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INFLUÊNCIA DA FLUOXETINA SOBRE A RESPOSTA IMUNO-INFLAMATÓRIA RELACIONADA À DOENÇA PERIODONTAL

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"Sem sonhos, a vida não tem brilho. Sem metas, os sonhos não têm alicerces. Sem prioridades, os sonhos não se tornam reais. Sonhe, trace metas, estabeleça prioridades e corra riscos para executar seus sonhos. Melhor é errar por tentar do que errar por omitir."

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RESUMO

A fluoxetina é um droga inibidora seletiva da recaptação de serotonina que apresenta propriedades imunomoduladoras e antiinflamatórias. O objetivo desse estudo foi avaliar os efeitos da fluoxetina sobre a resposta imuno-inflamatória relacionada à doença periodontal (DP). *In vitro*, avaliou-se a influência da fluoxetina sobre a capacidade das células dendríticas (DCs) em apresentar antígeno aos linfócitos T. As DCs foram obtidas da medula óssea de camundongos C57BL/6 e diferenciadas utilizando-se GM-CSF (20 ng/mL). As DCs foram tratadas com a fluoxetina (concentrações: 0,01, 0,1 ou 1 μM) para análise da produção de citocinas e quimiocinas, bem como da expressão de MHC-II e moléculas co-estimuladoras (CD80, CD86, PD-L1, ICOS-L) aos linfócitos T, utilizando-se ensaios de ELISA e citometria de fluxo respectivamente. Culturas de DCs e linfócitos T reativos ao *Aggregatibacter actinomycetemcomitans* (×*Aa*-T) foram utilizadas para avaliação de proliferação/ativação de linfócitos T. A desipramina, um inibidor seletivo da recaptação de norepinefrina, também foi incluída nos ensaios *in vitro* para comparação. *In vivo*, avaliou-se os efeitos da fluoxetina sobre a resposta inflamatória e a destruição tecidual utilizando-se modelo de DP induzida por ligadura. Ratos Wistar machos (SPF) foram submetidos à colocação de ligadura em torno dos primeiros molares inferiores e divididos em 3 grupos experimentais (n=10 animais/grupo): 1) ratos sem ligadura e sem tratamento (grupo controle); 2) ratos com ligadura e tratados com solução salina (grupo ligadura); 3) ratos com ligadura e tratados com a fluoxetina (20 mg/kg/dia, grupo ligadura + fluoxetina). Análises de reabsorção óssea na região de furca (lâminas coradas com H&E) e de colágeno no tecido conjuntivo da mesial dos primeiros molares (coloração de picrosirius) foram realizadas nos ratos submetidos a 15 dias de indução da DP. Tecidos gengivais de ratos submetidos a 3 dias de indução da DP foram submetidos às seguintes análises: expressão de IL-1β, COX-2, MMP-9 e iNOS utilizando-se RT-PCR e atividade da MMP-9 utilizando-se zimografia. Como resultados, a fluoxetina diminuiu a produção de IL-12, IL-1β, TNF-α, RANTES e MIP-1α pelas DCs estimuladas com LPS ($P < 0,05$, ANOVA, teste *t* de Student), bem como diminuiu significativamente a expressão de ICOS-L. Além disso, reduziu a proliferação de ×*Aa*-T estimulados com *Aa* pelas DCs. A serotonina (5- HT) aumentou a proliferação de ×*Aa*-T, indicando que os efeitos da

fluoxetina são independentes da 5-HT. A desipramina apresentou perfil semelhante à fluoxetina nos ensaios *in vitro*. No estudo *in vivo*, a fluoxetina reduziu a perda óssea em região de furca quando comparada ao grupo ligadura ($P < 0,05$ ANOVA, teste *t* de Student) e manteve a porcentagem de fibras colágenas com níveis similares ao grupo controle ($P > 0,05$). Ainda, a fluoxetina reduziu a expressão de IL-1β e COX-2 e a atividade da MMP-9 quando comparada ao grupo ligadura ($P < 0,05$). Em conjunto, os dados demonstram que a fluoxetina diminuiu a capacidade de apresentação de antígeno das DCs, bem como a resposta inflamatória, a reabsorção óssea e a perda de colágeno na DP, indicando que ela pode constituir uma abordagem terapêutica promissora como moduladora da resposta do hospedeiro na DP.

Palavras-chave: Fluoxetina, doença periodontal, células dendríticas, apresentação de antígeno, ICOS-L, serotonina, inflamação, reabsorção óssea, colágeno, modulação da resposta do hospedeiro

ABSTRACT

Fluoxetine is a selective serotonin reuptake inhibitor presenting immunomodulatory and anti-inflammatory properties. The aim of this study was to evaluate the fluoxetine effects on immunoinflammatory response associated with periodontal disease (PD). The *in vitro* study evaluated the effects of fluoxetine on antigen-presentation capacity of dendritic cells (DCs). Bone marrow DCs obtained from C57BL/6 wild type mice were differentiated using GM-CSF (20 ng/mL). DCs were treated with fluoxetine (concentrations of 0.01, 0.1 or 1 μM) for subsequent cytokine/chemokine assays (ELISA) and analysis of expression of MHC-class II and co-stimulatory molecules (CD80, CD86, PD-L1, ICOS-L) to T cell activation using flow cytometry. Fluoxetine was also applied to cultures with both DCs and *Aggregatibacter actinomycetemcomitans* (*Aa*)-reactive T cells (×*Aa*-T), which were used for analysis of T cells proliferation/activation using thymidine and ELISA assays. Desipramine, a selective norepinephrine reuptake inhibitor, was also tested *in vitro* for comparison to fluoxetine. *In vivo*, male Wistar rats received ligature placement around mandibular first molars and were randomly assigned into three experimental groups (n=10/group): 1) Control rats (without ligature); 2) rats with ligature + placebo (saline; oral gavage); 3) rats with ligature + fluoxetine (20 mg/kg/day in saline; oral gavage). Histometric and histological analyses were performed for measurement of loss of bone in furcation region (H&E stain) and collagen fibers (picrosirius red stain) in the connective tissue of rats submitted to 15 days of PD induction. Gingival tissues were collected from animals submitted to 3 days of PD induction for analyses of mRNA expression of IL-1β, COX-2, MMP-9 and iNOS using RT-PCR, measurement of total protein concentration and MMP-9 activity using zymogram. Fluoxetine suppressed IL-12, IL-1β, TNF-α, RANTES and MIP-1α production by LPS-stimulated DCs ($P < 0.05$, ANOVA, Student's *t* test), as well as significantly reduced the expression of ICOS-L. Fluoxetine suppressed the proliferation of ×*Aa*-T stimulated with *Aa*-antigen presentation by DCs from co-cultures. When applied to ×*Aa*-T/DCs co-cultures, serotonin (5-HT) increased T cell proliferation, indicating that fluoxetine effects are independent of 5-HT. Desipramine effects were similar to those of fluoxetine. In the *in vivo* study, fluoxetine reduced alveolar bone loss as compared to ligature group ($P < 0.05$, ANOVA, Student's *t* test) and maintained collagen

fibers levels similarly to control group ($P > 0.05$). Fluoxetine reduced IL-1β and COX-2 expression, as well as MMP-9 activity, from gingival tissues when compared to ligature group ($P < 0.05$). Altogether, data showed that fluoxetine can modulate the antigen-presentation capacity of DCs and reduce inflammatory response and loss of bone and collagen associated with PD. In conclusion, fluoxetine can modulate both immune and inflammatory responses on PD, suggesting that it may constitute a new therapeutic approach for modulation of host response in periodontal therapy.

Key words: Fluoxetine, periodontal disease, dendritic cells, antigen-presentation capacity, ICOS-L, serotonin, inflammation, bone resorption, collagen, host response modulation

SUMÁRIO

1. INTRODUÇÃO

A doença periodontal pode ser definida como uma infecção crônica de caráter inflamatório que leva à destruição dos tecidos de suporte dos dentes, com perda progressiva de inserção, reabsorção óssea e migração apical do epitélio juncional (Van Dyke & Serhan, 2003). Atualmente, a doença representa a principal causa de perda do elemento dental, além de agir como um fator modificador para a saúde sistêmica (Seymour *et al.*, 2007).

O biofilme dental é considerado o fator etiológico essencial para o início da doença. As bactérias presentes no biofilme associado à doença periodontal, tais como *Aggregatibacter actinomycetemcomitans* e *Porphyromonas gingivalis,* produzem fatores destrutivos e enzimas que degradam a matriz extracelular (como o colágeno) e membranas celulares do hospedeiro, a fim de obter nutrientes para seu crescimento e invasão tecidual. Ainda, os produtos e os componentes bacterianos (proteínas de membrana, fímbrias, lipopolissacarídeo, dentre outros) provocam a ativação da resposta imuno-inflamatória do hospedeiro, responsável pela maior parte da destruição tecidual observada na doença (Birkedal-Hansen, 1993; Salvi & Lang, 2005).

Os linfócitos T têm sido considerados como as principais células envolvidas na progressão da doença periodontal, especialmente por produzirem o fator de diferenciação de osteoclastos RANKL ("receptor activator of nuclear factor κb ligand"), assim como citocinas pró-inflamatórias (como o fator de necrose tumoral alfa – TNF-α) e antiinflamatórias (como a interleucina-10) relacionadas à doença periodontal (Taubman *et al*. 2005). Entretanto, a ativação dos linfócitos T depende da apresentação do antígeno pelas células dendríticas (DCs), que constituem as mais importantes células apresentadoras de antígeno do sistema imune (Banchereau & Steinman, 1998). Assim, tem-se documentado que a apresentação do antígeno pelas DCs é de importância crucial para a ativação das células T no contexto da doença periodontal (Cutler & Jotwani, 2004; Cutler & Teng, 2007).

A importância das DCs como células apresentadoras de antígeno está relacionada a algumas características: 1) elas expressam maiores níveis do complexo de histocompatibilidade maior (MHC) do que qualquer outra célula apresentadora de antígeno, bem como expressam moléculas co-estimuladoras (como CD80, CD86, PD-L1, ICOS-L),

sendo todos essenciais para a indução do receptor da célula T (TCR) e sua ativação; 2) em acréscimo à sua capacidade de apresentar antígeno, as DCs produzem importantes citocinas pró-inflamatórias e antiinflamatórias (i.e. interleucina-12, interleucina-1β, TNF-α, interleucina-10) e quimiocinas [tais como RANTES ("regulated on activation, normal T cell expressed and secreted" ou CCL5) e MIP-1α ("macrophage inflammatory protein 1α" ou CCL3)] em resposta ao estímulo bacteriano (Banchereau & Steinman, 1998). As respostas imunes mediadas pela apresentação do antígeno bacteriano pelas DCs aos linfócitos T são protetoras ao hospedeiro. No entanto, na infecção crônica da doença periodontal, estudos recentes sustentam a idéia de que respostas iniciadas pela atividade das DCs não controladas de forma suficiente podem causar danos teciduais pela produção de citocinas pró-inflamatórias e indução de células T hiper-reativas (Cutler & Teng, 2007).

Ainda, durante a ativação da resposta imuno-inflamatória, vários mediadores inflamatórios, incluindo citocinas pró-inflamatórias, prostanóides (prostaglandina E2 - PGE_2), metaloproteinases da matriz (MMPs) e óxido nítrico (NO), também são produzidos ou liberados pelas células imunes do infiltrado inflamatório ou células residentes do periodonto (Birkedal-Hansen, 1993; Kornman *et al*., 1997; Lappin *et al*., 2000). A citocina pró-inflamatória interleucina (IL)-1β constitui uma citocina de caráter multifuncional envolvida nas respostas imunes e inflamatórias (Gemmel *et al*., 1997). A IL-1β direciona e estimula uma cascata de eventos destrutivos nos tecidos periodontais, incluindo a produção de PGE_2 e de MMPs (Schwartz *et al*., 1997). A PGE_2 produzida pelas enzimas ciclooxigenases (COX) é um importante produto do metabolismo do ácido araquidônico relacionado aos danos teciduais (Schwartz *et al*., 1997). A IL-1β e a PGE_2, ambas produzidas por monócitos, macrófagos e fibroblastos ativados, têm sido consideradas importantes mediadores da reabsorção óssea na DP (Hofbauer & Heufelder, 2001; McCauley & Nohutcu, 2002), especialmente porque estimulam os osteoblastos e células do estroma a expressarem RANKL. Este, ao se ligar ao seu receptor RANK, induz o processo de diferenciação/ativação de osteoclastos, levando, assim, a um desequilíbrio do metabolismo ósseo e à conseqüente reabsorção (Kong *et al*., 1999; Wada *et al*., 2006). Além disso, a IL-1β e a PGE_2 estimulam fibroblastos e osteoclastos a produzirem MMPs (como a gelatinase B, MMP-9), as quais estão relacionadas à degradação de colágeno do

tecido conjuntivo e do osso alveolar (Kornman *et al*., 1997). Concomitantemente, a IL-1β e outras citocinas pró-inflamatórias estimulam a produção de óxido nítrico (NO), o qual tem sido considerado uma molécula fundamental em diversos processos inflamatórios, incluindo a doença periodontal (Lyons, 1995; Lappin *et al*., 2000). O NO é produzido por células inflamatórias e epiteliais nos sítios periodontais através da enzima óxido nítrico sintetase induzível (iNOS) (Lappin *et al*., 2000).

O emprego de agentes farmacológicos capazes de modular a resposta imuno-inflamatória do hospedeiro vem sendo considerado como uma importante terapia coadjuvante aos procedimentos mecânicos convencionais (Novak & Donley, 2002; Reddy *et al*., 2003). Até o momento, somente um fármaco foi aprovado pela "Food and Drug Administration" (FDA) para esta finalidade, a doxiciclina em doses sub-antimicrobianas (Periostat®) (Golub *et al*., 2001; Novak *et al.*, 2002; Emingil *et al.*, 2004). Nesse contexto, estudos recentes têm investigado o efeito de "novos" fármacos sobre as principais vias de modulação da resposta do hospedeiro, sendo observadas ações de algumas drogas sobre a síntese de PGE_2, a expressão de citocinas, a produção/atividade de MMPs, a síntese de NO e o metabolismo ósseo (Oringer, 2002; Reddy *et al*., 2003; Salvi & Lang, 2005). Em acréscimo à investigação da atividade dos agentes farmacológicos sobre a resposta inflamatória, considera-se, também, interessante investigar a influência de drogas sobre as respostas imunes celulares relacionadas à doença periodontal (Kirkwood *et al*., 2007).

A fluoxetina ($C_{17}H_{18}F_3NO.HCl$), uma droga inibidora seletiva da recaptação de serotonina, tem apresentado propriedades imunomoduladoras e antiiinflamatórias (Yaron *et al*., 1999; Abdel-Salam *et al*., 2003, 2004; Roumestan *et al*., 2007; Guemei *et al*., 2008), as quais fundamentaram o desenvolvimento do presente estudo. Assim, demonstrou-se que a fluoxetina diminui a produção de citocinas relacionas às respostas Th1 e a proliferação de células T estimuladas com agente mitogênico (Diamond *et al*., 2006). Recentemente, um estudo em ratos demonstrou que a fluoxetina é capaz de diminuir a ativação de linfócitos T (Fazzino *et al*., 2009). Entretanto, apesar desses efeitos já relatados na literatura sobre as células T, ainda não se sabe se a fluoxetina pode influenciar a apresentação do antígeno pelas DCs aos linfócitos T efetores via interação TCR/MHC-classe II.

Em relação à influência da fluoxetina sobre o processo inflamatório, verificou-se que ela diminui os níveis de citocinas pró-inflamatórias em modelos animais (Kubera *et al*., 2000; Roumestan *et al*., 2007; Guemei *et al*., 2008; Sacre *et al*., 2010), bem como os níveis de PGE_2 em exsudatos inflamatórios (Bianchi *et al*., 1995) e a inflamação periférica induzida por carragenina (Abdel-Salam *et al*., 2004). Em acréscimo, observou-se que a fluoxetina reduziu a produção de PGE_2 e NO por células sinoviais humanas (Yaron *et al*., 1999). Apesar de alguns mecanismos terem sido propostos para explicar os efeitos imunomoduladores e antiinflamatórios da fluoxetina (Bianchi *et al*., 1994; Diamond *et al*., 2006; Jin *et al*., 2009), os mecanismos precisos ainda não foram elucidados.

Embora os efeitos da fluoxetina sobre a ativação/proliferação de linfócitos T e de alguns efeitos sobre mediadores inflamatórios tenham sido investigados, esta droga ainda não havia sido estudada em relação à doença periodontal. Seu uso como terapia de modulação da resposta do hospedeiro foi considerado viável pela presente investigação pelas seguintes razões: 1) a fluoxetina tem sido largamente utilizada clinicamente no tratamento da depressão; 2) a fluoxetina é considerada segura e bem tolerada pelos pacientes, devido ao fato de ela não possuir afinidade por outros receptores relacionados a efeitos colaterais desagradáveis, tais como α_1-, α_2- e β-adrenérgicos, dopaminérgicos, histamínicos H_1, muscarínicos e receptores GABA (Calil, 2001); 3) os efeitos adversos relacionados à fluoxetina são transitórios e resolvidos espontaneamente com o tempo; 4) em função disso, o índice de descontinuação do uso devido aos efeitos colaterais é baixo (Zajecka *et al*., 1999; Calil, 2001); 5) devido à sua segurança e tolerabilidade pelos pacientes, a fluoxetina tem sido largamente utilizada para o tratamento de uma variedade de outras condições, inclusive de distúrbios crônicos e que exigem terapia de manutenção, como distúrbios do humor e da ansiedade, desordens pré-menstruais, bulimia e dor crônica (Calil, 2001).

Considerando-se as propriedades imunomoduladoras e antiinflamatórias da fluoxetina descritas na literatura, a sua segurança e o seu largo emprego clínico, considerou-se, no presente estudo, sua utilização como uma possível terapia adjunta às terapias convencionais para o controle da doença periodontal. Sendo assim, os objetivos do presente estudo foram: 1) avaliar a influência da fluoxetina sobre as respostas imunes

mediadas por células, especialmente sobre a capacidade das células dendríticas em apresentar antígeno aos linfócitos T durante a sinapse imunológica; 2) Avaliar a influência da fluoxetina sobre a resposta inflamatória e a perda óssea e de colágeno utilizando modelo animal de doença periodontal induzida por ligadura em ratos.

2. CAPÍTULOS

Esta dissertação está baseada na Deliberação CCPG/001/98/UNICAMP e na aprovação pela Congregação da Faculdade de Odontologia de Piracicaba em sua 105ª. Reunião Ordinária em 17/12/2003, que regulamenta o formato alternativo para tese de Doutorado e permite a inserção de artigos científicos de autoria do candidato.

Assim sendo, esta tese é composta de dois capítulos contendo artigos que se encontram em fase de submissão para publicação em revista científica, conforme descrito a seguir:

Capítulo 1

Artigo "Selective serotonin reuptake inhibitor attenuates the antigen presentation from dendritic cells to effector T lymphocytes".

Este artigo foi submetido ao periódico "FEMS Immunology & Medical Microbiology" (Anexo 3).

Capítulo 2

Artigo "Fluoxetine inhibits inflammatory response and bone loss in a rat model of ligature-induced periodontitis".

Este artigo será submetido ao periódico "Journal of Dental Research".

2.1. CAPÍTULO 1

Selective serotonin reuptake inhibitor attenuates the antigen presentation from dendritic cells to effector T lymphocytes

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Key words: Fluoxetine, serotonin (5-HT), dendritic cells, antigen presentation, ICOS-L
Running title: Fluoxetine suppresses dendritic cell/T cell response

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Abstract

Selective serotonin reuptake inhibitors (SSRIs) mediate their antidepressant effects by blocking serotonin transporter (SERT), which, in turn, increases the extracellular serotonin [5-hydroxytryptamine (5-HT)] at neuron synapse. Interestingly, fluoxetine, one of the SSRIs, has been found to possess immune modulation effects. However, it remains unclear if SSRIs can suppress the antigen-presenting function of dendritic cells (DCs). Therefore, fluoxetine was applied to a co-culture between *Aggregatibacter actinomycetemcomitans* (*Aa*)-reactive T cells (×*Aa*-T) isolated from *Aa*-immunized mouse and DCs, which resulted in suppressing the proliferation of ×*Aa*-T stimulated with *Aa*-antigen presentation by DCs. Fluoxetine increased the extracellular 5-HT in the ×*Aa*-T/DCs co-culture, whereas exogenously applied 5-HT promoted T cell proliferation in the ×*Aa*-T/DCs co-cultures, indicating that extracellular 5-HT is not responsible for fluoxetine-mediated suppression of ×*Aa*-T/DCs responses. Fluoxetine remarkably suppressed the expression of co-stimulatory molecule ICOS-L on DCs. Blocking of ICOS-L expressed on DCs with specific antibody down-modulated the antigen presentation from DCs to ×*Aa*-T cells. These results suggested that fluoxetine suppressed the ability of DCs to present bacterial antigens to T cells and resulting T cell proliferation in a SERT/5-HT-independent manner and that diminished expression of ICOS-L on DCs caused by fluoxetine might be partially associated with fluoxetine-mediated suppressions on DCs/T cell responses.

Introduction

Selective serotonin [5-hydroxytryptamine (5-HT)] reuptake inhibitors (SSRIs) are a class of antidepressant drugs used to treat major depression and other related neuronal disorders. In recent years, fluoxetine, a commonly prescribed SSRI that blocks the serotonin transporter (SERT) in the brain, was revealed to possess host beneficial side effects represented by peripheral anti-inflammatory and immunomodulatory properties (Yaron et al., 1999; Abdel-Salam et al., 2003; Roumestan et al., 2007). It was reported that fluoxetine down-regulates the Th1-type cytokine productions and proliferation of human blood T cells stimulated with a non-specific T cell mitogen, concanavalin A (Con A) (Diamond et al., 2006). Recently published *in vivo* studies using rats demonstrated that

SSRIs can down-regulate the activation of T lymphocytes (Fazzino et al., 2009). Although antigen presentation to memory T cells from dendritic cells (DCs) plays a critical role in the induction of adaptive immune responses to non-self organisms, especially to bacteria, it remains unclear if fluoxetine can affect antigen presentation from DCs to effector T lymphocytes via T cell receptor (TCR)/ major histocompatibility complex (MHC)-class-II engagement.

Periodontal disease (PD) is a chronic inflammatory disease triggered by bacterial infection that affects the attachment structures of the teeth. PD is one of the most important causes of tooth loss and has been considered a modifying factor of the systemic health of individuals (Seymour et al., 2007). The inflammatory products released by immune cells, such as dendritic cells (DCs) and T cells, after bacterial challenge are strongly related to host tissue destruction (Loesche and Grossman, 2001; Taubman et al., 2005). It is well documented that antigen presentation by DCs plays a pivotal role in regulating the activation of T cells by presenting bacterial antigens in the context of PD (Cutler and Jotwani, 2004; Cutler and Teng, 2007). DCs, which are well-equipped professional antigen-presenting cells, express higher levels of major histocompatibility complexes (MHC) than other antigen-presenting cells, along with permissive co-stimulatory molecules, for the induction of TCR/CD3 activation (Banchereau & Steinman, 1998). In addition to their roles in presenting bacterial antigens to T cells, DCs are also engaged in the production of important pro- and anti-inflammatory cytokines (i.e., IL-12, IL-1β, TNF-α and IL-10) and chemokines (i.e., RANTES and MIP-1α) in response to bacterial stimuli (Banchereau and Steinman, 1998). It has been thought that immune response to periodontal pathogens is host protective. However, in the chronic infection of PD, recent theory supports the idea that insufficiently controlled immune responses elicited by DCs can cause collateral tissue damage by their production of proinflammatory cytokines as well as by induction of overreacting T cells (Cutler and Teng, 2007). Given such possible pathogenic engagement mediated by DCs, they are considered to be interesting targets for the development of pharmacological regimens for chronic infection, especially PD.

Based on the above-noted evidence showing that fluoxetine can affect DCs and T cells, respectively, the aims of this study were to determine 1) whether fluoxetine can

affect the ability of DCs to present bacterial antigens to T cells in the immune synapse involving TCR and MHC-class-II and 2) whether blocking of SERT expressed in DCs by fluoxetine is responsible for its effects on antigen-presentation by DCs. Desipramine belongs to another class of antidepressant drug known as Norepinephrine Reuptake Inhibitors (NRIs). Since NRIs are also reported to possess immune suppressive effect (Roumestan et al., 2007; Hashioka et al., 2009), desipramine was included to compare its effects to fluoxetine. In this study, the periodontal pathogen *Aggregatibacter actinomycetemcomitans* (*Aa*) was used as a model bacterium which can elicit periodontal tissue destruction via activation of bacterial reactive T cells (Taubman et al., 2005; Teng et al., 2005; Kawai et al., 2007). Contrary to our expectation, the results demonstrated that fluoxetine suppresses the ability of DCs to present bacterial antigens to T cells and resulting T cell proliferation in a SERT/5-HT-independent manner.

Material and methods

Chemicals

Fluoxetine hydrochloride and desipramine hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in water at a high concentration (1 mM, respectively). The drugs were then diluted in fresh RPMI medium containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) to reach final concentrations (0.1, 1 or 10 μM) tested in the present study. Synthetic 5-HT (serotonin hydrochloride) was purchased from Acros Organics USA (Morris Plains, NJ).

Animals

C57BL/6 wild type mice (6–8 weeks old, male, Jackson Laboratory, Bar Harbor, ME) were housed in cages with water and food *ad libitum* in 12-hour dark-light cycles at constant temperature and maintained in the animal housing facility of The Forsyth Institute. All experiments were performed in compliance with protocols approved by the Forsyth Institutional Animal Care and Use Committee (IACUC).

Bacterial antigens

Aa strain Y4 (ATCC, Manassas, VA) was cultured in trypticase soy broth supplemented with 0.6% yeast extract (TSBY; Difco Laboratories, Detroit, MI) in humidified 5% CO_2 atmosphere at 37 ^{o}C. After cultivation, cells were fixed with formalin following the methods published previously (Kawai et al., 2007).

Development of CD11c^{+} DCs *ex vivo*

DCs were generated according to the method of Inaba et al. (1992) with modifications. Briefly, bone marrow was obtained from femurs and tibias of normal C57BL/6 wild type mice. The bone marrow mononuclear cells were isolated by density gradient centrifugation with Histopaque^{TM} (Sigma, St. Louis, MO) and cultured *ex vivo* with recombinant GM-CSF (20 ng/mL, Peprotech, Rocky Hill, NJ) in a complete DMEM medium that contains 10% FBS (Invitrogen, Carlsbad, CA), antibiotics (penicillin, streptomycin, and gentamicin; Invitrogen) and L-glutamine. At the third day, the complete DMEM medium with GM-CSF was partially (50%) replaced. After 7 days, CD11c^{+} DCs were isolated from the cultures using MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). For all experiments, CD11c^{+} DCs were cultured in a RPMI 1640 medium supplemented with 10% FBS (Invitrogen, Carlsbad, CA), 50 μmol/L of β-mercaptoethanol, antibiotics (penicillin, streptomycin, and gentamicin) and L-glutamine in 24- or 96-well plates.

Cytotoxicity assay

For evaluation of the drugs' cytotoxicity, DCs (2×10^4 cells/well in a 96-well plate) were incubated with fluoxetine or desipramine at concentrations of 0.1, 1 or 10 μM for 24 hours in RPMI medium, and the colorimetric MTT assay was performed. The stock MTT (3[4,5-dimethyl-thiazol-2yl]-2,5-diphenyl-tetrazolium bromide; Sigma-Aldrich) dissolved in PBS at 5 mg/mL was added to all wells (MTT stock 20 μL/90 μL culture medium containing DCs), followed by incubation for 4 hours at 37 °C, to form formazan crystals. In order to dissolve the crystals, 0.04 N HCl in propanol solution was added (120 μL/well). The plates were read after 30 minutes at 570 nm. The percentage of viability was calculated based on the control cells (non-treated) as having 100% of viability.

Co-culture of DCs and T cells

The *Aggregatibacter actinomycetemcomitans* (*Aa*)–reactive T cells were developed using C57BL/6 mice following the method published by our group (Kawai et al., 2007). The mononuculear cells were isolated from cervical lymph nodes of mice that received immunization with formalin-fixed *Aa* Y4 (injected 10^9 bacteria/100 μL in saline, subcutaneously at dorsal skin on days 0, 2 and 4) followed by booster immunization of subcutaneous injection into cheek region (10^9 bacteria/ 100 μL in saline on day 10). Mononuclear cells were isolated by density gradient centrifugation with Histopaque™ 1083 (Sigma, St. Louis, MO), and the resulting cells were passed through a nylon- and glass-wool column to enrich T cells.

Aa-reactive T cells were then co-cultured in the complete RPMI medium along with DCs in the presence or absence of *Aa* antigen (10^7 fixed bacteria/mL /well). The CD11c-positive DCs used in the co-cultures were obtained as described above ("Development of $CD11c^+$ DCs *ex vivo*") and submitted to one of the following treatments: (1) Post-treatment with drugs: DCs (2×10^4 cells/well) were incubated with mitomycin C (MMC, 20 μg/mL, 1 hour, 37°C) and co-cultured with *Aa*-reactive T cells (4×10^5 cells/well) in the presence or absence of fluoxetine or desipramine (1 μM) for 3 days; (2) Pre-treatment with drugs: DCs were pretreated with fluoxetine or desipramine (1 μM) for 24 h before culturing with T cells. After the pre-treatment period of 24 h, the DCs were treated with MMC (1 hour, 37°C). After extensive washing, the DCs were co-cultured (2×10^4 cells/well) with *Aa*-reactive T cells (4×10^5 cells/well) for 3 days. As noted above, *Aa* (10^7 fixed bacteria/mL /well) was applied to these co-culture systems as T cell antigen.

After 3 days, the supernatants were collected for the measurement of serotonin (5-HT) or TNF-α and IL-10 cytokines production by T cells using serotonin EIA kit (Immuno Biological Laboratories, Inc., Minneapolis, MN) and ELISA (ELISA development kits; PeproTech, Rocky Hill, NJ), respectively.

Proliferation of T cells was assessed as described previously (Kajiya et al., 2009). Briefly, [^{3}H] thymidine (0.5 μCi) was added to each well during the last 16 hours of a total 4-day culture. Cells were harvested, and the incorporated radioactivity in the cells under proliferation (cpm) was measured by a scintillation counter.

Proliferation of T cells from non-immunized mice was also assessed for comparison with proliferation of *Aa*-reactive T cells.

Detection of serotonin transporter (SERT) mRNA on DCs by RT-PCR

For RT-PCR analyses, total RNA was extracted from DCs cultures stimulated or not with LPS (*Escherichia coli*, 1 μg/mL, Sigma-Aldrich, St. Louis, MO) for 6 hours as well as from mouse brain (positive control), using RNA-bee™ reagent following the manufacturer's protocol (Tel. Test, Inc., Friendswood, TX). RT-PCR was performed as previously described (Han et al., 2009). Isolated RNA (1μg) was reverse transcribed with SuperScript-II (Invitrogen, Carlsbad, CA) in the presence of random primers. The resulting cDNA was used as the template DNA for the subsequent PCR performed by the High Fidelity Expand System (Roche, Indianapolis, IN). Designs of primers for serotonin transporter (SERT) and beta-actin are as follows: SERT (forward, 5'-acaacatcacctggacactccattc-3' and reverse, 5'-ccgcatatgtgatgaaaaggaggct-3'), beta-actin (forward 5'-gacggggtcacccacactgt-3', and reverse, 5'-aggagcaatgatcttgatcttc-3'). PCR conditions were as follows: 35 cycles of 94°C for 30 s; 55°C (beta-actin) or 58°C (SERT) for 30 s (optimized for each set of primer); 72°C for 1 min. PCR products were separated in 1.5% agarose gels stained with SYBR Safe™.

Enzyme immuno-assay to detect cytokines and chemokines from DCs cultures

To detect the concentration of IL-12, IL-1β, TNF-α, IL-10, RANTES (regulated on activation, normal T cell expressed and secreted or CCL5) and MIP-1α (macrophage inflammatory protein 1α or CCL3), the supernatants of DCs cultures stimulated with or without LPS (1 μg/mL) and treated with drugs (1 μM) for 24 hours were subjected to ELISA (ELISA development kits; PeproTech, Rocky Hill, NJ).

Flow cytometry to evaluate expression profile of cell surface molecules on DCs

The effects of fluoxetine and desipramine on the expression profiles of MHC-class II ($I\text{-}A^b$), CD80, CD86, ICOS-L and PD-L1 on immature DCs were determined using flow cytometry. The *ex vivo*-developed immature DCs were incubated in the presence or

absence of fluoxetine or desipramine (1 μM) for 24 hours. After incubation, 5×10^5 cells re-suspended in PBS containing 1% BSA and 0.02% NaN_3 were incubated with fluorescein isothiocyanate-conjugated anti-mouse CD11c (FITC-CD11c, BD Pharmingen, San Diego, CA, USA) along with phycoerythrin (PE)-conjugated anti-mouse MHC-class II (PE-I-A^b), PE-conjugated anti-mouse CD80 (PE-CD80) or PE-conjugated anti-mouse CD86 (PE-CD86) MAbs (all MAbs were from BD Pharmingen), with each antibody concentration at 10 μg/mL. After 1 hour of incubation, cells were washed twice and fixed with 2.5% formalin in PBS. For the staining of ICOS-L and PD-L1, the DCs were reacted with rat anti-mouse ICOS-L and anti-mouse PD-L1 MAbs (ICOS-L-MAb and PD-L1-MAb; eBioscience, San Diego, CA), followed by PE-conjugated anti-rat IgG (PE-anti-rat IgG; BD Pharmingen). After removal of PE-anti-rat IgG by washing DCs, FITC-CD11c were reacted to the DCs. Control isotype-matched rat IgG, PE-rat IgG and FITC-rat-IgG antibodies were also used to determine nonspecific staining. The expression profile of each molecule on DCs was determined by flow cytometry.

Statistical analysis

Results were submitted to the Student's *t* test. Values of $P < 0.05$ were considered statistically significant.

Results

Effects of fluoxetine on the antigen-specific T cell proliferation induced by DCs

The effects of fluoxetine and desipramine on the *Aa*-reactive T cell (×*Aa*-T) proliferation induced by co-culture with bone marrow-derived DCs and *Aa*-antigen were examined (Figure 1). The lymph node T cells isolated from control non-immunized mice showed little or no proliferation response to the co-culture with DCs and *Aa*-antigen (Figure 1A). However, in the presence of *Aa*-antigen, co-cultured between DC and the lymph node T cells isolated from *Aa*-immunized mice showed significantly elevated proliferative response. On the other hand, the addition of fluoxetine and desipramine suppressed such ×*Aa*-T cell proliferation in a dose-dependent manner (Figure 1A). Moreover, in response to antigen-presentation by DCs, ×*Aa*-T cells produced TNF-α

(Figure 1B), but not IL-4 (data not shown), suggesting that ×*Aa*-T cells are Th1-type. Importantly, fluoxetine and desipramine suppressed the TNF-α production from ×*Aa*-T cells co-cultured with DC and *Aa*-antigen (Figure 1B).

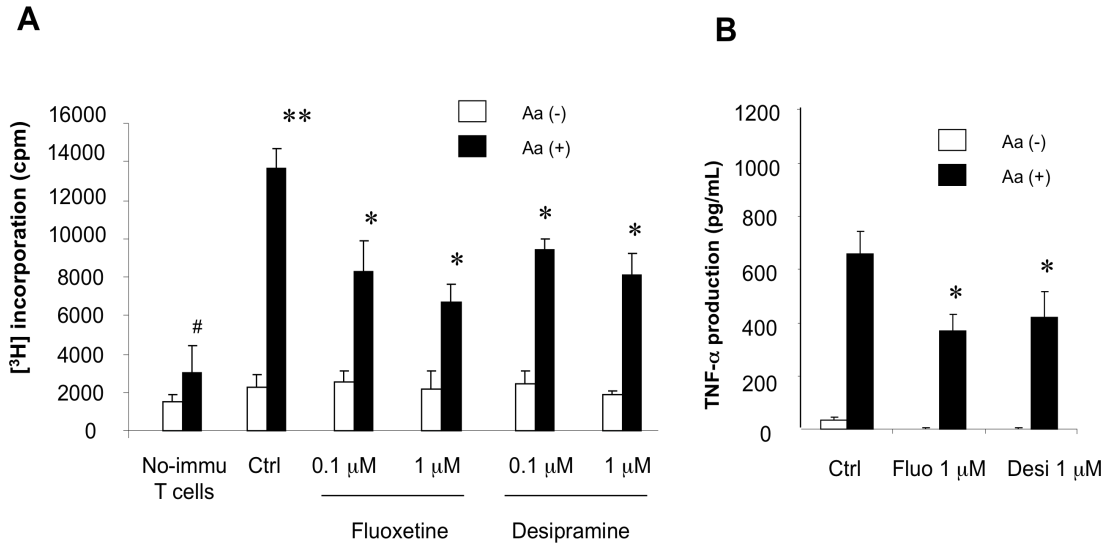

Figure 1. The effects of fluoxetine and desipramine on the bacteria (*Aa*)-antigen-reactive T cell (×*Aa*-T) responses induced by DCs.

(A and B) The effects of fluoxetine and desipramine on the *Aa*-antigen-specific ×*Aa*-T cell proliferation induced by co-culture with bone marrow derived DCs (treated with MMC) and *Aa*-antigen were examined. The lymph node T cells isolated from control non-immunized mice or *Aa*-immunized mice (4×10^5 cells/well, respectively) were co-cultured with DCs (2×10^4 cells/well, pretreated with MMC) in the presence or absence of *Aa*-antigen (10^7fixed bacteria/mL /well) for 4 days. Proliferation of T cells was determined by adding [^{3}H] thymidine (0.5 μCi) to each well during the last 16 hours of a total 4 day culture (A). Culture supernatants were collected 3 days after co-culture for the measurement of TNF-α production by ELISA (B). Results are expressed as the mean ± SD of incorporated [^{3}H] thymidine (cpm) or concentration of TNF-α (pg/mL). One representative result from three different experiments is shown. #, significantly higher than

non-immunized mice by Student *t* test ($P < 0.01$). *, significantly lower than control cultured with *Aa* by Student *t* test ($P < 0.01$).

In a separate assay, within the range of concentrations selected (0.1 – 10.0 μM), fluoxetine and desipramine did not affect the cell viability of DCs (Supplement figure 1), indicating that the diminished antigen-specific T cell proliferation mediated by fluoxetine and desipramine did not result from the cytotoxicity of these two drugs to DCs.

Pre-treatment of DCs with fluoxetine attenuated their ability to present antigen to T cells

When the DCs were pre-treated with fluoxetine or desipramine prior to the co-culture with ×*Aa*-T cells, antigen-specific T cell proliferation, as well as their TNF-α production, induced by such drug-pre-treated DCs were still significantly suppressed compared to the control DCs that were pre-incubated in the culture medium in the absence of drugs (Figure 2A and 2B). These results show that pre-treatment of DCs with either fluoxetine or desipramine can still diminish the antigen-specific T cell proliferation, suggesting that the change of immunological property of DCs caused by fluoxetine and desipramine resulted in the diminished ability of DCs to present bacterial antigen to T cells.

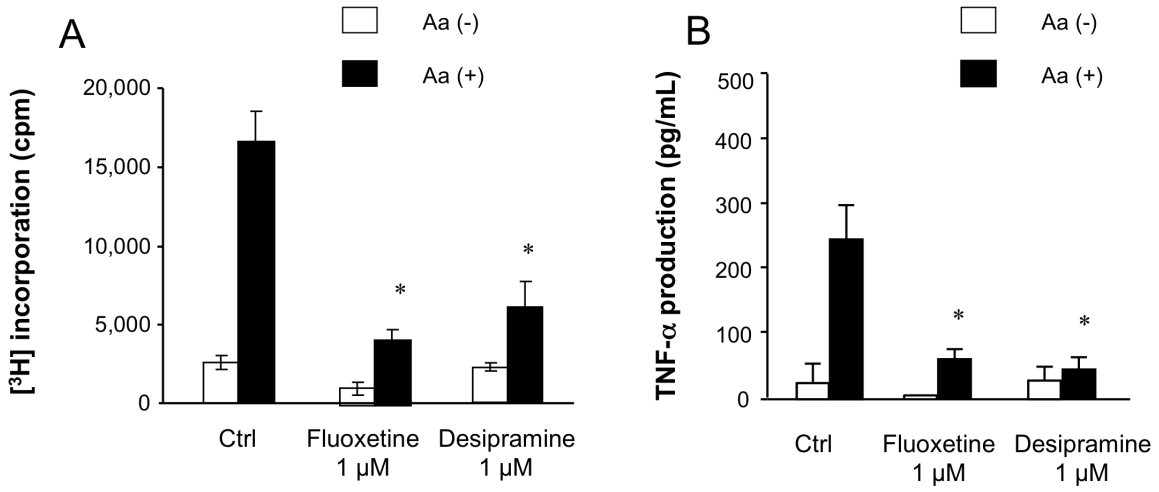

Figure 2. The effects of pre-treatment of DCs with fluoxetine or desipramine on DC's ability to present antigen.

(A and B) DCs were pre-treated with fluoxetine (1 μM) or desipramine (1 μM) for 24 hours. Subsequently, those DCs were further treated with MMC and co-cultured with ×*Aa*-T cells in the presence and absence of *Aa* antigen. Antigen-specific T cell proliferation (A) and their TNF-α production (B) were monitored following the protocol described in Figure 1. Results are expressed as the mean ± SD of incorporated [^{3}H] thymidine (cpm) or concentration of TNF-α (pg/mL). One representative result from three different experiments is shown. *, significantly lower than control (Ctrl) cultured with *Aa* by Student's *t* test ($P < 0.01$).

Lack of association of 5-HT in the fluoxetine-mediated suppression of antigen-specific T cell proliferation induced by DCs

A cutting-edge finding revealed that activated T cells can express 5-HT because of their expression of type 1 tryptophan hydroxylase (TPH-1), an enzyme involved in the synthesis of 5-HT (O'Connell et al., 2006). 5-HT released from T cells (which lack SERT) is up-taken by SERT expressed in DCs and such uptake of T cell-derived 5-HT by DCs is inhibited by fluoxetine (O'Connell et al., 2006). Subsequently, the 5-HT released back from DCs reduces the expression of cAMP in naïve T cells, suggesting that DCs can shuttle 5-HT produced from activated T cells to naïve T cells and thereby modulate proliferation of naïve T cells (O'Connell et al., 2006). According to these lines of published evidence, it was conceivable that SERT/5-HT system may be associated with fluoxetine-mediated suppression of proliferation of *Aa*-reactive T cells induced by antigen-presentation from DCs. To address possible involvement of SERT/5-HT system in fluoxetine-mediated suppression, following experiments were conducted.

The co-culture between ×*Aa*-T cells and DCs in the presence of *Aa* increased the level of extracellular 5-HT compared to the co-culture in the absence of *Aa*, suggesting that antigen-presentation from DCs induced 5-HT production by ×*Aa*-T cells (Figure 3A). Fluoxetine increased 5-HT production from co-culture with or without *Aa*-antigen, while the presence of *Aa*-antigen showed higher 5-HT production than no-*Aa* control. The

addition of desipramine also enhanced 5-HT production from the co-culture, while the presence or absence of *Aa* did not alter the level of 5-HT (Figure 3A).

The expression of SERT mRNA was confirmed in LPS-stimulated DCs, but not control non-stimulated DCs (Figure 3B), corresponding to the previous report (O'Connell et al., 2006). Very importantly, exogenously applied synthetic 5-HT to the ×*Aa*-T/DCs co-culture showed up-regulation of ×*Aa*-T cell proliferation induced by DCs (Figure 3C), indicating that 5-HT provided a co-stimulatory, instead of a suppressive, signal to the ×*Aa*-T cells.

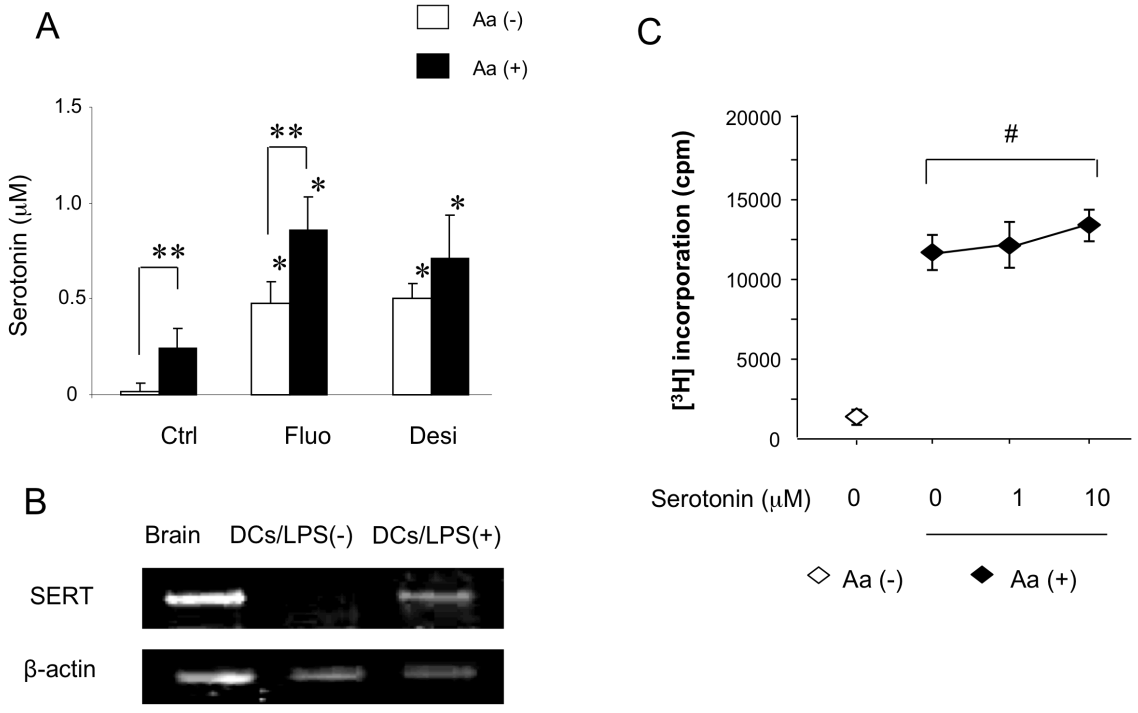

Figure 3. Expressions of serotonin (5-HT) in ×*Aa*-T/DCs co-cultures and SERT in DCs as well as the effects of synthetic 5-HT on the proliferative response of ×*Aa*-T cells.

(A) ×*Aa*-T cells (4 x 10^5 cells/well) were co-cultured with DCs (2 x 10^4 cells/well, pre-treated with MMC) in the presence and absence of *Aa* (10^7 fixed bacteria/mL /well). In addition, fluoxetine or desipramine (1 μM) was applied to the co-culture. After 3 days, the supernatants were collected for the measurement of 5-HT. *, higher than control co-culture

with *Aa* (Student's *t* test, $P < 0.05$), #, there is significant difference between the culture with and without *Aa* (Student's *t* test, $P < 0.05$). (B) mRNA for SERT expressed in DCs was monitored using RT-PCR. Total RNA isolated from DCs incubated with or without LPS (1 μg/mL) for 24 hours was subjected to RT-PCR using PCR primer set specific to SERT or beta-actin. As a positive control, brain tissue isolated from normal C57BL/6 mice was used. (C) Effects of synthetic 5-HT on the proliferative response of ×*Aa*-T cells in the co-culture with DC were evaluated. ×*Aa*-T cells (4×10^5 cells/well) were co-cultured with DCs (2×10^4 cells/well, pre-treated with MMC) in the presence and absence of *Aa* (10^7 fixed bacteria/mL /well). Synthetic 5-HT (1 and 10 μM) was applied to the co-culture. [^{3}H] thymidine (0.5 μCi) was applied to each well during the last 16 hours of a total 4-day culture. #, there is a significant difference between the culture with 5-HT (10 μM) and without 5-HT (Student's *t* test, $P < 0.05$)

These results suggested, in turn, that while fluoxetine can increase extracellular 5-HT in the co-culture between T cells and DCs, such increased 5-HT was not associated with fluoxetine-mediated suppression of DCs' ability to present bacterial antigens to T cells and resulting T cell proliferation, because it was shown that elevated 5-HT can up-regulate the proliferation of T cells, rather than suppressing it.

Effects of fluoxetine on IL-10 production from antigen-specific T cells proliferation induced by DCs

It was conceivable that fluoxetine or desipramine may act on T cells with regulatory property to induce their expression of IL-10, which, in turn, down regulated the antigen-specific T cell proliferation induced by DCs. However, the addition of fluoxetine into the co-culture between ×*Aa*-T cells and DCs did not alter the production of IL-10 in the culture (Figure 4). In fact, desipramine suppressed the production of IL-10 in this co-culture system (Figure 4). These results show that neither fluoxetine nor desipramine increased IL-10 production of ×*Aa*-T cells (Figure 4), suggesting that IL-10 produced by T cells may not be associated with the drug-mediated suppression of antigen-specific T cell proliferation induced by DCs.

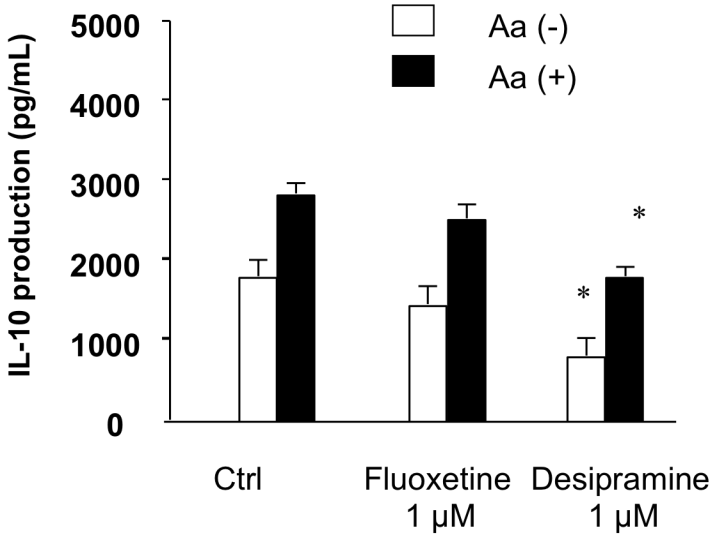

Figure 4. Effects of fluoxetine and desipramine on IL-10 production from antigen-specific T cell proliferation induced by DCs.

×*Aa*-T cells (4×10^5 cells/well) were co-cultured with DCs (2×10^4 cells/well, pre-treated with MMC) in the presence or absence of *Aa* (10^7 fixed bacteria/mL /well). In addition, fluoxetine or desipramine (1 μM) was applied to the co-culture. After 3 days, the supernatants were collected from the co-culture for the measurement of IL-10 using ELISA. Results are expressed as the mean ± SD of IL-10 (pg/mL) of triplicate cultures. *, significantly lower than control without drug by Student's *t* test ($P < 0.01$).

Effects of fluoxetine on the production of cytokine and chemokine by DCs

Fluoxetine and desipramine were reported to down-regulate the proliferation of human blood T cells stimulated with a non-specific T cell mitogen, Con A, and elevated trend of IL-10 produced in the blood cell culture was implicated for the mechanism of antidepressant-mediated suppression of DC/T cell responses (Diamond et al., 2006). Furthermore, IL-10 produced from immune regulatory DCs, such as tolerogenic DC, is considered to play a role in the suppression of antigen-specific T cell activation (Rutella et al., 2006). Therefore, effects of fluoxetine and desipramine on expression of immune-suppressive cytokine, IL-10, along with other proinflammatory cytokines from LPS-

stimulated DCs were monitored. In particular, LPS was used to stimulate DCs, because *Aa* is a Gram (-) pathogen which produces LPS and we reported that LPS can elicit *in vivo* antigen presentation to ×*Aa*-T cells (Kawai et al., 2000). The non-stimulated DCs showed modest basal expression of IL-1β, IL-12, MIP-1α and RANTES, whereas TNF-α and IL-10 expression were lower than the detection limit of the ELISA system. Both fluoxetine and desipramine suppressed basal level expression of IL-12 and RANTES, but not IL-1β or MIP-1α. Furthermore, while LPS stimulation markedly increased the production of TNF-α, IL-1β, IL-12, RANTES and MIP-1α in the DCs, addition of either fluoxetine or desipramine to the cultures resulted in significant suppression (Figure 5). The production of immune suppressive cytokine IL-10 induced by LPS was only suppressed by desipramine, but not by fluoxetine (Figure 5). These results showed that both fluoxetine and desipramine can suppress the LPS-induced production of anti- and proinflammatory cytokines as well as chemokines from DCs. In terms of the question addressed in this assay, fluoxetine and desipramine did not increase IL-10 expression from DCs irrespective of LPS-stimulation, indicating that IL-10 produced from DCs may not be associated with the fluoxetine- or desipramine-mediated suppression of proliferation of antigen-specific T cells induced by DCs.

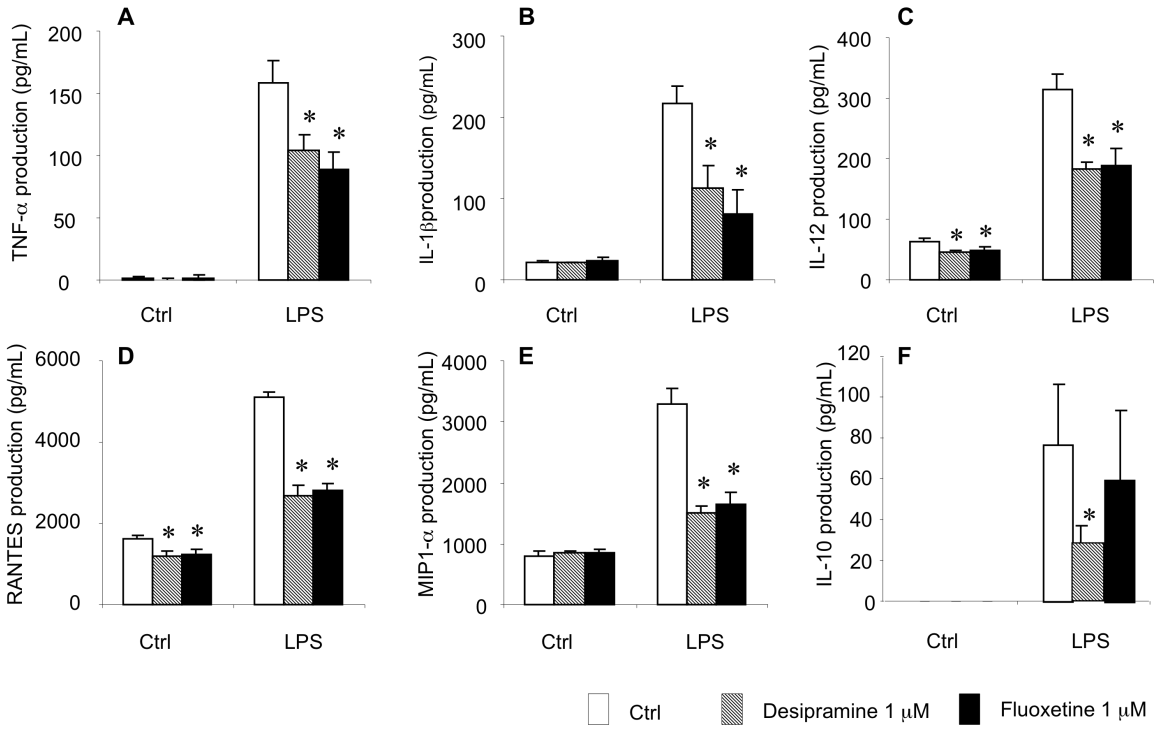

Figure 5. Effects of fluoxetine and desipramine on the production of cytokines and chemokines by LPS-stimulated DCs.

DCs were stimulated with or without LPS (1 μg/mL) in the presence or absence of fluoxetine or desipramine (1 μM) for 24 hours. The concentrations of TNF-α, IL-1β, IL-12, RANTES, MIP-1α and IL-10 in the culture supernatant were measured using ELISA. Results are expressed as the mean ± SD of cytokines/chemokines concentrations (pg/mL) of triplicate cultures. One representative result from three different experiments is shown. *, significantly lower than no drug treatment by Student's *t* test ($P < 0.01$).

Expression profile of DCs surface molecules after exposure to fluoxetine

The effects of fluoxetine and desipramine on the cell surface molecules that are considered to be involved in the antigen presentation by $CD11c^+$ DCs to T cells were monitored using flow cytometry (Figure 6). In the control non-stimulated DCs, the majority (more than 85%) of $CD11c^+$ cells were positive for CD80, MHC-class-II, ICOS-L and PD-L1, whereas about 30% of $CD11c^+$ cells expressed CD86, indicating that these $CD11c^+$

DCs induced in *ex vivo* culture display characteristics of immature DCs. The prevalence of CD86-positive cells in the $CD11c^+$ DCs was increased by addition of fluoxetine or desipramine. While both drugs appeared to possess marginal effects on the expression of MHC-class-II, PD-L1, and CD80, which showed slight increase, noticeable suppression of ICOS-L was induced by fluoxetine and desipramine (Figure 6).

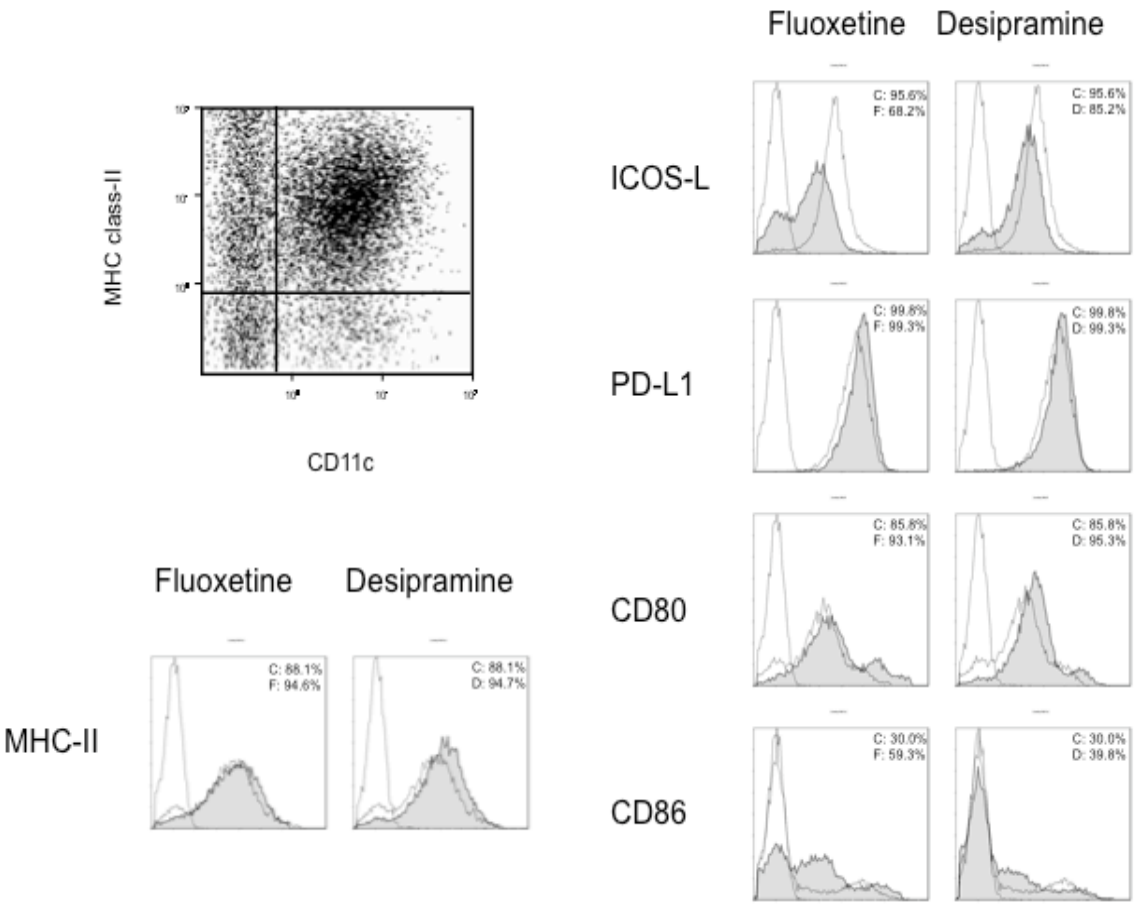

Figure 6. Influence of fluoxetine and desipramine on co-stimulatory molecules expressed on DCs.

DCs were developed from bone marrow cells by incubation *ex vivo* in the presence of GM-CSF for 7 days. The developed DCs were treated with or without fluoxetine or desipramine for 24 hours. The MHC-class-II and co-stimulatory molecules expression pattern on $CD11c^+$ DCs were monitored using flow cytometry. Without separation of $CD11c^+$ cells

using MACS beads, whole bone marrow cell culture containing developed DCs was subjected to flow cytometry. $CD11c^+$ cells were labeled by PE, while remaining cell markers, MHC-class-II, PD-L1, CD80, CD86, and ICOS-L, were labeled with FITC. # shows the non-stained control bone marrow cells. The open histogram indicates the FITC staining of PE positive ($CD11c^+$) DCs incubated in medium alone. The solid histograms display the FITC staining of PE positive ($CD11c^+$) DCs stimulated with fluoxetine (left column) or desipramine (right column), respectively. C: no drug treated control group. F: fluoxetine-treated group. D: desipramine-treated group.

Co-stimulatory signaling provided by ICOS-L was originally found to profoundly activate Th2-type T cells (Cutler and Jotwani, 2004; Taubman et al., 2005; Cutler and Teng, 2007). Since ×*Aa*-T cells are Th1-type T cells (Figure 1), it was questioned if ICOS-L expressed on DCs provides the co-stimulatory signals to ×*Aa*-T cells. To test such possibility, ×*Aa*-T cells were co-cultured with DCs in the presence or absence of *Aa*-antigen with or without anti-ICOS-L MAb or anti-CD80/CD86 MAbs. Anti-ICOS-L MAb suppressed antigen-specific T cell growth, albeit to a lesser extent (Student *t* test, $P < 0.05$) when compared to anti-CD80/CD86 MAbs that suppressed antigen-specific T cell growth remarkably (Student *t* test, $P < 0.01$) (Figure 7A). TNF-α production from ×*Aa*-T cells that were co-cultured with DCs and *Aa* antigen was diminished by anti-CD80/CD86 MAbs, but not by anti-ICOS-L MAb (Figure 7B). These results indicated that down-regulation of ICOS-L expressed on DCs by the anti-depressant drugs might be associated with the drugs' effects to suppress antigen-specific T cell proliferation, but not the production of TNF-α.

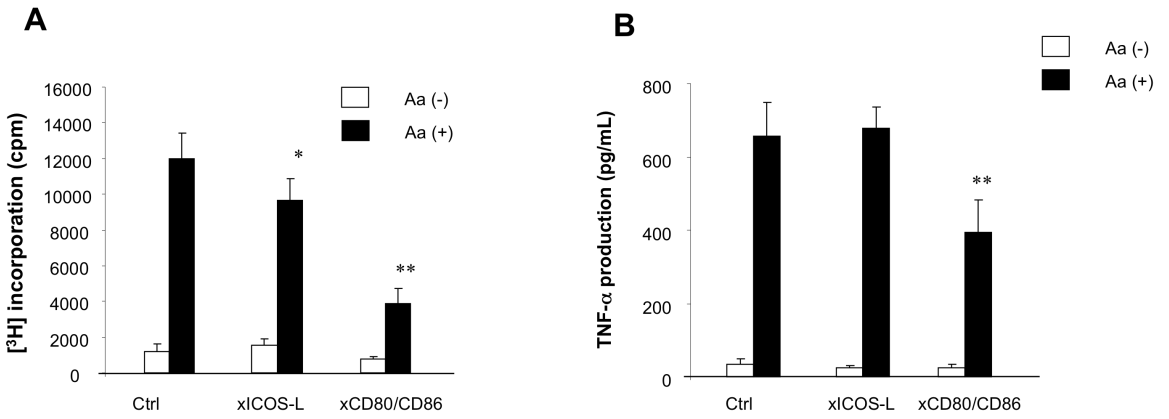

Figure 7. Engagement of ICOS-L co-stimulatory molecule in the ×*Aa*-T cell responses induced by antigen presentation from DCs.

×*Aa*-T cells (4×10^5 cells/well) were co-cultured with DCs (2×10^4 cells/well, pre-treated with MMC) in the presence or absence of *Aa* (10^7 fixed bacteria/mL /well). In addition, anti-mouse MHC-class II A^b MAb (10 μg/mL), anti-ICOS-L MAb (10 μg/mL), a mixture of anti-CD80 MAb and anti-CD86 MAb (10 μg/mL, respectively), or control rat IgG (10 μg/mL) was applied to the co-culture. Antigen-specific T cell proliferation (A) and their TNF-α production (B) were monitored following the protocol described in Figure 1. *, **, significantly lower than control cultured with *Aa* by Student's *t* test ($P < 0.05$, $P < 0.01$, respectively).

Discussion

The present study demonstrated that fluoxetine suppresses the ability of DCs to present bacterial (*Aa*) antigens to *Aa*-reactive T cells (×*Aa*-T cells) in a SERT/5-HT-independent manner, as monitored by T cell proliferation and their production of TNF-α, although activated T cells and DCs expressed 5-HT and SERT, respectively. SERT/5-HT independency is important, because of following evidence supported that fluoxetine mediated SERT/5-HT system is vitally affecting the interaction between ×*Aa*-T cells and DCs: 1) ligation of 5-HT receptors expressed on T cells is reported to activate T cells (Aune et al., 1993; Leon-Ponte et al., 2007), 2) activated DCs express SERT (Figure 3B) (O'Connell et al., 2006), and 3) fluoxetine increased 5-HT in the co-culture between ×*Aa*-T

cells and DC (Figure 3A), and 4) it is based on the finding that synthetic 5-HT applied to the co-culture of ×*Aa*-T cells and DCs increased the proliferation and TNF-α production from ×*Aa*-T cells (Figure 3C), indicating that extracellular 5-HT in the ×*Aa*-T/DCs co-culture can up-regulate T cell proliferation. However, fluoxetine applied to ×*Aa*-T/DCs co-culture suppressed T cell proliferation and their production of TNF-α by overwhelming the co-stimulatory effects of extracellular 5-HT on T cell response to antigen-presentation by DCs. Similar to fluoxetine, desipramine, one of the NRI drugs, also demonstrated suppressive effects on the antigen-presentation function by DCs, supporting a still undefined common nature among antidepressant drugs in the role they play to suppress the ability of DC to present bacterial antigens in a SERT-independent manner. Immune suppressive cytokine IL-10 appeared not to be associated with fluoxetine- or desipramine-mediated suppression of proliferation of bacteria-reactive T cells induced by antigen-presentation by DCs because neither drug increased the level of IL-10 produced in the ×*Aa*-T/DC co-culture. Diminished expression of co-stimulatory molecule ICOS-L on DCs caused by fluoxetine, as well as desipramine, appeared to be partially associated with their suppression of antigen-presenting function by DCs.

Initially, the ICOS co-stimulatory molecule was implicated to play a role in Th2-prone T cell activation based on the study using an ICOS-knockout mice (Dong et al., 2001; McAdam et al., 2001; Tafuri et al., 2001). However, subsequent studies demonstrated that ICOS co-stimulation is, indeed, required for both Th1 and Th2 responses (Ozkaynak et al., 2001; Rottman et al., 2001; Smith et al., 2003; Smith et al., 2006). More recent studies indicated a role of ICOS in supporting memory and effector T cell responses (Shiao et al., 2005; Mahajan et al., 2007). Therefore, it is plausible that the diminished expression of ICOS-L on the fluoxetine- or desipramine-treated DCs decreases its ability to present antigen to ×*Aa*-reactive effector Th1 T cells. However, the level of suppression of antigen-specific T cell growth mediated by anti-ICOS-L MAb was marginal and not as strong as the suppression caused by addition of fluoxetine to ×*Aa*-T/DC co-culture. Therefore, it is speculated that fluoxetine or desipramine would affect other co-stimulatory molecules or molecules in DC whose engagement is required for antigen presentation to ×*Aa*-reactive effector Th1 T cells.

While this study demonstrated that fluoxetine and desipramine act on DCs to suppress their ability to present antigen, it is still conceivable that these drugs also affect T cells directly and suppress their response to antigen-presentation by DCs through TCR/MHC-class-II engagement. Indeed, fluoxetine and desipramine significantly inhibited the proliferation of T cells as well as their production of TNF-α in response to TCR/CD28 stimulation (Supplement figure 2). Furthermore, the addition of 5-HT (10 μM) to the TCR/CD28-stimulated T cells did up-regulate T cell proliferation in response to stimulation with anti-TCR/CD28 MAbs (Supplement figure 3), suggesting that 5-HT can provide additional co-stimulation to the T cell activation induced by TCR/CD28 engagement, but that 5-HT is not responsible for the drug-mediated suppression of TCR/CD28-activated T cells. These results indicated that fluoxetine and desipramine appear to down-regulate not only the antigen presenting function of DCs but also suppress the TCR/CD28 elicited T cell activation process by overwhelming the T cell co-stimulatory effects of extracellular 5-HT produced and accumulated by drug-mediated inhibition of 5-HT from SERT.

Fluoxetine displayed inhibitory effects on the production of TNF-α, IL-1β and IL-12 by immature DCs that were stimulated with LPS (Figure 5). Similar suppressive effects were also detected when desipramine was applied to the LPS-stimulated immature DCs (Figure 5). Since ×*Aa*-T cells were Th1 type and since overactivation of Th1-type T cells is associated with RANKL-mediated periodontal bone loss (Taubman et al., 2005), drug-mediated suppression of IL-12, which functions to promote the development and activation of the Th1 type T cells, is considered to contribute to the down-regulation of pathogenic Th1 type responses. These results of fluoxetine- and desipramine-mediated suppression of proinflammatory cytokine production by DCs were in accordance with data from previous studies evaluating the influence of NRIs or SSRIs on immune cells (Xia et al., 1996; Maes et al., 1999; Kubera et al., 2001; Roumestan *et al.*, 2007; Guemei et al., 2008). It was also reported that these two drugs can down-regulate the production of pro-inflammatory cytokines from LPS-stimulated monocytes (Roumestan et al., 2007). However, effects of 5-HT on cytokine production by DCs are rather complex. For example, 5-HT decreased TNF-α and IL-12, but promoted IL-1β production, in human DCs (Idzko et al., 2004), which cannot account for the fluoxetine- and desipramine-mediated suppression of all three

proinflammatory cytokines, i.e., TNF-α, IL-1β, and IL-12, produced by DCs (Figure 5). Indeed, some studies have suggested that there is another possible mechanism inducing fluoxetine suppression of immune responses in a 5-HT-independent manner (Diamond et al., 2006; Frick et al., 2008). We support the latter theory, i.e., that fluoxetine suppresses proinflammatory cytokine production from activated DCs in a 5-HT-independent fashion because the addition of exogenous 5-HT to LPS-stimulated DCs did not alter their expression pattern of TNF-α and IL-1β (data not shown).

The present study also analyzed the effects of fluoxetine and desipramine on the production of chemokines. Fluoxetine and desipramine reduced the RANTES and MIP-1α production by LPS-stimulated DCs. RANTES and MIP-1α are produced by variety of immune cells including DCs (Lore et al., 1998), and these two chemokines in concert with other chemotactic factors control chemotaxis of T cells and other leukocytes (Miller and Krangel, 1992; Dieu-Nosjean *et al.*, 1999). Furthermore, chemotaxis of lymphocytes induced by chemokines appears to be engaged in the antigen presentation from DCs to T cells (Caux et al., 2000; Vicari et al., 2004). Therefore, diminished expression of RANTES and MIP-1α from activated DCs in response to fluoxetine and desipramine may be also associated with the drug-mediated suppression of antigen-presentation from DCs to T cells.

Based on evidence that 1) antigen-presentation is the key rate-limiting step in the generation of an immune/inflammatory response (Yoshimura et al., 2001) and 2) over-activation of bacteria-reactive Th1-type T cells possibly results in host periodontal tissue destruction (Kawai et al., 2007), regulation of antigen-presenting by DCs, which are the most robust antigen-presenting cells, might be a valid strategy for the down-regulation of tissue destruction caused by immune-associated periodontal disease (Cutler and Jotwani, 2004). The present study demonstrated that fluoxetine and desipramine can be potent drugs in down-regulating the activation of bacteria-reactive T cells by suppressing the antigen-presenting function of DCs. Our preliminary study also showed that systemic administration of fluoxetine inhibited the development of periodontal bone resorption along with the suppression of immune response elicited to oral bacteria (*Pasteurella pneumotropica*) induced in a mouse model of periodontal disease (data not shown) following the previously published protocol (Kawai et al., 2007).

In conclusion, the present study demonstrated that fluoxetine and desipramine suppressed the ability of DCs to present bacterial antigens to T cells and down-regulated the resulting T cell proliferationin a SERT/5-HT-independent manner and that diminished expression of ICOS-L on DCs caused by these two drugs appeared to be partially associated with their suppression of antigen-presenting function by DCs. These findings clearly suggest an interesting potential of such drugs for modulation of the T cell immune responses to bacterial infection in the context of periodontal disease and other conditions, e.g., colitis, where overreaction of T cells to bacteria is thought to be related to host tissue damage.

Acknowledgments

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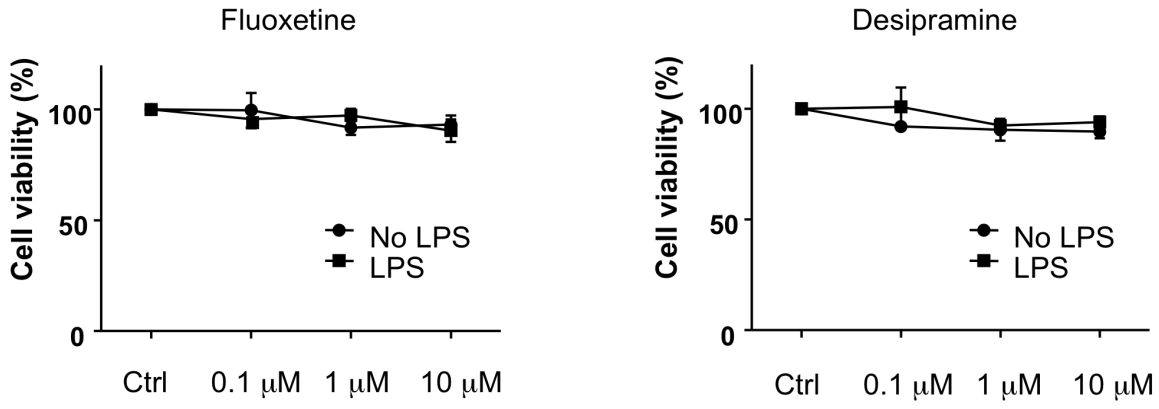
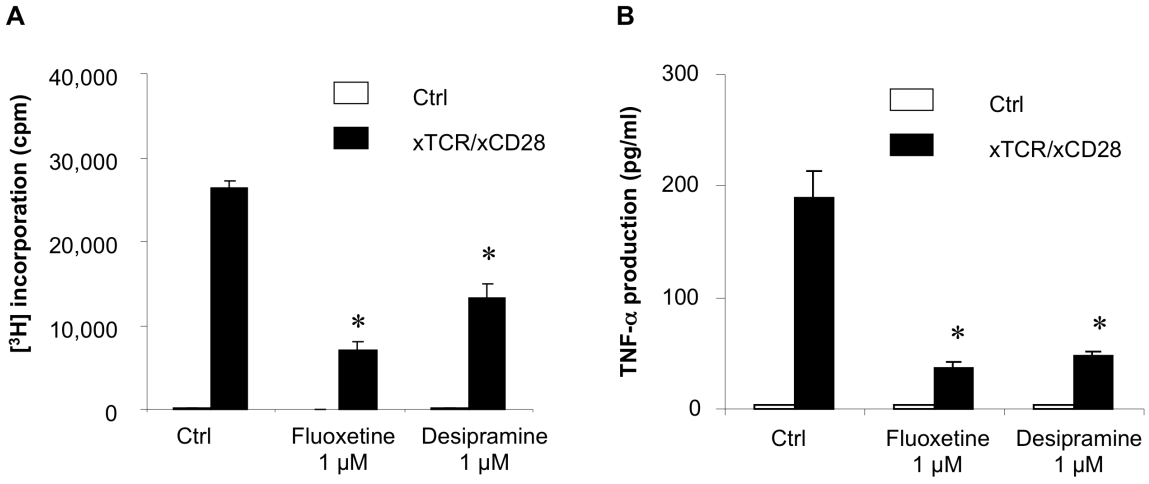
SUPPLEMENT FIGURES



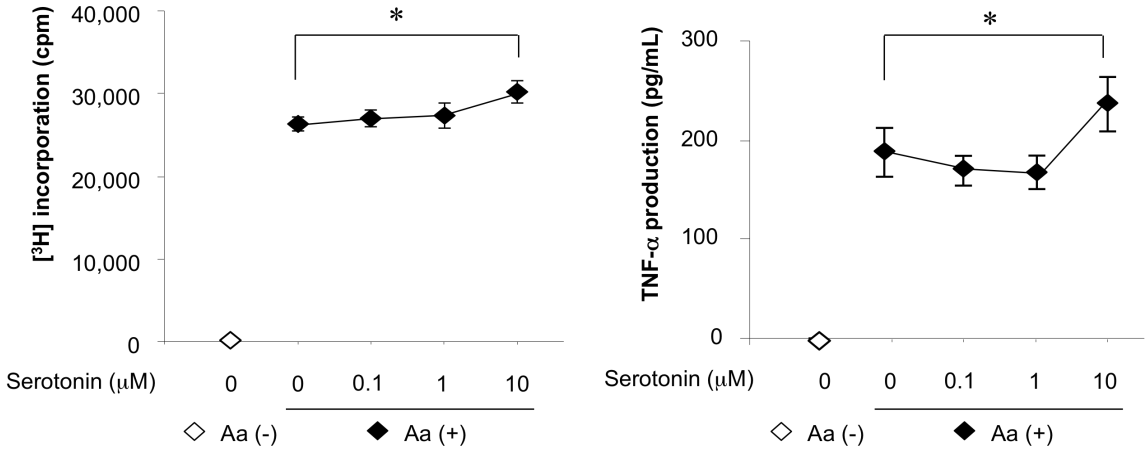
Supplement figure 1. Effects of fluoxetine and desipramine treatments on dendritic cells (DCs) viability.

DCs (2×10^4 cells/well in a 96-well plate) were incubated with fluoxetine or desipramine at concentrations of 0.1, 1 or 10 μM for 24 hours in RPMI medium, and the colorimetric MTT assay was performed. The percentage of viability was calculated based on the control cells (non-treated) as having 100% of viability.



Supplement figure 2. Effects of fluoxetine and desipramine on TCR/CD28 elicited T cell activation.

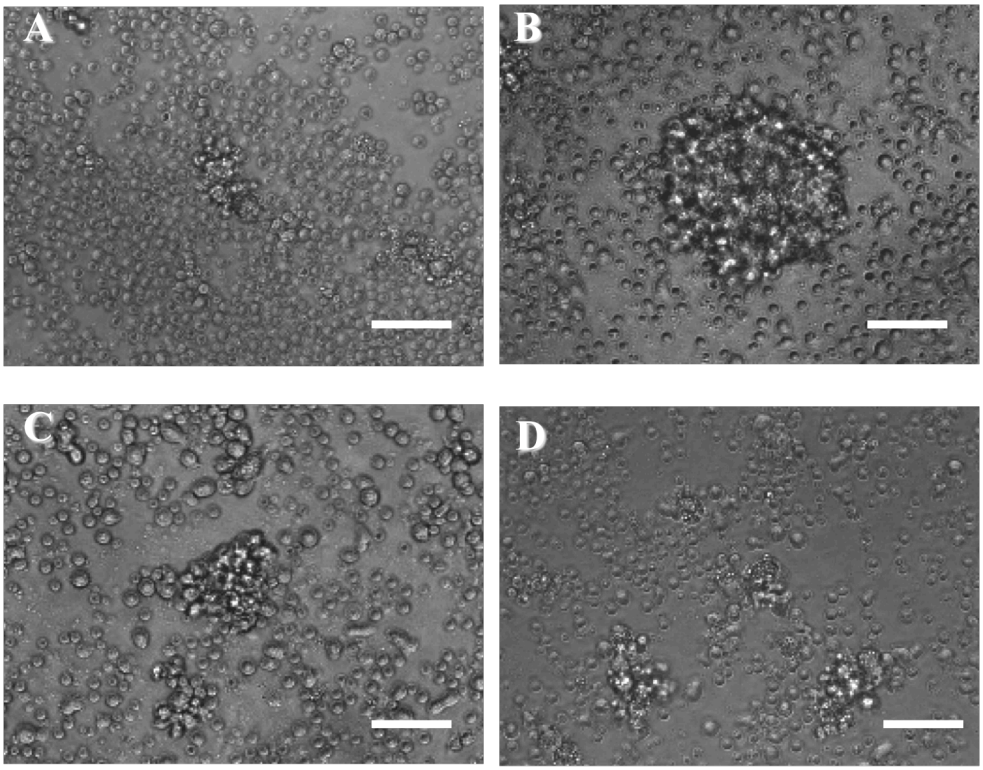
×*Aa*-T cells (4 × 10^5 cells/well) were stimulated with anti-TCR/CD28 MAbs and treated with fluoxetine or desipramine (1 μM) for 24 hours. Proliferation of T cells was determined by adding [^{3}H] thymidine (0.5 μCi) to each well during the last 16 hours of a total 4 day culture. Culture supernatants were collected after 3 days for the measurement of TNF-α production by ELISA. Fluoxetine and desipramine significantly inhibited the proliferation of T cells (A) as well as their production of TNF-α (B) in response to TCR/CD28 stimulation. Results are expressed as the mean ± SD of incorporated [^{3}H] thymidine (cpm) or concentration of TNF-α (pg/mL). One representative result from three different experiments is shown. *, significantly lower than control by Student *t* test ($P < 0.01$).



Supplement figure 3. Effects of serotonin (5-HT) on TCR/CD28 elicited T cell activation.

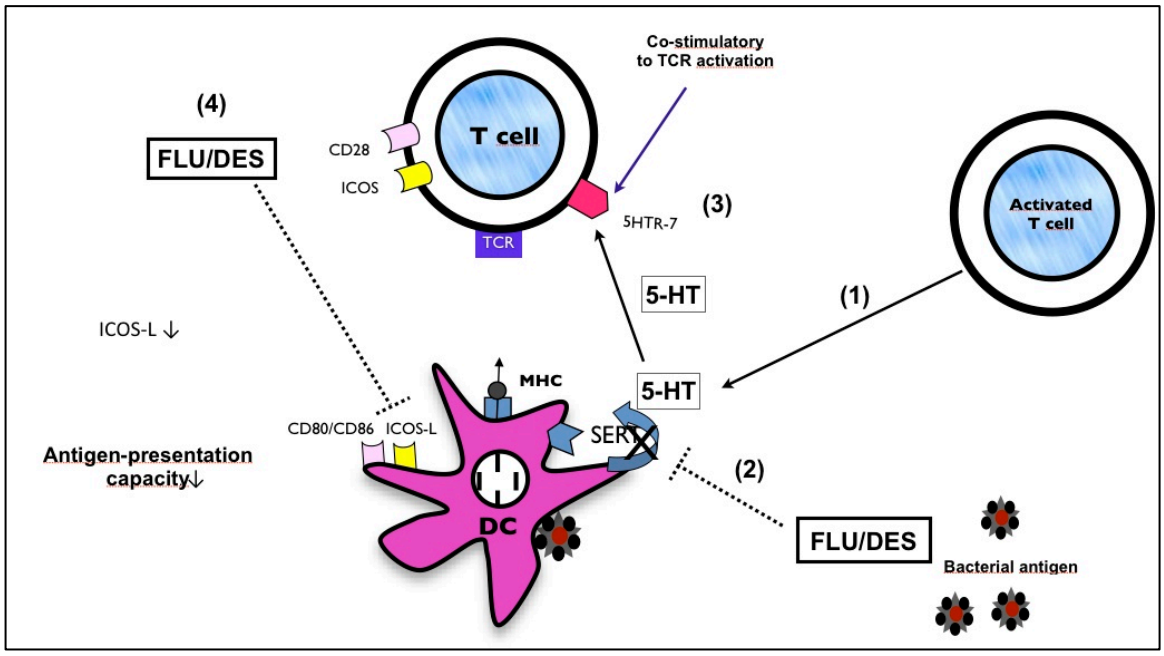
×*Aa*-T cells (4 × 10^5 cells/well) were stimulated with anti-TCR/CD28 MAbs and treated with serotonin (0.1, 1 and 10 μM) for 24 hours. Proliferation of T cells was determined by adding [^{3}H] thymidine (0.5 μCi) to each well during the last 16 hours of a total 4 day culture. Culture supernatants were collected after 3 days for the measurement of TNF-α production by ELISA. The addition of 5-HT to the TCR/CD28-stimulated T cells did up-regulate T cell proliferation and TNF-α production (10 μM) in response to stimulation with anti-TCR/CD28 MAbs, suggesting that 5-HT can provide additional co-stimulation to the T

cell activation induced by TCR/CD28 engagement, but that 5-HT is not responsible for the drug-mediated suppression of TCR/CD28-activated T cells. One representative result from three different experiments is shown. *, significantly different from control by Student *t* test ($P < 0.05$).



Supplement figure 4. Cell-cell aggregation of co-cultures of *Aa*-reactive T cells (×*Aa*-T cells) and dendritic cells (DCs) during immunological synapse.

(A) ×*Aa*-T cells + DCs in the absence of *Aa*; (B) ×*Aa*-T cells + non-treated control DCs in the presence of *Aa* without any drug treatments; (C) ×*Aa*-T cells + fluoxetine-treated (1 μM, 24 hours) DCs in the presence of *Aa*; (D) ×*Aa*-T cells+ desipramine-treated (1 μM, 24 hours) DCs stimulated with *Aa*. Bar = 30 μm.



Supplement figure 5. Fluoxetine (FLU) and desipramine (DES) supression mechanism schema.

2.2. CAPÍTULO 2

Fluoxetine inhibits inflammatory response and bone loss in a rat model of ligature-induced periodontitis

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Abstract

Fluoxetine, a selective serotonin reuptake inhibitor, has recently been found to possess anti-inflammatory properties. The present study investigated the effects of fluoxetine on inflammatory tissue destruction in a rat model of ligature-induced periodontitis (PD). Male Wistar rats were randomly assigned into three groups (n=10/group): 1) Control rats (without ligature); 2) rats with ligature + placebo (saline; oral gavage); 3) rats with ligature + fluoxetine (20 mg/kg/day in saline; oral gavage). Histological analyses were performed on the furcation region and mesial of mandibular first molars of rats sacrificed at 15 days after ligature-induced PD. Compared to the ligature + placebo group, alveolar bone loss was reduced in the fluoxetine group ($P < 0.05$), and the integrity of collagen fibers in gingival tissue was maintained. Moreover, in gingival tissue sampled 3 days after ligature attachment, fluoxetine administration reduced IL-1β and COX-2 mRNA expression. Fluoxetine down-regulated MMP-9 activity, without affecting MMP-9 mRNA expression induced by ligature, compared to the ligature + placebo group ($P < 0.05$). These data suggested that fluoxetine suppressed proinflammatory responses, as well as proteolytic enzyme activity induced by ligature. Therefore, fluoxetine both suppresses inflammatory response and protects against periodontal bone resorption and destruction of collagen fibers, thus constituting a novel and promising therapeutic approach for periodontal disease.

Key words: Fluoxetine, inflammation, periodontal disease, bone resorption, collagen fibers, host modulation therapy

Introduction

Although the etiology of periodontal disease (PD) is related to putatively virulent microorganisms in the biofilm, it has also been demonstrated that significant tissue destruction derives from immune/inflammatory responses of a PD-susceptible host to microbial challenge (Kornman *et al*., 1997; Han *et al*., 2007). The persistent presence of these bacteria in the gingival crevice disrupts homeostatic balance between periodontal bacteria and host cells, which results in synthesis and release of proinflammatory cytokines,

proteolytic enzymes and chemical inflammatory mediators by a variety of resident and infiltrating host cells in the periodontium (Kornman *et al*., 1997; Lappin *et al*., 2000).

A better understanding of these host mechanisms have led to the search for new therapeutic agents aimed at modulation of host response to bacteria through the inhibition of immune/inflammatory mediators. Some pharmacological agents, such as inhibitors of cyclooxygenase (COX)-2, matrix metalloproteinases (MMPs) and nitric oxide (NO), have been reported to reduce inflammatory parameters and bone resorption *in vivo* (Golub *et al*., 1998; Holzhausen *et al*., 2002; Reddy *et al*., 2003; Leitão *et al*., 2005). Therefore, these pharmacological agents could offer a valuable adjunct strategy to periodontal treatment when the use of conventional therapies alone fails to control the progression of PD.

Fluoxetine is a selective serotonin reuptake inhibitor used as an antidepressant drug (Calil, 2001). However, recent studies have shown additional analgesic, anti-inflammatory and immunomodulatory effects by fluoxetine (Yaron *et al*., 1999; Abdel-Salam *et al*., 2003; Roumestan *et al*., 2007). Especially, anti-inflammatory activity of fluoxetine has been presented in several reports. For example, studies using animal models have shown that fluoxetine can decrease proinflammatory cytokine levels in inflammatory diseases, including rheumatoid arthritis (Roumestan *et al*., 2007; Guemei *et al*., 2008; Sacre *et al*., 2010). Fluoxetine was also found to reduce prostaglandin-E2 (PGE_2) levels in subcutaneous exudates and paw edema in carrageenan-induced inflammation (Bianchi *et al*., 1995; Abdel-Salam *et al*., 2003). Moreover, fluoxetine can reduce both PGE_2 and NO production by human synovial cells (Yaron *et al*., 1999).

Although fluoxetine can reduce the production of important inflammatory mediators, no studies have thus far evaluated its impact on PD. Given its anti-inflammatory effects, safety and high medical prescription rate in clinical practice, we asked if fluoxetine might affect the development of PD. To test this hypothesis, we assessed the effects of systemically administered fluoxetine on inflammatory host responses, bone resorption and collagen destruction using a rat model of ligature-induced periodontitis.

Material and Methods

Animals

Male Wistar rats (60-day-old) obtained from CEMIB (University of Campinas, SP, Brazil) were housed in plastic cages with food and water given *ad libitum* and maintained in a specific pathogen-free facility at the Piracicaba Dental School. The protocol used for this rat experiment was approved by the Ethical Committee on Animal Research (Protocol #1499-1) at the University of Campinas. The behavior and physical appearance of the animals were monitored daily, and their weight was assessed at the beginning and end of each experimental period.

Induction of periodontal disease (PD) and treatment

To induce PD, rats were first anesthetized with an intramuscular injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). A cotton ligature was placed in a subgingival position around the cervix of both sides of mandibular first molars in each animal (Irie *et al*., 2008). In order to immobilize the ligature, two knots were made at mesial aspect of the first molars.

After ligature placement, animals were randomly assigned to three experimental groups (n=10 animals/group): 1) Control rats (without ligature); 2) rats with ligature + placebo (saline); 3) rats with ligature + fluoxetine (20 mg/kg/day in saline, Roumestan *et al*., 2007). Fluoxetine hydrochloride was obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in saline solution (vehicle). All treatments (saline or fluoxetine) were given orally (gavages) 1 hour before the attachment of ligature and daily during experimental periods.

Rats were euthanized under general anesthesia after 3 or 15 days from the attachment of ligature, respectively, according to the protocols established by Rodini *et al*. (2008) and Holzhausen *et al*. (2002). At Day 3, gingival tissue samples of the same size were collected from the mandibular first molars regions, immediately frozen, and kept at -80°C until processing for RT-PCR analysis and protein assays. Mandibular alveolar bone specimens of rats collected at Day 15 were submitted to histological analysis.

Histological analysis

The alveolar bone specimens were immediately fixed with 10% neutral buffered formalin and then decalcified with 10% EDTA aqueous solution for 60 days. The

decalcified specimens were dehydrated and embedded in paraffin. Serial sections obtained in a mesiodistal direction (5 μm thickness) were stained with hematoxylin and eosin (H&E) for measurement of bone loss or reacted with picrosirius red for the evaluation of collagen content.

Measurement of periodontal bone loss

The images of five semi-serial sections stained with H&E were digitized at a magnification of x50. The influence of fluoxetine on periodontal bone loss was histometrically assessed by measuring the area (mm^2) of bone resorption in the furcation region, according to a method previously reported (Nociti *et al*., 2000). Evaluation was performed by a single examiner blind to the treatment assignment, using Image-Pro® (Media Cybernetics, Silver Spring, MD, USA). Alveolar bone specimens from control group (no ligature) were also measured to compare the results from both ligature groups.

Collagen assessment in the connective tissue

To evaluate the effects of fluoxetine on the inflammatory change of collagen fibers in the connective tissue, three equidistant sections were obtained from the region corresponding to the mesial of first molars and then stained with picrosirius red (Rich and Whittaker, 2005). The images of the sections were obtained by polarization microscopy and digitized at x400 magnification. First, the images of collagen fibers that displayed red hue were selected using AdobePhotoshop 7.0.1 image processing software (Adobe Systems Incorporated, San Jose, CA). The selected images were then transferred to ImageJ software, version 1.31p (National Institutes of Health, Bethesda, MD), where they were binarized, and the percentage of area filled by collagen fibers was calculated. The sections from control group were also evaluated.

RNA isolation

Total RNA was isolated from gingival tissues using TRIzol® reagent following the manufacturer's instructions. The isolated RNA was resuspended in ultrapure DNase/RNase free water and stored at -80°C. The RNA concentration and quality were determined using

a Nanodrop Spectrophotometer (ND-3300, NanoDrop Technologies, Wilmington, DE). DNAse I (Invitrogen, Carlsbad, CA) was used to eliminate DNA contamination.

Detection of IL-1β, COX-2, MMP-9 and iNOS mRNA using RT-PCR

Isolated total RNA (0.5 μg) was reverse transcribed with the SuperScript synthesis system in the presence of random primers (Invitrogen). Subsequently, resultant complementary DNA (cDNA) was amplified by PCR with *Taq* DNA polymerase (Invitrogen). The primer sequences used for amplification of target mRNA genes were: IL-1β, sense 5'-TCCATGAGCTTTGTACAAGG-3', antisense 5'-GGTGCTGATGTACCAGTTGG-3', 237 bp; COX-2, sense 5'-TGATGACTGCCCAACTCCCATG-3', antisense 5'-AATGTTGAAGGTGTCCGGCAGC-3', 702 bp; MMP-9, sense 5'-GGATTACCTGTACCGCTATGGTTA-3', antisense 5'-TTGGATCCAATAGGTGATGTTATG-3', 241 bp; iNOS, sense 5'-ACAACAGGAACCTACCAGCTCA-3', antisense 5'-GATGTTGTAGCGCTGTGTGTCA-3', 651 bp. Amplification of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (sense 5'-ACCACAGTCCATGCCATCAC-3', antisense 5'-TCCACCACCCTGTTGCTGTA-3', 450 bp) was used as an internal control.

PCR conditions were 30–35 cycles at 94°C for 30 s; 55–60°C for 30 s and 72°C for 1 min. The size of the PCR products was determined by comparison with the 100 bp ladder (Invitrogen). The agarose gels containing the amplified products were scanned and analyzed by ImageJ, which provided numeric values that allowed a semi-quantitative comparison between target genes and the internal control gene.

Preparation of gingival tissue homogenates

The gingival tissues collected from mandibular first molars were homogenized in a lysis buffer [25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1% SDS, 1% NP-40, (Sigma®)] using a tissue homogenizer (model MA-102, Marconi Piracicaba, SP, Brazil). After the centrifugation of homogenized samples, the supernatants were removed and stored at -80°C for further analyses. Measurement of total

protein in the supernatants was performed using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA), following the manufacturer's instructions (de Brito Penna Forte *et al*., 2010).

Gelatinase activity of MMP-9 using zymography

The measurement of MMP-9 activity was performed using gelatin zymography (Marques *et al*., 2009). Aliquots of gingival tissue homogenates containing the same protein amount were run under non-reducing conditions without heat denaturation onto 10% sodium dodecyl sulfate–polyacrylamide gels (SDS-PAGE) co-polymerized with 1.6 mg/mL of gelatin (Sigma®) as substrate. After SDS-PAGE, the gels were washed twice in 2% Triton X-100 (Sigma®) for 30 min each, and then the gels were incubated overnight in activation buffer (50 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$) for 16 h at 37°C. The MMP-9 gelatinolytic activity was detected after staining the gels with Coomassie™ Brilliant Blue G-250 (USB Corporation, Cleveland, OH, USA). A gelatinase zymography standard was used in all gels (CHEMICON International, Inc). The intensities of the bands in the photographed gels were analyzed to determine the gelatinase activity using the ImageJ (NIH) software.

Statistical analysis

Data from assays are presented as means ± standard deviation (SD). The results were subjected to one-way analysis of variance (ANOVA), and statistical differences among the three groups were analyzed using the Student's *t*-test at a significance level of 5%. Data were analyzed using statistical software (BioEstat 5.0, Sociedade Civil Mamirauá, Belém-PA, Brazil).

Results

The effects of fluoxetine on ligature-induced bone resorption in rats were evaluated. According to histometric analysis (Figure 1 A), the experimental group treated with fluoxetine showed significantly lower levels of ligature-induced bone loss in the furcation region when compared to the ligature-alone group ($P < 0.05$, Figure 1). Histo-

morphological images of the furcation region show that the pronounced bone loss caused by ligature (Figure 1 D) compared to control (Figure 1 C) was abolished by treatment with fluoxetine (Figure 1E). Furthermore, the group treated with fluoxetine maintained an amount of collagen comparable to control ($P > 0.05$, Figure 1 B). In contrast, the ligature-alone group showed a significantly lower amount of collagen compared to either control or the fluoxetine-treated group ($P < 0.05$, Figure 1 B). The configuration of collagen fibers determined by fluorescent immuno-histochemistry showed less picrosirius red staining pattern in the ligature-alone group compared to either control or the fluoxetine-treated group (Figure 1, F: control, G: ligature-alone group, and H: ligature + fluoxetine). These results suggested that fluoxetine inhibited periodontal bone resorption and destruction of collagen caused by ligature attachment.

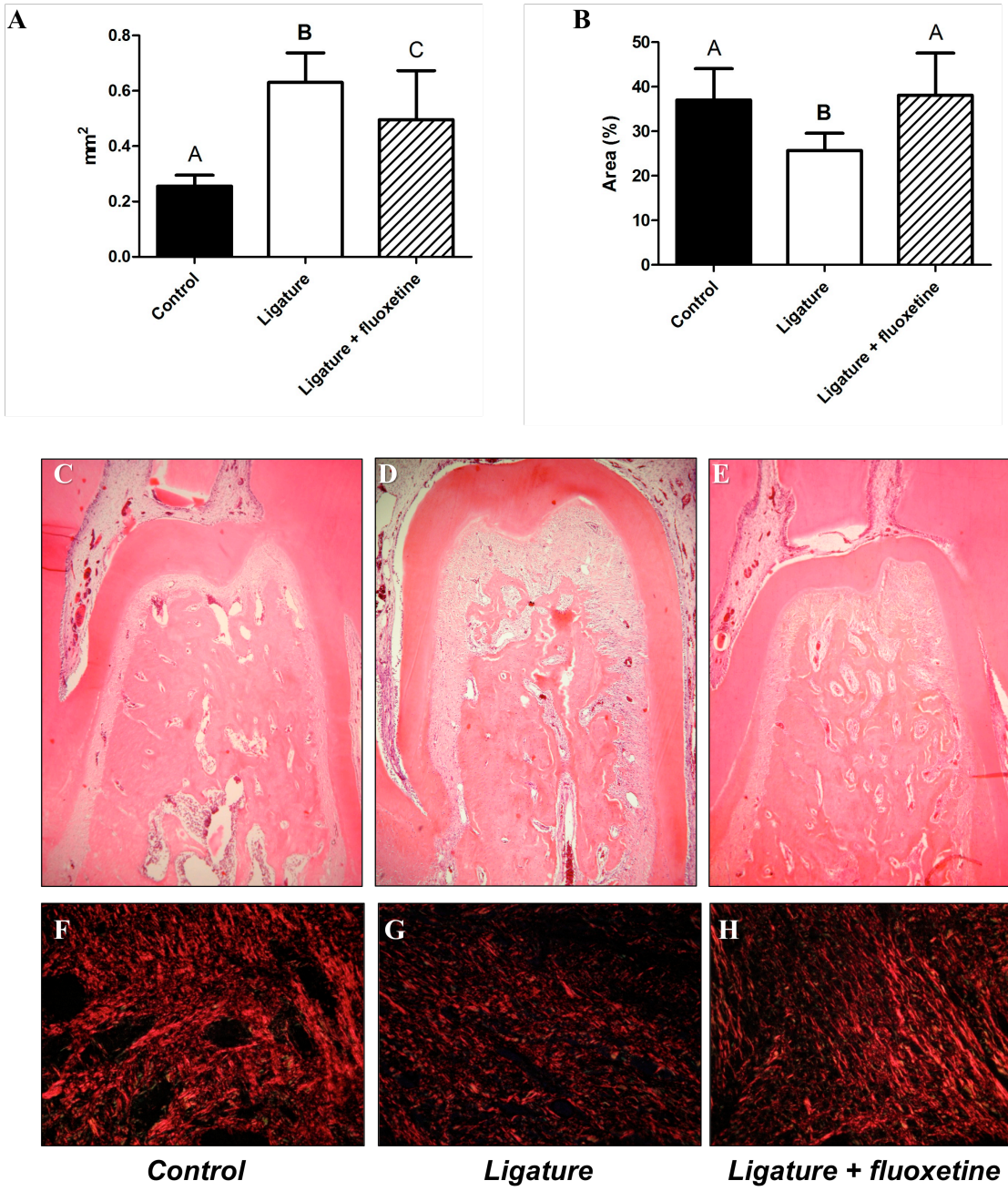

Figure 1. **Effects of fluoxetine treatment on alveolar bone loss and collagen content in a rat model of ligature-induced periodontitis.**

Data represent the mean ± standard deviation (SD) of 10 rats for each group. A) Measurement of bone loss (mm^2) in the furcation region of first molars of control (rats having no ligature; healthy sites), ligature (saline-treated rats with ligature), and ligature +

fluoxetine (fluoxetine-treated rats, 20 mg/kg/day) groups after 15 days of periodontal disease induction. B) Quantitative analysis of red collagen fibers (% area) in the connective tissue immediately above the bone crest in the mesial of the mandibular first molars of control, ligature-alone and ligature + fluoxetine groups after 15 days of periodontal disease induction. Fluoxetine reduced alveolar bone loss as compared to ligature-alone group ($P < 0.05$) and maintained collagen fiber levels similarly to the control group ($P > 0.05$). Different letters on the top of each bar indicate statistical differences (Figure 1 A: A vs. B, $P < 0.01$; A vs. C, $P < 0.05$; B vs. C, $P < 0.01$; Figure 1 B: A vs. B, $P < 0.01$, ANOVA followed by Student's *t* test). C, D, E are the images of histochemical staining at the furcation region of control, ligature and ligature + fluoxetine groups, respectively (hematoxylin and eosin staining, magnification of x50). F, G, H are fluorescent images of the collagen fibers stained with picrosirius red in the connective tissue immediately above the bone crest in the mesial of mandibular first molars of control, ligature and ligature + fluoxetine groups, respectively (picrosirius red stain, magnification of x400).

The influence of fluoxetine on the expressions of mRNA for COX-2, IL-1β, MMP-9 and iNOS were compared among the groups tested (Figure 2). All four mRNA expressions were elevated in the ligature-alone group compared to control, indicating that inflammatory response was induced by attachment of ligature around tooth. However, fluoxetine treatment significantly reduced IL-1β and COX-2 mRNA expression induced in the gingival tissues in comparison to the ligature-alone group ($P < 0.05$). At the same time, no differences were found for MMP-9 and iNOS expression induced by the attachment of ligature when fluoxetine was compared to ligature-alone group ($P > 0.05$).

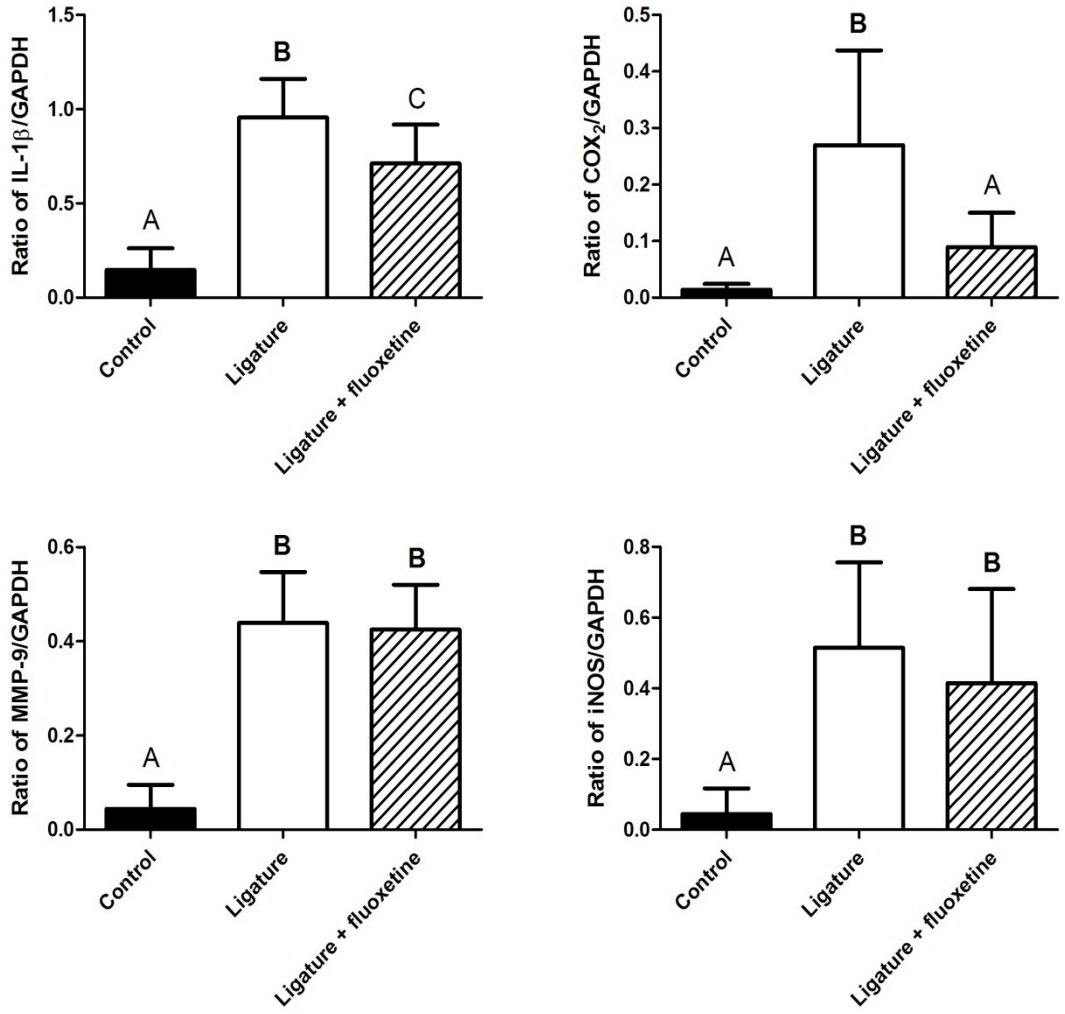

Figure 2. Effects of fluoxetine treatment on expression of IL-1β, COX-2, MMP-9 and iNOS mRNA levels monitored in gingival tissues.

mRNA expression levels (ratio of PCR products for target gene/GAPDH measured in agarose gel) of respective genes, IL-1β, COX-2, MMP-9 and iNOS in the gingival tissues were calculated. The column and bar indicate mean ± SD. Data were collected from the samples isolated from the gingival tissues of 1) control, 2) ligature-alone and 3) ligature + fluoxetine on Day-3. Different letters on the top of each bar indicate statistical differences (IL-1β: A vs. B, $P < 0.01$; A vs. C, $P < 0.05$; B vs. C, $P < 0.01$. COX-2: A vs. B, $P < 0.01$; MMP-9: A vs. B, $P < 0.01$; iNOs: A vs. B, $P < 0.01$. ANOVA followed by Student's t test).

Based on MMP-9's activities to digest type-IV and type-V collagens, it is well established that MMP-9 induced in the periodontal lesion contributes to the destruction of collagen fibers (McCauley and Nohutcu, 2002). While significant destruction of collagen fibers was induced by ligature, fluoxetine inhibited such collagen degradation. Still, enhanced expression of MMP-9 mRNA was not suppressed by fluoxetine. Therefore, using gelatin gel zymography, we examined if fluoxetine could affect the enzymatic activity of MMP-9. Testing showed that MMP-9 activity in gingival tissue was remarkably up-regulated by ligature attachment and that fluoxetine suppressed such MMP-9 activity in the ligature-induced periodontal disease (Figure 3). This result suggested that fluoxetine's suppressive effects on MMP-9 enzymatic activity contributed to the inhibition of destruction of collagen fibers.

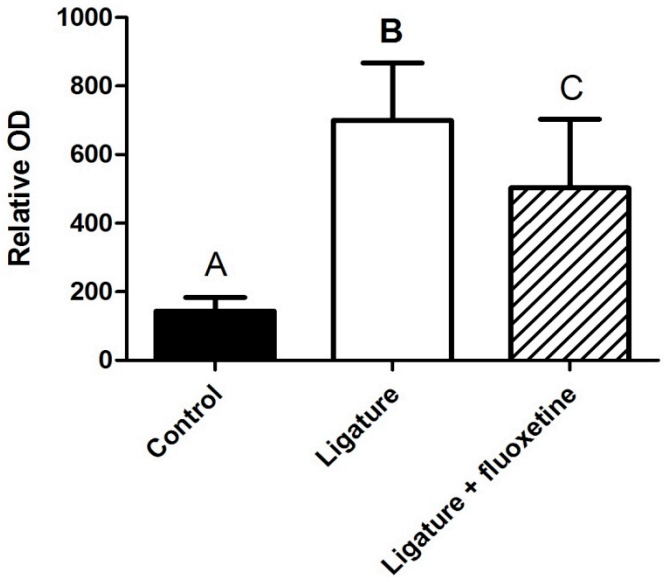

Figure 3. Effects of fluoxetine treatment on gelatinase activity of matrix metalloproteinase (MMP)-9.

Equal amounts of protein contained in the gingival tissue homogenates of control, ligature-alone and fluoxetine + ligature groups were subjected to gelatinase activity assay, as shown in the Material and Methods section. Different letters on the top of each bar indicate statistical differences (A vs. B, $P < 0.01$; A vs. C, $P < 0.05$; B vs. C, $P < 0.01$. ANOVA

followed by Student's *t* test).

Discussion

The present study showed that oral treatment with fluoxetine positively modulated important key periodontal inflammatory mediators in rats submitted to a ligature-induced PD model, which mimics very well the PD process (Nociti *et al*., 2000; Holzhausen *et al*., 2002; Graves *et al*., 2008). Furthermore, fluoxetine reduced alveolar bone resorption and presented a positive effect over collagen breakdown in gingival tissues. Altogether, these findings not only confirm the potential anti-inflammatory capacity of fluoxetine, but also bring the first information about its effects on PD.

The modulation of the local inflammatory host responses by fluoxetine observed in this investigation [i.e., reduction of IL-1β and COX-2 expression, total protein concentration (data not shown) and MMP-9 activity in gingival tissues] seems to be responsible for its positive influence on bone resorption. Among all these inflammatory indicators, we can speculate that most fluoxetine inhibitory effects can be attributed to the reduction of IL-1β expression. Noting that IL-1β levels significantly decrease after successful periodontal therapy (Hou *et al*., 1995), it is still one of the most powerful pro-inflammatory factors (McCauley and Nohutcu, 2002). In fact, multifunctional IL-1β orchestrates a cascade of destructive events in periodontal tissues, triggering the production of an array of inflammatory mediators/enzymes, including PGE_2 and MMP-9 (Schwartz *et al*., 1997), both of which were also affected by fluoxetine in the present study.

PGE_2 has been extensively related to inflammation and bone resorption, and biosynthesis of PGE_2 in the context of inflammation is regulated by COX-2 (Offenbacher *et al*., 1993). In this context, inhibitors of both PGE_2 production and COX-2 expression can markedly reduce alveolar bone resorption *in vivo* (Reddy *et al*. 2003). The effects of fluoxetine on PGE_2 have only been scarcely reported (Bianchi *et al*., 1995; Yaron *et al*., 1999; Jin *et al*., 2009); therefore, studies, such as the present work, are still necessary to strengthen reporting about this specific anti-inflammatory activity of fluoxetine. In this investigation, COX-2 mRNA expression was reduced in the gingival tissues after drug treatment, suggesting that fluoxetine possibly down-modulated the PGE_2 generation by

suppressing the production of COX-2 protein in the inflamed sites, thus contributing to the bone loss reduction observed here.

The positive influence of fluoxetine on collagen from the gingival connective tissue encouraged the authors of this study to perform additional analysis aimed at clarifying if fluoxetine could affect the gelatinolytic activity of MMP-9 (gelatinase B, type IV collagenase). Because significant MMP-9 activity is found in progressive diseased sites, a measurable level of influence on this activity is relevant (Teng *et al*., 1992). MMP-9 can efficiently degrade collagen fibers in concert with MMP-1 and MMP-3, which results in tissue destruction during inflammation (Opdenakker *et al*., 2001). We found that fluoxetine could down-modulate MMP-9 enzymatic activity without affecting expression of MMP-9 mRNA induced by ligature-mediated inflammation. Thus, in the context of periodontitis, our finding is of considerable therapeutic interest in that the only drug approved in the U.S. FDA for clinical use as a host modulator is a collagenase (or MMP)-inhibitory drug: doxycycline (Golub *et al*., 1998). Therefore, similarly to doxycycline, fluoxetine could be used for the purpose to suppress connective tissue destruction occurring in the context of periodontal disease.

Fluoxetine has shown inhibitory effects on NO produced by synovial cells from patients with rheumatoid arthritis (Yaron *et al*., 1999). Considering the importance of NO in the pathogenesis of periodontal disease (Lappin *et al*., 2000) and the fact that NO inhibitors have been shown to reduce periodontal bone resorption (Leitão *et al*., 2005), the present study also evaluated the effects of fluoxetine on gingival iNOS expression. Our investigation, however, failed to demonstrate any effect of fluoxetine on iNOS, suggesting that the bone resorption reduction observed here was probably unrelated to NO. Nonetheless, since the effects of fluoxetine on iNOS/NO are not well known and since existing reports have shown contradictory results (Ha *et al*., 2006; Roman *et al*., 2009), we suggest that more studies are needed to evaluate whether fluoxetine can influence NO in the context of PD.

Some mechanisms have been proposed to explain the anti-inflammatory properties of fluoxetine (Bianchi *et al*., 1994, 1995; Jin *et al*., 2009). Diamond *et al*. (2009) suggested that the effects of fluoxetine might be mediated by cyclic adenosine monophosphate.

Moreover, fluoxetine has been reported to reduce the transcription activity of nuclear factor (NF)-κB (Roumestan *et al*., 2007; Jin *et al*., 2009), which can, to some extent, explain the anti-inflammatory effect observed in the present study. Therefore, our findings and those from previous studies led us to consider a relevant suppression effect of fluoxetine on the inflammation induced in the rat model of ligature-induced periodontal disease.

From the perspective of therapeutic value, some tolerance issues have arisen from the use of fluoxetine, including gastrointestinal disorders and weight loss, but such side effects are transitory and dissipate quickly, if appropriate dosage is used (Zajecka *et al*., 1999; Serretti & Mandelli, 2010). Indeed, in the present study, the weight loss observed in the ligature + fluoxetine group in comparison to the other groups (data not shown) could be explained by these side effects. While findings have merit with respect to periodontal therapy, the present investigation can be useful in research aimed at new therapeutic strategies to treat other peripheral or central nervous disorders (Hartung and Kieseier, 2000), as well as important inflammatory conditions and autoimmune disorders, such as rheumatoid arthritis, if possible side effects of fluoxetine can be managed with adjustment of dosage.

In conclusion, our results suggest that fluoxetine may be considered a promising new therapeutic approach to treat PD. To better understand the mechanisms by which fluoxetine modulates host response, further studies must be conducted to test its effects on immune cells in the pathogenesis of PD. We believe that further clinical trials should be conducted to clarify the benefits of fluoxetine (and other selective serotonin reuptake inhibitors) as an adjunct regimen in periodontal therapy.

Acknowledgments

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3. CONCLUSÃO

Considerando-se a influência da fluoxetina sobre a capacidade das células dendríticas em apresentar antígeno aos linfócitos T, bem como os seus efeitos observados sobre a resposta inflamatória, a reabsorção óssea e o colágeno do tecido conjuntivo em ratos com doença periodontal induzida, os resultados do presente estudo sugerem que a fluoxetina é capaz de modular a resposta imuno-inflamatória relacionada à doença periodontal, podendo constituir uma abordagem terapêutica promissora como terapia de modulação da resposta do hospedeiro.

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ANEXOS

ANEXO 1: Certificado do comitê de ética ("Institutional Animal Care & Use Comittee, The Forsyth Institute") referente aos animais utilizados no estudo apresentado no Capítulo 1.

THE FORSYTH INSTITUTE

Institutional Animal Care & Use Committee

September 17, 2008

To: Dr. Toshi Kawai

From: Susan Rittling, Chair

Re: Animal Protocol Renewal Application # 06-020

The above 2nd year renewal application for the use of vertebrate animals in research:

Submitted: 9/15/08

Entitled: Treg cells in mouse model of periodontal disease

has been reviewed and given FULL APPROVAL by the Executive Committee of the Forsyth Institute's Animal Care and Use Committee on September 19, 2008

Protocol Approval Date: September 19, 2006

Animal Assurance Number: A3051-01

The protocol approval date and animal assurance number must appear on your grant application.

Protocol Integrity

You are reminded that, as Principal Investigator of this Protocol, you are required to abide by all of the terms and conditions you have included in the protocol and/or developed in collaboration with the IACUC. Should you find it necessary to deviate from the protocol, you should file an "Amendment" form with the IACUC detailing the changes you propose. You may not undertake any such changes until approval is given by IACUC, unless the change involves the immediate life and/or welfare of the animals. Failure to abide by this policy can result in administrative action, including termination of the study.

Enclosed is a copy of the signed renewal for your records.

SRR:om
cc Animal Quarters

ANEXO 2: Certificado do Comitê de Ética na Experimentação Animal (CEEA/UNICAMP) referente ao estudo *in vivo* apresentado no Capítulo 2.

UNICAMP

CEEA/Unicamp

Comissão de Ética na Experimentação Animal
CEEA/Unicamp

CERTIFICADO

Certificamos que o Protocolo nº **1499-1**, sobre **"Estudo *in vivo* da atividade da fluoxetina e da desipramina sobre a modulação da resposta imuno-inflamatória do hospedeiro na doença periodontal"**, sob a responsabilidade de **Prof. Dr. Pedro Luiz Rosalen / Prof. Dr. Gilson César Franco / Luciana Salles Branco de Almeida**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **28 de abril de 2008**.

CERTIFICATE

We certify that the protocol nº **1499-1**, entitled **"*In vivo* evaluation of the activity of fluoxetine and desipramine in host response in periodontal disease"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **April 28, 2008**.

Campinas, 28 de abril de 2008.

Profa. Dra. Ana Aparecida Guaraldo
Presidente

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ANEXO 3: Comprovante de submissão do artigo científico referente ao Capítulo 1 ("Selective serotonin reuptake inhibitor attenuates the antigen presentation from dendritic cells to effector T lymphocytes").

From: onbehalfof+femsim+fems-microbiology.org@manuscriptcentral.com [onbehalfof+femsim+fems-microbiology.org@manuscriptcentral.com] On Behalf Of femsim@fems-microbiology.org [femsim@fems-microbiology.org]
Sent: Wednesday, November 03, 2010 10:37 AM
To: Kawai, Toshi; Kajiya, Mikihito
Subject: Manuscript submitted - FEMSIM-10-11-0247

03-Nov-2010

Dear Prof. Toshihisa Kawai,

The manuscript you submitted to our journal, Selective serotonin reuptake inhibitors attenuate the antigen presentation from dendritic cells to effector T lymphocytes, by Almeida, Luciana; Kajiya, Mikihito; Cardoso, Cristina; Silva, Marcelo; Ohta, Kouji; Rosalen, Pedro; Franco, Gilson; Han, Xiaozhe; Taubman, Martin; Kawai, Toshihisa, has been uploaded to Manuscript Central.

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