O papel das quimiocinas CCL3, CCL2 e seus receptores na movimentação dentária ortodôntica

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“Viva como se fosse morrer amanhã.

Aprenda como se fosse viver para sempre".

Mahatma Gandhi

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SUMÁRIO

LISTA DE ABREVIATURAS

CCL – *CC chemokine ligand*

CCR – *CC chemokine receptor*

CSF – Fator estimulador de colônia

CGRP - Peptídeo relacionado ao gene da calcitonina

COL-1 – Colágeno tipo 1

DNA - Ácido desoxirribonucleico

EGF - Fator de crescimento epidermal

FGF-2 - Fator de crescimento de fibroblastos 2

HIV – Vírus da imunodeficiência adquirida

IFN- γ – Interferon gama

IGF-1 - Fator de crescimento do tipo insulina 1

IL- Interleucina

MMP – Metaloproteinase da matriz

MCP-1 – Proteína Quimiotática para Monócitos -1

MIP-1α – Proteína Inflamatória de Macrófago – 1 alfa

M-CSF – Fator estimulador de colônia de macrófago

OCN – Osteocalcina

OPG – Osteoprotegerina

PCR – Reação em cadeia da polimerase

PGE2 - Prostaglandina E 2

RANK – *Receptor activator of NFkB* / Receptor ativador de NF-kappa-B

RANKL – *Receptor activator of NFkB ligand* / Ligante do receptor ativador de NF-kappa-B

RANTES – *Regulated upon activation, normal T-cell expressed, and secreted*

RUNX2 – *Runt-related transcription factor* 2

TGF-β - *Transforming growth factor beta*

TNF- α – Fator de necrose tumoral alfa

TRAP – Fosfatase ácida resistente ao tartarato

VEGF - fator de crescimento endotelial vascular

WT – *Wild-type* / selvagem

RESUMO

O movimento dentário ortodôntico (MDO) é obtido pela remodelação do ligamento periodontal (LP) e osso alveolar em resposta à carga mecânica. Este processo é regulado por mediadores pró-inflamatórios, como citocinas e quimiocinas. Entre as quimiocinas, CCL2, CCL3 e CCL5 têm um papel importante na osteoclastogênese e seus níveis são aumentados nos tecidos periodontais após a aplicação de uma força ortodôntica. Como o efeito destas quimiocinas é mediado pela ligação aos seus receptores, nesta tese objetivou-se investigar o papel das quimiocinas e receptores CCL3/CCR1/CCR5 e CCL2/CCR2 no recrutamento e ativação dos osteoclastos durante a MDO. Para tal, um aparelho ortodôntico foi instalado em camundongos selvagens (WT) e animais deficientes para os receptores CCR5 ($CCR5^{-/-}$), CCR1 ($CCR1^{-/-}$), e CCR2 ($CCR2^{-/-}$), para a quimiocina CCL3 ($CCL3^{-/-}$) e animais tratados com Met-RANTES (antagonista dos receptores CCR1 e CCR5), com P8A (análogo de CCL2) e com veículo (PBS). O número de osteoclastos TRAP-positivos e a quantidade de movimentação ortodôntica foram quantificados histomorfometricamente. Além disso, *real-time PCR* foi utilizado para avaliar a expressão dos mediadores envolvidos na remodelação óssea. Nossos resultados demonstraram que o número de células TRAP-positivas, a quantidade de MDO e a expressão de RANKL, Catepsina K e MMP13 aumentaram significativamente nos camundongos $CCR5^{-/-}$. Por outro lado, o número de osteoclastos e a MDO foram reduzidos nos animais $CCL3^{-/-}$ e $CCR1^{-/-}$ comparados aos WT, bem como nos tratados com Met-RANTES em relação aos tratados com veículo. Estes resultados foram consistentes com a menor expressão de RANK, RANKL e TNF-α no grupo $CCL3^{-/-}$. O tratamento com o Met-RANTES resultou ainda

na redução da expressão de Catepsina K e MMP13. Os resultados sugerem que o CCR5 tem um papel anti-reabsortivo, enquanto o receptor CCR1 apresenta função pró-reabsortiva. Além disso, a ação do CCR1 é dependente, ao menos em parte, de sua ligação à quimiocina CCL3. Os resultados também mostraram que o número de células TRAP-positivas e a quantidade de MDO diminuíram nos camundongos $CCR2^{-/-}$ e nos animais tratados com P8A. Paralelamente, a diminuição da expressão do eixo RANKL/RANK foi observada no grupo $CCR2^{-/-}$. Estes dados sugerem que o eixo CCL2/CCR2 está relacionado ao recrutamento e ativação de osteoclastos, durante a MOD.

ABSTRACT

Orthodontic tooth movement (OTM) is achieved by the remodeling of periodontal ligament (PDL) and alveolar bone in response to mechanical loading. This process is regulated by pro-inflammatory mediators, such as cytokines and chemokines. CCL2, CCL3 and CCL5 are chemokines involved in osteoclastogenesis and are upregulated in periodontium after mechanical loading. As their cellular effects are mediated by binding to receptors, this study aimed to investigate the role of the chemokines and receptors CCL3/CCR1/CCR5 and CCL2/CCR2 in osteoclast recruitment and activation during OTM. An orthodontic appliance was placed in wild-type mice (WT), CCR5-deficient mice ($CCR5^{-/-}$), CCR1-deficient mice ($CCR1^{-/-}$), CCL3-deficient mice ($CCL3^{-/-}$), CCR2-deficient mice ($CCR2^{-/-}$) and mice treated with Met-RANTES (antagonist of CCR1 and CCR5), P8A (analog of CCL2) and vehicle (PBS). The number of TRAP-positive osteoclasts and the amount of OTM were quantified histomorphometrically. Moreover, the expression of mediators involved in bone remodeling was evaluated by *Real-time PCR*. Our data showed that the number of TRAP-positive cells, the amount of OTM and RANKL, Cathepsin K and MMP13 levels were significantly higher in $CCR5^{-/-}$ compared to WT mice. On the other hand, the number of osteoclasts and the amount of OTM were significantly diminished in $CCL3^{-/-}$ mice, $CCR1^{-/-}$ mice and Met-RANTES treated mice when compared to WT and vehicle treated mice, respectively. In accordance with these results, the levels of RANK, RANKL and TNF-α decreased in $CCL3^{-/-}$ mice. Moreover, the treatment with Met-RANTES also reduced the expression of Cathepsin K and MMP13. These results suggest that CCR1 is one of the main pro-resorbing chemokine receptors, while CCR5 is an anti-resorbing receptor involved in

OTM. In addition, the CCR1 action is dependent, at least in part, on CCL3 binding. Furthermore, TRAP-positive cells and the amount of OTM were significantly decreased in $CCR2^{-/-}$ and P8A-treated mice, when compared to WT and vehicle treated mice, respectively. In agreement with these data, the expression of the RANKL/RANK axis was lower in $CCR2^{-/-}$ than in WT mice. In summary, our results suggest that the CCL2/CCR2 axis might be involved in osteoclast activity and recruitment during OTM.

1. SÍNTESE BIBLIOGRÁFICA

1.1 Biologia do Movimento Dentário Ortodôntico

O movimento dentário ortodôntico é obtido pela remodelação do ligamento periodontal e osso alveolar em resposta à carga mecânica. Portanto, quando uma força ortodôntica é aplicada sobre o dente surgem mudanças macroscópicas e microscópicas significativas nos tecidos periodontais. Dentre estas alterações, pode-se observar a formação de duas áreas distintas e opostas no ligamento periodontal: a de compressão e a de tensão. Na área de compressão, a raiz dentária é deslocada contra o osso alveolar, comprimindo o ligamento periodontal adjacente. Nesta região, ocorre, então, reabsorção óssea por osteoclastos e consequente movimentação dentária em direção ao osso reabsorvido. Na área oposta, uma força de tensão é aplicada nas fibras do ligamento periodontal e novo tecido ósseo é depositado neste local por osteoblastos (Cattaneo *et al*., 2005; Krishnan e Davidovitch, 2006; Wise e King, 2008; Krishnan e Davidovitch, 2009).

O processo de movimentação dentária, seguido da aplicação de força ortodôntica, realiza-se em três fases: inicial, com movimento instantâneo do dente; platô, na qual nenhum movimento visível do dente ocorre; e linear ou aceleração, quando o dente volta a movimentar-se com maior velocidade (Krishnan e Davidovitch, 2006; Andrade Jr. *et al*., 2007a; Wise e King, 2008). Na fase inicial, o movimento rápido do dente é observado imediatamente após a aplicação da força ortodôntica. Neste momento, o dente se desloca dentro do espaço do ligamento periodontal, resultando no movimento do fluido periodontal da área de compressão

para a área de tensão (Krishnan e Davidovitch, 2006; Wise e King, 2008). Em virtude desta movimentação dentária, no lado de compressão, as fibras periodontais são desorganizadas, os vasos sanguíneos são comprimidos (Andrade Jr. *et al*., 2007a; Krishnan e Davidovitch, 2006; Wise e King, 2008), e as células e os tecidos são danificados. Isto resulta em hipóxia, na formação de um processo inflamatório agudo e no desenvolvimento de uma área acelular denominada área de hialinização (Krishnan e Davidovitch, 2006; Wise e King, 2008). Desta forma, quando as células e matriz extracelular dos tecidos periodontais sofrem estresse, a transdução direta da força mecânica ortodôntica para o núcleo da célula estressada é realizada através do citoesqueleto, processo este denominado mecanotransdução (Krishnan e Davidovitch, 2009). Esta sinalização intracelular leva à ativação de genes específicos e, consequentemente, produção e liberação de várias citocinas [interleucina 1 beta (IL-1β), interleucina 6 (IL-6), interleucina 8 (IL-8), fator de necrose tumoral alfa (TNF-α)], fator de crescimento [fator de crescimento endotelial vascular (VEGF)], prostaglandina E2 (PGE2) e neuropeptídios [peptídeo relacionado ao gene da calcitonina (CGRP) e substância P] para o meio extracelular (Masella e Meister, 2006; Krishnan e Davidovitch, 2006). Estas substâncias interagem direta ou indiretamente com a população de células periodontais residentes, promovem dilatação dos vasos sanguíneos e consequente extravasamento do plasma e migração, por diapedese, dos leucócitos para o espaço extravascular, caracterizando a formação de um infiltrado inflamatório agudo (Krishnan e Davidovitch, 2006; Wise e King, 2008).

Estudos relatam que a área de hialinização, formada durante este processo inicial, é responsável por interromper o movimento dentário por alguns dias (Bohl *et al*., 2004; Krishnan e Davidovitch, 2006). Este período de intervalo, com taxa

relativamente baixa ou ausente de deslocamento do dente, caracteriza a segunda fase do movimento dentário ortodôntico, denominado platô (Bohl *et al*., 2004; Krishnan e Davidovitch, 2006; Andrade Jr. *et al*., 2007a; Wise e King, 2008). Em humanos, o movimento do dente alcança um platô que dura cerca de 4 a 20 dias (Krishnan e Davidovitch, 2006), variando de acordo com a magnitude de força aplicada e tipo de movimento dentário, enquanto que o período é de 3 a 4 dias em camundongos (Yoshimatsu *et al*., 2006; Andrade Jr. *et al*., 2007a). Nenhum movimento adicional ocorre até que o tecido acelular e o osso alveolar adjacente sejam removidos (Bohl *et al*., 2004; Andrade Jr. *et al*., 2007a).

A eliminação do tecido hialinizado se inicia um ou dois dias depois da aplicação da força ortodôntica, quando a fase aguda da inflamação é diminuída e substituída por um processo inflamatório crônico (Krishnan e Davidovitch, 2006). Desta forma, os leucócitos, que migraram para o tecido periodontal na fase inicial da movimentação dentária, sintetizam e liberam moléculas de sinalização específicas, que estão envolvidas no processo de reabsorção óssea (citocinas, quimiocinas, fatores de crescimento, fator estimulador de colônia (CSF) e metabólitos do ácido araquidônico) (Masella e Meister, 2006; Krishnan e Davidovitch, 2006; Krishnan e Davidovitch, 2009). As interações dos vários tipos de células com estas substâncias desencadeiam o recrutamento de células fagocíticas, tais como células da linhagem de monócitos/macrófagos e precursores de osteoclastos (Rody *et al*., 2001; Krishnan e Davidovitch, 2006; Wise e King, 2008). Estas células sofrem diferenciação em macrófagos e osteoclastos maduros e, posteriormente, removem o tecido acelular da área do ligamento periodontal comprimido e osso alveolar adjacente, respectivamente, permitindo que o dente continue o seu movimento (Bohl *et al*., 2004; Krishnan e Davidovitch, 2006). O movimento dentário ortodôntico passa,

então, para a terceira fase, denominada fase de aceleração ou linear, a qual é caracterizada pelo deslocamento propriamente dito da unidade dentária (Krishnan e Davidovitch, 2006; Wise e King, 2008).

Na área de tensão, é observada deposição óssea pelos osteoblastos (Krishnan e Davidovitch, 2006; Wise e King, 2008; Krishnan e Davidovitch, 2009). Este processo é iniciado quando os osteócitos (mecanosensores) “percebem” alterações estruturais, por meio de alteração no fluxo dos canais e canalículos ósseos, causadas pela força de tensão e transmitem esta mensagem para os osteoblastos locais desempenharem sua função (Krishnan e Davidovitch, 2009). Além disso, as células periodontais estiradas (outro mecanosensor) estimulam a replicação celular e a produção de quimiocinas, citocinas e fatores de crescimento específicos para recrutamento e diferenciação dos osteoblastos e, consequentemente, para a formação óssea (Krishnan e Davidovitch, 2006). Ao mesmo tempo, fatores de crescimento [*Transforming growth factor beta* (TGF-β) e fator de crescimento do tipo insulina 1 (IGF-1)] estimulam a proliferação de células do ligamento periodontal e a síntese de colágeno, resultando na manutenção do aparato do tecido periodontal (Palioto *et al*., 2004; Fujii *et al.,* 2010).

Desta forma, fica claro que os osteoclastos e osteoblastos desempenham papel importante no processo de reabsorção e formação de osso alveolar durante a movimentação dentária ortodôntica. Portanto, entender melhor os mecanismos envolvidos na diferenciação e na ativação destas células durante a remodelação óssea induzida por força mecânica faz-se necessário.

1.2 Biologia dos Osteoclastos

Os osteoclastos são células multinucleadas derivadas de células tronco hematopoiéticas ou de células da linhagem de monócitos/macrófagos (Sims e Gooi, 2008). Estas células são marcadas positivamente pela fosfatase ácida resistente ao tartarato (TRAP) (Faust *et al.*, 1999; Liu *et al*., 2003; Pan *et al*., 2005) e expressam catepsina K e metaloproteinases (MMP), que participam da degradação de colágeno tipo I da matriz óssea (Nakamura *et al*., 2004). A osteoclastogênese ocorre seguindo uma seqüência ordenada de eventos, como: (1) divisão das células tronco e proliferação de precursores de osteoclastos em tecido hematopoiéticos (medula óssea de ossos longos ou baço) ou de medula óssea do osso alveolar adjacente, (2) migração do precursor de osteoclasto para o local de reabsorção óssea, (3) diferenciação do precursor de osteoclasto em pré-osteoclasto, e (4) fusão dos pré-osteoclastos para formar osteoclastos multinucleados (Udagawa *et al*., 1999; Graves *et al*., 1999; Rody *et al*., 2001; Cumano e Godin, 2007; Sims e Gooi, 2008).

Os osteoblastos desempenham papel importante no recrutamento, na diferenciação e na regulação da atividade dos osteoclastos, por meio de sinalização parácrina ou interação direta entre estes dois tipos celulares (Sims e Gooi, 2008; Matsuo e Iries, 2008; Boyce e Xing, 2008). Estudos demonstraram que osteoblastos, estimulados por IL-1 e TNF-α, são fontes de quimiocinas (CCL2, CCL3, CCL5), que por sua vez atuam no recrutamento dos precursores de osteoclastos (Graves *et al*., 1999; Yu *et al*., 2004; Yano *et al*., 2005) para o sítio de reabsorção óssea, no qual irão se diferenciar em osteoclastos maduros. Além disso, PGE2 e citocinas, tais como IL-1, IL-6, IL-8 e TNF-α, estimulam os osteoblastos/células estromais a produzirem os principais reguladores da diferenciação de osteoclastos: o CSF

derivado de macrófagos (M-CSF) (Boyle *et al*., 2003; Boyce *et al*., 2006) e o ligante para o receptor ativador de NF-kappa B (RANKL) (Boyle *et al*., 2003; Boyce *et al*., 2006; Sims e Gooi, 2008; Boyce e Xing, 2008). Este processo de diferenciação de osteoclasto é realizado quando M-CSF e RANKL ligam-se aos seus respectivos receptores c-Fms e receptor ativador do NF-kappa B (RANK) expressos nos precursores de osteoclastos (Sims e Gooi, 2008; Boyce e Xing, 2008). Além da diferenciação, a expressão do RANKL pelos osteoblastos também modula positivamente a atividade dos osteoclastos maduros (Udagawa *et al*., 1999; Boyle *et al*., 2003; Sims e Gooi, 2008; Boyce e Xing, 2008). Portanto, pode-se afirmar que a interação RANK-RANKL favorece a reabsorção óssea. Por outro lado, o osteoblasto pode regular negativamente a reabsorção óssea ao expressar a osteoprotegerina (OPG). A OPG é uma molécula que pode se apresentar na forma solúvel ou ligada à membrana e que, ao se ligar ao RANKL, inibe a interação RANK-RANKL (Sims e Gooi, 2008; Boyce e Xing, 2008; Aoki *et al*., 2010). É importante ressaltar também que não somente o RANKL, mas também outras citocinas (IL-1β, TNF-α, IL-6, IL-11), fatores de crescimento [fator de crescimento de fibroblastos-2 (FGF-2), fator de crescimento epidermal (EGF)] e quimiocinas (CCL2, CCL3, CCL5, CCL7, CCL9, IL-8) podem, direta ou indiretamente, aumentar a diferenciação, sobrevida, e atividade dos osteoclastos (Kawaguchi *et al*., 2000; Yu *et al*., 2004; Yano *et al*., 2005; Wei *et al*., 2005; Silva *et al*., 2007; Yao *et al*., 2008; Alves *et al*., 2009).

Como o osteoblasto desempenha importante papel na diferenciação e ativação de osteoclastos e na formação óssea, a avaliação da expressão dos marcadores de atividade e diferenciação dos osteoblastos pode contribuir para o entendimento da remodelação óssea induzida por força mecânica. O *Runt-related transcription factor 2* (RUNX2) é o fator transcricional relacionado à diferenciação de

osteoblastos e formação de osso (Franceschi *et al*., 2007). Uma vez diferenciado, os osteoblastos produzem proteínas tais como osteocalcina (OCN) e colágeno tipo 1 (COL-1) para formação da matriz óssea, sendo, então, considerados importantes marcadores de osteoblastos maduros e formação óssea (Franceschi *et al*., 2007).

Diante do exposto, o recrutamento de precursores de osteoclastos, diferenciação em osteoclastos maduros e ativação destas células são mediados por citocinas, fatores de crescimento, M-CSF e quimiocinas (Boyle *et al*., 2003; Boyce *et al*., 2006; Sims e Gooi, 2008; Boyce e Xing, 2008). Estudos adicionais devem ser realizados para aprofundamento dos mecanismos envolvidos na reabsorção óssea em resposta à carga mecânica ortodôntica. O modelo de movimentação ortodôntica em camundongos possibilita estudar os mediadores inflamatórios que regulam o recrutamento e atividade dos osteoclastos, tais como as citocinas e quimiocinas, e seus receptores. O entendimento a respeito da função destas moléculas na reabsorção óssea pode contribuir futuramente para modular a movimentação dentária e evitar danos nos tecidos periodontais adjacentes. Além disso, este conhecimento pode ser empregado no entendimento de outros processos como o remodelamento ósseo fisiológico e processos patológicos como osteoporose.

1.3 Quimiocinas na Remodelação Óssea

As quimiocinas são proteínas de baixo peso molecular que pertencem à grande família de citocinas quimiotáticas (Yadav *et al*., 2010; Schall e Proudfoot, 2011). Estas moléculas são subdivididas em quatro subfamílias: C, CC, CXC e CX3C. Estes tipos de quimiocinas são classificados de acordo com a estruturação das cisteínas residuais que se localizam próximas à região N terminal das proteínas.

As quimiocinas que apresentam as cisteínas separadas por aminoácido são denominadas CXC ou CX3C, enquanto que as não separadas são denominadas CC ou C (Zlotnik e Yoshie, 2000; Yadav *et al*., 2010; Schall e Proudfoot, 2011). Os receptores de quimiocinas apresentam 7 domínios transmembrana e são acoplados à proteína G. Estas moléculas são nomeadas de acordo com a família de seus ligantes, e as duas principais subfamílias são CCR ou CXCR (Zlotnik e Yoshie, 2000; Yadav *et al*., 2010). Muitas das quimiocinas da família CC apresentam capacidade de interagir com diferentes receptores de quimiocina, e um mesmo receptor pode se ligar a diferentes quimiocinas (Yadav *et al*., 2010; Schall e Proudfoot, 2011). A interação quimiocina/receptor é responsável pelas diferentes funções exercidas por estas moléculas (Yadav *et al*., 2010; Schall e Proudfoot, 2011). Funcionalmente, as quimiocinas desempenham papel importante em diversos processos homeostáticos e patológicos (Yadav *et al*., 2010).

Na remodelação óssea, as quimiocinas controlam não somente o recrutamento, como também, a proliferação, diferenciação, ativação e sobrevida das células ósseas envolvidas neste processo, tais como osteoclastos e osteoblastos (Watanabe *et al*., 2004; Yu *et al*., 2004; Yano *et al*., 2005; Lee *et al.*, 2007). O movimento dentário ortodôntico é um exemplo de remodelamento do osso alveolar, que ocorre após a aplicação de um estímulo mecânico e o consequente desenvolvimento de processo inflamatório transitório (Krishnan e Davidovitch, 2006; Wise e King, 2008). Recentemente, alguns estudos mostraram aumento do nível de quimiocinas, tais como CCL2, CCL3, CCL5, CXCL12, na movimentação dentária ortodôntica em modelo animal (Alhashimi *et al*., 1999; Andrade *et al.*, 2007b; Andrade *et al.*, 2009) e em humanos (Garlet *et al.*, 2008). Porém, pouco se conhece a respeito da participação destas moléculas no processo de

reabsorção/remodelação óssea após aplicação de força ortodôntica. **Portanto, o objetivo geral deste trabalho foi investigar o papel de quimiocinas e receptores: CCL3/CCR1/CCR5 e CCL2/CCR2, no recrutamento e atividade dos osteoclastos durante a movimentação dentária ortodôntica.**

1.3.1 Eixo CCL3/CCR1/CCR5

Proteína Inflamatória de Macrófago-1α (MIP-1α)/CCL3 é uma quimiocina importante no desenvolvimento do processo inflamatório, na inibição da proliferação de células tronco e da infecção pelo vírus da imunodeficiência humana (HIV) (Menten *et al*., 2002). Esta quimiocina interage com os receptores CCR1, CCR3 e CCR5 (Menten *et al*., 2002; Allen *et al*., 2007; Sallusto e Baggiolini, 2008). CCL3 é composta por 92 aminoácidos tanto em ratos quanto em humanos. Além disso, esta quimiocina em ratos apresenta homologia com a de humanos (69% de similaridade). Diversas células secretam CCL3, incluindo monócitos, macrófagos, neutrófilos, células T, eosinófilos, basófilos, osteoclastos e osteoblastos. Esta quimiocina participa da quimioatração de linfócitos T, monócitos, células dentríticas imaturas, basófilos e eosinófilos, como também desempenha papel importante na modulação da produção de citocinas tais como Interferon gama (IFN- γ) pelas células T *helper* (Menten *et al*., 2002).

CCL3 também desempenha um importante papel na reabsorção óssea (Yu *et al.*, 2004; Watanabe *et al*., 2004; Oba *et al*., 2005; Lee *et al*., 2007; Tsubaki *et al*., 2007). Esta quimiocina, quando se liga aos receptores CCR1 (Han *et al*., 2001; Yu *et al.*, 2004; Okamatsu *et al*., 2004; Oba *et al*., 2005; Lee *et al*., 2007) e CCR5 (Han *et al*., 2001; Oba *et al*., 2005; Lee *et al*., 2007), expressos nos precursores de

osteoclastos, promove a quimiotaxia destas células (Scheven *et al*., 1999; Yu *et al.*, 2004). No processo de diferenciação dos precursores de osteoclastos induzido por RANKL, CCL3 aumenta a formação dos osteoclastos em número e tamanho (Han *et al*., 2001; Okamatsu *et al*., 2004; Yu *et al.*, 2004; Watanabe *et al*., 2004; Oba *et al*., 2005; Lee *et al*., 2007; Tsubaki *et al*., 2007), estimula a atividade dos osteoclastos (Okamatsu *et al*., 2004) e prolonga a sobrevida destas células maduras (Okamatsu *et al*., 2004; Lee *et al*., 2007). Além disso, RANKL estimula a produção de CCL3 pelos próprios osteoclastos em diferenciação, o que sugere uma sinalização parácrina e autócrina durante a osteoclastogênese (Yu *et al*., 2004; Kim *et al*., 2006b; Lee *et al*., 2007).

Além dos osteoclastos, os osteoblastos também expressam os receptores CCR1 e CCR5 (Yano *et al.,* 2005), e produzem níveis elevados de CCL3 e CCL5 quando estimulados por IL-1 e TNF-α (Yano *et al*., 2005). A liberação destas quimiocinas pelos osteoblastos pode, significativamente, contribuir para o recrutamento e desenvolvimento dos osteoclastos no sítio de osteólise, exacerbando, desta forma, a perda óssea (Yu *et al*., 2004; Yano *et al*., 2005). Além disso, CCL3 também está envolvida de forma indireta na diferenciação do osteoclasto, uma vez que esta quimiocina estimula o aumento da expressão de RANKL pelo osteoblasto (Tsubaki *et al*., 2007) e induz interação entre o osteoclasto e o osteoblasto (Watanabe *et al*., 2004).

Estudos *in vitro* e *in vivo* evidenciaram o aumento da expressão de CCL3 em doenças ósseas inflamatórias, tais como doença periodontal (Ryu *et al*., 2007; Repeke *et al*., 2010), artrite reumatóide (Toh *et al*., 2004), e osteólise associada ao mieloma múltipo (Han *et al*., 2001*;* Abe *et al*., 2002; Abe *et al*., 2009). Esta quimiocina pode desempenhar importante função na osteoclastogênese e aumento

da reabsorção óssea nestas doenças (Han *et al.,* 2001*;* Abe *et al*., 2002; Toh *et al*., 2004; Ryu *et al*., 2007; Abe *et al*., 2009). Além disso, o nível de CCL3 também se apresenta aumentado durante a movimentação dentária ortodôntica (Garlet *et al*., 2008; Andrade Jr *et al*., 2009), porém, estudos adicionais devem ser realizados com o intuito de avaliar o papel do CCL3 neste processo. **Portanto, um dos objetivos específicos deste trabalho foi avaliar o impacto da deleção da quimiocina CCL3 no modelo de movimentação dentária ortodôntica.**

Estudos sugerem que a quimiocina *Regulated upon activation, normal T-cell expressed, and secreted* (RANTES)/CCL5, pode também contribuir efetivamente para o processo de reabsorção óssea (Yu *et al*., 2004; Yano *et al*., 2005). Assim como a quimiocina CCL3, CCL5 pode ligar-se aos receptores CCR1 e CCR5 expressos nos osteoclastos e osteoblastos (Han *et al*., 2001; Okamatsu *et al*., 2004; Yu *et al*., 2004; Yano *et al*., 2005; Oba *et al*., 2005; Lee *et al*., 2007). Deste modo, esta interação receptor/CCL5 estimula tanto a quimiotaxia dos precursores de osteoclastos, quanto a diferenciação destas células em osteoclastos maduros (Yu *et al*., 2004). CCL5 induz também recrutamento e evita a apoptose dos osteoblastos (Yano *et al*., 2005). Além disso, estas células secretam níveis elevados de CCL5, promovendo sinalização autócrina e ao mesmo tempo parácrina para os osteoclastos, o que pode resultar em aumento da reabsorção óssea (Yu *et al*., 2004; Yano *et al*., 2005). Portanto, pode-se sugerir que CCL5 é uma importante molécula de comunicação entre os osteoclastos e osteoblastos durante a remodelação óssea (Yano *et al*., 2005).

Alguns estudos demonstraram o envolvimento da quimiocina CCL5 na progressão de doenças inflamatórias crônicas caracterizadas por perda óssea, como artrite reumatóide, doenças periodontais e osteomielite (Wright e Friedland, 2002;

Lisignoli *et al.*, 2002; Repeke *et al*., 2010). Por isso, surge o questionamento se CCL5 também está envolvido no processo de reabsorção óssea gerada por forças mecânicas exercidas por aparelhos ortodônticos. Foi demonstrado que a expressão do CCL5 aumenta durante o movimento dentário ortodôntico (Alhashimi *et al.*, 1999; Andrade Jr. *et al*., 2007b; Garlet *et al*., 2008; Andrade Jr. *et al*., 2009). Além disso, a expressão desta quimiocina é reduzida nos sítios periodontais de animais deficientes do receptor p55 que apresentaram reabsorção óssea diminuída durante este processo (Andrade Jr. *et al*., 2007b). Desta maneira, é importante investigar o papel dos receptores desta quimiocina na movimentação dentária ortodôntica.

Estudos *in vitro* e *in vivo* também demonstraram que os receptores CCR1 e CCR5 estão envolvidos na patogênese de doenças ósseas, tais como mieloma múltiplo (Oba *et al*., 2005; Menu *et al*., 2006; Vallet *et al*., 2007), doença periodontal (Repeke *et al*., 2010; Ferreira Jr. *et al*., 2011), artrite reumatóide (Shahrara *et al*., 2005) e lesão periapical (Rossi *et al*., 2008). O bloqueio ou ausência do CCR1 e/ou CCR5 resulta em redução da osteólise no mieloma múltiplo, artrite reumatóide e doença periodontal (Oba *et al*., 2005; Shahrara *et al*., 2005; Menu *et al*., 2006; Vallet *et al*., 2007; Repeke *et al*., 2010; Ferreira Jr. *et al*., 2011; Repeke *et al*., 2011). Em contraste, o CCR5 é um receptor anti-reabsortivo na lesão periapical (Rossi *et al*., 2008), e atua controlando a resolução da inflamação na artrite experimental (Doodes *et al*., 2009). Portanto, como a função do CCR5 e CCR1 na reabsorção/remodelação óssea ainda não está bem definida, o modelo de movimentação dentária ortodôntica pode ser utilizado para auxiliar no entendimento deste processo. Neste sentido, o Met-RANTES, uma proteína CCL5 recombinante, apresenta capacidade de se ligar aos receptores CCR1 e CCR5, impedindo sinalização e resposta celular (Proudfoot *et al*., 1996), representando assim uma importante ferramente para o estudo destes

receptores. **Desta forma, outro objetivo específico deste trabalho foi avaliar o papel dos receptores CCR1 e CCR5 na reabsorção/remodelação óssea durante a movimentação dentária ortodôntica.**

1.3.2 Eixo CCL2/CCR2

A Proteína Quimiotática para Monócitos-1 (MCP-1)/CCL2 tem um importante papel no recrutamento de monócitos e macrófagos (Yadav *et al*., 2010). Esta quimiocina é sintetizada por monócitos, células endoteliais, células musculares lisas, células epiteliais, osteoblastos e osteoclastos (Graves *et al.*, 1999; Kim *et al*., 2005; Kim *et al*., 2006a; Kim *et al*., 2006b; Yadav *et al*., 2010). Os efeitos celulares do CCL2 podem ser mediados pela ligação desta quimiocina ao receptor CCR2 (Allen *et al*., 2007; Yadav *et al*., 2010).

Em relação ao processo de remodelação óssea, os precursores de osteoclastos expressam CCR2 (Kim *et al*., 2006b; Silva *et al.*, 2007; Binder *et al*., 2009) e a interação entre CCL2/CCR2 é capaz de promover o recrutamento destas células para o tecido ósseo (Silva *et al.*, 2007; Binder *et al*., 2009). CCL2 está também envolvida no processo de diferenciação dos precursores de osteoclastos em osteoclastos maduros, promovendo fusão destas células (Kim *et al*., 2005; Kim *et al*., 2006a; Kim *et al*., 2006b; Miyamoto *et al*., 2009; Binder *et al*., 2009). Além disso, estudos *in vitro* demonstraram que a reabsorção óssea é aumentada na presença de CCL2 (Kim *et al*., 2005; Kim *et al*., 2006a; Binder *et al*., 2009). Porém, estas funções, via interação CCL2/CCR2, são dependentes da ligação RANK/RANKL (Kim *et al*., 2005; Kim *et al*., 2006a; Kim *et al*., 2006b; Miyamoto *et al*., 2009; Binder *et al*., 2009).

Níveis aumentados de CCL2 são observados em condições inflamatórias associadas com reabsorção óssea *in vivo*, tais como artrite reumatóide (Iwamoto *et al*., 2008), metástase óssea (Lu e Kang, 2009), doença periodontal (Kurtis *et al*., 2005; Pradeep *et al*., 2009), osteólise periapical (Garlet *et al*., 2010) e movimentação dentária ortodôntica (Alhashimi *et al*., 1999; Andrade Jr. *et al.*, 2007b; Andrade Jr. *et al.*, 2009; Garlet *et al.*, 2008). Além disso, o bloqueio ou ausência do receptor CCR2 previne reabsorção óssea na artrite experimental (Brühl *et al*., 2004; Brodmerkel *et al*., 2005), na osteoporose (Binder *et al*., 2009) e no reparo de fratura óssea (Xing *et al*., 2010). Neste sentido, o P8A também tem sido apresentado como uma estratégia terapêutica eficaz para evitar reabsorção óssea na artrite experimental (Shahrara *et al*., 2008). Esta molécula é um análogo do CCL2 que apresenta efeito inibitório na migração celular dependente de CCR2 (Handel *et al.*, 2008). Porém, apesar de estudos anteriores evidenciarem o aumento da expressão de CCL2 na movimentação dentária ortodôntica (Andrade Jr. *et al*., 2007b), o papel do eixo CCL2/CCR2 neste processo de remodelação óssea ainda não foi demonstrado. **Desta maneira, um dos objetivos específicos deste trabalho foi avaliar o impacto da deleção do receptor CCR2 e o uso do P8A no modelo de movimentação dentária ortodôntica.**

2. ARTIGOS

2.1 Artigo 1

CCR5 down-regulates osteoclast function in orthodontic tooth movement.

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CCR5 Down-regulates Osteoclast Function in Orthodontic Tooth Movement

ABSTRACT

During orthodontic tooth movement, there is local production of chemokines and an influx of leukocytes into the periodontium. CCL5 plays an important role in osteoclast recruitment and activation. This study aimed to investigate whether the CCR5-receptor influences these events and, consequently, orthodontic tooth movement. An orthodontic appliance was placed in wild-type mice (WT) and CCR5-deficient mice ($CCR5^{-/-}$). The expression of mediators involved in bone remodeling was evaluated in periodontal tissues by Real-time PCR. The number of TRAP-positive osteoclasts and the expression of cathepsin K, RANKL, and MMP13 were significantly higher in $CCR5^{-/-}$. Meanwhile, the expression of two osteoblastic differentiation markers, RUNX2 and osteocalcin, and that of bone resorption regulators, IL-10 and OPG, were lower in $CCR5^{-/-}$. Analysis of the data also showed that $CCR5^{-/-}$ exhibited a greater amount of tooth movement after 7 days of mechanical loading. The results suggested that CCR5 might be a down-regulator of alveolar bone resorption during orthodontic movement.

KEY WORDS: orthodontic tooth movement, bone remodeling, CCR5, chemokines.

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INTRODUCTION

Orthodontic tooth movement has been defined as the result of a biological response to mechanical stimulus applied to a tooth, and it is achieved by periodontal ligament (PDL) and alveolar bone remodeling. Bone is resorbed by osteoclasts on the pressure sites, and it is formed by osteoblasts on the tension sites (Krishnan and Davidovitch, 2006; Wise and King, 2008).

Bone remodeling is also regulated by endogenous inflammatory mediators, such as prostaglandins, cytokines, and chemokines (Alhashimi *et al.*, 1999; Andrade *et al.*, 2007; Garlet *et al.*, 2007, 2008; Maeda *et al.*, 2007; Boyce and Xing, 2008; Wise and King, 2008). Chemokines are a large family of low-molecular-weight chemotactic cytokines (Allen *et al.*, 2007; Sallusto and Baggiolini, 2008). These molecules provide key signals for trafficking and homing of osteoclasts and osteoblasts (Fuller *et al.*, 1995; Yu *et al.*, 2004; Yano *et al.*, 2005). Moreover, chemokines may also control proliferation, differentiation, activation, and survival of bone cells (Han *et al.*, 2001; Watanabe *et al.*, 2004; Yu *et al.*, 2004; Yano *et al.*, 2005; Lee *et al.*, 2007).

Recent studies have suggested that CC chemokines may play a role in animal models (Alhashimi *et al.*, 1999; Andrade *et al.*, 2007) and human orthodontic tooth movement (Maeda *et al.*, 2007; Garlet *et al.*, 2008). However, little is known about which chemokines or corresponding receptors are involved in the resorption and formation of bone during orthodontic tooth movement. Some reports indicated that both CCL3 (Fuller *et al.*, 1995; Han *et al.*, 2001; Watanabe *et al.*, 2004; Yu *et al.*, 2004; Oba *et al.*, 2005; Lee *et al.*, 2007) and CCL5 (Yu *et al.*, 2004; Yano *et al.*, 2005) promote recruitment and differentiation of osteoclasts. Both chemokines bind to CCR1 and CCR5 receptors (Oba *et al.*, 2005; Yano *et al.*, 2005; Allen *et al.*, 2007; Sallusto and Baggiolini, 2008), which are expressed in osteoclasts (Han *et al.*, 2001; Yu *et al.*, 2004; Yano *et al.*, 2005; Lee *et al.*, 2007) and osteoblasts (Yano *et al.*, 2005). Some results implicated CCR5 in osteolysis *in vitro* and *in vivo* (Oba *et al.*, 2005; Menu *et al.*, 2006, Rossi *et al.*, 2008), although its function in bone resorption has not yet been defined. It has also been found that interference with CCR5, by systemic administration of an antagonist, resulted in the abrogation of multiple-myeloma-induced osteolysis (Menu *et al.*, 2006) and amelioration of arthritis-related bone loss (Vierboom *et al.*, 2005; Okamoto and Kamatani, 2006).

A better understanding of cellular and molecular responses to mechanical loading is crucial for future improvements in orthodontic treatment. In this present study, we applied a tooth movement model to CCR5-deficient mice to investigate the role of CCR5 in orthodontic tooth movement.

MATERIALS & METHODS

Experimental Animals

Thirty ten-week-old wild-type mice (WT) (C57BL6/J) and 30 ten-week-old CCR5-deficient mice ($CCR5^{-/-}$) were used in this experiment. All animals were treated under ethical regulations for animal experiments, defined by the Institutional Ethics Committee. Each animal's weight was recorded throughout the experimental period, and there was no significant loss of weight.

Experimental Protocol

The experimental protocol was based on previous work (Andrade *et al.,* 2007). The mice were anesthetized i.p. with 0.2 mL of a solution containing xylazine (0.02 mg mL^{-1}) and ketamine (50 mg mL^{-1}). An orthodontic appliance consisted of a Ni-Ti 0.25 x 0.76 mm (Lancer Orthodontics, San Marcos, CA, USA) coil spring, bonded by a light-cured resin (Transbond, Unitek/3M, Monrovia, CA, USA) between the maxillary right first molar and the incisors (Appendix Fig. 1). The left side was used as the control. The force magnitude was calibrated by a tension gauge (Shimpo Corp., Tokyo, Japan) to exert a force of 0.1 N applied in the mesial direction. The amount of force produced by the activation of the coil (1 mm = 0.1 N) was based on our previous experiments, which were adapted from the method proposed by Pavlin *et al*. (2000). There was no reactivation during the experimental period. The animals were divided into 2 groups: control group (non-operated animals) and experimental group (with activated coil spring). Mice were killed with an overdose of anesthetic at the following times: 7 and 12 days for histological measurements, and 3 and 7 days for biochemical analysis. For every set of experiments (histological and biochemical measurements), 5 animals were used for each time-point.

Histology

The right and left halves of the maxillae, including first, second, and third molars, were dissected and fixed in 10% buffered formalin (pH 7.4) and rinsed in distilled water. After fixation, each hemi-maxilla was decalcified in 14% EDTA (pH 7.4) for 14 days and embedded in paraffin. The samples were cut into vertical sections of 4 µm thickness. The selection was based on morphological criteria such as the position of the disto-buccal root, where it appeared to be as long as possible. The sections were stained for tartrate-resistant acid phosphatase (TRAP; Sigma-Aldrich, St. Louis, MO, USA), counterstained with hematoxylin, and used for histological examination. The distal-buccal root, on the coronal two-thirds of the mesial periodontal site, was used for the osteoclast counts on 5 sections *per* animal. Osteoclasts were identified as TRAP-positive, multinucleated cells on the bone surface. The total number of osteoclasts was determined in 5 consecutive microscopic fields (x 40).

Measurement of Tooth Movement

Image J software (National Institutes of Health) was utilized for morphometric evaluation of the amount of tooth movement. We measured the distance between the cementum-enamel junctions (CEJs) from the first molar and the second molar (1^{st}- and 2^{nd}-molar distance) in 5 vertical sections *per* animal using an Axioskop 40 (Carl Zeiss, Göttingen, Germany) equipped with a digital camera (PowerShot A620, Canon, Tokyo, Japan), according to a previous study (Mavragani *et al.,* 2005). Three measurements were conducted for each evaluation, and the variability was below 5% in all cases. To validate the consistency of the measurement, two examiners measured 20 successive slides until an r^2 of at least 0.85 was repeatedly obtained.

RNA Extraction and Real-time PCR

Using a stereomicroscope, we extracted periodontal ligament and surrounding alveolar bone samples from the areas adjacent to the upper first molars. The gingivae, oral mucosa, and teeth were dissected and discarded. The tissues were subjected to RNA extraction with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized with 2 µg of RNA through a reverse-transcription reaction (Superscript II, Invitrogen). Real-time PCR analysis was performed in an ABI Prism 7000 with the SYBR-green fluorescence quantification system (Applied Biosystems, Foster City, CA, USA). Standard PCR conditions were 95ºC (10 min), and then 40 cycles of 94ºC (1 min), 58ºC (1 min), and 72ºC (2 min), followed by the standard denaturation curve. Primer sequences were as follows for mouse β-actin: OPG, RANKL, TRAF6, MMP13, cathepsin K, RUNX2, OCN, CCL2, CCL3, CCL5, CCR1, and IL-10 (Appendix Table 1).

We used the mean Ct values from duplicate measurements to calculate expression of the target gene, with normalization to an internal control (β-actin) using the 2-ΔDCt formula.

Statistical Analysis

The evaluation of each group was expressed as the mean ± SEM. Comparison among the groups was statistically analyzed by one-way analysis of variance (ANOVA), followed by the Newman-Keuls multiple comparison test. $P < 0.05$ was considered statistically significant.

RESULTS

Tooth Movement, TRAP Activity, and the Number of TRAP-positive Cells Increased in $CCR5^{-/-}$ Mice

The results demonstrated a greater amount of tooth movement in $CCR5^{-/-}$ mice after 7 and 12 days of mechanical loading when compared with that in WT mice at the same time-points (Fig. 1A).

In the control group, TRAP activity was found on the distal side of the alveolar bone surface, and no activity was observed in the mesial region of the periodontium (Fig. 1B). On day 7, TRAP activity appeared to increase on the mesial periodontium of the disto-buccal root, and to decrease on the distal side of this root in WT mice. In $CCR5^{-/-}$ mice, there appeared to be a similar decrease on the distal side, but a greater increase of TRAP activity on the mesial side. On day 12, TRAP activity appeared to increase more extensively in $CCR5^{-/-}$ mice (Fig. 1C), which presented a greater

alveolar bone resorption area than did WT mice (Fig.1D). Overall, there was a good correlation between the intensity of TRAP activity, as evaluated qualitatively, and the number of TRAP-positive osteoclasts. Indeed, the quantification of TRAP-positive osteoclasts in WT mice increased steadily until day 12. However, the number of TRAP-positive osteoclasts was greater in CCR5$^{-/-}$ than in WT mice (Fig. 1E).

Expression of Osteoclast Markers Cathepsin K, Receptor Activator of NF-kappaB Ligand (RANKL), and Metalloprotease 13 (MMP13) Increased in CCR5$^{-/-}$ Mice

There was an increase in TNF receptor-associated factor 6 (TRAF6) levels in CCR5$^{-/-}$ mice after orthodontic force, but the results were not statistically significant compared with those in WT mice (Fig. 2A). However, when compared with WT mice, CCR5$^{-/-}$ mice exhibited a significant increase in MMP 13 (Fig. 2B), cathepsin K (Fig. 2C), and RANKL levels (Fig. 2D) after 3 and 7 days of mechanical loading.

Levels of CCL2, CCL3, and CCL5 Did Not Change Significantly in CCR5$^{-/-}$ Mice

A significant increase in mRNA expression of CCL2, CCL3, and CCL5 was observed in the periodontal tissue of WT and CCR5$^{-/-}$ mice after 3 and 7 days of mechanical loading (Appendix Figs. 2A-2C). However, there was no significant difference between the WT and CCR5$^{-/-}$ experimental groups. In contrast, there was a significant increase of CCR1 expression in CCR5$^{-/-}$ mice after 3 and 7 days of orthodontic force at the same time-points (Appendix Fig. 2D, Appendix Tables 2 and 3).

Expression of Osteoblast Markers Runt-related Transcription Factor 2 (RUNX2) and Osteocalcin (OCN) Diminished in CCR5$^{-/-}$ Mice

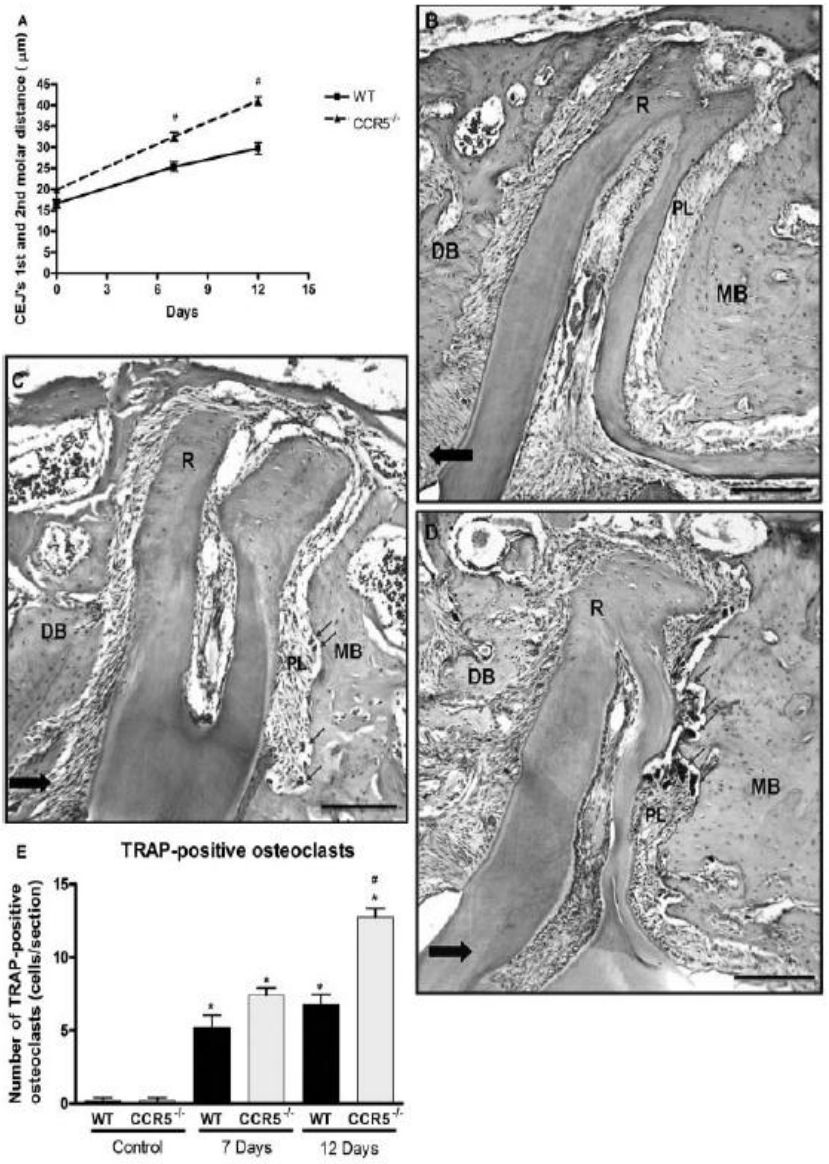

Figure 1. Tooth movement and TRAP-positive cells. **(A)** Time-course of changes in the amount of tooth movement between WT and CCR5$^{-/-}$ mice, morphometrically evaluated by measurement of the distance between the cementum-enamel junctions (CEJs) from the first molar and the second molar. WT (black line) and CCR5$^{-/-}$ (dotted line). (B,C,D) Histological changes related to orthodontic tooth movement in WT and CCR5$^{-/-}$ mice. Vertical sections (4-μm thickness) of the periodontium around the disto-buccal root of the first molar stained with TRAP. **(B)** Control group (before mechanical loading). **(C)** TRAP activity increased on the mesial alveolar bone and decreased on the distal alveolar bone in WT mice. **(D)** TRAP activity was greater in CCR5$^{-/-}$ mice. TRAP-positive osteoclasts are indicated by blue arrows. MB, mesial alveolar bone; DB, distal alveolar bone; PL, periodontal ligament; R, root. The black arrows indicate orthodontic tooth movement. Original magnification, x100. **(E)** Number of TRAP-positive osteoclasts. The total number of positive cells in the disto-buccal root was determined in 5 consecutive microscopic fields (x40). There were 5 animals in each group on each day. The data are expressed as the mean ± SEM. *$P < 0.05$ compared the control with the experimental group, with the same animal type. #$P < 0.05$ compared WT experimental groups and CCR5$^{-/-}$ experimental groups, at the same moment. One-way ANOVA and Newman-Keuls multiple-comparison test. Bar = 10 μm.

There was a significant increase in RUNX2 and OCN mRNA expression in WT periodontal tissue after 3 and 7 days of mechanical loading in comparison with that in the control group. Meanwhile, the levels of these 2 osteoblastic markers were significantly lower in CCR5$^{-/-}$ experimental groups when compared with those in WT mice at the same time-points (Appendix Figs. 3A, 3B).

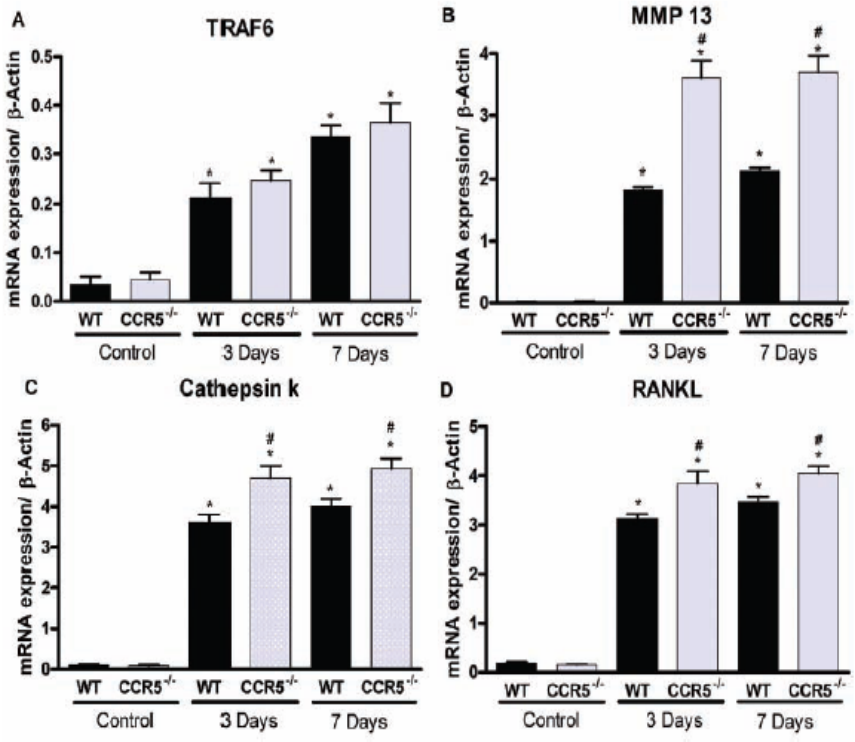

Figure 2. mRNA expression of TRAF 6 **(A)**, MMP 13 **(B)**, cathepsin K **(C)**, and RANKL **(D)** in the mouse periodontium (WT and CCR5$^{-/-}$) after 3 and 7 days of mechanical loading. There were 5 animals in each group on each day. The data are expressed as the mean ± SEM. *$P < 0.05$ compared the control group with the experimental group (after 3 or 7 days of orthodontic force). #$P < 0.05$ compared WT experimental groups and CCR5$^{-/-}$ experimental groups, at the same moment. One-way ANOVA and Newman-Keuls multiple-comparison test.

Levels of Negative Osteoclast Regulators Diminished in CCR5$^{-/-}$ Mice

When compared with WT mice, there was a significant decrease in osteoprotegerin (OPG) levels in CCR5$^{-/-}$ mice after 3 days of orthodontic force (Appendix Fig. 3C). Meanwhile, the expression of interleukin 10 (IL-10) after 7 days of mechanical loading was significant diminished in CCR5$^{-/-}$ compared with WT animals at the same time-points (Appendix Fig. 3D).

DISCUSSION

In the current study, the results demonstrated a greater amount of tooth movement in CCR5$^{-/-}$ mice, which was correlated with increased alveolar bone resorption and high numbers of TRAP-positive osteoclasts. The Real-time PCR analysis revealed that mRNA levels of the osteoclast activity markers MMP13, cathepsin K, and RANKL (Nakamura *et al.*, 2004; Blair *et al.*, 2005; Garlet *et al.*, 2007; Boyce and Xing, 2008) were higher in CCR5$^{-/-}$ mice than in WT controls. In accordance with the latter results, a recent report showed that, in the absence of CCR5, periapical bone resorption was significantly increased (Rossi *et al.*, 2008).

Some reports have investigated the expression and function of CC chemokine receptors in osteoclasts (Han *et al.*, 2001; Yu *et al.*, 2004; Yano *et al.*, 2005; Lee *et al.*, 2007). It has been reported that both CCR1 and CCR5 may be used by CCL3 and CCL5 to induce chemotaxis and the formation of osteoclasts *in vitro* (Yu *et al.*, 2004; Oba *et al.*, 2005; Lee *et al.*, 2007). CCR1 and CCR5 are also involved in osteoclast activity and, consequently, in the induction of osteolysis *in vitro* (Oba *et al.*, 2005; Menu *et al.*, 2006) and *in vivo* (Menu *et al.*, 2006). In line with these studies, analysis of the data showed that CCR1 expression increased after mechanical loading. Moreover, expression of CCR1 was greater in CCR5$^{-/-}$ than in WT mice. Further research is now required to confirm the role of CCR1 in orthodontic tooth movement.

The results also revealed that CCL3 and CCL5 expression was increased in periodontal tissues of mice subjected to orthodontic tooth movement. This is in accordance with recent reports that demonstrated increased levels of these chemokines associated with orthodontic movement in animal models (Alhashimi *et al.*, 1999; Andrade *et al.*, 2007) and humans (Maeda *et al.*, 2007; Garlet *et al.*, 2008). It is noteworthy that the absence of CCR5 did not result in significant changes in expression of their ligands CCL3 and CCL5.

It has been shown that osteoblast/osteoclast interaction chiefly regulates bone remodeling (Yu *et al.*, 2004; Yano *et al.*, 2005; Boyce and Xing, 2008). The results demonstrated that the osteoblast differentiation markers RUNX2 and OCN (Franceschi *et al.*, 2007; Garlet *et al.*, 2008) were increased during bone remodeling after orthodontic force in WT mice. This is in accordance with a recent report of high levels of osteocalcin after mechanical loading (Garlet *et al.*, 2007, 2008). In contrast, osteoblast differentiation markers were diminished in CCR5$^{-/-}$ mice. In parallel, it was observed that the levels of IL-10 and OPG, negative regulators of bone resorption, were decreased in CCR5$^{-/-}$ animals (Boyce and Xing, 2008; Rossi *et al.*, 2008). Taken together, the results suggested that a diminished differentiation of osteoblasts in CCR5$^{-/-}$ mice led to a reduction of inhibitory signals for osteoclasts, resulting in increased alveolar bone resorption and greater orthodontic tooth movement. This finding is supported by the results of a study in a periapical lesion model that implicated CCR5 as a negative regulator of bone resorption (Rossi *et al.*, 2008). It is worth considering that the absence of CCR5 may also result in a differential leukocyte subset migration which may ultimately determine greater bone resorption (Okamoto and Kamatani, 2006; Yurchenko *et al.*, 2006;

Moreira *et al.*, 2008). In spite of the broad expression of CCR5 by leukocytes (Yurchenko *et al.*, 2006; Moreira *et al.*, 2008), the decreased IL-10 levels suggested that the migration of regulatory T-cells (which characteristically produce IL-10) could be impaired, therefore contributing to increased bone resorption activity. In fact, regulatory T-cells have been shown to suppress osteoclast formation (Zaiss *et al.*, 2007). Further studies are nonetheless needed to clarify the latter hypothesis.

In conclusion, the present study suggests that CCR5 might be a down-regulator of alveolar bone resorption during orthodontic tooth movement. Furthermore, analysis of our data provides a new insight into the development of future therapeutic interventions with the CCR5 antagonist, which could prevent inflammatory bone loss.

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RESEARCH REPORTS
Biological

CCR5 Down-regulates Osteoclast Function in Orthodontic Tooth Movement

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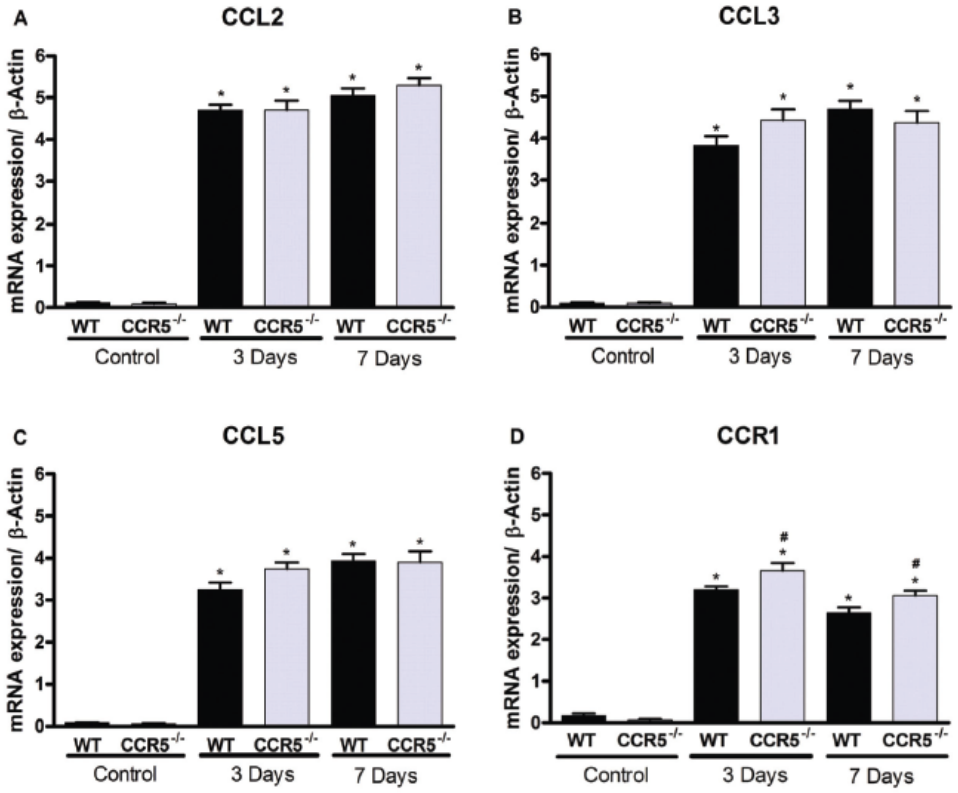
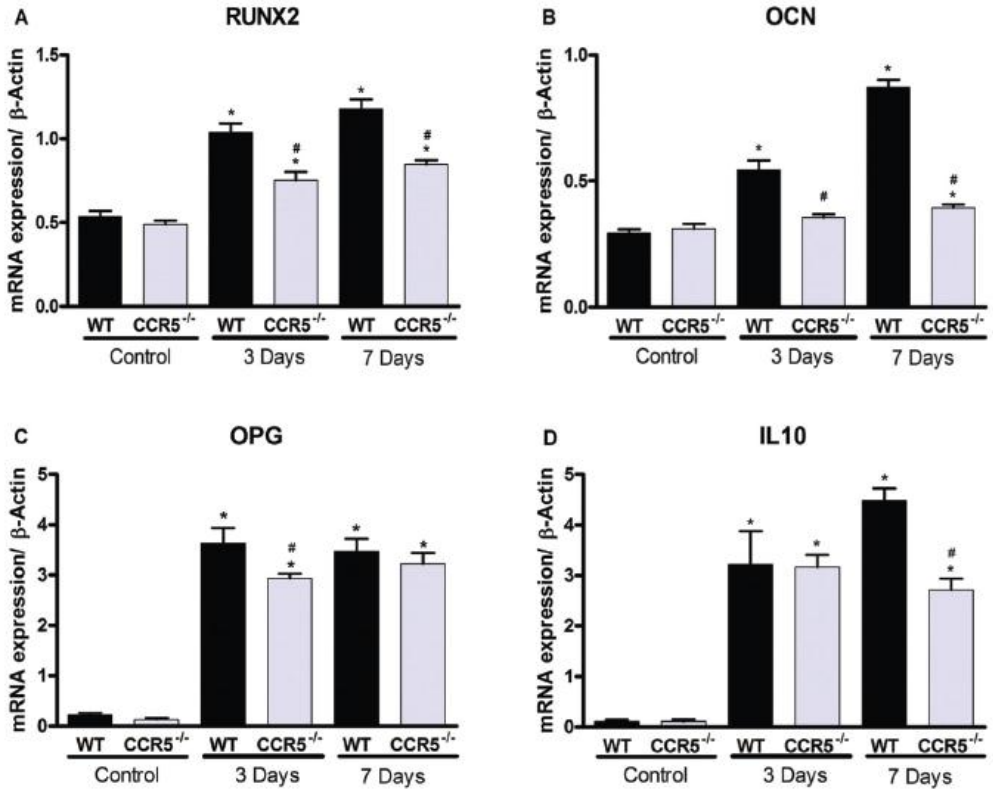
APPENDIX

Appendix Figure 1. Coil spring bonded between maxillary right first molar and incisors.



Appendix Figure 2. mRNA expression of CCL5 **(A),** CCL2 **(B),** CCL3 **(C),** and CCR1 **(D)** in the mouse periodontium (WT and $CCR5^{-/-}$) after 3 and 7 days of mechanical loading. The data are expressed as the mean ± SEM. *P < 0.05 comparing the control group with the experimental group (after 3 or 7 days of orthodontic force), in the same animal type. #P < 0.05 comparing WT experimental groups and $CCR5^{-/-}$ experimental groups, at the same moment. One-way ANOVA and Newman-Keuls multiple-comparison test.



Appendix Figure 3. mRNA expression of RUNX2 **(A),** OCN **(B),** OPG **(C),** and IL-10 **(D)** in the mouse periodontium (WT and CCR5$^{-/-}$) after 3 and 7 days of mechanical loading. There were 5 animals in each group on each day. The data are expressed as the mean ± SEM. *$P < 0.05$ compared the control group with the experimental group (after 3 or 7 days of orthodontic force). #$P < 0.05$ compared WT and CCR5$^{-/-}$ experimental groups, at the same moment. One-way ANOVA and Newman-Keuls multiple-comparison test.

Appendix Table 1. Primer Sequences and Reaction Properties

Target	Sense and Anti-sense Sequences	At* (°C)	Mt (°C)	bp
CCL2	AGGAAGATCTCAGTG CAGAG AGTCTTCGGAGTTTGCCTTTG	62	82	177
CCL3	TTCTGCTGACAAGCTCACCCT ATGGCGCTGAGAAGACTTGGT	60	79	322
CCL5	TTCCCTGTCATCGCTTGCTCT CGGATGGAGATGCCGATTTT	60	81	433
CCR1	TGCAGGTGACTGAGGTGATTG TGAAACAGCTGCCGAAGGTAC	58	79	103
CCR5	TTCCCTGTCATCGCTTGCTCT CGGATGGAGATGCCGATTTT	60	81	433
IL-10	Agatc tccgagatgc cttca Ccgtggagcaggtgaagaat	58	85	307
RUNX2	AACCACAGAACCACAAGTGCG AAATGACTCGGTTGGTCTCGG	58	80	119
OCN	AAGCCTTCATGTCCAAGCAGG TTTGTAGGCGGTCTTCAAGCC	60	78	170
OPG	GGAACCCCAGAGCGAAATACA CCTGAAGAATGCCTCCTCACA	57	77	225
RANKL	CAGAAGATGGCACTCACTGCA CACCATCGCTTTCTCTGCTCT	65	73	203
TRAF6	ATATGAACTCCCCAACCCCCT AAACCACGGCAGGAAACGA	58	75	124
Cathepsin K	CTCCCTCTCGATCCTACAGTAATGA TCAGAGTCAATGCCTCCGTTC	58	80	307
MMP-13	AGAGATGCGTGGAGAGTCGAA AAGGTTTGGAATCTGCCCAGG	65	85	162
β-actin	Atgtttgagaccttcaaca CACGTCAGACTTCATGATGG	56	75	495

*At, annealing temperature; Mt, melting temperature; bp, base pairs of amplicon size.

Appendix Table 2. Mean Values and Standard Deviations for Numbers of Osteoclasts Observed and Amounts of Tooth Movement

	WT		SHAM		$CCR5^{-/-}$	
	Mean	SD	Mean	SD	Mean	SD
Number of TRAP Cells						
0 day	0	0	0	0	0	0
3 day	0.8000	0.4472	0.6000	0.5477		
7 day	5.200	1.924	5.200	1.304	7.400	1.158
12 day	6.800	1.483	7.400	0.8944	12.76	1.257
Tooth Movement						
0 day	16.59	2.146	16.74	2.280	19.39	1.051
7 day	25.57	2.593	25.14	1.710	32.52	2.387
12 day	29.68	3.192	29.90	2.802	41.00	2.100

Appendix Table 3. CCR1 Expression Difference in WT and CCR5$^{-/-}$ Mice—ANOVA and Newman-Keuls Test

Parameter	Value		
Table Analyzed			
CCR1			
One-way analysis of variance			
P value	P < 0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	6		
F	277.4		
R-squared	0.9761		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	19.29		
P value	0.0017		
P value summary	**		
Do the variances differ signif.? (P < 0.05)	Yes		
ANOVA Table	SS	Df	MS
Treatment (between columns)	91.05	5	18.21
Residual (within columns)	2.232	34	0.06565
Total	93.29	39	
Newman-Keuls Multiple-comparison Test	Mean Diff.	Q	P value
CCR5$^{-/-}$ control vs. WT control	–0.1063	1.173	P > 0.05
WT 7 days vs. CCR5$^{-/-}$ 7 days	–0.4117	3.935	P < 0.01
WT 3 days vs. CCR5$^{-/-}$ 3 days	–0.4650	4.445	P < 0.01

2.2. Artigo 2

The effect of CCL3 and CCR1 in bone remodeling induced by mechanical loading

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(Artigo em preparação para submissão)

The effect of CCL3 and CCR1 in bone remodeling induced by mechanical loading

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ABSTRACT

Bone remodeling is affected by mechanical loading and inflammatory mediators, such as chemokines. Of these, CCL3 is involved in bone remodeling, being its cellular effects mediated by binding to CCR1 and CCR5 receptors, expressed in osteoclasts and osteoblasts. Our group has previously demonstrated that CCR5 down-regulates strain-induced bone resorption. Thus, the present study aimed to investigate the role of CCR1 and CCL3 in bone remodeling induced by mechanical loading. An orthodontic appliance, consisting of a Ni-Ti coil spring, was placed between incisors and first molar of CCL3-deficient mice ($CCL3^{-/-}$), wild type (WT) mice, mice treated with Met-RANTES (an antagonist of CCR5 and CCR1) and CCR1-deficient mice ($CCR1^{-/-}$). Histomorphometric analysis was used to determine the amount of orthodontic tooth movement and number of osteoclasts after 6 and 12 days of mechanical loading. The expression of bone remodeling markers was evaluated by Real-time PCR. Bone remodeling was significantly decreased in $CCL3^{-/-}$ mice, in $CCR1^{-/-}$ mice and in Met-RANTES-treated group. In accordance, the mRNA levels of RANK, RANKL, TNF-α and RANKL/OPG ratio were diminished in periodontium of $CCL3^{-/-}$ mice and mice treated with Met-RANTES. The Met-RANTES treatment also reduced the levels of Cathepsin K and MMP13. The expression of osteoblast markers was also affected by CCL3 deficiency and Met-Rantes treatment. Altogether, these findings suggest that CCR1 is pivotal for bone remodeling induced by mechanical loading and these actions depend, at least in part, on CCL3.

Key-words: CCL3, CCR1, bone remodeling, mechanical loading

INTRODUCTION

Osteoimmune response and mechanical loading are intimately related to the activity of bone resorbing osteoclasts and bone forming osteoblasts. Imbalances on this process of bone remodeling may lead to clinical disorders such as osteoporosis (Jones *et al*., 2011; Papachroni *et al.*, 2009). Several *in vitro* studies identify possible mechanisms through which mechanical loading is converted to biological responses (Cheung *et al*., 2011; Al-Dujaili *et al*., 2011). Nevertheless, there is a lack of data regarding the evaluation of *in vivo* consequences triggered by strain. In this context, the compression strain induces necrosis, hypoxia, physical damage to cells and bone resorption. In contrast, the tension force promotes angiogenesis, stretch of matrix cell and bone formation (Krishnan and Davidovitch, 2006; Krishnan and Davidovitch, 2009). These characteristics are not completely simulated in *in vitro* studies. Therefore, the use of *in vivo* models, which allow the evaluation of the effect of inflammation on bone remodeling induced by mechanical loading, such as the model of orthodontic tooth movement (OTM) (Andrade *et al*., 2009; Taddei *et al*., 2011), may be useful to study the connection between bone and immune system.

This inflammatory response induced by mechanical loading in periodontium is characterized by the early release of specific inflammatory mediators. These molecules induce bone resorption or formation around the teeth, depending on kind of strain applied (Krishnan and Davidovitch, 2006; Krishnan and Davidovitch, 2009). Among these, chemokines have pivotal role in strain-managed bone remodeling (Andrade *et al.*, 2009; Taddei *et al.*, 2011). Accordingly, it has been shown that the expression of CCL3 and its receptor CCR1 is increased in bone and soft tissues under mechanical loading (Andrade Jr. *et al*., 2009). As CCL3 is directly associated to the recruitment and activation of osteoclast precursor cells and of osteoblasts (Yu

et al., 2004; Yano *et al*., 2005; Silva *et al.*, 2007), new studies should investigate the role of this chemokine on bone remodeling induced by mechanical loading.

In this regard, CCR1 and CCR5 (receptors of CCL3) seem to exert significant pro-resorptive roles in infectious conditions involving bone loss, such as periodontal disease (Repeke *et al.*, 2010; Ferreira Jr. *et al.*, 2011). In contrast, CCR5 plays an opposite function in non-infectious scenario, controlling the resolution of inflammation in experimental arthritis (Doodes *et al*., 2009) and reducing bone resorption during OTM (Andrade Jr. *et al*., 2009). Therefore, it is important to investigate the effect of CCL3 and CCR1 in non-infectious bone remodeling induced by mechanical stimulus. This background would be useful to modulate side effects of OTM (Taddei *et al*., 2011) and to control non-infectious pathological bone loss in diseases such as osteoporosis and rheumatoid arthritis (Papachroni *et al.*, 2009; Shahrara *et al*., 2005).

Therefore, the aim of this study was to investigate the role of CCL3 and CCR1 on bone remodeling triggered by application of mechanical loading.

MATERIALS and METHODS

Experimental Animals

Twenty five ten-week-old wild-type (WT) (C57BL6/J), 25 CCL3 deficient mice ($CCL3^{-/-}$), 10 CCR1 deficient mice ($CCR1^{-/-}$) obtained from the Jackson Laboratory (Bar Harbor, ME, USA), five vehicle- (PBS) treated mice (Vehicle) and 25 Met-RANTES- (an antagonist of CCR1 and CCR5) treated mice (Met) were used in this experiment. All animals were treated under the ethical regulations for animal experiments, defined by the Institutional Ethics Committee. Each animal's weight was recorded throughout the experimental period, and there was no significant loss of weight.

Experimental Protocol

Induction of tooth movement was performed as previously described (Taddei *et al.*, 2011). Briefly, an orthodontic appliance consisting of a Ni-Ti 0.25 x 0.76 mm coil spring (Lancer Orthodontics, San Marcos, CA, USA) was bonded between maxillary right first molar and the incisors, exerting a force of 0.35 N applied in the mesial direction. There was no reactivation during the experimental period. This study was divided in 3 parts. In the first one, 2 groups were compared: WT and $CCL3^{-/-}$ mice. In the second part, it was evaluated vehicle- in relation to Met-RANTES-treated (s.c., 0,5 mg/Kg/day) mice. Moreover, $CCR1^{-/-}$ mice were compared to Wt mice. For histomorphometric analysis, the left side without appliance of maxilla was used as control. Two sub-groups were achieved for molecular analysis: control (mice without appliance) and experimental (with activated coil spring) groups. For histopathological analysis, mice were killed with an overdose of anesthetic after 6 and 12 days of

mechanical loading. For molecular examination, these groups were sacrificed at 0, 12 and 72 hr. For every set of experiments, 5 animals were used for each time-point.

Histopathological Analysis

The right and the left maxillae halves, including first, second and third molars were dissected and fixed in 10% buffered formalin (pH 7.4). After fixation, each hemimaxillae were decalcified in 14% EDTA (pH 7.4) for 20 days and embedded in paraffin. Samples were cut into sagittal sections of 5 µm thickness. Sections were stained for tartrate resistant acid phosphatase (TRAP; Sigma-Aldrich, Saint Louis, MO, USA), counterstained with hematoxylin, and used for histological examination. The first molar distal-buccal root, on the mesial periodontal site, was used for the osteoclasts counts, on 5 sections per animal. Osteoclasts were identified as TRAP-positive multinucleated cells sited on the bone surface. The slides were counted by two examiners blind of group status.

Measurement of Tooth Movement

Image J software (National Institutes of Health) was used to quantify the amount of tooth movement, as previously described (Taddei *et al*., 2011). Tooth movement was obtained through the difference between the distance of the cementum-enamel-junction's (CEJ's) of the first molar and the second molar (1st and 2nd molar distance) of the experimental side (right hemi-maxilla) in relation to the control side (left hemi-maxilla) of the same animal. Five vertical sections per animal were evaluated under a microscope Axioskop 40 (Carl Zeiss, Göttingen, Niedersachsen, Germany) adapted to a digital camera (PowerShot A620, Canon, Tokyo, Honshu,

Japan). Three measurements were conducted for each evaluation and the variability was below 5%.

RNA Extraction and Real-time PCR

Using a stereomicroscope, periodontal ligament and surrounding alveolar bone samples were extracted from the upper first molars. Gingival tissue, oral mucosa and tooth were discarded. These tissues were submitted to RNA extraction using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized using 2 µg of RNA through a reverse transcription reaction (Superscript II, Invitrogen). Real-time PCR analysis was performed in MiniOpticon (BioRad, Hercules, CA, USA) using SYBR-green fluorescence quantification system (Applied Biosystems, Foster City, CA, USA). Standard PCR conditions were 95ºC (10 min), and then 40 cycles of 94ºC (1 min), 58^{o}C (1 min) and 72 ºC (2 min), followed by the standard denaturation curve. Primer sequences are described in Table I.

The mean Ct values from duplicate measurements were used to calculate expression of the target gene, with normalization to a housekeeping gene (β-actin) using the $2^{-\Delta\Delta Ct}$ formula.

Statistical Analysis

Results in each group were expressed as the mean ± SEM. The differences among the groups were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. $P < 0.05$ was considered statistically significant.

RESULTS

The role of CCL3 in alveolar bone remodeling induced by mechanical loading

To understand the functions of CCL3 in bone metabolism, we used a model of bone remodeling induced by mechanical loading in $CCL3^{-/-}$ mice. Our first step was to analyze the alveolar bone histologic phenotype expressed by WT and $CCL3^{-/-}$ mice during OTM. The amount of tooth movement (Fig. 1a) and numbers of TRAP-positive osteoclasts (Fig. 1b) were increased after 6 and 12 days of orthodontic force in WT mice ($P < 0.05$). In comparison to WT mice, these histomorphometric analyses showed diminished tooth movement (Fig. 1a) and fewer TRAP-positive cells (Fig. 1b) in $CCL3^{-/-}$ mice at the same time points ($P < 0.05$). Moreover, alveolar bone morphology without orthodontic appliance (control side) presented increased TRAP activity on the distal side of the alveolar bone surface, while no activity was noted in the mesial region of the periodontium in both mice groups (Fig 1c and f), representing the physiological tooth movement in distal direction. On the other hand, the mechanical loading applied on the tooth in mesial direction after 6 days induced increased TRAP activity in the mesial site, reducing this parameter analyses in distal region. On day 12, TRAP activity seemed to increase more extensively in WT mice (Fig. 1d and g), which presented a greater alveolar bone resorption area than did $CCL3^{-/-}$ mice (Fig. 1e and h). These findings indicated that the bone resorption and osteoclast recruitment, induced by mechanical loading, are positively modulated by CCL3 chemokine.

Expression of bone remodeling-related markers in CCL3$^{-/-}$ mice

In view of CCL3$^{-/-}$ mice altered alveolar bone microscopic phenotype, we characterized the mRNA expression pattern of markers involved in bone resorption. The mechanical loading induced an increase of receptor activator of nuclear factor kappa-B (RANK) (Fig. 2a), receptor activator of nuclear factor kappa-B ligand (RANKL) (Fig. 2b) and tumor necrosis factor alpha (TNF-α) (Fig. 2c) mRNA levels in WT and CCL3$^{-/-}$ mice ($P < 0.05$). However, the expression of these molecules was reduced in CCL3$^{-/-}$ when compared with WT mice ($P < 0.05$) (Fig. 2a-c). There was no significant change in the mRNA levels of Cathepsin K (Fig. 2d) and metalloproteinase 13 (MMP13) (Fig. 2e) between both groups.

In addition, we further investigated if the lack of CCL3 could interfere with the transcriptional level of osteoblast markers and negative regulators of bone resorption-related markers. The expression levels of runt-related transcription factor 2 (RUNX2) (a transcription factor considered to be an early marker of osteoblast differentiation) was upregulated in periodontium of both groups after 12 hr of mechanical loading in WT mice, but it was reduced in CCL3$^{-/-}$ mice after 72 hr (Fig. 3a) ($P < 0.05$). There was no difference between the levels of osteocalcin (OCN) (a later marker of osteoblast differentiation and activity) (Fig. 3b), interleukin 10 (IL-10) (Fig. 3c) and osteoprotegerin (OPG) (Fig. 3d) in both groups. Moreover, the RANKL/OPG ratio was decreased in CCL3$^{-/-}$ mice after 12 hr of mechanical loading ($P < 0.05$), confirming the pro-resorptive role of CCL3 in this process (Fig. 3e). Therefore, these data demonstrated that CCL3 is involved in osteoclast and osteoblast differentiation during bone remodeling induced by orthodontic force.

The blockade of CCR1 and CCR5 alters alveolar bone microscopic phenotype after mechanical loading

Since CCL3 is ligand of CCR1 and CCR5, our next step was to blockage these receptors with Met-RANTES treatment, to evaluate its effect on bone remodeling incuced by mechanical loading. It was observed a reduction in amount of OTM (Fig. 4a) and numbers of TRAP-positive osteoclasts (Fig. 4b) in mice treated with Met-RANTES than in untreated mice ($P < 0.05$). The qualitative analysis of alveolar bone confirmed the diminished TRAP activity and bone resorption caused by Met-RANTES treatment (Fig. 4e and h).

Distinct expression of bone remodeling-related markers in mice treated with Met-RANTES

In order to elucidate if CCR1 alters the expression of bone resorption- and bone formation-related markers during OTM, we next measured the mRNA levels of these molecules in peridontium of Met-RANTES-treated mice. The results showed that the treatment with Met-RANTES reduced the expression of RANK (Fig. 5a), RANKL (Fig. 5b), TNF-α (Fig. 5c), Cathepsin K (Fig. 5d) and MMP13 (Fig. 5e) in periodontium of mice submitted to orthodontic force when compared with untreated mice in the same conditions ($P < 0.05$). Then, our next question was if this receptor also influenced osteoblast markers expression. The level RUNX2 (Fig. 6a) was reduced in Met-RANTES-treated mice only after 72 hr of mechanical loading ($P < 0.05$), whereas this treatment induced increasing expression of OCN after 12 and 72 hr (Fig. 6b) ($P < 0.05$). Unexpectedly, the expression of IL-10 (Fig. 6c) and OPG (Fig. 6d) was reduced in mice treated with Met-RANTES ($P < 0.05$). However, the RANKL/OPG

ratio was reduced with Met-RANTES treatment after 72 hr of mechanical loading ($P < 0.05$), confirming the anti-resorptive action of this drug (Fig. 6e).

CCR1 plays an important role in mechanical loading-induced bone resorption

Our previous study demonstrated that CCR5 has been associated with down regulation of bone resorption (Andrade Jr. *et al*., 2009). As the blockage of both CCR1 and CCR5 resulted in lower amount of OTM and numbers of TRAP-positive osteoclasts, our next step was to confirm if CCR1 is the key receptor of bone resorption induced by mechanical loading. For that, we used $CCR1^{-/-}$ mice. The amount of OTM (Fig. 7a) and numbers of TRAP-positive osteoclasts (Fig. 7b) were lower in $CCR1^{-/-}$ mice than in WT mice ($P < 0.05$). These results suggest that CCR1 might be the receptor responsible for osteoclast recruitment and bone resorption induced by mechanical loading.

DISCUSSION

Bone remodeling is a lifelong process, which involves the equilibrium between bone resorption and formation. This process might be modulated by osteoimmune response and mechanical loading (Jones *et al*., 2011; Papachroni *et al.*, 2009). In this context, chemokines have pivotal role in strain-induced bone remodeling (Andrade *et al.*, 2009; Taddei *et al.*, 2011). As the levels of CCL3 and CCR1 were increased in periodontium after orthodontic force (Andrade Jr. *et al*., 2009), the aim of present study was to evaluate the role of these chemokine and receptor in this scenario. Ours major findings demonstrated that the CCL3/CCR1 axis plays an important role in osteoclast recruitment, differentiation and activity during bone remodeling induced by mechanical loading. Moreover, the blockage of CCR1 was effective to control bone loss.

Our data demonstrated that CCL3 is a pro-resorptive chemokine in mechanical loading-induced bone remodeling. In accordance, previous studies demonstrated the role of CCL3 in osteoclast recruitment (Yu *et al.*, 2004), in increase of osteoclasts number and size during RANKL-induced osteoclastogenesis (Yu *et al.*, 2004; Okamatsu *et al*., 2004; Tsubaki *et al*., 2007) and in osteoclast activity (Okamatsu *et al*., 2004). In contrast, CCL3 does not affect the bone loss involved in the periodontal disease (Repeck *et al*., 2010). Therefore, it is important to note that the triggering factors (i.e., microbial factors vs. mechanical loading), the nature of inflammatory processes (i.e., chronic vs. transitory inflammation) can change the function of some inflammatory mediators in bone remodeling process (Ferreira Jr. *et al*., 2011). Reinforcing this hypothesis, recent studies demonstrated that CCR5 up-regulates infectious-related bone loss in periodontal diseases (Repeke *et al*., 2010; Ferreira Jr.

e*t al*., 2011), while this same receptor inhibits the bone resorption induced by mechanical loading (Andrade Jr. *et al*., 2009).

In line with the reduced bone resorption, the levels of pro-resorptive markers, such as RANK, RANKL and TNF-α, were decreased in CCL3$^{-/-}$ mice after mechanical loading. *In vitro* studies demonstrated that CCL3 increases the expression of RANKL by osteoblasts and induces osteoclast-osteoblast interaction, increasing osteoclast differentiation and consequently bone resorption (Watanabe *et al*., 2004; Tsubaki *et al*., 2007). In parallel, TNF-α is widely known to stimulate the progression of disorders associated with bone loss (Queiroz-Junior *et al*., 2011) and mechanical loading-induced bone resorption (Andrade Jr. *et al.,* 2007). It also triggers the release of other inflammatory mediators in stimulated tissues, including chemokines (Yu *et al*., 2004; Silva *et al.*, 2007). In this context, TNF-α has already been demonstrated to stimulate CCL3 production by osteoblasts (Yu *et al*., 2004). On the other hand, our findings showed that the transcription of TNF-α was also up-regulated by CCL3, showing other mecanism by which CCL3 contributes to strain-induced bone resorption.

To further strengthen our data, we used a pharmacological strategy with Met-RANTES, a CCL5 recombinant molecule, which specifically binds to CCR1 and CCR5, impairing the subsequent signaling and cellular response (Proudfoot *et al*., 1996). In the present study, we demonstrated that the blockage of CCR1 and CCR5 by Met-RANTES presented higher effectiveness when compared with the absence of CCL3 in the attenuation of bone resorption phenotype after mechanical loading. In this context, our results showed that Met-RANTES treatment not only reduced levels of the RANK/RANKL axis and TNF-α in bone resorption scenario like observed in CCL3$^{-/-}$ mice, but also decreased the Cathepsin K and MMP13 (proteases that

degrade bone matrix) expression. In accordance, Met-RANTES treatment results in reduced TNF-α and RANKL expression and osteolysis in bone lytic diseases, such as rheumatoid arthritis and periodontal disease (Shahrara *et al*., 2005, Repeke *et al*., 2011).

With these results, we hypothesize that CCR1 may positively modulate bone resorption, since previous data from our group indicated that CCR5 is a down-regulator receptor of bone resorption induced by mechanical loading (Andrade *et al*., 2009). Confirming this hypothesis, it was observed diminished amount of OTM and number of osteoclast in $CCR1^{-/-}$ mice. Thus, it seems that the interaction between CCL3 and CCR1 is the responsible axis for inducing bone resorption after mechanical loading. This is in line with the role of CCR1 in physiologic bone remodeling (Hoshino *et al.*, 2010), in bone loss associated with multiple myeloma metastasis (Vallet *et al*., 2007) and periodontal disease (Repeke *et al*., 2010).

Besides osteoclast, osteoblast also expresses CCR1 and CCR5 receptors (Yano *et al*., 2005). As differentiation and function of osteoblast are essential to bone remodeling process, we investigated the expression of osteoblast markers, RUNX2 and OCN (Liu *et al*., 2001). We observed a reduction in the levels of RUNX2 in $CCL3^{-/-}$ and Met-RANTES-treated mice. Moreover, the treatment with Met-RANTES concomitantly increased OCN expression. Then, our findings suggested that the blockage of both CCR1 and CCR5 receptors and absence of CCL3 affect the expression of osteoblast differentiation markers expression.

Our results indicated a reduction in the expression of IL-10 and OPG after treatment with Met-RANTES. However, this effect was not sufficient to induce greater tooth movement, probably, because the expression of pro-resorptive mediators (RANKL, RANK, TNF-α) was also impaired concomitantly. Reinforcing this hypothesis, the

reduced RANKL/OPG ratio confirms the anti-resorptive scenario after blockage of CCR1 receptor.

In summary, CCR1 is a pivotal receptor involved in osteoclast recruitment, differentiation and activity, resulting in development of a pro-resorptive bone scenario induced by mechanical loading. These actions are dependent, at least in part, on CCL3. Moreover, the blockage of CCR1 and CCR5, using Met-RANTES, might be a therapeutic strategy for reducing bone resorption, without affecting bone homeostasis. Therefore, an adequate pharmacological therapy coupled with mechanical loading-based treatments may modulate osteoclast and osteoblast activity and, thus, enhance the effectiveness of bone remodeling therapies.

ACKNOWLEDGMENTS

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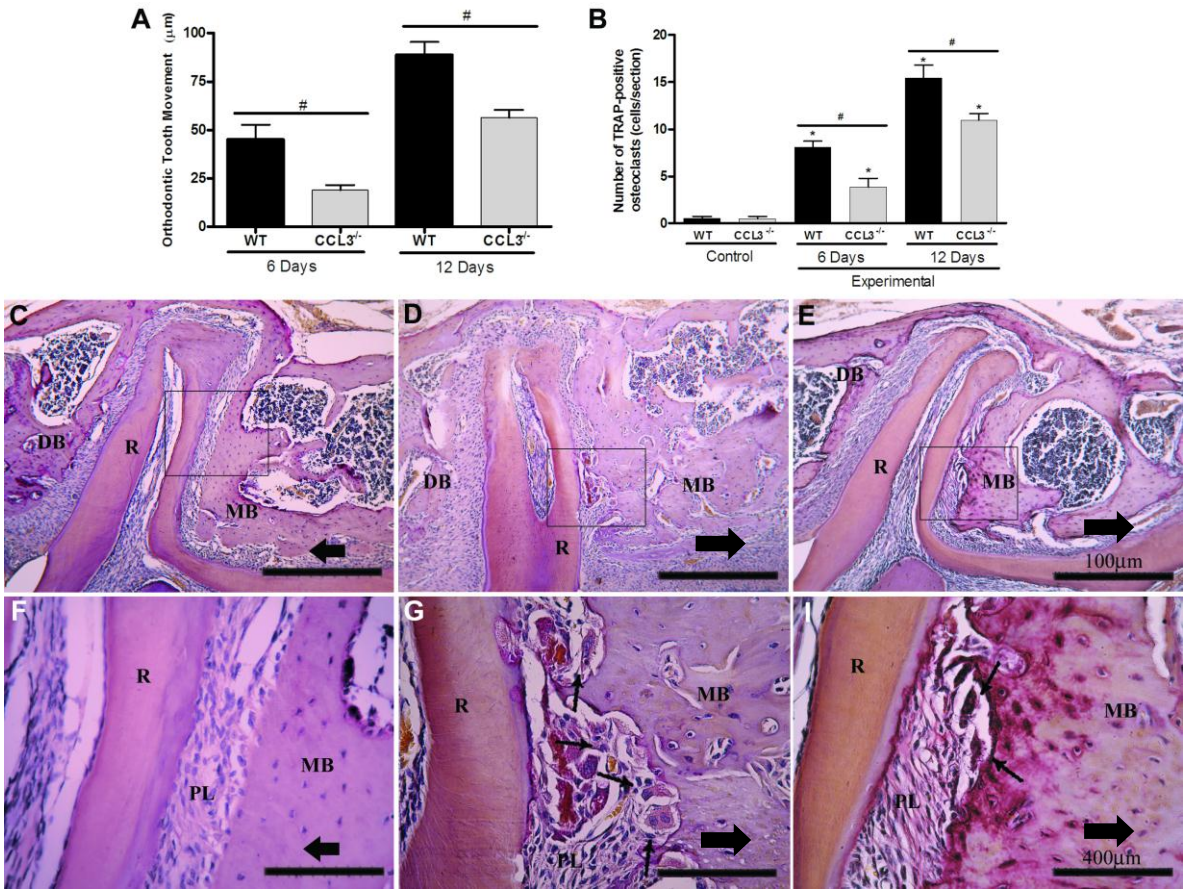

Fig. 1. (a) Time course of changes in the amount of tooth movement between WT and CCL3$^{-/-}$ mice. (b) Number of TRAP-positive osteoclasts. (c-h) Histological changes related to orthodontic tooth movement in WT and CCL3$^{-/-}$ mice. Sections of the periodontium around the disto-buccal root of the first molar were stained with TRAP. (c) control group (without mechanical loading). (d) WT and (e) CCL3$^{-/-}$ experimental group (12 days after mechanical loading). Panels (f), (g) and (h) represent the higher view of the identified area in (c), (d) and (e), respectively. Small arrows indicate TRAP-positive osteoclasts. MB, mesial alveolar bone; DB, distal alveolar bone; PL, periodontal ligament; R, root. Large arrows to left indicate the direction of physiological tooth movement. Large arrows to right indicate the direction of orthodontic tooth movement. Data are expressed as the mean ± SEM. *P < 0.05 comparing the control group to the respective experimental group. $^{\#}$P < 0.05 comparing WT and CCL3$^{-/-}$ experimental groups. One-way ANOVA and Newman-Keuls multiple comparison test. Bar = 100 μm or 400 μm.

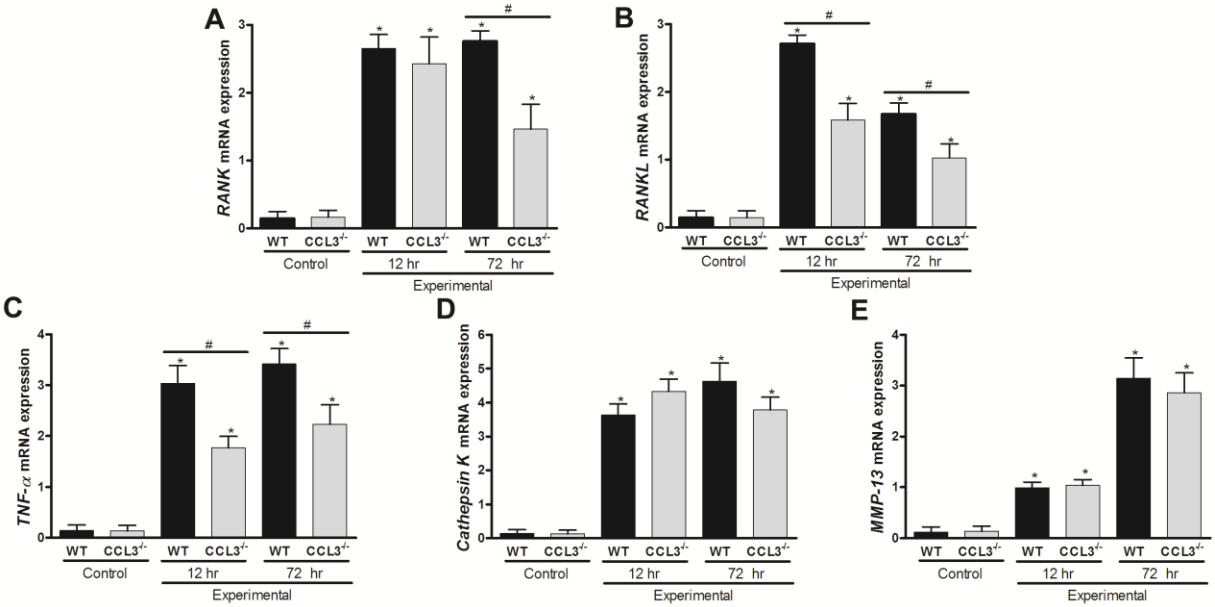

Fig. 2. mRNA expression of axis RANK (a)/ RANKL (b); TNF-α (c); and osteoclast-related markers Cathepsin K (d) and MMP13 (e) in WT and $CCL3^{-/-}$ periodontium after 12 and 72 hr of mechanical loading. Data are expressed as mean ± SEM. *P < 0.05 comparing control to the respective experimental group. $^{\#}$P < 0.05 comparing WT and $CCL3^{-/-}$ experimental groups. One-way ANOVA and Newman-Keuls multiple comparison test.

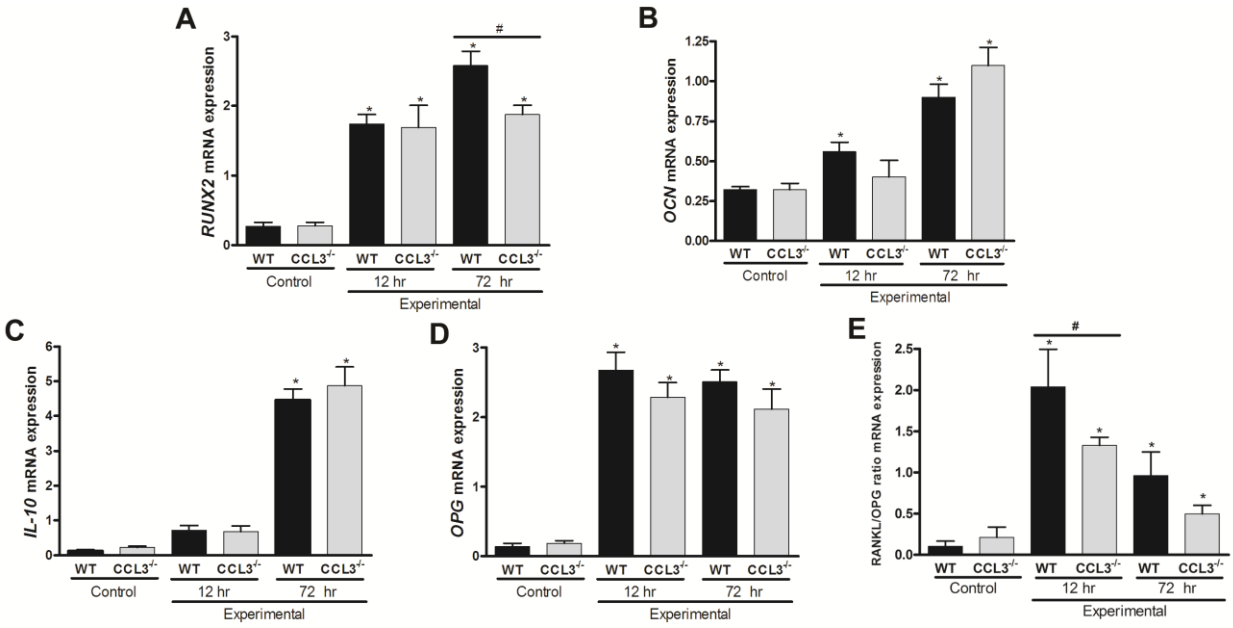

Fig. 3. mRNA expression of osteoblast-related markers RUNX2 (a) and OCN (b); down regulators of bone resorption-related markers IL-10 (c) and OPG (d); and RANKL/OPG ratio (e) in periodontium of WT and CCL3$^{-/-}$ mice after 12 and 72 hr of mechanical loading. Data are expressed as mean ± SEM. *P < 0.05 comparing control group to the respective experimental group. $^{\#}$P < 0.05 comparing WT and CCL3$^{-/-}$ experimental groups. One-way ANOVA and Newman-Keuls multiple comparison test.

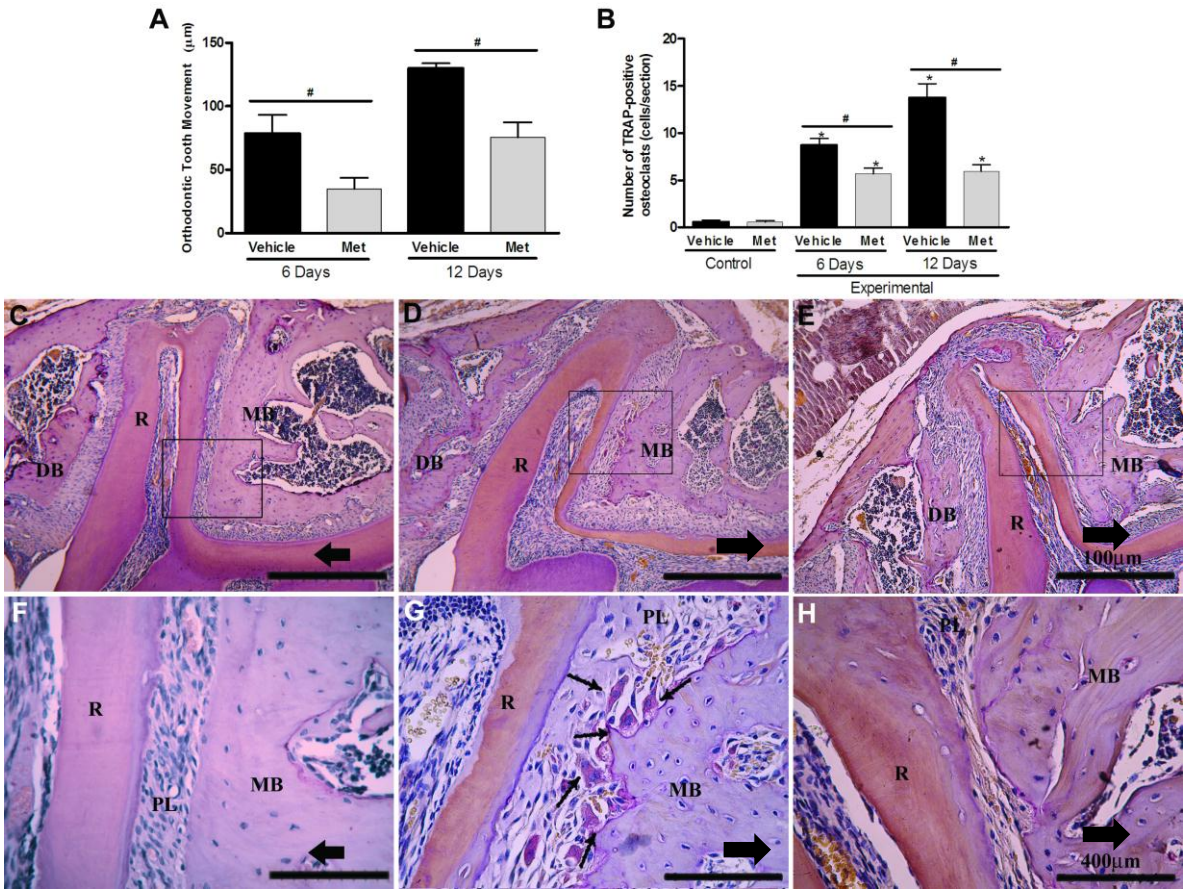

Fig. 4. (a) Time course of changes in the amount of tooth movement between vehicle- and Met-RANTES-treated mice. (b) Number of TRAP-positive osteoclasts. (c-h) Histological changes related to orthodontic tooth movement in vehicle- and Met-RANTES-treated mice. Sections of the periodontium around the disto-buccal root of the first molar were stained with TRAP. (c) control group (without mechanical loading). (d) vehicle and (e) Met-RANTES experimental group (12 days after mechanical loading). Panels (f), (g) and (h) represent the higher view of the identified area in (c), (d) and (e), respectively. Small arrows indicate TRAP-positive osteoclasts. MB, mesial alveolar bone; DB, distal alveolar bone; PL, periodontal ligament; R, root. Large arrows to left indicate the direction of physiological tooth movement. Large arrows to right indicate the direction of orthodontic tooth movement. Data are expressed as the mean ± SEM. *P < 0.05 comparing the control group to the respective experimental group. $^{\#}$P < 0.05 comparing vehicle and Met-RANTES experimental groups. One-way ANOVA and Newman-Keuls multiple comparison test. Bar = 100 µm or 400 µm.

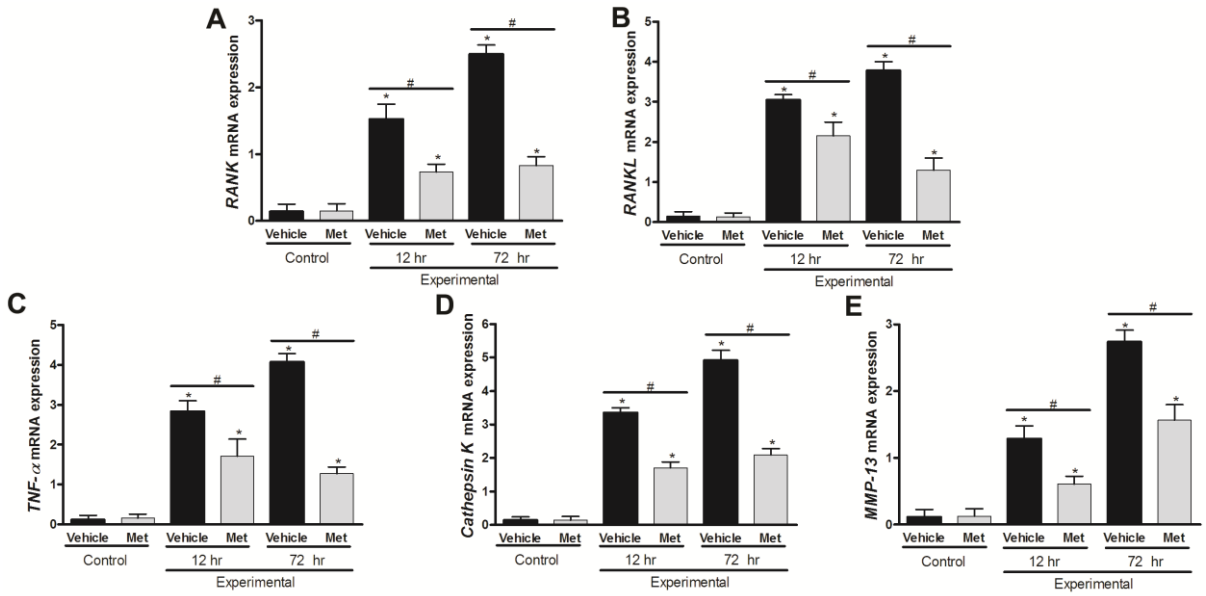

Fig. 5. mRNA expression of axis RANK (a)/ RANKL (b); TNF-α (c); and osteoclast-related markers Cathepsin K (d) and MMP13 (e) in periodontium of vehicle- and Met-RANTES-treated mice after 12 and 72 hr of mechanical loading. Data are expressed as mean ± SEM. *P < 0.05 comparing control to the respective experimental group. $^{\#}$P < 0.05 comparing vehicle and Met-RANTES experimental groups. One-way ANOVA and Newman-Keuls multiple comparison test.

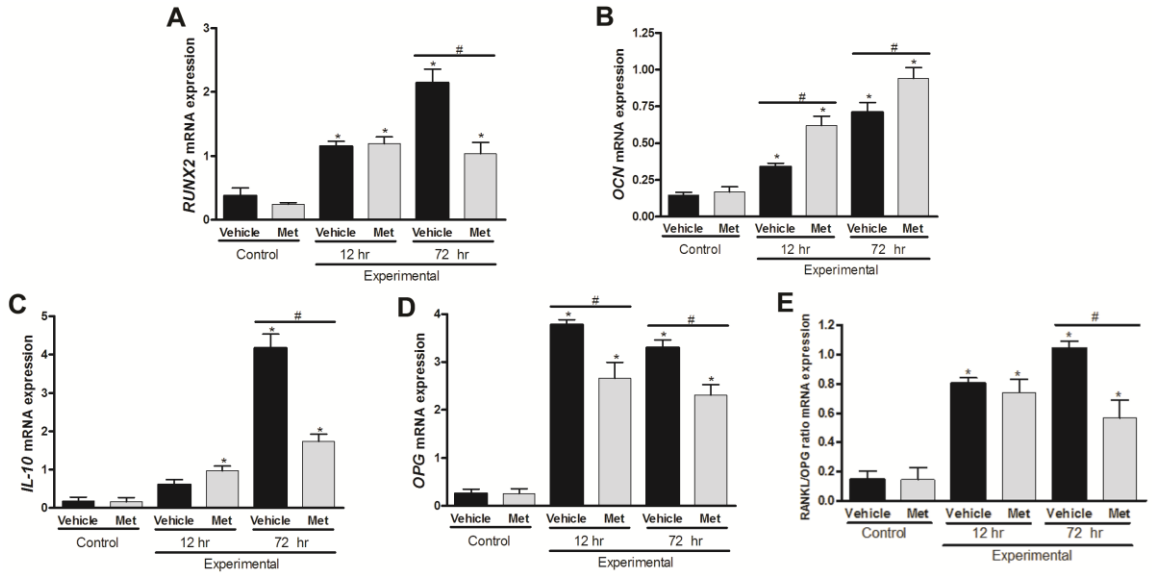

Fig. 6. mRNA expression of osteoblast-related markers RUNX2 (a) and OCN (b); down regulators of bone resorption-related markers IL-10 (c) and OPG (d); and RANKL/OPG ratio (e) in periodontium of vehicle- and Met-RANTES-treated mice after 12 and 72 hr of mechanical loading. Data are expressed as mean ± SEM. *P < 0.05 comparing control group to the respective experimental group. #P < 0.05 comparing vehicle and Met-RANTES experimental groups. One-way ANOVA and Newman-Keuls multiple comparison test.

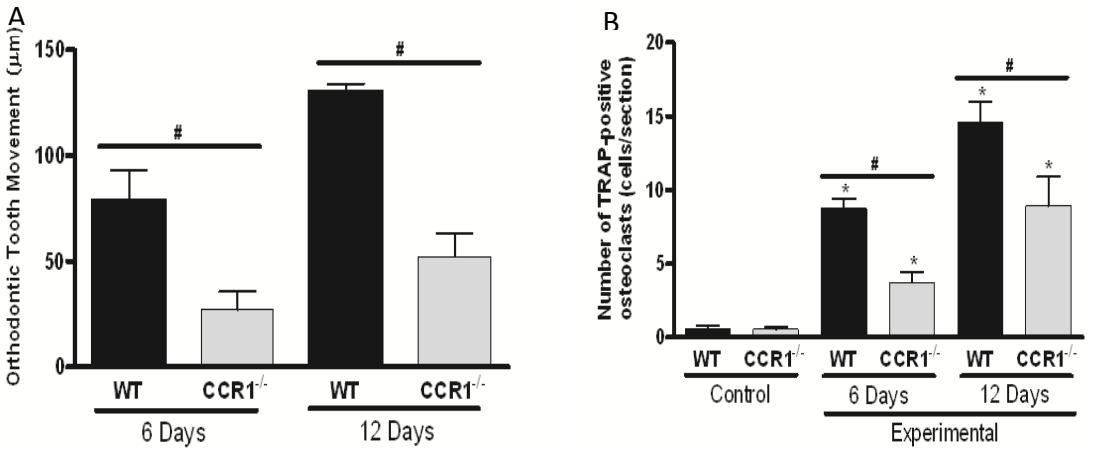

Fig. 7. (a) Time course of changes in the amount of tooth movement between WT and CCR1$^{-/-}$ mice. (b) Number of TRAP-positive osteoclasts. Data are expressed as the mean ± SEM. *P < 0.05 comparing the control group to the respective experimental group. $^{\#}$P < 0.05 comparing WT and CCR1$^{-/-}$ experimental groups. One-way ANOVA and Newman-Keuls multiple comparison test.

Table I. Primer sequences and reaction properties.

Target	*Sense and anti-sense sequences*	At (°C)	Mt (°C)	Bp
IL-10	AGATC TCCGAGATGC CTTCA CCGTGGAGCAGGTGAAGAAT	58	85	307
RUNX2	AACCACAGAACCACAAGTGCG AAATGACTCGGTTGGTCTCGG	58	80	119
OCN	AAGCCTTCATGTCCAAGCAGG TTTGTAGGCGGTCTTCAAGCC	60	78	170
OPG	GGAACCCCAGAGCGAAATACA CCTGAAGAATGCCTCCTCACA	57	77	225
RANKL	CAGAAGATGGCACTCACTGCA CACCATCGCTTTCTCTGCTCT	65	73	203
RANK	CAAACCTTGGACCAACTGCAC GCAGACCACATCTGATTCCGT	60	84	76
Cathepsin K	CTCCCTCTCGATCCTACAGTAATGA TCAGAGTCAATGCCTCCGTTC	58	80	307
MMP13	AGAGATGCGTGGAGAGTCGAA AAGGTTTGGAATCTGCCCAGG	65	85	162
TNF-α	TGT GCT CAG AGC TTT CAA CAA CTT GAT GGT GGT GCA TGA GA	58	80	124
β-actina	ATGTTTGAGACCTTCAACA CACGTCAGACTTCATGATGG	56	75	495

At: annealing temperature; Mt: Melting temperature; Bp: base pairs of amplicon size.

2.3 Artigo 3

Role of CCR2 in orthodontic tooth movement

Taddei SRA, Andrade Jr. I, Queiroz-Junior CM, Garlet TP, Garlet GP, Cunha FQ, Teixeira MM, Silva TA

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Thank you for resubmitting the revised version of your manuscript. I sent the most recent revision back to the original referees, who are now satisfied that all necessary changes have been made and they recommend acceptance and publication of your research in the AJO-DO. Congratulations.

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With kind regards,

Vincent G. Kokich
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Role of CCR2 in Orthodontic Tooth Movement

ABSTRACT

Introduction: Cytokines and chemokines regulate bone remodeling during orthodontic tooth movement (OTM). CC chemokine ligand 2 (CCL2) is involved in osteoclast recruitment and activity and its expression is increased in periodontal tissues under mechanical loading. This study investigated whether the CC chemokine receptor 2 (CCR2)/CCL2 axis influences OTM. **Methods:** A coil spring was placed in CCR2 deficient ($CCR2^{-/-}$), wild-type (WT), vehicle treated (vehicle), and P8A (CCL2 analog) treated mice. In a histopathological analysis, the amount of OTM and numbers of osteoclast were determined. The expression of mediators involved in bone remodeling was evaluated by Real-Time PCR. **Results:** OTM and the number of TRAP-positive cells were significantly decreased in $CCR2^{-/-}$ and P8A mice in relation to wild-type (WT) and vehicle treated mice, respectively. The expression of RANKL, RANK and osteoblasts markers (COL-1 and OCN) was lower in $CCR2^{-/-}$ than in WT. No significant difference was found in OPG levels between the groups. **Conclusions:** These data suggested a reduction of osteoclast and osteoblast activities in the absence of CCR2. In conclusion, CCR2/CCL2 axis is positively associated to osteoclast recruitment, bone resorption and OTM. Therefore, the blockage of CCR2/CCL2 axis might be used in the future for modulating the extent of OTM.

Key words: orthodontic tooth movement – bone remodeling – chemokines

INTRODUCTION

Orthodontic tooth movement is achieved by remodeling of periodontal ligament and alveolar bone in response to mechanical stimulation. Bone is resorbed by osteoclasts on the pressure sites and it is formed by osteoblasts on the tension sites.[1,2] This process is regulated by no-infectious and transient inflammatory response that is characterized by releasing of several mediators, such as cytokines and chemokines.[3-8]

Chemokines, a large family of chemotactic cytokines, provide key signals for trafficking, differentiation and activity of bone cells.[9,10] The CC chemokine ligand 2 (CCL2, formerly known as monocyte chemotatic protein-1, MCP-1) has been found to promote chemotaxis, differentiation and activation of osteoclasts.[11-16] The cellular effects of CCL2 are mediated by its engagement with the CC chemokine receptor 2 (CCR2),[17] which is expressed by osteoclast precursors.[13-15] In addition, CCL2 expression is greatly increased in periodontal tissues submitted to orthodontic loading,[3,4,6,7] as well as in other inflammatory conditions such as rheumatoid arthritis,[18] bone cancer metastasis,[19] periodontal disease[20,21] and periapical osteolysis.[22]

Studies *in vitro* and *in vivo* demostrated that the blockage or absence of CCR2 significantly prevents bone resorption in experimental arthritis,[23,24] osteoporosis[15] and bone fracture healing.[25] Although the expression of CCL2 has been shown in periodontium submitted to orthodontic force,[3,4,6,7] the functional role of CCL2 and CCR2 in orthodontic tooth movement is not known. This study aimed to investigate the role of CCR2/CCL2 axis in osteoclast recruitment and activity using a well-established mouse model of orthodontic tooth movement. We hypothesized that the

CCR2/CCL2 axis would contribute significantly to osteoclast recruitment and consequently, to orthodontic tooth movement.

MATERIALS and METHODS

Experimental Animals

Twenty five ten-week-old wild-type (WT) (C57BL6/J), 25 CCR2 deficient mice ($CCR2^{-/-}$) obtained from the Jackson Laboratory (Bar Harbor, ME), five vehicle (PBS) treated mice (Vehicle) and 15 P8A (a monomeric variant of the chemokine CCL2 able to inhibit CCR2-mediated leukocyte recruitment) treated mice (P8A) were used in this experiment. CCR2 knockout mice have been previously bred into the C57BL/6 background for 9 generations. In the genome of CCR2 knockout mice, the entire coding region except the first 39 nucleotides and 5' untranslated region of the CCR2 gene in chromosome 9 were recombined with the neomycin-resistant gene (the coding region and 3' untranslated region are replaced with a polII-neo cassette). No expression of CCR2 has been observed in this mouse. Absence of transcript was confirmed by RT-PCR using mRNA isolated from spleens and thioglycolate elicited peritoneal exudate cells of homozygous mutant animals. Overall, mice that are homozygous for the targeted mutation are viable, fertile, normal in size and do not display any gross physical or behavioral abnormalities.[26]

All animals were treated under the ethical regulations for animal experiments, defined by the Institutional Ethics Committee. Each animal's weight was recorded throughout the experimental period, and there was no significant loss of weight.

Experimental Protocol

Induction of tooth movement was performed as previously described.[7] Briefly, mice were anesthetized i.p. with 0.2 mL of a solution containing xylazine (0.02 mg/mL) and ketamine (50 mg/mL). An orthodontic appliance consisting of a Ni-Ti 0.25 x 0.76 mm coil spring (Lancer Orthodontics, San Marcos, CA, USA) was bonded by a light cured resin (Transbond, Unitek/3M, Monrova, CA, USA) between maxillary right first molar and the incisors (Fig 1). The magnitude of force was calibrated by a tension gauge (Shimpo Instruments, Itasca, IL, USA) to exert a force of 35 g applied in the mesial direction. There was no reactivation during the experimental period. This study was divided in 2 parts. In the first one, named general, 2 groups were compared: WT and $CCR2^{-/-}$ mice. In the second part, named specific, 4 groups were evaluated: vehicle and P8A injected groups at 3 different doses (administration s.c. of 0.5, 1.5 and 3.0 mg/kg/day)). For histomorphometric analysis, the left side without appliance of maxilla was used as control. Two sub-groups were achieved for molecular analysis: control (mice without appliance) and experimental (with activated coil spring) groups. For histopathological analysis, WT and $CCR2^{-/-}$ groups were killed with an overdose of anesthetic after 6 and 12 days of mechanical loading (Appendix Table I). For molecular examination, these groups were sacrificed at 0, 12 and 72 hr (Appendix Table II). Vehicle and P8A treated groups (at the 3 different doses) were killed after 12 days of orthodontic force for histomorphometric analysis (Appendix Table I). For every set of experiments, 5 animals were used for each time-point.

Histopathological Analysis

The right and the left maxillae halves, including first, second and third molars were dissected and fixed in 10% buffered formalin (pH 7.4). After fixation, each

hemimaxillae were decalcified in 14% EDTA (pH 7.4) for 20 days and embedded in paraffin. Samples were cut into sagittal sections of 5 µm thickness. Sections were stained for tartrate resistant acid phosphatase (TRAP; Sigma-Aldrich, Saint Louis, MO, USA), counterstained with hematoxylin, and used for histological examination. The first molar distal-buccal root, on the mesial periodontal site, was used for the osteoclasts counts, on 5 sections per animal. Osteoclasts were identified as TRAP-positive, multinucleated cells sited on the bone surface. The slides were counted by two examiners, and the intraclass correlation coefficient showed average measures of 0.977, validating the measurement.

Measurement of Tooth Movement

Image J software (National Institutes of Health) was used to quantify the amount of tooth movement, as previously described.[7] Tooth movement was obtained through the difference between the distance of the cementum-enamel-junction's (CEJ's) of the first molar and the second molar (1st and 2nd molar distance) of the experimental side (right hemi-maxila) in relation to the control side (left hemi-maxila) of the same animal. Five vertical sections per animal were evaluated under a microscope Axioskop 40 (Carl Zeiss, Göttingen, Niedersachsen, Germany) adapted to a digital camera (PowerShot A620, Canon, Tokyo, Honshu, Japan). Three measurements were conducted for each evaluation and the variability was below 5%.

RNA Extraction and Real-time PCR

Using a stereomicroscope, periodontal ligament and surrounding alveolar bone samples were extracted from the upper first molars. Gingival tissue, oral mucosa and tooth were discarded. These tissues were sujbected to RNA extraction and Real

Time-PCR to evaluate the expression of molecules known to regulate osteoclast function (receptor activator of nuclear factor kappa-B (RANK), receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG)) and osteoblast markers (osteocalcin (OCN) and colagen-1 (COL-1)). RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized using 2 µg of RNA through a reverse transcription reaction (Superscript II, Invitrogen). Real-time PCR analysis was performed in MiniOpticon (BioRad, Hercules, CA, USA) using SYBR-green fluorescence quantification system (Applied Biosystems, Foster City, CA, USA). Standard PCR conditions were 95 ºC (10 min), and then 40 cycles of 94 ºC (1 min), 58 ºC (1 min) and 72 ºC (2 min), followed by the standard denaturation curve. Primer sequences are described in the Appendix Table III.

The mean Ct values from duplicate measurements were used to calculate expression of the target gene, with normalization to a housekeeping gene (β-actin) using the $2^{-\Delta\Delta Ct}$ formula.

Statistical Analysis

Results in each group were expressed as the mean ± SEM. As the data sets presented a normal distribution, differences among the groups were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. $P < 0.05$ was considered statistically significant.

RESULTS

The Amount of Tooth Movement and The Number of TRAP-positive Cells

Histomorphometric results showed that the amount of tooth movement (Fig 2, A and Table I) and numbers of TRAP-positives osteoclasts (Fig 2, B) were increased after 6 and 12 days of mechanical loading in WT mice ($P < 0.05$). On the other hand, a diminished tooth movement (Fig 2, A and Table I) and fewer TRAP-positive cells (Fig 2, B) were observed in $CCR2^{-/-}$ mice after 12 days ($P < 0.05$). There was no significant difference between the groups after 6 days of mechanical loading. Moreover, microscopic analysis revealed that, in the control side, TRAP activity was found on the distal side of the alveolar bone surface, and no activity was observed in the mesial region of the periodontium (Fig 2, C). After 6 days of orthodontic loading, there appeared to be an increase in TRAP activity on the mesial periodontium of the distobuccal root (the side of pressure) and a reduction on the distal side of this root (the side of tension). On day 12, TRAP activity appeared to increase more extensively in WT mice (Fig 2, D and F), which presented a greater alveolar bone resorption area than did $CCR2^{-/-}$ mice (Fig 2, E and G). In contrast, a wide hyalinized area on the mesial side was observed in $CCR2^{-/-}$ mice (Fig 2, F and G).

In order to investigate the importance of CCR2 ligand (CCL2) in this model, we further analyzed whether P8A, a CCL2 monomeric variant that is able to inhibit CCR2-mediated leukocyte recruitment, would also change the amount of tooth movement and osteoclast recruitment. Tooth movement (Fig 3, A) and the number of TRAP-positive osteoclasts (Fig 3, B) were reduced in P8A-treated mice in a dose-dependent way (Fig 3, A and B) when compared to mice treated with vehicle ($P < 0.05$).

Expression of Osteoclast and Osteoblast Markers

To understand the mechanisms involved in the altered bone remodeling of $CCR2^{-/-}$ mice during orthodontic tooth movement, we also evaluated the expression of osteoclasts regulators (RANK, RANKL and OPG) and osteoblast markers (OCN and COL-1). RANK and RANKL mRNA levels were significantly increased after mechanical loading ($P < 0.05$), but were smaller in $CCR2^{-/-}$ than in WT mice ($P < 0.05$) (Fig 4, A and B). The mechanical stress up-regulated OPG levels but the increase was similar in WT and $CCR2^{-/-}$ mice (Fig 4, C). Expression of OCN and COL-1 was significantly increased after mechanical loading ($P < 0.05$), but it was of lower in $CCR2^{-/-}$ than in WT mice ($P < 0.05$) (Fig 5, A and B).

DISCUSSION

We have previously observed increased CCL2 expression during orthodontic tooth movement.[4,7] In the present study, the functional role of CCL2 and CCR2 was evaluated. Our major findings were a reduced number of osteoclasts and a diminished tooth movement when CCL2 interactions were absent (CCR2$^{-/-}$ mice) or antagonized (P8A-treated mice). CCR2 deficiency was associated with lower expression of RANKL, RANK and osteoblasts markers (COL-1 and OCN), reinforcing the role of CCL2/CCR2 interactions in driving bone remodeling during orthodontic tooth movement.

Several studies have shown increased CCL2 expression during orthodontic tooth movement[3,4,6,7] as well as in other sites of bone remodeling, such as rheumatoid arthritis,[18] periodontal disease[20,21] and bone metastasis,[19] in which osteoclastogenesis is highly stimulated. As the cellular effects of CCL2 might be mediated by CCR2,[17] its absence might interfere with osteoclast differentiation and, consequently, with bone remodeling.[15] Our results suggest that not only CCL2 is expressed but also the CCL2/CCR2 axis plays a significant role in osteoclast recruitment and bone resorption in orthodontic tooth movement. Although previous studies have already shown increased bone mineral density in CCR2$^{-/-}$ mice, which makes these animals more resistant to compressive loadings,[15] this does not seem to fully explain the decreased orthodontic tooth movement in these animals. Indeed, similar results were observed after treatment with a CCL2 inhibitor in WT mice, arguing that an innate effect in bone physiology could not explain the results observed. In contrast, the diminished CCL2/CCR2-mediated osteoclast recruitment

and differentiation could account for our observations, an effect also seen in a model of osteoporosis model *in vivo*.[15]

The results obtained in animals treated with P8A, a monomeric variant of CCL2 which inhbits CCR2-dependent cell migration *in vivo*,[27] suggested that CCL2 is the most important CCR2 ligand in this model. The results reported a smaller amount of tooth movement and fewer numbers of TRAP-positive osteoclasts in P8A treated mice after mechanical loading. In accordance, P8A reduced bone lesions in rats with arthritis.[28] Moreover, $CCL2^{-/-}$ mice have been shown to present a high bone mass phenotype owing to a smaller number of osteoclasts.[15] Taken all together, these data confirm that the CCL2/CCR2 axis is involved in the recruitment of osteoclast precursors and, consequently, in orthodontic tooth movement. Therefore, this effect does not seem to result from the differences in the bone density observed in $CCR2^{-/-}$ mice, but from the reduction of osteoclast recruitment due to the lack of CCR2, since WT mice treated with P8A and $CCR2^{-/-}$ mice presented similar results.

Osteoclastogenesis and bone resorption activity are up-regulated by RANKL, produced by osteoblast/stroma cells, through their binding to their receptor RANK expressed in osteoclast progenitor cells.[29] This process can be inhibited by the decoy receptor OPG, which avoids RANK-RANKL engagement.[29,30] In order to understand the molecular basis of the impaired osteoclast differentiation and activity in the absence of CCR2, these osteoclast regulators were analyzed. The expression of both RANKL and RANK was decreased in $CCR2^{-/-}$ mice when compared to WT mice, while there was no significant difference in the OPG levels between both groups. In accordance, previous studies showed that CCR2 deficiency decreases RANK expression by preosteoclasts,[15] and reduces osteoclastic bone resorption *in vitro* and *in vivo* models.[15,25] Moreover, treatment with P8A reduced RANKL levels and bone

erosion in rats with arthritis.[28] Taken together, our results suggest that the observed reduction in the number of osteoclasts, led to less bone resorption and, consequently, to a diminished amount orthodontic tooth movement in CCR2$^{-/-}$, which might be related to a down-regulation of RANKL/RANK gene expression.

Considering that osteoblasts may interact with osteoclasts and regulate bone remodeling,[29,30] we also evaluated the expression of osteoblasts markers. In agreement with other studies,[5,6,7] our results demonstrated a significant increase of COL-1 and OCN mRNA expression in periodontal tissues of WT mice after orthodontic tooth movement. Nevertheless, COL-1 and OCN expression was lower in CCR2$^{-/-}$ mice than in WT mice, suggesting that osteoblast differentiation and activity were decreased in the absence of CCR2. This reduced osteoblast activity may be linked to a decrease of osteoclasts stimulatory signals (as RANKL), resulting in diminished bone resorption in CCR2$^{-/-}$ mice. On the other hand, in an osteoporosis model, both bone formation and OCN levels in serum were not changed in CCR2$^{-/-}$ mice when compared to WT mice.[15] Further research is required to confirm the role of CCR2 in differentiation and activity of osteoblasts.

CONCLUSIONS

1. The absence of CCR2 decrease osteoclast chemoattraction and decreased osteoclast and osteoblast activities, leading to reduced tooth movement. This is the first demonstration that CCR2 plays an important role in bone remodeling during orthodontic tooth movement.
2. CCL2 is the primary CCR2 ligand, and plays a central role in osteoclast recruitment and, consequently, in orthodontic tooth movement.

3. The blockade of CCR2/CCL2 axis might be used for future therapeutic interventions, limiting inflammatory bone loss diseases, such as osteoporosis and rheumatoid arthritis, or modulating the extent of orthodontic tooth movement.

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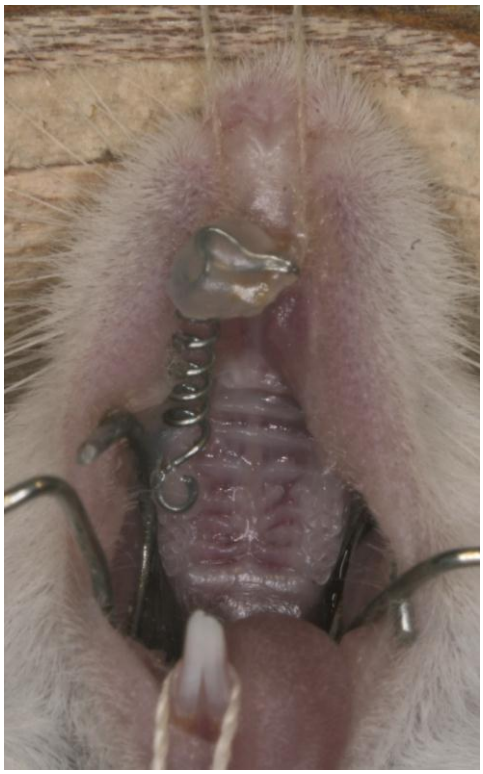

Fig 1. Occlusal view of a nickel-titanium (Ni-Ti) open coil spring placed between the upper right first molar and the incisors.

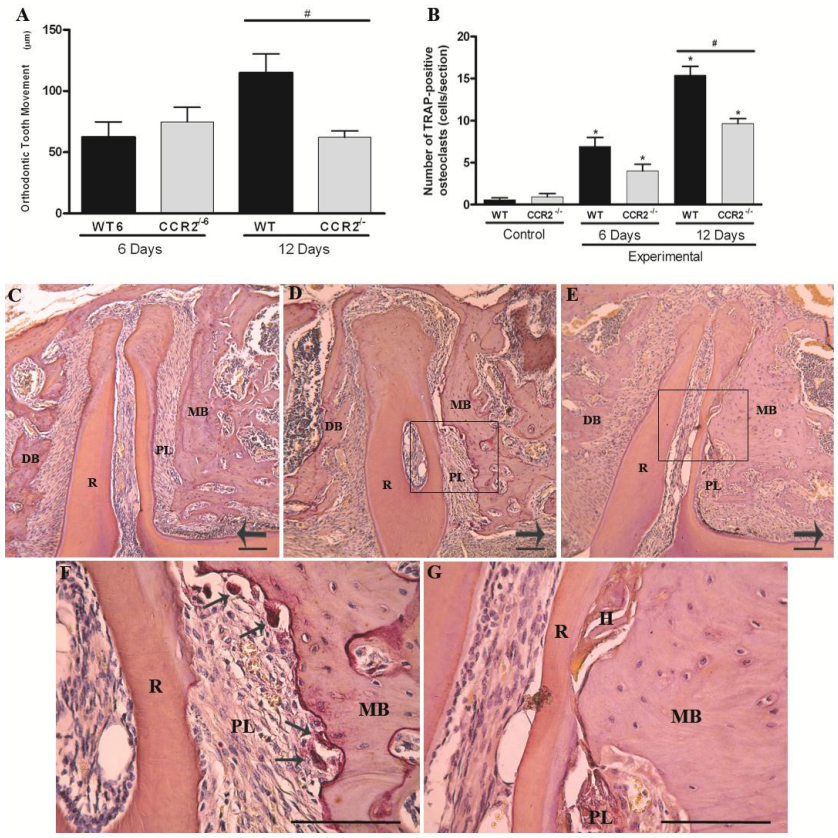

Fig 2. (A) Time course of changes in the amount of tooth movement between WT and CCR2$^{-/-}$ mice. (B) Number of TRAP-positive osteoclasts. (C-G) Histological changes related to orthodontic tooth movement in WT and CCR2$^{-/-}$ mice. Sections of the periodontium around the disto-buccal root of the first molar were stained with TRAP. (C) control group (without mechanical loading). (D) WT and (E) CCR2$^{-/-}$ experimental group (12 days after mechanical loading). Higher view of the identified area in D (F) and in E (G). Small arrows indicate TRAP-positive osteoclasts. MB, mesial alveolar bone; DB, distal alveolar bone; PL, periodontal ligament; R, root; H, hyalinized area. Large arrows indicate the direction of tooth movement. Data are expressed as the mean ± SEM. *P < 0.05 comparing the control group to the respective experimental group. $^{\#}$P < 0.05 comparing WT and CCR2$^{-/-}$ experimental groups. One-way ANOVA and Newman-Keuls multiple comparison test. Bar = 100 µm.

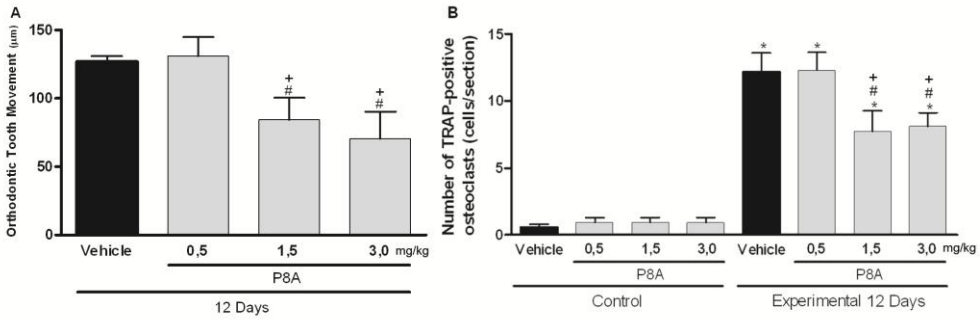

Fig 3. (A) Effect of different doses of P8A in the amount of tooth movement and (B) number of TRAP-positive osteoclasts. Data are expressed as the mean ± SEM. *P < 0.05 comparing the control group to the respective experimental group. #P < 0.05 compared vehicle and P8A experimental groups. +P < 0.05 comparing P8A 0.5 mg/Kg/day to the two other P8A-treated groups (1.5 and 3.0 mg/kg/day). One-way ANOVA and Newman-Keuls multiple comparison test.

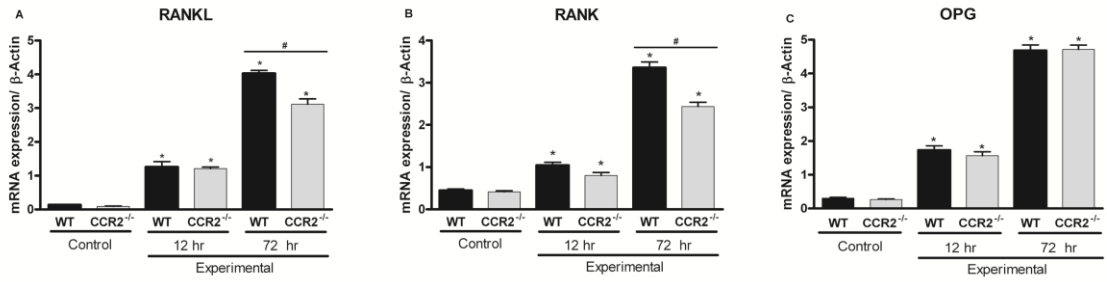

Fig 4. mRNA expression of osteoclast differetiation and activity markers RANKL (A), RANK (B) and OPG (C) in WT and CCR2$^{-/-}$ periodontium after 12 and 72 hr of mechanical loading. Data are expressed as mean ± SEM. *P < 0.05 comparing control to the respective experimental group. #P < 0.05 comparing WT and CCR2$^{-/-}$ experimental groups. One-way ANOVA and Newman-Keuls multiple comparison test.

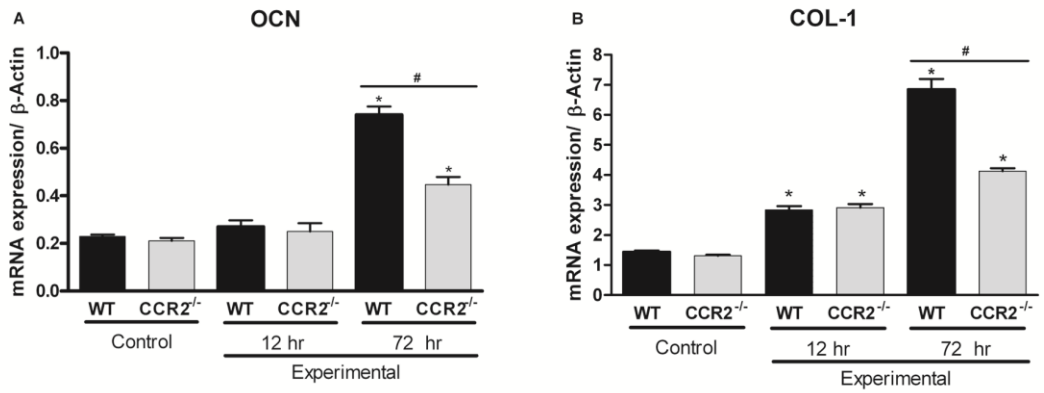

Fig 5. mRNA expression of osteoblastic markers OCN (A) and COL-1 (B) in WT and CCR2$^{-/-}$ periodontium after 12 and 72 hr of mechanical loading. Data are expressed as mean ± SEM. *P < 0.05 comparing control group to the respective experimental group. $^{\#}$P < 0.05 comparing WT and CCR2$^{-/-}$ experimental groups. One-way ANOVA and Newman-Keuls multiple comparison test.

Table I. Time course of changes in the amount of tooth movement between WT and CCR2$^{-/-}$ mice.

	Orthodontic Tooth Movement		
	WT (mean ± SEM)	CCR2$^{-/-}$ (mean ± SEM)	P valor
6 Days	62.5 ± 12.5	75 ± 11.9	P > 0.05
12 Days	115 ± 15.5	62.5 ± 4.7	P < 0.05

Appendix Table I. Number of mice in each group for histopathological analysis.

	Histopathological Analysis					
	WT	CCR2$^{-/-}$	Vehicle	P8A 0,5 mg/Kg	P8A 1,5 mg/Kg	P8A 3,0 mg/Kg
6 Days	**5**	**5**	**-**	**-**	**-**	**-**
12 Days	**5**	**5**	**5**	**5**	**5**	**5**

Appendix Table II. Number of mice in each group for molecular analysis.

	Real Time-PCR	
	WT	CCR2$^{-/-}$
0 hr	**5**	**5**
12 hr	**5**	**5**
72 hr	**5**	**5**

Appendix Table III. Primer sequences and reaction properties.

Target/ GI	Forward or Reverse sequences	At(°C)	Mt(°C)	Bp
RANK GI:110350008	(F) 5'-CAAACCTTGGACCAACTGCAC-3' (R) 5'-GCAGACCACATCTGATTCCGT-3'	60	84	76
OPG GI:2072182	(F) 5'-GGAACCCCAGAGCGAAATACA-3' (R) 5'-CCTGAAGAATGCCTCCTCACA-3'	57	77	225
RANKL GI:114842414	(F) 5'-CAGAAGATGGCACTCACTGCA-3' (R) 5'-CACCATCGCTTTCTCTGCTCT-3'	65	73	203
OCN GI:508299	(F) 5'-AAGCCTTCATGTCCAAGCAGG-3' (R) 5'-TTTGTAGGCGGTCTTCAAGCC-3'	60	78	170
COL-1 GI:118131144	(F) 5'-AATCACCTGCGTACAGAACGG-3' (R) 5'-CAGATCACGTCATCGCACAAC-3'	62	84	114
β-actin GI:145966868	(F) 5'-ATGTTTGAGACCTTCAACA-3' (R) 5'-CACGTCAGACTTCATGATGG-3'	56	75	495

At: annealing temperature; Mt: Melting temperature; Bp: base pairs of amplicon size.

3. DISCUSSÃO

A remodelação óssea é um processo fisiológico, responsável pela manutenção do *turnover ósseo*. Este processo ocorre por meio de uma ação coordenada das células ósseas, resultando em reabsorção óssea por osteoclastos e deposição óssea por osteoblastos. Um desequilíbrio entre estes dois processos pode resultar em diversas alterações ósseas (Jones *et al*., 2011; Papachroni *et al.*, 2009). Os estudos apresentados nesta tese pertencem à linha de pesquisa intitulada "**Mecanismos envolvidos na reabsorção/remodelação óssea alveolar em modelos experimentais**". O desenvolvimento desta linha de pesquisa iniciou-se em 2005 com a criação do modelo experimental de movimentação dentária ortodôntica em camundongos (Andrade Jr. *et al*., 2007a). A movimentação dentária ortodôntica é realizada através do remodelamento do osso alveolar e ligamento periodontal (Krishnan e Davidovitch, 2006; Krishnan e Davidovitch, 2009). A alteração da homeostase no periodonto, provocada pela força ortodôntica, desencadeia a formação de um ambiente pró-reabsortivo nas áreas de pressão e anti-reabsorptivo nas áreas de tensão, induzindo reabsorção e formação óssea alveolar, respectivamente (Krishnan e Davidovitch, 2006; Krishnan e Davidovitch, 2009). Esta remodelação óssea é resultado do surgimento do processo inflamatório asséptico transitório no ligamento periodontal, caracterizada pela liberação de vários mediadores, tais como: neurotransmissores, fatores de crescimento, quimiocinas e citocinas (Krishnan e Davidovitch, 2006). O aumento do nível de quimiocinas e citocinas na movimentação dentária ortodôntica tem sido demonstrado em modelo animal (Alhashimi et al., 1999; Andrade et al., 2007b; Andrade et al., 2009) e em humanos (Garlet et al., 2007; Garlet et al., 2008), tais como TNF-α, IL-10, CCL2,

CCL3, CCL5, CXCL12. Porém, poucos estudos têm investigado o papel específico de cada citocina e/ou quimiocina neste processo. Portanto, o objetivo geral desta tese foi avaliar o papel das quimiocinas e receptores CCL3/CCR1/CCR5 e CCL2/CCR2 na reabsorção/remodelação óssea induzida por força mecânica.

É importante salientar que a remodelação óssea pode ser regulada pela resposta osteoimune e pela força mecânica (Jones *et al*., 2011; Papachroni *et al.*, 2009). Portanto, os tratamentos para doenças osteodesgenerativas buscam utilizar terapias farmacológicas para neutralizar a ação de mediadores pro-reabsortivos (Repeke *et al.*, 2011; Saharara *et al.*, 2005), associadas com tratamentos baseados em força mecânica, visando aumento da massa óssea (Papachroni *et al.*, 2009). Neste contexto, alguns estudos *in vitro* vêm investigando quais são as vias moleculares ativadas pelo estímulo mecânico em células ósseas (Cheung *et al*., 2011; Al-Dujaili *et al*., 2011). Entretanto, os estudos *in vitro* não simulam, ao mesmo tempo, algumas condições observadas *in vivo,* como a hipóxia, necrose e o dano físico da célula desencadeados pela força mecânica compressiva, assim como angiogênese e remodelamento da matriz celular induzidos pela força de tensão (Krishnan e Davidovitch, 2006; Krishnan e Davidovitch, 2009). Portanto, o modelo *in vivo* de movimentação dentária ortodôntica permitiu estudar o papel de mediadores pro- e anti-reabsortivos na remodelação óssea induzida por força mecânica (Andrade *et al*., 2009; Taddei *et al*., 2011). Este conhecimento pode ser empregado em estratégias terapêuticas para modular a movimentação dentária ortodôntica e outras doenças osteodegenerativas.

3.1 O Papel da Quimiocina e Receptores CCL3/CCR1/CCR5 na Reabsorção/Remodelação Óssea Durante Movimentação Dentária Ortodôntica

Estudos *in vitro* demonstraram que a quimiocina CCL5 (ligante dos receptores CCR1 e CCR5) estimula o recrutamento e diferenciação de osteoclastos e osteoblastos, estando, então, envolvida no processo de remodelação óssea (Yu *et al*., 2004; Yano *et al*., 2005). Além disso, o primeiro estudo realizado por nosso grupo demonstrou que CCL5 pode ser uma molécula importante na reabsorção óssea induzida por força ortodôntica (Andrade Jr. *et al*., 2007b). Considerando estes achados, o primeiro estudo desta tese investigou o papel do CCR5 na movimentação dentária ortodôntica, realizando experimentos em animais deficientes para o receptor CCR5 ($CCR5^{-/-}$). Verificamos que os animais $CCR5^{-/-}$ apresentaram maior movimentação ortodôntica e número de osteoclastos TRAP-positivos, além de aumento da expressão de marcadores da atividade osteoclástica (catepsina K, RANKL e MMP13) em relação aos animais selvagens. Os nossos resultados sugerem que o receptor CCR5 atua como regulador negativo da reabsorção óssea alveolar induzida por força mecânica (*Andrade Jr. et al., 2009*). Consistente com estes achados, a reabsorção óssea periapical e a artrite experimental foi exarcebada na ausência de CCR5 (Rossi *et al*., 2008; Doodes *et al*., 2009).

Já que os osteoblastos expressam também o receptor CCR5 (Yano *et al*., 2005) e são essenciais para o processo de remodelação óssea, nós investigamos a expressão dos marcadores de diferenciação e atividade destas células, tais como RUNX2 e OCN (Andrade Jr. *et al*., 2009). Os resultados mostraram que RUNX2 e OCN foram diminuídas em animais $CCR5^{-/-}$, sugerindo que a diferenciação e

atividade dos osteoblastos podem ser reguladas pelo receptor CCR5. Entretanto, o papel do CCR5 na diferenciação e atividade dos osteoblastos deve ser confirmado, empregando-se outros métodos/modelos de estudo.

Em paralelo, observou-se que animais CCR5$^{-/-}$ também apresentaram os níveis de IL-10 e OPG (reguladores negativos da reabsorção óssea) reduzidos (Andrade Jr. *et al*., 2009). Como o osteoblasto é uma das fontes de OPG (Boyce e Xing, 2008), estes achados sugerem que a redução na diferenciação de osteoblastos possa ter resultado na diminuição dos sinais inibitórios para os osteoclastos, causando aumento da reabsorção do osso alveolar e maior movimentação dentária ortodôntica. Um estudo prévio demonstrou que as células T regulatórias podem ser responsáveis pela produção de IL-10 e supressão da formação de osteoclastos (Zaiss *et al*., 2007). Neste contexto, como a migração destas células é dependente do receptor CCR5 (Yurchenko *et al.,* 2006), nossos dados podem sugerir um papel destas células na reabsorção óssea (Andrade Jr. *et al*., 2009). Entretanto esta hipótese deve ser melhor investigado empregando-se sistemas in vivo e in vitro.

Ao contrário do CCR5, CCR1 é o receptor que apresenta maior expressão nos precursores de osteoclastos quando estimulados por RANKL (Yu et al., 2004). Além disso, CCR1 está envolvido na atividade dos osteoclastos e, conseqüentemente, na indução de osteólise *in vitro* (Oba *et al*., 2005; Menu *et al*., 2006; Hoshino *et al*., 2010) e *in vivo* (Menu *et al*., 2006). Portanto, nós investigamos a expressão de CCR1 nos animais CCR5$^{-/-}$. O nível de CCR1 foi aumentado no periodonto de camudongos WT após aplicação de força ortodôntica, ao mesmo tempo em que ocorreu o aumento do nível de RANKL (Andrade Jr. *et al*., 2009). Além disso, somado ao fenótipo de reabsorção óssea aumentada (observação

microscópica), os animais CCR5$^{-/-}$ apresentaram maior expressão de CCR1 e RANKL (Andrade Jr. *et al*., 2009), sugerindo que CCR1 possivelmente está envolvido na formação deste ambiente pró-reabsortivo.

Além do aumento da expressão de CCR1, foram observados níveis aumentados de CCL3 (ligante dos receptores CCR1 e CCR5) após aplicação de força ortodôntica (Andrade Jr*. et al.,* 2009). Estes receptores e esta quimiocina podem modular a reabsorção óssea por promoverem quimiotaxia, recrutamento e atividade dos osteoclastos *in vitro* (Yu *et al., 2004*). Portanto, investigamos, em seguida, o papel do CCR1 e CCL3 na reabsorção/remodelação óssea induzida por força mecânica (Taddei *et al., sd*). Foi observado menor movimentação dentária e número de osteoclastos TRAP-positivos nos camundongos CCL3$^{-/-}$, CCR1$^{-/-}$ e tratados com Met-RANTES. Então, nossos principais achados demonstraram que o eixo CCL3/CCR1 desempenha um importante papel no recrutamento, atividade e diferenciação dos osteoclastos durante a reabsorção/remodelação óssea induzida por força mecânica (Taddei *et al*., *sd*). Estes achados corroboram com a função pró-reabsortiva do CCR1 demonstrada na remodelação óssea fisiológica (Hoshino *et al.*, 2010), na perda óssea de mieloma múltiplos (Vallet *et al*., 2007) e na doença periodontal (Repeke *et al*., 2010). Entretanto, é importante ressaltar que a reabsorção óssea induzida por força ortodôntica não é apenas dependente da quimiocina CCL3 (Taddei *et al*., *sd*), já que outra quimiocina ligante do CCR1, CCL5, pode ser importante neste processo (Andrade Jr. *et al*., 2007b).

Em concordância com a reabsoção óssea reduzida, os níveis de marcadores pró-rebasortivos, tais como RANK, RANKL and TNF-α, foram diminuídos em camundongos CCL3$^{-/-}$ durante movimentação dentária ortodôntica (Taddei *et al*., *sd*). Como descrito anteriormente em estudos *in vitro*, CCL3 aumenta

a expressão de RANKL pelos osteoblastos (Tsubaki *et al*., 2007) e induz interação osteoblasto-osteoclasto (Watanabe *et al*., 2004), o que pode resultar no aumento de diferenciação de osteoclastos e consequentemente reabsorção óssea. Em paralelo, nossos achados mostraram também que a produção de TNF-α, uma citocina pró-reabsortiva, parece ser dependente de CCL3 (Taddei *et al*., *sd*). A redução na expressão de TNF-α pode ter contribuído para menor reabsorção óssea observada em camundongos $CCL3^{-/-}$.

Além disso, não somente os níveis de RANK/RANKL e TNF-α foram reduzidos em animais tratados com Met-RANTES, mas também a expressão de catepsina K e MMP13. Em concordância com nossos achados, o tratamento com Met-RANTES resultou em expressão reduzida de TNF-α e RANKL, e diminuição da perda óssea em doenças como artrite reumatoide e doença periodontal (Shahrara *et al*., 2005, Repeke *et al*., 2011). É importante ressaltar também que o bloqueio utilizando Met-RANTES foi mais efetivo para controlar a perda óssea que a deleção genética de CCL3, sendo demonstrado pela redução na expressão demarcadores de reabsorção óssea, catepsina K e MMP13, observada após o tratamento com Met-RANTES (Taddei *et al*., *sd*). Estudos prévios, demonstraram a eficácia do tratamento com Met-RANTES na redução de perda óssea na doença periodontal (Repeke *et al*., 2011) e artrite reumatoide (Shaharara *et al*., 2005). Neste contexto, o Met-RANTES deve ser testado como uma estratégia terapêutica para reduzir reabsorção óssea em outras doenças, como na osteoporose, já que o bloqueio de receptores de quimiocina não interfere tanto na homeostase do osso, quanto os tratamentos que impedem a função do sistema RANKL/RANK (Binder *et al*., 2009).

Os resultados deste estudo demonstraram também que a expressão de RUNX2 foi diminuída nos animais $CCL3^{-/-}$ e nos animais tratados com Met-RANTES,

porém ocorreu aumento de OCN após tratamento com Met-RANTES (Taddei *et al*., *sd*). Estes dados sugerem que o bloqueio de ambos CCR1 e CCR5 e a deleção genética de CCL3 afetam a expressão dos marcadores de osteoblastos. Em resumo, CCR1 parece funcionar como receptor pró-reabsortivo, enquanto o CCR5 é anti-reabsortivo na remodelação óssea induzida por força mecânica. A ação do CCR1 é dependente, pelo menos em parte, da quimiocina CCL3. Além disso, o bloqueio de CCR1 e CCR5, usando Met-RANTES, pode ser uma estratégia terapêutica para doenças ósseas.

3.2. O Papel do CCL2 e CCR2 na Movimentação Dentária Ortodôntica

Em nossos estudos anteriores (Andrade Jr. *et al*., 2007b; Andrade Jr. *et al*., 2009) observamos que a expressão de CCL2 apresentou-se aumentada durante a movimentação dentária ortodôntica, tão bem quanto em outros sítios de remodelação óssea, tais como artrite reumatoide (Iwamoto *et al*., 2008), doença periodontal (Kurtis *et al*., 2005; Pradeep *et al*., 2009) e metástase ósseas (Lu e Kang , 2009), nas quais osteoclastogênese é altamente estimulada. Estudos *in vitro* têm demonstrado que CCL2 promove quimiotaxia, diferenciação e ativação dos osteoclastos (Kim *et al*., 2005; Kim *et al*., 2006a; Kim *et al*., 2006b; Silva *et al*., 2007; Binder *et al*., 2009; Miyamoto *et al*., 2009), ao se ligar ao receptor CCR2 (Yadav *et al*., 2010). Portanto, nosso próximo objetivo foi investigar o papel do eixo CCL2/CCR2 na movimentação dentária ortodôntica. Os resultados mostraram que a quantidade de movimentação dentária e o número de células TRAP-positivas foram significativamente diminuídos nos camundongos deficientes para o receptor CCR2 e tratados com P8A (análogo de CCL2). Em concordância com estes dados, a

expressão dos marcadores de atividade e diferenciação dos osteoclastos (RANKL e RANK) e dos osteoblastos (COL-1 e OCN) foi menor nos camundongos CCR2$^{-/-}$ que nos WT. Estes dados sugerem que o eixo CCL2/CCR2 está positivamente envolvido no recrutamento de osteoclastos, atividade dos osteoclastos e osteoblastos, reabsorção óssea e movimentação dentária ortodôntica (Taddei *et al.* 2011).

Estudos prévios mostraram que a densidade óssea do camundongo CCR2$^{-/-}$ é aumentada, o que faz deste animal mais resistente à força compressiva (Binder *et al*., 2009). Entretanto, este efeito inato da fisiologia óssea não parece explicar completamente a menor movimentação dentária observada neste animal, já que resultados similares foram observados após tratamento com um inibidor para CCL2/CCR2 (P8A) em camundongos selvagens. Ao contrário, a redução do recrutamento dos osteoclastos, regulado pelo eixo CCL2/CCR2, pode justificar a menor reabsorção óssea e movimentação dentária nos animais CCR2$^{-/-}$ observadas neste estudo (Taddei *et al.* 2011). Além disso, nossos resultados sugerem que o fenótipo histológico, apresentado pelos camundongos CCR2$^{-/-}$ durante a movimentação dentária, parece estar relacionado com a redução da expressão do eixo RANKL/RANK (Taddei *et al.* 2011). Estudos prévios corroboram nossos achados, mostrando que na ausência de CCR2 ocorre diminuição da expressão de RANK nos precursores de osteoclastos (Binder *et al*., 2009), e redução da reabsorção óssea em modelos *in vitro* e *in vivo* (Binder *et al*., 2009; Xing *et al*., 2010). Além disso, o tratamento com P8A reduziu os níveis de RANKL e reabsorção óssea em ratos com artrite (Shahrara *et al*., 2008).

Os resultados obtidos em animais tratados com P8A, uma variável monomérica do CCL2, a qual inibe a migração de células dependentes de CCR2 *in vivo* (Handel *et al*., 2008), sugere que a função de CCR2 ocorre principalmente via

ligação ao CCL2 neste modelo (Taddei *et al.,* 2011). Semelhante aos nossos resultados, a ausência de CCL2 resulta em menor número de osteoclastos (Binder *et al*., 2009) e o tratamento com P8A reduz as lesões ósseas em ratos com artrite (Shahrara *et al*., 2008).

A expressão de COL-1 e OCN foi diminuída em camundongos $CCR2^{-/-}$ neste estudo, sugerindo que a diferenciação e atividade de osteoblastos foram reduzidas na ausência de CCR2 (Taddei *et al.* 2011). Esta menor diferenciação de osteoblastos pode estar relacionada a uma redução dos sinais estimulatórios (como RANKL), resultando em menor reabsorção óssea nos animais $CCR2^{-/-}$ (Taddei *et al.* 2011). Por outro lado, em um modelo de osteoporose, tanto a formação do osso quando os níveis de OCN não foram alterados em camundongos $CCR2^{-/-}$ (Binder *et al*., 2009). Estudos adicionais podem confirmar o papel do CCR2 na diferenciação e atividade dos osteoblastos.

Em resumo, a quimiocina CCL2 e o receptor CCR2 regulam positivamente o recrutamento e atividade dos osteoclastos durante a movimentação dentária. Além disso, o bloqueio do eixo CCL2/CCR2 pode ser usado para futuras intervenções terapêuticas, limitando a progressão de perda óssea inflamatória em doenças tais como osteoporoses e artrite reumatoide, ou modulando a extensão da movimentação dentária ortodôntica (Taddei *et al.* 2011).

4. CONCLUSÕES

Os resultados deste trabalho permitem-nos concluir que:

1) O receptor CCR5 interfere negativamente no recrutamento e atividade dos osteoclastos, diminuindo a movimentação dentária ortodôntica.
2) O eixo CCL3/CCR1 induz o recrutamento e ativação de osteoclastos, sendo uma via pró-reabsortiva durante a movimentação dentária ortodôntica.
3) O eixo CCL2/CCR2 regula positivamente o recrutamento e atividade dos osteoclastos durante a movimentação dentária induzida pela força ortodôntica.

5. PERSPECTIVAS

O papel das quimiocinas e receptores CCL3/CCR1/CCR5 e CCL2/CCR2 na reabsorção óssea durante a movimentação dentária foram abordados nos 3 tópicos deste trabalho de tese. Entretanto, alguns pontos merecem aprofundamento:

- Confirmar o papel do CCR1 na remodelação óssea induzida por força mecânica, utilizando animais deficientes para este receptor (estes foram adquiridos recentemente pelo nosso grupo).
- Avaliar o papel da IL-10 na movimentação dentária ortodôntica.
- Investigar o papel do CCR5 na diferenciação e atividade das células ósseas *in vitro*.
- Desenvolver um modelo de osteoporose experimental induzida por deficiência de estrógeno, para extrapolar nossos conhecimentos a respeito da reabsorção/remodelação óssea patológica. Portanto, outra perspectiva é investigar o papel do CCR1 e CCR5 na reabsorção/remodelamento ósseo neste processo. Além disso, testar novas estratégias terapêuticas para osteoporose, empregando-se Met-RANTES e P8A.

Diante deste conhecimento obtido, surgiu o interesse de extrapolar nossas investigações para a área clínica da Ortodontia. Portanto, outras perspectivas são:

- Determinar a cinética de expressão das citocinas, quimiocinas e de marcadores de reabsorção/remodelação óssea no ligamento periodontal após movimentação dentária ortodôntica em humanos, empregando-se Real-Time PCR.

- Avaliar a cinética de expressão das citocinas, quimiocinas e de marcadores de reabsorção/remodelação óssea no fluido gengival após força ortodôntica em humanos e correlacionar estes dados com a expressão destes marcadores no ligamento periodontal.

Estas informações obtidas terão potencial para utilização clínica como marcadores do processo de remodelação óssea, com o propósito de uma identificação precoce dos efeitos indesejáveis causados pelo uso de força ortodôntica excessiva, os quais podem apenas ser detectados quando em estágio avançado. Cumpre-nos salientar que estudos dessa natureza também podem contribuir fornecendo dados importantes sobre os mediadores que regulam a reabsorção óssea e, consequentemente, determinam o sucesso da terapia ortodôntica e ampliam, sobremaneira, as estratégias terapêuticas para o tratamento de doenças ósseas.

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