infection. Nat Rev Immunol. 2007;7:975-87.

Aranami T, Yamamura T. Th17 Cells and Autoimmune Encephalomyelitis (EAE/MS). Allergol Int. 2008;57:115-20.

Awasthi A, Murugaiyan G, Kuchroo VK. Interplay between effector Th17 and regulatory T cells. J Clin Immunol. 2008;28:660-70.

Bacchetta R, Gambineri E, Roncarolo MG. Role of regulatory T cells and FOXP3 in human diseases. J Allergy Clin Immunol. 2007;120:227-35.

Back JF. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med. 2002; 347: 911-20.

Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet. 2001;27:20-1.

Bernard CC, Johns TG, Slavin A, Ichikawa M, Ewing C, Liu J, et al. Myelin oligodendrocyte glycoprotein: a novel candidate autoantigen in multiple sclerosis. J Mol Med. 1997;75:77-88.

* International Committee of Medical Journal Editors. Uniform Requirements for Manuscripts Submitted to Biomedical Journal: sample references.[homepage on the Internet]. Bethesda: U.S. National Library of Medicine; 2003[last updated 2003 July 09; cited 2005 Jun 01]. Available from:http://www.nlm.nih.gov/bsd/uniform_requirements.html National Library of Medicine. List of journals indexed in Index Medicus. Washington, 2003. 240p.

Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet. 2001; 27:68-73.

Cooke A, Tonks P, Jones FM, O'Shea H, Hutchings P, Fulford AJ, et al. Infection with *Schistosoma mansoni* prevents insulin dependent diabetes mellitus in non-obese diabetic mice. Parasite Immunol. 1999; 21:169-76.

Cools N, Ponsaerts P, Van Tendeloo VF, Berneman ZN. Regulatory T cells and human disease. Clin Dev Immunol. 2007; doi: 10.1155/2007/89195.

Corthay A. How do regulatory T cells work? Scand J Immunol. 2009; 70:326-36.

Costa-Cruz JM. *Strongyloides stercoralis*. In: Neves DP, editor. Parasitologia humana. Rio de Janeiro: Atheneu; 2005. p.275-84.

de Veer MJ, Kemp JM, Meeusen EN. The innate host defence against nematode parasites. Parasite Immunol. 2007;29:1-9.

Elloso MM, Phiel K, Henderson RA, Harris HA, Adelman SJ. Suppression of experimental autoimmune encephalomyelitis using estrogen receptor-selective ligand. J Endocrinol. 2005;185:243-52.

Espinoza-Jiménez A, Rivera-Montoya I, Cardenas-Arreola R, Morán L, Terrazas LI. *Taenia crassiceps* infection attenuates multiple low-dose streptozotocin-induced diabetes. J Biomed Biotechnol. 2010; doi:10.1155/2010/850541.

Fleming J, Fabry Z. The hygiene hypothesis and multiple sclerosis. Ann Neurol. 2007;61:85-9.

Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4(+)CD25(+) regulatory T cells. Nat Immunol. 2003;4:330-6.

Furuzawa-Carballeda J, Vargas-Rojas MI, Cabral AR. Autoimmune inflammation from the Th17 perspective. Autoimmun Rev. 2007;6:169-75.

Genta RM. Global prevalence of strongyloidiasis: critical review with epidemiologic insights into the prevention of disseminated disease. Rev Infect Dis. 1989;11:755-67.

Gershon RK, Cohen P, Hencin R, Liebhaber SA. Suppressor T cells. J Immunol. 1972;108:586-90.

Gold R, Linington C, Lassmann H. Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. Brain. 2006;129:1953-71.

Gold R, Wolinsky JS. Pathophysiology of multiple sclerosis and the place of teriflunomide. Acta Neurol Scand. 2010;doi:10.1111/j.1600-0404.2010.01444.x.

Gruden-Movsesijan A, Ilic N, Mostarica-Stojkovic M, Stosic-Grujicic S, Milic M, Sofronic-Milosavljevic Lj. Trichinella spirallis: Modulation of experimental autoimmune encephalomyelitis in DA rats. Exp Parasitol. 2008;118:641-7.

Haas J, Hug A, Viehover A, Fritzsching B, Falk CS, Filser A, et al. Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. Eur J Immunol. 2005;35:3343–52.

Helmby H, Bickle Q. Immune modulation by helminth infections. Parasite Immunol. 2006; 28:479-81.

Helmby H. Helminths and our immune system: Friend or foe? Parasitol Int. 2009; 58:121-7.

Hohlfeld R. Multiple sclerosis: Human model for EAE? Eur J Immunol. 2009;39: 1991-2058.

Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003;299:1057-61.

Huan J, Culbertson N, Spencer L, Bartholomew R, Burrows GG, Chou YK, et al. Decreased FOXP3 levels in multiple sclerosis patients. J Neurosci Res. 2005; 81:45–52.

Hübner MP, Stocker JT, Mitre E. Inhibition of type 1 diabetes in filarial-infected non-obese diabetic mice is associated with a T helper type 2 shift and induction of Foxp3+ regulatory T cells. Immunology. 2008;127:512-22.

Imitola J, Chitnis T, Khoury SJ. Cytokines in multiple sclerosis: from bench to bedside. Pharmacol Ther. 2005;106:163-77.

Ishizu T, Osoegawa M, Mei FJ, Kikuchi H, Tanaka M, Takakura Y, et al. Intrathecal activation of the IL-17/IL-8 axis in opticospinal multiple sclerosis. Brain. 2005;128:988-1002.

Klinkert WE, Kojima K, Lesslauer W, Rinner W, Lassmann H, Wekerle H. TNF-alpha receptor fusion protein prevents experimental auto-immune encephalomyelitis and demyelination in Lewis rats: an overview. J Neuroimmunol. 1997;72:163-8.

Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, et al. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J Immunol. 2006;177:566-73.

Kreider T, Anthony RM, Urban JF Jr, Gause WC. Alternatively activated macrophages in helminth infections. Curr Opin Immunol. 2007;19:448-53.

Lafaille JJ, Nagashima K, Katsuki M, Tonegawa S. High incidence of spontaneous autoimmune encephalomyelitis in immunodeficient anti-myelin basic protein T cell receptor transgenic mice. Cell. 1994;78:399-408.

La Flamme AC, Ruddenklau K, Backstrom BT. Schistosomiasis decreases central nervous system inflammation and alters the progression of experimental autoimmune encephalomyelitis. Infect Immun. 2003;71:4996-5004.

Lawrence CE. Is there a common mechanism of gastrointestinal nematode expulsion? Parasite Immunol. 2003;25:271-81.

Link H, Xiao B. Rat models as tool to develop new immunoterapies. Immunol Rev. 2001;184:117-28.

Liu Q, Sundar K, Mishra PK, Mousavi G, Liu Z, Gaydo A, et al. Helminth infection can reduce insulitis and type 1 diabetes through CD25- and IL-10-independent mechanisms. Infect Immun. 2009;77:5347-58.

Lock C, Hermans G, Pedotti R, Brendolan A, Schadt E, Garren H, et al. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. Nat Med. 2002;8:500-8.

Lucchinetti C, Brück W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: implication for the pathogenesis of demyelination. Ann Neurol. 2000;47:707-17.

Maizels RM, Balic A, Gomez-Escobar N, Nair M, Taylor MD, Allen JE. Helminth parasites-master of regulation. Immunol Rev. 2004;201:89-116.

Maloy KJ, Antonelli LR, Lefevre M, Powrie F. Cure of innate intestinal immune pathology by CD4+CD25+ regulatory T cells. Immunol Lett. 2005;97:189-92.

Marques KB, Scorisa JM, Zanon R, Freria CM, Santos LMB, Damasceno BP, et al. The immunomodulator glatiramer acetate influences spinal motoneuron plasticity during the course of multiple sclerosis in an animal model. Braz J Med Biol Res. 2009;42:179-88.

McGeachy MJ, Stephens LA, Anderton SM. Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. J Immunol. 2005;175:3025–32.

Mucida D, Park Y, Cheroutre H. From the diet to the nucleus: vitamin A and TGF-beta join efforts at the mucosal interface of the intestine. Semin Immunol. 2009;21:14-21.

Namer IJ, Steibel J, Klinguer C, Trifilieff E, Mohr M, Poulet P. Magnetic resonance imaging of PLP-induced experimental allergic encephalomyelitis in Lewis rats. J Neuroimmunol. 1998;92:22-8.

Ozdemir C, Akdis M, Akdis CA. T regulatory cells and their counterparts: masters of immune regulation. Clin Exp Allergy. 2009;39:626-39.

Peron JP, Yang K, Chen ML, Brandao WN, Basso AS, Commodaro AG, et al. Oral tolerance reduces Th17 cells as well as the overall inflammation in the central nervous system of EAE mice. J Neuroimmunol. 2010; 227:10-7. Epub 2010 jul 2.

Roncarolo MG, Gregori S. Is FOXP3 a bona fide marker for human regulatory T cells? Eur J Immunol. 2008;38:925-7.

Rook GAW, Adams V, Hunt J, Palmer R, Martinelli R, Brunet LR. Mycobacteria and other environmental organisms as immunomodulators for immunoregulatory disorders. Springer Semin Immunol. 2004;25:237-55.

Rook GA. The hygiene hypothesis and the increasing prevalence of chronic inflammatory disorders. Trans R Soc Trop Med Hyg. 2007;101:1072-4.

Rudick RA. Esclerose Múltipla e patologias desmielinizantes do sistema nervoso central. In: Goldman L, Ausello D, editors. Tratado de Medicina Interna. Rio de Janeiro: Elsevier; 2005. p.2713-21.

Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 1995;155:1151-64.

Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, et al. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. Immunol Rev. 2001;182:18-32.

Sewell D, Qing Z, Reinke E, Elliot D, Weinstock J, Sardor M, et al. Immunomodulation of experimental autoimmune encephalomyelitis by helminth ova immunization. Int Immunol. 2003;15: 59-69.

Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol. 2002; 2:389-400.

Siddiqui AA, Berk SL. Diagnosis of *Strongyloides stercorallis* infection. Clin Infect Dis. 2001; 33:1040-7.

Siddiqui AA, Berk SL. Strongyloidiasis. Curr Treat Option Infect Dis. 2003;5:283-9.

Silberberg DH. Doenças desmielinizantes. In: Wyngaarden JB, Smith LH, Bennett JC, editors. Tratado de medicina interna. Rio de Janeiro: Medsi;1992. p.2243-9.

Sospedra M, Martin R. Immunology of multiple sclerosis. Annu Rev Immunol. 2005;23:683-747.

Steinman L. Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. Cell. 1996;85:299-302.

Strachan DP. Hay fever, hygiene, and household size. BMJ. 1989;299:1259-60.

Swanborg RH. Experimental autoimmune encephalomyelitis in the rat: lessons in T-cell immunology and autoreactivity. Immunol Rev. 2001;184:129-35.

Tang Q, Henriksen KJ, Bi M, Finger EB, Szot G, Ye J, et al. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. J Exp Med. 2004;199:1455-65.

Tindall N, Wilson PA. Criteria for a proof of migration routes of immature parasites inside hosts exemplified by studies of *Strongyloides ratti* in the rat. Parasitology. 1988;96:551-63.

Urquhart GM, Armour J, Duncan JL, Dunn AM, Jennings FW. Parasitologia veterinária. Rio de Janeiro: Guanabara Koogan S.A.; 1998. 273p.

Varriale S, Béraud E, Barbaria J, Galibert R, Bernard D. Regulation of experimental autoimmune encephalomyelitis: inhibition of adoptive experimental autoimmune encephalomyelitis by 'recovery-associated suppressor cells'. J Neuroimmunol. 1994;53:123-31.

Venken K, Hellings N, Hensen K, Rummens JL, Medaer R, D'Hooghe M, et al. Secondary progressive in contrast to relapsing-remitting multiple sclerosis patients show a normal CD4+CD25+ regulatory T-cell function and FOXP3 expression. J Neurosci Res. 2006;83:1432-46.

Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J Exp Med. 2004;199:971-9.

Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nat Rev Immunol. 2008;8:523-32.

Workman CJ, Szymczak-Workman AL, Collison LW, Pillai MR, Vignali DA. The development and function of regulatory T cells. Cell Mol Life Sci. 2009;66:2603-22.

World Health Organization [Internet]. Neurological disorders a public health approach. Geneva: WHO; 2006 [cited 2008 out 06]. Available from: http://www.who.int/mental_health/neurology/chapter_3_a_neuro_disorders_public_h_challenges.pdf.

Zaccone P, Burton O, Miller N, Jones FM, Dune DW, Cooke A. *Schistosoma mansoni* egg antigens induce Treg that participate in diabetes prevention in NOD mice. European J Immunol. 2009;39:1098-107.

Zheng X, Hu X, Zhou G, Lu Z, Qiu W, Bao J, et al. Soluble egg antigen from *Schistosoma japonicum* modulates the progression of chronic progressive experimental autoimmune encephalomyelitis via TH2-shift response. J Neuroimmunol. 2008;194:107-14.

Zorzella SFG, Seger J, Martins DR, Pelizon AC, Sartori A. Resistance to experimental autoimmune encephalomyelitis development in Lewis rats from conventional animal facility. Mem Inst Oswaldo Cruz. 2007;102:931-6.

3. Objetivos

3. OBJETIVOS

3.1 Objetivo Geral

Caracterizar a infecção por *Strongyloides venezuelensis* em ratos Lewis e avaliar se a mesma modula as características clínicas, imunológicas e histopatológicas da EAE nestes animais.

3.2 Objetivos Específicos

1- Acompanhar a cinética da infecção por *S. venezuelensis* em ratos Lewis para determinar as fases aguda e de recuperação e para avaliar os padrões de resposta imune nestas duas fases;

2- Avaliar o efeito da infecção primária por *S. venezuelensis* (fase aguda e de recuperação) no desenvolvimento (características clínicas, imunológicas e histopatológicas) da EAE;

3- Avaliar o efeito de múltiplas infecções por este helminto no desenvolvimento da EAE;

4- Determinar a frequência de células T reguladoras no baço e nos linfonodos (inguinais e poplíteos) após infecção única (fase aguda e de recuperação) ou múltiplas infecções com *S. venezuelensis*.

4. Resultados e Discussão

4. RESULTADOS E DISCUSSÃO

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

4.1 Artigo científico I: Recovery from *Strongyloides venezuelensis* infection in Lewis rats is associated with a strong Th2 response

4.2 Artigo científico II: *Strongyloides venezuelensis* infection did not prevent full experimental autoimmune encephalomyelitis evolution: implications for hygiene hypothesis

4.3 Artigo científico III: Experimental autoimmune encephalomyelitis evolution was not modified by multiple infections with *Strongyloides venezuelensis*

4.1 Artigo Científico I

Parasite Immunology

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Brief Definitive Report

Recovery from *Strongyloides venezuelensis* infection in Lewis rats is associated with a strong Th2 response

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SUMMARY

In this study, we investigated the characteristics of the infection and subsequent immunity induced by Strongyloides venezuelensis in *Lewis rats. Animals were infected with 4000 L3 of* S. venezuelensis *and number of eggs per gram of faeces indicated an acute phase around day 8 and a recovery phase around day 32 after infection. A strong Th2 polarization during recovery phase was ascertained by a significant increase in IgG1 and IgE compared with that in the acute period. A shift in the cytokine profile confirmed these findings. A predominant production of IFN-γ during the acute phase was followed by IL-10 production during recovery. Together these findings show that experimental infection of Lewis rats with* S. venezuelensis *presents a kinetics of parasite establishment and immunity similar to that described in other models of helminthic infection.*

Keywords *Lewis rats,* Strongyloides venezuelensis, *Th2 cells*

INTRODUCTION

Strongyloidiais is a parasitosis caused by *Strongyloides stercoralis.* Infection of rodents with *Strongyloides venezuelensis*, a gastrointestinal nematode that naturally infects wild rats, is an experimental model to study Strongyloidiais. The immune response to *Strongyloides spp.* is characterized by the production of Th2-type cytokines, such as IL-3, IL-4, IL-5 and IL-10 (1–3), increased levels of serum IgE (4) and IgG1 (3,5), tissue and blood eosinophilia (6) and intestinal mastocytosis (7). However, different kinds of immune response can be observed with different strains of *Strongyloides spp.* Recently, a study comparing two heterologous strains of *S. venezuelensis* showed that the strains differed in the stimulation of humoral immune response (3). The dynamics of *S. venezuelensis* infection, especially concerning the kinetics of egg elimination, the induced immunity and the tissue migration route, are already known in Wistar rats (8,9) and in several mice strains (10), but not in Lewis rats. Thus, the aim of this study was to determine the kinetics of *S. venezuelensis* infection and to characterize the immune specific response during acute and recovery phases in Lewis rats.

MATERIALS AND METHODS

Adult female Lewis rats were allocated into four experimental groups containing five animals each. Two groups were used as controls and the others were infected with 4000 *S. venezuelensis* infective filiform larvae by subcutaneous route. At the 8th day after infection (acute phase), one control group and one infected group were euthanized. The other groups were euthanized at the 32nd day after infection (recovery phase). Larvae were obtained as previously described elsewhere (9). Infection intensity was

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determined by counting the number of eggs per gram of faeces (EPG) daily using a modified Cornell McMaster method (11) and by counting the number of parthenogenetic female worms found in the first third portion of the small intestine. Eosinophils, specific antibody levels, total IgE and cytokine production were evaluated at the 8th and 32nd day after infection. Parasite-specific IgG1 and IgG2b were estimated by ELISA. Parasite antigen preparation and ELISA methodology were performed according to the procedure described by Fernandes *et al.*, 2008 (12). Total IgE was determined in blood samples diluted 1 : 10 also by ELISA according to the manufacturer's instructions (Immunology Consultants Laboratory, Inc; Newberg, OR, USA). The sensitivity of this assay was 0·5 ng/mL. Spleen and lymph node (popliteal + inguinal) cells were collected and adjusted to 5×10^6 cells/mL and $2{\cdot}5 \times 10^6$ cells/mL, respectively. Cells were cultured in RPMI supplemented with 10% FCS, 2 mM of L-glutamine and 40 mg/L of gentamicin, in the presence of 100 μg/mL of *S. venezuelensis* L3 antigen or 5 μg/mL of concanavalin A (ConA, Sigma; St. Louis, MO, USA). Cytokine levels were evaluated in culture supernatants collected 72 h later by ELISA according to the manufacturer's instructions (R & D Systems; Minneapolis, MN, USA). ELISA sensitivity for IFN-γ and IL-10 was 19 and 31 pg/mL, respectively. Data were expressed as mean ± SD. Comparisons between groups were made by Student's *t*-test for parameters with normal distribution and by Mann–Whitney test for parameters with nonnormal distribution. Statistical analysis was accomplished with SigmaStat for Windows v 3·5 (Systat Software Inc, San Jose, CA, USA).

RESULTS

Parasite eggs were detected in the faeces for the first time at day 6 of infection. Maximal egg number (42 300 EPG) was observed at day 8 post-infection and this period was referred to as acute phase. A second peak (21 300 EPG) was also observed at 11 days post-infection. From this period on, the egg number decreased steadily until day 21 when EPG varied from 0 to 100 (Figure 1a). This very low level of infection was detected until day 32 and was considered the recovery phase. As expected, a significantly higher number of parthenogenetic females was recovered at the acute phase in comparison with that of the recovery period (Figure 1b). Differences in antibody specific levels, eosinophil counts and cytokine production were observed by comparing these two phases. IgG1 (Figure 1c) and IgG2b (Figure 1d) specific levels were significantly higher in the acute phase compared with that in the noninfected control group. Production of specific IgG1 significantly increased during the recovery phase, whereas IgG2b levels remained similar to the levels reached during the acute phase. Total IgE was significantly more elevated in infected animals in comparison with that in the control ones in both the acute and recovery phases (Figure 1e). However, a significantly increased IgE level was observed at the recovery period comparing with that in the acute phase. Acute phase was also characterized by a significant increase in blood eosinophils (control = $0{\cdot}02 \times 10^6$/mL ($\pm 0{\cdot}04 \times 10^6$/mL), infected = $0{\cdot}24 \times 10^6$/mL ($\pm 0{\cdot}16 \times 10^6$/mL), $P < 0{\cdot}05$). IFN-γ induced by Con A or *S. venezuelensis* L3 antigen stimulation was evaluated in spleen cell cultures. IFN-γ levels stimulated by Con A were lower in infected animals, in both the acute and recovery phases (Figure 2b,f). However, a significant decrease was observed in splenic cell cultures during the recovery phase (Figure 2f). Specific stimulation with *S. venezuelensis* L3 antigen did not induce IFN-γ production by lymph node cells from the acute and recovery phases (data not shown). However, significantly higher levels of this cytokine were detected in splenic cell cultures during the acute phase (Figure 2a). Interestingly, IFN-γ concentration decreased to basal levels during the recovery phase (data not shown). Only cultures from lymph node cells showed differences in IL-10 production between infected and normal rats. No IL-10 was detected in cultures stimulated with specific antigen during the acute phase (not shown). However, there was IL-10 induction by specific stimulation during recovery (Figure 2d). On the other hand, IL-10 levels induced by Con A were reduced in both phases, being statistically significant only in the acute period of infection (Figure 2c).

DISCUSSION

This investigation was carried out to establish if inoculation of *S. venezuelensis* in Lewis rats triggers an infection and a subsequent immunity similar to that described in other rodents and also in human infections by *S. stercoralis.* In Lewis rats subcutaneously infected with 4000 L3, parasite eggs were detected in the faeces for the first time at day 6 post-infection, but the maximal egg number was observed at day 8 post-infection. A second peak in the egg number was observed at 11 days post-infection, which decreased steadily thereafter. This kinetics in egg number coincided with the amount of parthenogenetic females recovered from the small intestine. The highest amount was detected during the acute phase, whereas a very low number was found at the recovery phase. Considering these findings, the acute phase occurred around the 8th day and the recovery phase around the 32nd day of infection. This infection kinetics indicates a profile that is similar to infections caused by *S. venezuelensis* (8) and also by *S. ratti* in Wistar rats (13). Immunity against *Strongyloides spp.* is characterized by a

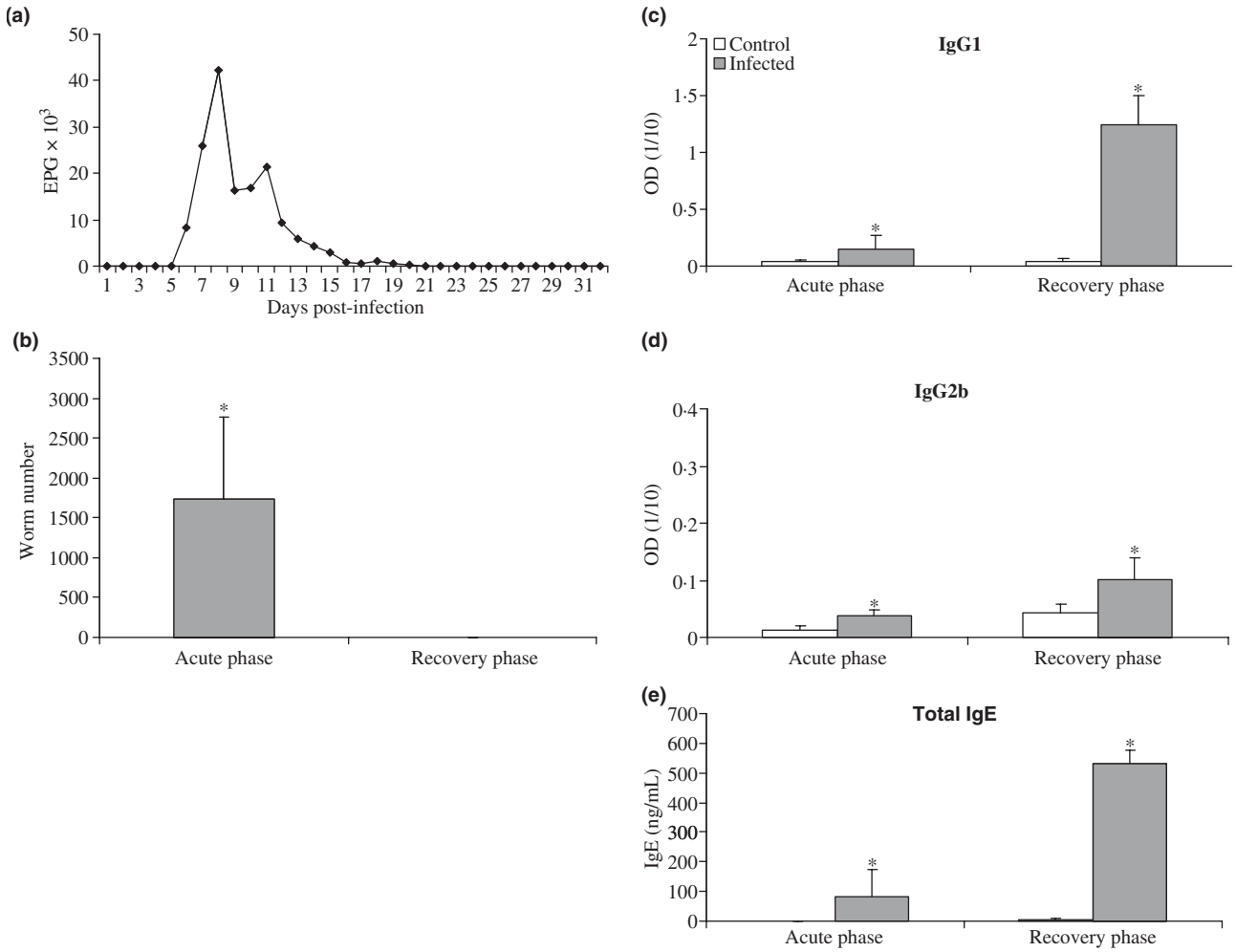

Figure 1 Kinetics of *Strongyloides venezuelensis* infection and induced humoral immunity. Eggs per gram of faeces (a) and number of female adult worms recovered from the small intestine (b). In the acute phase, the number of female adult worms varied from 97 to 2979. Seric levels of specific IgG1 (c), IgG2b (d) and total IgE (e) in infected Lewis rats. The number of eggs per gram of faeces was determined in a faecal pool. Results are expressed as mean ± SD of five animals per group. $*P < 0{\cdot}05$.

typical Th2 pattern with a predominant production of IL-3, IL-4, IL-5 and IL-10 (1,3). Elevated levels of IgG1, IgE, eosinophils and intestinal mastocytosis have been abundantly described (3–7). In this study, both IgG1 and IgG2b specific antibodies were significantly elevated at the acute phase. However, a much higher increase in IgG1 concentration already suggested a stronger Th2 polarization at this period. This tendency became evident at the recovery phase when IgG1 but not IgG2b presented a significant increase compared with that in the acute infection. These results are similar to the ones described in mice infected with *S. venezuelensis* (3) and Wistar rats infected with *S. ratti* (5). Wilkes *et al.*, 2007 (5), even called attention for a finding that was very similar to our results, i.e. that there was a significant elevation of IgG1 specific levels during the recovery phase compared with that at the acute phase. They also stressed the fact that IgG1 higher levels coincided with worm elimination. Total IgE was significantly elevated in both the acute and recovery phases. Interestingly, IgE levels were significantly higher in the recovery phase compared with that at the acute period of infection. Although IgE levels have been a hallmark in helminthic infections, its contribution to control these parasites has been, at least, controversial (14). Elevated IgE levels have been reported in both *S. venezuelensis* and *S. ratti* experimental infections (5,12). A significant rise in eosinophil number was detected in Lewis rats during the acute phase of *S. venezuelensis* infection. These counts returned to basal levels during the recovery phase. These findings are in accordance with the literature reports that showed increased number of blood eosinophils following helminthic infections (15). Their subsequent disappearance from the blood has been attributed to migration to the site of

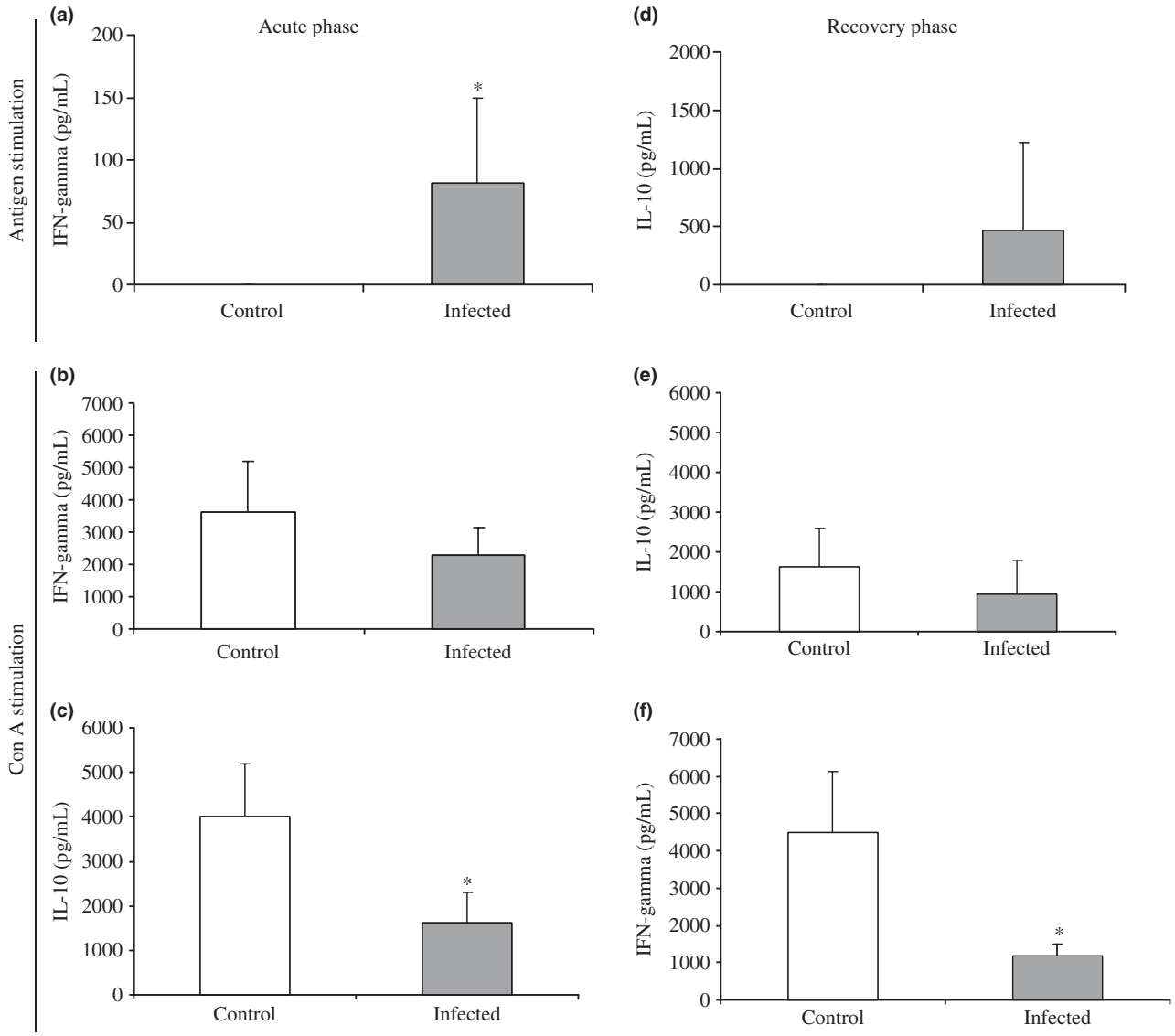

Figure 2 IFN-γ and IL-10 production in infected Lewis rats: acute phase (a, b and c) and recovery phase (d, e and f). Spleen (a, b and f) and lymph node (c, d and e) cells were stimulated with Con A or *S. venezuelensis* L3 antigen. Results are expressed as mean ± SD of five animals per group. $*P < 0{\cdot}05$.

the infection where they degranulate, releasing eosinophil secondary granule proteins (16). Production of cytokines by secondary lymphoid organ cultures stimulated with specific antigens and Con A was used to characterize cellular immunity. Considering IFN-γ induction by specific stimuli, a significant production was detected during the acute phase but not at the recovery phase. The opposite happened with IL-10 production, i.e. absence of this cytokine at the acute period and presence of detectable levels during the recovery phase. Analysing these data together with antibody levels (IgG subclasses and IgE), we could suggest that an initial mixed pattern (Th1/Th2) at the acute phase was followed predominantly by a Th2 polarization during the recovery phase. Production of IFN-γ and IL-10 stimulated by polyclonal activation with Con A showed a similar pattern, i.e. a general decreased production of these mediators by cultures of spleen and lymph nodes. A theoretical explanation for this finding is that T lymphocytes capable of producing these cytokines migrate from lymphoid organs to the places of temporary (lungs) or final (intestine) establishment of the worm. This possibility is supported by recent literature reports (3,8,17). Together these results show that experimental inoculation of Lewis rats with *S. venezuelensis* triggers an infection that is similar in terms of kinetics of parasite

establishment and immunity to experimental strongyloidiasis in other rodents and also in human *S. stercoralis* infection.

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REFERENCES

1 Kobayashi T, Tsuchiya K, Hara T, *et al.* Intestinal mast cell response and mucosal defence against *Strongyloides venezuelensis* in interleukin-3 hyporesponsive mice. *Parasite Immunol* 1998; **20**: 279–284.
2 Machado ER, Ueta MT, Lourenço EV, *et al.* Leukotrienes play a role in the control of parasite burden in murine Strongyloidiasis. *J Immunol* 2005; **175**: 3892–3899.
3 Machado ER, Ueta MT, Lourenço EV, Anibal FF, Roque-Barreira MC & Faccioli LH. Comparison of immune response in mice infected with different strains of *Strongyloides venezuelensis*. *Parasite Immunol* 2007; **29**: 549–557.
4 Korenaga M, Nawa Y & Tada I. IgE response in *Strongyloides ratti*-infected rats with special reference to the life cycle of the parasite. *Z Parasitenk* 1986; **72**: 213–220.
5 Wilkes CP, Bleak C, Paterson S & Viney ME. The immune response during a *Strongyloides ratti* infection of rats. *Parasite Immunol* 2007; **29**: 339–346.
6 Ovington KS, Mckie K, Matthaei KI, Young IG & Behm CA. Regulation of primary *Strongyloides ratti* infections in mice: a role for interleukin-5. *Immunology* 1998; **95**: 488–493.
7 Tegoshi T, Okada M, Nishida M & Arizono N. Early increase of gut intraepithelial mast cell precursors following *Strongyloides venezuelensis* infection in mice. *Parasitology* 1997; **114**: 181–187.
8 Silveira MR, Nunes KP, Cara DC, *et al.* Infection with *Strongyloides venezuelensis* induces transient airway eosinophilic inflammation, an increase in immunoglobulin E, and hyperresponsiveness in rats. *Infect Immun* 2002; **70**: 6263–6272.
9 Nakai ES & Amarante AFT. Infecção experimental de camundongos (Mus musculus) e ratos (Rattus norvegicus) com *Strongyloides venezuelensis*. *Rev Bras Parasitol Vet* 2001; **10**: 1–6.
10 Amarante AFT & Oliveira-Siqueira TCG. *Strongyloides venezuelensis* infection susceptibility of seven inbred strains of mice. *Arq Bras Med Vet Zootec* 2002; **54**: 273–278.
11 Gordon HM & WhitlocK HV. A new technique for couting nematode eggs in sheep faeces. *J Coun Scient Ind Res* 1939; **12**: 50–52.
12 Fernandes A, Pereira ATM, Eschenazi PD, *et al.* Evaluation of the immune response against *Strongyloides venezuelensis* in antigen-immunized or previously infected mice. *Parasite Immunol* 2008; **30**: 139–149.
13 Kimura E, Shintoku Y, Kadosaka T, Fujiwara M, Kondo S & Itoh M. A second peak of egg excretion in *Strongyloides ratti*-infected rats: its origin and biological meaning. *Parasitology* 1999; **119**: 221–226.
14 Cooper PJ, Ayre G, Martin C, Rizzo JA, Ponte EV & Cruz AA. Geohelminth infections: a review of the role of IgE and assessment of potential risks of anti-IgE treatment. *Allergy* 2008; **63**: 409–417.
15 Ganley-Leal LM, Mwinzi PN, Cetre-Sossah CB, *et al.* Correlation between eosinophils and protection against reinfection with *Schistosoma mansoni* and the effect of human immunodeficiency virus type 1 coinfection in humans. *Infect Immunol* 2006; **74**: 2169–2176.
16 Antony RM, Rutitzky LI, Urban JF Jr, Stadecker MJ & Gause WC. Protective immune mechanisms in helminth infection. *Nat Rev Immunol* 2007; **7**: 975–987.
17 Negrão-Corrêa D, Souza DG, Pinho V, Barsante MM, Souza ALS & Teixeira MM. Platelet-activating factor receptor deficiency delays elimination of adult worms but reduces fecundity in *Strongyloides venezuelensis*-infected mice. *Infect Immun* 2004; **72**: 1135–1142.

4.2 Artigo Científico II

Strongyloides venezuelensis **infection did not prevent full experimental autoimmune encephalomyelitis evolution: implications for hygiene hypothesis**

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Abstract

Prevalence of allergic and autoimmune pathologies is clearly increasing in developed countries. This has been attributed to a decreased exposure to certain microorganisms and been referred as hygiene hypothesis. Epidemiological and experimental evidences are accumulating in support of this hypothesis. In this study we evaluated if a previous infection with *Strongyloides venezuelensis* would alter the progression of experimental autoimmune encephalomyelitis (EAE) in Lewis rats. The animals were initially infected with 4000 L3 infective larvae of *S. venezuelensis* by subcutaneous route. Encephalomyelitis was then induced during acute or recovery phases of this infection by immunization with myelin emulsified with Complete Freund Adjuvant plus *Mycobacterium butyricum*. Former infection with this worm was not able to change EAE progression. Neither clinical, weight and clinical score, nor histopathological differences were observed. Cytometric analysis with antibodies specific for CD4+CD25+Foxp3+ T cells indicated that the frequency of these cells in both, spleen and regional lymph nodes, was not changed during acute or chronic phases of the infection. This finding could partly explain the failure of this worm to protect against EAE progression. In spite of this, the infection generally downmodulated cytokine production induced by both, myelin and concanavalin A. Together these results demonstrated that *S. venezuelensis* infection was not able to modify EAE progression in Lewis rats. In the context of the hygiene hypothesis, these results reinforce the need for a comparative approach among different helminth species to identify the ones with immunoregulatory competence.

Keywords: hygiene hypothesis, experimental autoimmune encephalomyelitis, *S. venezuelensis*

1. Introduction

A steady increase in the incidence of autoimmune, allergic and inflammatory diseases has been reported in the most developed countries (Sawczenko et al., 2001; Back, 2002). This higher incidence has been attributed, at least partially, to a lack of contact between humans and certain environmental agents and has been denominated hygiene hypothesis. According to this hypothesis, organisms as lactobacillus, mycobacteria and helminths are endowed with immunoregulatory properties that are able to regulate immune-mediated diseases (Rook et al., 2004; Rook, 2007). At least three immunoregulatory mechanisms have been described in association with these agents as polarization of immune response towards Th2 (Zheng et al., 2008), induction of regulatory T cells (Zaccone et al., 2009) and more recently, induction of alternatively activated macrophages (Espinoza-Jiménez et al., 2010).

This theory has been supported by both, epidemiological and experimental data (Okada et al., 2010) being multiple sclerosis (MS) one of the autoimmune diseases that seems to be restrained by contact with these environmental agents. MS is a devastating disease that affects more than 1 million people worldwide by seriously compromising motor and sensory function through demyelination and axonal loss. Even though Th1 cells have been classically described as the main responsible for the central nervous system (CNS) destruction, emerging data suggest that Th17 cells also contribute to CNS autoimmunity in both, MS and the corresponding experimental autoimmune encephalomyelitis (EAE) model (El-behi et al., 2010).

Epidemiological data is suggestive of a protective effect of helminth infections on MS. MS increased frequency in the last century, as happened with other autoimmune diseases was coincidental with clear improved sanitation. This more hygienic lifestyle precluded exposure to helminths whose colonization was nearly universal prior to the 1930s (La Flame et al., 2004; Cabre et al., 2005; Elliot et al., 2007). This preventive effect of helminths in MS was more recently reinforced by experimental studies. For example, *Trichinella spirallis* infection and immunization with *Schistosoma* antigens were able to reduce EAE severity in Dark-Agouti rats and C57BL/6 mice, respectively (Sewell et al., 2003; Gruden-Movesijan et al., 2008; Zheng et al., 2008). Clinical trials also indicate that exposure to helminths reduces disease activity in patients with ulcerative colitis and Crohn's disease (Summers et al., 2005a; Summers et al., 2005b). Even though this kind of investigation was not conducted in MS patients recent reports indicate that parasite-infected MS patients, showed a significantly lower number of relapses, more discrete changes in disability scores and also significantly lower magnetic resonance imaging activity. Interestingly, these improved clinical manifestations were related to increased frequency of CD4+CD25+Foxp3+ T cells (Correale & Farez, 2007).

One of the most common helminth found in the human population is *Strongyloides stercoralis*. The estimated number of people colonized with this worm is around 100 million (Genta, 1989; Siddiqui & Berk, 2003). Much of the knowledge related to this parasite was obtained from rodent experimental infections with *S. venezuelensis* (Marra et al., 2010a; Marra et al., 2010b).

The current study was designed to evaluate the effect of a previous infection with *S. venezuelensis* on experimental autoimmune encephalomyelitis.

2. Materials and Methods

2.1 Animals

Female Lewis rats weighing 110-130 g and with 4-6 weeks old were purchased from the CEMIB (UNICAMP, Campinas, SP, Brazil). The animals received sterilized food and water *ad libitum* and were manipulated in compliance with the ethical guidelines adopted by the Brazilian College of Animal Experimentation, being the experimental protocol approved by the local Ethics Committee (protocol 607).

2.2 Experimental design

Rats were infected with *S. venezuelensis* infective third-stage larvae (L3). At the 8^{th} (acute phase) and 32^{nd} (recovery phase) days after infection animals were submitted to EAE induction by immunization with myelin. Animals were daily evaluated for weight loss and clinical score and euthanized during EAE recovery phase to assess the immune response and inflammatory infiltration at the CNS. Cytokine production and antibody levels were determined by ELISA. The frequency of $CD4^{+}CD25^{+}$Foxp3+ T cells obtained from regional lymph nodes and spleen were analyzed during acute and recovery phases of infection.

2.3 Parasite and infection

S. venezuelensis strain employed in this study was isolated from wild rats in 1980. It has been maintained in Wistar rats, routinely infected at the Parasitology Laboratory of the Univ Estadual Paulista (UNESP). For experimental infections,

infective third-stage larvae (L3) of *S. venezuelensis* were obtained from faecal cultures using sterilized horse manure as substrate. The cultures were incubated at 25 °C for 72 h and L3 were collected and concentrated by using a Baermann apparatus. Recovered larvae were washed in phosphate-buffered saline (PBS), the number of viable infective larvae was estimated under optical microscopy and 4000 L3 were subcutaneously inoculated in each animal.

2.4 EAE induction and evaluation

Rats were immunized with 25 μg of myelin basic protein (MBP - Sigma) emulsified with Complete Freund's Adjuvant containing 5 mg/mL of *Mycobacterium butyricum*. Animals were injected in the hind left footpad with 50 μL of the emulsion and daily evaluated for weight loss and clinical score. Signs of disease were graded as 0 (zero): no disease; 1: loss of tonicity in the distal portion of the tail; 2: total loss of tail tonicity; 3: hind limb weakness (partial paralysis); 4: complete hind limb paralysis and urinary incontinence and 5: moribund.

2.5 Quantification of inflammatory infiltrates

The histological analysis was performed during EAE recovery phase, i.e., 20 days after immunization with myelin. After euthanasia, brain and lumbar spinal cord samples were removed and fixed in a 10% solution of buffered formalin. Paraffin slides with 4-5 μm, were routinely stained with hematoxylin and eosin (H&E) and analyzed with a Nikon microscope. Quantitative evaluation of perivascular inflammatory infiltrates was performed in a computerized system for image analysis (Qwin Lite 3.1, Leica Microsystems, Wetzlar, Germany). The total section area of each brain and lumbar spinal cord was measured to avoid any inter-animal variance. Further, perivascular mononuclear infiltrate areas of whole

sections were assessed by point-counting morphometry, as described elsewhere (Bock et al., 2003). The values were expressed as μm^2 of mononuclear infiltrate per mm^2 of organ section ($\mu m^2/mm^2$).

2.6 Anti-myelin antibody levels

Serum samples were collected at the recovery EAE phase and tested by ELISA for the presence of antibodies against MBP. Briefly, plates were coated with 5 μg/mL of antigen in coating solution (Na_2CO_3 / $NaHCO_3$; pH 9.6) overnight at 4 ºC. Non-specific antibody binding was blocked by incubation with 0.05% Tween 20, 10% fetal calf serum in PBS (200 μL per well) for 1 h at 37 ºC. Subsequently the plates were incubated overnight at 4 ºC with dilutions (1/1000) of rat serum samples. For detection of specific serum IgG1 and IgG2b, the plates were incubated with class specific biotinylated mouse anti-rat antibodies (Oxford Biotechnology). Plates were then incubated for 30 min at room temperature with Strept AB (kit from Dako, Carpinteria) and revealed by adding H_2O_2 + OPD (Sigma). Color development was stopped with H_2SO_4 and optical density was measured at 492 nm.

2.7 IFN-γ, TNF-α and IL-10 production

Spleen and lymph node (popliteal + inguinal) cells were collected and adjusted to $5x10^6$ cells/mL and $2.5x10^6$ cells/ mL, respectively. Cells were cultured in complete RPMI medium (RPMI supplemented with 10% of fetal calf serum, 2 mM L-glutamine and 40 mg/L of gentamicin), in the presence of 10 μg/mL of myelin or 5 μg/mL of concanavalin A (ConA, Sigma, St. Louis, MO, USA). Cytokine levels were evaluated 72 h later in culture supernatants by ELISA according to manufacturer instructions (R & D Systems, Minneapolis, MN, USA).

Sensitivity of ELISA for IFN-γ, TNF-α and IL-10 was 19; 31 and 31 pg/mL, respectively.

2.8 Frequency of $CD4^+CD25^+Foxp3^+$ Tcells in *S. venezuelensis* infected rats

Spleen and lymph node (popliteal + inguinal) cells were collected and the red blood cells were lysed with Hank's buffer containing NH_4Cl. The cell suspension was washed once in RPMI 1640 and adjusted to $2.5x10^6$ cells. Cells were then incubated with 0.5 μg of fluorescein isothiocianate (FITC) anti-rat CD4 (clone OX35) and 0.25 μg of allophycocyanin (APC) anti-rat CD25 (clone OX39) for 20 min at room temperature. A staining for Foxp3 was then performed utilizing the phycoerythrin (PE) anti-mouse/rat Foxp3 Staining Set (eBioscience, San Diego, CA, USA) according to the manufacturer instructions. After incubation, the cells were fixed in paraformaldehyde 1%. The cells were analyzed by flow cytometry using the FACSCanto II (Becton Dickinson, San Jose, CA) and FlowJo software (TreeStar, Ashland, OR, EUA).

2.9 Statistical analysis

Data were expressed as mean ± SE or with interquartile (25-75%) ranges. Comparisons between groups were made by Student`s t test or one way ANOVA with post-hoc Holm-Sidak test for parameters with normal distribution, and by Mann-Whitney U test or Kruskal-Wallis test for parameters with non-normal distribution. Significance level was $p< 0.05$. Statistical analysis was accomplished with SigmaStat for Windows v 3.5 (Systat Software Inc).

3. Results

3.1 Weight variation and clinical score

Lewis rats immunized with myelin associated with CFA developed encephalomyelitis characterized by weight loss and elevated clinical score (figure 1). Prior infection of the animals with L3 from *S. venezuelensis* did not alter weight loss (figure 1a and 1b) nor clinical score (figure 1c and d). This non interference in clinical manifestations was always observed, being independent of the period of the infection with *S. venezuelensis* (acute or recovery) during which EAE was induced.

3.2 Inflammation in the CNS was not prevent by *S. venezuelensis* infection

Accentuated weight loss and high clinical scores that occurred during EAE progression were associated with a clear inflammatory reaction in the CNS. Brain and lumbar spinal cord sections showed typical inflammatory foci presenting mainly mononuclear cells that were preferentially localized around small vessels (figure 2). This inflammatory scenario was not changed by earlier contact with *S. venezuelensis*. Morphometric analysis indicated that the perivascular inflammatory infiltrate in the CNS was quantitatively similar in the three experimental groups: Control EAE, EAE induced during the acute phase of *S. venezuelensis* infection and EAE induced during the recovery phase of the infection (Table 1).

3.3 Cytokine production was modulated by infection with *S. venezuelensis*

Humoral immune response against myelin was not affected by former infection (acute or recovery phases) (figure 3a and 3e) with *S. venezuelensis*. However, significant alterations were observed in the production of some cytokines induced by specific or polyclonal stimulation. Induction of EAE during the acute phase (8

days after infection) determined more pronounced alterations in the production of cytokines induced by myelin as increased production of IFN-γ (figure 3b) by splenic cells and decreased production of IFN-γ (figure 3c) and IL-10 (figure 3d) by lymph node cells. No differences were observed in IFN-γ (spleen and lymph nodes) (figures 4a and 4c, respectively) and IL-10 (lymph nodes) (figure 4 e) production induced by Con A. However, previously infected mice produced less TNF-α (spleen and lymph nodes) (figures 4b and 4d, respectively) is response to Con A stimulation. Induction of EAE during the recovery phase (32 days after infection) determined alterations only on cytokine production induced by Con A. Spleen and lymph node cells from formerly infected mice produced less IFN-γ (figures 4f and 4h) and TNF-α (figures 4g and 4i), with no changes in IL-10 levels (figure 4j).

3.4 Frequency of Foxp3+ cells in rats infected with *S. venezuelensis*

The frequency of Foxp3+ cells was analyzed in CD4+CD25+ T cells population obtained from lymph nodes (popliteal + inguinal) (figure 5a) and spleen (figure 5b). As can be observed, similar amounts of Foxp3+ cells were present in both organs from control animals (non-infected). During both phases of the infection (acute and recovery) the % of these cells remained unchanged in these organs.

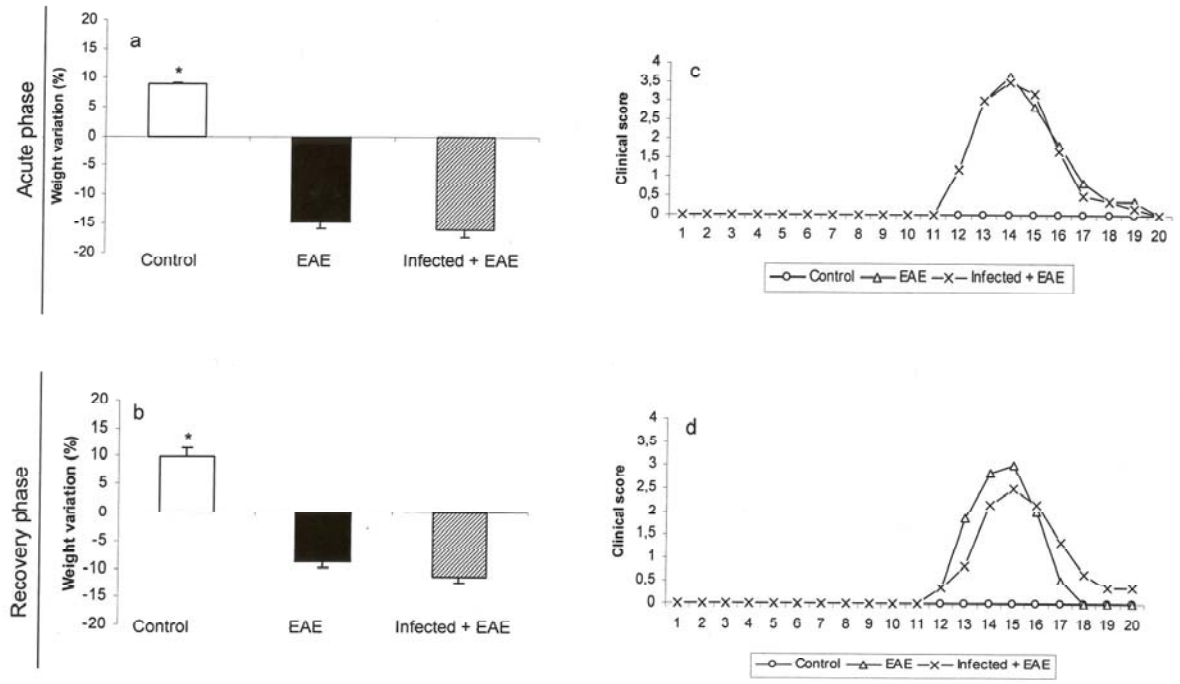

Figure 1 - Effect of previous infection with *S. venezuelensis* on EAE development. Female Lewis rats were infected with *S. venezuelensis* and then submitted to EAE induction during the acute or the recovery infection phases. Weight variation (a and b) and clinical score (c and d) were daily evaluated. Data were presented by mean±SE (a and b) or mean (c and d) of 5-6 rats. *$p<0.05$

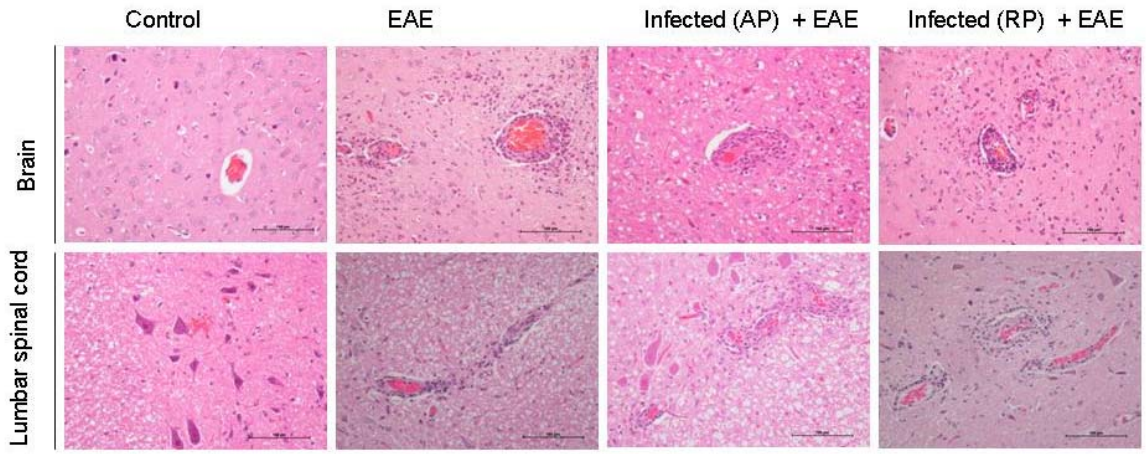

Figure 2 - Effect of previous infection with *S. venezuelensis* on CNS inflammation. Female Lewis rats were infected with *S. venezuelensis* and EAE was then induced during the acute (AP) or the recovery (RP) infection phases. Brain and spinal cord inflammatory infiltrates were evaluated 20 days after EAE induction.

Table 1 - Morphometric analysis of perivascular inflammatory infiltrates in the central nervous system

	EAE	Infected (AP) + EAE	Infected (RP) + EAE
Brain*	1,5 (1,0 – 1,8)	1,0 (0,8 – 1,1)	2,5 (1,3 – 6,0)
Lumbar spinal cord *	3,2 (2,5 – 4,1)	5,1 (2,6 – 7,7)	4,8 (3,4 – 7,8)

AP: Acute phase; RP: Recovery phase
* μm^2 of mononuclear infiltrate/mm^2 of organ section

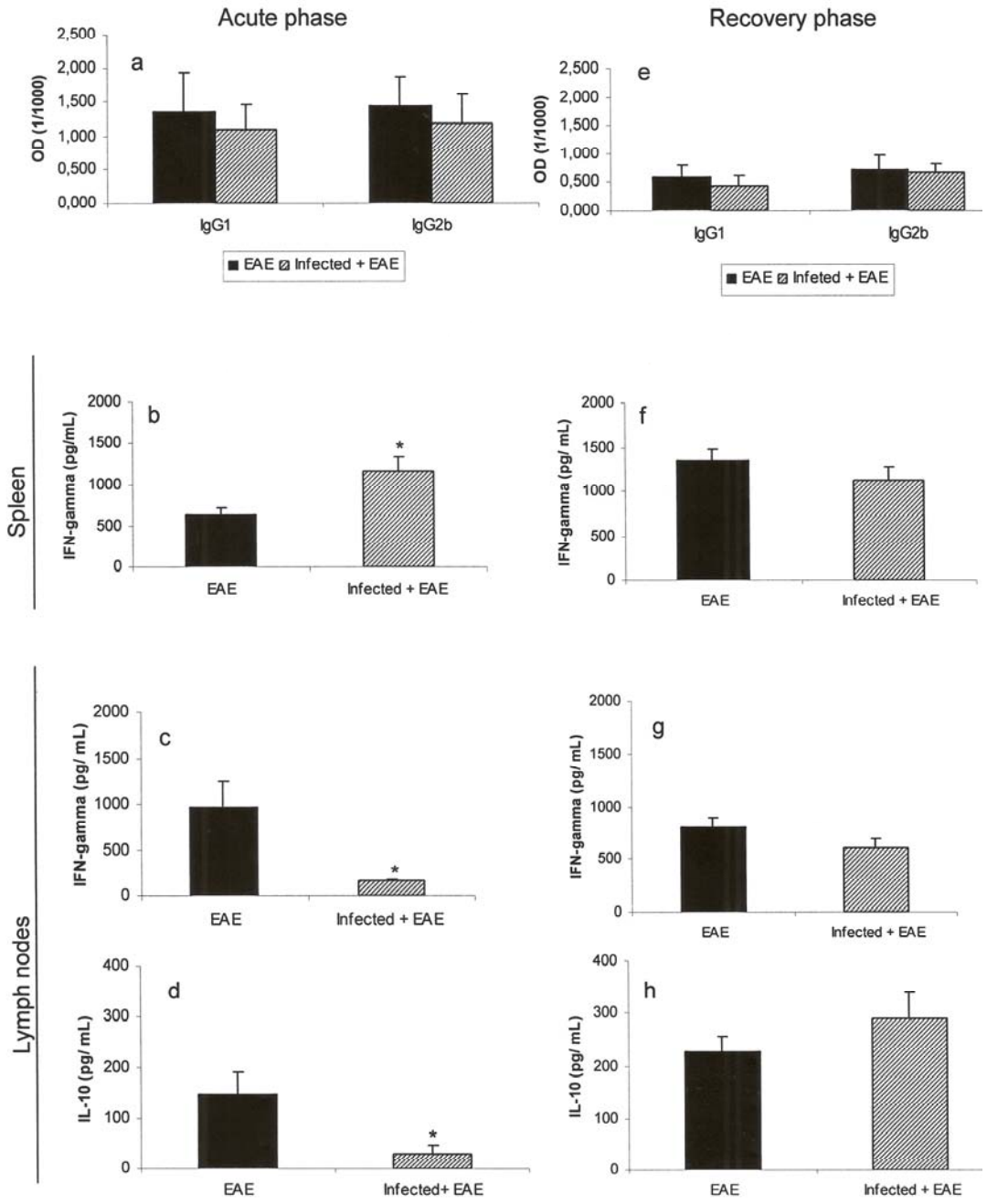

Figure 3 - Effect of previous infection with *S. venezuelensis* on myelin-specific immune response. Female Lewis rats were infected with *S. venezuelensis* and EAE was then induced during the acute or recovery phase of the infection. Antibody and cytokine production were assayed 20 days after EAE induction. Seric levels of IgG1 and IgG2b (a and e). IFN-γ production by spleen (b and f) and lymph node cells (c and g). IL-10 production by lymph node cells (d and h). Data were presented by mean±SE of 5-6 rats. *$p<0.05$

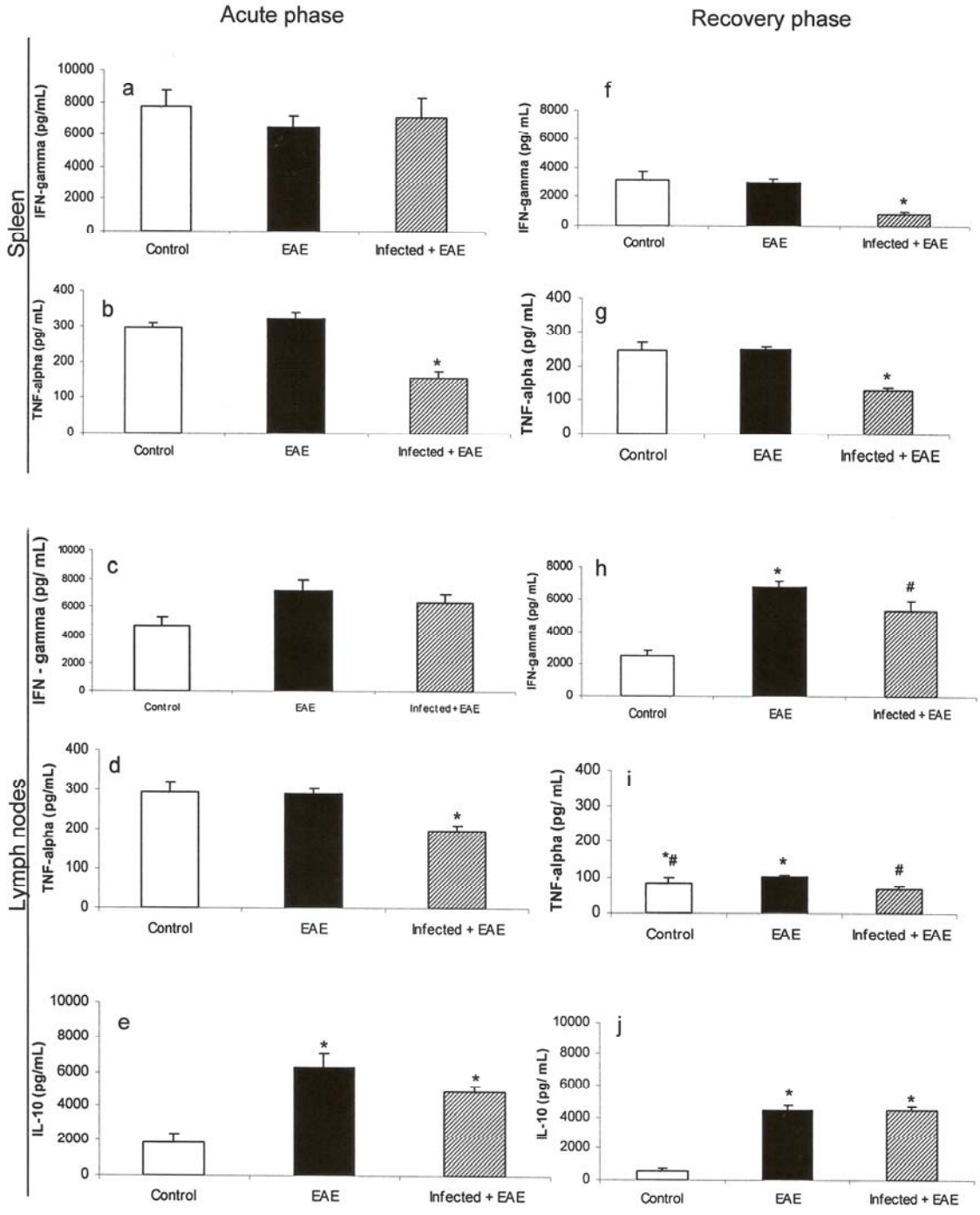

Figure 4 - Effect of previous infection with *S. venezuelensis* on Con A cytokine induction. IFN-γ production by spleen (a and f) and lymph node cells (c and h), TNF-α production by spleen (b and g) and lymph node cells (d and i) and IL-10 production by lymph node cells (e and j). Data were presented by mean±SE of 5-6 rats. Different symbols indicate significant difference between groups $p < 0.05$

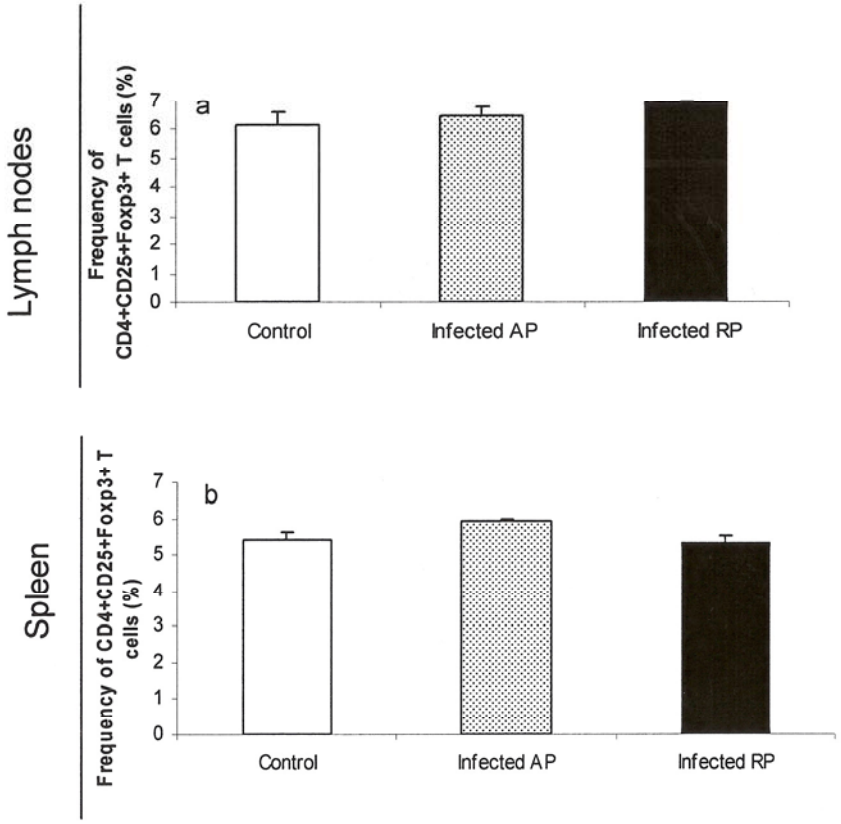

Figure 5 - Frequency of CD4+CD25+Foxp3+ T cells in lymph node (popliteal + inguinal) (a) and spleen (b) cells. Female Lewis rats were infected with *S. venezuelensis* and the percentage of CD4+CD25+Foxp3+ T cells was determined during the acute and recovery phases of the infection by flow cytometric analysis. Data were presented by mean±SE of 5-6 rats.

4. Discussion

In this investigation we evaluated if a previous infection with *S. venezuelensis* could interfere with development of EAE in Lewis rats. Rats immunized with myelin associated with CFA developed the expected signs of encephalomyelitis as weight loss and paralysis. They also presented clear inflammatory infiltrates in both, brain and spinal cord as we observed before (Zorzella-Pezavento et al., 2010). Contrary to what was hypothesized, previous infection with *S. venezuelensis* did not avoid full development of EAE. Lewis rats were infected with 4000 L3 by subcutaneous route and encephalomyelitis was induced during acute (8 days after infection) or recovery (32 days after infection) phases of infection. This correspondence of the 8th and 32nd days to acute and recovery phases, respectively, was previously determined by our group (Chiuso-Minicucci et al., 2010). This prior contact with the worm was not able to alter the encephalomyelitis, neither clinically nor histopathologically. Similar weight losses and clinical scores were present in both experimental groups. In addition, a quantitative analysis of inflammatory infiltrates indicated similar amounts of mononuclear cells in brain and lumbar spinal cord, independently of the contact with the helminth.

As one of the hallmarks of the hygiene hypothesis is based on induction of T regulatory cells by these environmental microorganisms, a flow cytometric analysis was done to analyze if infection would trigger an increased % of CD4+CD25+Foxp3+ T cells. Interestingly, the level of these regulatory T cells (Treg) was not changed neither at the acute nor at the recovery periods of the infection. These findings were initially interpreted as evidence that infection of

Lewis rats with *S. venezuelensis* is not able to induce a significant expansion of this cell population. Therefore, in a very simplistic line of thought, this lack of Treg expansion could explain the absence of an immunoregulatory effect over EAE development. However, it is important to stress that in some experimental parasitic models, Treg cells preferentially accumulate at the infected site as was described by Belkaid et al., (2002) for *Leishmania major* and Taylor et al., (2005) for filarial parasites. For the best of our knowledge, this kind of evaluation was not done with *S. venezuelensis*. It is therefore possible that Treg cells could be induced by this helminth but would be locally housed, as in the mesenteric lymph nodes for example (Finney et al., 2007; Liu et al., 2009).

If Treg cells were not induced or expanded during infection of Lewis rats with *S. venezuelensis*, we could still expect that the helminth-evoked Th2 response during the recovery phase could be able to delay or decrease EAE severity. In a previous report we demonstrated a clear Th2 profile during the recovery phase of *S. venezuelensis* infection in Lewis rats (Chiuso-Minicucci et al., 2010). Differently from these results, numerous investigators have shown that infection with a variety of helminths can slow down disease progression in murine model systems, by skewing cytokine production towards a Th2 profile. This protective immunomodulation has been triggered, for example, by *S. mansoni* eggs in allergen-induced airway hiper-responsiveness (Mangan et al., 2006) and in TNBS- induced colitis (Elliott et al., 2003); by *Heligmosomoides polygyrus* in experimental colitis (Elliott et al., 2008) and type 1 diabetes in NOD mice (Liu et al., 2009) and by *Nippostrongyloides brasiliensis* in arthritis (Salinas-Carmona et al., 2009). Some investigations explored the potential protective role of helminths

on EAE (La Flamme et al., 2003; Sewell et al., 2003; Gruden-Movsesijan et al., 2008; Zheng et al., 2008). Even though the described protection was attributed to a Th2 polarization, we can speculate that other mechanisms were required to act in concert with this to determine a detectable effect as for example, Treg induction (Wilson et al., 2005; Hübner et al., 2008) or alternatively activated macrophages (Espinoza-Jiménez et al., 2010). Based on the hygiene hypothesis, whose assumption is the contact with environment agents, we could also hypothesize that protection can be triggered by both, isolated agents or special associations of them, depending upon their antigens, life cycles and interplay with the host. These possibilities were not experimentally examined yet.

Many aspects could explain the failure of this helminth infection to reduce EAE development. First, it has been demonstrated that a protective effect can be time-and burden-dependent, with only chronically or heavily infected mice protected from ovalbumin-evoked allergic reaction (Smits et al., 2007). In this sense, higher *S. venezuelensis* burden, multiple previous infections or a constant contact with this parasite would be worthwhile to test. Second, this ability to modulate immunomediated diseases seems to be dependent on the helminth spp. There is not a systematic investigation to establish and compare the ability of different helminth spp. to modulate autoimmune pathologies. This approach would allow a better understanding of the factors that determine this immunomodulatory potential as for example helminth species involved, infection intensity, the frequency and timing of infection and also the acute or chronic nature of the infection (Helmby, 2009). On occasion, infection was even found to worsen disease in animal models. A meta-analysis of 30 studies concluded that overall

parasitic infections did not protect against asthma and that infection with *A. lumbricoides* was, in fact, associated with an increased risk for developing asthma (Leonardi-Bee et al., 2006).

Even though infection with *S. venezuelensis* was not able to clinically modify EAE in Lewis rats, it clearly modulated cytokine production. Acute phase of the infection determined more pronounced alterations in specific myelin induced cytokines whereas recovery phase determined alterations only in cytokine production induced by Con A stimulation. When this effect was present, it was usually down-regulatory, i.e., previously infected rats produced lower cytokine levels. These results are fully coherent with the undoubted helminth`s ability to down-regulate the host immune system at both the antigen-specific and polyclonal levels (Maizels & Yazdanbakhsh, 2008). At least three distinct mechanisms have been proposed to explain this down-regulatory ability: a modified Th2 response characterized by intact IL-4 and IL-10 but reduced IL-5, induction of regulatory cells as Tr1 and Foxp3+Tregs and alternatively activated macrophages.

Overall, this investigation indicates that a previous infection with *S. venezuelensis* was not able to modify EAE progression in Lewis rats. We don't see these results as a drawback to the hygiene hypothesis. We rather believe that they reinforce the need for a careful and systematical comparison of the immunoregulatory potential among the diverse helminth species.

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5. References

Back JF. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med. 2002;347:911-20.

Belkaid Y, Piccirilo AC, Mendez S, Shevack E, Sacks DL. CD4+CD25+ regulatory T cells control *Leishmania major* persistence and immunity. Nature. 2002;420:502-7.

Bock T, Pakkenberg B, Buschard K. Increased islet volume but unchanged islet number in ob/ob mice. Diabetes. 2003;*52*:1716-22.

Cabre P, Signate A, Olindo S, Merle H, Caparros-Lefebvre D, Bera O, et al. Role of return migration in the emergence of multiple sclerosis in the French West Indies. Brain. 2005;128:2899–910.

Chiuso-Minicucci F, Marra NM, Zorzella-Pezavento SF, França TG, Ishikawa LL, Amarante MR, et al. Recovery from *Strongyloides venezuelensis* infection in Lewis rats is associated with a strong Th2 response. Parasite Immunol. 2010;32:74-8.

Correale J, Farez M. Association between parasite infection and immune responses in multiple sclerosis. Ann Neurol. 2007;61:97-108.

El-behi M, Rostami A, Ciric B. Current views on the roles of Th1 and Th17 cells in experimental autoimmune encephalomyelitis. J Neuroimmune Pharmacol. 2010;5:189-97.

Elliott DE, Li J, Blum A, Metwali A, Qadir K, Urban JF Jr, et al. Exposure to schistosome eggs protects mice from TNB-induced colitis. Am J Physiol Gastrointest Liver Physiol. 2003;284:G385-G391.

Elliott DE, Summers RW, Weinstock JV. Helminths as governors of immune-mediated inflammation. Int J Parasitol. 2007;37:457-64.

Elliott DE, Metwali A, Leung J. Colonization with *Heligmosomoides polygyrus* suppresses mucosal IL-17 production. J Immunol. 2008;181:2414-9.

Espinoza-Jiménez A, Rivera-Montoya I, Cardenas-Arreola R, Morán L, Terrazas LI. *Taenia crassiceps* infection attenuates multiple low-dose streptozotocin-induced diabetes. J Biomed Biotechnol. 2010; doi:10.1155/2010/850541.

Finney CAM, Taylor MD, Wilson MS, Maizels RM. Expansion and activation of CD4+CD25+ regulatory T cells in *Heligmosomoides polygyrus* infection. Eur J Immunol. 2007;37:1874-86.

Genta RM. Global prevalence of strongyloidiasis: critical review with epidemiolgic insights into the prevention of disseminated disease. Rev Infect Dis. 1989;11:755-67.

Gruden-Movsesijan A, Ilic N, Mostarica-Stojkovic M, Stosic-Grujicic S, Milic M, Sofronic-Milosavljevic Lj. *Trichinella spirallis*: Modulation of experimental autoimmune encephalomyelitis in DA rats. Exp Parasitol. 2008;118:641-7.

Helmby H. Helminths and our immune system: friend or foe? Parasitol Int. 2009;58:121-7.

Hübner MP, Stocker JT, Mitre E. Inhibition of type 1 diabetes in filarial-infected non-obese diabetic mice is associated with a T helper type 2 shift and induction of Foxp3+ regulatory T cells. Immunology. 2008;127:512-22.

La Flamme AC, Ruddenklau K, Backstrom BT. Schistosomiasis decreases central nervous system inflammation and alters the progression of experimental autoimmune encephalomyelitis. Infect Immun. 2003;71:4996-5004.

La Flamme AC, Canagasabey K, Harvie M, Bäckström BT. Schistosomiasis protects against multiple sclerosis. Mem Inst Oswalso Cruz. 2004;99:33-6.

Leonardi-Bee J, Pritchard D, Britton J. Asthma and current intestinal parasite infection. Systematic review and meta-analysis. Am J Respir Crit Care Med. 2006;174:514-23.

Liu Q, Sundar K, Mishra PK, Mousavi G, Liu Z, Gaydo A, et al. Helminth infection can reduce insulitis and type 1 diabetes through CD25- and IL-10-independent mechanisms. Infect Immun. 2009;77:5347-58.

Maizels RM, Yazdanbakhsh M. T-cell regulation in helminth parasite infections: Implications for Inflammatory Diseases. Chem Immunol Allergy. 2008,94:112-23.

Mangan NE, van Rooijen N, McKenzie AN, Fallon PG. Helminth-modified pulmonary immune response protects mice from allergen-induced airway hyperresponsiveness. J Immunol. 2006;176:138-47.

Marra NM, Chiuso-Minicucci F, Machado GC, Zorzella-Pezavento SF, França TG, Ishikawa LLW, et al. Faecal examination and PCR to detect *Strongyloides venezuelensis* in experimentally infected Lewis rats. Mem Inst Oswaldo Cruz. 2010a;105:57-61.

Marra NM, Chiuso-Minicucci F, Machado GC, Zorzella-Pezavento SFG, França TGD, Ishikawa LLW, et al. Migratory route of *Strongyloides venezuelensis* in Lewis rats: Comparison of histological analyses and PCR. Exp Parasitol. 2010b. Epub 2010 aug 27.

Okada H, Kuhn C, Feillet H, Bach JF. The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. Clin Exp Immunol. 2010;160:1-9.

Rook GAW, Adams V, Hunt J, Palmer R, Martinelli R, Brunet LR. Mycobacteria and other environmental organisms as immunomodulators for immunoregulatory disorders. Springer Semin Immunol. 2004;25:237-55.

Rook GAW. The hygiene hypothesis and the increasing prevalence of chronic inflammatory disorders. Trans R Soc Trop Med Hyg. 2007;101:1072-4.

Salinas-Carmona MC, de la Cruz-Galicia G, Pérez-Rivera I, Solís-Soto JM, Segoviano-Ramirez JC, Vázquez AV, et al. Spontaneous arthritis in MRL/lpr mice is aggravated by *Staphylococcus aureus* and ameliorated by *Nippostrongylus brasiliensis* infections. Autoimmunity. 2009;42:25-32.

Sawczenko A, Sandhu BK, Logan RF, Jenkins H, Taylor CJ, Mian S, et al. Prospective survey of childhood inflammatory bowel disease in the British Isles. Lancet. 2001;357:1093-4.

Sewell D, Qing Z, Reinke E, Elliot D, Weinstock J, Sardor M, et al. Immunomodulation of experimental autoimmune encephalomyelitis by helminth ova immunization. Int Immunol. 2003;15:59-69.

Siddiqui AA & Berk SL. Strongyloidiasis. Curr Treatment Option. 2003; 5:283-9.

Smits HH, Hammad H, van Nimwegen M, Soullie T, Willart MA, Lievers E, et al. Protective effect of *Schistosoma mansoni* infection on allergic airway inflammation depends on the intensity and chronicity of infection. J Allerg Clin Immunol. 2007;120:932-40.

Summers RW, Elliott DE, Urban JF Jr, Thompson R, Weinstock JV. *Trichuris suis* therapy in Crohn's disease. Gut. 2005a;54:87-90.

Summers RW, Elliott DE, Urban JF Jr, Thompson RA, Weinstock JV. *Trichuris suis* therapy for active ulcerative colitis: a randomized controlled trial. Gastroenterology. 2005b;128:825-32.

Taylor MD, LeGoff L, Harris A, Malone E, Allen JE, Maizels RM. Removal of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance in vivo. J Immunol. 2005;174:4924-33.

Wilson MS, Taylor MD, Balic A, Finney CA, Lamb JR, Maizels RM. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. J Exp Med. 2005;202:1199-212.

Zaccone P, Burton O, Miller N, Jones FM, Dune DW, Cooke A. *Schistosoma mansoni* egg antigens induce Treg that participate in diabetes prevention in NOD mice. European J Immunol. 2009;39:1098-107.

Zheng X, Hu X, Zhou G, Lu Z, Qiu W, Bao J, et al. Soluble egg antigen from *Schistosoma japonicum* modulates the progression of chronic progressive experimental autoimmune encephalomyelitis via TH2-shift response. J Neuroimmunol. 2008;194:107-14.

Zorzella-Pezavento SF, Chiuso-Minicucci F, França TG, Ishikawa LL, Martins DR, Silva CL, et al. Immunization with pVAXhsp65 decreases inflammation and modulates immune response in experimental encephalomyelitis. Neuroimmunomodulation. 2010;17:287-97.

4.3 Artigo Científico III

Experimental autoimmune encephalomyelitis evolution was not modified by multiple infections with *Strongyloides venezuelensis*

Effect of *S. venezuelensis* on encephalomyelitis

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SUMMARY

According to the hygiene hypothesis, the increased incidence of allergic and autoimmune diseases in developed countries is mainly explained by the decreased contact between the human population and certain environmental agents as lactobacillus, mycobacteria and helminths. In this study we evaluated the effect of multiple infections with *S. venezuelensis* on the development of experimental autoimmune encephalomyelitis (EAE) in Lewis rats. Multiple infections before EAE induction were not able to change the evolution of the disease. No alterations were observed in weight loss, clinical score and inflammation intensity at the Central Nervous System. These multiple infections were able to induce a strong Th2 polarization characterized by significant IgG1 but not IgG2b parasite specific antibodies. However, the percentage of $Foxp3^+$ T cells was not changed, being their levels in the spleen and lymph node of infected rats, comparable to the ones found in normal animals. These results suggest that a Th2 polarized response without concomitant expansion of Foxp3+ regulatory T cells was not able to modify EAE progression. Even though these results do not threaten the hygiene hypothesis, they suggest that this paradigma might be an oversimplification. They also emphasize the need of a comparative immunoregulatory study among the diverse helminth spp.

Keywords: hygiene hypothesis, *Strongyloides venezuelensis*, experimental autoimmune encephalomyelitis, Th2 response

INTRODUCTION

Multiple sclerosis (MS) is considered the most common inflammatory demyelinating disease, affecting approximately one million adults. Different cell types, including Th1, Th17, Tc, B and regulatory T cells are involved in the inflammatory reaction that damages the myelin sheat (1). Strong evidence has been provided for a potential functional defect of CD4+CD25+Foxp3+ regulatory T cells in patients with relapsing-remmitting MS (2). Animal models have been extraordinarily useful, providing a deeper insight into the immunopathogenesis of MS (3). These models indicated, for example, that regulatory T cells can prevent induced EAE and also contribute to genetic EAE resistance (4). Within this scenario, the possible modulation of autoimmunity and allergy by certain environmental agents, as lactobacillus, mycobacteria and helminths, has been associated with activation and/or expansion of regulatory T cells (5) and induction of strong Th2 polarization (5,6) . This study was designed to evaluate the type of response (Th2 polarization and/or Foxp3+ T cells) induced by multiple infections with *S. venezuelensis* and its effect on EAE progression induced in Lewis rats.

MATERIALS AND METHODS

Female Lewis were infected four times (once a week) with 4000 *S. venezuelensis* infective filiform larvae by subcutaneous route at the abdominal region. Infection intensity was determined by counting the number of eggs per gram of faeces (EPG) by a modified Cornell McMaster method (7). Two procedures were adopted fifteen days after last *S. venezuelensis* inoculation: evaluation of immune response (by specific antibody and $Foxp3^+$ T cells determination) and EAE induction. Parasite-specific IgG1 and IgG2b were estimated by ELISA by using antigen

obtained as previously described (8). The frequency of $Foxp3^+$ T cells was determined in a $CD4^+CD25^+$ cell populations obtained from spleen and regional lymph nodes (popliteal + inguinal). Cells were adjusted to $2.5x10^6$ and then surface-labelled with fluorescein isothiocianate (FITC) anti-rat CD4 (0.5μg) and allophycocyanin (APC) anti-rat CD25 (0.25μg). After this step, a staining for Foxp3 by using the phycoerythrin (PE) anti-mouse/rat Foxp3 Staining Set (eBioscience, San Diego, CA, USA) was performed according to the manufacturer instructions. After incubation, the cells were fixed in paraformaldehyde 1% and analyzed with a FACSCanto II (BD Biosciences) flow cytometer and Flow Jo software (TreeStar, Ashland, OR, EUA). EAE was induced by inoculation of 25 μg of myelin basic protein (MBP- Sigma) emulsified with Complete Freund's Adjuvant (CFA) containing 5 mg/mL of *Mycobacterium butyricum*, in the hind left footpad. Animals were daily evaluated for weight loss and clinical score. Signs of disease were graded as 0 (zero): no disease; 1: loss of tonicity in the distal portion of the tail; 2: total loss of tail tonicity; 3: hind limb weakness (partial paralysis); 4: complete hind limb paralysis and urinary incontinence and 5: moribund. Presence and amount of brain and spinal cord inflammatory infiltrates were assessed during EAE recovery phase (20 days after immunization) as previously described (9). IFN-gamma and IL-10 production were also determined at this phase. For this, lymph node (popliteal + inguinal) cells were collected and adjusted to $2.5x10^6$ cells/ mL in RPMI supplemented with 10% fetal calf serum, 2 mM L-glutamine and 40 mg/L of gentamicin, in the presence of 10 μg/mL of myelin or 5 μg/mL of concanavalin A (ConA, Sigma, St. Louis, MO, USA). Cytokine levels were evaluated in culture supernatants collected 72h later by ELISA according to manufacturer instructions

(R & D Systems, Minneapolis, MN, USA). ELISA sensitivity for IFN-γ and IL-10 was 19 and 31 pg/mL, respectively. Data were expressed as mean ± SE. Comparisons between groups were made by Student`s t test or one way ANOVA with post-hoc Holm-Sidak test for parameters with normal distribution, and by Mann-Whitney U test or Kruskal-Wallis test for parameters with non-normal distribution. Significance level was $p < 0.05$. Statistical analysis was accomplished with SigmaStat for Windows v 3.5 (Systat Software Inc).

RESULTS

1) Multiple infections with *S. venezuelensis*: kinetics, antibody production and frequency of Foxp3+ regulatory T cells

A high number of EPG was detected 8 days after the first worm inoculation. The amount of eggs decreased by day 13 and was very low at days 20 and 27. No more eggs were detected 34 days after initial infection. (figure 1a). Evaluation of specific antibody levels by ELISA indicated significant production of IgG1 but not IgG2b (figure 1b). The frequency of cells expressing the regulatory foxp3 marker was determined in spleen and lymph node cells. The percentage of Foxp3+ cells in CD4+CD25+ T cell population, from both origins, was comparable in control and infected rats (figure 1c).

2) Weight variation, clinical score and cytokine production

Lewis rats immunized with myelin developed EAE characterized by accentuated weight losses and elevated clinical scores. Multiple infections with *S. venezuelensis* before EAE induction were not able to modify disease clinical manifestations (figure 2a and 2b). This previous contact with the worm was also

not able to modulate IL-10 and IFN-γ production by regional lymph node cell cultures stimulated with myelin (figure 2c) or Con A (figure 2d).

3) Inflammation at the Central Nervous System (CNS)

The earlier contact with *S. venezuelensis* was also not capable to modify the extension of inflammation in the CNS (figure 2e). Morphometric analysis in the brain (EAE= 1.3±0.3 $\mu m^2/mm^2$, Infected + EAE= 1.02±0.03 $\mu m^2/mm^2$) and the spinal cord sections (EAE= 12.2±2 $\mu m^2/mm^2$; Infected + EAE= 9.3±2.2 $\mu m^2/mm^2$) indicated perivascular infiltrates with similar intensities in both experimental groups.

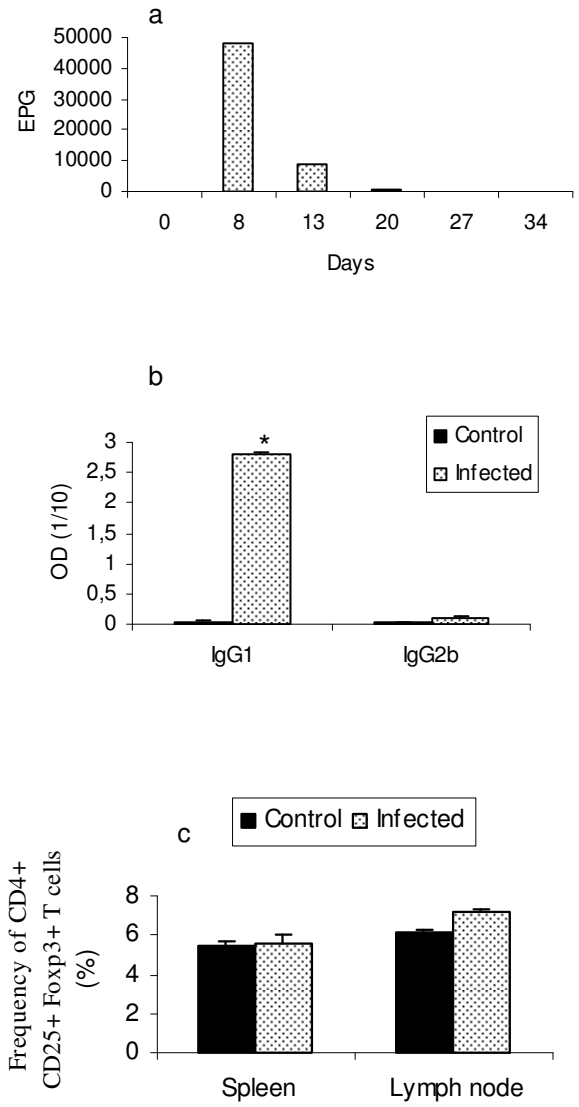

Figure 1 Multiple infections with *S. venezuelensis*: kinetics, antibody production and frequency of Foxp3+ regulatory T cells. Eggs per gram of faeces (a), serum levels of specific IgG1 and IgG2b (b) and frequency of $CD4^{+}CD25^{+}$Foxp3+ T cells (c) in lymph nodes and spleen cells. The number of egg per gram of faeces was determined in a faecal pool. Data were presented by mean±SE of five rats. *$p<0.05$

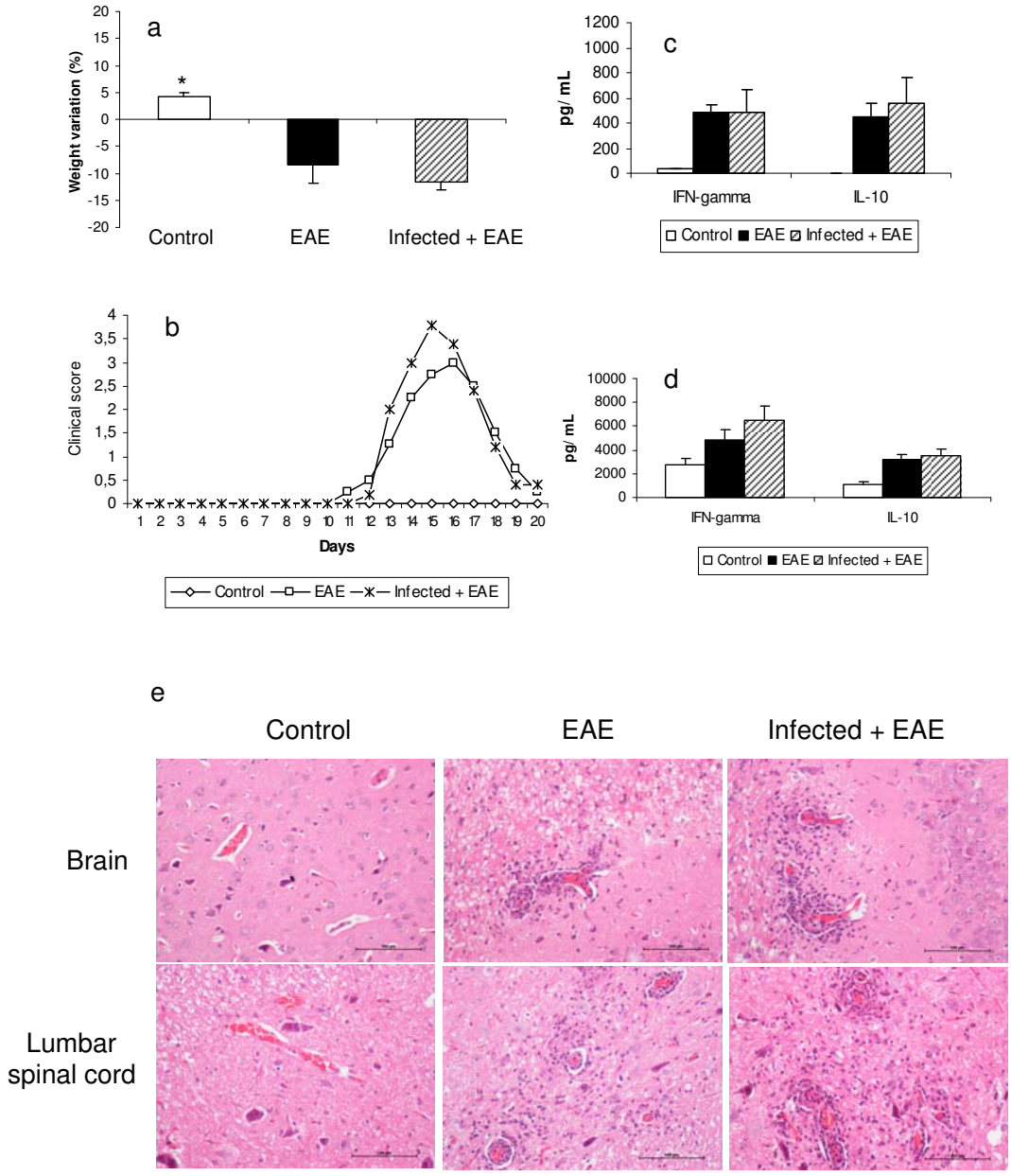

Figure 2 Effect of infections with *S. venezuelensis* on weight variation, clinical score, cytokine production and inflammation at the CNS in Lewis rats submitted to EAE induction. Weight variation (a) and clinical score (b). IFN-γ and IL-10 production by lymph node cell cultures stimulated with MBP (c) or Con A (d). Histophatological analysis of brain and lumbar spinal cord in Lewis rats submitted to EAE induction (e). Data were presented by mean±SE (a, c and d) or mean (b) of five rats. *p<0.05

DISCUSSION

This investigation was carried out to determine if a previous and prolonged contact with the helminth *S. venezuelensis* was able to modify EAE. To mimicry a constant contact with the worms, adult female Lewis rats were weekly infected with 4000 L3 of *S. venezuelensis* by subcutaneous route at the abdominal region. As expected, a higher number of eggs was detected 8 days after the first inoculation. The first worm dose already determined a state of resistance characterized by a continuous decrease in egg numbers in spite of the ensuing worm doses. These findings suggest that Lewis rats constitute a non-permissive host for this parasite (10). The establishment of a Th2 polarized response after multiple infections was indicated by a significant increase in IgG1, but not IgG2b, specific antibodies. In a previous report we also observed an elevated production of total IgE after a single inoculation of *S. venezuelensis* (11), reinforcing the expected ability of this worm to induce a Th2 type of response, as widely described for other helminths (12,13).

Many reports have emphasized the association of helminth infections with expansion of CD4+CD25+Foxp3+ regulatory T cells (14-16). However, the multiple infection protocol with *S. venezuelensis*, employed in this investigation, was not able to trigger expansion of these cells in lymph nodes (inguinal and popliteal) or spleen. The most straightforward explanation for this finding would be by the non induction of regulatory T cells by this helminth. Theoretically, this seems reasonable because the first contact with *S. venezuelensis* was already determining a state of resistance. Therefore, as all or the majority of the parasites were eliminated, there was no need for regulatory T cells expansion to regulate a

potential immunopathological reaction. The possibility that regulatory T cell expansion is occurring at the mesenteric lymph nodes, at the Peyer patches or even at other sites cannot be excluded. There are reports of regulatory T cell expansion in different sites as in the periphery of the granuloma (17), the infection site and the draining lymph node (18) and also around the muscle-encysted *Trichinella spiralis* larvae (19).

Even in the absence of T regulatory cells, Th2 polarization could still provide an environment capable to modify EAE development as has been reported for diabetes (20) and arthritis (21). To test this possibility, fifteen days after last *S. venezuelensis* inoculation, experimental encephalomyelitis was induced by inoculation of myelin emulsified with CFA. Contrary to hygiene hypothesis, the clinical evolution of the neurological disease was very similar in the two experimental groups, i.e., non-infected and previously infected with *S. venezuelensis*. They equally lost weight, the average clinical score was the same and acute and remission phases also occurred at comparable time periods. This was confirmed by further histopathological evaluation, whose quantitative analysis of the inflammatory infiltrates indicated similar values at the brain and lumbar spinal cord, independently of a former contact with the helminth.

These results are comparable to a recently investigation from our group describing that a single infection with *S. venezuelensis* was also not able to change the overall development of EAE (submitted). These findings are different from many reports that demonstrate the ability of helminth infections to protect against diabetes (22), arthritis (23) and also EAE (24).

The explanation for this seemingly contradictory finding could be an intrinsic characteristic of the helminth spp. itself. In this sense, a systematic evaluation of the immunoregulatory potential associated with distinct helminth spp. would be certainly enlightening.

In spite of this absence of protection, we believe that these findings will contribute to elucidate the limits of the hygiene hypothesis. In this sense, a comparative search with different helminth spp. must be done to disclose the biological and biochemical characteristics that are associated with protection against immunomediated diseases.

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REFERENCES

1Holmøy T & Hestvik AL. Multiple sclerosis: immunopathogenesis and controversies in defining the cause. *Curr Opin Infect Dis* 2008; **21**: 271-278.

2 Venken K, Hellings N, Liblau R & Stinissen P. Disturbed regulatory T cell homeostasis in multiple sclerosis. *Trends Mol Med* 2010; **16**: 58-68.

3 Mix E, Meyer-Rienecker H, Hartung HP & Zettl UK. Animal models of multiple sclerosis-Potentials and limitations. *Prog Neurobiol* 2010 (in press).

4 Vandenbark AA & Offner H. Critical evaluation of regulatory T cells in autoimmunity: are the most potent regulatory specificities being ignored? *Immunology* 2008; **125**: 1-13.

5 Rook GAW. The hygiene hypothesis and the increasing prevalence of chronic inflammatory disorders. *Trans R Soc Trop Med Hyg* 2007; **101**: 1072-1074.

6 Zaccone P, Burton OT, Gibbs S, *et al*. Immune modulation *by Schistosoma mansoni* antigens in NOD mice: effects on both innate and adaptive immune systems. *J Biomed Biotechnol* 2010 (in press).

7 Gordon HM & WhitlocK HV. A new technique for couting nematode eggs in sheep faeces. *J Coun Scient Ind Res* 1939; **12**: 50-52.

8 Fernandes A, Pereira AT, Eschenazi PD, *et al*. Evaluation of the immune response against *Strongyloides venezuelensis* in antigen-immunized or previously infected mice. *Parasite Immunol* 2008; **30**: 139-49.

9 Seger J, Zorzella-Pezavento SF, Pelizon AC, Martins DR, Domingues A & Sartori A. Decreased production of TNF-alpha by lymph node cells indicates experimental autoimmune encephalomyelitis remission in Lewis rats. *Mem Inst Oswaldo Cruz* 2010; **105**: 263-268.

10 McKay DM. The therapeutic helminth? *Trends Parasitol* 2009; **25**:109-114.

11 Chiuso-Minicucci F, Marra NM, Zorzella-Pezavento SF, *et al*. Recovery from *Strongyloides venezuelensis* infection in Lewis rats is associated with a strong Th2 response. *Parasite Immunol* 2010; **32**:74-78.

12 de Oliveira Fraga LA, Torrero MN, Tocheva AS, Mitre E & Davies SJ. Induction of type 2 responses by schistosome worms during prepatent infection. *J Infect Dis* 2010; **201**:464-472.

13 Everts B, Smits HH, Hokke CH & Yazdanbakhsh M. Helminths and dendritic cells: sensing and regulating via pattern recognition receptors, Th2 and Treg responses. *Eur J Immunol* 2010; **40**:1525-1537.

14 Finney CA, Taylor MD, Wilson MS & Maizels RM. Expansion and activation of CD4(+)CD25(+) regulatory T cells in *Heligmosomoides polygyrus* infection. *Eur J Immunol* 2007; **37**:1874-1886.

15 Pacífico LG, Marinho FA, Fonseca CT, *et al*. *Schistosoma mansoni* antigens modulate experimental allergic asthma in a murine model: a major role for CD4+ CD25+ Foxp3+ T cells independent of interleukin-10. *Infect Immun* 2009; **77**: 98-107.

16 Walsh KP, Brady MT, Finlay CM, Boon L & Mills KHG. Infection with a helminth parasite attenuates autoimmunity through TGF-{beta}-mediated suppression of Th17 and Th1 responses. *J Immunol* 2009; **183**: 1577–1586.

17 Layland LE, Mages J, Loddenkemper C, *et al*. Pronounced phenotype in activated regulatory T cells during a chronic helminth infection. *J Immunol* 2010; **184**: 713-724.

18 Taylor MD, van der Werf N, Harris A, *et al*. Early recruitment of natural CD4+ Foxp3+ Treg cells by infective larvae determines the outcome of filarial infection. *Eur J Immunol* 2009; **39**: 192-206.

19 Beiting DP, Gagliardo LF, Hesse M, Bliss SK, Meskill D & Appleton JA. Coordinated control of immunity to muscle stage Trichinella spiralis by IL-10, regulatory T cells, and TGF-beta. *J Immunol* 2007; **178**: 1039-1047.

20 Zaccone P, Fehérvári Z, Jones FM, *et al*. *Schistosoma mansoni* antigens modulate the activity of the innate immune response and prevent onset of type 1 diabetes. *Eur J Immunol* 2003; **33**: 1439-1449.

21 Salinas-Carmona MC, de la Cruz-Galicia G, Pérez-Rivera I, *et al*. Spontaneous arthritis in MRL/lpr mice is aggravated by *Staphylococcus aureus* and ameliorated by *Nippostrongylus brasiliensis* infections. *Autoimmunity* 2009; **42**: 25-32.

22 Liu Q, Sundar K, Mishra PK, et al. Helminth infection can reduce insulitis and type 1 diabetes through CD25- and IL-10-independent mechanisms. *Infect Immun* 2009; **77**: 5347-5358.

23 Osada Y, Shimizu S, Kumagai T, Yamada S & Kanazawa T. *Schistosoma mansoni* infection reduces severity of collagen-induced arthritis via down-regulation of pro-inflammatory mediators. *Int J Parasitol* 2009; **39**: 457-464.

24 Gruden-Movsesijan A, Ilic N, Mostarica-Stojkovic M, Stosic-Grujicic S, Milic M & Sofronic-Milosavljevic Lj. *Trichinella spirallis*: Modulation of experimental autoimmune encephalomyelitis in DA rats. *Exp Parasitol* 2008; **118**: 641-647.