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A modulação da resposta imune na colite experimental induzida por TNBS em camundongos da linhagem BALB/c: efeitos da tolerância oral e da transferência adotiva de células dendríticas $CD11c^{+}$

Lisiery Negrini Paiatto

Dissertação apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas (Biologia Celular e Molecular).

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LISIERY NEGRINI PAIATTO

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Orientadora: Profª. Drª. Patrícia Ucelli Simioni

Coorientadora: Profª. Drª. Marcia Regina Brochetto Braga

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AUTORA: LISIERY NEGRINI PAIATTO
ORIENTADORA: PATRICIA UCELLI SIMIONI

Aprovada como parte das exigências para obtenção do Título de Mestra em CIÊNCIAS BIOLÓGICAS (BIOLOGIA CELULAR E MOLECULAR), pela Comissão Examinadora:

Profa. Dra. PATRICIA UCELLI SIMIONI
Departamento de Genética, Evolução e Bioagentes / UNICAMP - Universidade Estadual de Campinas/ Instituto de Biologia - SP

Profa. Dra. PATRICIA PASQUALI PARISE MALTEMPI
Departamento de Biologia / UNESP - Instituto de Biociências de Rio Claro - SP

Profa. Dra. DANIELA MAIRA CARDOZO
Departamento de Patologia Clínica / UNICAMP - Universidade Estadual de Campinas - SP

Rio Claro, 31 de agosto de 2017

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RESUMO

A quebra da tolerância imunológica a antígenos próprios é um mecanismo gerador comum às respostas imunes deletérias. Várias estratégias têm sido propostas para modular respostas autoimunes, entre as quais se destacam a administração oral de antígenos relacionados à doença, a transferência adotiva de células tolerogênicas e/ou o tratamento com citocinas reguladoras. Nesse contexto, tem sido mostrado que a ingestão de antígenos proteicos presentes na dieta pode gerar efeitos indiretos sobre o sistema imune do hospedeiro, caracterizados pela supressão da resposta imune a proteínas antigenicamente não relacionadas, conhecidos como supressão *bystander*. O presente projeto teve por objetivo analisar os efeitos indiretos da tolerância oral induzida pela ingestão de ovalbumina (OVA) e a transferência adotiva de células dendríticas isoladas de animais tolerantes a OVA (tDC) na colite induzida por TNBS em camundongos. Os resultados mostraram que o tratamento de camundongos com OVA por via oral, antes ou após a indução da colite e a transferência adotiva de tDC, foram capazes de reduzir sinais da doença, tais como a perda de peso, bem como preservar parcialmente a integridade do tecido colônico, quando comparados aos animais colíticos não tratados com OVA (controles). A supressão *bystander* relacionada ao consumo de OVA foi associada à expansão da frequência de células T reguladoras (regs) e de células T secretoras de interleucina (IL) -10, possíveis mecanismos de regulação das manifestações clínicas da colite induzida por TNBS. As DC obtidas de animais tolerantes a OVA apresentaram expressão aumentada de CD80, compatível com perfil tolerogênico. A transferência dessa população de células para animais colíticos foi capaz de reduzir os sinais clínicos e histológicos da colite, mimetizando os efeitos da tolerância oral. A transferência adotiva de tDC levou a redução da frequência de células Th17, redução de secreção de IL-17 e IL-9 e aumento de secreção de IL-10 e IL-4 *in vitro*. Até onde é de nosso conhecimento, não existem dados na literatura mostrando o efeito da tolerância oral e a transferência adotiva de tDC no tratamento de colite.

Palavras-chaves: Doenças autoimunes, colite, tolerância, supressão *bystander*, ovalbumina, TNBS, células dendríticas e camundongos.

ABSTRACT

The breakdown of immune tolerance to self antigens is a common mechanism for deleterious immune responses. Several interventions have been proposed to modulate autoimmune responses, such as oral administration of disease-related antigens, adoptive transfer of tolerogenic cells and/or treatment with regulatory cytokines. In this context, it has been demonstrated that the ingestion of protein antigens can generate indirect effects on the immune system of the host, characterized by suppression of the immune response to antigenically unrelated proteins, known as *bystander* suppression. The present project aims to analyze the indirect effects of oral tolerance induced by ovalbumin (OVA) and by adoptive transfer of tolerant dendritic cells (tDC) in TNBS induced colitis in mice. Our results showed that the treatment of oral OVA mice before or after induction of colitis and the adductive transfer of tDC were able to reduce the signs of the disease, such as weight loss, as well as partially preserve the integrity of the Compared to non-OVA treated animals (controls). The bystander suppression related to OVA consumption appears to favor the expansion of regulatory (regs) T and interleukin (IL)-10 secreting T cells responsible for reducing the clinical manifestations of TNBS-induced colitis. On the other hand, DC obtained from OVA-tolerant animals showed increased expression of CD80. Administration of this cells population to colitic animals was able to reduce the clinical and histological signs of colitis, possibly by reducing Th17 cells, reduction of secretion of Il-17 and IL-9 and augment of IL-10 and IL-4. To the best of our knowledge, there are no data in the literature showing the effect of oral tolerance and the adoptive transfer of tDC in the treatment of colitis.

Keywords: Autoimmune diseases, colitis, tolerance, suppression *bystander*, ovalbumin, TNBS, dendritic cells and mice.

LISTA DE SIGLAS, ABREVIAÇÕES E SÍMBOLOS.

ACF – Adjuvante complexo de Freund

APC – Célula apresentadora de antígeno profissional

CIA – *Collagen- induced arthritis*

CTLA-4 – Proteína associada a linfócitos T

DCs – Células Dendríticas

DII – Doenças inflamatórias intestinais

EAE – Encefalomielite experimental

Foxp3+ - *Forkhead box protein 3*

IEL – Linfócito intraepitelial

IgE – Imunoglobulina do tipo E

IL – Interleucina

IFN-γ – Interferon gama

MHC-I – Complexo principal de histocompatibilidade de classe I

MHC-II – Complexo principal de histocompatibilidade de classe II

OVA – Ovalbumina

tDC – Células dendríticas tolerantes

Th17 – Linfócitos T *helper* do tipo 17

Treg – Linfócitos T reguladores

Tr1 – Linfócitos T reguladores do tipo 1

Tr2 – Linfócitos T reguladores do tipo 2

TNBS – 2,4,6 - ácido trinitrobenzenosulfônico

TNF-α – Fator de Necrose Tumoral alfa

TGF-β – Fator transformador de crescimento beta

µL – microlitros

M – Molar

µM – micromolar

mL– mililitros

nm – nanômetros

rpm – rotações por minutos

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1 INTRODUÇÃO

As doenças inflamatórias intestinais (DII), tais como a doença de Crohn e a colite ulcerativa tem sido grande foco de estudo devido ao aumento da incidência dessas nas populações humanas (COSNES et al., 2011; MOLODECKY et al., 2012). Essas doenças podem surgir em decorrência da quebra da tolerância a antígenos próprios na mucosa intestinal. Essa quebra pode ser associada a fatores como injúria ou predisposição genética e, por sua vez, pode levar o surgimento de doenças inflamatórias intestinais entre outras autoimunidades (FARIA; WEINER, 2005; OLIVEIRA et al., 2015). Considerando que o trato gastrointestinal (TGI) está constantemente exposto aos antígenos da dieta, a indução da tolerância oral pela administração de alta dose de determinado antígeno pode gerar o estado de anergia nas células T e baixas doses induz células T reguladoras em camundongos (PABST; MOWAT, 2012; WEINER et al., 2011).

Um modelo muito utilizado para estudo das DII é a indução de colite experimental em camundongos BALB/c pela instilação intrarretal de 2,4,6 - ácido trinitrobenzenosulfônico (TNBS) (MAJEWSKA-SZCZEPANIK et al., 2012; PAIATTO et al., 2017; ZHANG et al., 2006). Esse ácido é capaz de gerar lesões, sinais clínicos e imunológicos semelhantes aos observados na doença inflamatória intestinal que ocorre em humanos (MAJEWSKA-SZCZEPANIK et al., 2012; NEURATH et al., 1995a, 1996b; TE VELDE; VERSTEGE; HOMMES, 2006; WU et al., 2013).

Com o intuito de modular o sistema imunológico frente às doenças autoimunes diversos autores têm estudado um mecanismo conhecido como supressão *bystander*, no qual relacionado à tolerância oral tem como definição quando a resposta imune para um determinado epítopo suprime a resposta para outro epítopo administrado concomitantemente ou imediatamente após o primeiro (CANTARUTI et al., 2017; GOTSMAN et al., 2001; MADDALONI et al., 2015; SINHA et al., 2009).

Nesse contexto, as células dendríticas são células fagocíticas que possuem importante papel na resposta imune no trato gastrointestinal, pois constantemente apresentam determinantes antigênicos que podem ou não estimular respostas inflamatórias (WEINER et al., 2011). Essas células possuem mecanismos, como, apresentação de antígenos aos linfócitos T, indução de células T reguladoras e supressão da resposta imune. Quando essa célula é ativada fornece diversos sinais, entre eles, a secreção de citocinas, interação entre moléculas co-estimulatórias

para estimular a proliferação e diferenciação de células T *naives* em células T efetoras (THOMÉ et al., 2012; WALKER, 2013; WEINER et al., 2011). Alguns antígenos possuem epítopos similares no cólon e promove a supressão em células T que secretam citocinas anti-inflamatórias (GOTSMAN et al., 2001). É sabido que a inflamação do trato gastrointestinal associada as DII é capaz de induzir a secreção de citocinas pró-inflamatórias, as quais ativam subpopulações de linfócitos T de perfil inflamatório, alterando a homeostase do organismo (GOTSMAN et al., 2001; HANAUER, 2006; MA et al., 2016). As células dendríticas tolerantes têm sido um grande foco de estudo em diversas doenças experimentais, pela sua capacidade de suprimir a resposta imunológica através de moléculas co-estimulatórias presentes em sua superfície (HINTERLEITNER; JABRI, 2016; SILVA et al., 2015; WALKER, 2013).

2 REVISÃO DA LITERATURA

2.1 DOENÇA INFLAMATÓRIA INTESTINAL

Embora em constante contato com antígenos e com microrganismos do TGI, o sistema imunológico tem a habilidade de diferenciar os antígenos próprios dos não próprios e tolerar substâncias potencialmente benéficas ao organismo, como proteínas alimentares e antígenos da microbiota intestinal. Essa capacidade de tolerância possibilita a homeostase do organismo. Entretanto, caso essa tolerância não se desenvolva, ou seja quebrada, o sistema imunológico irá responder contra antígenos próprios, por meio de linfócitos autorreativos e auto anticorpos que desencadeiam reações de autoimunidade e doenças autoimunes (ALIBERTI, 2016; DE MATTOS et al., 2015). Entre essas doenças autoimunes estão às doenças inflamatórias intestinais (DII) que são classificadas em dois tipos principais: doença de Crohn e colite ulcerativa em humanos. A doença de Crohn e a colite ulcerativa são doenças distintas que apresentam algumas semelhanças como o fato de serem caracterizadas por uma inflamação crônica no TGI, resultado de uma resposta exacerbada do sistema imunológico contra auto antígenos e/ou contra a microbiota comensal (COSNES et al., 2011; DE MATTOS et al., 2015; GEREMIA et al., 2014; KUMAR et al., 2006; MOLODECKY et al., 2012; YAMADA et al., 2016).

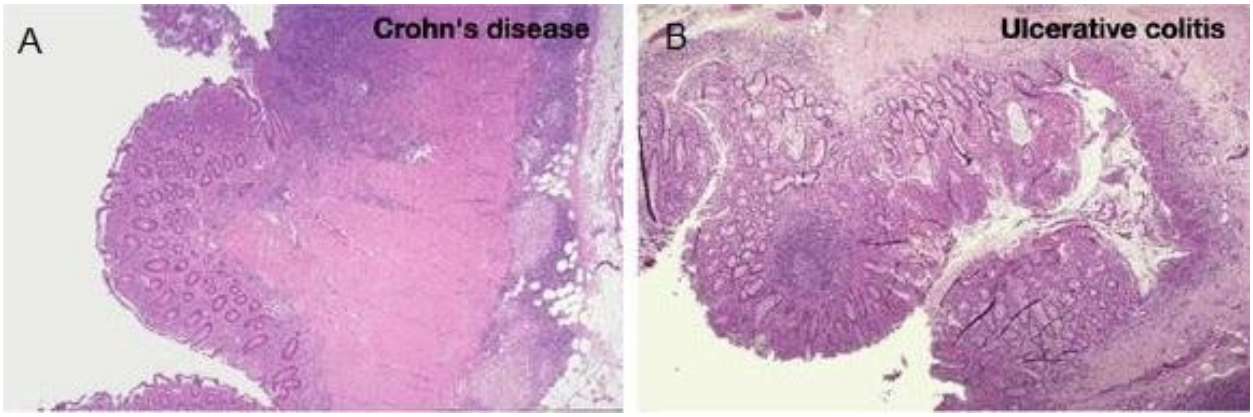
A etiologia dessas doenças não está totalmente esclarecida, mas sabe-se que são alterações de caráter multifatorial, que envolvem fatores imunológicos como falhas na regulação da resposta imune, fatores ambientais como tabagismo, administração hormonal, fatores associados ao hospedeiro como susceptibilidade genética e desbalanço da microbiota intestinal (BLUMBERG,

2009; DE MATTOS et al., 2015; DE SOUZA; FIOCCHI, 2016; HANAUER, 2006; KOTREDES; THOMAS; GAMERO, 2017). Nesse contexto, sabe-se que alguns antígenos da dieta conseguem alterar a função da microbiota intestinal, pela sinalização dos receptores de reconhecimento padrão (PRRs) do tipo *Toll-like receptor* (TLRs) que se ligam aos padrões moleculares associados aos patógenos (PAMPs), gerando uma resposta inflamatória (ANDERSON et al., 2016; DE SOUZA; FIOCCHI, 2016). Além disso, o fenômeno da epigenética está relacionado diretamente com alterações da homeostase do TGI, o que sugere uma provável explicação para aumento de casos DII na população mundial (RAPOZO; BERNARDAZZI; DE SOUZA, 2017). O uso de probióticos também pode afetar o crescimento e desenvolvimento da microbiota indígena e tem se mostrado eficaz na prevenção e no tratamento de infecções, alergias e doenças inflamatórias intestinais, por atuar na manutenção da integridade das células epiteliais (DE SOUZA; FIOCCHI, 2016; KALLIOMAKI et al., 2010; RIJKERS et al., 2010).

A doença de Crohn e a colite ulcerativa geralmente iniciam-se com uma inflamação crônica, com aparecimento de sintomas nas primeiras décadas de vida, com picos de bem-estar e recaída. A doença de Crohn acomete mulheres em maior frequência, enquanto a colite ulcerativa acomete maior número de homens (BAUMGART; SANDBORN, 2012; HANAUER, 2006). A sintomatologia dessas DII inclui perda de peso, desnutrição, diarreia sanguinolenta e dor abdominal (RUFO; BOUSVAROS, 2006).

A doença de Crohn ocorre principalmente na região terminal do íleo, mas pode acometer qualquer outra região do TGI, desde a boca até o ânus, com quadro de espessamento da parede intestinal por inflamação transmural, caracterizada pela presença de granulomas, fístulas, ulcerações profundas e estenoses (Figura 1 A) (FREEMAN, 2014; SPEKHORST et al., 2014). Já a colite ulcerativa limita-se a ocorrer no cólon e no reto, sendo caracterizados por ulcerações superficiais, abscessos nas criptas e infiltrações de neutrófilos na lâmina própria, como apresentado na fotomiscroscopia de luz da Figura 1 B (SPEKHORST et al., 2014; XAVIER; PODOLSKY, 2007).

Figura 1 – Fotomicroscopia histológica de portadores de doença de Crohn (A) e de colite ulcerativa (B).



Fonte: Retirado de BOUMA; STROBER (2003).

Uma metodologia frequentemente utilizada em modelos experimentais para indução de inflamação semelhante à encontrada nas DII é a administração de agentes químicos. Uma substância muito utilizada para indução de enterocolite é o hapteno 2,4,6 – ácido trinitrobenzenosulfônico (TNBS), instilado por via intrarretal. Embora esse modelo reproduza de forma idêntica as DII em humanos, a administração desse ácido promove uma resposta imune mediada por células (RANDHAWA et al., 2014) semelhante à encontrada na doença de Crohn (ERI et al., 2008; OKAMOTO; UEMOTO; TABATA, 2012; SRINIVASAN; SUMMERLIN, 2009). Outros agentes químicos são utilizados para induzir colite experimental como oxalazona e sulfato de sódio de dextrano (DSS). Enquanto a administração de TNBS induz preferencialmente um padrão de resposta T *helpe*r do tipo 1 (Th1), oxalazona induz preferencialmente a resposta de células T *helper* do tipo 2 (Th2) (RANDHAWA et al., 2014) e o DSS causa regeneração lenta do epitélio colônico e inflamação crônica que exibe mudanças de citocinas das subpopulações Th1 e Th2 na mucosa (DIELEMAN et al., 1998; RANDHAWA et al., 2014).

O TNBS diluído em álcool promove a quebra da barreira da mucosa intestinal, pela alteração da permeabilidade celular. O hapteno TNBS se associa às proteínas teciduais e

desencadeia uma resposta imunológica, promovendo uma inflamação que se inicia rapidamente, com aumento de expressão de moléculas de adesão, como as integrinas presente nas superfícies celulares, cuja função é intermediar e reorganizar a comunicação entre o citoesqueleto celular à células apresentadoras de antígeno profissionais (APC) e proteínas plasmáticas, responsáveis pela retenção de leucócitos no endotélio a migrar pela membrana basal até chegar no local da inflamação, através de quimiotaxia de células. As integrinas são essenciais para interações das APC com os linfócitos T. Além disso, o aumento da expressão de selectina pelos leucócitos, plaquetas e endotélio ativado por citocinas, promovem a adesão de leucócitos às células endoteliais e a rolagem dos leucócitos sanguíneos até o local da inflamação para produzir citocinas pró-inflamatórias (FERRAZ; FERNANDEZ, 2014; MORRIS et al., 1989; WIRTZ et al., 2007; WITAICENIS, 2010; ZHENG; GAO; WANG, 2000). Essa resposta apresenta preferencialmente um perfil de células Th1 (RANDHAWA et al., 2014) que secretam citocinas como o interferon gama (IFN-γ) e fator de necrose tumoral (TNF) (STROBER; FUSS, 2011). Essas citocinas atuam nos macrófagos, estimulando a secreção de citocinas pró-inflamatórias, tais como IL-6, TNF-α, IL-1β e IL-18 (STROBER; LÚDVÍKSSON; FUSS, 1998) favorecendo a inflamação transmural como observado na doença de Crohn (NEURATH; FINOTTO; GLIMCHER, 2002). Entretanto, estudo mostrou que mesmo na ausência de IFN-γ, a colite experimental induzida por TNBS pode ser ativada pelo perfil de células Th2 em camundongos BALB/c (DOHI et al., 2000). Essa subpopulação secreta principalmente as citocinas IL-4, IL-5, IL-9 e IL-13 que estão primordialmente associadas com alterações nas criptas intestinais e inflamação superficial no cólon como observado na doença colite ulcerativa em humanos (NEURATH; FINOTTO; GLIMCHER, 2002). As citocinas IL-12 e IL-4 controlam a diferenciação entre as subpopulações Th1 e Th2 respectivamente, e inibem a indução da subpopulação oposta (NEURATH; FINOTTO; GLIMCHER, 2002).

Existem diferente linhagens de camundongos estudados na colite experimental induzida por TNBS, entre elas, a linhagem SJL que é altamente susceptível a indução de colite (TE VELDE; VERSTEGE; HOMMES, 2006). Já os camundongos da linhagem BALB/c são frequentemente utilizados para indução de colite por administração de TNBS pelo fato desses animais apresentarem suscetibilidade a doença, porém, de forma mais branda que os animais da linhagem SJL, mas com sinais clínicos e imunológicos ainda relevantes (LINDSAY et al., 2002; SRINIVASAN; SUMMERLIN, 2009; TE VELDE; VERSTEGE; HOMMES, 2006). Os animais

de linhagem SJL submetidos à colite experimental induzido por TNBS desenvolvem diarreia severa, sangramento, perda de peso e prolapso retal (SCHEIFFELE; FUSS, 2002; WIRTZ et al., 2007).

A tolerância oral por ingestão de ovalbumina em modelo experimental tem se mostrado um método alternativo e eficaz para estudo de diversas enfermidades, entre as quais a artrite e asma. O mecanismo envolvido nos processos de tolerância a antígenos externos está relacionado à indução de células T reguladoras (Treg) (RUSSO et al., 2001; THOMÉ et al., 2012). Partindo desse pressuposto, não foram encontrados estudos prévios na literatura que demonstrem os efeitos da tolerância induzida pela administração oral de ovalbumina em modelo de colite experimental induzida por TNBS em camundongos BALB/c.

2.2 TOLERÂNCIA IMUNOLÓGICA

2.2.1 TOLERÂNCIA ORAL

O trato gastrointestinal está em contato constante com proteínas da dieta, microrganismos comensais e microrganismos potencialmente patogênicos. Para garantir a manutenção da homeostase do organismo, o sistema imunológico da mucosa intestinal deve ser capaz de gerar respostas tolerogênicas para as proteínas da dieta e para os antígenos da microbiota, bem como respostas protetoras contra antígenos danosos para o próprio organismo. A capacidade do tecido linfoide associado à mucosa intestinal de suprimir a resposta imune sistêmica para antígenos proteicos previamente administrados por via oral, conhecidos como tolerância oral, tem sido demonstrada em diferentes modelos experimentais e para diferentes antígenos (CANTARUTI et al., 2017; HYUN; BARRETT, 2006; PABST; MOWAT, 2012). Nesse contexto, trabalhos realizados mostraram que só é possível induzir tolerância oral em animais que apresentam um repertório normal de receptores clonais para o antígeno alvo, mas não em linhagens transgênicas como a linhagem DO11.10 (SIMIONI et al., 2004, 2010). Cerca de 80% dos linfócitos T periféricos do camundongo transgênico da linhagem DO11.10 portam um transgene para as cadeias α e β de TCR que reconhece o peptídeo 323-339 da ovalbumina (OVA), apresentado no contexto de MHC II-IA^d (MURPHY; HEIMBERGER; LOH, 1990), o que torna o seu repertório de TCR bastante limitado. Embora esses animais possam ser imunizados por via parenteral com diferentes proteínas, como a albumina do soro bovino (BSA) e a imunoglobulina humana (dados

não publicados), não foi possível estabelecer a tolerância oral à OVA nesse animal em quaisquer dos diferentes regimes testados (SIMIONI et al., 2004) e nas diferentes faixas etárias estudadas (SIMIONI et al., 2010).

Diversos mecanismos parecem estar envolvidos na indução de tolerância, entre os quais a anergia, deleção clonal e a indução de células T reguladoras (GARSIDE; MOWAT, 2001; PABST; MOWAT, 2012; WEINER et al., 2011). Dados na literatura mostram que células T reguladoras podem desempenhar um papel relevante nas doenças autoimunes (OKAMOTO; UEMOTO; TABATA, 2012; THOMÉ et al., 2012) e a expansão dessa população pode auxiliar no controle de suas manifestações clínicas (MATTEOLI et al., 2010; NEURATH, 2014). Por outro lado, as células Th17 apresentam um papel potencial na indução das doenças inflamatórias intestinais e sua redução também deve contribuir para a melhoria dos quadros clínicos dessas doenças (NEURATH, 2014).

Estudos sugerem que os mecanismos que atuam na indução de tolerância oral estão diretamente relacionados ao regime de administração de antígeno utilizado. Assim, a administração de altas doses de antígenos de uma única vez levaria a deleção clonal ou a anergia, enquanto doses múltiplas de antígeno em baixas concentrações seriam capazes de promover a supressão mediada por células T supressoras/reguladoras (FARIA et al., 2003; FARIA; WEINER, 2006; WEINER et al., 2011).

Contudo, Siewert e colaboradores (2008), encontraram elevada frequência do marcador de supressão denominado *Forkhead box protein 3 (*Foxp3^{+}) em células T de animais submetidos a um protocolo de tolerância oral induzida por alta dose de antígeno, indicando que a dose elevada não levou a deleção clonal, mas à geração de respostas supressoras associadas com a tolerância oral. Por outro lado, resultados recentes do Laboratório de Inflamação e Imunologia Celular (LIIC); da Universidade Estadual de Campinas (UNICAMP, Campinas, SP, Brasil) mostraram que a ingestão continuada de doses baixas de OVA, de fato, interfere na distribuição fenotípica de linfócitos intraepiteliais (IELs) do intestino delgado de camundongos selvagens BALB/c e DO11.10, bem como no padrão de expressão de citocinas por essas células. Após a ingestão de OVA, camundongos selvagens BALB/c apresentaram aumento da frequência de linfócitos T reguladores CD4^{+}Foxp3^{+} e da expressão de citocinas reguladoras. O mesmo tratamento, no entanto, resultou em alterações de natureza inflamatória no intestino delgado de camundongos DO11.10, agravadas pelo desafio parenteral com o antígeno. A ingestão de OVA pelos

transgênicos resultou em IELs com níveis aumentados de mRNA para citocinas inflamatórias, particularmente TNF-α, IL-6 e IL-17 (RUBERTI et al., 2012).

O grupo do LIIC demonstrou que o consumo de OVA por um período de 7 dias foi capaz de reduzir significativamente a severidade da artrite induzida pela administração de colágeno tipo II + OVA em camundongos BALB/c, conforme observado pela redução do edema da pata, bem como a preservação das estruturas ósseas e dos parâmetros imunológicos mais relevantes nos animais tolerantes. Nesse trabalho, nosso grupo demonstrou que a supressão *bystander* induzida pela tolerância oral foi capaz de modificar o curso da artrite, com participação das células dendríticas nesse processo (THOMÉ et al., 2012).

2.3 SUPRESSÃO *BYSTANDER*

A apresentação de antígenos de origem proteica que se inicia na mucosa intestinal tem sido associada com a geração local de células Th3 secretoras de TGF-β (LAP^+), células T reguladoras do tipo 1 (Tr1) secretoras de IL-10 e células Treg $CD8^+$, além de células $TCD4^+CD25^+Foxp3^+$ (COTTREZ et al., 2000; SUN et al., 2007; WEINER et al., 2011). Essas células T reguladoras geradas no intestino migram para órgãos linfoides secundários, tais como placas de Peyer e linfonodos mesentéricos, onde inibem a geração de células efetoras não específicas, por mecanismo conhecido como supressão *bystander* (FARIA; WEINER, 2006).

Os efeitos da supressão *bystander* estão relacionados à liberação de citocinas tais como TGF-β, IL-4 e IL-10, e à ação de células T reguladoras da resposta imune (BAYRAK; MITCHISON, 1998; GOTSMAN et al., 2001; MADDALONI et al., 2015; SHLOMAI et al., 2001; SINHA et al., 2009). Intervenções que levem à supressão *bystander* podem ser de grande interesse para a regulação de autoimunidades (MADDALONI et al., 2015; NOGUCHI et al., 1998). Desta forma, a resposta supressora iniciada pela administração mucosa de antígenos proteicos da dieta associados a antígenos próprios vem ganhando espaço como proposta de intervenção preventiva ou terapêutica nessas enfermidades (BAYRAK; MITCHISON, 1998; WEINER et al., 2011). Nesse sentido, diversos autores têm mostrado que a tolerância oral à antígenos da dieta pode prevenir ou inibir a progressão de doenças autoimunes sistêmicas ou órgão-específicas (CANTARUTI et al., 2017; MADDALONI et al., 2015; OEFNER et al., 2012; THOMÉ et al., 2012; WANG et al., 2013).

O fenômeno de supressão *bystander* associado à tolerância oral foi primeiramente descrito pelo grupo de Weiner (MILLER, 1991). Esses autores mostraram que o TGF-β liberado por células T reguladoras antígeno-específicas reestimuladas *in vitro* era capaz de suprimir a resposta de células T antígeno-específicas e não específicas em sistema de cultura de células com técnica de *transwell*. Da mesma forma, demonstraram que animais alimentados com OVA tornavam-se resistentes à indução da encefalomielite experimental (EAE), quando a proteína básica de mielina e adjuvante completo de Freund (ACF) e a OVA foram administradas simultaneamente em sítios subcutâneos próximos. Em estudo posterior realizado em roedores alimentados com OVA e imunizados com uma mistura de OVA e albumina humana, também foram observados níveis reduzidos de IgE e da resposta de hipersensibilidade tardia para albumina humana comparados com os controles não tratados com OVA por via oral (DAHLMAN-HÖGLUND et al., 1995). Ainda, dados do grupo do LIIC mostraram que é possível proteger camundongos contra a artrite experimental (CIA) induzida por colágeno e OVA em ACF pela tolerização oral com OVA, tanto de forma preventiva como terapêutica, embora os antígenos envolvidos na tolerização e no desencadeamento da doença tenham sido administrados de modo independente, tanto no que se refere à via como ao intervalo de tempo entre ambos (THOMÉ et al., 2012). O aumento da frequência de células T $CD4^{+}Foxp3^{+}$ e $TCD4^{+}IL10^{+}$ também foi observado em análise *ex-vivo* e após a reestimulação *in vitro* de células esplênicas dos animais protegidos. Foi verificado ainda que a transferência adotiva de células dendríticas de animais tolerantes à OVA também foi capaz de conferir proteção contra a CIA. Esses resultados, assim como os trabalhos de Vaz e colaboradores (CANTARUTI et al., 2017; CARVALHO et al., 1994; CARVALHO; VAZ, 1990, 1996; CARVALHO; VERDOLIN; VAZ, 1997) mostraram efeitos indiretos da tolerância oral que não se encaixam perfeitamente no modelo de supressão *bystander*. Os dados desses autores mostraram que é possível obter redução da resposta a antígeno não relacionado àquele utilizado para induzir tolerância oral, mesmo quando sua administração é feita vários dias após a ingestão do tolerógeno e do desafio parenteral com o antígeno tolerado. Trabalho do grupo de Vaz mostra ainda que a expansão de células T reguladoras $TCD4^{+}Foxp3^{+}$ e T $CD4^{+}LAP^{+}$ inespecíficas na fase precoce da exposição parenteral ao antígeno usado no tratamento oral, o que não se observa no animal não tolerante submetido ao mesmo tratamento (CASTRO-JUNIOR et al., 2012).

2.4 LINFÓCITOS T *HELPER* (TH) E CITOCINAS RELACIONADAS

No final da década de 1980 foi identificado que as populações de células T *helper näive* conseguiam se diferenciar em células, Th1 e Th2, sendo essas populações definidas pelos seus perfis de secreção de citocinas específicas após serem estimuladas (MOSMANN et al., 1986). Mais recentemente, um novo subconjunto de linfócitos T *helper* foi identificado, os linfócitos T *helper* 17 (Th17), os quais secretam IL-17 e IL-23 (STROBER; FUSS, 2011). Já os linfócitos T reguladores, células que expressam o marcador $CD4^+$, a cadeia α do receptor da IL-2 ($CD25^+$) e o fator de transcrição $Foxp3^+$, se desenvolvem no timo (DE SOUZA; FIOCCHI, 2016) e possuem função primordial na homeostase do organismo e na prevenção de doenças autoimunes (KO et al., 2010; YAMADA et al., 2016).

As células Treg têm sido muito empregadas na busca de tratamento de diversas doenças autoimunes, entre elas as DII (KRAUS; MAYER, 2005). Essa população de células possui potencial para supressão de outras populações celulares da resposta imune (STEINMAN; HAWIGER; NUSSENZWEIG, 2003). Algumas citocinas características secretadas pelas células Treg ($CD4^+CD25^+$) são interleucina-10 (IL-10) e TGF- β (NAKAMURA; KITANI; STROBER, 2001).

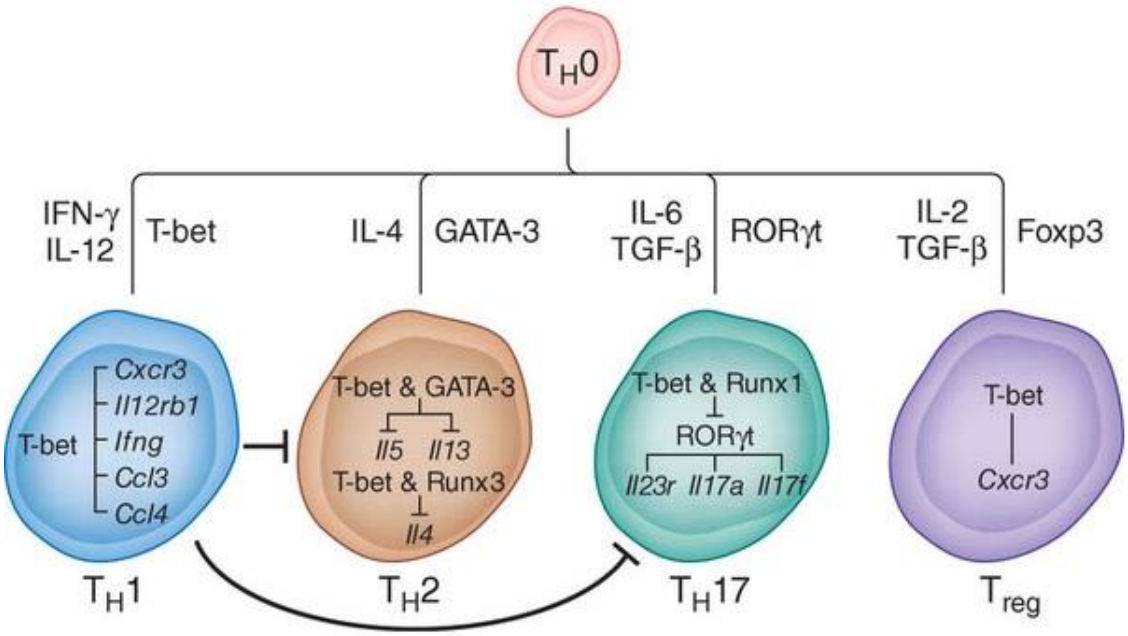
Ainda, populações de células reguladoras expressam altos níveis do antígeno 4 associados ao linfócito T citotóxico (CTLA-4), uma molécula co-estimulatória que compete com o receptor CD28 pela ligação com a molécula B7. CTLA-4 tem maior afinidade de ligação pelo B7 do que o receptor CD28 e a ligação entre CTLA-4 e B7 bloqueia a reposta imunológica, pela inibição do segundo sinal e supressão da sinalização desencadeada pela ligação do receptor de linfócitos T (TCR) (WALKER, 2013).

Estudo utilizando camundongos *knockout* para IL-10, que não expressam o fator de transcrição $Foxp3^+$ comprovou que essa citocina inibe a função das células dendríticas e de macrófagos (MURAI et al., 2009). Já o TGF- β inibe a função efetora dos linfócitos e dos macrófagos (MCGEACHY et al., 2007; NEURATH et al., 1996b).

Estudo recente mostrou que pacientes com DII apresentam um número aumentado de células Th17, bem como de fatores de transcrição, entre eles, T-bet, GATA-3, STAT3 e STAT4 além de diminuição de Treg (YAMADA et al., 2016). O fator de transcrição T-bet, expresso nas populações de células Th1, induz a secreção de IFN-γ. O fator de transcrição GATA-3, associado à população Th2, está relacionado com a secreção de IL-4, IL-5 e IL-13. Já a subpopulação Th17

é orquestrada pelo fator de transcrição RORγt, como mostra a Figura 3 (LAZAREVIC; GLIMCHER, 2011; NEURATH; FINOTTO; GLIMCHER, 2002). Sabe-se que a administração combinada das citocinas TGF-β e IL-6 ou IL-21 são capazes de estimular a geração *in vitro* de células Th17 (KORN et al., 2009) e *in vivo* (GUO, 2016; KORN et al., 2009; LAZAREVIC; GLIMCHER, 2011; MANGAN et al., 2006). O desenvolvimento de células Th17 e Treg estão interligados, visto que o TGF- β induz a diferenciação de células T *näive* Foxp3^{+}. A secreção de IL-6 suprime essa ativação dessa população reguladora. Já a combinação dessas duas citocinas, que possuem funções opostas, é capaz de promover a diferenciação de Th17 e a IL-21 amplifica a frequência de células Th17 (KORN et al., 2009).

Figura 3 - Diferenciação de células T *helper*. Th1: T *helper* 1; Th2: T *helper* 2; Th17: T *helper* 17; Treg: T reguladores; CXCR3: receptor de quimiocina; IL12rb1: subunidade β2 do receptor de IL-12; CCL3 e CCL4: quimiocinas; T-bet; fator de transcrição do Th1; GATA-3: fator de transcrição do Th2; RORγt: fator de transcrição do Th17; Foxp3: fator de transcrição de Treg; Runx1 e Runx3: fatores de transcrição; IL: interleucina; IFN-γ: interferon gama; TGF-β: fator transformador de crescimento beta.



Fonte: Retirado de LAZAREVIC; GLIMCHER (2011).

Além das subpopulações relatadas acima foi identificada recentemente a subpopulação de células T *helper* 9, que secreta a citocina característica IL-9. O fator de transcrição, denominado PU.1 que caracteriza a secreção de IL-9 é induzido pela estimulação das células T pela citocina TGF-β. O PU.1 é capaz de regular negativamente a diferenciação de células do perfil Th2. Estudo mostra que células T efetoras Th9 que expressam PU.1 são essenciais para a indução da colite, sendo a manipulação dessa população uma possível abordagem para o tratamento de DII (GERLACH et al., 2014).

A inflamação nas DII se deve à secreção de citocinas pró-inflamatórias pelos leucócitos (HANAUER, 2006). O desequilíbrio entre as citocinas pró-inflamatórias e anti-inflamatórias encontradas na mucosa intestinal determina a intensidade e a duração da resposta inflamatória na colite experimental (KOTREDES; THOMAS; GAMERO, 2017; NEURATH et al., 1995b; SHLOMAI et al., 2001). Essa secreção exacerbada de citocinas pró-inflamatórias no intestino ativa células Th1, Th2 e Th17 da mucosa (HANAUER, 2006), impedindo o término da inflamação e induzindo a destruição tecidual que resulta na DII (NEURATH, 2014).

Os mecanismos envolvidos nas autoimunidades são diversos e não mutuamente exclusivos, podendo incluir uma combinação de fatores como a produção de IL-10, IL-35, TGF-β, CTLA-4, gene de ativação de linfócito-3 (LAG-3), indoleamina-2,3-dioxigenase (IDO), perforinas/granzimas e antagonistas do receptor do fator de necrose tumoral induzido por glicocorticoide (GITR) (MIYARA; SAKAGUCHI, 2007). Dados recentes mostram que células T *helper* 17 e outras células produtoras de IL-17 desempenham um papel crucial nas manifestações inflamatórias intestinais. A IL-17, juntamente com a IL-22, parece estar relacionada com a indução de colite, já que estas citocinas iniciam e amplificam os sinais inflamatórios locais e promovem a ativação de mecanismos contra regulatórios diretamente nas células do epitélio intestinal (MONTELEONE et al., 2012). Além dessas citocinas, a IL-23 liberada por células dendríticas e macrófagos da mucosa intestinal inflamada ativa o fator de transcrição STAT4 em linfócitos T de memória presentes no local, estimulando a produção de IFN-γ. Por sua vez, o IFN-γ induz a produção de citocinas inflamatórias por células do sistema imune inato, que contribuem para o aumento da inflamação tecidual na colite (GEREMIA; JEWELL, 2012). A IL-21 junto com o TGF-β fazem parte de um ciclo de realimentação para as células do subgrupo Th17 (KORN et al., 2009).

A doença de Crohn apresenta um perfil celular caracterizado principalmente por linfócitos Th1, com aumento das citocinas IFN-γ, TNF-α e IL-2. Essas citocinas estimulam a liberação de IL-1, IL-6, IL-8, IL-12 e IL-18 por APC, tornado essa resposta retroalimentada (DE MATTOS et al., 2015; STROBER; FUSS, 2011). Na colite ulcerativa, as subpopulações de células T mais comumente encontrados são de células Th2 que por sua vez, secretam com maior frequência IL-4, IL-5 e IL-9. Estudo recente mostra que células Th9, produtoras de IL-9, prejudicam a função da barreira intestinal e impede a cicatrização da mucosa. (GERLACH et al., 2014). Outro estudo realizado em pacientes asmáticos revela que a inibição da IL-9 pode possibilitar o desenvolvimento de tratamentos contrarreações alérgicas como a asma. A população Th9 apresenta um perfil de citocinas pró-inflamatórias presente em doenças autoimunes e na inflamação. A citocina IL-9 possui ação pleiotrópica sobre as células do sistema imune (ARAÚJO, 2013).

Ainda nesse contexto, a literatura indica que, além dos linfócitos, as células dendríticas (DCs) também estão implicadas tanto na indução como na manutenção da tolerância, se tornando um alvo terapêutico no tratamento das autoimunidades (NAKAMURA; KITANI; STROBER, 2001; RAMSDELL, 2003; THORSTENSON; KHORUTS, 2001).

2.5 CÉLULAS DENDRÍTICAS

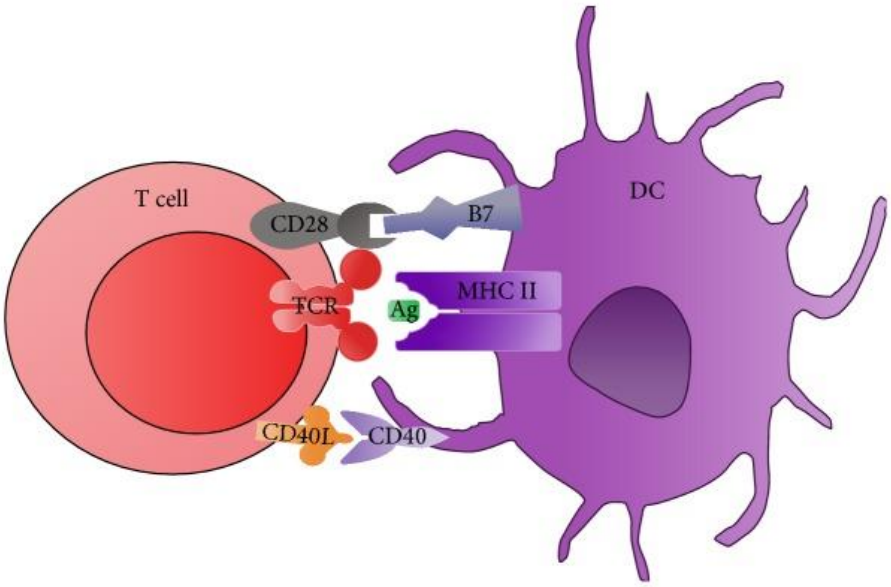
A resposta imune inata e a resposta imune adaptativa possuem células efetoras em comum, entre elas, DCs que são células que auxiliam na apresentação de antígenos. Essas células são derivadas da medula óssea e encontradas em tecidos epiteliais e linfoides, se tornando madura após a captura do antígeno (RUBERTI et al., 2012; THOMÉ et al., 2012).

As DCs, assim como os macrófagos e os linfócitos B, são APC que expressam moléculas do complexo principal de histocompatibilidade de classe II (MHC-II) em suas superfícies, que conhecidamente apresentam antígenos para os linfócitos $TCD4^{+}$, além de expressar a molécula MHC-I que apresentam antígeno para $TCD8^{+}$, presente em todas as células nucleadas, gerando o primeiro sinal para o processo de ativação. Expressam ainda moléculas co-estimulatórias, CD80 e CD86 (família da B7) e CD40, como ilustrado na Figura 2 (SILVA et al., 2015; YOO; HA, 2016). A ausência dessas moléculas co-estimulatórias que fornece o segundo sinal de ativação pode gerar a indução de tolerância, pela interação entre as moléculas da família B7 presente nas

DCs com CTLA-4 nos linfócitos T (RUBERTI et al., 2012; WALKER, 2013). Entre as APC, as DCs são as únicas que conseguem apresentar antígenos para linfócitos TCD4^{+} *näive*; sendo capazes de apresentar antígenos próprios e não próprios (MOREAU et al., 2012; SILVA et al., 2015).

A maturação da célula dendrítica transforma uma simples célula captadora de antígeno em eficiente célula apresentadora profissional, caracterizada por alta expressão de MHC-II, CD80, CD86, CD40, receptor de quimiocina CCR7 e receptores de Fc, encontrada em regiões rica em linfócitos T, enquanto que célula dendrítica imatura possui altos níveis de receptores de quimiocinas como, CCR1, CCR5, CCR6, baixos níveis de CCR7 e das moléculas co-estimulatórias CD80, CD86 e CD40 e reside principalmente na pele, no epitélio gastrointestinal e respiratório (STEINMAN; BANCHEREAU, 2007; STEINMAN; HAWIGER; NUSSENZWEIG, 2003). Estudo mostrou que defeitos no gene do receptor de quimiocina CCR7 e integrinas estão associados com o surgimento de DII, como resultado de migração defeituosa de células Treg (YAMADA et al., 2016).

Figura 2 - Representação esquemática da interação de células T e DCs: as principais moléculas co-estimuladoras. DC: células dendríticas; MHC II: complexo principal de histocompatibilidade II; TCR: receptor de células T; CD40L: ligante de CD40.



Fonte: Retirado de SILVA et al (2015).

As DCs têm sido grande alvo de estudo em diversas áreas, por desempenharem papel fundamental na imunidade e na tolerância (SILVA et al., 2015). Dados da literatura mostram que a indução do perfil tolerogênico em DCs (tDC) manipuladas *ex vivo* para tratamento de rejeição de transplante e doenças autoimunes promoveu estado de anergia nos linfócitos T. Ainda, as DCs podem atuar na indução da tolerância de células T pela indução de células T reguladoras (Treg) (MOREAU et al., 2012; YOO; HA, 2016) e deleção clonal (STEINMAN; HAWIGER; NUSSENZWEIG, 2003). As DCs produzem elevadas concentrações de ácido retinóico, no qual está associado à transformação de um linfócito T *näive* em T reg, a fim de produzir TGF-β (FARIA; WEINER, 2005; STEINMAN; HAWIGER; NUSSENZWEIG, 2003).

A despeito do grande interesse despertado pelo assunto, os efeitos da tolerância oral e a participação de células dendríticas tolerogênicas na colite ainda não estão completamente elucidados (MATTEOLI et al., 2010; NEURATH et al., 1995; PEDERSEN et al., 2007; SHAN-SHAN; YU-LAN, 2009).

3 OBJETIVOS

- Avaliar os efeitos da tolerância oral e da transferência de células tolerogênicas na modulação da resposta inflamatória encontrada na colite experimental.

3.1 OBJETIVOS ESTRATÉGICOS

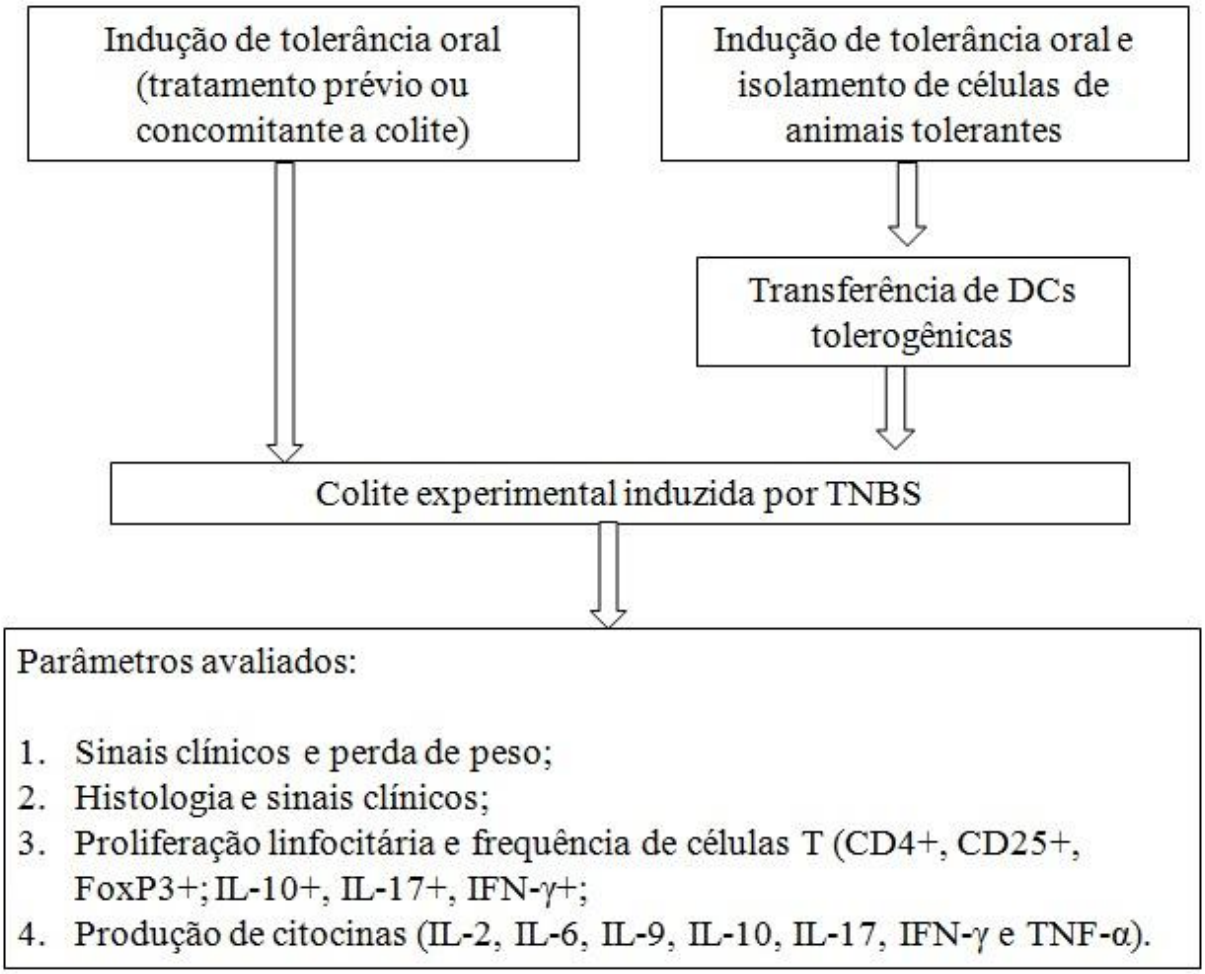
✓ Para avaliar os efeitos da tolerância oral no curso da colite experimental induzida por TNBS, camundongos BALB/c foram tratados com OVA por via oral, antes e concomitantemente com a indução de colite;

✓ Para avaliar os efeitos da transferência adotiva de células dendríticas tolerogênicas no curso da colite experimental induzida por TNBS, células dendríticas isoladas de camundongos BALB/c tratados com OVA por via oral foram administradas por via intravenosa a camundongos BALB/c antes da indução de colite.

Em todos os grupos e nos respectivos controles os seguintes parâmetros foram avaliados:

- Sinais clínicos
- Variação de peso
- Histologia do tecido colônico
- Proliferação celular frente a estímulo inespecífico (Concanavalina A)
- Frequência de células secretoras de citocinas IL-10, IL-17, IFN-γ e de células $CD4^{+}CD25^{+}Foxp3^{+}$.
- Níveis de citocinas (IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ e IL-9) em sobrenadantes de cultura de células de baço estimuladas com Concanavalina A.

4 DESIGN EXPERIMENTAL



5 MATERIAL E MÉTODOS

5.1 ANIMAIS

Os camundongos da linhagem BALB/c, fêmeas com quatro semanas de idade, foram obtidos no CEMIB (Centro Multidisciplinar para Investigação Biológica na Área da Ciência de Animais de Laboratório – UNICAMP, Campinas, SP, Brasil). Esses animais foram mantidos em microisoladores no Biotério do Departamento de Microbiologia e Imunologia (DMI) do Instituto de Biologia (IB), com água e ração autoclavados em livre demanda e ambiente com temperatura e fotoperíodo controlados. Os grupos experimentais foram compostos por um mínimo de 5 animais (n=5). Este estudo foi aprovado pelo Comitê de Ética do Uso de Animal (CEUA) da Universidade Estadual de Campinas (UNICAMP), protocolo nº 3077-1 (ANEXO 1).

5.2 TOLERÂNCIA ORAL

Para a indução de tolerância oral, foi utilizado protocolo de administração de OVA por adição à água de beber, como descrito anteriormente (SIMIONI et al., 2004), com algumas modificações. Camundongos BALB/c receberam ovalbumina (OVA) da marca Rhoster (Comércio e Indústria Rhoster. LTDA., Vargem Grande Paulista, SP, Brasil), na concentração de 4mg/mL, misturada a água de beber, por 7 dias consecutivos. Os camundongos do grupo controle receberam apenas água.

5.3 ISOLAMENTO E TRANSFERÊNCIA ADOTIVA DE DC DO BAÇO

Células esplênicas de camundongos tolerantes a OVA e do grupo controle foram isoladas e incubadas em solução salina balanceada de Hanks (HBSS, HyClone, Sigma) livre de cálcio e magnésio, contendo 0,5mg/mL de colagenase (tipo 1A – Sigma) por 15 minutos em temperatura ambiente. Em seguida, as células foram maceradas com ajuda de peneira e lavadas por duas vezes com PBS-BSA, 0,5% por centrifugação a 200g por 10 minutos à 4ºC (5804 R - Centrifugue Eppendorf). Em seguida, as hemácias foram lisadas com uso de tampão de lise (solução de Ack 1%) por 2 minutos. Para interrupção de lise celular, adicionou-se 10mL de PBS-BSA 0,5% e as suspensão celular foi submetida à nova centrifugação. O *pellet* celular foi ressuspendido em 1mL de PBS-BSA 0,5%. Os agregados celulares foram removidos por filtração em seringa de 1mL. Após contagem e ajuste de concentração para 1.10^6 células por mL foi adicionado 100µL de

beads imunomagnéticos recobertos com anticorpos anti-CD11c (130-052-001, Miltenyi Biotec, Alemanha) conforme recomendações do fabricante. As populações de células CD11c foram isoladas em coluna de separação (MS MACS, Miltenyi Biotec, Alemanha) (THOMÉ et al., 2012). Alíquotas de $3x10^5$ DCs, purificadas como acima descrito, foram administradas por via intravenosa (i.v.) em 3 doses, nos camundongos tratados e controles, nos dias -5, -3 e 1 do início da indução da colite.

5.4 INDUÇÃO DA COLITE EXPERIMENTAL

Os camundongos experimentais do grupo colítico ou controle foram previamente anestesiados com solução de cetamina (60 mg/kg) e xilazina (6mg/kg). Em seguida, uma cânula uretral foi introduzida cuidadosamente no lúmen do cólon até 4cm proximais do ânus, para administração do agente indutor da colite ou do veículo. Para a indução da colite, os camundongos receberam 100 µL de 2,4,6 - ácido trinitrobenzenosulfônico (TNBS, Sigma, USA) na concentração de 1,0mg/mL diluído em etanol a 50% (para quebrar a barreira do epitélio intestinal) segundo indicações de Neurath e colaboradores (1995), com modificações. Os animais foram mantidos em posição vertical por 30 segundos. Animais do grupo controle receberam 100µL de etanol 50%. Os animais de todos os grupos foram pesados individualmente e acompanhados clinicamente, diariamente, até o sacrifício. Os sinais clínicos como diarreia, prolapso retal, hemorragia e caquexia foram registrados e foram atribuídas pontuações, variando de 0 a 2, sendo 0: sem alteração, 1: ligeira alteração e 2: alteração grave.

5.5 HISTOLOGIA DOS INTESTINOS

Camundongos (n=3) submetidos aos diferentes tratamentos foram sacrificados cinco dias após a instilação do TNBS. A porção distal dos intestinos foi removida e lavada cuidadosamente com PBS 0,02M e pH 7,3. Os tecidos foram fixados com paraformoldeído 4% em PBS 0,02M pH 7,2. As peças foram mantidas em solução sob agitação por 24h. Em seguida, as mesmas foram lavadas com PBS 0,02M pH 7,2 por 3 vezes de 30 minutos e com água destilada por 30 minutos. Os tecidos foram diafanizados e incluídos em parafina (Paraplast Plus Sigma P3683). Os cortes finos (5µm) foram obtidos em micrótomo (modelo Jung Biocut 2035, Leica) e corados com Hematoxilina e Eosina (Merck). Duas porções distais dos intestinos (1 a 2 cm e 2 a 3 cm do reto) foram avaliadas quanto a presença de hemorragia (escore de 0 a 2, sendo, 0- nenhum, 1-

hemorragia presente, 2– grande hemorragia), pregas (escore de 0 a 5, sendo, 0 – pregas normais e evidentes, 1 – pregas levemente disformes, 2 – pregas disformes, 3- pregas com forma prejudicada, 4- redução drástica das pregas e grande infiltrado, 5- inexistência de pregas e alto comprometimento nos tecidos), dilatação da mucosa (escore de 0 a 2, sendo, 0 – nenhum ou reduzidos vazios, 1- vazios aparentes e 2 – elevado número de vazios) e infiltrado linfocitário na mucosa, submucosa e mesentério (escore de 0 a 4, sendo, 0- nenhum, 1- reduzido infiltrado, 2- médio infiltrado, 3- considerável infiltração com desorganização da submucosa e 4 – imenso infiltrado). O espessamento da parede do colón foi medido em micrômetros, em duas porções distais, com o programa Infinity Analyze Nikon H600L (aumento de 100X).

5.6 PROLIFERAÇÃO CELULAR

5.6.1 OBTENÇÃO DE SUSPENSÃO CELULAR

Para os ensaios de proliferação, parte dos animais (n=6) foi sacrificada e os baços foram removidos em condições assépticas, em fluxo laminar e colocados a placas de Petri contendo 2mL de meio RPMI 1640 (Sigma–Aldrich Chemic). Os baços foram macerados individualmente com auxílio de peneira e, em seguida, as suspensões celulares foram transferidas para tubos cônicos e centrifugadas a 1500 rpm por 10 minutos a 4ºC (Centrifugue 5804 R - Eppendorf). O sobrenadante foi descartado e o *pellet* de células esplênicas foi ressuspendido em 2mL de tampão de lise por 2 minutos. Para interrupção da lise celular, adicionou-se 10mL de meio RPMI estéril. Após novo ciclo de lavagem por centrifugação, as células foram ressuspendidas em 5mL de RPMI. As suspensões tiveram sua concentração ajustada para 1 x 10^6 células/mL em meio de cultura RPMI contendo 10% de Soro Fetal Bovino (SBF, Nutricell, Campinas, SP, Brasil) e gentamicina (Sigma) a 50µg/mL.

5.6.2 MARCAÇÃO COM CFSE

Uma alíquota de 1mL de suspensão de células de baço foi removida para marcação com *Carboxyfluorescein succinimidyl ester* (CFSE, Invitrogen, USA). Para tanto, adicionou-se 25µM de CFSE. As suspensões celulares foram estimuladas com 2,5µg/mL de Concanavalina A (ConA, Sigma) e foram semeadas em placas de 96 poços (Corning) em um volume de 100 µL, em sextuplicata. Para determinar o valor máximo de incorporação da sonda, alíquotas das suspensões celulares marcadas com CFSE foram fixadas no dia 0 com PBS contendo 1% de formaldeído e

reservadas para análise por citometria de fluxo (FACSCalibur, BD Pharmingen). As placas foram incubadas a 37ºC em estufa a 5% de CO_2 por 72 horas. Após o período de incubação todas as células foram fixadas com PBS contendo 1% de formaldeído. Foi realizada contagem e ajuste de concentração celular, ressuspendendo-se o *pellet* celular em PBS conforme descrito anteriormente (SIMIONI et al., 2010; THOMÉ et al., 2012). As populações foram transferidas para tubos apropriados para análise da proliferação celular em citômetro de fluxo.

5.6.3 AVALIAÇÃO DO PERFIL FENOTÍPICO DAS DC

Os perfis fenotípicos das DCs foram avaliados por citometria de fluxo, por marcação com anticorpos anti-CD11c APC (Clone: N418) ou (Clone: HL3), anti-MHC-II PE (130-091-368), anti-CD80 FITC (Clone: 16-10A1), anti-CD86 FITC (Clone: GL1) e anti-CD40 FITC (Clone: 3/23) em concentrações previamente padronizadas e em seguida incubadas por 30 minutos à 4ºC. As suspensões celulares foram lavadas com o tampão de lavagem do kit e fixadas com PBS contendo 1% de formaldeído. As aquisições foram realizadas em citômetro de fluxo (FACScalibur, Becton-Dickinson, Franklin Lakes, NJ, USA) localizado no Instituto de Biologia da UNICAMP. As análises das populações foram realizadas utilizando-se o software FCS Express para análise

5.6.4 AVALIAÇÃO DO PERFIL FENOTÍPICO DOS LINFÓCITOS T

Após o cultivo celular, a frequência de células T foi avaliada por ensaio de imunofluorescência e leitura por citometria de fluxo. Para tanto, as suspensões celulares foram marcadas com os anticorpos anti $CD4^+$ PE (Clone: GK1.5) e anti $CD25^+$ FITC (Clone: 7D4) em concentrações previamente padronizadas e em seguida incubadas por 30 minutos à 4ºC. As suspensões celulares foram lavadas e as células foram permeabilizadas pela adição tampão de fixação/permeabilização (*Cytofix/cytoperm fixation/permeabilization* kit, Becton-Dickinson, BD) e marcadas com os anticorpos anti $Foxp3^+$ APC (Clone: FJK-16s), anti $IL\text{-}17^+$ APC (Clone: eBIO17B7) ou Alexa Fluor 647 (Clone: TC11-18410), anti IFN-γ^+ APC (Clone: XMG1.2), IL-10^+APC (Clone: JESS-16E3), anti T-bet Alexa 647 (Clone: O4-46), anti GATA3 Alexa Fluor 647 (Clone: L50-823) e anti ROR-GT Alexa Fluor 647 (Clone: Q21-378). Posteriormente, as suspensões foram incubadas a 4ºC por 50 minutos e então lavadas com o tampão de lavagem do kit. As suspensões foram novamente, incubadas por 50 minutos à 4ºC, lavadas e fixadas com

PBS contendo 1% de formaldeído. Para as aquisições das populações foi utilizado o citômetro de fluxo FACScalibur (Becton-Dickinson, Franklin Lakes, NJ, USA) localizado no Instituto de Biologia da UNICAMP. As análises foram realizadas com o software *FCS Express 5 Plus Research Edition* (FCS Express Launcher).

5.6.5 DOSAGEM DE CITOCINAS

As citocinas IL-2, IL-4, IL-6, TNF-α, IFN-γ, IL-17 e IL-10 foram quantificadas nos sobrenadantes das culturas de células esplênicas por ensaio de citometria de fluxo, utilizando o kit de CBA Mutiplex (BD, Cytometric Bead Array Th1/Th2/Th17, San Diego, USA) segundo instruções do fabricante. A determinação da IL-9 foi realizada com o kit Flex CBA (BD Cytometric Flex Set Th9, San Diego, USA), segundo instruções do fabricante. Resumidamente, foram adicionados *beads* de captura das citocinas em tampão diluente do kit e, em seguida, foi adicionada a amostra de sobrenadante de cultura a ser quantificada (ou o padrão de cada citocina), seguido do reagente de detecção. As reações foram incubadas por 2 horas em temperatura ambiente, protegidas da luz, e em seguida centrifugadas e ressuspendidas em tampão de lavagem, como indicado pelo fabricante. As amostras foram adquiridas em equipamento FACScalibur (Becton-Dickinson) e analisadas com o software FCAP Array ™ Software Version 3.0 (BD).

5.7 ANÁLISE ESTATÍSTICA

A análise estatística dos resultados foi realizada através do teste ANOVA sendo utilizado o teste Bonferroni a *posteriori*, utilizando o programa GraphPad Prism 5, a fim de comparar os grupos (n=6). Foram considerados os valores significativos de $p < 0,05$.

6 RESULTADOS

Os resultados obtidos permitiram a elaboração de dois manuscritos. Um artigo científico, descrito no capítulo 1 e apresentado no anexo 2, foi previamente publicado em revista indexada internacional (Plos One, com fator de impacto 3,05 segundo The Thomson Reuters Impact Factor). Um segundo manuscrito foi submetido à avaliação para publicação para a mesma revista no mês de julho de 2017 (capítulo 2 e anexo 3).

6.1 CAPÍTULO I

Artigo publicado a revista Plos One (Anexo 2)

Title: Oral Tolerance Induced by OVA Intake Ameliorates TNBS-Induced Colitis in Mice

Lisiery N. Paiatto[1,2]; Fernanda G. D. Silva[3]; Julia Bier[1], Márcia R Brochetto-Braga [2], Áureo T. Yamada[4], Wirla M. S. C. Tamashiro[1]; Patricia U. Simioni[1, 2, 5 *]

[1]Department of Genetics, Evolution and Bioagents, Institute of Biology, State University of Campinas, UNICAMP, Campinas, SP, Brazil.

[2] Institute of Biosciences, Universidade Estadual Paulista, UNESP, Rio Claro, SP, Brazil.

[3]Faculty of Food Engineering, University of Campinas, UNICAMP, Campinas, SP, Brazil.

[4]Department of Histology and Embryology, Institute of Biology, State University of Campinas, UNICAMP, Campinas, SP, Brazil.

[5]Department of Biomedical Science, Faculty of Americana, FAM, Americana, SP, Brazil.

[*] Corresponding author

E-mail: psimioni@gmail.com (PUS)

Resumo

Introdução: Os dados da literatura mostraram que o consumo de proteínas na dieta pode causar efeitos moduladores no sistema imunológico do hospedeiro, processo denominado tolerância oral por supressão *bystander*. Tem sido demonstrado que a supressão *bystander* induzida por proteínas dietéticas pode melhorar doenças inflamatória tais como artrite experimental. Aqui, avaliamos os efeitos da tolerância oral induzida pela ingestão de ovalbumina (OVA) na colite induzida por TNBS em camundongos, um modelo experimental para a doença de Crohn humana. **Métodos e Resultados:** A colite foi induzida em camundongos BALB/c por instilação de uma dose única de TNBS (100 mg / kg) em etanol para o cólon. Os camundongos tolerizados receberam OVA (4mg/mL) dissolvida na água potável durante sete dias consecutivos, antes ou concomitantemente com a instilação intrarretal. Os grupos de controlo receberam água isenta de proteínas e etanol por via intrarretal. Observou-se que a indução prévia ou concomitante de tolerância oral foi capaz de reduzir a gravidade da colite observada pela recuperação do ganho de peso corporal, melhora dos sinais clínicos e redução das anormalidades histológicas. A proliferação *in vitro* de células de baço a partir de camundongos colíticos tolerantes foi inferior à dos camundongos de controle, o mesmo que as frequências de células T $CD4^+$ que secretam IL-17 e IFN-γ. As frequências de células T reguladoras e células T que secretam IL-10 aumentaram significativamente em camundongos tratados oralmente com OVA. Os níveis de citocinas inflamatórias (IL-17A, TNF-α, IL-6 e IFN-γ) foram menores nos sobrenadantes das células de camundongos colíticos tolerantes, enquanto os níveis de IL-10 foram maiores. **Conclusão:** Os dados mostram que a modulação da resposta imune induzida pela tolerância oral reduz a gravidade da colite experimental. Tal modulação pode ser parcialmente atribuída ao aumento de

células Treg e à redução de citocinas pró-inflamatórias em órgãos linfoides periféricos de camundongos tolerantes por supressão *bystander*.

Abstract

Introduction: Literature data have shown that the consumption of dietary proteins may cause modulatory effects on the host immune system, process denominated oral tolerance by bystander suppression. It has been shown that the bystander suppression induced by dietary proteins can improve inflammatory diseases such as experimental arthritis. Here, we evaluated the effects of oral tolerance induced by ingestion of ovalbumin (OVA) on TNBS-induced colitis in mice, an experimental model for human Crohn's disease. **Methods and Results**: Colitis was induced in BALB/c mice by instilling a single dose of TNBS (100 mg/kg) in ethanol into the colon. Tolerized mice received OVA (4mg/mL) dissolved in the drinking water for seven consecutive days, prior to or concomitantly with the intrarectal instillation. Control groups received protein-free water and ethanol by intrarectal route. We observed that either the prior or concomitant induction of oral tolerance were able to reduce the severity of colitis as noted by recovery of body weight gain, improvement of clinical signs and reduction of histological abnormalities. The *in vitro* proliferation of spleen cells from tolerant colitic mice was lower than that of control mice, the same as the frequencies of $CD4^+$ T cells secreting IL-17 and IFN-γ. The frequencies of regulatory T cells and T cells secreting IL-10 have increased significantly in mice orally treated with OVA. The levels of inflammatory cytokines (IL-17A, TNF-α, IL-6 and IFN-γ) were lower in supernatants of cells from tolerant colitic mice, whereas IL-10 levels were higher. **Conclusion:** Our data show that the modulation of immune response induced by oral tolerance reduces the severity of experimental colitis. Such modulation may be partially attributed to the increase of

Treg cells and reduction of pro-inflammatory cytokines in peripheral lymphoid organs of tolerant mice by bystander suppression.

Introduction

Recently, inflammatory bowel disease (IBD) has been receiving more attention from physicians and researchers due to the increase in its incidence in human populations(COSNES et al., 2011; DE MATTOS et al., 2015; ENGEL; NEURATH, 2010; GEREMIA et al., 2014; MOLODECKY et al., 2012). IBD comprises a set of related diseases, collectively called Crohn's Disease (CD) and Ulcerative Colitis (UC), which origin has been attributed to the breakdown of tolerance to self-antigens in the intestinal mucosa(FARIA; WEINER, 2005). The mechanisms involved in these autoimmunities are diverse and cannot be considered mutually exclusive. They may include a combination of factors among which one can highlight the unbalanced production of cytokines and interleukins such as interleukin (IL)-9 IL-10, IL-35, transforming growth factor (TGF)-β. Several other molecules, transcription factors and receptors are associated with colitis, including Cytotoxic T-Lymphocyte Antigen (CTLA)-4, Leukocyte Activation Gene (LAG) -3, indoleamine (IDO), perforin/antagonists and Glucocorticoid-Induced Tumor Necrosis Factor Receptor (GITR) (BANKS et al., 2003; KOBAYASHI et al., 2008; MIYARA; SAKAGUCHI, 2007; SHEN; DURUM, 2010; TAKAMATSU et al., 2013).

Recent literature, however, emphasizes the role of Th17 cells in inflammatory diseases such as colitis. T helper (Th) 17 cells and other IL-17-producing-cells play a crucial role in intestinal inflammatory diseases. IL-17 together with IL-22 appear to be related to induction of colitis, since these cytokines initiate and amplify the local inflammatory signs and promote the activation of regulatory mechanisms directly against the cells of the intestinal epithelium (HARBOUR et al., 2015; JIANG et al., 2014). In turn, IFN-γ induces the production of inflammatory cytokines

by cells of the innate immune system, contributing to an increased tissue inflammation seen in colitis (GEREMIA et al., 2014; GUO, 2016; HARBOUR et al., 2015; OH et al., 2014). Therefore, modulation of Th17 and INF-γ-secreting cells may affect the inflammation in colitis.

On the other hand, IL-10 produced by macrophages and regulatory T cells may skew the response into a regulatory one, leading to a reduction in inflammation(GUO, 2016). In addition, several results suggest that tolerogenic dendritic cells (DC) lead to the generation of induced regulatory T (Treg) cells ($CD4^{+}CD25^{+}Foxp3^{+}$) and an increase in the number of lymphocytes expressing the suppressor molecule CTLA-4 (HUANG et al., 2010; KO et al., 2010; MATTEOLI et al., 2010a; PLETINCKX et al., 2011).

Accordingly, several studies have attempted to modulate the inflammatory response observed in experimental colitis by the induction of a tolerogenic response (FARIA; WEINER, 2006; HYUN; BARRETT, 2006; KRAUS; MAYER, 2005; MATTEOLI et al., 2010a; SHAN-SHAN; YU-LAN, 2009). Specifically, oral tolerance by dietary antigens administration is a tool to the modulation of the immune system. The suppression of immune response on oral tolerance is due to the low responsiveness of local or systemic immune system, triggered by such protein antigens (BARONE et al., 1998; FUJIHASHI; MCGHEE, 2004; MATTEOLI et al., 2010a; PABST; MOWAT, 2012; WHITACRE et al., 1996). Despite the great interest in the subject, the effects of oral tolerance by bystander suppression in colitis are not yet fully understood (DUAN et al., 2012; MATTEOLI et al., 2010a; NEURATH et al., 1995a; SHAN-SHAN; YU-LAN, 2009).

The aim of this study was to evaluate the effects of oral tolerance on TNBS-induced colitis in mice of BALB/c. Tolerance was induced by oral administration of ovalbumin (OVA) in the previous and subsequent induction of colitis. Parameters as body weight, histology of target tissues, cell proliferation, cytokine production and expansion of regulatory CD25+Foxp3+ T cells

and Th17 cells on spleen cell cultures were evaluated here. Our data suggested that oral tolerance to OVA modulates the immune responses against TNBS in BALB/c mice by bystander suppression. Suppression of the immune response is reflected in the increase in regulatory T cells and decreased production of pro-inflammatory cytokines that together markedly ameliorated the severity of the colitis.

Material and Methods

Animals

SPF female BALB/c mice (four weeks old) were obtained from the Multidisciplinary Center for Biological Research (CEMIB) of the University of Campinas (UNICAMP) Campinas, SP, Brazil, and housed in plastic cages in groups of five. They were maintained in specific pathogen-free environment at 25o C ±1 and photoperiod of 12/12 hours, and fed with autoclaved Nuvilab CR-diet and water *ad libitum* for at least 4 weeks before being used in experiments. The methods described in this manuscript were carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals', as promoted by the Brazilian College of Animal Experimentation (COBEA), and was approved by the Ethics Committee for Animal Experimentation of University of Campinas. (CEUA/UNICAMP. Protocol #3077-1). All experimental procedures were performed under proper anesthesia and all efforts were made to minimize animal suffering. Mice general health was daily monitored for signs of inflammation such as rectal swelling, rectal bleeding, soft stool or weight loss. On day 5 after TNBS instillation, all mice were sacrificed by cervical dislocation after anesthesia with a mixture of ketamine (60 mg/kg, Ketalar; Pfizer) and xylazine (6 mg/kg, Rompun; Bayer) (i.p.).

Oral tolerance

The induction of oral tolerance to OVA was performed as described elsewhere (SIMIONI et al., 2004) and depicted in Fig 1. Briefly, 4mg/mL OVA (Rhoster Commerce and Industry Ltda, Vargem Grande Paulista, SP, Brazil) was added to water supply of BALB/c mice for 7 consecutive days. The control mice received protein-free water.

Induction of experimental colitis

Experimental colitis was induced in BALB/c mice according to indications of Neurath and colleagues (NEURATH et al., 1995a, 1996a). Mice were anesthetized and instilled with 100 μL of 1 mg/mL TNBS (2,4,6 - trinitrobenzenesulfonic acid; Sigma, USA) solution in 50% ethanol by intrarectal route. Control animals received 100 μL of 50% ethanol (Fig 1).

Evaluation of clinical signs of colitis

All groups were weighed daily until sacrifice. Clinical signs such as diarrhea, rectal prolapse, bleeding and cachexia were registered and assigned as scores, ranging from 0 to 2, with 0: no change, 1: slight change, and 2: severe change.

Histological analysis

On 5 days after TNBS instillation, mice were euthanized and the distal portion of the large intestines were removed and fixed with 4% paraformaldehyde. The pieces were cleared and embedded in paraffin. The sections were stained with hematoxylin and eosin. For the histological analysis, the sections were evaluated for the presence of folds, hemorrhage, and infiltration of leukocytes on two distal portions of the intestines (1 to 2 cm and 2 to 3 cm from the rectum). The thickening of the wall of the colon was measured in micrometers in distal portions, with Infinity Analyze Nikon H600L program (100X). A score ranging from 0 to 20 was assigned (NEURATH et al., 1996a, 1995b).

Spleen cell proliferation

On day 5 after colitis induction, mice of all groups were sacrificed and spleens were aseptically removed. The spleens were macerated individually and erythrocytes in cell suspensions were lysed. Cells were pelleted at 200 g for 10 min and cell concentration was adjusted to 1 x 10^6 cells/mL in RPMI medium (Sigma, USA) supplemented with 10% fetal bovine serum (Cultilab). Cells were stained with 1.25μM Carboxyfluorescein diacetate succinimidyl ester (CFSE) according to manufacturer's instructions (Invitrogen, USA). To determine the maximum uptake, aliquots of the cell suspensions stained with CFSE were fixed with 1% formaldehyde and analyzed by flow cytometer. Stained cells were seeded at $4x10^5$ cell/well in sextuplicate, and Concanavalin A (ConA) was added to each well at final concentration of 2.5μg/mL. Plates were incubated at 37°C in humidified incubator, with 5% CO_2 for 72 hours. After the incubation period, cells were fixed with 1% formaldehyde and proliferation was assessed in $CD4^+CSFE^+$ cells by flow cytometer (THOMÉ et al., 2012). Acquisitions were performed with FACScalibur flow cytometer (Becton-Dickinson) and analyzes were done with the FCS Express 5 Plus Research Edition software (FCS Express Launcher). Results were expressed as proliferation index (fold change) calculated in relation to control group. Cells not stained with CFSE were also cultured in the presence of ConA and their supernatants were collected for dosage of cytokines.

Phenotypic profile of T lymphocytes

The frequencies of T $CD4^+CD25^+$ $Foxp3^+$ (Treg cells), T $CD4^+IL17^+$, T $CD4^+IFN\gamma^+$ and T $CD4^+IL\text{-}10^+$ cells in the cultures were assessed by flow cytometer. Briefly, cell suspensions were washed and initially stained with anti-CD4-PE (Clone GK1.5) and anti-CD25-FITC (Clone 7D4). Following, cells were permeabilized by the addition of fixation/permeabilization buffer (Cytofix / Cytoperm fixation/permeabilization kit, Becton-Dickinson, BD) and stained with anti-Foxp3-

APC (clone FJK-16S), anti-IL-17-APC (clone eBIO17B7) or Alexa Fluor 647 (Clone TC11-18410), anti-IFNγ-APC (Clone XMG1.2), IL-10-APC (clone JESS-16E3) according to manufacturer's instructions. Acquisitions were performed with FACSCalibur flow cytometer and analyzes were done with the FCS Express 5 Plus, Research Edition software.

Th1/Th2/Th17 cytokines determination

IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ and TNF-α were quantified in culture supernatants of spleen cells by flow cytometry by using Multiplex CBA kit (BD Cytometric Bead Array Th1/Th2/Th17, San Diego, USA) according to manufacturer's instructions. Cells were acquired in FACSCalibur cytometer and analyzed with FCAP Array TM Software Version 3.0 (BD).

Statistical analysis

The statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The statistical significance of differences between control and experimental groups was determined by one-way ANOVA, followed by multiple comparisons *Bonferroni's* test. Results were expressed as mean ± Standard Error Mean (SEM). Values were considered significant at $P > 0.05$. All data are representative of at least three independent experiments.

Results and Discussion

The gastrointestinal tract is in constant contact with dietary proteins, commensal microorganisms and potentially pathogenic microorganisms. To ensure the maintenance of homeostasis of the organism, the immune system of the intestinal mucosa must be able to tolerate antigens from diet and commensal microbiota and generate protective responses against harmful antigens. The ability of intestinal mucosa-associated lymphoid tissue (MALT) to suppress systemic immune

response against ingested proteins is known as oral tolerance. Oral tolerance has been demonstrated in various experimental models and for different antigens (FUJIHASHI; MCGHEE, 2004; HYUN; BARRETT, 2006; MOWAT et al., 2004; PABST; MOWAT, 2012; WANG et al., 2013). Nowadays, other routes are under evaluation in order to develop oral tolerance strategies for immunotherapy. In this sense, eye-induced tolerance is a promising therapeutic tool in the treatment of complicated autoimmune diseases such as CIA (FAROOQ; ASHOUR, 2012; FAROOQ; KUMAR; ASHOUR, 2014) or EAE (FAROOQ; ASHOUR, 2013, 2014; FAROOQ; ELKHATIB; ASHOUR, 2014). Several tolerance protocols are being proposed for controlling inflammatory manifestations, in particular autoimmune diseases such as colitis (BAYRAK; MITCHISON, 1998; DA CUNHA; WEINER, 2012; MIN et al., 2006; RODRIGUES et al., 2006; WEINER, 1997; WEINER et al., 1994b, 2011).

The TNBS-induced colitis is a widely used experimental model for the study of inflammatory bowel disease (IBD), since their clinical symptoms and immunological response are similar to those observed in human intestinal diseases (ERI et al., 2008; MAJEWSKA-SZCZEPANIK et al., 2012; NEURATH et al., 1995b, 1996b; OKAMOTO; UEMOTO; TABATA, 2012; SRINIVASAN; SUMMERLIN, 2009; TE VELDE; VERSTEGE; HOMMES, 2006; WU et al., 2013). BALB/c mice are often used for induction of colitis by TNBS administration because they develop a milder disease than SJL strain, but with significant clinical and immunological signals (LINDSAY et al., 2002; SRINIVASAN; SUMMERLIN, 2009; TE VELDE; VERSTEGE; HOMMES, 2006).

Data presented here show that the consumption of OVA, either prior or concomitant to colitis induction, led to the reduction of the weight (Fig 2A) and of typical clinical signs of TNBS-induced colitis, such as diarrhea, rectal prolapse, bleeding and cachexia (Fig 2B). The subsequent

intraperitoneal challenge with OVA led to decreased levels of serum antibodies in animals fed protein, indicating a systemic effect of oral treatment (Fig 2C).

To evaluate the possible effects of OVA consumption on the intestinal mucosa, the distal segments of the large intestine of mice from all groups were collected on the fifth day after the induction of colitis and evaluated histologically. As summarized in Fig 3, the oral treatment with OVA prior to or concomitant with induction of colitis was able to partially preserve the integrity of the colonic tissue of mice with colitis. This can be observed by the significant reduction of histological changes (bends, hemorrhage and leukocyte infiltration) in the tissues of animals tolerized to OVA (Figs 3A and 3B) as well as the preservation of the wall thickness of the colon (Fig 3C) compared to the control animals. Together, these results indicate a possible bystander suppression of the immune response in mice tolerized with OVA, able to alter the course of experimental disease.

Previous studies conducted in our laboratory showed that it is possible to induce oral tolerance to OVA in animals that exhibit a normal repertoire of receptors to the target antigen (SIMIONI et al., 2004, 2010). Several mechanisms seem to be involved in the induction of tolerance, including anergy, clonal deletion and induction of Treg cells (GARSIDE; MOWAT, 2001; GOMES-SANTOS et al., 2012; GOUBIER et al., 2008; MARTH; STROBER; KELSALL, 1996; PABST; MOWAT, 2012). Weiner and colleagues (FARIA; WEINER, 2006; WEINER et al., 1994a, 2011) suggested that the mechanisms that operate in the induction of oral tolerance are directly related to the antigen administration regimen. Thus, administration of high doses of antigens at once would lead to clonal deletion or anergy, while multiple doses of antigen in low concentrations would be capable of promoting the suppression mediated by suppressor/regulators T cells. However, Siewert et al (SIEWERT et al., 2008) found a high rate of $Foxp3^+$ T cells from oral tolerant mice that had been induced by high dose of antigen. This finding indicates that

higher doses of antigen did not lead to clonal deletion, but the generation of suppressive responses associated with oral tolerance. Some cytokines, such as granulocyte macrophage colony stimulating factor (GM-CSF) can modulate DC towards a tolerogenic profile acting as an anti-inflammatory or regulatory modulator in autoimmune diseases, depending on its dose and the presence of other cytokines. Tolerogenic DCs can increase the frequency and function of regulatory T-cells (BHATTACHARYA et al., 2011, 2015a, 2015b; GATHUNGU et al., 2013; HADDAD et al., 2016; ROWIN et al., 2012).

Data from our laboratory have shown that continued ingestion of low doses of OVA can interfere with the phenotypic distribution of intraepithelial lymphocytes (IELs) in the small intestine of wild type BALB/c. After ingestion of OVA, BALB/c mice showed increased frequency of $CD4^{+}Foxp3^{+}$ regulatory T cells and increased expression of regulatory cytokines on IELs (RUBERTI et al., 2012).

To investigate the systemic effects of OVA intake on cellular immune response of colitic mice induced by TNBS, spleen cells were collected on the fifth day after the induction of colitis and assessed for their proliferative capacity and for the expansion of $CD4^{+}$ T lymphocytes with regulatory and effector profiles. The results summarized in Fig 4 and in S1 Fig show that the proliferation of spleen cells from mice fed with OVA was significantly lower than that observed in cultures of spleen cells from the control group (Fig 4A), whereas the frequency of Treg cells ($TCD4^{+}CD25^{+}$ $Foxp3^{+}$) and expression of Foxp3 was higher in cultures of cells from tolerized mice (Figs 4B and 4C). It was also observed an increase in the frequency of $CD4^{+}IL\text{-}10^{+}$ T cells in spleen cell cultures of mouse fed with OVA (Fig 4D) as well as a reduced frequency of $CD4^{+}IFN\text{-}\gamma^{+}$ T cells and $CD4^{+}IL17^{+}$ T cells (Figs 4F 4H) compared with non-tolerant group.

Assessment of cytokines in the supernatants of spleen cells revealed no statistically significant differences in IL-2 secretion in the experimental groups (Fig 5A), but showed an increase in IL-10 levels (Fig 5B) and reduction in levels of IFN-gamma (Fig 5C) and IL-17 (Fig 5D) in the spleen cell cultures from mice tolerized with OVA compared to the non-tolerant group. The results presented here are consistent with data recently obtained by our group in the experimental model of arthritis (THOMÉ et al., 2012).

Corroborating our data, the literature shows that regulatory T cells may play an important role in autoimmune diseases (CARDOSO et al., 2008; GUO, 2016; KIESLER; FUSS; STROBER, 2015; LEE et al., 2006; OKAMOTO; UEMOTO; TABATA, 2012; RAMSDELL, 2003; THOMAS; BAUMGART, 2012) and the expansion of this population can aid in the control of clinical manifestations (BODEN; SNAPPER, 2008; MATTEOLI et al., 2010a; NEURATH, 2014; WALKER, 2013). Moreover, Th17 cells have a potential role in the induction of inflammatory bowel disease and its reduction must also contribute to the improvement of the clinical picture in several autoimmune conditions(FENG et al., 2011; HARBOUR et al., 2015; LIU et al., 2009; NEURATH, 2014).

It is already known that $CD4^{+}CD25^{+}Foxp3^{+}$ cells on the intestinal mucosa seems to be able to induce local generation of Th3 cells secreting TGF-β (LAP^{+}), type 1 regulatory T (Tr1) cells and $CD8^{+}$T reg cells (SUN et al., 2007; WEINER et al., 2011). These regulatory T cells generated in the intestine migrate to secondary lymphoid organs such as Peyer's patches and mesenteric lymph nodes, which inhibit the generation of nonspecific effector cells, by a mechanism known as bystander suppression (FARIA; WEINER, 2006; VON HERRATH; HARRISON, 2003). In addition, the imbalance between proinflammatory and anti-inflammatory cytokines released by the intestinal mucosa determines the intensity and duration of the inflammatory response seen in

experimental colitis (ARDIZZONE et al., 2009; GUO, 2016; MUZES et al., 2012; NEURATH et al., 1995b; OH et al., 2014; SCHEIFFELE; FUSS, 2002; TALERO et al., 2008).

Data from our laboratory has shown that it is possible to protect mice against experimental arthritis (CIA) by oral tolerization with OVA as a preventive or therapeutic intervention, although the antigens involved in tolerization and triggering the disease had been administered independently (THOMÉ et al., 2012).

An increased frequency of both $CD4^{+}FoxP3^{+}$ and $CD4^{+}$ $IL10^{+}$ lymphocytes was also observed after *in vitro* restimulation of spleen cells from tolerized arthritic animals. These results, however, as well as those of Vaz and colleagues (CARVALHO et al., 1994; CARVALHO; VAZ, 1990, 1996; CARVALHO; VERDOLIN; VAZ, 1997) show indirect effects of oral tolerance that do not fit perfectly in bystander suppression model.

By definition, the bystander suppression occurs when the immune response to a particular epitope suppresses the response to another epitope administered concurrently or immediately after the first one. The effects of bystander suppression is related to the secretion of cytokines such as TGF-β, IL-4 and IL-10, and by the action of regulatory T cells(BAYRAK; MITCHISON, 1998; GOTSMAN et al., 2001; SINHA et al., 2009).

Interventions leading to bystander suppression may be of great interest in autoimmunity regulation. Thus, the suppressive response initiated by mucosal administration of dietary proteins associated with self-antigens has been gaining ground as a preventive or therapeutic intervention proposed for autoimmune diseases (BAYRAK; MITCHISON, 1998; PABST; MOWAT, 2012; WEINER et al., 2011). In this respect, several authors have shown that the oral tolerance to dietary antigens may prevent or inhibit the progression of systemic or organ-specific autoimmune diseases (OEFNER et al., 2012; THOMÉ et al., 2012; WANG et al., 2013). In this regard, data from our and others laboratories have shown that it is possible to reduce the immune response to

a different antigen from that used to induce oral tolerance, even if this antigen is administered several days after ingestion of tolerogen and parenteral challenge with tolerated antigen (FAROOQ; ASHOUR, 2012; THOMÉ et al., 2012). Recent work from Vaz group have shown that there is an expansion of regulatory T $CD4^{+}Foxp3^{+}$ and T $CD4^{+}LAP^{+}$ non-specific cells in the early stage of parenteral exposure to the antigen used in the oral treatment, phenomenon that is not seen in non-tolerant animals subjected to the same treatment (CASTRO-JUNIOR et al., 2012). Similarly to what we have shown with our work, other authors have demonstrated that increased IL-10 production correlates with the ability to reduce inflammatory responses associated with human and experimental colitis (GLOCKER et al., 2011; GUO; LI, 2014).

Conclusions

Our results indicate that the development of oral tolerance to OVA was able to reduce the signs and the immune response in TNBS-induced colitis. The immunomodulation observed can be attributed to the expansion of regulatory T cells and IL-10 producing cells, by a mechanism known as bystander suppression. Further studies are in progress to evaluate the role of dendritic cells in the protection afforded by oral tolerance in this colitis model.

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FIGURES

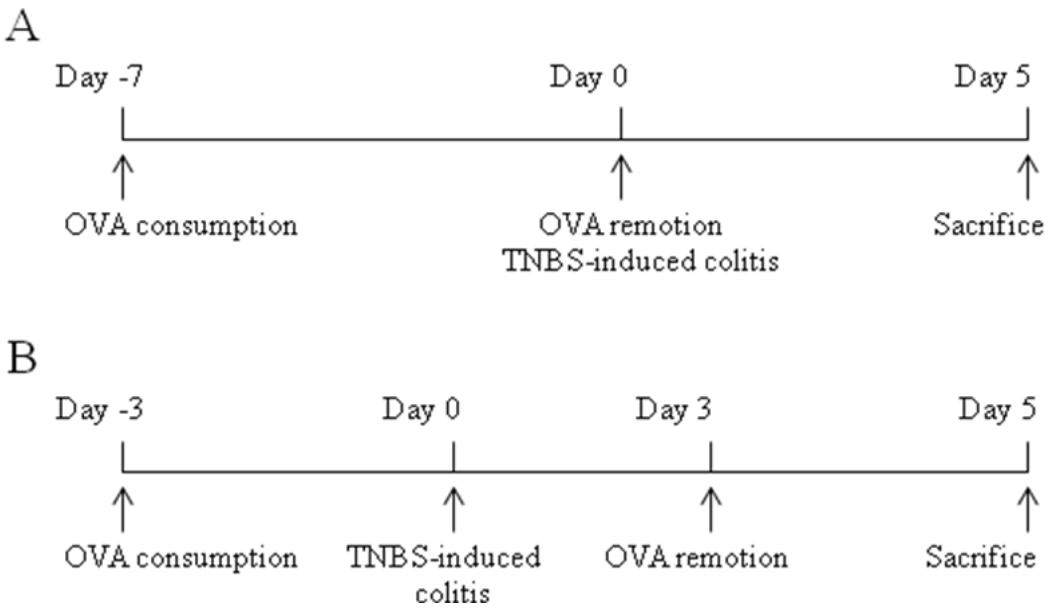


Figure 1. Flowchart of the TNBS-induced colitis and oral tolerance design. Panel A: Previous oral tolerance. Panel B: Concomitant oral tolerance

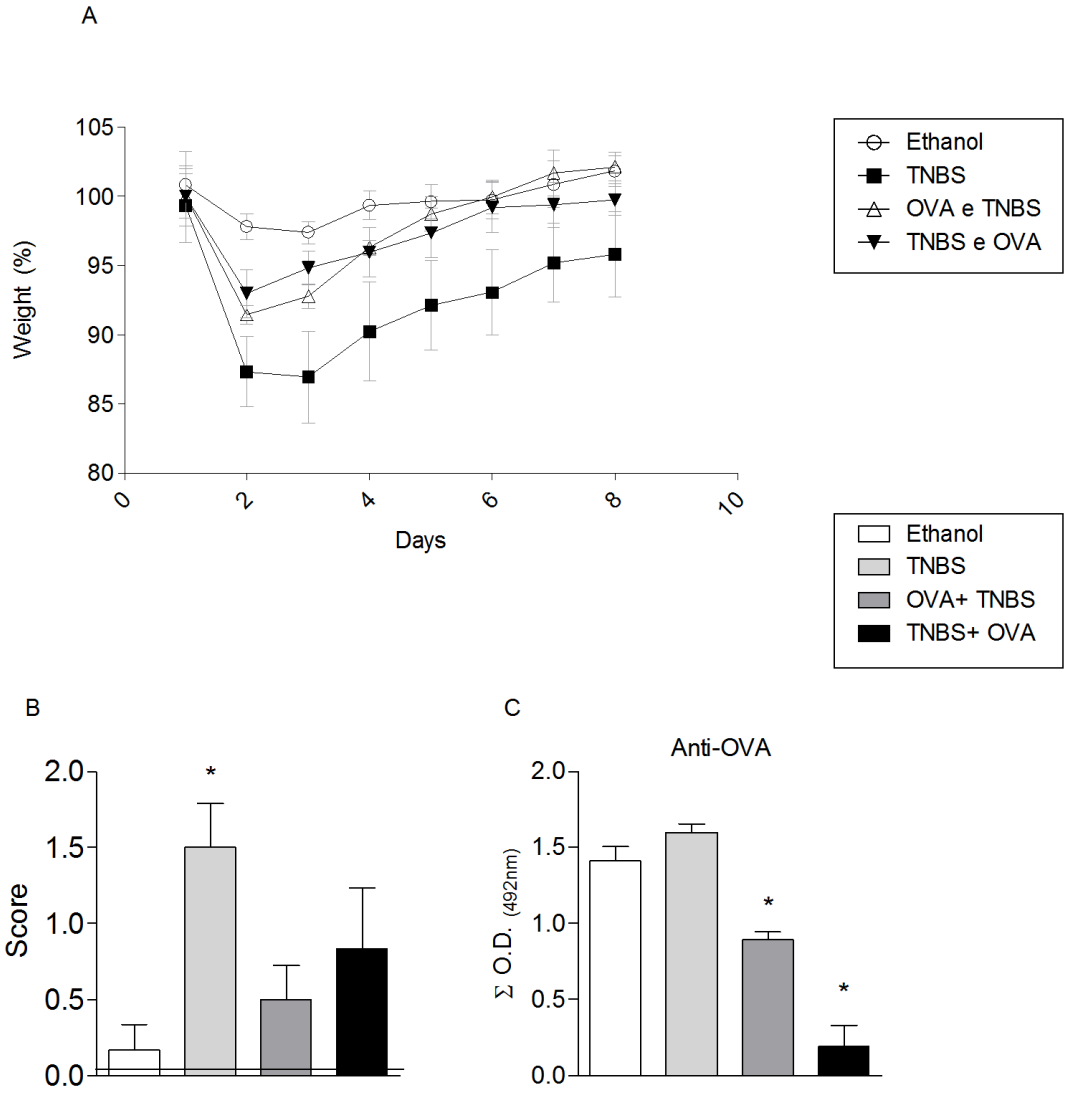


Figure 2. OVA intake improves the severity of TNBS-induced colitis in mice. For induction of tolerance an OVA solution (4mg / ml) was provided in drinking water to BALB/c mice (n = 5) for seven consecutive days, either 3 or 10 days before colitis induction. The experimental colitis was induced in BALB/c mice by intrarectal instillation of TNBS in 50% ethanol (1 mg / mL, 100 μL). Two control groups were included: mice that received protein-free water and were instilled with either ethanol (white bar) or TNBS (light gray bar). Panel A: Temporal changes in body weight, in percentage. All mice were weight daily and the weight alteration was represented by percentage in comparison with the mean initial value found on day 1 and represented as 100%. Body weights in orally treated mice were significantly higher than in non-tolerant mice. Panel B: Clinical signs were evaluated for the presence of diarrhea, rectal prolapse, bleeding and cachexia, assigning a score ranging from 0 to 2, with 0: no change, 1: slight change, and 2: severe change. Panel C: Oral tolerance to OVA modulates antibody production. Oral tolerance and experimental colitis was induced as illustrated in Fig 1. Five days after TNBS instillation, mice of all groups were immunized with OVA (10 mg) in aluminum hydroxide (1 mg) by intraperitoneal route, and 14 days later challenged i.p. with 10mgOVA in saline solution. On day 21, sera were collected,

diluted 1: 100 to 1: 12,800 and tested by ELISA for anti-OVA antibodies (A) and anti-TNP (B). Values are means ±SEM, sum of the optical densities (O.D.) Data are representative of three independent experiments (n = 5). ANOVA followed by Bonferroni post-test were used to determine statistical significance ($p<0.05$).

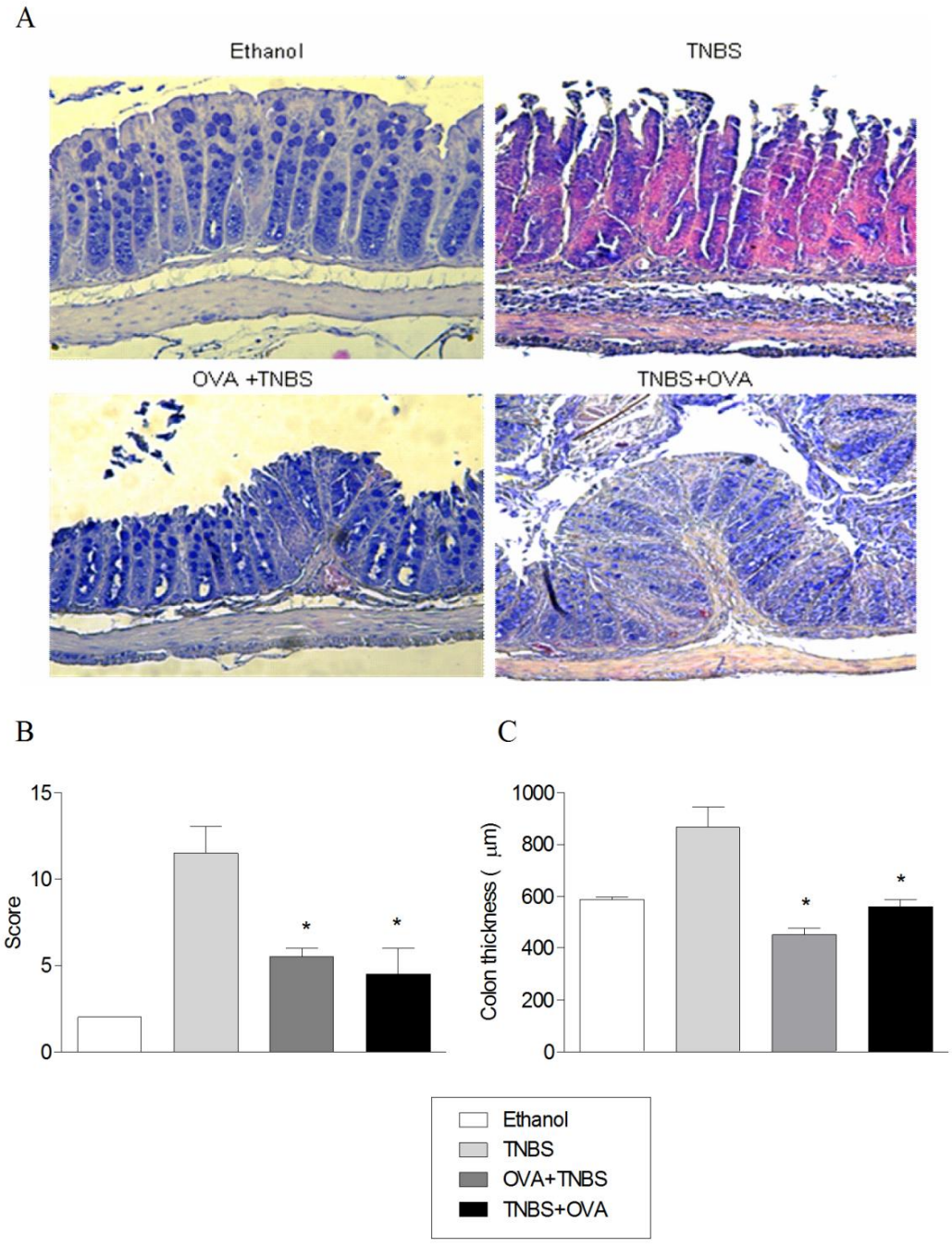


Figure 3. The oral tolerance to OVA reduces histopathological changes in the intestine of mice induced by instillation of TNBS. BALB / c mice subjected to the treatments described in

Figure 2 were sacrificed 5 days after induction of colitis and sections of the distal part of large intestine were fixed in paraffin for histological processing. Panel A: Histological sections of 5μm were stained with hematoxylin and eosin and examined by light microscopy (200X). Panel B: Sections were assessed for the presence of folds, hemorrhage, and infiltration of leukocytes at two distal portions of the intestines (1 to 2 cm and 2 to 3 cm from the rectum), assigning a score ranging from 0 to 20. Panel C: Thickening of the colon wall, measured in micrometers in two distal portions, with Infinity Analyze Nikon H600L program (100X). Two independent analyzes were performed.

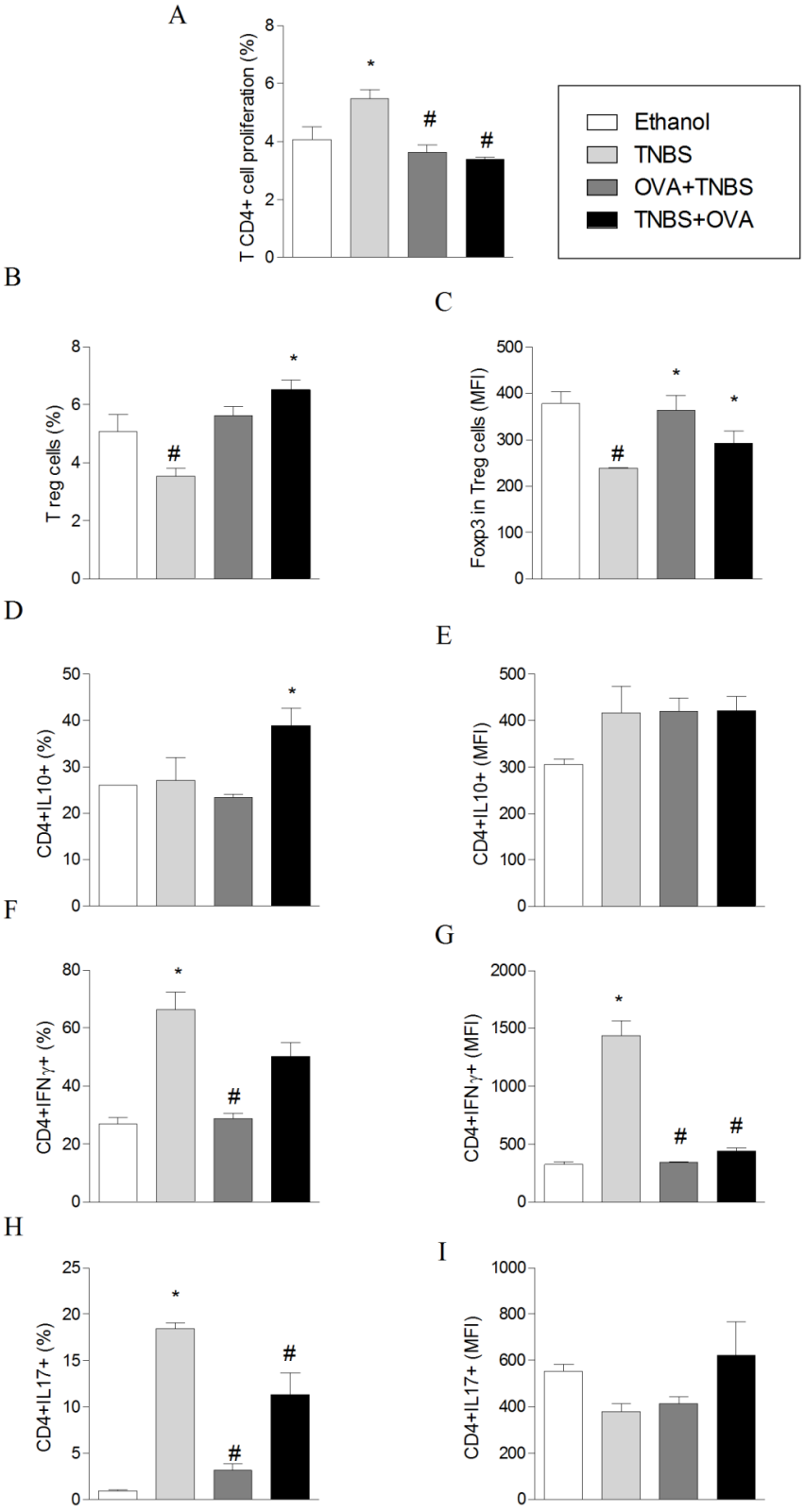


Fig 4. The oral tolerance to OVA alters the proliferative response and cytokine producing cells from mice with TNBS-induced colitis. The induction of tolerance and colitis was

performed as described in Fig 2. Mice were sacrificed five days after TNBS instillation. Spleens were aseptically removed; cells were labeled with CFSE and cultured at a concentration of 2x106 cells / ml in the presence of Concanavalin A (ConA; 2,5µg / ml) for 72 hours at 37˚C and5%CO2. Panel A: Spleen cell proliferation. Cells were fixed in1%formaldehyde and the readings performed in flow cytometer (FACSCalibur, BD). Proliferation was calculated using the software FCS Express and represents the inverse of the ratio of the fluorescence exhibited by the cells after 72 hours of culture and those immediately after labeling with CFSE. The cell frequency (Panel B) and the mean fluorescence intensity (MFI) of T regulatory CD25+Foxp3+ cells (Panel C), and the frequency of cytokine producing cells (Panels D-I) were monitored within the CD4+ T cell gate. The values correspond to the mean ± S.E.M. of two independent experiments (n = 5). ANOVA followed by Bonferroni a *posteriori* test was used to determine statistical significance.

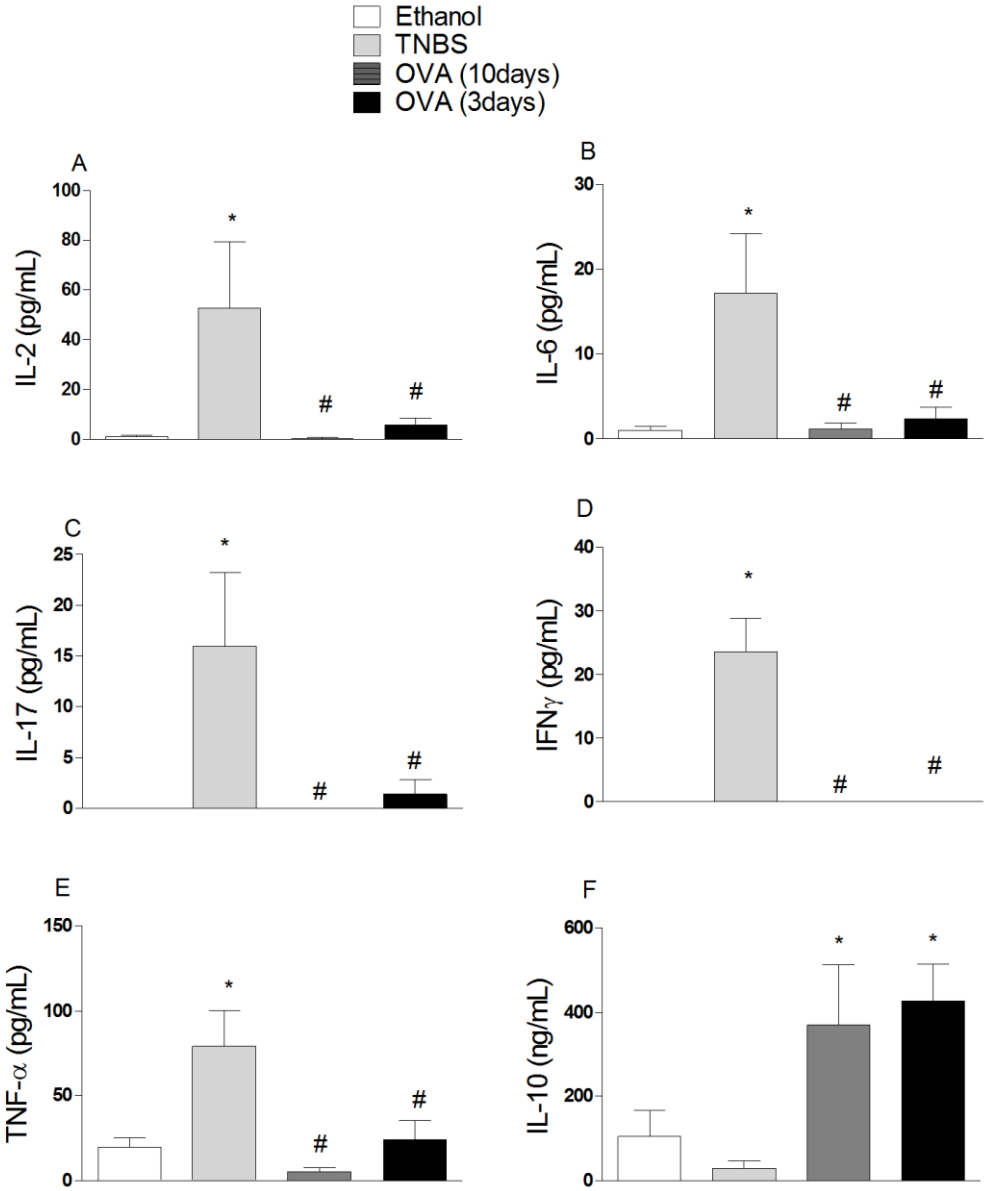
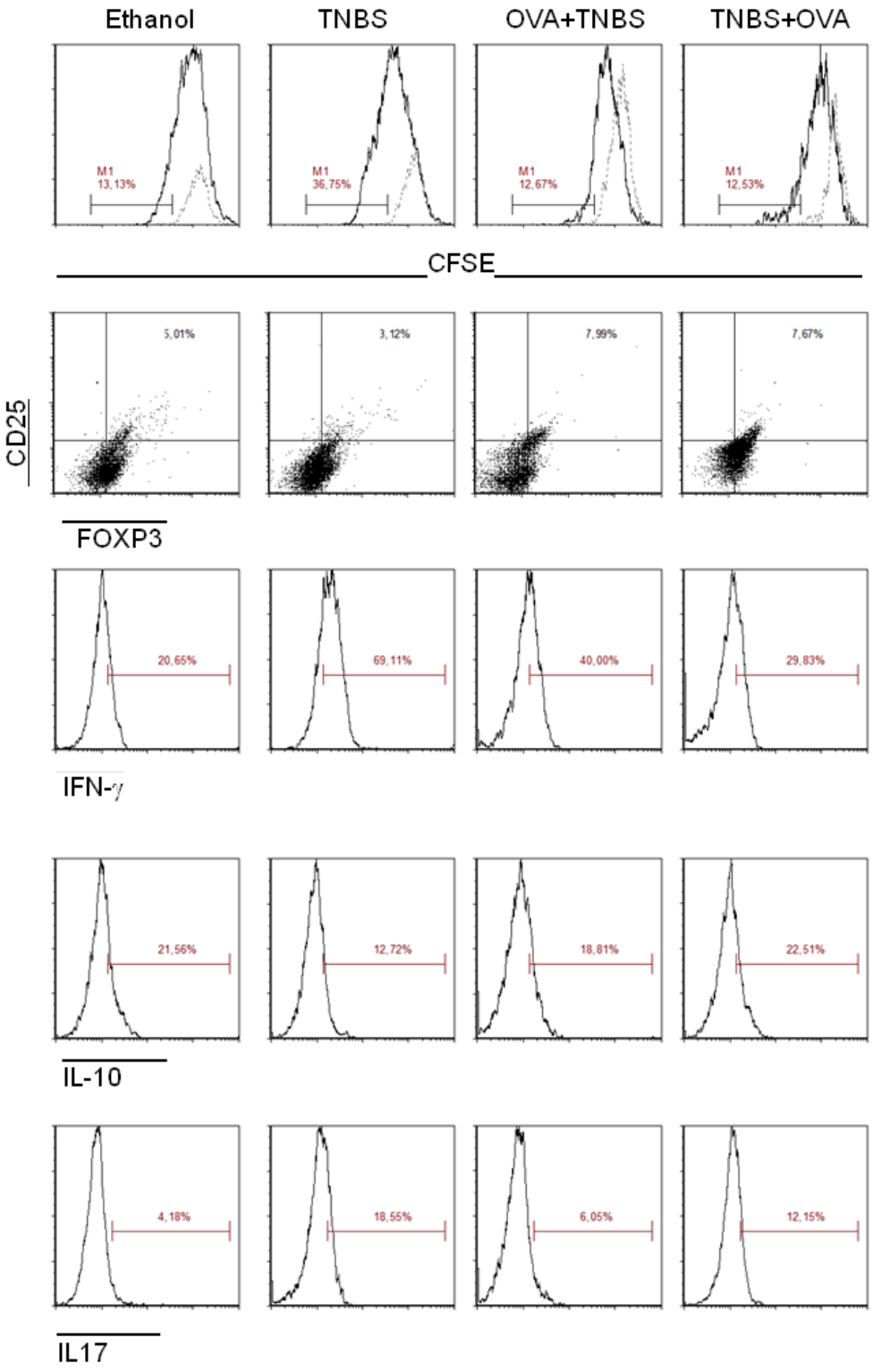


Fig 5. The oral tolerance to OVA alters cytokine levels in supernatants of ConA-stimulated spleen cells from mice with TNBS-induced colitis. Cultures of spleen cells were carried out as described in Fig 4. Cytokine levels were evaluated in the culture supernatants by using the CBA Multiplex kit (Cytometric Bead Array Th1 / Th2 / Th17, BD) and readings were performed in a flow cytometer (FACSCalibur, BD). Cytokine concentrations were determined using the array FCAPTM Version 3.0 Software (BD). Results were expressed as means ± S.E.M. obtained from two independent experiments (n = 5).ANOVA followed by Bonferroni a posteriori test was used to determine statistical significance.



S1 Fig. Oral tolerance reduces the proliferation of T lymphocytes, increases the proportion of CD25+Foxp3+Tregs and reduces IL-17-and INF-γ- producing-T cells on colitic mice. Five days after the TNBS instillation, leukocytes were harvested from spleens of mice that

received OVA by oral route, as described in Fig 2. Leukocytes from spleens of naïve and untreated TNBS mice were used as controls. The spleen cells were stained with CFSE and cultured at a concentration of 2x106 cells / mL in the presence of Concanavalin A (ConA; 2,5μg/ mL) for 72 hours at 37˚C and 5% CO2. CFSE: Cells were fixed in 1% formaldehyde and the readings performed in flow cytometer (FACSCalibur, BD). Cell proliferation was determined using flow cytometry and assessed by fluorescence decay of the probe in the gate of CD4+ cells. The frequency of CD25+ Foxp3+ Tregs in the different groups was evaluated in the gate of CD4+ cells. IFN-γ-, IL-10-, and IL-17-producing cells were evaluated in the gate of CD4+ cells as well. Histograms and plots are derived from a representative animal from two independent experiments (n = 5/each assay).

6.2 CAPÍTULO II

Artigo submetido à avaliação para publicação para revista Plos One (Anexo 3).

Title: Adoptive transfer of tolerogenic dendritic cells reduces severity of experimental colitis in BALB/c mice.

Lisiery N. Paiatto[1,2]; Fernanda G. D. Silva[3]; Áureo T. Yamada[4], Wirla M. S. C. Tamashiro[1]; Patricia U. Simioni[1, 2, 5 *]

[1]Department of Genetics, Evolution and Bioagents, Institute of Biology, State University of Campinas, UNICAMP, Campinas, SP, Brazil.

[2]Institute of Biosciences, Universidade Estadual Paulista, UNESP, Rio Claro, SP, Brazil.

[3]Faculty of Food Engineering, University of Campinas, UNICAMP, Campinas, SP, Brazil.

[4]Department of Histology and Embryology, Institute of Biology, State University of Campinas, UNICAMP, Campinas, SP, Brazil.

[5]Department of Biomedical Science, Faculty of Americana, FAM, Americana, SP, Brazil.

[*] Corresponding author

E-mail: psimioni@gmail.com (PUS)

E-mail addresses:

Lisiery Negrini Paiatto: lisy_paiatto@hotmail.com

Fernanda Drummond: fer.dru@gmail.com

Áureo Tatsumi Yamada: yamadat@unicamp.br

Wirla Maria da Silva Cunha Tamashiro: wirlatam@unicamp.br

Patricia Ucelli Simioni: psimioni@gmail.com

Resumo

Introdução: Além das terapias convencionais, várias estratégias têm sido propostas para modular as respostas autoimunes, incluindo a transferência adotiva de células. Neste contexto, tem sido demonstrado que a transferência adotiva de células dendríticas tolerogênicas (DCs) é uma terapia promissora. O presente estudo teve como objetivo analisar os efeitos da transferência adotiva de DCs obtida de camundongos tolerantes à ovalbumina (OVA) na severidade da colite induzida por TNBS bem como os eventuais mecanismos de proteção. **Métodos e Resultados:** Para induzir tolerância oral camundongos BALB/c receberam OVA a 4mg/mL na água de beber, por sete dias consecutivos. As DCs foram isoladas do baço de camundongos tolerantes (tDC) e *naives* (nDC), e transferida adotivamente para camundongos singênicos. Após três dias, a colite foi induzida nesses camundongos por instilação intrarretal de 100 µg de ácido 2,4,6 – trinitobenzenosulfônico (TNBS) dissolvido a etanol 50%. Os controles receberam apenas instilação intrarretal de solução de TNBS ou do veículo. Os camundongos de todos os grupos foram eutanasiados após 5 dias e prossegui-se com avaliações dos parâmetros. Os resultados mostram que o tratamento prévio com tDC foi capaz de reduzir sinais clínicos da colite, como perda de peso, bem como de preservar parcialmente a integridade do tecido colônico. A proliferação *in vitro* de células de baço de camundongos tratados com DCs foi significativamente reduzida em comparação com a proliferação de células de animais dos grupos controles. Embora não tenha sido observada diferença significativa nas frequências de células Treg nos diferentes grupos experimentais, a frequência de células $Th17^{+}$ e a secreção de IL-17 e IL-9 foram reduzidas nas culturas de células esplênicas de camundongos tratados com DCs, enquanto que os níveis de IL-10 e IL-4 foram aumentados. Os níveis de citocinas inflamatórias também foram menores nos sobrenadantes de células de camundongos tratados com tDC. **Conclusão:** Os resultados permitem concluir que a

transferência adotiva de tDC foi capaz de reduzir os sinais clínicos e imunológicos da colite induzida por TNBS. A imunomodulação observada pode estar relacionada à redução de células Th17 e de secreção de citocinas inflamatórias. A administração de DCs tolerantes foi capaz de alterar as respostas proliferativas de células T provavelmente devido à redução significativa da expressão da molécula CD80. As DCs podem ser uma ferramenta terapêutica para a modulação de respostas imunes em desordens inflamatórias, especificamente em colite humana. Até onde é de nosso conhecimento, não existem dados prévios na literatura que mostrem os efeitos protetores das DCs tolerantes à OVA no tratamento da colite experimental.

Abstract

Introduction: In addition to conventional therapies, several new strategies have been proposed to modulate autoimmune diseases, including the adoptive transfer of immunological cells. In this context, tolerogenic dendritic cells (DCs) appear to be one of the most promising treatments to autoimmune disorders. The present study aimed to evaluate the effects of adoptive transfer of DCs obtained from ovalbumin (OVA) tolerant mice on the severity of TNBS induced colitis, and analyses the eventual protective mechanisms. **Methods and Results:** To induce oral tolerance, BALB/c mice were fed with 4mg/mL OVA solution for seven consecutive days. Spleen DCs were isolated from tolerant (tDCs) and naïve (nDCs) mice, and then adoptively transferred to syngeneic mice. Three days later, colitis was induced in DC treated mice by intrarectal instillation of 100μg 2,4,6-trinitrobenzenesulfonic acid (TNBS) dissolved in 50% ethanol. Controls received only intrarectal instillation of either TNBS solution or vehicle. Five days later, mice from all groups were euthanized and examined for physiologic and immunologic parameters. Our results show that previous treatment with tDC is able to reduce signs of the colitis, such as weight loss, as well as partially preserve the integrity of the colonic tissue. In vitro proliferation of spleen cells from DC-treated mice was significantly lower than the proliferation of spleen cells from mice of the control groups. Although no significant difference was observed in the frequencies of Treg cells in the different experimental groups, the frequency of $Th17^{+}CD4^{+}$cells and the secretion of Il-17 and IL-9 were reduced in the cultures of spleen cells from DC treated mice, whereas the levels of IL-10 and IL-4 were augmented. The levels of inflammatory cytokines were also lower in supernatants of cells from tDC treated mice. **Conclusion:** The results allow concluding that the adoptive transfer of tDC is able to reduce the clinical and immunological signs of the drug-induced colitis. The immunomodulation observed

here can be related to the reduction of Th17 cells and inflammatory cytokine secretion, as well as the augment of anti-inflammatory interleukins. Tolerogenic DC altered the proliferative and T cell immune responses probably due to the significant reduction of CD80 expression. DC can be a therapeutic toll for modulating immune responses in inflammatory disorders, specifically in human colitis. To the best of our knowledge, there are no previous data in the literature showing the protective effects of OVA tolerant DCs in the treatment of colitis.

Introduction

Imbalance of the intestinal microbiota as well as genetic and environmental factors can trigger chronic inflammation in the gastrointestinal tract, called inflammatory bowel diseases (IBD). Ulcerative colitis (UC) and Crohn's disease (CD) are the main IBD in humans. The pathophysiological mechanisms of IBD, however, are not yet fully understood. Thus, experimental models that mimic human disease are important tools for studying these diseases (DUCHMANN et al., 1996; ENGEL; NEURATH, 2010; WIRTZ et al., 2007).

Experimental models of colitis targeting the development of new therapeutic agents include chemical induction, immune cell transfer, or genetic manipulation of laboratory animals (ARIJS et al., 2011; ERI et al., 2008; LINDSAY et al., 2002; OWCZAREK et al., 2009; PEDERSEN et al., 2007; PERRIER; RUTGEERTS, 2011; WU et al., 2013). Among the experimental models most used in the study of colitis, the intrarectal administration of TNBS in mice stands out for releasing signs and symptoms very similar to those observed in humans (RANDHAWA et al., 2014; STROBER et al., 1997; WHITTEM; WILLIAMS; WILLIAMS, 2010).

Mechanisms enrolled in the physiopathology of IBD include unbalance of cytokines such as interleukin (IL)-9, IL-10, IL-35, transforming growth factor (TGF)-β as well as enzymes, cell receptors and transcription factors such as Indoleamine-2,3-dioxygenase (IDO), Cytotoxic T-Lymphocyte Antigen (CTLA)-4, Leukocyte Activation Gene (LAG) -3, perforin/antagonists and Glucocorticoid-Induced Tumor Necrosis Factor Receptor (GITR) (BANKS et al., 2003; KOBAYASHI et al., 2008; MIYARA; SAKAGUCHI, 2007; SHEN; DURUM, 2010; TAKAMATSU et al., 2013). Several new therapies are under evaluation to treat and reduce the signs and progression of IBDs. Among the options for conventional treatments, adoptive transfer

of tolerogenic cells such as DC and Treg cells emerges as a promising alternative under evaluation (PABST; MOWAT, 2012; THOMÉ et al., 2012).

Dendritic cells (DCs) are the major professional antigen presenting cells (APC) that play a relevant role in the activation of naive T lymphocytes (MATTEOLI et al., 2010b; PEDERSEN et al., 2007). The DCs that inhabit the gastrointestinal tract, however, have a proven involvement in the modulation of peripheral tolerance, by the secretion of anti-inflammatory cytokines and stimulation of Treg cells (GORDON et al., 2014; KALANTARI et al., 2011; NIESS; REINECKER, 2006; YAMAZAKI; STEINMAN, 2009). In this sense, studies from our group and others suggest that oral tolerance can generate DCs with tolerogenic profile in peripheral lymphoid organs, thus leading to the increase of regulatory T cells (PAIATTO et al., 2017; THOMÉ et al., 2012).

The aim of this study is to evaluate the effects of adoptive transfer of dendritic cells obtained from OVA tolerant BALB/ c mice in the experimental colitis induced by TNBS in syngeneic mice.

Material and methods

Animals

BALB/c female mice (20–25 g) at four weeks of age were obtained from the Multidisciplinary Center for Biological Research (CEMIB) of the University of Campinas (UNICAMP), Campinas, SP, Brazil. They were maintained in specific pathogen-free environment at 25^{o} C ±1 and photoperiod of 12/12 hours. Mice were fed with autoclaved Nuvilab CR-diet and water *ad libitum* for 2-4 weeks before being used in experiments. The methods described in this manuscript were carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals', as promoted by the Brazilian College of Animal Experimentation (COBEA), and was approved by the Ethics Committee for Animal Experimentation of University of Campinas (CEUA/UNICAMP. Protocol #3077-1). All experimental procedures were performed under proper anesthesia and all efforts were made to minimize animal suffering. The experimental groups consisted of at least five animals. Mice general health was daily monitored for signs of inflammation such as rectal swelling, rectal bleeding, soft stool or weight loss.

Oral tolerance

The induction of oral tolerance to OVA was performed as described previously (PAIATTO et al., 2017; SIMIONI et al., 2004). Briefly, 4mg/mL OVA (Rhoster Commerce and Industry Ltda, Vargem Grande Paulista, SP, Brazil) was added to water supply of BALB/c mice for 7 consecutive days. The control mice received protein-free water.

Isolation of DCs

Dendritic cells (DCs) were isolated from spleens of OVA tolerant (tDC) or naïve (nDC) mice as previously described (SIMIONI; FERNANDES; TAMASHIRO, 2016; THOMÉ et al., 2012). To

performing DC isolation, spleen cells were incubated with magnetic beads coated with anti-CD11c antibodies (Clone 130-052-001, Miltenyi Biotec, Germany), and using column (MS MACS, 130-042-201, Miltenyi Biotec), with Mini MACS Separation Unit (421-02, Miltenyi Biotec), according to the manufacturer's recommendations.

Phenotypic profile of DCs

The phenotypic profiles of DCs were evaluated by flow cytometry, by labeling with anti-mouse CD11c - APC (Clone: HL3), anti-mouse MHC-II-PE (130-091-368, Miltenyi Biotec), anti-mouseCD80-FITC (Clone: 16-10A1), anti-mouseCD86- FITC (Clone: GL1) and anti-mouseCD40-FITC (Clone: 3/23) according to the manufacturer's instructions. The readings were performed using the FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ, USA) flow cytometer located at the Institute of Biology of UNICAMP, using FCS Express 5 Plus, Research Edition software.

Syngeneic adoptive transfer of DCs

Three doses of $3x10^5$tDCs isolated from OVA tolerant BALB/c mice or nDCs isolated from naïve BALB/c mice were injected intravenously into naive syngeneic mice on days -5, -3 and 1 of TNBS intrarectal instillation, as previously described (SIMIONI et al., 2004; SIMIONI; FERNANDES; TAMASHIRO, 2016; THOMÉ et al., 2012).

Induction of experimental colitis

Colitis was induced by intrarectal administration of a single dose of 2,4,6-trinitrobenzenesulphonic acid (TNBS), as described elsewhere (NEURATH et al., 1995b, 1996b)**,** with modifications. Briefly, mice were anesthetized and instilled with 100µL of 1.0mg/mL TNBS (2,4,6 - trinitrobenzenesulfonic acid; Sigma, USA) dissolved in 50% ethanol into the lumen of the colon. To ensure the agent enter entire colon, mice were held in a vertical position for 30 s. Two

control groups of mice that did not receive dendritic cells were used: 1) animals inoculated intrarectally with 100μL 50% ethanol; 2) animals inoculated intrarectally with 100μL TNBS dissolved in 50% ethanol (Fig 1).

Fig 1: Flowchart of obtaining dendritic cells and inducing colitis. (Panel A) Obtaining dendritic cells: Naive and tolerant mice were used to obtain DCs. The animals in the tolerant group received a solution of 4mg/mL OVA as drinking water for seven consecutive days. The naïve animals received protein-free water (tap water). Animals from each group were sacrificed for isolation of splenic DCs using magnetic beads coated with anti-CD11c on days 1, 3, and 5 after discontinuation of oral treatment. (Panel B) Treatment with DCs and colitis induction: Three doses of $3x10^5$ of naïve or tolerant DCs were injected intravenously in naïve BALB/c mice on days -5, -3 and 1 in relation to the day colitis induction. Twenty-four hours after the last dose, 100 μL of 1mg/mL TNBS solution was instilled by intrarectal route in treated BALB/c mice. The control groups consisted of mice without any treatment (naïve mice) and of mice challenged with TNBS (colitic mice).

Evaluation of clinical symptoms of TNBS induced colitis

Animals in all groups were weighed daily until sacrifice on the fifth day after the induction of colitis. Weight variation was calculated as percentage. Clinical symptoms such as diarrhea, rectal prolapse, bleeding and cachexia were registered and assigned as scores, ranging from 0 to 2, with 0: no change, 1: slight change, and 2: severe change (PAIATTO et al., 2017).

Histological analysis of colitis

Mice were euthanized and the distal portions of the large intestines were removed. Two colon segments with 1 cm each were taken 4 cm from the anus, fixed in 4% buffered formalin, dehydrated with grade ethanol solution, and embedded in paraffin (Paraplast Plus Sigma P3683).

Slices of 5µm were obtained on microtome (Leica - model Jung Biocut 2035), mounted on clean glass slides, deparaffinized, rehydrated, and dyed with hematoxylin and eosin (Merck). Sections were evaluated for the presence of folds, hemorrhage, mucosal dilatation and infiltration of leukocytes on two distal portions (from 1 to 2 cm and from 2 to 3 cm from the rectum) of the intestines.

Scores were attributed to the presence of mucosa folds (from 0 to 5, 0 - normal and evident folds; 1 - slightly deformed folds; 2 - deformed folds; 3 - folds with impaired shape; 4 - drastic reduction of folds and large infiltration; 5 - nonexistence of folds and high tissue compromise), hemorrhage (0- none; 1-hemorrhage present; 2-large hemorrhage), mucosal dilatation (0 - none or reduced voids; 1 apparent voids; 2 - high voids) and lymphocytic infiltrate in the mucosa, submucosa and mesentery (0- none; 1- reduced infiltrate; 2 considerable infiltration with disorganization of the submucosa; 3 - intense infiltrate). (NEURATH et al., 1995b, 1996b)

Spleen cell proliferation

Spleens OVA tolerant (tDC) or naïve (nDC) mice were collected aseptically from the mice of all experimental groups, macerated individually, suspended in lysis buffer and pelleted by centrifugation at 200 g for 10 min. Cell concentration was adjusted to $1x10^6$ cells/mL in RPMI medium (Sigma, USA) supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil). After washing, spleen cell suspensions were incubated with 25µM carboxyfluoresceinsuccinimidyl probe ester (CFSE) in RPMI complete medium at room temperature for 5 min, according manufacturer's recommendations (Invitrogen, USA). Cells were then pelleted by centrifugation and suspended in RPMI complete medium. To determine the maximum uptake of CFSE, aliquots of the cell suspensions were fixed with 1% formaldehyde in PBS and analyzed by flow cytometer. CFSE -labeled cells were seeded in 96-well plates

(Corning), in sextuplicate, and incubated in the presence of 2.5μg/mL ConA for 72 hours at 37 ºC. Cell cultures in absence of stimuli were used as control.

The proliferation of T lymphocytes in cultures was assessed in gate of $CD4^+CFSE^+$ cells. Acquisitions were performed with FACSCalibur flow cytometer (FACSCalibur flow cytometer, BD Becton Dickinson, San Jose, CA) (THOMÉ et al., 2012). The results were analyzed with the software FCS Express Plus Research Edition software (FCS Express Launcher). Results were expressed as proliferation index (fold change), calculated in comparison to control group (PAIATTO et al., 2017). Cells not stained with CFSE were also cultured in the presence of ConA and supernatants from spleen cell cultures of all experimental and respective control groups were collected for dosage of cytokines.

Phenotypic profile of T-cells

The frequencies of $TCD4^+CD25^+$ $Foxp3^+$ (Treg cells), $TCD4^+IL17^+$, $TCD4^+IFN\gamma^+$ and $TCD4^+IL\text{-}10^+$ cells in the cultures were assessed by flow cytometer. Briefly, cell suspensions were washed and initially stained with anti-CD4-PE (Clone GK1.5) and anti-CD25-FITC (Clone 7D4). Then, cells were permeabilized by the addition of fixation/permeabilization buffer (Cytofix / Cytoperm fixation/permeabilization kit, Becton-Dickinson, BD) and stained with anti-Foxp3-APC (clone FJK-16S), anti-IL-17-APC (clone eBIO17B7) or Alexa Fluor 647 (Clone TC11-18410), anti-IFN-γ-APC (Clone XMG1.2), IL-10-APC (Clone JESS-16E3), anti-T-bet Alexa 647 (Clone O446), anti-GATA3 Alexa Fluor 647 (Clone L50-823) and anti-ROR-GT Alexa Fluor 647 (Clone Q21-378), according to manufacturer's instructions. Acquisitions were performed with FACScalibur flow cytometer and analyzes were done with the FCS Express 5 Plus, Research Edition software (PAIATTO et al., 2017).

Determination of Th1, Th2, Th17 and Th9 cytokines

IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ and TNF-α were quantified in culture supernatants of spleen cells by flow cytometer, using Multiplex CBA kit (BD Cytometric Bead Array Th1/Th2/Th17, San Diego, USA) according to manufacturer's instructions. Cells were acquired in FACSCalibur cytometer and analyzed with FCAP Array TM Software Version 3.0 (BD). IL-9 determination was assayed with CBA flex set (BD Cytometric Flex Set Th9, San Diego, USA).

Statistical analysis

The statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The statistical significance of differences between control and experimental groups were determined by one-way ANOVA, followed by Bonferroni's test for multiple comparisons. The results were expressed as mean ± Standard Error of the Mean (S.E.M). Values were considered significant at $P > 0.05$. All data presented are representative of at least three independent experiments.

Results and discussion

The self-destructive immune response observed in IBD correlates positively with the loss of microbiota tolerance, the activation of immune cells and the presence of intense local inflammatory infiltrate. Although the mechanisms triggering the disease are not well understood, the presentation of enteric microorganism antigens by dendritic cells located in the lamina propria of the intestinal mucosa and in Peyer's plaques seems to represent a preponderant role in the etiology of these diseases (ANDRADE; VAZ; FARIA, 2003; FENG et al., 2010; NIESS; REINECKER, 2006; RUTELLA; LOCATELLI, 2011).

In previous studies of our group, we hypothesized that the establishment of oral tolerance by

ingestion of dietary proteins could restore, at least in part, the tolerogenic environment of the intestinal mucosa, with important reflexes to the systemic immune response. In this regard, we have previously shown that induction of oral tolerance to ovalbumin was able to prevent antigen-induced arthritis as well as TNBS-induced colitis in BALB / c mice (PAIATTO et al., 2017; THOMÉ et al., 2012). We have also shown that arthritis induced by administration of OVA in complete Freund's adjuvant in BALB/c mice can be prevented by the adoptive transfer of DCs from syngeneic OVA-tolerant animals (THOMÉ et al., 2012).

The intrarectal administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS) is a protocol well established in the literature for studying Crohn's disease, since it closely mimics this human disorder (DE MATTOS et al., 2015; LIU et al., 2009; MATRICON, 2008; PAIATTO et al., 2017; TONTINI et al., 2015). In the present study, we evaluated the effects of adoptive transfer of DCs collected from OVA-tolerant mice on the course of TNBS-induced colitis in syngeneic animals, as well as on the ex vivo response of their splenic immune cells.

As depicted in Fig 2 (Panel A), prior treatment with oral OVA is able to reduce the percentage of DCs expressing CD80, CD86 and CD40 surface markers compared to DCs from untreated mice, confirming data that we obtained previously (THOMÉ et al., 2012). The clinical signs and body weight variation were analyzed in dayly bases for five days after TNBS instillation. Clinical symptoms evaluated include cachexia, rectal prolapse, diarrhea, and bleeding. As illustrated in Fig 2B, the weight loss associated with TNBS-induced colitis was completely prevented by adoptive transfer of DCs with a tolerogenic profile (tDC), but not by the other treatments. Likewise, there was a clear reduction of clinical signs associated with TNBS-induced colitis in mice treated with tDCs in comparison to control groups, although they were not statistically significant (Fig 2C).

As previously observed in OVA-tolerant mice (PAIATTO et al., 2017), pretreatment with tolerogenic DCs was also able to reduce histological damage of the colonic tissue induced by TNBS instillation (Fig 3). Similarly, the reduction of the inflammatory manifestations in osteoarthritis was also found previously in OVA-fed mice as well as in syngeneic mice treated with tolerogenic DCs (THOMÉ et al., 2012). Together with our previous results, the present study reinforces our hypothesis that the promotion of tolerogenic environment in the intestinal mucosa can result in considerable systemic benefits.

Fig 2: Adoptive transfer of $CD11c^+$ dendritic cells isolated from tolerant mice reduces severity of experimental colitis. (Panel A) Characterization of DCs isolated from spleens of naive and tolerant mice (nDCs and tDCs, respectively): The frequency of the $CD11c^+$ $MHCII^+$ cells in DC preparations and the fluorescence intensity (MFI) geometric means of CD40, CD80 and CD86 expressed in those cells were assessed by flow cytometry. (Panel B) Temporal changes in body weight: Body weight was taken in daily base from 1 to 5 days after TNBS instillation and is represented as mean percentage in relation to the mean initial value (100%). It is possible to note that body weights in both colitic and nDC-treated mice dropped after treatment by intrarectal route whereas remained stable in tDC treated mice. (Panel C) Clinical signs: Clinical signs of colitis were evaluated for the presence of diarrhea, rectal prolapse, bleeding and cachexia, assigning a score ranging from 0 to 2, with 0: no change, 1: slight change, and 2: severe change. Data are representative of three independent experiments (n = 5). ANOVA followed by Bonferroni post-test were used to determine statistical significance ($p < 0.05$).

Fig 3: Transfer of DCs obtained from OVA-treated mice reduces histopathological damages in the large intestine caused by TNBS-induced colitis. BALB/c mice were exposed to the treatments described in Fig 1 and sacrificed 5 days after induction of colitis. Distal portions of large gut were collected and fixed in paraffin for histological processing. Panels Ethanol, TNBS, nDC+TNBS and tDC+TNBS represent sections obtained from mice treated with ethanol, colitic, pretreated with nDC and pretreated with tDC. Histological sections of 5μm were stained with hematoxylin and eosin and examined by light microscopy (200X).

Since the adoptive transfer of tolerogenic DCs significantly reduce the clinical signs and the local inflammatory manifestations of TNBS-induced colitis, we then sought to assess the effect of this treatment on the systemic immune responses of the treated mice. Initially, we investigate the proliferative responses of spleen cells cultured in the presence of Con-A. The spleen cells were previously labeled with CFSE and lectin-induced proliferation was assessed by flow cytometry. As shown in Fig 4, proliferative responses of spleen cells obtained from tDC-treated mice were similar to those observed in the cultures of naive spleen cells and spleen cells from mice treated with nDCs. However, its proliferative responses were significantly lower than those observed in the cultures of spleen cells collected from colitic control mice. Results similar to these had also been observed after the adoptive transfer of tDCs in the experimental arthritis model (THOMÉ et al., 2012).

Fig 4: Adoptive transfer of tolerogenic DCs alters the proliferative response, cytokine producing and transcription factor expressing-cells in cultures of spleen cells from mice with TNBS-induced colitis. Transfer of DCs and induction of colitis were performed as described in Fig 1. Mice were sacrificed five days after TNBS instillation. Spleens were aseptically removed; cells were labeled with CFSE and cultured at a concentration of $2x10^6$

cells/ml in the presence of Concanavalin A (ConA; 2,5μg/ml) for 72 hours at 37^{o}C and 5% CO2. (Panel A) Spleen cell proliferation: Cells were fixed in 1% formaldehyde and the readings performed in flow cytometer (FACSCalibur, BD). Proliferation was calculated using the software FCS Express and represents the inverse of the ratio of the fluorescence exhibited by the cells after 72 hours of culture and those immediately after labeling with CFSE. The cell frequency of T regulatory $CD25^{+}Foxp3^{+}$ cells and the frequency of cytokine producing cells and transcription factors (GATA3, Tbet, and RORc) expressing cells were monitored within the $CD4^{+}$ T cell gate. The values correspond to the mean ± S.E.M. of two independent experiments (n = 5). ANOVA followed by Bonferroni a posteriori test was used to determine statistical significance

After culturing with Con-A, the spleen cells were further labeled with specific antibodies to evaluate the expansion of T helper cells (Th1, Th2, Th17) and Treg cells, as well as the expression of the transcription factors T-bet, GATA3 and RORc in $TCD4^{+}$ cells. Fig 4 shows that the frequency of Th17 ($TCD4^{+}IL\text{-}17^{+}$) cells was significantly more reduced in the splenic cell cultures of mice treated with tDCs than in the splenic cell cultures of control colitic mice. In contrast, the frequency of Th2 ($TCD4^{+}$ $IL\text{-}10^{+}$) cells was higher in the cultures of spleen cells from group treated with tDCs than in the other groups. Fig 4 shows that the frequency of Th17 cells was significantly more reduced in the splenic cell cultures of mice treated with tDCs than in the splenic cell cultures of control colitic mice. In contrast, the frequency of $TCD4^{+}$ $IL\text{-}10^{+}$ cells was higher in the cultures of spleen cells from group treated with tDCs than in the other groups. Although it is possible to observe a reduction in the frequency of T cells expressing T-bet, GATA-3 and RORc transcription factors in splenic cell cultures from DC-treated mice (nDCs and tDCs) compared to the control group, the results were not significant. The same occurred with respect to Treg cell frequencies. The same occurred with respect to Treg cell frequencies.

The production of cytokines measured in the supernatants of the splenic cell cultures was closely related to the variations observed in the different populations of $TCD4^+$ cells examined here (Fig 5). In this sense, it was possible to verify a significant reduction in IL-17 levels in supernatants of the splenic cell cultures of the tDC-treated mice compared to the control group. Although there was a tendency for higher levels of IL-4 and IL-10 in the cell cultures of the tDC-treated group, the results were not statistically significant. Significant variations were also not observed in TNF-α levels among the different groups studied. A reduction was observed in IFN-γ levels in nDC-treated mice. These results resemble those obtained by our group in the collagen-OVA-induced arthritis model in BALB/c, indicating that the modulation of inflammatory disease by tolerogenic DCs produced by OVA-fed animals is due to the increased production of anti-inflammatory cytokines and the consequent reduction of Th17 cells (THOMÉ et al., 2012). As we have postulated, other authors have also suggested that increased production of IL-10 is essential to suppress the exacerbated activation of inflammatory cytokines that result in inflammation and development of colitis (GLOCKER et al., 2011; GUO; LI, 2014).

Fig 5: Adoptive transfer of DC alters cytokine levels in supernatants of ConA-stimulated spleen cells from mice with TNBS-induced colitis. Cultures of spleen cells were carried out as described in Fig 4. Cytokine levels were evaluated in the culture supernatants by using the CBA Multiplex kit (Cytometric Bead Array Th1/Th2/Th17, BD Bioscience), CBA kit (Cytometric Bead Array Th19, BD) and readings were performed in a flow cytometer (FACSCalibur, BD). Cytokine concentrations were determined using the array FCAP TM Version 3.0 Software (BD). Results were expressed as means ± S.E.M. obtained from two independent experiments (n = 5).ANOVA followed by Bonferroni a posteriori test was used to determine statistical significance.

IL-9, a cytokine newly associated with IBD, may act as an inflammatory or regulatory cytokine because of its effects on both Th17 cells and Treg cells (DEFENDENTI et al., 2015; GOSWAMI; KAPLAN, 2011) . It has been shown that IL-9 synergizes with TGF-beta in the differentiation of Th17 cells (MILLER et al., 1991; MILLINGTON, MOWAT; GARSIDE, 2004; THOMÉ et al., 2012; WILDNER; THURAU, 1995; ZONNEVELD-HUIJSSOON Et al., 2011). On the other hand, Th17 cells can secrete IL-9 with regulatory effects in vivo (Refs). In vitro results confirm that IL-9 acts on Treg cells ($CD4^{+}FoxP3^{+}$ T cells), increasing its suppressor function through signaling pathways linked to the transcription factor STAT3 and STAT5 (ELYAMAN et al., 2009). For this reason, IL-9 has been identified as a cytokine capable of initially inducing and then regulating tissue inflammation. Taken together, these findings led us to investigate the presence of IL-9 in the supernatants of splenic cell cultures of mice from the different experimental groups studied here. Our results show a clear trend of reducing IL-9 levels in spleen cell cultures collected from DCs treated (tDCs and nDCs) compared to the control group (Fig 5). Recent literature data has been shown a positive correlation between disease severity and IL-9 production in patients with Crohn's disease, but not in patients with ulcerative colitis (DEFENDENTI et al., 2015; ELYAMAN et al., 2009; GERLACH et al., 2014). Although TNBS-induced colitis is considered a model of the Crohn's disease, mimicking its clinical and inflammatory aspects, we observed in this study that the production of cytokines in the experimental disease, in particular IL-9, differs somewhat from that seen in human disease. An earlier report in the literature showed that the adoptive transfer of tolerogenic DCs could modulate colitis induced by the inoculation of $CD4^{+}$ $CD25^{+}$ T cells (Treg $CD4^{+}$ T cells) into SCID mice. The tolerogenic DCs employed in the study were differentiated from bone marrow

cells cultured in the presence of IL-10 and pulsed with enteric bacteria extract, and their administration resulted in a significant reduction of the clinical and inflammatory signals associated with IBD in immunodeficient mice (PEDERSEN et al., 2007). The results obtained in the present study corroborate previous data from our group about the ability of DCs generated by induction of oral tolerance to a dietary protein (THOMÉ et al., 2012). The potential of DCs obtained from tolerant animals (tDC) in suppressing immune reactivity has also been demonstrated in other murine models such as allograft, autoimmune experimental encephalomyelitis (EAE), myasthenia gravis and type 1 diabetes (LUTZ et al., 2002; LUTZ; SCHULER, 2002 MÜLLER et al., 2002), which gives a greater degree of reliability to our data.

Conclusion

Our results indicate that the adoptive transfer of tolerogenic is able to reduce the signs and the immune response in TNBS-induced colitis. The immunomodulation observed can be related to the reduction in the number ofTh17 cells and the secretion of IL-17 and IL-9, as well as to the increased production of IL-10 and IL-4. Tolerogenic DC can alter the proliferative and immune response of T cells due to the significant reduction of CD80 expression. Further studies are necessary to track and evaluate the *in vivo* effects of adoptive transfer of tolerogenic DC, but these cells can be DC can a therapeutic toll for modulating immune responses in inflammatory disorders, specifically in human colitis.

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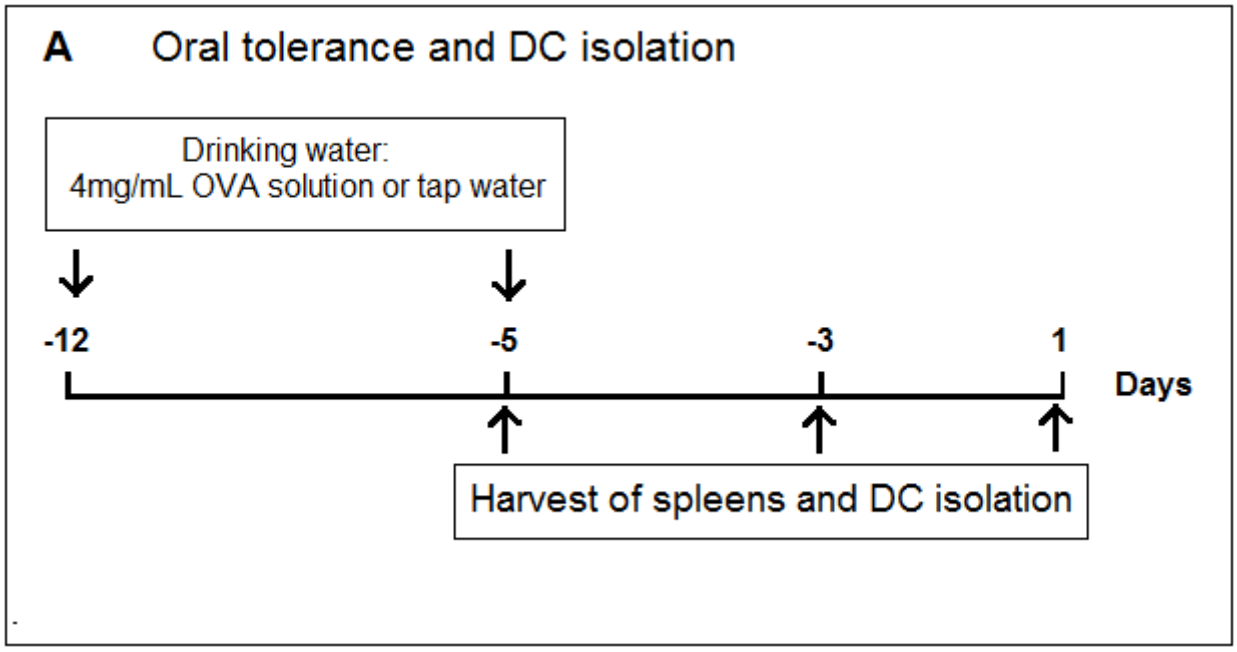

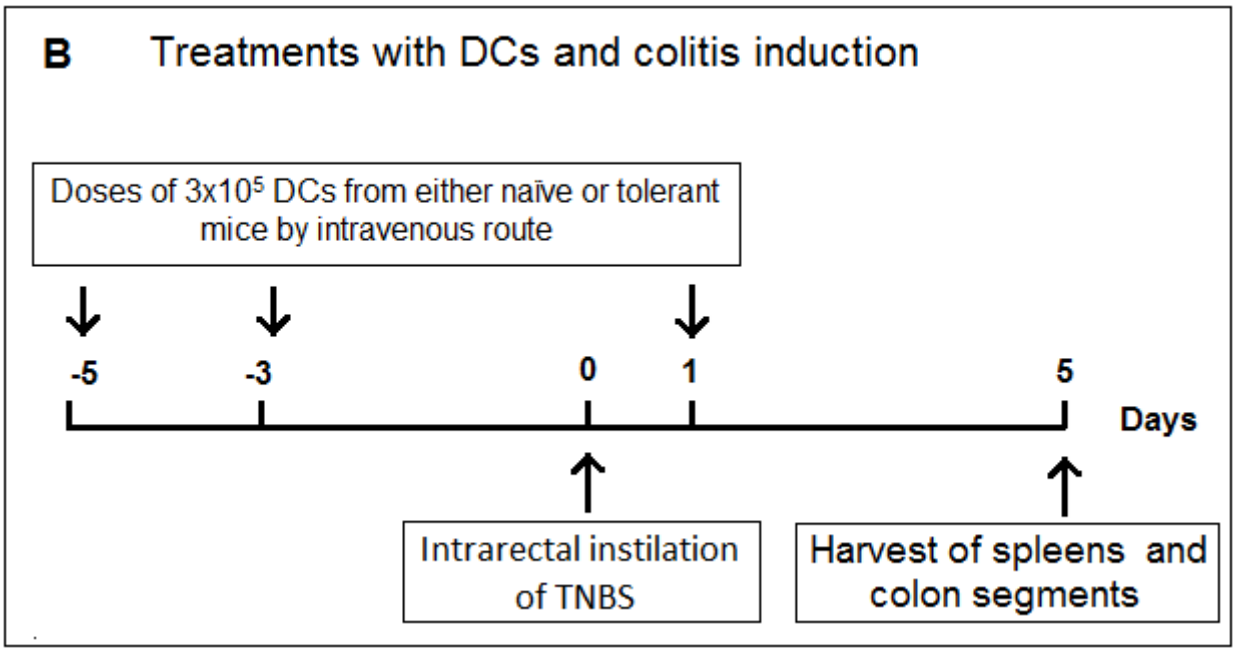

Fig 1: Flowchart of obtaining dendritic cells and inducing colitis. (Panel A) Obtaining dendritic cells: Naive and tolerant mice were used to obtain DCs. The animals in the tolerant group received a solution of 4mg/mL OVA as drinking water for seven consecutive days. The

naïve animals received protein-free water (tap water). Animals from each group were sacrificed for isolation of splenic DCs using magnetic beads coated with anti-CD11c on days 1, 3, and 5 after discontinuation of oral treatment. (Panel B) Treatment with DCs and colitis induction: Three doses of $3x10^5$ of naïve or tolerant DCs were injected intravenously in naïve BALB/c mice on days -5, -3 and 1 in relation to the day colitis induction. Twenty-four hours after the last dose, 100 µL of 1mg/mL TNBS solution was instilled by intrarectal route in treated BALB/c mice. The control groups consisted of mice without any treatment (naïve mice) and of mice challenged with TNBS (colitic mice).

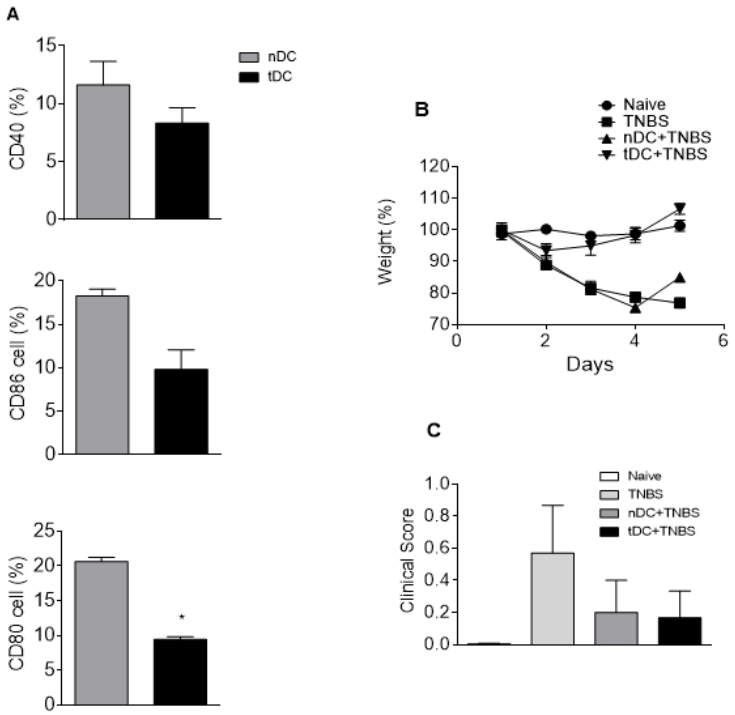

Fig 2: Adoptive transfer of CD11c$^+$ dendritic cells isolated from tolerant mice reduces severity of experimental colitis. (Panel A) Characterization of DCs isolated from spleens of

naive and tolerant mice (nDCs and tDCs, respectively): The frequency of the $CD11c^{+}$ $MHCII^{+}$ cells in DC preparations and the fluorescence intensity (MFI) geometric means of CD40, CD80 and CD86 expressed in those cells were assessed by flow cytometry. (Panel B) Temporal changes in body weight: Body weight was taken in daily base from 1 to 5 days after TNBS instillation and is represented as mean percentage in relation to the mean initial value (100%). It is possible to note that body weights in both colitic and nDC-treated mice dropped significantly after treatment by intrarectal route whereas remained stable in tDC treated mice. (Panel C) Clinical signs: Clinical signs of colitis were evaluated for the presence of diarrhea, rectal prolapse, bleeding and cachexia, assigning a score ranging from 0 to 2, with 0: no change, 1: slight change, and 2: severe change. Data are representative of three independent experiments (n = 5). ANOVA followed by Bonferroni post-test were used to determine statistical significance ($p < 0.05$).

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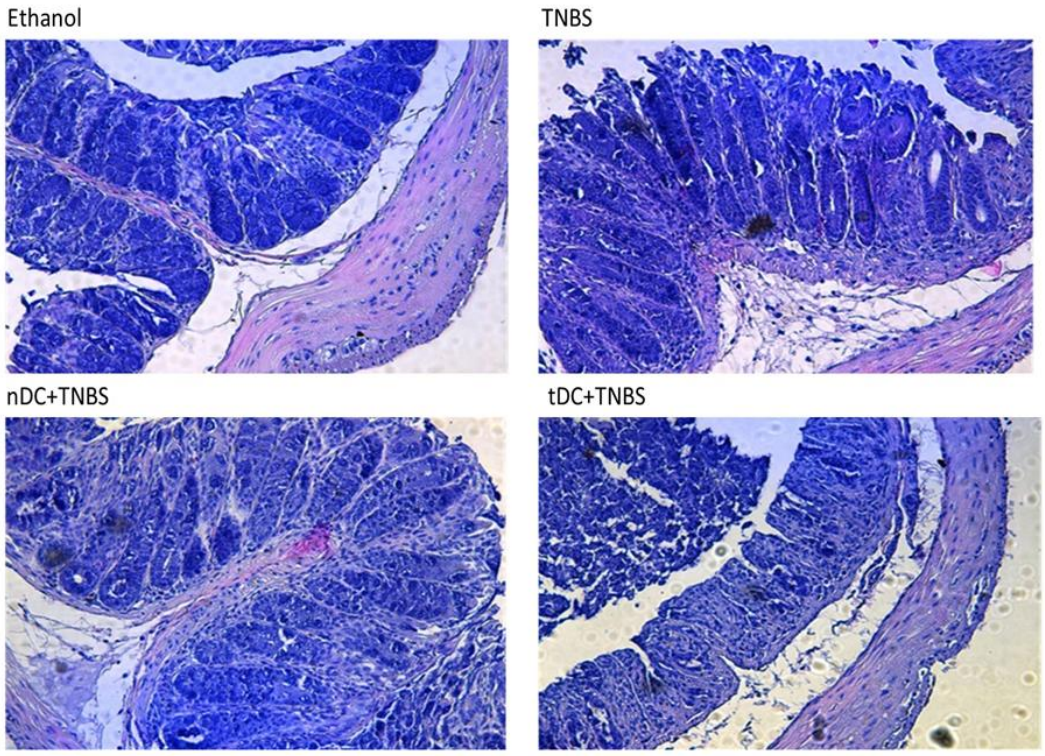

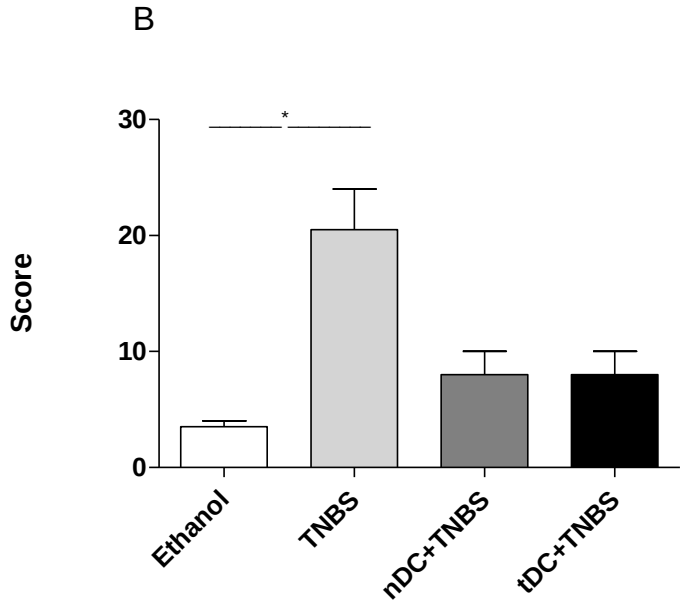

Fig 3: Transfer of DCs obtained from OVA-treated mice reduces histopathological damages in the large intestine caused by TNBS-induced colitis. Panel A: BALB/c mice were exposed to the treatments described in Fig 1 and sacrificed 5 days after induction of colitis. Distal portions of large gut were collected and fixed in paraffin for histological processing. Panels Ethanol, TNBS, nDC+TNBS and tDC+TNBS represent sections obtained from mice treated with ethanol, colitic, pretreated with nDC and pretreated with tDC. Histological sections of 5μm were stained with hematoxylin and eosin and examined by light microscopy (200X). Panel B: Sections were assessed for the presence of folds, hemorrhage, and infiltration of leukocytes at two distal portions of the intestines, assigning a score ranging from 0 to 20.

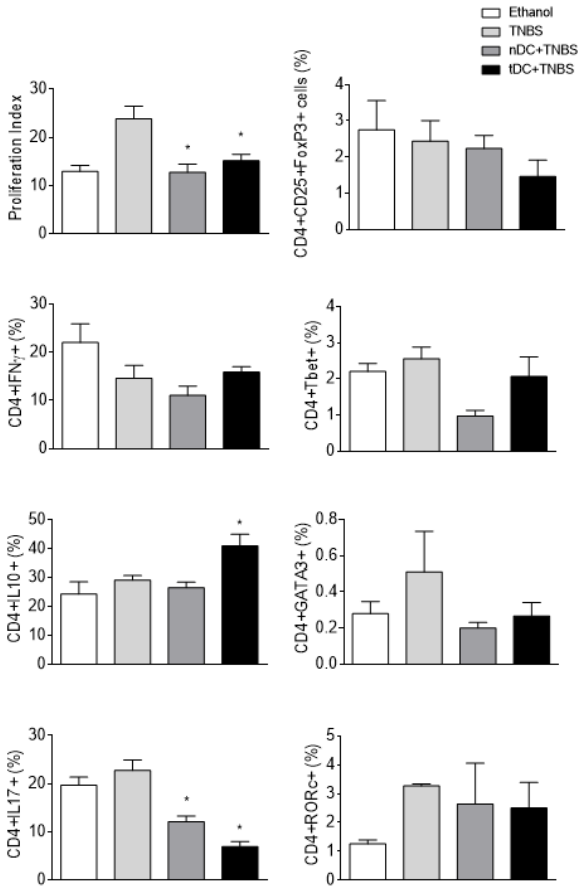

Fig 4: Adoptive transfer of tolerogenic DCs alters the proliferative response, cytokine producing and transcription factor expressing-cells in cultures of spleen cells from mice with TNBS-induced colitis. Transfer of DCs and induction of colitis were performed as described in Fig 1. Mice were sacrificed five days after TNBS instillation. Spleens were aseptically removed; cells were labeled with CFSE and cultured at a concentration of $2x10^6$ cells / ml in the presence of Concanavalin A (ConA; 2,5µg/ml) for 72 hours at 37°C and 5% CO2. (Panel A) Spleen cell proliferation: Cells were fixed in 1% formaldehyde and the readings performed in flow cytometer (FACSCalibur, BD). Proliferation was calculated using the software FCS Express and represents the inverse of the ratio of the fluorescence exhibited by the cells after 72 hours of culture and those immediately after labeling with CFSE. The cell frequency of T regulatory $CD25^+Foxp3^+$ cells and the frequency of cytokine producing cells and transcription

factors (GATA3, Tbet, and RORc) expressing cells were monitored within the $CD4^+$ T cell gate. The values correspond to the mean ± S.E.M. of two independent experiments (n = 5). ANOVA followed by Bonferroni a posteriori test was used to determine statistical significance.

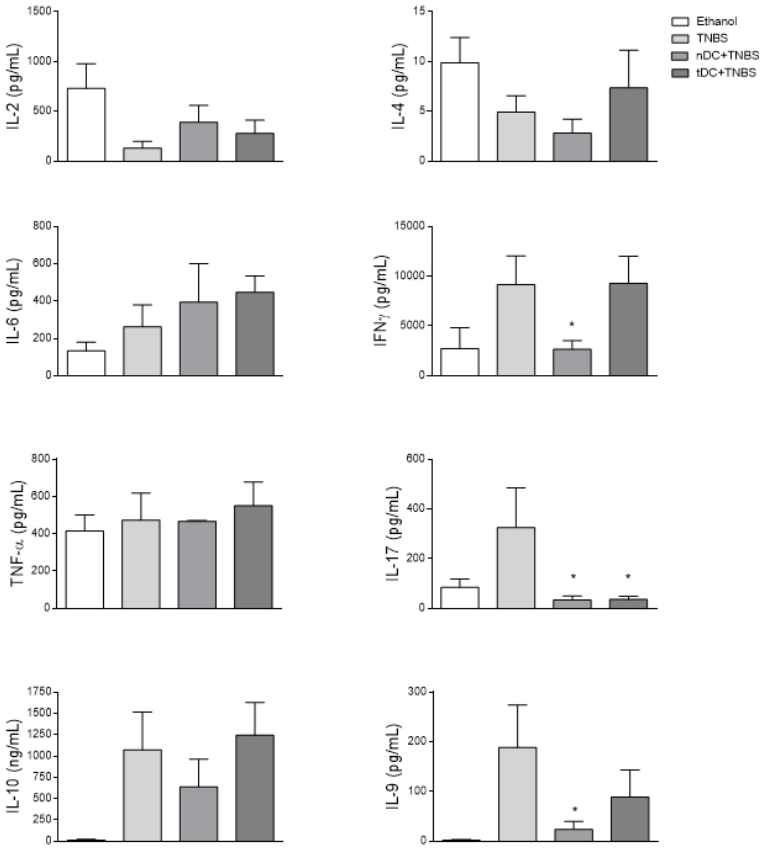

Fig 5: Adoptive transfer of DC alters cytokine levels in supernatants of ConA-stimulated spleen cells from mice with TNBS-induced colitis. Cultures of spleen cells were carried out as described in Fig 4. Cytokine levels were evaluated in the culture supernatants by using the CBA Multiplex kit (Cytometric Bead Array Th1/Th2/Th17, BD Bioscience), CBA kit (Cytometric Bead Array Th19, BD) and readings were performed in a flow cytometer (FACSCalibur, BD). Cytokine concentrations were determined using the array FCAP TM Version 3.0 Software (BD). Results were expressed as means ± S.E.M. obtained from two independent experiments (n =

5).ANOVA followed by Bonferroni a posteriori test was used to determine statistical significance.

7 CONSIDERAÇÕES FINAIS

Os resultados demonstram que a supressão *bystander* associada à ingestão de antígenos da dieta foi capaz de amenizar os efeitos da colite experimental em camundongos BALB/c, como, comprovado pela redução da perda de peso e dos sinais clínicos e pela preservação da integridade do tecido colônico.

O tratamento com OVA por via oral foi capaz de modular a resposta inflamatória no modelo de colite induzida experimentalmente por TNBS, com redução dos danos no tecido intestinal. Essa modulação está relacionada à expansão de células T regulatórias e à produção aumentada de IL-10.

A modulação da resposta imune observada na tolerância oral pode ser transferida pela administração de DCs tolerogênicas. A transferência de DCs obtidas de camundongos tolerantes a OVA foi capaz de reduzir os danos associados à colite, bem como de suprimir a resposta proliferativa *in vitro*. Isso pode ser associado à redução de expressão da molécula co-estimulatória CD80 em DCs isoladas de animais tratados com OVA oral. A administração de DCs tolerogênicas para animais colíticos possibilitou à redução da frequência de células Th17 e da secreção de citocinas IL-17 e IL-9, bem como o aumento de citocinas reguladoras como IL-10 e IL-4 *in vitro*.

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ANEXOS

1. Protocolo de Comitê de Ética em Experimentação Animal.

2. Artigo do capítulo 1 publicado: Plos One, 12: (1), 2017.

3. Artigo do capítulo 2 submetido: Plos One, 2017.

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

CERTIFICADO

Certificamos que o projeto "**Avaliação dos efeitos da tolerância oral à ovalbumina e de células dendríticas tolerogênicas sobre o curso da resposta imune em doenças autoimunes experimentais**" (protocolo nº **3077-1**), sob a responsabilidade de **Dra. Patricia Ucelli Simioni / Profa. Dra. Wirla Maria da Silva Cunha Tamashiro**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **03 de junho de 2013**.

Campinas, 23 de janeiro de 2015.

2ª. VIA

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
Presidente

Fátima Alonso
Secretária Executiva

PLOS ONE

RESEARCH ARTICLE

Oral Tolerance Induced by OVA Intake Ameliorates TNBS-Induced Colitis in Mice

Lisiery N. Paiatto[1,2], Fernanda G. D. Silva[3], Julia Bier[1], Márcia R. Brochetto-Braga[2], Áureo T. Yamada[4], Wirla M. S. C. Tamashiro[1], Patricia U. Simioni[1,2,5]*

1 Department of Genetics, Evolution and Bioagents, Institute of Biology, State University of Campinas, UNICAMP, Campinas, São Paulo, Brazil, **2** Institute of Biosciences, Universidade Estadual Paulista, UNESP, Rio Claro, São Paulo, Brazil, **3** Faculty of Food Engineering, University of Campinas, UNICAMP, Campinas, São Paulo, Brazil, **4** Department of Histology and Embryology, Institute of Biology, State University of Campinas, UNICAMP, Campinas, São Paulo, Brazil, **5** Department of Biomedical Science, Faculty of Americana, FAM, Americana, São Paulo, Brazil

* psimioni@gmail.com

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Abstract

Introduction

Literature data have shown that the consumption of dietary proteins may cause modulatory effects on the host immune system, process denominated oral tolerance by bystander suppression. It has been shown that the bystander suppression induced by dietary proteins can improve inflammatory diseases such as experimental arthritis. Here, we evaluated the effects of oral tolerance induced by ingestion of ovalbumin (OVA) on TNBS-induced colitis in mice, an experimental model for human Crohn's disease.

Methods and Results

PLOS ONE

Adoptive transfer of tolerogenic dendritic cells reduces severity of experimental colitis in BALB/c mice

--Manuscript Draft--

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Article Type:	Research Article
Full Title:	Adoptive transfer of tolerogenic dendritic cells reduces severity of experimental colitis in BALB/c mice
Short Title:	Adoptive transfer of dendritic cells reduces severity of colitis in mice
Corresponding Author:	Patricia U. Simioni, Ph.D. Universidade Estadual Paulista Julio de Mesquita Filho Rio Claro, São Paulo BRAZIL
Keywords:	Colitis; dendritic Cell; tolerance; autoimmunity; CD80; CD86; CD40; IL-17; IL-10; Treg; T-bet; GATA-3; ROR-c; Oral tolerance; Experimental colitis; cytokine; culture; inflammatory bowel disease; ovalbumin; adoptive transfer; il-9; immuneregulation; gastrointestinal tract; TNBS; Crohn's disease
Abstract:	Introduction: In addition to conventional therapies, several new strategies have been proposed to modulate autoimmune diseases, including the adoptive transfer of immunological cells. In this context, tolerogenic dendritic cells (DCs) appear to be one of the most promising treatments to autoimmune disorders. The present study aimed to evaluate the effects of adoptive transfer of DCs obtained from ovalbumin (OVA) tolerant mice on the severity of TNBS induced colitis, and analyses the eventual protective mechanisms. Methods and Results: To induce oral tolerance, BALB/c mice were fed with 4mg/mL OVA solution for seven consecutive days. Spleen DCs were isolated from tolerant (tDCs) and naïve (nDCs) mice, and then adoptively transferred to syngeneic mice. Three days later, colitis was induced in DC treated mice by intrarectal instillation of 100µg 2,4,6-trinitrobenzenesulfonic acid (TNBS) dissolved in 50% ethanol. Controls received only intrarectal instillation of either TNBS solution or vehicle. Five days later, mice from all groups were euthanized and examined for physiologic and immunologic parameters. Our results show that previous treatment with tDC is able to reduce signs of the colitis, such as weight loss, as well as partially preserve the integrity of the colonic tissue. In vitro proliferation of spleen cells from DC-treated mice was significantly lower than the proliferation of spleen cells from mice of the control groups. Although no significant difference was observed in the frequencies of Treg cells in the different experimental groups, the frequency of Th17+CD4+cells and the secretion of Il-17 and IL-9 were reduced in the cultures of spleen cells from DC treated mice, whereas the levels of IL-10 and IL-4 were augmented. The levels of inflammatory cytokines were also lower in supernatants of cells from tDC treated mice. Conclusion: The results allow concluding that the adoptive transfer of tDC is able to reduce the clinical and immunological signs of the drug-induced colitis. The immunomodulation observed here can be related to the reduction of Th17 cells and inflammatory cytokine secretion, as well as the augment of anti-inflammatory interleukins. Tolerogenic DC altered the proliferative and T cell immune responses probably due to the significant reduction of CD86 expression. DC can be a therapeutic toll for modulating immune responses in inflammatory disorders, specifically in human colitis. To the best of our knowledge, there are no previous data in the literature showing the protective effects of OVA tolerant DCs in the treatment of colitis.
Order of Authors:	Lisiery Negrini Paiatto
	Fernanda G D Silva
	Áureo T Yamada
	Wirla M S C Tamashiro
	Patricia U. Simioni, Ph.D.
Opposed Reviewers:	