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



EFEITO DE CEPAS TOXIGÊNICAS DE *Staphylococcus aureus*
NO DESENVOLVIMENTO DA ENCEFALITE AUTOIMUNE
EXPERIMENTAL

THAIS GRAZIELA DONEGÁ FRANÇA

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

Orientadora: *Profa Dra Alexandrina Sartori*

**BOTUCATU – SP
2013**



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“Julio de Mesquita Filho”

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FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO DE AQUIS. E TRAT. DA INFORMAÇÃO
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CAMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: **ROSEMEIRE APARECIDA VICENTE**

França, Thais Graziela Donegá.

Efeito de cepas toxigênicas de *Staphylococcus aureus* no desenvolvimento da encefalite autoimune experimental : estudo experimental em camundongos /Thais Graziela Donegá França. – Botucatu : [s.n.], 2013

Tese (doutorado) - Universidade Estadual Paulista, Instituto de Biociências de Botucatu

Orientador: Alexandrina Sartori

Coorientador: Maria de Lourdes Ribeiro de Souza da Cunha

Capes: 21102007

1. Encefalite. 2. Doenças autoimunes. 3. Superantígenos. 4. Camundongo como animal de laboratório. 5. Estafilococos aureus. 6. Esclerose múltipla.

Palavras-chave: Encefalite autoimune experimental; Doenças autoimunes, *S. aureus*; Superantígenos.

“(...) Persiga um sonho, mas não deixe ele viver sozinho.

Procure, sempre procure o fim de uma história, seja ela qual for.

Se achar que precisa voltar, volte!

Se perceber que precisa seguir, siga!

Se estiver tudo errado, comece novamente.

Se estiver tudo certo, continue.

Se sentir saudades, mate-a.

Se perder um amor, não se perca!

Se achá-lo, segure-o!”

Fernando Pessoa

Dedicatória

Dedicatória

Ao meu esposo Luis Gustavo, por percorrer junto comigo os caminhos traçados pela vida. Obrigada pelo carinho, dedicação, compreensão e companheirismo. Você incentiva a conquistas dos meus sonhos, me fortalece quando caio e traz tranquilidade para enfrentar as dificuldades.

Amo você!

Aos meus pais Alceu e Maria, pelo amor e compreensão concedidos em todos os momentos de minha vida. Tenho por vocês eterna gratidão por tudo que me deram com muito amor e carinho.

E ao meu irmão Patrick, por estar sempre por perto auxiliando na criação e educação. Obrigada pelo amor e carinho concedidos.

Amo vocês!

Agradecimientos

Agradeço especialmente,

À minha orientadora Profa Dra Alexandrina Sartori que me abriu portas e mostrou caminhos. Agradeço pelos valiosos ensinamentos, pela paciência e disponibilidade que possibilitou a realização desse trabalho. Enfim, agradeço pela imensurável contribuição para minha formação profissional e por me incentivar a querer sempre mais.

À minha co-orientadora Profa Dra Maria de Lourdes Ribeiro de Souza da Cunha pelos valiosos ensinamentos, pela disponibilidade, tranquilidade e sutileza na execução deste trabalho. Sou grata por me incentivar a continuar neste fantástico mundo da pesquisa.

Muito obrigada!

Agradeço,

Primeiramente a DEUS, pois sem ele presente em minha vida, nada seria possível!

A todos os meus familiares pelo apoio dispensado.

Às minhas companheiras e grandes amigas, Fernanda Chiuso, Larissa Ishikawa, Larissa Camargo, Priscila Colavite e Sofia Zorzella, pelas horas de trabalhos, pelas valiosas discussões, pelas inesquecíveis risadas, enfim, agradeço pelo auxílio imensurável para a realização deste trabalho e pela participação inesquecível em minha vida.

Aos meus companheiros de pesquisa, Bianca Balbino, Thais Fraga e Luiza Mimura pela contribuição valiosa na realização deste trabalho.

Às minhas amigas, Mariana Fávero, Lígia Abraão, Katheryne Benini, Larissa Doddi e Tatiana Bachiega pelas

valiosas sugestões e pelos momentos agradáveis de descontração proporcionados.

À profa Dra Maria Terezinha Serrão Peraçoli, profa Lúcia Helena O'Dwyer de Oliveira e Dra Fernanda Chiuso Minicucci pelas valiosas sugestões durante meu exame de qualificação.

À bióloga Camila Marques e a Dra. Maura Rosane Valerio Ikoma do Laboratório de Citometria de Fluxo da Fundação Dr. Amaral Carvalho da cidade de Jaú pelo auxílio, disponibilidade e atenção dispensada para realização deste trabalho.

Aos professores do Departamento de microbiologia e Imunologia pela grandiosa contribuição para minha formação profissional.

Aos pós-graduandos do Departamento de Microbiologia e Imunologia pelo apoio e pelo conhecimento compartilhado.

Aos funcionários do Departamento de Microbiologia e Imunologia, em especial ao Lula, pela ajuda no manuseio dos animais do biotério e ao Luiz, pelos preparos de soluções.

Aos professores e funcionários da seção de Pós-Graduação em Biologia Geral e Aplicada do Instituto de Biociências de Botucatu pela atenção sempre dispensada.

Aos funcionários da biblioteca da UNESP de Botucatu.

À FAPESP pela bolsa concedida (n.2009/53175-7).

Enfim, a todos que de forma direta ou indiretamente contribuíram para o desenvolvimento desse trabalho e para minha formação profissional.

Muito Obrigada!!!

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Resumo

RESUMO

A esclerose múltipla (EM) é uma doença desmielinizante que afeta o sistema nervoso central (SNC). A encefalite autoimune experimental (EAE) é o modelo animal mais empregado para investigar mecanismos imunopatogênicos, terapia e efeito de agentes infecciosos na EM. Neste contexto, utilizamos este modelo para avaliar o efeito de cepas produtoras de superantígenos (SAGs) no desenvolvimento da EAE. Para isto, camundongos C57BL/6 foram infectados com cepas distintas de *S. aureus* e 3 dias após foram submetidos à indução de EAE por imunização com MOG (*myelin oligodendrocyte glycoprotein*). O efeito das infecções foi avaliado através dos seguintes parâmetros: perda de peso corporal, escore clínico, inflamação do SNC e produção de citocinas por células esplênicas ou isoladas do SNC. A presença de células T CD4+CD25+Foxp3+ (Tregs) foi determinada em alguns experimentos. Os resultados obtidos foram organizados em 3 manuscritos. Na primeira etapa, comparamos 2 cepas de *S. aureus* quanto às suas habilidades de causar bacteremia, migração para o cérebro e efeito da infecção sobre a fase aguda da EAE. Foram escolhidas as cepas de *S. aureus* ATCC 51650 que é produtora do superantígeno TSST-1 (TSST-1+) e ATCC 43300 (TOX-) que não produz nenhum tipo de SAG. As 2 cepas causaram bacteremia e atingiram o cérebro, entretanto, só a cepa TOX- causou inflamação local. As 2 cepas reduziram os sintomas clínicos da EAE durante a fase aguda mas a cepa produtora de TSST-1+ determinou efeito mais acentuado. O efeito protetor de ambas foi associado com modulação da produção local e periférica de citocinas. No segundo manuscrito investigamos se o efeito protetor da infecção prévia com *S. aureus* era mantido na fase crônica da EAE. Constatamos que o efeito protetor perdurou até a fase crônica sendo também caracterizado por menor incidência da

doença, menor perda de peso e escores clínicos mais baixos. O efeito da cepa produtora de TSST-1+ também foi mais acentuado nesta fase. As infecções prévias com as 2 cepas modularam a produção local e periférica de citocinas mas só a cepa TOX- modulou a porcentagem de células Tregs. No último trabalho mostramos que a cepa ATCC 14458, produtora de enterotoxina SEB (SEB+), também foi protetora determinando redução na perda de peso, no escore clínico, na incidência da doença e também no processo inflamatório presente no SNC. Este efeito protetor esteve associado com redução na produção de IL-17. Juntos estes resultados mostraram que infecções prévias com diferentes cepas de *S. aureus*, inclusive com 2 cepas produtoras de SAgS, determinaram efeito protetor na EAE. Um entendimento mais aprofundado dos mecanismos imunológicos envolvidos nesta proteção poderá resultar em medidas preventivas de possível aplicação na EM.

Palavras-chave: encefalite autoimune experimental, doença autoimune, *S. aureus*, superantígenos

Abstract

Abstract

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS). Experimental autoimmune encephalomyelitis (EAE) is a widely accepted model used to investigate immunopathogenic mechanisms, therapy and the effect of infectious agents on MS. In this context, this model was employed to evaluate the effect of *S. aureus* superantigen (SAg) producer strains on EAE development. For this C57BL/6 mice were infected with distinct *S. aureus* strains and three days after infection they were submitted to EAE induction by immunization with MOG (myelin oligodendrocyte glycoprotein). The effect of infections was evaluated by body weight loss, clinical score, inflammation of the CNS and cytokine production by spleen cells or CNS cells. The presence of CD4⁺CD25⁺Foxp3⁺ T cells (regulatory T cells) was determined in some experiments. The results were organized into 3 manuscripts. Initially, we compared the ability of 2 *S. aureus* strains to cause bacteremia, to migrate to the brain and their effect in the acute phase of EAE. The *S. aureus* strains ATCC 51650 that is a TSST-1 producer (TSST-1⁺) and ATCC 43300 that does not produce any SAg (TOX⁻) were used in this work. Both *S. aureus* strains were capable to cause bacteremia and migrate of the brain, however, only TOX⁻ group caused inflammation in the brain. Both *S. aureus* strains were able to decrease the severity of EAE during the acute phase, but TSST-1⁺ strain triggered a more accentuated protective effect. Protective activity of both *S. aureus* strains was associated with local and peripheral cytokine modulation. In the second manuscript we investigated if the protective effect of the previous infection with *S. aureus* remained during the chronic disease phase. The results indicated that protection persisted during chronic EAE phase and was characterized by

lower disease incidence, smaller body weight loss and also reduced clinical scores. The effect of the TSST-1+ was also more accentuated in this phase. Modulation of local and peripheral cytokine production was also triggered by both strains but only the TOX- strain downmodulated the percentage of regulatory T cells in the CNS. In the last work we demonstrated that *S. aureus* ATCC 14458, that is a SEB producer strain (SEB+), was able to partially avoid EAE progression. Previous infected animals presented reduction in body weight loss, in clinical scores and also in disease incidence. They also showed less inflammation in the CNS. This protective activity was associated with reduced production of IL-17 by spleen cell culture. These results demonstrated that previous infection with different *S. aureus* strains partially avoided EAE development in C57BL/6 mice. A deeper investigation to understand the immunological mechanisms involved in this protective ability will be necessary to eventually result in a preventive measure against MS.

Key-words: experimental autoimmune encephalomyelitis, autoimmunity disease, *S. aureus*, superantigens

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 com início das manifestações clínicas entre 20 a 40 anos de idade (Hohlfeld, 2009, Mulvey et al., 2010). 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 proporção 2:1 (WHO, 2006; Constantinescu et al., 2011). 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). O tratamento da EM é realizado com corticosteróides, drogas imunomoduladoras como o IFN- β e acetato de glatiramer e anticorpos monoclonais (Constantinescu et al., 2011).

Causa e patogênese da EM não são completamente conhecidas, mas ainda se acredita que esta seja, fundamentalmente, uma doença autoimune mediada por células Th1 com especificidade para antígenos do SNC. As citocinas como TNF- α , IFN- γ e IL-17 desempenham um papel importante no processo inflamatório e consequente degeneração axonal, morte dos oligodendrócitos e disfunção neuronal (Steinman 2001; Elloso et al., 2005). As principais características histopatológicas da EM são presença de infiltrado inflamatório composto por linfócitos T e B e macrófagos; desmielinização ocasionada pela destruição da bainha de mielina ou pela morte dos oligodendrócitos, células responsáveis pela produção de mielina;

dano ou perda dos axônios; além da gliose, que se caracteriza por aumento do número de células da glia na substância branca em resposta ao dano no SNC (Constantinescu et al., 2011).

A causa exata da EM permanece desconhecida, entretanto existem evidências de que fatores genéticos e ambientais contribuam para o desenvolvimento da doença. Evidências epidemiológicas indicam que infecções bacterianas e virais contribuem para o desenvolvimento da EM. Os patógenos podem induzir autoimunidade através do fenômeno denominado mimetismo antigênico, que ocorre quando as células T específicas para peptídeos derivados de patógenos possuem reatividade cruzada com auto-antígenos (Sospedra & Martin, 2005). A imunopatogênese da EM também tem sido atribuída a defeitos na atividade funcional de células T reguladoras (CD4+CD25+Foxp3+). 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 (Sakaguchi et al., 1995; Tang et al., 2004). Apesar de alguns trabalhos mostrarem níveis normais dessas células em pacientes com EM (Venken et al., 2006) tem sido descrita redução na atividade funcional das mesmas *in vitro* (Huan et al., 2005).

1.2 – Encefalite autoimune experimental

A encefalite autoimune experimental (EAE) é uma doença desmielinizante do SNC cujas características clínicas e histológicas se assemelham às encontradas na EM, sendo utilizada como modelo experimental para o estudo da doença humana. Esta doença é induzida principalmente em camundongos e ratos 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) (Libbey & Fujinami, 2011).

A EAE é mediada por células T específicas para mielina que são ativadas na periferia e migram para o SNC após a permeabilização da barreira hemato-encefálica. No SNC estes linfócitos T são reativados por contato com as células apresentadoras de antígenos (APCs) presentes no local, resultando em processo inflamatório, desmielinização e dano axonal. Dependendo do protocolo de imunização e das características genéticas da cepa de camundongo utilizada, a EAE pode ser caracterizada como uma doença aguda monofásica, crônica progressiva ou com um decurso clínico composto por recidivas e remissões (Fletcher et al., 2010). 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).

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 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.

Os primeiros sintomas clínicos da doença são geralmente observados dentro de 9 a 12 dias após a indução, no entanto, o decurso clínico subsequente depende da espécie que está sendo estudada e do tipo de imunização (Gold et al., 2006). 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 o 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). As lesões são caracterizadas por áreas confluentes de infiltrado inflamatório mononuclear, composto principalmente por macrófagos e linfócitos T CD4+ e desmielinização na substância branca periférica (Day, 2005). Modelos experimentais empregando ratos e camundongos são amplamente empregados em estudos de imunopatogênese e desenvolvimento de novas terapias ('t Hart et al., 2011).

1.3 – Imunopatogênese da EAE

A EAE tem sido classicamente 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). A contribuição das células Th1 na EAE foi comprovado pela observação de que camundongos *knockout* para IL-12p40 eram resistentes à indução da EAE. Como a IL-12 é necessária na diferenciação do padrão Th1, esses camundongos não produziam IFN- γ e outras citocinas associada com este perfil. Altos níveis de IFN- γ são detectados no CNS durante a fase aguda da EAE, havendo um declínio na fase de recuperação, o que comprova a participação dessa citocina na patogênese dessa doença.

Mais recentemente, ficou demonstrado que outros tipos de células T efetoras tais como Tc e Th17 também podem contribuir para o desenvolvimento desta patologia. A expressão de IL-17 tem sido associada com diversas doenças autoimunes como artrite

reumatóide, lúpus eritematoso sistêmico, psoríase e EM (Matusevicius et al., 1999; Kolls & Lindén, 2004). Diversos estudos indicam que a IL-17 tem um papel relevante tanto na EM como na EAE. Em modelo experimental foi observado que animais deficientes em IL-17 desenvolvem EAE mais tardiamente e com sintomatologia mais branda (Komiya et al., 2006). Além disso, tem sido constatado que infiltração de células T e inflamação no SNC de animais com EAE só ocorre 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). A principal função da IL-17 na imunopatogênese da EAE é a sua capacidade de interferir na barreira hemato-encefálica. Recentemente, Huppert e colaboradores em 2010 demonstraram que a IL-17 estimula a produção de espécies reativas de oxigênio nas células endoteliais do cérebro e desta forma, ativa a contração de células endoteliais acarretando maior permeabilidade da barreira hemato-encefálica. Além disso, as espécies reativas de oxigênio aumentam a expressão de moléculas de adesão endoteliais resultando em maior migração de células inflamatórias através desta barreira.

Ainda não se sabe qual subtipo de linfócito T CD4 é mais importante na patogênese da EAE, visto que tanto camundongos deficientes em ROR γ t como em T-bet são resistentes à indução da EAE, confirmando que tanto linfócitos Th17 quanto Th1 estão envolvidos no processo de desmielinização do SNC (Bettelli et al., 2004; Ivanov et al., 2006). Além disso, pesquisadores caracterizaram o fenótipo das células T infiltrantes no SNC de animais com EAE e verificaram a presença tanto de células Th1 quanto Th17 (Korn et al., 2007; Langrish et al., 2005).

O que dificulta ainda mais a distinção do papel imunopatogênico do IFN- γ e da IL-17 na EAE é a plasticidade do fenótipo Th17. As células Th1 são relativamente estáveis

enquanto as células Th2 podem alterar a sua configuração para um fenótipo Th1 dependendo do microambiente (Lee et al., 2009). As células Th17 também parecem ser pouco estáveis podendo co-expressar IFN- γ e IL-17 ou expressar apenas IFN- γ (Flecher et al., 2010). Células que secretam ambas as citocinas são frequentemente detectadas no SNC de camundongos com EAE (Abromson-Leeman et al, 2009).

É possível que o desenvolvimento da EM esteja relacionado com defeitos na atividade funcional de células T reguladoras. Em 1995, Sakaguchi e colaboradores, 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 de então estas células 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).

As células T reguladoras CD4⁺CD25⁺ também expressam o gene regulador Foxp3 que codifica o fator de transcrição Foxp3; este 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 (Brunkow et al., 2001) ressalta a importância desse fator como fundamental no desenvolvimento destas células. Atualmente, o Foxp3 é considerado o melhor marcador para células T reguladoras de camundongos.

As células T reguladoras são classificadas como naturais ou adaptativas. As células T reguladoras naturais (T CD4⁺CD25⁺Foxp3⁺) são originadas no timo, enquanto as induzidas são geradas na periferia. A conversão de células TCD4 *naive* 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 e colaboradores em 2009, a expressão de Foxp3 é observada nas células Th3, mas não nas células Tr1, no entanto outros pesquisadores afirmam que as células reguladoras Th3 e Tr1 não expressam Foxp3 (Curotto de Lafaille & Lafaille, 2009).

Os mecanismos de supressão empregados pelas células T reguladoras podem ser divididos em quatro tipos distintos. O primeiro é a supressão mediada por citocinas inibitórias, como IL10, TGF- β e IL-35. O segundo é a supressão mediada por citólise que é dependente de granzima A e perforina nos humanos e de granzima B em camundongos. O terceiro mecanismo é a supressão por distúrbio metabólico, como é o caso, por exemplo, da apoptose da célula efetora por privação de IL-2 (Vignali et al., 2008; Workman et al., 2009). O último mecanismo envolve 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. Se sabe atualmente que tanto as células T reguladoras naturais quanto as adaptativas regulam o processo inflamatório na EM e na EAE. Estudos realizados na EAE induzida por MOG mostraram que a transferência de células T CD4⁺ CD25⁺ reduziu a gravidade da doença e que as mesmas suprimiram as células específicas para MOG *in vitro* (Kohm et al., 2002). Esse efeito regulador é mediado por IL-10, visto que em animais *knockout* para IL-10 a transferência de células T reguladoras não conferiu proteção na EAE (Zhang et al., 2004).

Quando se avalia o decurso clínico natural da EAE se observa acúmulo de células T reguladoras no SNC durante a fase de recuperação da doença e a sua depleção inibe a resolução da sintomatologia dos animais (McGeachy et al., 2005). A fase de recuperação da EAE tem sido também associada à indução de TGF- β (Zhang et al., 2006) e IL-10 (McGeachy et al., 2005), sendo que essas citocinas podem ser produzidas tanto por células T reguladoras naturais quanto por células do perfil Tr1. Um dado interessante foi publicado por Korn e colaboradores, em 2007, demonstrando que embora células T reguladoras estejam presentes no SNC durante o pico da EAE, essas células são incapazes de suprimir totalmente as células T efetoras presentes no local. Esses estudos sugerem que a autoimunidade depende de um balanço delicado entre as respostas de perfil regulador e inflamatório. Neste contexto, durante o início da doença predominaria uma resposta inflamatória enquanto que na fase de resolução haveria um aumento das células T reguladoras.

1.4- *Staphylococcus aureus* e superantígenos

Membros do gênero *Staphylococcus* são cocos Gram-positivos que se apresentam isolados ou em arranjos irregulares lembrando cachos de uva. São imóveis, não formadores de esporos, catalase positivos e a maioria das espécies é anaeróbia facultativa (Larkin et al., 2009). O gênero *Staphylococcus* é composto de 40 espécies, sendo que a maioria delas é coagulase-negativa. A síntese de coagulase ocorre nas espécies *S. aureus*, *S. schleiferi* subsp. *coagulans*, *S. intermedius*, *S. hyicus* e *S. delphini* (Trülsch et al., 2002). Esses microrganismos se encontram amplamente distribuídos na natureza, sendo principalmente encontrados na pele e nas membranas mucosas de mamíferos e animais (Larkin et al., 2009). A espécie *S. aureus* é uma bactéria oportunista que coloniza principalmente o interior

das narinas em mais de 20% da população (Foster, 2005). Essa relação geralmente é benigna ou simbiótica com seus hospedeiros. Contudo se a barreira cutânea for rompida por trauma, por exemplo, esses microrganismos podem apresentar comportamento patogênico (Larkin et al., 2009). Os *S. aureus*, em especial as cepas resistentes à meticilina (MRSA), são os que mais se destacam como causadores de morbidade e mortalidade em humanos (Larkin et al., 2009; Watkins et al., 2012). Essa espécie causa inúmeras doenças tais como intoxicação alimentar, infecções de pele, osteomielite, endocardite, pneumonia, enterocolite, choque tóxico, bacteremia, miocardite, meningite e recentemente vem sendo também descrita como responsável pelo desencadeamento de doenças autoimunes (Larkin et al., 2009).

Apesar de ser combatida por barreiras como pele e mucosas e também por uma série de mecanismos imunológicos, tanto não específicos quanto específicos, o *S. aureus* dispõe de uma série de mecanismos de escape que lhe permitem evadir às defesas do hospedeiro. Os principais mecanismos de evasão descritos incluem inibição de quimiotaxia de neutrófilos (Murdoch & Finn, 2002); liberação de toxinas que matam os leucócitos (Montoya & Gouaux, 2003); resistência à fagocitose (Palmqvist et al, 2002); inativação do sistema complemento (Rooijackers et al, 2005) e liberação de moléculas imunomoduladoras as quais podem determinar imunossupressão (Foster, 2005). Entre estas moléculas associadas com os mecanismos de escape estão descritas várias toxinas. Entre estas toxinas se destaca um grupo de enterotoxinas que podem causar síndromes semelhantes a choques e lesões gastrointestinais. Uma característica muito interessante destas moléculas é que as mesmas podem atuar como superantígenos (SAGs), ou seja, são capazes de estimular um grande número de células T, independentemente de suas especificidades (Foster, 2005; Ortega et al., 2010).

Os SAGs bacterianos são proteínas produzidas predominantemente por *S. aureus* e *Streptococcus piogenes* as quais estão envolvidas em várias patologias (McCormick et al., 2001; Ortega et al., 2010). SAGs diferem dos antígenos convencionais em vários aspectos. Antígenos convencionais precisam ser inicialmente processados de tal forma que sejam gerados peptídeos. Estes peptídeos são encaixados na fenda presente em moléculas de histocompatibilidade (MHC) de classe I ou de classe II e este complexo é então apresentado aos linfócitos T específicos. Os linfócitos T, por sua vez, utilizam a região variável de seus receptores para antígeno (TCR) para reconhecer esta associação entre peptídeo e MHC. De forma diferente, os SAGs atuam como moléculas intactas e se ligam diretamente nas moléculas de MHC de classe II expostas na superfície de células apresentadoras de antígeno (APCs), sem que ocorra um processamento prévio (Mollick et al., 1989). Os SAGs se ligam às moléculas de classe II em um local distinto da fenda no qual se liga o peptídeo de antígeno convencional (Russel et al., 1991). Este complexo SAG/MHC II interage diretamente com a região variável da cadeia β ($v\beta$) do TCR determinando ativação das APCs e dos linfócitos (Callahan et al., 1990). Os linfócitos ativados desta forma podem produzir citocinas, se tornar anérgicos ou sofrer um processo de apoptose (Lin et al., 2007; Arad et al., 2011; Lee et al., 2012). Teoricamente esta resposta exacerbada pode desencadear desenvolvimento de doenças autoimunes ou então agravar a sintomatologia já existente (Krakauer, 1999; Mulvey et al., 2010).

Os SAGs bacterianos incluem as enterotoxinas estafilocócicas (ES) e a toxina 1 da síndrome do choque tóxico (TSST-1) (Lina et al., 2004). Existe uma variedade de ES (A, B, C, D, E, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V e X) e elas são

definidas pela sua capacidade de causar patologias como intoxicação alimentar (Mulvey et al., 2010).

As características dos SAGs SEB e da TSST-1 que possam justificar o estudo de sua contribuição na EAE são sucintamente descritas abaixo. A SEB é constituída por um único polipeptídeo de 28000 Da enovelado sob a forma de dois domínios (Swaminathan et al., 1992). Devido à sua estrutura terciária muito compacta, esta enterotoxina é altamente resistente à clivagem por proteases. Classicamente a SEB tem sido associada com intoxicação alimentar (Mantis, 2005). Entretanto, mais recentemente, tem sido demonstrado que a mesma pode desencadear ativação sistêmica acentuada do sistema imunológico quando administrada por outras vias tais como nasal, conjuntiva ou através do canal vaginal (Rajagopalan et al., 2007). Em termos de interação com as células T, a SEB reconhece as seguintes subclasses de $v\beta$ humanos: $v\beta 3$, 12, 13.2, 14, 15, 17 e 20 (Mantis et al., 2005). Uma avaliação sistemática do efeito da SEB em doenças autoimunes não tem sido realizada. Entretanto, alguns trabalhos mostram o efeito preventivo desta ou de outras enterotoxinas estafilocócicas. Por exemplo, Kawamura e colaboradores em 1993, constataram que a administração de várias enterotoxinas de *S. aureus* preveniu o diabetes em camundongos NOD. Os autores sugeriram que este tratamento induziu células T reguladoras. Gonzalo et al., 1994, também observaram que uma única inoculação de enterotoxina B foi capaz de reduzir a autoimunidade em camundongos MRL/lpr. Por outro lado, alguns protocolos estão associados com indução de autoimunidade. Ellerman & Like, 1992, demonstraram que células esplênicas estimuladas com enterotoxinas estafilocócicas foram capazes de transferir diabetes adotivamente. A SEB também desencadeou agravamento da

uveoretinite experimental via ativação de células T CD4⁺ vβ 8 (+) (Kohno et al., 2009), e causou recidiva da EAE (Constantinescu et al., 1998).

A TSST-1 é traduzida como uma proteína precursora contendo 234 aminoácidos e secretada após a clivagem de uma sequência aminoterminal de 40 aminoácidos. A proteína madura é constituída por uma única cadeia polipeptídica com um peso molecular de 22000 Da. Apesar de conter uma alta porcentagem de aminoácidos hidrofóbicos, a TSST-1 é hidrossolúvel e geralmente também resiste ao calor e proteólise (Dinges et al., 2000; Larkin et al., 2009). A TSST-1 é um potente SAg que interage com a subpopulação de células T vβ 2 e 4 sendo a causa mais comum da Síndrome do Choque Tóxico (TSS). A TSS é uma doença aguda e potencialmente fatal, caracterizada por febre alta, *rash* difuso escamoso, hipotensão e envolvimento de três ou mais órgãos (Larkin et al., 2009). Esta toxina é produzida por aproximadamente 20% das amostras de *S. aureus* isoladas de pacientes humanos. Uma possível participação da TSST-1 nas patologias autoimunes tem sido avaliada de forma mais direta só em modelos de artrite. Por exemplo, a cepa LS-1, que é produtora de TSST-1, é utilizada para desencadear artrite séptica quando administrada por via intravenosa em camundongos (Sakinienė & Tarkowski, 2002). A real contribuição da TSST-1 tem sido sugerida pela maior gravidade da artrite causada pelas cepas produtoras desta toxina, em comparação com as não produtoras (Abdelnour et al., 1994). Além disto, a TSST-1 acelera a artrite induzida por colágeno em camundongos DBA (Kageyama et al., 2001).

1.5- Efeito do *Staphylococcus aureus* na EM / EAE

Múltiplos fatores contribuem para o desenvolvimento da resposta imune contra antígenos próprios. Os fatores ambientais são responsáveis por até 70% do

risco de desenvolver EM e entre eles, destacam-se as infecções por vírus e bactérias que foram descritos como capazes de iniciar ou agravar esta patologia (Delogu et al., 2012).

Uma possível associação de *S. aureus* com EM foi inicialmente sugerida por Aasjord et al., 1986, pela demonstração de que alguns pacientes com EM apresentavam anticorpos específicos para antígenos estafilocócicos no líquido. Como descrevemos anteriormente, os estafilococos secretam moléculas denominadas SAGs capazes de causar doenças graves, em parte devido ao efeito sobre as células do sistema imunológico. A possibilidade de que estas moléculas estejam envolvidas no desencadeamento de doenças autoimunes como EM, granulomatose de Wegener e artrite reumatóide vem sendo investigada (Xu & McCormick, 2012). No caso específico da EM foi demonstrado, por exemplo, que superantígenos bacterianos são capazes de ativar e expandir clones de células T autorreativas para mielina (Burns et al., 1992). Além disto, foi observado que a enterotoxina B estafilocócica foi capaz de exacerbar e de causar recidivas em camundongos com EAE em remissão ou doença subclínica (Brocke et al., 1993). Estes efeitos têm, de forma geral, sido atribuídos à proliferação e produção de citocinas pelas células T ativadas. Recentemente foi descrito por Mulvey e colaboradores, em 2010, que *S. aureus* produtor de enterotoxina A (SEA) pode ser utilizado como um possível marcador para exacerbação da EM. Esta hipótese baseia-se em um estudo utilizando swabs nasais para a detecção de cepas de *S. aureus* produtoras de SEA, SEB e TSST-1 em pacientes portadores de EM. Os resultados demonstraram maior prevalência de SEA em pacientes que apresentaram recidiva da doença. Entretanto, também tem sido constatado que SAGs podem anergizar e deletar subpopulações de T com determinados domínios V β . Isto dependeria do momento de exposição ao

SAGs (Rellahan et al., 1990; Kawabe & Ochi, 1991). Por exemplo, recidivas de paralisia clínica podem ser induzidas pela administração das enterotoxinas A e B em camundongos previamente imunizados com MBP (Schiftenbauer et al., 1993; Brocke et al., 1993; Soos et al., 1995). Por outro lado, a administração de SEB antes da imunização com MBP preveniu o desenvolvimento da doença devido à anergia/deleção da subpopulação T V β 8 (Soos et al., 1993; Kalman et al., 1993).

Além destes efeitos mais conhecidos, relatos recentes revelam uma conexão muito interessante, mas bastante complexa, entre os SAGs e as células T reguladoras. Por um lado os SAGs parecem inibir a atividade imunorreguladora das células T. Por exemplo, SEB inibiu células Treg através da indução de uma proteína ligante de GITR (Cardona et al., 2006). Por si só isto já seria relevante no contexto das doenças autoimunes, mas foi constatado que este efeito ocorre juntamente com uma polarização no sentido Th17 (Zehn et al., 2007), cuja contribuição na patogênese da EM e EAE está bem estabelecida (Kolls & Lindén, 2004). Por outro lado, na dependência do protocolo empregado, os SAGs também são capazes de ativar células Tregs. Erokhmanoff e colaboradores, em 2009, mostraram indução de tolerância, mediada por aumento da frequência de células Foxp3+, após imunização repetida com SEB.

Só existem dois relatos antigos sugerindo uma possível conexão entre TSST-1 e EM. Stinissen e colaboradores, em 1995, constataram que células T $\gamma\delta$ isoladas de pacientes com EM eram ativadas e adquiriam atividades citotóxicas específicas em presença da TSST-1. Este grupo de pesquisadores também observou que células T específicas para mielina eram ativadas e sofriam expansão clonal na presença da TSST-1 e outros SAGs (Zhang et al., 1995).

1.6- Racional do projeto

Apesar da causa e da patogênese da EM não serem completamente conhecidas, sabe-se que fatores genéticos e ambientais são relevantes para o desenvolvimento desta patologia. Entre os fatores ambientais destacam-se as infecções por bactérias, vírus e outros patógenos. No contexto destas infecções destacam-se as causadas pelo *S. aureus*, pois este agente secreta uma série de substâncias, entre elas, os SAGs que podem afetar o desenvolvimento da EM. A literatura é escassa quanto ao efeito das cepas de *S. aureus* produtoras de SAGs na EM. Isto ocorre não só pela inerente dificuldade de se avaliar doenças humanas diretamente, mas também porque existem cepas de *S. aureus* produtoras de SAGs distintos e também cepas produtoras de vários SAGs ao mesmo tempo. Além disto, os poucos trabalhos disponíveis sugerem que este assunto é bastante complexo. Por estas razões, propomos neste trabalho, investigar o efeito de infecções prévias com 3 cepas distintas de *S. aureus* no desenvolvimento da EAE que é um modelo largamente utilizado para estudar EM. Nossa hipótese de trabalho prevê uma exacerbação da sintomatologia clínica associada com aumento do processo inflamatório no SNC.

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Objetivos

3 – OBJETIVOS

3.1 – Objetivo Geral

Avaliar o efeito da infecção com diferentes cepas toxigênicas de *S. aureus* nas características clínicas, imunológicas e histopatológicas da EAE.

3.2 – Objetivos Específicos

- 1- Avaliar o efeito da infecção prévia com uma cepa de *S. aureus* produtora de TSST-1 e outra cepa não produtora de toxinas no desenvolvimento da EAE (fase crônica da doença).
- 2- Avaliar o efeito da infecção prévia com uma cepa de *S. aureus* produtora de TSST-1 e outra não produtora de toxinas no desenvolvimento da EAE (fase aguda da doença).
- 3- Avaliar o efeito da infecção prévia com uma cepa de *S. aureus* produtora de SEB no desenvolvimento da EAE (fase aguda da doença).

Resultados e discussão

4 – RESULTADOS E DISCUSSÃO

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

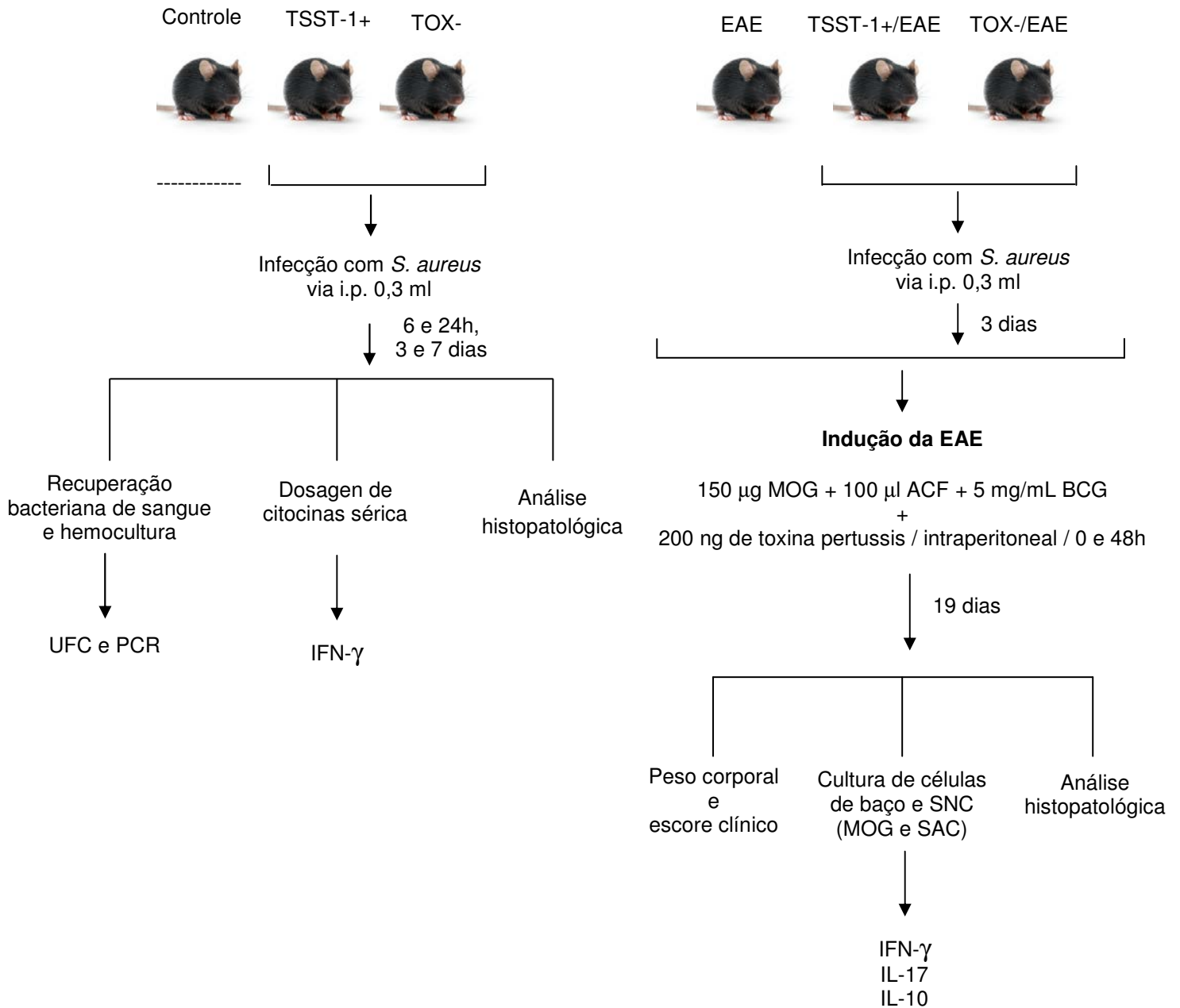
4.1- Artigo científico I: *Staphylococcus aureus* invades the brain but does not worsen experimental encephalomyelitis in C57BL/6 mice

4.2- Artigo científico II: Ability of *Staphylococcus aureus* to suppress Experimental Autoimmune Encephomyelitis is more accentuated in a TSST-1-positive strain

4.3- Artigo científico III: Previous infection with enterotoxin B-positive *S. aureus* strain decreases severity of encephalomyelitis in mice

Artigo Científico I

4.1 – Artigo científico I: *Staphylococcus aureus* invades the brain but does not worsen experimental encephalomyelitis in C57BL/6 mice



***Staphylococcus aureus* invades the brain but does not worsen experimental encephalomyelitis in C57BL/6 mice**

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Abstract

Staphylococcus aureus has been linked to autoimmune diseases mainly by its ability to secrete superantigens (SAGs). In this work we compared 2 *S. aureus* strains in terms of their infectivity to C57BL/6 mice and also their effect on experimental autoimmune encephalomyelitis (EAE). Initially C57BL/6 female mice were infected with *S. aureus* ATCC 51650 (TSST-1+) or ATCC 43300 (TOX-). Both infections caused bacteremia that was associated with weight loss and high seric levels of IFN- γ . Both strains reached the brain but only TOX- strain caused brain inflammation. We then tested the effect of these 2 strains on EAE progression. For this, C57BL/6 mice were infected and 3 days later they were submitted to EAE induction by immunization with MOG associated with Complete Freund Adjuvant (CFA). Both infections clearly affected EAE development. In previous infected mice the paralysis was less severe and disease incidence was also smaller. This protective effect was also associated with a less intense inflammation in the brain. Previous infection determined immunomodulation in cytokine production by both, local (central nervous system) and peripheral (spleen cells) cultures. These findings indicate that previous infection with these 2 *S. aureus* strains reached the brain but did not increased the severity of EAE.

Key-words: *Staphylococcus aureus*, superantigen, TSST-1, experimental autoimmune encephalomyelitis, inflammatory infiltrate

Introduction

Multiple sclerosis (MS) is a chronic neurological disease that is characterized by inflammation, demyelination and also by axonal deterioration (Constantinescu et al., 2011; Comabelle & Khoury, 2012). Although the etiology of MS is still unknown, many reports point towards a very prominent role of the immune system in the

pathogenesis of this disease (Hemmer et al., 2006). Th1 (IFN- γ producing CD4+ T cells) and Th17 (IL-17 producing CD4+ T cells) are considered to perform a critical role on the development of this disease (Chen et al., 2012). Much of the available knowledge on MS is coming from its corresponding model designated experimental autoimmune encephalomyelitis (EAE). Even though EAE is considered a complex model, it is widely accepted that in terms of immuno and neuropathological mechanisms it resembles much of the inflammation, demyelination, axonal loss and gliosis that characterize MS (Mix et al., 2010; Constantinescu et al., 2011). In addition to genetic factors, environmental circumstances play a major role on autoimmunity triggering. Special emphasis is being given to the contribution of infections by virus, bacteria and other pathogens (Delogu et al., 2011). Mechanisms by which an infection can lead to an autoimmune pathology have been extensively investigated in experimental models. Briefly, the main mechanisms for activation of self-reactive T and B lymphocytes during infections include molecular mimicry, activation of autoreactive T cells by bacterial or viral superantigens (SAGs), enhanced processing and presentation of self-antigens, bystander activation at inflammatory sites and lymphocyte activation by lymphotropic viruses (Wucherpfennig, 2001). *Staphylococcus aureus* has been linked to autoimmune diseases mainly through its ability to produce superantigens (Sags). *S. aureus* enterotoxins (A, B, C, D, E and Toxic shock syndrome toxin 1) are the prototypic SAGs and were the first molecules of this family to be more deeply characterized (Torres et al., 2001). SAGs bind directly to MHC class II molecules on the surface of antigen presenting cells being this binding at a site outside the antigen-binding groove (Russell et al., 1990). This complex MHC/SAGs is able to directly interact with the beta chain ($\nu\beta$) of the T cell receptor on T cells. This interaction is followed by T cell activation (Callahan et al.,

1990). As is well known there are many distinct $v\beta$ families. Interestingly, different SAGs bind and activate different $v\beta$ subsets. For example, TSST-1 from *S. aureus* interacts with human T cells bearing $v\beta$ 2 whereas staphylococcal enterotoxin B (SEB) activates human T cells expressing $v\beta$ 3, 12, 14, 15, 17 and 20 (Marrack et al., 1990). The contribution of *S. aureus* TSST-1 to MS or EAE development is not thoroughly investigated. Zhang et al., 1995, evaluated the reactivity of myelin specific T cells, obtained from MS patients and also healthy donors, to SEA, SEB and TSST-1. The majority of the clones responded to at least one of the tested SAGs. The clones reactive to SEA and SEB expressed various $v\beta$ genes whereas T cell clones reactive to TSST-1 correlated with the $v\beta$ 2 expression. In this context, the main objectives of this study are to compare 2 *S. aureus* strains in terms of infectivity to C57BL/6 mice and also their effect on EAE development. The main difference between the strains is that one of them produces TSST-1 whereas the other does not produce any SAG.

Material and Methods

Experimental design

In the first step of this work C57BL/6 mice were infected with *S. aureus* strains ATCC 51650 (TSST-1+) or ATCC 43300 (TOX-) and the presence of bacteria and IFN- γ seric levels were tested 6 and 24 hours and also 3 and 7 days after infection. Fourteen days after infection the animals were perfused and the presence of bacteria and inflammatory infiltrates were evaluated in the brain. In the second step, we tested the effect of previous infection with these 2 strains on EAE development. C57BL/6 mice were then infected and 3 days later they were submitted to EAE induction by immunization with MOG emulsified with complete Freund's adjuvant

(CFA). The effect in EAE development was evaluated by clinical follow-up (weight variation and clinical score) and also by histopathological analysis of the brain. The immunoregulatory effect of the infection was checked by cytokine production by spleen cells and also by cells isolated from the CNS stimulated with MOG or *Staphylococcus aureus* Cowan I (SAC).

Animals

Female C57BL/6 mice (4-6 weeks old) were purchased from CEMIB (UNICAMP, São Paulo, SP, Brazil). The animals were fed with sterilized food and water *ad libitum* and were manipulated in accordance with the ethical guidelines adopted by the Brazilian College of Animal Experimentation. All experimental protocols were approved by the local Ethics Committee (Ethics Committee for Animal Experimentation, Bioscience Institute, Univ. Estadual Paulista).

Bacterial suspension

Two *Staphylococcus aureus* strains obtained from the American Type Culture Collection (ATCC) were used in this work. The ATCC 51650 strain is characterized by TSST-1 production while the ATCC 43300 does not produce any toxin. These strains were cultured and incubated at 37°C for 24h. Isolated colonies were inoculated into brain heart broth (BHI, Merck) and incubated at 37°C for 24h. Bacteria were collected by centrifugation, washed three times and resuspended in cold sterile saline as previously described by França et al., 2009. The bacterial suspension was spectrophotometrically adjusted to 0.3 at 520 nm, as described by Nakane et al., 1996. The animals were infected by intraperitoneal route with 300µl of the bacteria suspension containing 10^7 - 10^{12} CFU/ml.

Quantification of bacteria

Bacteria were quantified in the blood by determining the number of colony forming units (CFU) and also by PCR. CFU were directly determined in blood samples that were inoculated in baird parker agar and incubated at 37 °C. The number of CFU was stimulated by standard plating procedures. For PCR analysis, blood samples were inoculated in brain heart infusion (BHI) broth (hemoculture) and incubated for 7 days at 37 °C. DNA was then extracted using the GFX kit (Amersham Pharmacia Biotech) as described by Pereira et al., 2009. PCR was carried out in 0.5 ml microcentrifuge tubes in a total volume of 25 µl containing 1 µM of each primer, 2.0 U Taq polymerase, 100 µM deoxyribonucleotide triphosphates, and 150 µg nucleic acid. The two *S. aureus*-specific primers were used: Sa442-1 (5'-AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG-3') and Sa442-2 (5'-CGT ATT GAG ATT TCA GTA GAT AAT ACA ACA-3'). The size of the amplified product is 102 bp. PCR was carried out in an appropriate thermocycler using the following parameters as described by Martineau et al., 1998, 3 min at 96 °C and then 30 or 40 cycles of 1 second at 95 °C for the denaturation step and 30 seconds at 55 °C for the annealing-extension step. The efficiency of the amplification reactions was evaluated by electrophoresis on 2% agarose gel prepared in 0.5X TBE buffer and stained with ethidium bromide. The size of the amplified products was compared with the 100-bp standard and the gels were photographed under UV illumination.

EAE induction

MOG35–55 peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Proteimax, São Paulo, Brazil. EAE was induced as previously described (Peron et al., 2010). Briefly, mice were immunized subcutaneously with 150µg of MOG35–55 peptide emulsified with CFA containing 400 µg of BCG. Mice also received 2 doses,

0 and 48 h after immunization, of 200 ng of *Bordetella pertussis* toxin (Sigma) intraperitoneally. Animals were daily inspected and disease intensity was graded as: 0 - no disease, 1 - limp tail, 2 - weak/partially paralyzed hind legs, 3 - completely paralyzed hind legs, 4 - complete hind and partial front leg paralysis, 5 - complete paralysis/ death.

Isolation of mononuclear cells infiltrated in the CNS

Mice were anesthetized with ketamine/xylazine and perfused with 10 mL of saline solution. Brain and cervical spinal cords were excised, macerated and maintained in 4 mL of RPMI (Sigma) supplemented with 2.5% collagenase D (Roche®) at 37°C, 5% CO₂ incubator. 45 min later suspensions were washed in RPMI and centrifuged at 450xg for 15 min at 4°C. Cells were then resuspended in percoll (GE Healthcare) 37% and gently laid over percoll 70% in tubes of 15 mL. The tubes were centrifuged at 950xg for 20 min with centrifuge breaks turned off. After centrifugation the ring containing mononuclear cells was collected, washed in RPMI and centrifuged at 450xg for 5 min. Cellular pellets were then resuspended in complete RPMI medium and adjusted 2x10⁵ cells/ml. Cytokine levels were evaluated 48 hours by enzyme-linked immunosorbent assay (ELISA) in supernatants from cultures stimulated *in vitro* with MOG (50µg/ml) and SAC (1/2500). IFN-γ and IL-10 BD OptEIA Sets (Becton Dickinson) and IL-17 Duoset (R&D Systems, Minneapolis, MN, USA) were performed according to the manufacturer's instruction.

Spleen cell cultures

Mice were submitted to EAE induction and evaluated 19 days after. Spleen cells were collected and adjusted to 5x10⁶ cells/ml. Cells were cultured in complete RPMI medium (RPMI supplemented with 5% of fetal calf serum - FCS, 20mM glutamine and 40 IU/ml of gentamicin) in the presence of MOG (20 µg/ml) or SAC

(PANSORBIN[®], EMD) (1/2500). Cytokine levels were evaluated 48 h later by enzyme-linked immunosorbent assay (ELISA) in culture supernatants the kits described above.

Evaluation of inflammatory infiltrates in the CNS

Histological analysis was performed in the brain during 2 time periods: 14th in infected animals and 19th day in animals with EAE that were previously infected. After euthanasia and blood withdrawal, brain samples were removed and fixed in 10% formaldehyde. Tissues were dehydrated in graded ethanol and embedded in a 100% paraffin block. Five micron thick sections were mounted over glass slides, stained with hematoxylin and eosin and then analyzed with a Nikon microscope. Presence of bacteria in the brain was evaluated in brain imprints submitted to Gram staining.

Statistical analysis

Data were expressed as mean \pm SE. Comparisons between groups were made by one way ANOVA with post-hoc Holm-Sidak method for parameters with normal distribution, and by Kruskal-Wallis post-hoc Dunn's method or Tukey test for parameters with non-normal distribution. The disease incidence was evaluated by Chi-square test ($p=0,008$). Significance level was $p<0.05$. Statistical analysis was accomplished with SigmaStat for Windows v 3.5 (Systat Software Inc).

Results

Bacterial recovery from C57BL/6 mice infected with *S. aureus* strains

Bacterial recovery from blood samples was analyzed by PCR and CFU. PCR assay was initially performed with DNA directly extracted from blood samples. As they resulted negative they were carried out again from hemocultures of the infected animals. PCR was done with a genus primer identified as SA442 that allows the

production of a 102bp band that was present in all samples collected 6 and 24 hours after infection in comparison to a control sample collected from a normal mouse (sample 16 in the picture), as shown in figure 1a. However, this band was not found in samples obtained 3 and 7 days after infection (not shown). The presence of bacteria was also checked by determination of CFU directly from blood samples. As shown in figure 1b, the two strains were isolated from blood after 6 and 24 hours and also after 3 and 7 days of infection.

Weight loss and IFN-gamma seric levels in infected mice

As can be observed in figure 2a, animals infected with anyone of the two strains lost body weight. This loss was more accentuated during the 2nd and 3th days of infection. Six to 7 days after infection the animals already recovered their original weight. The highest percentage of weight loss presented by each strain is illustrated in figure 2b. No IFN- γ was detected in blood samples from non-infected mice (not shown). Analysis made 6 hours after infection showed that IFN- γ levels were significantly higher in TSST-1+ infected mice in comparison to TOX- infected ones. However, the levels of this cytokine were similar at the other time periods of evaluation (24h, 3 and 7 days). IFN- γ production steadily decayed from 24h to 7 days. These results can be observed in figure 2c.

Presence of *S. aureus* and inflammatory infiltrates in the brain

Fourteen days after i.p. infection mice were perfused with buffered saline solution to exclude blood from the CNS. Brain imprints stained with GRAM showed structures very similar to GRAM positive bacteria in TSST-1+ infected (figure 3c and d) and also in TOX- (figure 3e and f) infected animals. These structures were not found in non-infected mice (figure 3a and b). Even though typical of *S. aureus* growth, these structures seemed partially destroyed. In brain sections stained with H&E there were

no signs of inflammation in normal (figure 4a and b) and also in mice previously infected with the TSST-1 strain (figure 4c and d). Contrarily, an exuberant inflammatory infiltrate was detected in mice infected with the TOX- strain (figure 4e and f).

Acute disease is reduced by previous infection

The EAE control group comprising C57BL/6 mice immunized with MOG showed, as expected, a very elevated degree of paralysis. These animals reached an average clinical score of 2.5 at the peak of the disease that occurred around days 16 and 17 after EAE induction. Previous infection with both strains clearly reduced these clinical scores being the effect of the TSST-1+ strain more accentuated. These findings can be observed at figure 5a and b. Disease incidence followed this same pattern. At day 19 disease incidence was 92, 69 and 33% in EAE, TOX-/EAE and TSST-1+/EAE groups, respectively, as showed in table 1.

Inflammation at the brain during the acute phase

The degree of brain inflammation was visually inspected. As shown in figure 6b, a conspicuous inflammation with the characteristic perivascular localization was observed in the EAE control group. Infection with both *S. aureus* strains before EAE induction determined a partial reduction in inflammation as shown in figures 6c and 6d for TSST-1+ and TOX-, respectively. No inflammatory infiltrates were observed in control normal animals (figure 6a).

Cytokines production

Stimulation with MOG determined similar cytokine profiles in cultures from spleen and CNS infiltrating cells. Previous infections with *S. aureus* strains did not change IFN- γ and IL-10 production. However, the production of IL-17 was lower in previously infected animals. Even though both strains downmodulated the production of this

cytokine, only animals infected with the TOX- strain showed a significant decrease in IL-17 production. In spleen cell cultures stimulated with SAC, production of IFN- γ and IL-10 was higher in mice previously infected and IL-17 production was lower in mice infected with TOX- strain (figure 8). In CNS infiltrating cell cultures stimulated with SAC, production of IFN- γ was also higher in previously infected mice (figure 7). In these conditions there was no difference in IL-17 production and the levels of IL-10 were lower in previously infected mice, even though without statistical difference.

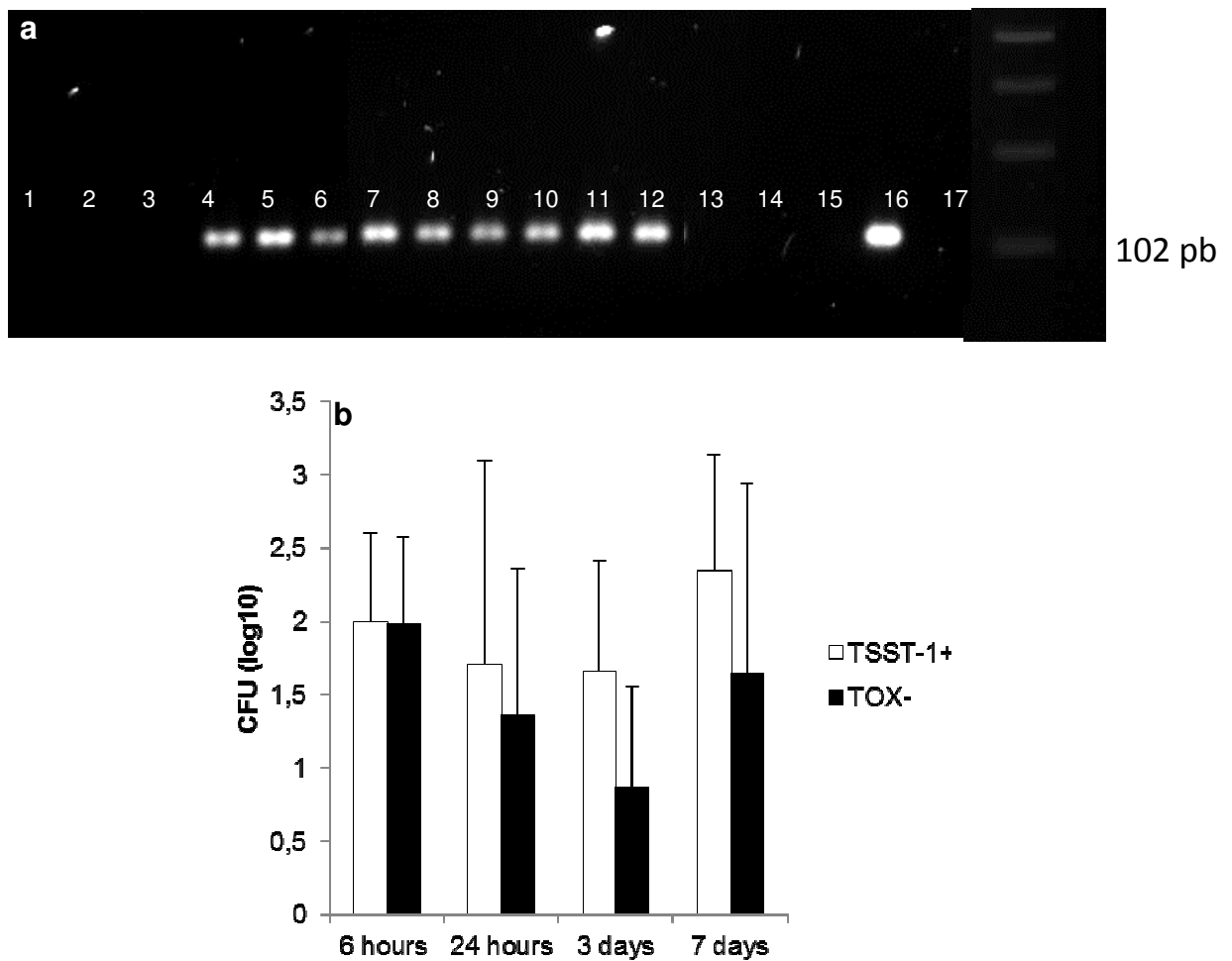


Figure 1: Bacterial recovery in C57BL/6 mice infected with *S. aureus*. PCR analysis in hemoculture samples evaluated 6h after infection (a). Non infected animals (1-3), infected with *S. aureus* TSST-1+ (4-6) and infected with *S. aureus* TOX- (7-9). PCR analysis in hemoculture samples evaluated 24h after infection. Animals infected with *S. aureus* TSST-1+ (10-12) and infected with *S. aureus* TOX- (13-15) in comparison to positive control (16) and negative control (H₂O - 17). CFU determination in blood samples from animals infected with different *S. aureus* strains (b).

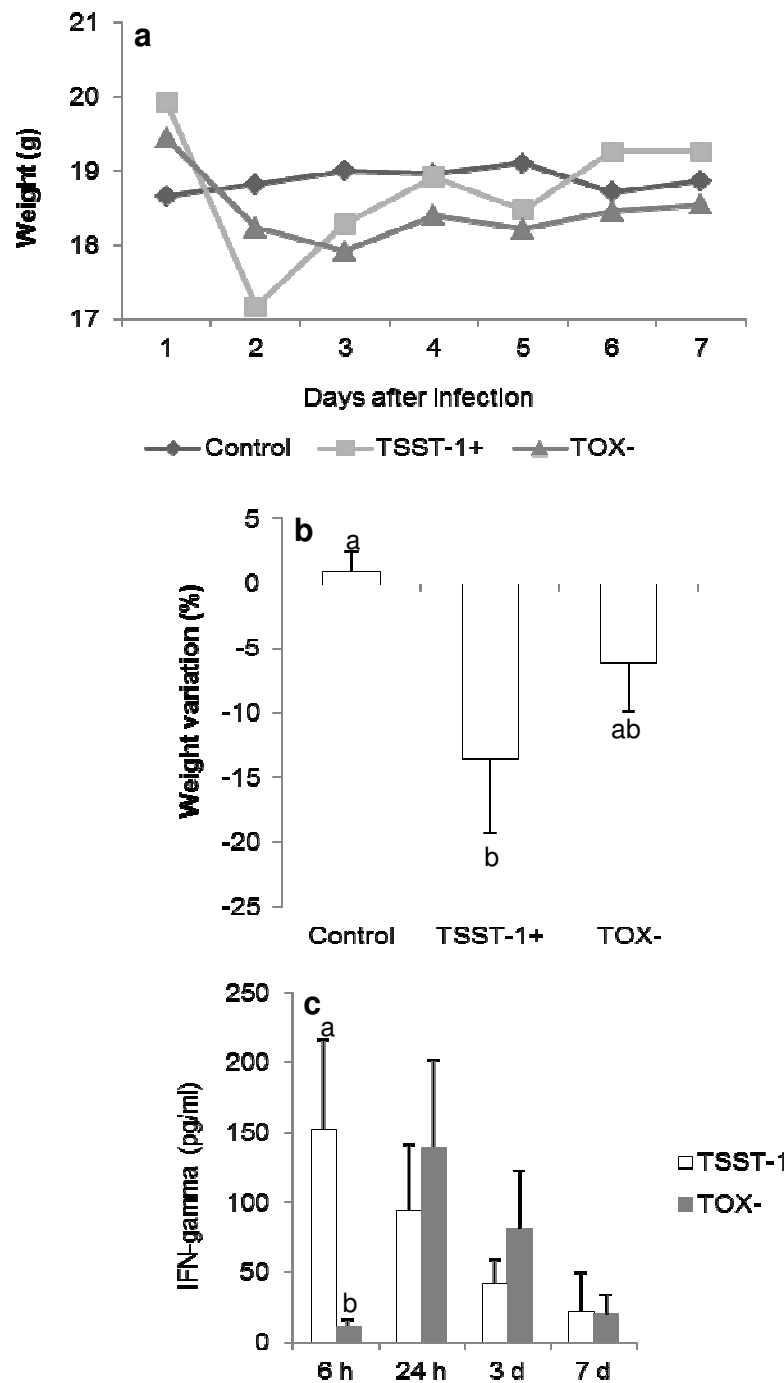


Figure 2: Body weight and IFN- γ seric levels in C57BL/6 mice infected with *S. aureus* strain. C57BL/6 were infected with 2 distinct *S. aureus* strains by i.p. route. Body Weight was daily evaluated (a); weight variation at 2nd day (b). IFN- γ levels evaluated during in different periods (c). Data were presented by mean \pm SE of 4-10 mice and representative of two independent experiments, $p < 0.05$.

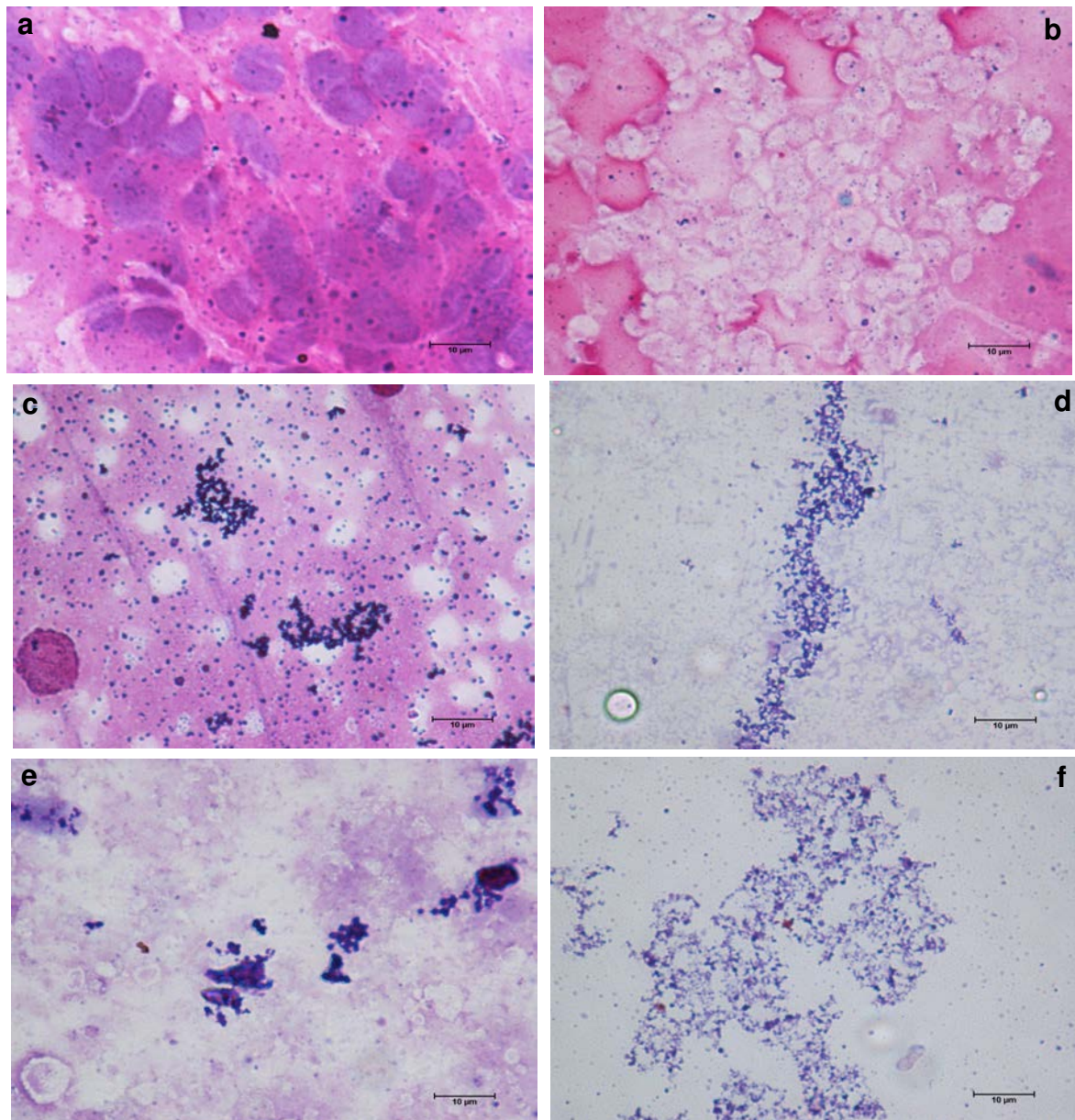


Figure 3: Dissemination of *S. aureus* to the brain. C57BL/6 mice were submitted to infection with 2 distinct *S. aureus* strains. The presence of bacteria in the brain was analysed in brain imprints stained with Gram. Non-infected mice (a, b), mice infected with TSST-1+ (c, d) and mice infected with TOX- (e, f) were evaluated 14 days after of infection. Pane shows samples obtained from 2 animals. Other mice from each showed similar results.

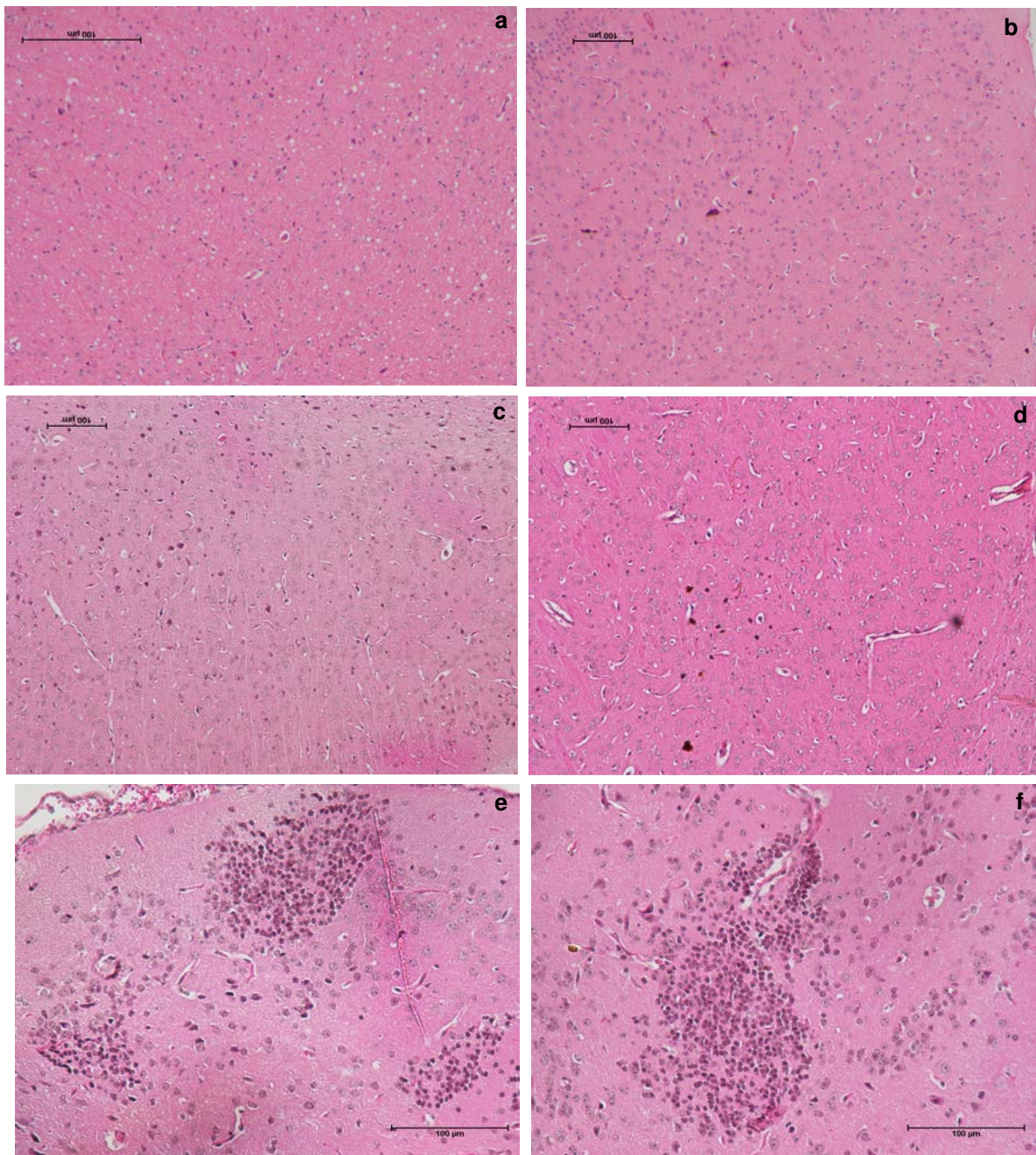


Figure 4: Brain histopathological analysis in mice infected with *S. aureus* strains. C57BL/6 mice were infected with *S. aureus* TSST-1+ strain and *S. aureus* TOX- strain. Fourteen days after infection, the brain were collected and sections were stained with H&E. Normal non-infected mice (a, b), mice previously infected with TSST-1+ strain (c, d) and mice infected with TOX- strain (e, f). Panel shows samples obtained from 2 animals. Other mice from each group presented similar results.

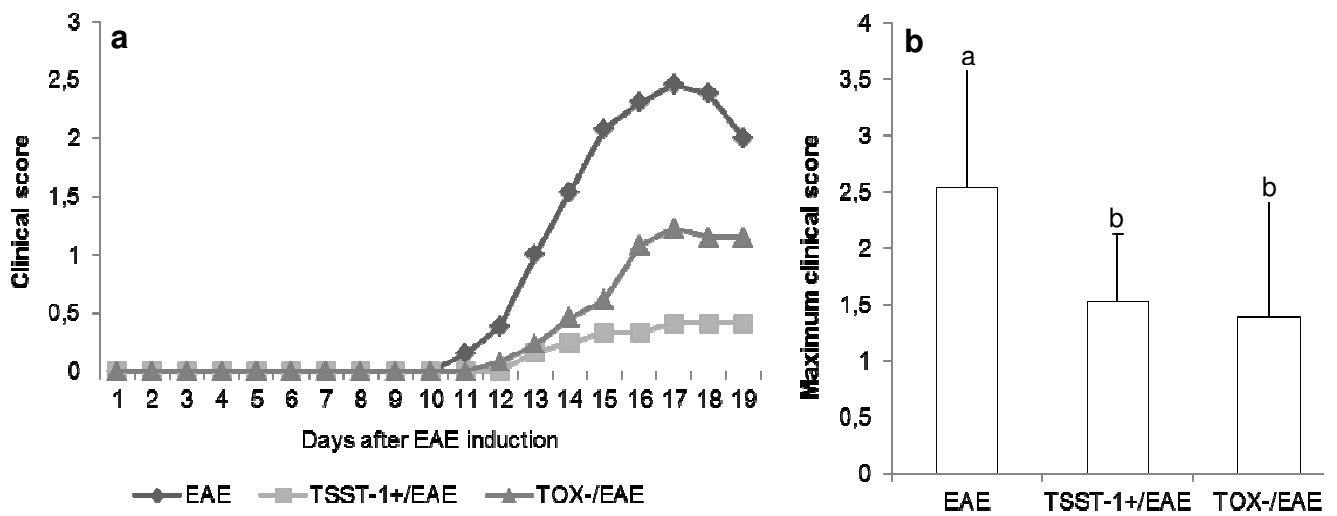


Figure 5: Effect of previous infection with *S. aureus* on EAE development.

C57BL/6 mice were infected 3 days before EAE induction. Clinical scores daily evaluated (a) and maximum clinical score (b). Data were presented by mean \pm SE of 5-7 mice and representative of two independent experiments, $p < 0.05$.

Table 1: Disease incidence in mice submitted to EAE induction (%)

	Number of sick animals	Percentage of sick animals (%)	p
EAE (n=13)	12/13	92	p= 0,008
TSST-1+/EAE (n=12)	4/12	33	
TOX-/EAE (n=13)	9/13	69	

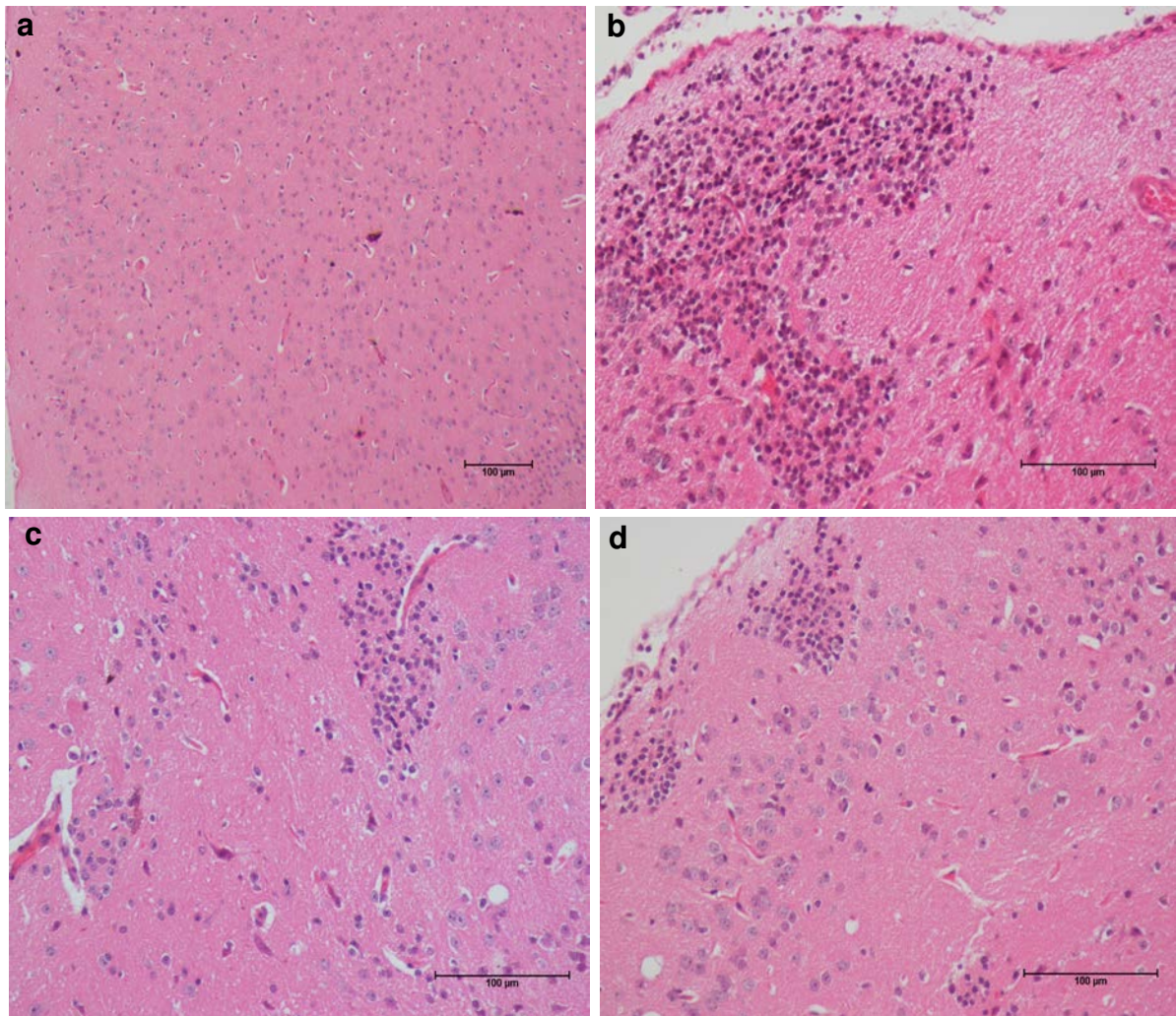


Figure 6: Brain inflammation in mice with EAE: effect of previous infection with *S. aureus* strains. C57BL/6 mice were infected with *S. aureus* strains and 3 days later they were submitted to EAE induction. Brain histopathology was analysed during the acute phase. Sample from normal mice (a); sample from mice with EAE (b); sample from mice infected with TSST-1+ strain before EAE induction (c) and sample from mice infected with TOX- strain before EAE induction (d). Panel shows samples from one animal from each group.

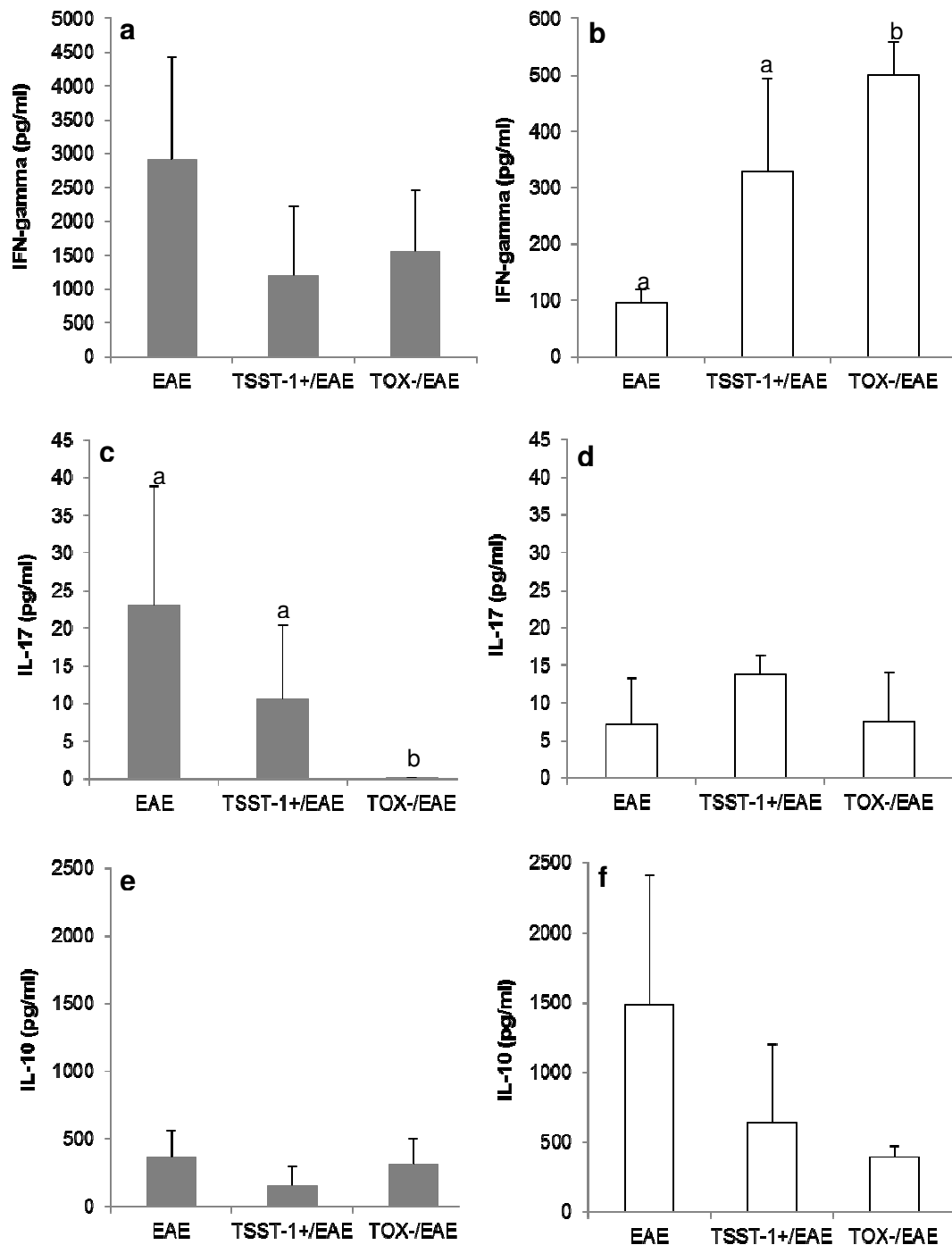


Figure 7: Cytokines produced by CNS cells in mice infected with *S. aureus* strain before EAE induction. C57BL/6 mice were infected with *S. aureus* strains and 3 days later they were submitted to EAE induction. Cytokine production by CNS infiltrating cells was tested 19 days after EAE induction. IFN- γ (a), IL-17 (c) and IL-10 (e) in cultures stimulated with MOG; IFN- γ (b), IL-17 (d) and IL-10 (f) in cultures stimulated with SAC. Data were presented by mean \pm SE of 5-7 mice, $p < 0.05$.

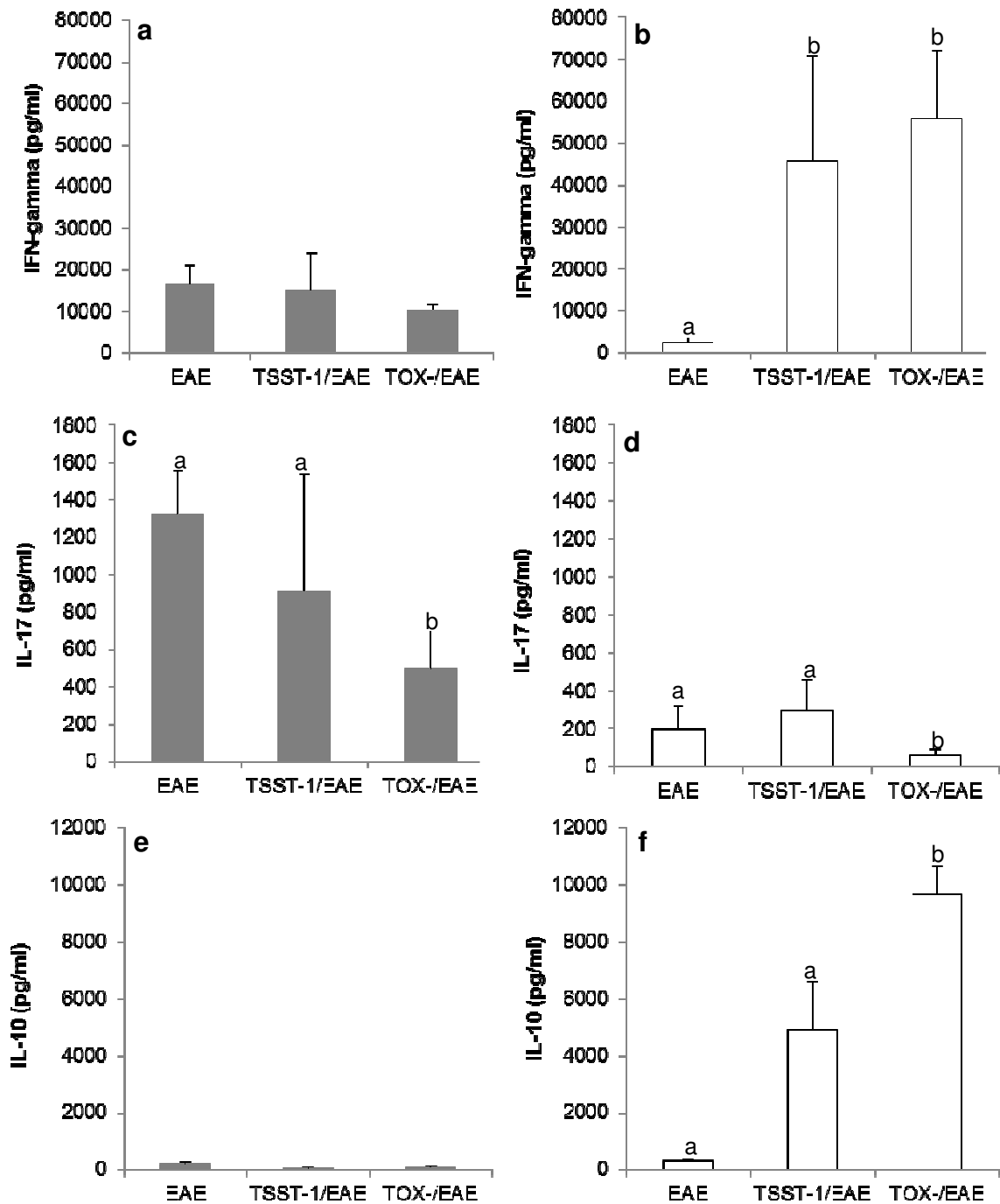


Figure 8: Cytokines produced by spleen cells in mice infected with *S. aureus* strain before EAE induction. C57BL/6 mice were infected with *S. aureus* strains and 3 days later they were submitted to EAE induction. Cytokine production by spleen cells was tested 19 days after EAE induction. IFN- γ (a), IL-17 (c) and IL-10 (e) in cultures stimulated with MOG; IFN- γ (b), IL-17 (d) and IL-10 (f) in cultures stimulated with SAC. Data were presented by mean \pm SE of 5-7 mice, $p < 0.05$.

Discussion

Experimental autoimmune encephalomyelitis (EAE) is one of the best studied autoimmunity models. As a model for multiple sclerosis (MS), it mimics the autoimmune attack against myelin that characterizes this disease (Constantinescu et al., 2011). Multiple factors are thought to contribute to generation of immune response against self-components, including differences in the genetic background, hormonal milieu and environmental factors. Among the environmental factors known to play a role in autoimmunity, the most important seems to be infections with virus, bacteria or other pathogens (Striso et al., 2010). *S. aureus* is one of these infectious agents that has been linked to autoimmune induction (Torres & Johnson, 1998). *S. aureus* is characteristically able to synthesize a multitude of virulence factors, including many toxins (Xu & McComirck, 2012). It can, for example, produce superantigens (SAGs) that are able to activate, non-specifically, CD4+ T cells reactive to myelin (Zhang et al., 1995; Mulvey et al., 2010). In addition, it has been recently described that *S. aureus* is capable to cross the blood-brain barrier (Sheen et al., 2010). In this context, we asked if SAGs production affects both, the ability to reach the brain and the effect on EAE development. To do this investigation we selected two *S. aureus* strains: the ATCC 51650 strain that is characterized by the production of TSST-1 but not of other SAGs and the ATCC 43300 that is described as not able to produce any type of toxin. Then, we initially infected C57BL/6 female mice to evaluate the presence of these bacteria in the blood. Bacteremia was checked by both, PCR employing a staphylococcus genus primer and determination of the number of colony forming units (CFU) in blood samples. Considering the two methodologies, both strains caused bacteremia. Even though PCR was expected to be more sensitive, it detected bacterial DNA only at the beginning (6 and 24h) of the

infection. On the other hand, the classical CFU methodology revealed presence of both strains in all evaluated periods. These results together with weight loss indicated that both *S. aureus* strains were able to cause an experimental infection. These results are similar to the ones described by Gjertsson et al., 2012 that demonstrated that mice intravenously inoculated with wild-type *S. aureus* strain RN4220 also lost a significantly amount of weight. Even though body weight loss was more accentuated in TSST-1+ strain, blood bacterial load was comparable in both infections, during all periods of evaluation. In spite of being equally infective, these two strains elicited different amounts of IFN- γ production *in vivo*. Six hours after infection, the level of this cytokine was much higher in TSST-1+ infected mice. As IFN- γ is described as one of the cytokines preferentially induced by TSST-1 in both, humans (Pichereau, 2012) and mice (Kageyama et al., 2001), we attributed this higher IFN- γ level to TSST-1 secretion. This possibility is supported by the work of Gjertsson et al., 2003. These authors demonstrated that TSST-1 acts as the most potent restimulator of IFN- γ production *in vitro*. To evaluate if these strains were able to again access to the CNS, we looked for *S. aureus* in brain imprints and homogenates. These tests were done 14 days after initial infection; animals were submitted to perfusion to exclude a possible contamination of the brain with blood. Even though no bacteria was recovered from brain homogenates, clusters of grape like structures were visualized in imprints from infected but not normal animals. These findings need confirmation but they suggest that both bacterial strain are reaching the CNS as has already being described in humans (Sheen et al., 2010) and mice (Lo et al., 1994). A recent publication highlighted the mechanism of this brain penetration by showing with *in vitro* experiments that lipoteichoic acid mediated penetration in human brain

microvascular endothelial cells (Sheen et al., 2010). Interestingly, the observed aggregates in brain imprints seemed to have a lot of cellular debris what could suggest that they are formed by alive and dead bacteria and also by apoptotic debris from infected fagocytic cells (Fraunholz & Sinha, 2012). Brain sections stained with H&E showed inflammatory infiltrates in the TOX- infected animals but in the TSST-1+ infected ones. The morphological appearance of the majority of infiltrated cells suggest that they were polymorphonuclear cells. Additional studies will be necessary to confirm both, the presence of bacteria in the brain and also this differential ability to induce inflammation in the brain. In addition, we cannot exclude that these *S. aureus* strains present a differential inflammatory kinetics in the brain. To test their effect on EAE progression, mice were initially infected with *S. aureus* strains and then submitted to EAE induction. We would like to highlight that encephalomyelitis was induced at the third day of infection, i. e, when bacteria was still present in blood circulation. Both strains were able to significantly decrease the maximum clinical scores and therefore, they were able to decrease the encephalomyelitis severity. Nonetheless, the TSST-1 strain was endowed with a more powerful protective ability being able to also significantly decrease disease incidence. To try to get some insight in this protective mechanism we evaluated cytokine production by both, CNS isolated mononuclear cells and spleen cells restimulated *in vitro* with MOG and SAC. Considering cytokine induced by SAC, we observed that specific cells were highly expanded at the periphery. Some of these cells reached the CNS in both strains, as can be implied from IFN- γ levels in CNS cells stimulated with SAC. This elevated local IFN- γ production could be responsible for apoptosis of encephalotogenic T cell clones as described by Quin et al., 2004. In this scenario we could imagine that IFN- γ

is determining apoptosis of Th17 cells as suggested by the lower level of this cytokine in CNS cells from previously infected animals. Very high IL-10 levels were induced by SAC in spleen cells from infected mice, however this was not associated with elevation of these cells in the CNS. These findings could suggest that the protective effect of *S. aureus* strains is not associated with migration of IL-10 producer cells to the CNS. Alternatively, we could hypothesize that this massive peripheral IL10 disponibility is avoiding the activation of encephalytogenic T cells clones in the periphery. Cytokine evaluation did not allow, however, to explain the most evident protective effect of the TSST-1+ strain.

Acknowledgements

The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) that supported this study with grants.

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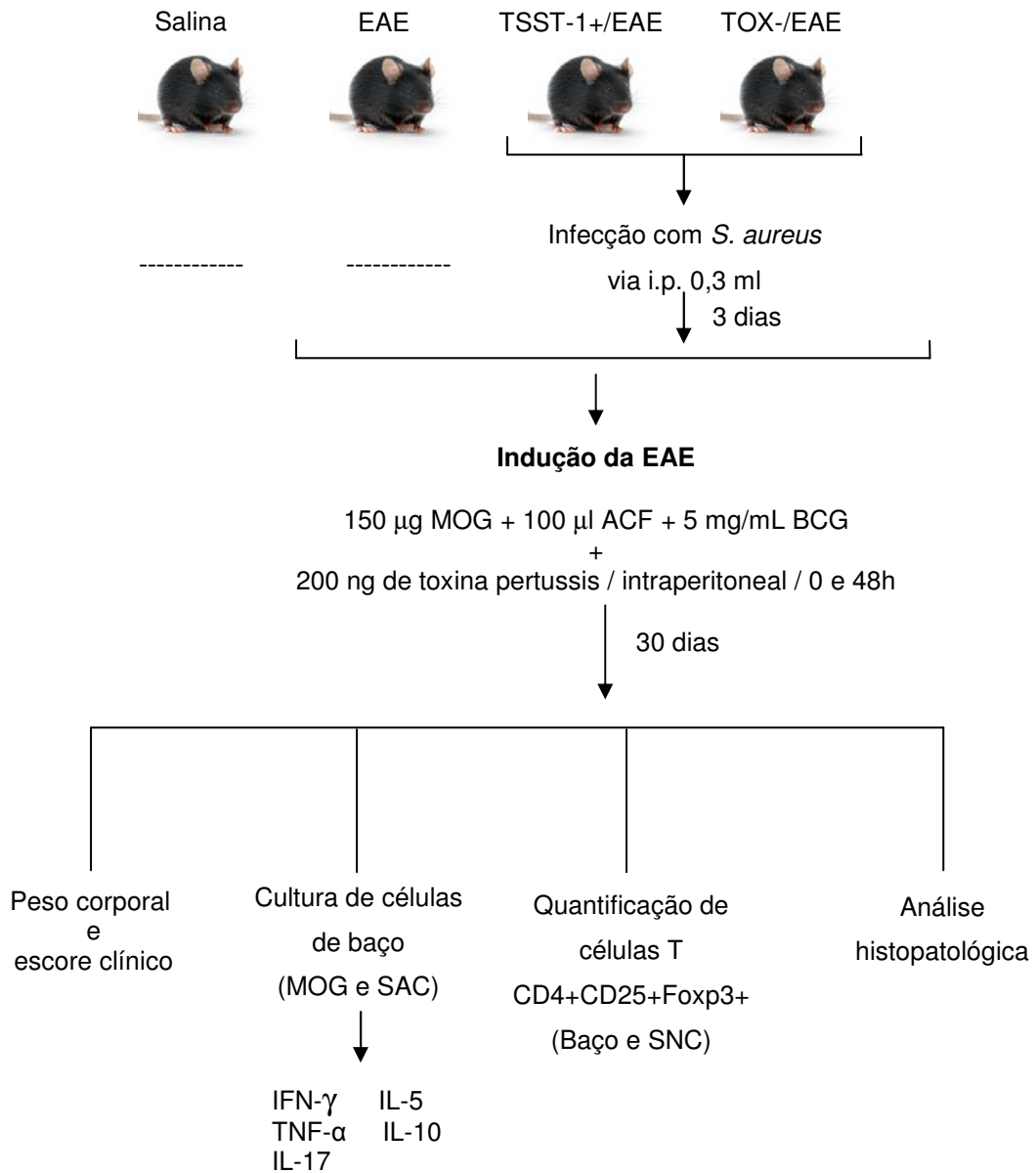
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Artigo Científico II

4.2 – Artigo científico II: Ability of *Staphylococcus aureus* to suppress Experimental Autoimmune Encephomyelitis is more accentuated in a TSST-1-positive strain



**Ability of *Staphylococcus aureus* to suppress Experimental Autoimmune
Encephomyelitis is more accentuated in a TSST-1-positive strain**

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Abstract

Bacterial superantigens are potent T cell activators that could non-specifically activate T cells with specificity for antigens of the central nervous system (CNS). In this study we compared the effect of 2 *S. aureus* strains on experimental autoimmune encephalomyelitis (EAE) development. C57BL/6 female mice were infected with *S. aureus* ATCC 51650 that produces TSST-1 (TSST-1+) and *S. aureus* ATCC 43300 that does not produce toxins (TOX-). Three days later these animals were submitted to EAE induction by immunization with myelin oligodendrocyte glycoprotein (MOG) emulsified in complete Freund's adjuvant (CFA). Weight variation, disease incidence and clinical score were daily recorded. Presence of inflammation in the CNS and cytokine production were evaluated during the chronic disease phase and inflammation and percentage of Foxp3+ regulatory T cells were assayed during the acute period of the disease. Previous infection with both bacteria strains decreased disease severity but the effect of the TSST-1+ strain was much more pronounced and associated with a striking reduction of inflammation in the CNS during the chronic disease phase. In MOG stimulated cultures there was a reduced production of IL-5 and IL-10 in the TOX- and TSST-1+ groups, respectively. In *S. aureus* stimulated cultures there was an increased production of IFN- γ , TNF- α and IL-10 in both infected groups and an increased level of IL-5 in TSST-1+ group. The frequency of Foxp3+ T cells was reduced in the TOX- group. These results indicated that previous infection with a *S. aureus* strain TSST-1+ was highly protective against EAE development, suggesting that this superantigen plays a major role in this effect.

Key-words: *S. aureus*; superantigen; experimental autoimmune encephalomyelitis; regulatory T cells

Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) mainly mediated by T cells specific for myelin self-antigen. These autoreactive T cells generated in the periphery cross the blood-brain barrier and arrive to the brain parenchyma where they initiate an autoimmune attack to the myelin sheath (Mulvey et al., 2010; Constantinescu et al., 2011). MS has been histopathologically characterized by four main findings: inflammation, demyelination, axonal damage and loss and gliosis. Much of the achieved knowledge in MS has been derived from studies in EAE. This experimental disease can be induced in a variety of animals, specially in rodents, providing models of acute monophasic, relapsing remitting and chronic progressive CNS inflammation (Constantinescu et al., 2011). Although many gaps still exist in the understanding of MS immunopathogenesis, it is widely believed that this complex pathology involves both host genetic and environmental factors (Mulvey et al., 2010). Infectious disease agents can modulate autoimmune diseases in many different ways, being able to trigger these pathologies or contrarily, to avoid their development (Sewel et al., 2002; Mix et al., 2010). *S. aureus* is one of the most prevalent pathogens in the human population and is mainly colonizing the anterior nares of 20-60% of the people. These Gram positive bacteria can cause superficial skin infections such as abscesses and impetigo, or serious invasive infections such as septic arthritis, osteomyelitis, endocarditis (Lowy, 1998; Peacock et al., 2001; Foster, 2005) and food poisoning syndrome or foodborne outbreaks (Kérouanton et al., 2007). The proteins secreted by *S. aureus* are important virulence factors, including surface proteins that promote adhesion to damage tissue and to the surface of host cells (Foster & Hook, 1998), that bind proteins in blood to help evade immune responses, and that promote iron uptake (Skaar & Schneewund 2004). Most strains

express a polysaccharide capsule (O`Riordian & Lee, 2004). This microorganism can secrete an array of extracellular enzymes such as proteases, hyaluronidase, lipase, hemolysin and nuclease that facilitate tissue destruction and spreading, membrane damaging toxins that cause cytolytic effects on host cells and tissue damage (Dinges et al., 2000; Normanno et al., 2007). At present, 20 serologically distinct staphylococcus superantigens (Sags) have been described, comprising TSST-1, the enterotoxins A-E and G-J (Ortega et al., 2010). *S. aureus* SAGs are capable of activating T cells due to their ability to bind to both MHC class II molecules in antigen presenting cells and specific V- β regions of the T cell receptor (Krakauer, 1999; Mulvey et al., 2010). This activation results in exacerbation of the immune response with a polyclonal stimulation of T cells and an elevated production of proinflammatory cytokines (Larkin et al., 2009; Mulvey et al., 2010). Experimental and epidemiological evidences support the theory that SAGs producing *S. aureus* may be implicated in the genesis of MS (Brock et al., 1993; Schiffenbauer et al., 1993; mulvey et al., 2010). In this context, the main objective of this investigation was to evaluate the effect of a *S. aureus* TSST-1-positive strain on EAE development.

Material and Methods

Experimental design

Mice were infected with *S. aureus* strains and 3 days later they were submitted to EAE induction by immunization with MOG in CFA. The effect of infection in EAE development was evaluated by clinical follow-up (weight variation and clinical score) and also by histopathological analysis of the CNS. The immunoregulatory effect of the infection was checked by cytokine production by spleen cells stimulated with

MOG or *S. aureus* Cowan I (SAC) and also by quantification of regulatory T cells in the spleen and CNS.

Animals

Female C57BL/6 mice (4-6 weeks old) were purchased from CEMIB (UNICAMP, São Paulo, SP, Brazil). The animals were fed with sterilized food and water *ad libitum* and were manipulated in accordance with the ethical guidelines adopted by the Brazilian College of Animal Experimentation. All experimental protocols were approved by the local Ethics Committee (Ethics Committee for Animal Experimentation, Bioscience Institute, Univ. Estadual Paulista).

Bacterial suspension

Two *Staphylococcus aureus* strains obtained from the American Type Culture Collection (ATCC) were used in this work. The ATCC 51650 strain is characterized by TSST-1 production (TSST-1+) while the ATCC 43300 does not produce any toxin (TOX-). These strains were initially cultured in blood agar and incubated at 37°C for 24h. Isolated colonies were inoculated into brain heart broth (BHI, Merck) and incubated at 37°C for 24h. Bacteria were collected by centrifugation, washed three times and resuspended in cold sterile saline as previously described by França et al., 2009. The bacterial suspension was spectrophotometrically adjusted to 0.3 at 520 nm, as described by Nakane et al., 1996. The animals were infected by intraperitoneal route with 300µl of the bacterial suspension containing 10⁷-10¹²CFU/ml.

EAE induction

MOG35–55 peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Proteimax, São Paulo, Brazil. EAE was induced as previously described (Peron et al., 2010). Briefly, mice were immunized subcutaneously with 150µg of MOG35–55

peptide emulsified in CFA containing 400 µg of BCG. Mice also received 2 doses, 0 and 48 h after immunization of 200 ng of *Bordetella pertussis* toxin (Sigma) intraperitoneally. Animals were daily inspected and disease intensity was graded as: 0 - no disease, 1 - limp tail, 2 - weak/partially paralyzed hind legs, 3 - completely paralyzed hind legs, 4 - complete hind and partial front leg paralysis, 5 - complete paralysis/ death.

Cell culture conditions and cytokine assay

Animals were euthanized 30 days after EAE induction. Spleen cells were collected and adjusted to 5×10^6 cells/ml. Cells were cultured in complete RPMI medium (RPMI supplemented with 5% of fetal calf serum - FCS, 20mM glutamine and 40 IU/ml of gentamicin) in the presence of MOG (20 µg/ml) or SAC (PANSORBIN[®], EMD) (1/2500). Cytokine levels were evaluated 48 h later by enzyme-linked immunosorbent assay (ELISA) in culture supernatants by using IFN- γ , IL-5 and IL-10 BD OptEIA Sets (Becton Dickinson) and IL-17 and TNF- α DuoSet (R&D Systems, Minneapolis, MN, USA). The assays were performed according to the manufacturer's instruction.

Evaluation of inflammatory infiltrates in the CNS

A histological analysis was performed in the CNS at the 30th day after EAE induction. After euthanasia and blood withdrawal, brain and lumbar spinal cord samples were removed and fixed in 10% formaldehyde. Tissues were dehydrated in graded ethanol and embedded in a 100% paraffin block. Five micron thick sections were mounted over glass slides, stained with hematoxylin and eosin and analyzed with a Nikon microscope.

Isolation of mononuclear cells infiltrated in the CNS

Mice were anesthetized with ketamine/xylazine and perfused with 10 mL of saline solution. Brain and cervical spinal cords were excised, macerated and maintained in

4 mL of RPMI (Sigma) supplemented with 2.5% collagenase D (Roche®) at 37°C, 5% CO₂ incubator. 45 min later suspensions were washed in RPMI and centrifuged at 450xg for 15 min at 4°C. Cells were then resuspended in percoll (GE Healthcare) 37% and gently laid over percoll 70% in tubes of 15 mL. The tubes were centrifuged at 950xg for 20 min with centrifuge breaks turned off. After centrifugation the ring containing mononuclear cells was collected, washed in RPMI and centrifuged at 450xg for 5 min. Cellular pellets were then resuspended in complete RPMI medium, counted and the proportion of regulatory T cells was determined as described below.

Percentage of CD4+CD25+Foxp3+ T cells in spleen and CNS

Spleen cells were collected and the red blood cells were lysed with Hank's buffer containing NH₄Cl. Mononuclear cells infiltrated in the brain and cervical spinal cord were isolated as described before. Cell suspensions were washed once in RPMI 1640 and adjusted to 2.5x10⁶ cells. Cells were then incubated with 0.5 µg of fluorescein isothiocyanate (FITC) anti-mouse CD4 (clone GK1.5) and 0.25 µg of allophycocyanin (APC) anti-mouse CD25 (clone PC61.5) 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's instructions. After incubation, the cells were fixed in paraformaldehyde 1% and later analyzed by flow cytometry using the FACSCalibur (Becton Dickinson, San Jose, CA) and FlowJo software (TreeStar, Ashland, OR, EUA).

Statistical analysis

Data were expressed as mean ± SE. Comparisons between groups were made by one way ANOVA with post-hoc Holm-Sidak methods for parameters with normal distribution, and by Kruskal-Wallis post-hoc Dunn's method for parameters with non-

normal distribution. The disease incidence was evaluated by Chi-square test ($p=0,051$). Significance level was $p<0.05$. Statistical analysis was accomplished with SigmaStat for Windows v 3.5 (Systat Software Inc).

Results

Clinical disease severity is reduced by previous infection with *S. aureus*

The EAE control group comprising C57BL/6 mice immunized with MOG associated with CFA, without a previous infection with *S. aureus*, presented the expected clinical alterations. These animals showed a significant reduction in body weight (figure 1a) that coincided with the highest clinical scores observed during the acute phase of the disease (figure 2a). Previous infection with the 2 *S. aureus* strains avoided this reduction in body weight (figure 1b) and also decreased clinical scores (figure 2b). The 2 strains provoked a similar qualitative effect. This protective effect on disease severity was also noted when disease incidence was examined. As shown in table 1, 89% of the animals from the control group got sick whereas the incidence in TOX- and TSST-1+ was 67, and 36%, respectively.

Inflammation at the CNS during the chronic phase

Typical lesions characterized by intense inflammatory infiltrates were observed in the brain (figure 3a) and lumbar spinal cord (figure 3b) from the EAE control group. A very similar picture was found in animals that were infected with the TOX- strain before EAE induction (figures 3e and 3f, respectively). Differently from these results, the group previously infected with the TSST-1+ strain presented a very discrete infiltration in the spinal cord but not in the brain (figures 3d and 3c, respectively). In this case the observed infiltrates were circumscribed to the perivascular region.

Cytokine production induced by MOG and *S. aureus*

Production of IFN- γ , TNF- α and IL-17 induced by MOG was statistically similar, independently of the previous infection with the 2 bacterial strains. However a slight decrease in IL-17 production by the TSST-1+ experimental group was observed (figure 4c). Significant reduction of IL-5 (figure 4d) and IL-10 (figure 4e) were observed in the groups previously infected with TOX- and TSST-1+, respectively. Some clear differences were observed in the level of these cytokines when splenic cultures were stimulated with *S. aureus* antigen (SAC). IFN- γ , TNF- α and IL-10 production by the 2 groups infected with *S. aureus* before EAE induction, were significantly elevated in comparison with the EAE group (figures 5a, b and c, respectively). IL-5 production by the TSST-1+ group was also significantly higher comparing to EAE and TOX-/EAE groups (figure 5d). Similar levels of IL-17 were found in the three experimental groups (figure 5c).

Inflammation at the brain during acute phase

As expected an exuberant inflammatory infiltrate was found in the brain of the EAE control group during the acute phase of the disease (figure 6c). Even though not so copious, similar infiltrates were found in the brains of mice that were infected with *S. aureus* TSST-1+ (figure 6d) and TOX- (figure 6e) before EAE induction.

Quantification of CD4+CD25+Foxp3+ T cells

Animals with EAE presented a significant increase in the frequency of CD4+CD25+Foxp3+ T cells in the spleen during the acute phase in comparison with the control group (figure 6a). Previous infection with TSST-1+ and TOX- did not alter the percentage of these T cells in this peripheral lymphoid organ (figure 6a). At this same period of the infection, we compared the % of these regulatory T cells in the CNS. As showed in figure 6b, the amount of CD4+CD25+Foxp3+ T cells was similar

in EAE and TSST-1+ groups but it was significantly lower in the group previously infected with the TOX- strain.

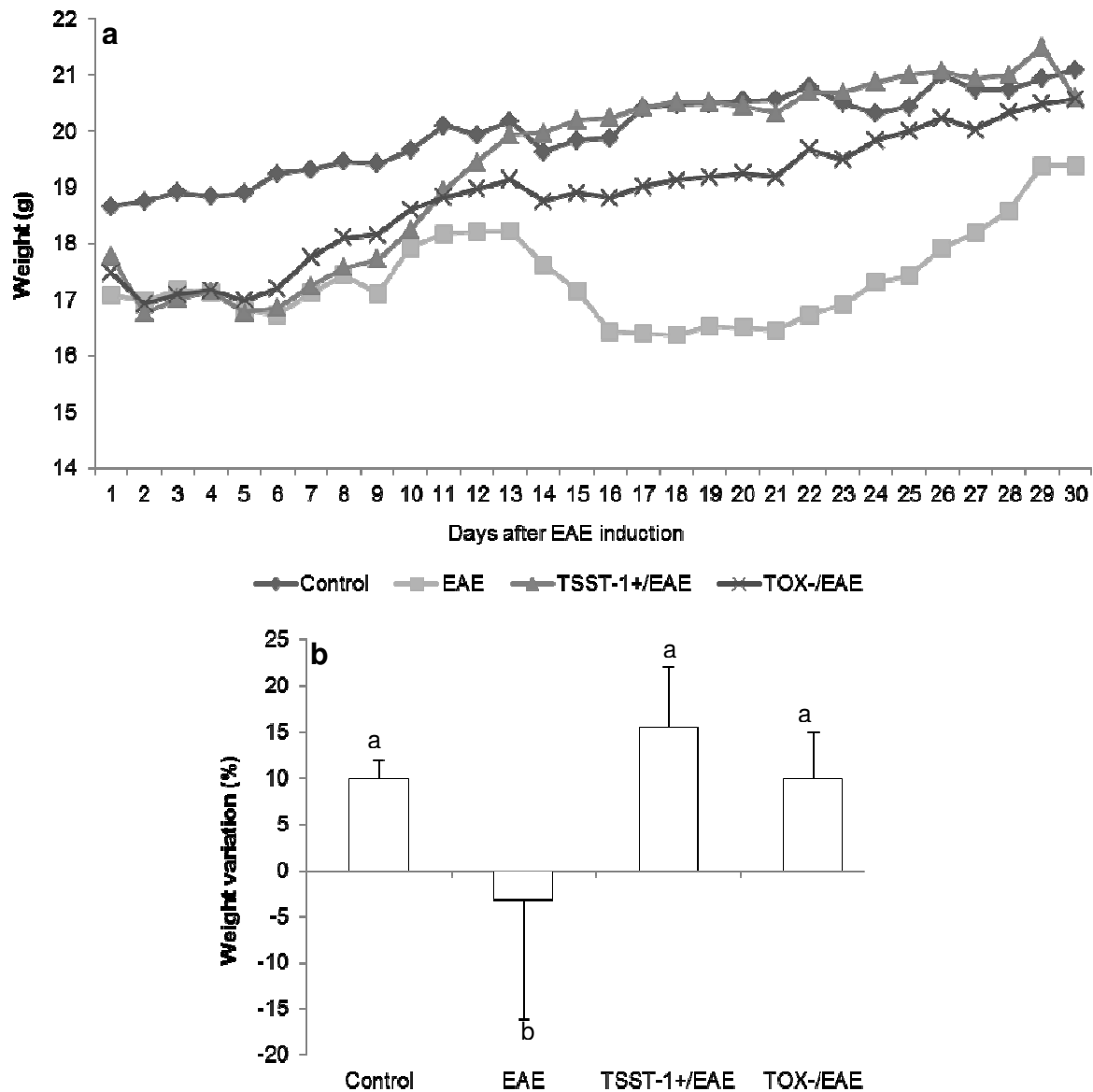


Figure 1: Effect of previous infection with *S. aureus* on body weight. C57BL/6 mice were infected with *S. aureus* TSST-1+ and TOX-. Three days later the animals were submitted to EAE induction and body weight was daily evaluated. Profile of weight variation during 30 days (a) and body variation between the initial weights and the ones found at the acute disease phase (b). Data were presented by mean \pm SE of 9-12 mice and representative of two independent experiments, $p < 0.05$.

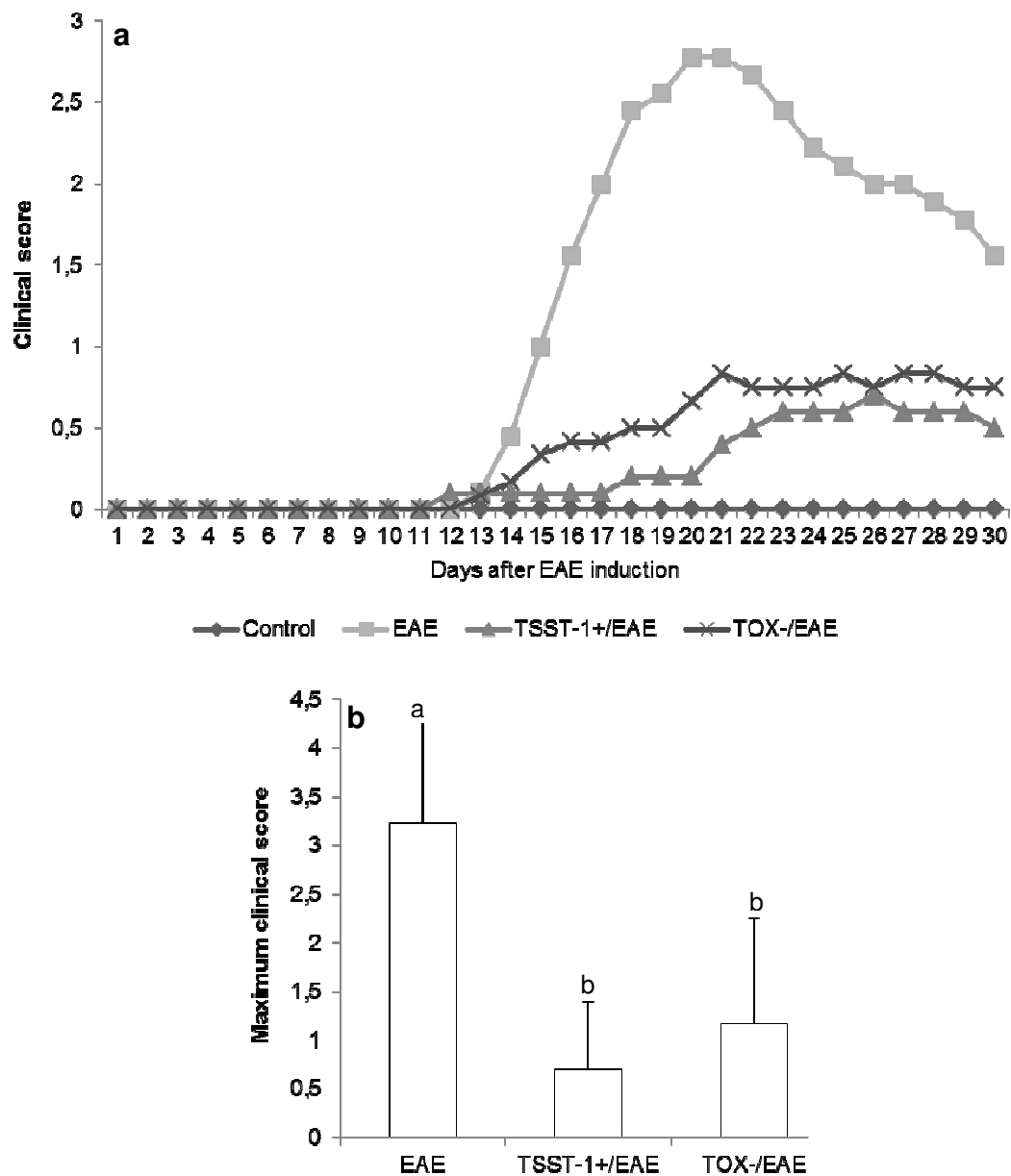


Figure 2: Effect of previous infection with *S. aureus* clinical score. C57BL/6 mice were infected with *S. aureus* TSST-1+ and TOX-. Three days later the animals were submitted to EAE induction and clinical score was daily evaluated. Profile of clinical score during 30 days (a) and maximum clinical score (b). Data were presented by mean \pm SE of 9-12 mice and representative of two independent experiments, $p < 0.05$.

Table 1: Disease incidence in mice submitted to EAE induction (%)

	Number of sick animals	Percentage of sick animals	P
EAE (n= 9)	8/9	89	p= 0,051
TSST-1 ⁺ /EAE (n=10)	4/10	36	
TOX ⁺ /EAE (n=12)	8/12	67	

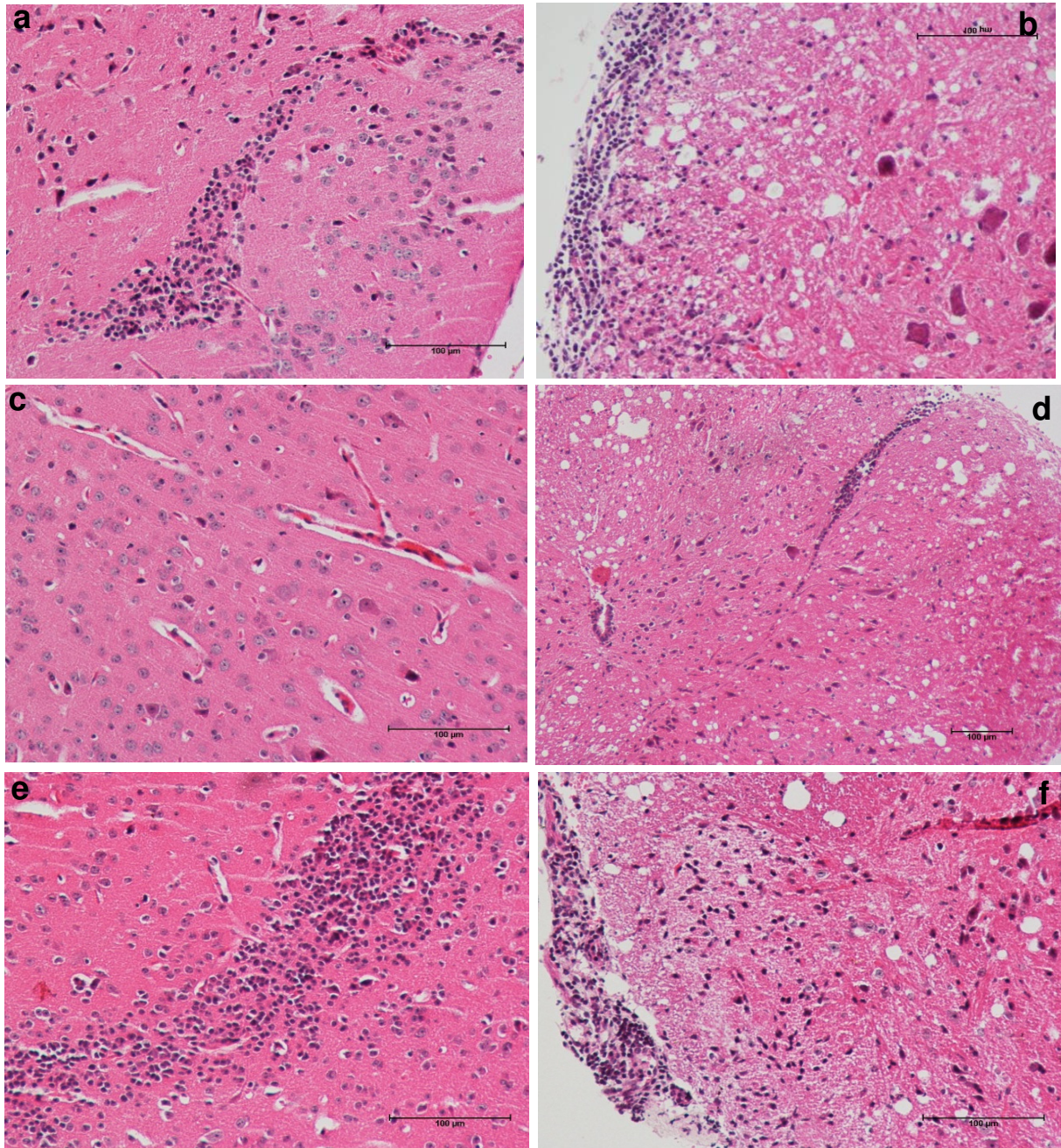


Figure 3: Inflammation in the CNS of mice with EAE: effect of previous infection with *S. aureus*. C57BL/6 mice were infected with *S. aureus* TSST-1+ and TOX- and 3 days later they animals were submitted to EAE induction. Brain and spinal cord inflammatory infiltrates in mice with EAE (a, b), mice previously infected with *S. aureus* TSST-1 strain submitted to EAE (c, d) and mice previously infected with *S. aureus* TOX- strain submitted to EAE (e, f) were evaluated 30 days after disease induction. Panel is representative of 6-7 animals/group.

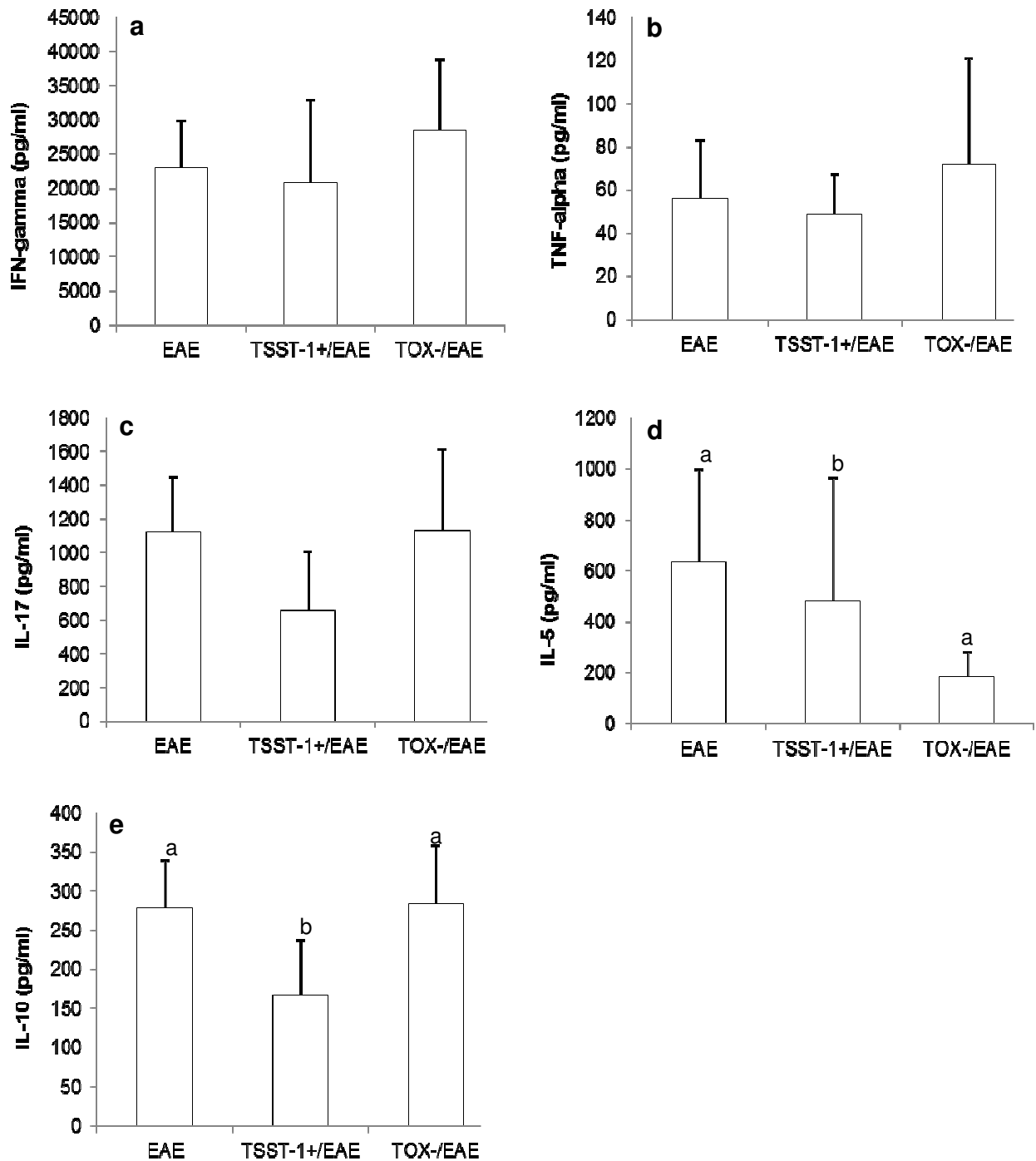


Figure 4: Production of cytokines by spleen cells from mice with EAE: effect of previous infection with *S. aureus*. C57BL/6 mice were infected with *S. aureus* strains and 3 days later they were submitted to EAE induction. Cytokine production was tested 30 days after EAE induction. IFN- γ (a), TNF- α (b), IL-17 (c), IL-5 (d) and IL-10 (e) production were assayed in spleen cells cultures re-stimulated *in vitro* with MOG. Data were presented by mean \pm SE of 5-12 mice, p<0.05.

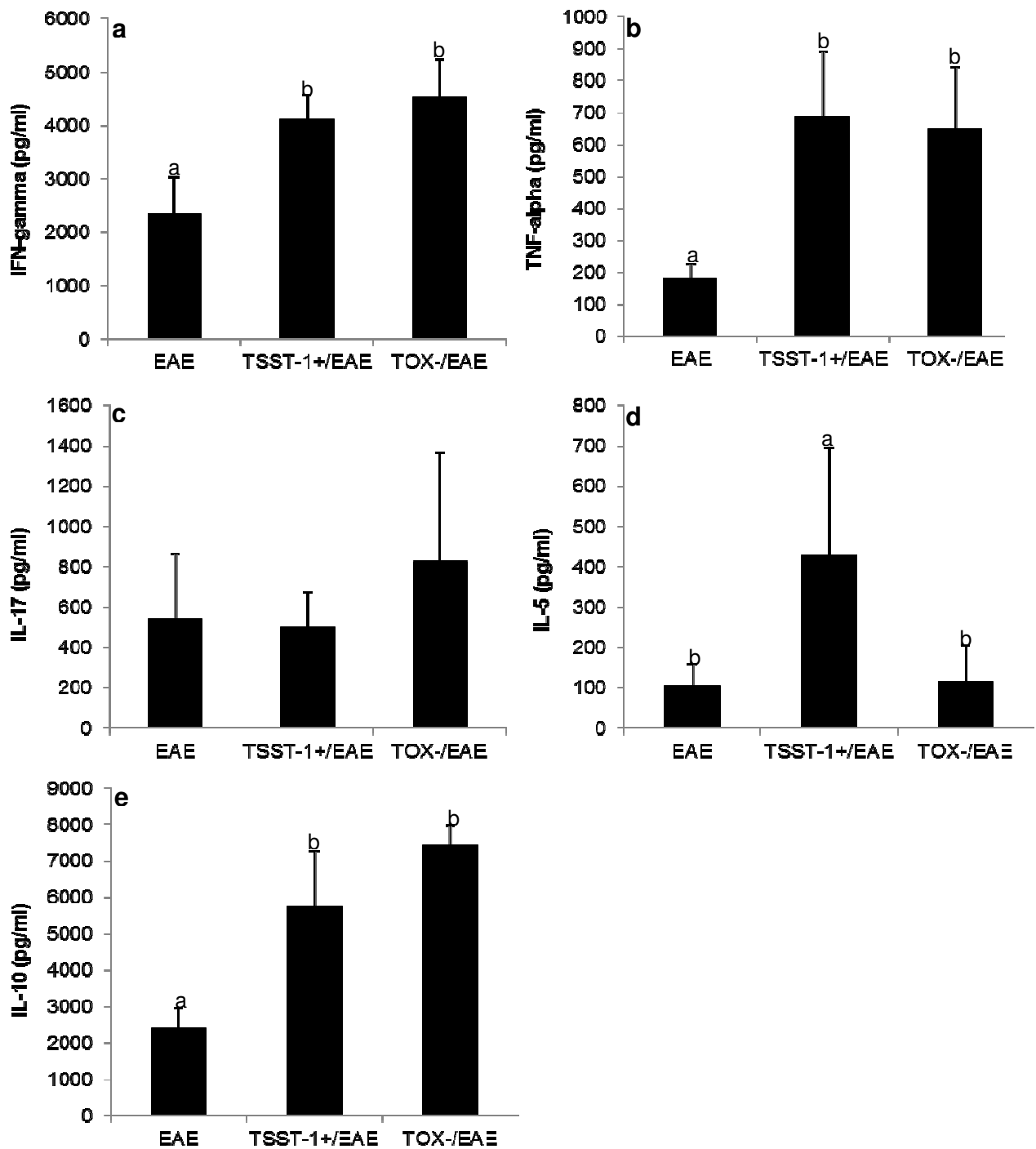


Figure 5: Production of cytokines by spleen cells from mice with EAE: effect of previous infection with *S. aureus*. C57BL/6 mice were infected with *S. aureus* strains and 3 days later they were submitted to EAE induction. Cytokine production was tested 30 days after EAE induction. IFN- γ (a), TNF- α (b), IL-17 (c), IL-5 (d) and IL-10 (e) production were assayed in spleen cells cultures re-stimulated *in vitro* with SAC. Data were presented by mean \pm SE of 5-12 mice, $p < 0.05$.

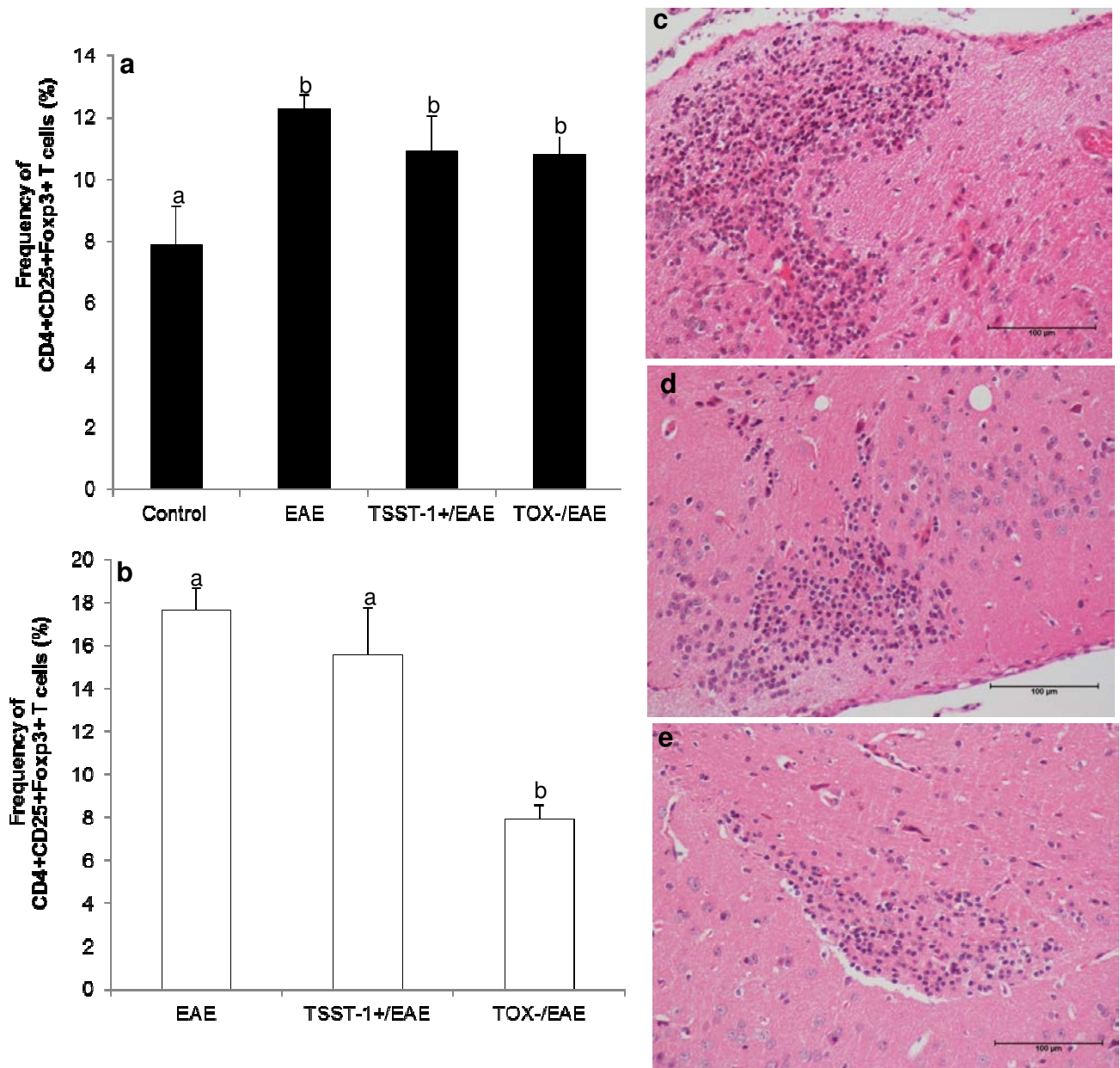


Figure 6: Presence of regulatory T cells in the spleen and CNS of mice with EAE: effect of previous infections with *S. aureus*. The percentage of CD4+CD25+Foxp3+ T cells was evaluated in the spleen (a) and CNS (b). Brain inflammatory infiltrates in mice with EAE (c), mice previously infected with *S. aureus* TSST-1 strain submitted to EAE (d) and mice previously infected with *S. aureus* TOX- strain submitted to EAE (e) were evaluated 19 days after induction of disease. Data were presented by mean \pm SE of 6-9 mice, $p < 0.05$. Panel is representative of 6-7 animals/group.

Discussion

Multiple sclerosis (MS) is a demyelinating disease of the CNS that is characterized by an autoimmune inflammatory process involving myelin antigens (Constantinescu et al., 2011). Experimental autoimmune encephalomyelitis (EAE) is still the most widely accepted animal model to investigate this pathology. Epidemiological studies have identified several environmental risk factors that contribute to the development of this disease as viral infections, smoking and vitamin D serum levels (Zawada, 2012). Even though superantigens (Sags) have been implicated in the pathogenesis of different autoimmune diseases as type I diabetes, Kawasaki disease and MS (Kotzin, 1994), their effects have not being systematically tested in these pathologies. In the present investigation, we evaluated the effect of previous infections with *S. aureus* on EAE development. For this female C57BL/6 mice were infected with 2 distinct *S. aureus* strains, one that was a producer of the TSST-1 toxin (ATCC 51650) and the other that did not produce this neither other SAGs (ATCC 43300). Three days after infection, the animals were submitted to EAE induction and disease evolution was compared with a control group not infected before EAE induction. Previous inoculation with both strains clearly determined a protective effect characterized by the appearance of a much more benign disease. Formerly infected animals did not lose weight during the acute phase and also presented a less severe sickness with lower clinical scores. In addition to these less serious clinical manifestations, there was also a lower disease incidence. The comparison of all these parameters suggested that the TSST-1+ strain was more protective than the TOX- bacteria. In addition, the first signals of paralysis in the TSST-1+ infected group were delayed. In this group the symptoms of paralysis appeared only at the 21st day after EAE

induction, in comparison to the EAE control group that was already sick by the 14th day. We cannot make a direct comparison of these results with the literature because there are no experimental approaches similar to the one employed by us. This is therefore the first direct demonstration that an *S. aureus* infection was able to decrease the severity of EAE. The effect of SAGs on MS, EAE and other human or experimental autoimmune diseases is complex and needs additional investigation to be fully elucidated. Theoretically, these molecules could exacerbate or, contrarily, protect against autoimmunity, being the possibility to be deleterious much more predominant. The first evidences suggesting contribution of SAGs derived from *S. aureus* to trigger MS came from its experimental model. Brocke et al., 1993 demonstrated that purified toxin was able to induce relapsing paralysis and Soos et al., 1993, described that staphylococcal enterotoxin B was able to reactivate EAE. Evidences also support the theory that SAGs producing *S. aureus* may be implicated in the etiology of MS. For example, Stinissen et al., 1995, demonstrated that very small amounts of *S. aureus* Sags were able to activate gamma delta T cell clones isolated from MS patients and control. More recently, Mulvey et al., 2010, suggested that *S. aureus* harbouring enterotoxin A could be a risk factor for MS exacerbation. The protective effect of TSST-1+ strain over EAE was associated with a clear reduction in both, brain and lumbar spinal cord inflammation. Even though the TOX-strain was also able to downmodulate the disease, this experimental group presented an unexpected high inflammatory infiltrate in the CNS, similar to the one found in the control (non-infected) EAE group.

To understand the mechanism by which *S. aureus* was downmodulating this disease, we evaluated the production of cytokines by spleen cells stimulated with MOG. A possible reduction in the production of encephalotogenic cytokines (IFN- γ , TNF- α and

IL-17) was not detected. However, we observed a significant downmodulation of IL-5 and IL-10 levels in the TOX-/EAE and TSST-1+/EAE groups, respectively. It is tempting to hypothesize that the reduced production of these anti-inflammatory cytokines in this peripheral lymphoid organ is due to their migration to the CNS where somehow they turn down the activation of encephalogenic T cells. A theoretical basis for this possibility is found in the literature. Initially described as a product of Th2 cells, IL-10 is now recognized as being secreted by almost every cell type of the immune system and able to restrain the inflammatory process (Banchereau et al., 2012). In the context of MS and EAE, IL-10 elicits beneficial effects on disease. In MS patients, for example, IL-10 levels are increased in the serum during disease remission (Waubant et al., 2001). Additionally, the efficacy of IFN- β and glatiramer acetate, two widely used MS treatments, is partially attributed to induction of IL-10 (Repre et al., 1996; Waubant et al., 2001; Pul et al., 2012). Also genetic studies using the EAE model showed that IL-10 deletion enhances EAE disease severity while over-expression of this molecule protected mice (Bettelli et al., 1998). Concerning IL-5 it has been demonstrated that treatment of EAE with a herpes simplex virus type 1 vector expressing IL-5 ameliorated EAE and decreased the number of infiltrating lymphocytes in the brain (Nygardas et al., 2012). Similarly, treatment of experimental autoimmune neuritis with recombinant IL-5 markedly reduced clinical paralysis, weight loss, demyelination and also infiltration of CD4⁺ (Th1 and Th17), CD8⁺ T cells and macrophages in nerves (Tran et al., 2012).

Other findings could also contribute to explain the protective *S. aureus* effect. For example, the production of cytokines by spleen cells stimulated with *S. aureus* particulate antigens (Cowan I strain) indicated a strong peripheral immune stimulation characterized by elevated production of IFN- γ , TNF- α and IL-10 in both

infected groups. IFN- γ and TNF- α could contribute to the observed protection by causing apoptosis of encephalotogenic activated T cells as has been demonstrated with diabetogenic T cells in NOD mice (Qin et al., 2004). The elevated production of IL-10 induced by both, MOG and SAC and the described ability of this cytokine to maintain Foxp3 expression (Murai et al., 2009) lead us to look for the presence of CD4⁺CD25⁺Foxp3⁺ T cells in both, the spleen of infected mice and also in cells eluted from the CNS of infected mice with EAE. After 14 days of infection we found no difference in the percentage of regulatory T cells. However, differently from expected, the group previously infected with the TOX- strain presented a decreased amount of these cells in the CNS. This could explain, at least partially, the less protective effect of the TOX- strain effect in comparison to the TSST-1+ strain. Alternatively, we could hypothesize that these two *S. aureus* strains are protecting the animals from EAE development by using different molecular mechanisms or more than one immunomodulatory via. In this scenario we could imagine that the TSST-1 is more effective because it is employing a stronger or more than one mechanism. Literature concerning the effects of SAGs on EAE is clearly towards disease aggravation (Schiffenbauer et al., 1993; Zhang et al., 1995; Constantinescu et al., 1998; Soos et al., 2002). Only a few reports have suggested or more recently demonstrated that an *S. aureus* component is able to protect against EAE development. Xie et al., 2006, demonstrated that the extracellular adherence protein (Eap) secreted by *S. aureus* is able to prevent development of EAE by inhibiting infiltration of inflammatory cells into the CNS. Interestingly, the crystal structures of Eap domains from *S. aureus* revealed an unexpected homology to bacterial SAGs (Geisbrecht et al., 2005). Further studies are necessary to unravel the complex effect

of this pathogen and its components on EAE and, therefore, to shed some light on the role of *S. aureus* infection on MS.

Acknowledgements

The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) that supported this study with grants.

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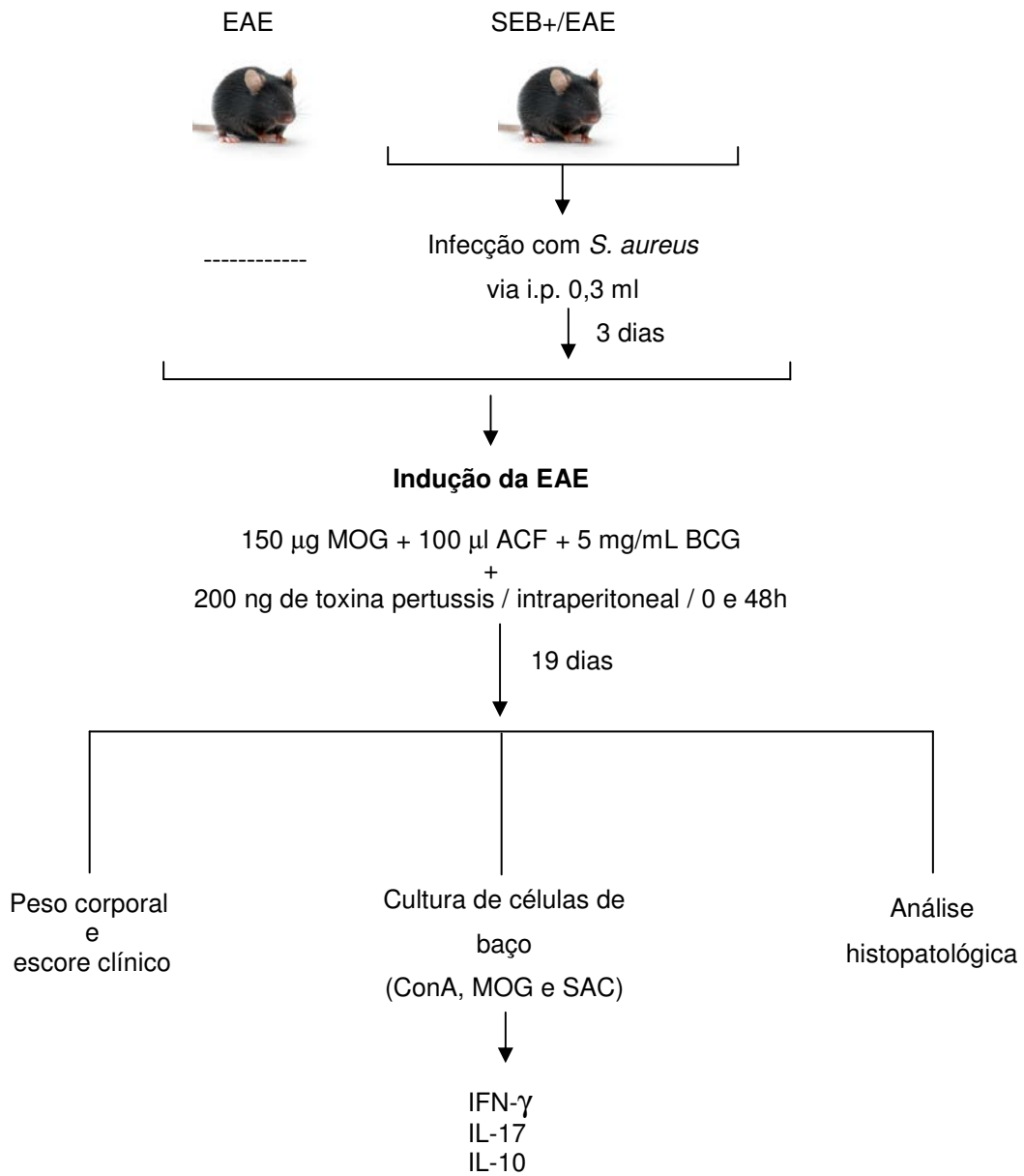
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Artigo Científico IIII

4.3 – Artigo científico III: Previous infection contact with *S. aureus* SEB decreases severity of encephalomyelitis in mice



**Previous infection with SEB positive *S. aureus* decreases severity of
encephalomyelitis in C57BL/6 mice**

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Abstract

Previous data indicated that superantigens (SAGs) like SEA, SEB and TSST-1 were able to activate and expand human myelin basic protein reactive T cells being, therefore, able to contribute to multiple sclerosis development. Here we investigated the effect of a previous infection with a ATCC 14458 (SEB+) *S. aureus* strain on experimental autoimmune encephalomyelitis (EAE). C57BL/6 female mice were then infected with SEB+ and 3 days later they were submitted to EAE induction. Previously infected animals presented reduction in body weight loss, in clinical scores and also in disease incidence. They also presented less inflammation at the central nervous system. Production of IL-17 by spleen cells stimulated with the cognate self-antigen was also lower in previously infected animals in comparison to the non-infected EAE control group. Contrarily to what could be expected, our data indicated that a previous contact with *S. aureus* able to produce SEB, was able to partially protect mice against EAE development.

Key-words: *Staphylococcus aureus*, enterotoxin B, experimental autoimmune encephalomyelitis, IL-17

Introduction

Multiple sclerosis (MS) is a chronic, complex neurological disease that has been mainly attributed to an autoimmune inflammatory attack of the central nervous system (CNS). MS is characterized by demyelination, multifocal inflammation, reactive gliosis and oligodendrocyte and also axonal loss (Nosenwortly et al., 2000; Comabella & khoury, 2011). Much of our knowledge on MS, including immunopathogenesis and development of new therapies, is being generated with the help of its corresponding model called experimental autoimmune encephalomyelitis

(EAE). Active EAE is induced by immunization of rodents, specially mice, with CNS tissue or myelin peptides together with Complete Freund Adjuvant (CFA) (Constantinescu et al., 2011). There is a general consensus that MS is highly dependent upon genetic and environmental factors as infection, smoking and vitamin D uptake (Lin et al., 2012; Koch et al., 2013). *S. aureus* is one of the most common pathogens and it is estimated that 20% of the human population is colonized with this bacteria, mainly at the skin and nasal cavity. This Gram positive bacteria has been pointed as a possible cofactor for development of CNS autoimmune diseases (Visser et al., 2005; Roghmann et al., 2007). Increasing evidence suggest that superantigens (SAGs) produced by *S. aureus* and other pathogens play a role in autoimmunity and other immunomediated pathologies. SAGs are proteins with an intrinsic ability to bind to MHC class II molecules present on the surface of antigen presenting cells (APCs). They have affinity for a site located outside of the antigen-binding cleft (Torres & Johnson, 1998). This SAGs/MCH complex subsequently binds to the variable region of certain β chains of the T cell antigen receptor (Dellabona et al., 1990) provoking a polyclonal activation of as many as 20% of the T cell repertoire (Torres & Johnson, 1998). This initial activation is commonly associated with an expressive cytokine secretion and later succeeded by lymphocyte's apoptosis or anergy (Xu & McCormick, 2012; Pinchuk et al., 2010; Stow et al., 2010). Some epidemiological and experimental evidences support the possible connexion between *S. aureus* infections and MS. For example, in a recent report the presence of *S. aureus* harbouring enterotoxin A was proposed as a possible risk factor for MS (Mulvey et al., 2011). Formerly data indicated that SEA, SEB and TSST-1 SAGs were all able to activate and expand human myelin basic protein reactive T cells (Zhang et al., 1995). In addition, these staphylococcal enterotoxins were able to induce accelerated

pathology, reactivation or relapses in EAE (Soos et al., 1995; Brocke et al., 1993; Constantinescu et al., 1998). However, in certain conditions, SAGs were able to prevent EAE development. This was initially demonstrated by Kuschnaroff et al., 1999. These authors observed that previous inoculation of purified SEB partially protected mice against EAE progression. These observations together with the fact that there are no studies testing the effect of infections with *S.aureus* prompted us to investigate the effect of a previous infection with an *S. aureus* SEB producer strain on EAE. We also evaluated the production of encephalotogenic (IFN- γ and IL-17) and antiinflammatory (IL-10) cytokines.

Material and Methods

Experimental design

Mice were infected with *S. aureus* and 3 days later they were submitted to EAE induction by immunization with MOG in complete Freund's adjuvant (CFA). The effect of the infection in EAE development was evaluated by clinical follow-up (weight variation and clinical score) and also by histopathological analysis of the CNS. The immunoregulatory effect of the infection was checked by cytokine production by spleen cells stimulated with ConA, MOG or *Staphylococcus aureus* Cowan I (SAC).

Animals

Female C57BL/6 mice (4-6 weeks old) were purchased from CEMIB (UNICAMP, São Paulo, SP, Brazil). The animals were fed with sterilized food and water *ad libitum* and were manipulated in accordance with the ethical guidelines adopted by the Brazilian College of Animal Experimentation. All experimental protocols were approved by the local Ethics Committee (Ethics Committee for Animal Experimentation, Biosciences Institute, Univ. Estadual Paulista).

Bacterial suspension

The ATCC 14458 strain that was originally obtained from the American Type Culture Collection is characterized by SEB production (SEB+). This strain was cultured in blood agar and incubated at 37 °C for 24h. Isolated colonies were inoculated into brain heart broth (BHI, Merck) and incubated at 37 °C for 24h. Bacteria collected by centrifugation, washed three times and resuspended in cold sterile saline, as described by França et al., 2009. The bacteria suspension were spectrophotometrically adjusted to 0.3 at 520 nm, as described by Nakane et al., 1996. The animals were infected by intraperitoneal route with 300µl of the bacteria suspension containing 10^7 - 10^{12} CFU/ml.

EAE induction

MOG35–55 peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Proteimax, São Paulo, Brazil. EAE was induced as previously described (Peron et al., 2010). Briefly, mice were immunized subcutaneously with 150µg of MOG35–55 peptide emulsified in CFA containing 400 µg of BCG. Mice also received 2 doses, 0 and 48 h after immunization of 200 ng of *Bordetella pertussis* toxin (Sigma) intraperitoneally. Animals were daily inspected and disease intensity was graded as: 0 - no disease, 1 - limp tail, 2 - weak/partially paralyzed hind legs, 3 - completely paralyzed hind legs, 4 - complete hind and partial front leg paralysis, 5 - complete paralysis/ death.

Cell culture conditions and cytokine assay

Animals were euthanized 19 days after EAE induction. Spleen cells were collected and adjusted to 5×10^6 cells/ml. Cells were cultured in complete RPMI medium (RPMI supplemented with 5% of fetal calf serum - FCS, 20mM glutamine and 40 IU/ml of gentamicin) in the presence of MOG (20 µg/ml) or SAC (PANSORBIN[®], EMD)

(1/2500). Cytokine levels were evaluated 48 h later by enzyme-linked immunosorbent assay (ELISA) in culture supernatants by using IFN- γ and IL-10 BD OptEIA Sets (Becton Dickinson) and IL-17 DuoSet (R&D Systems, Minneapolis, MN, USA). The assays were performed according to the manufacturer's instruction.

Evaluation of inflammatory infiltrates in the CNS

A histological analysis was performed in the CNS at the 19th day after EAE induction. After euthanasia and blood withdrawal, brain and lumbar spinal cord samples were removed and fixed in 10% formaldehyde. Tissues were dehydrated in graded ethanol and embedded in a 100% paraffin block. Five micron thick sections were mounted over glass slides, stained with hematoxylin and eosin and analyzed with a Nikon microscope.

Statistical analysis

Data were expressed as mean \pm SE. Comparisons between groups were made by one way ANOVA with post-hoc Holm-Sidak methods for parameters with normal distribution, and by Kruskal-Wallis post-hoc Dunn's method or Tukey test for parameters with non-normal distribution. The disease incidence was evaluated by Chi-square test ($p=0,073$). Significance level was $p<0.05$. Statistical analysis was accomplished with SigmaStat for Windows v 3.5 (Systat Software Inc).

Results

Experimental autoimmune encephalomyelitis development

Mice submitted to experimental encephalomyelitis presented the expected clinical manifestations characterized by significant weight loss and accentuated hind leg paralysis. Body weight variation between the original weight and the values detected

at the 17th day, when the highest clinical scores were observed, are shown in figure 1b. Kinetics of the clinical scores and the maximal detected score observed in the 2 experimental groups are shown in figures 2a and 2b, respectively. Disease incidence was significantly lower in the previously infected group as showed in table 1.

Brain and spinal cord inflammation

As expected no inflammatory infiltrates were present in brain sections obtained from normal mice (figure 3a). Animals submitted to EAE induction exhibited an exuberant inflammatory process mainly localized around blood vessels (figure 3b). Infection with the *S.aureus* SEB positive strain clearly reduced the amount of local inflammation (figure 3c).

Cytokine production by spleen cells

In cultures stimulated with the cognate antigen, i. e, MOG peptide (35-55), there was a significant production of IL-17 and IL-10 in mice infected with the SEB positive strain before EAE induction in comparison with the EAE control group. Concerning IFN- γ levels no differences were observed between these 2 groups. A very similar cytokine profile was detected in cultures polyclonally activated with ConA. A very distinct pattern was, however, observed when spleen cells were stimulated with SAC (heat-killed and formalin-hardened *S. aureus* Cowan I). In these conditions, the levels of IFN- γ and IL-10 were extremely elevated in the group infected with the SEB positive *S. aureus* before EAE induction whereas IL-17 levels were comparable in these 2 experimental groups. These results can be observed in figure 4.

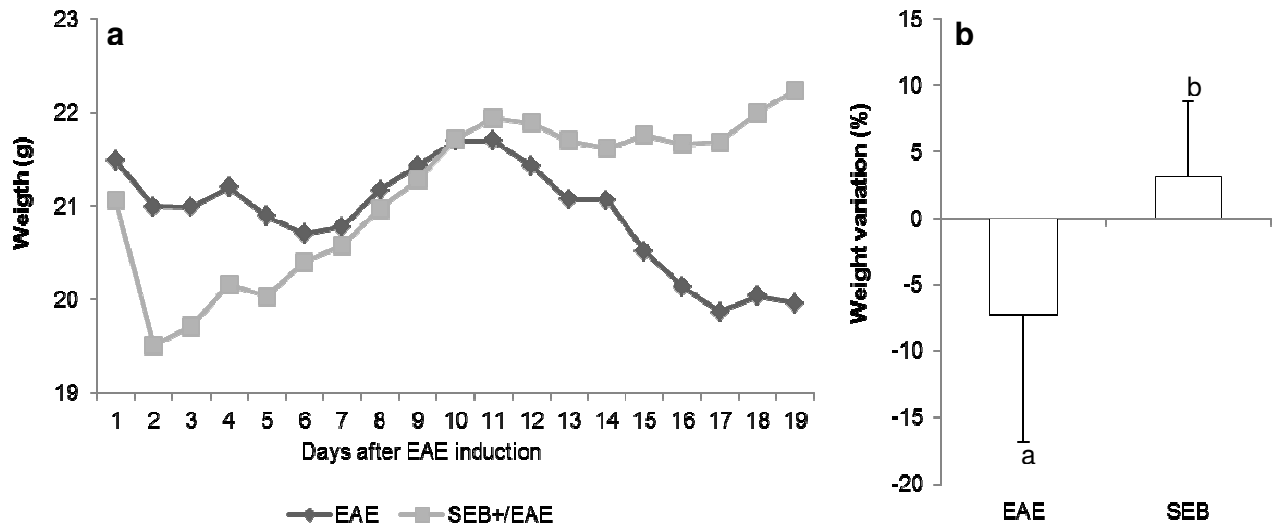


Figure 1: Effect of previous infection with *S. aureus* SEB positive body weight.

C57BL/6 mice were infected 3 days before EAE induction. Body weight (a) was daily evaluated and weight variation (b). Data were presented by mean \pm SE of 13 mice and representative of two independent experiments, $p < 0.05$.

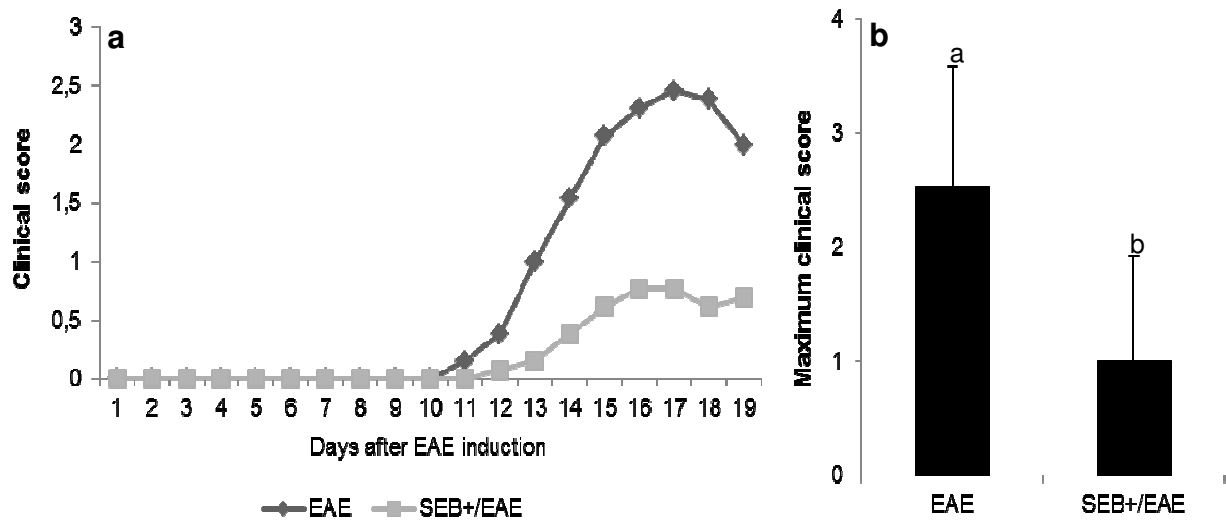


Figure 2: Effect of previous infection with *S. aureus* SEB-positive on EAE clinical scores. C57BL/6 mice were infected 3 days before EAE induction. Clinical score (a) was daily evaluated and maximum clinical score (b). Data were presented by mean±SE of 13 mice and representative of two independent experiments, p<0.05.

Table 1: Effect of previous infection with SEB-positive *S. aureus* on EAE

	incidence induction (%)		p
	Number of sick animals	Percentage of sick animals	
EAE (n=13)	12/13	92	p= 0,073
SEB+/EAE (n=13)	7/13	54	

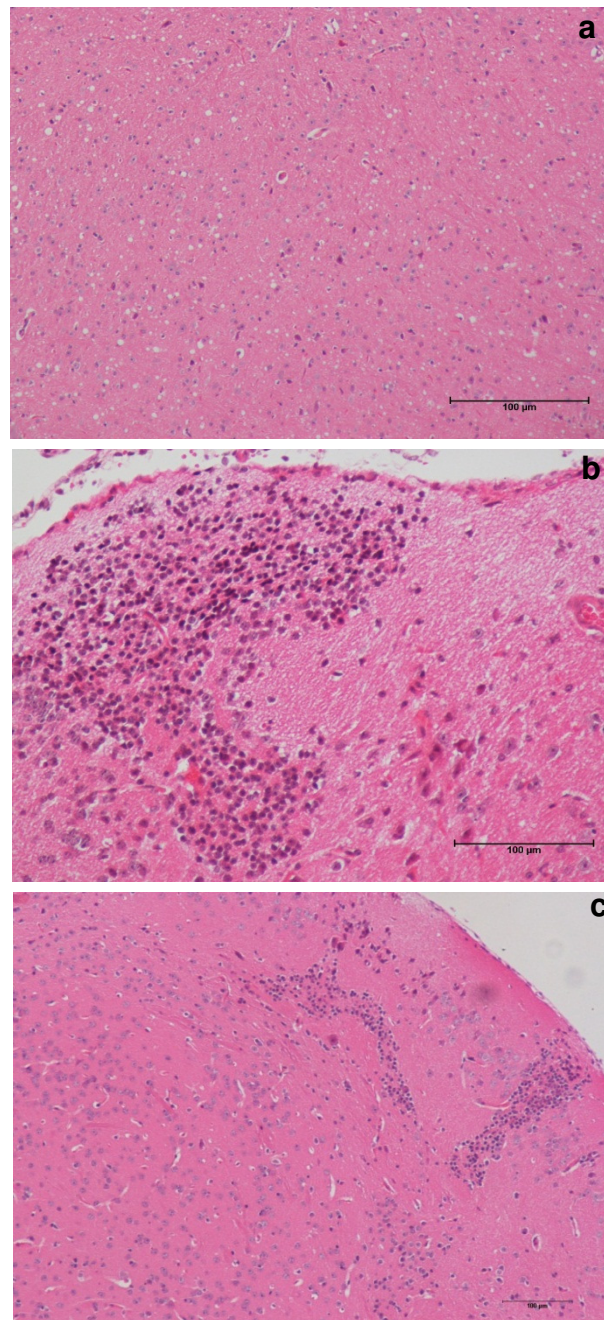


Figure 3: Brain inflammation in mice with EAE: effect of previous infection with SEB-positive *S. aureus*. C57BL/6 mice were infected with *S. aureus* and 3 days later they were submitted to EAE induction. Brains were collected 19 days after disease induction and sections were stained with H&E. Normal control (a); EAE control (b); infected before EAE induction (c).

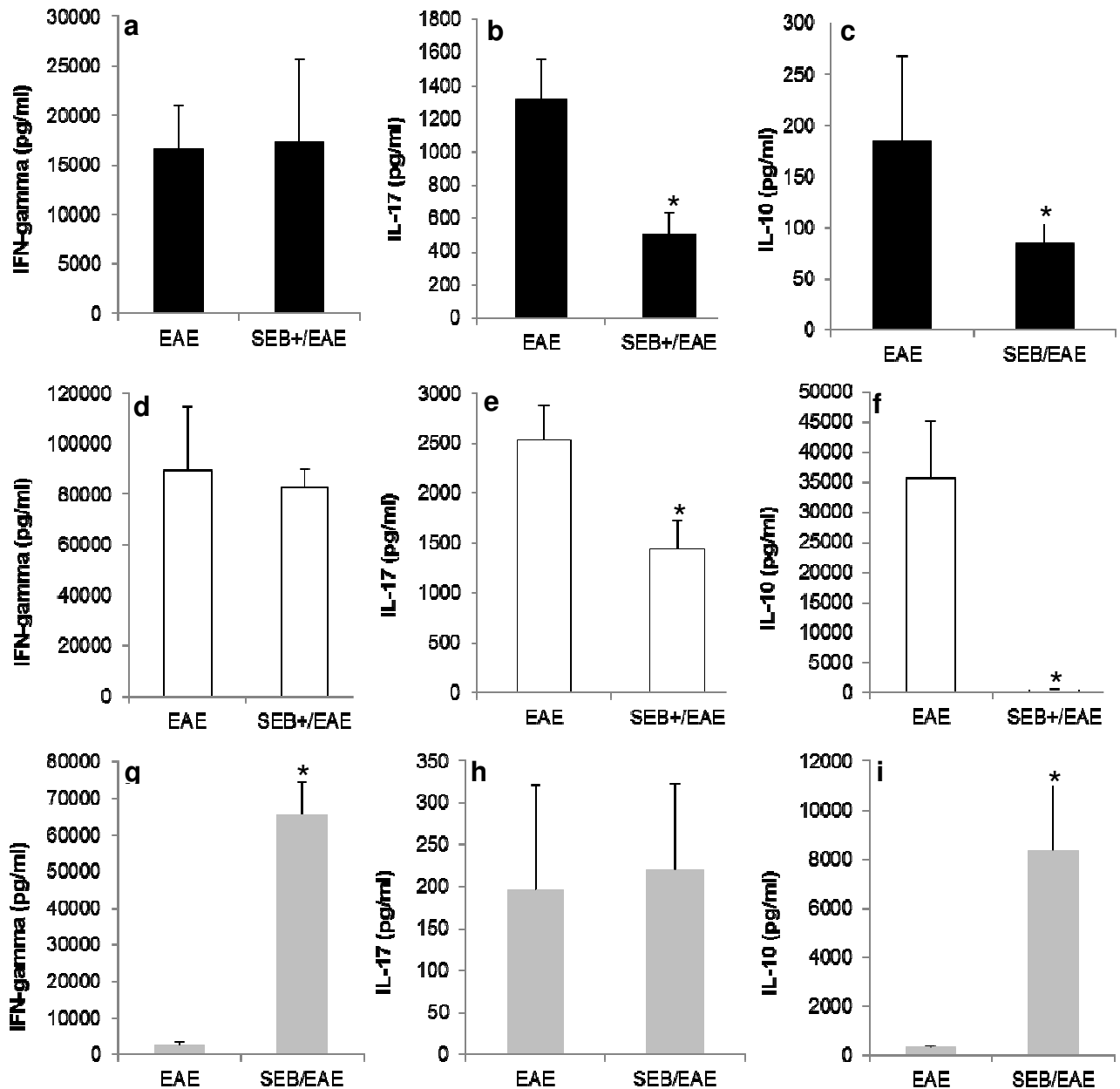


Figure 4: Production of cytokines by mice with EAE: effect of previous infection with SEB-positive *S. aureus*. C57BL/6 mice were infected with *S. aureus* and 3 days later submitted to EAE induction. Cytokine production was tested 19 days after EAE induction. Cytokine production was assayed cultures re-stimulated *in vitro* with MOG, IFN- γ (a), IL-17 (b) and IL-10 (c), ConA IFN- γ (e), IL-17 (f) and IL-10 (g) and SAC IFN- γ (g), IL-17 (h) and IL-10 (i). Data were presented by mean \pm SE of 5-7 mice, $p < 0.05$.

Discussion

Multiple sclerosis (MS) is a devastating autoimmune inflammatory disease whose incidence is increasing in the more developed countries. This raised incidence has been partially attributed to improvement in health and sanitary conditions what avoids contact with many environmental agents (Zilber & Kahana, 1996; Zawada, 2012). *S. aureus* is one of the most predominant agents that colonizes from 20-30% of the human population. It can be pathogenic but almost always it is found as non-pathogenic bacteria in skin and nasal mucosa (Foster, 2005). In this work we used an artificial experimental infection to evaluate the effect of *S. aureus* on EAE. C57BL/6 female mice immunized with MOG plus CFA developed an accentuated paralysis together with an also significant body weight loss as has been widely described for this model (Zhang et al., 2012). Infection with an *S. aureus* SEB producer strain 3 days before EAE induction triggered a protective activity characterized by a significant decrease in weight loss, clinical scores and also in disease incidence. Brain examination showed the presence of very discrete inflammatory infiltrates in mice formerly infected in comparison to non-infected ones. For the best of our knowledge this is the first demonstration that infection with this enterotoxin B producer strain is protective against EAE development. We believe that this effect is at least partially, due to production of this SA_g because Kuschnaroff et al., 1999 demonstrated that infection of purified SEB one week before EAE induction decreased EAE severity in PL/J mice. To try to get some understanding on the mechanism of this protection we analysed the peripheral production of some of the most relevant cytokines (IFN- γ , IL-17 and IL-10) involved in the immunopathogenesis of MS and EAE. When spleen cells were stimulated with the cognate antigen (MOG) there was no difference in IFN- γ production between the 2 experimental groups.

However, there was a significant reduction in the production of IL-17 and IL-10. Exactly the same profile was observed when the cultures were stimulated with ConA. Considering the low levels of IL-17 and IL-10 induced by MOG it is conceivable that this is due to a smaller percentage of myelin specific cells in infected mice. In this context, a smaller number of specific cells could generate a smaller pool of memory cells, explaining the lower production of these cytokines. The fact that *in vivo* inoculation of SEB causes an expansion of $v\beta 8+$ cells followed 4 days later by their deletion or anergy, gives some support to this possibility (Kawabe & Ochi, 1991; Watson et al., 2012). Still reinforcing this hypothesis, the *in vivo* response to ConA stimulation was similarly affected by previous infection. The very discrete inflammation in the brain of infected animals could be, therefore, consequence of a smaller T cell activation in the periphery. On the other hand, spleen cells stimulated with a particulate *S. aureus* antigen produced a very distinct profile characterized by a very elevated production of IFN- γ and IL-10 with no difference in IL-17 levels. These elevated levels of IFN- γ and IL-10 in the periphery could contribute to lower brain inflammation by acting at the peripheral activated T cells and also locally, in the brain after crossing the blood brain barrier. Some papers give support to this possibility. It has been clearly demonstrated, for example, that IFN- γ was able to determine apoptosis of diabetogenic T cells in NOD mice (Qin et al., 2004). In addition, IL-10 is widely accepted as an important suppressive cytokine, produced by a large number of immune cells and relevant to control inflammation to minimize damage to self (O'Garra et al., 2004). The possible contribution of regulatory T cells (Tregs) must be also considered. Treg cells, classically characterized by the expression of forkhead transcription factor (Foxp3) and the IL-2R α -chain CD25, play

a central role in maintaining tolerance to self and prevention of an overexuberant inflammatory response to infection (Ziegler, 2006; Sakaguchi et al., 1995). Recent investigations indicated that bacteria can use SAGs to induced Tregs and therefore to avoid confrontation with the immune system (Taylor & Llewelyn, 2010). Other studies support the notion that SAGs have a complex effect over subsets of Tregs. For example, it has been demonstrated that both, CD4+CD25+ and CD4+CD25- are associated with tolerance induced by SAGs (Feunou et al., 2003; Grundstron et al., 2003; Taylor & Llewelyn, 2010). It is also described that SAGs can be used as a biological tool to trigger *in vivo* preactivation of endogenous Tregs to enhance their suppressive potency (Tanriver et al., 2009). A possible contribution of other mechanisms, independent of SAGs production and activity, cannot be excluded. For example, Xie et al., 2006, reported that extracellular adherence protein (Eap) from *S. aureus* inhibits EAE in mice. Interestingly, they also demonstrated that Eap reduced adhesion of peripheral blood T cells to immobilized ICAM-1, explaining therefore the decreased infiltration of T cells to the CNS. A possible practical application of our finding is illustrated by the work of Lönnqvist et al., 2009. These authors demonstrated that BALB/c mice exposed to Staphylococcal enterotoxin A as neonates were more efficiently tolerised when they become adults. In this same line of thinking, we could hypothesize that contact with certain *S. aureus* strains, as this SEB-positive one, could educate the immune system to promote tolerance to myelin.

Acknowledgements

The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) that supported this study with grants.

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