Hyperocclusion upregulates CCL3 expression in CCL2- and CCR2-deficient mice

T. Tsutsumi[1,2], H. Kajiya[1*], K. T. Goto[3], Y. Takahashi[2] and K. Okabe[1]

[1]Department of Physiological Science and Molecular Biology, Fukuoka Dental College, Fukuoka 8140193, Japan

[2]Department of Oral Rehabilitation, Fukuoka Dental College, Fukuoka 8140193, Japan

[3]Department of Dental Hygiene, Fukuoka College of Health Sciences, Fukuoka 8140193, Japan

***Corresponding author**: Hiroshi Kajiya, Department of Physiological Science and Molecular Biology, Fukuoka Dental College, 2-15-1 Tamura, Sawara-ku, Fukuoka 8140193, Japan.

Phone: +81-92-801-0411 ext. 660; Fax: +81-92-801-4909

E-mail: kajiya@college.fdcnet.ac.jp

Short title: Hyperocclusion up-regulates CCL3.

Key words: CC chemokine ligand 3, hyperocclusion, mechanical stress, PDL cells, bone resorption

Number of words in abstract: 196

Number of words in manuscript and abstract: 2,680

Number of references: 30

Number of figures: 1 gray scale; 3 color

Number of appendices to be published online: 1 figure

Conflicts of interest: The authors have declared that no conflicts of interest exist.

Category: Original Research Report

Abbreviations: MS, mechanical stress; PDL, periodontal ligament; CCL2, CC chemokine ligand 2 (MCP-1; monocyte chemoattractant protein-1); CCR2, CC chemokine receptor 2; CCL3, CC chemokine ligand 3 (MIP-1α); CCL5, CC chemokine ligand 5 (RANTES)

ABSTRACT

Excessive mechanical stress (MS) during hyperocclusion is known to result in disappearance of the alveolar hard line, enlargement of the periodontal ligament (PDL) space, and destruction of alveolar bone, leading to occlusal traumatism. We have recently reported that MS induces predominantly C-C chemokine ligand (CCL) 2 expression in PDL tissues, leading, *via* C-C chemokine receptor (CCR) 2, to MS-dependent osteoclastogenesis in alveolar bone. Thus, we hypothesize that ablation of the CCL2/CCR2 signaling pathway should suppress MS-induced osteoclastogenesis-associated chemokines and alleviate occlusal traumatism. We examined the effect of MS on chemokine expression and osteoclastogenesis using *in vivo* and *in vitro* hyperocclusion models with CCL2-deficient ($CCL2^{(-/-)}$) and CCR2-deficient ($CCR2^{(-/-)}$) mice. Compared with that in wild type mice, expression of CCL3 in PDL cells and TRAP-positive cells in alveolar bone from $CCL2^{(-/-)}$ and $CCR2^{(-/-)}$ mice was up-regulated, even in the absence of MS. Furthermore, the expression of CCL3 and TRAP-positive cells were significantly increased after both four and seven days of hyperocclusal MS loading in $CCL2^{(-/-)}$ and $CCR2^{(-/-)}$ mice. Hyperocclusion induced compensatory CCL3 expression and promoted osteoclastogenesis to counterbalance deficient CCL2/CCR2 signaling, suggesting that co-expression of CCL3 with CCL2 may precipitate synergistic, MS-dependent alveolar bone destruction during occlusal traumatism.

INTRODUCTION

Premature occlusal contact and lateral jiggling of abutment teeth in partial dentures results in excessive forces being exerted on periodontal tissues. Excessive mechanical stress (MS) during hyperocclusion has been shown to induce in disappearance of the alveolar hard line, enlargement of the periodontal ligament (PDL) space, and damage to periodontal tissues, resulting in bone resorption and tooth loss (Yoshinaga *et al.*, 2007; Walker *et al.*, 2008). In contrast, MS produced by normal occlusion has an inhibitory effect on unopposed eruption and physiological drifting of teeth (Walker *et al.*, 2008).

Chemokines are a large family of chemotactic cytokines that direct the trafficking and homing of specific subpopulations of leukocytes and other cells, in both physiologic and pathologic processes (Ross and Zlotnik, 2000; Gerard and Rollins, 2001). Among the chemokine family, CCL2 (MCP-1), CCL3 (MIP-1α) and CCL5 (RANTES) have been identified as typical osteoclastogenesis-associated chemokines. These contribute to osteoclast recruitment and activation (Chae *et al.*, 2002; Graves *et al.*, 2002; Toh *et al.*, 2004). A recent study has shown that CCL2, CCL3 and CCL5 are involved in human osteoclast differentiation from osteoclast precursors *in vitro* (Kim *et al.*, 2006). Several studies have also reported that MS induced the expression of cytokines and chemokines in alveolar bone resorption during periodontitis and orthodontic tooth movement (Andrade *et al.*, 2007; Silva *et al.*, 2007; Garlet *et al.*, 2008). We have recently reported that excessive MS with hyperocclusion predominantly induces CCL2 expression in rat PDL tissues, leading to MS-dependent osteoclastogenesis in alveolar bone in *in vitro* and *in vivo* rodent models of hyperocclusion (Goto *et al.*, 2011).

Thus, we hypothesize that ablation of the CCL2/CCR2 signaling pathway should inhibit the expression of osteoclastogenesis-associated chemokines in the PDL, preventing

occlusal traumatism. We have examined the effects of MS on chemokine expression and osteoclastogenesis using *in vivo* and *in vitro* hyperocclusion models in which either CCL2 ($CCL2^{(-/-)}$) or CCR2 ($CCR2^{(-/-)}$) are deficient compared to wild type (WT) mice.

MATERIALS & METHODS

Experimental animals

Five-week old C57BL/6 mice were used unmodified (WT) or with CCL2 ($CCL2^{(-/-)}$) or CCR2 ($CCR2^{(-/-)}$) knocked out. Mice were purchased from Kyudo Animal Co, Ltd. (Tosu, Saga, Japan) and Jackson Lab (Sacramento, CA, USA). All animals were treated according to the ethical regulations for animal experimentation defined by the Animal Care Committee of the Fukuoka Dental College, Fukuoka, Japan.

Cell culture

Primary cultures of mouse PDL cells were established from WT, $CCL2^{(-/-)}$ and $CCR2^{(-/-)}$ mice. Mice were killed by injecting an excess of isoflurane anesthesia. Mandibles from five mice were hemisected into left and right halves by incision through the midline symphysis, dissected from surrounding tissues, rinsed twice in phosphate-buffered saline and stored in serum-free Dulbecco's Modified Eagle Medium (D-MEM, Sigma-Aldrich, St. Louis, MO, USA). Using a stereomicroscope, first molars were extracted by incising the periodontal ligament attaching them to the surrounding alveolar bone; teeth were then rinsed and stored in PBS. Collagenase and trypsin were used to remove PDL cells enzymatically from the surface of the molars. For this procedure, teeth were placed into a centrifuge tube containing DMEM augmented with 2 mg/ml collagenase (Wako Co Ltd., Osaka, Japan) and 0.25% trypsin (Wako) and incubated for 15 min at 37 °C. Additional DMEM containing collagenase and trypsin was then added to the tube and incubated for a further 2 h. PDL cells were then collected by centrifugation at 400g for 5min, re-suspended in DMEM containing 20% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin and cultured in a

dish at 37 °C in a humidified gas mixture of 5% CO_2/95% air.

In vitro intermittent stretching model of mechanical stress

Mice PDL cells were cultured in a 50-cm^2 silicon chamber at a density of 5×10^5 cells/cm^2. The silicon chamber was attached to a stretching apparatus (STB-140, Strex Co. Ltd., Osaka, Japan) and subjected to intermittent uniaxial stretching (60 s/return; 29 s resting time; 1.6 mm stretch length; 105% stretch ratio) for 1–4 days.

In vivo Hyperocclusion Model

Anesthesia was achieved using diethyl ether and pentobarbital (50 mg/kg, Kyoritsu, Tokyo, Japan). To create a hyperocclusive state, the three right maxillary molars were modified to accept a stainless steel wire bonded with methylmethacrylate resin (Super-Bond; Sun Medical Inc., Shiga, Japan). Stainless steel wire (0.3 mm diameter) was bonded to the occlusal surfaces of the right maxillary molars in mice accoding to privous our study (Goto *et al.*, 2011).

RNA Isolation, Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from PDL cells using TRIzol reagent. First-strand cDNA was synthesized using 3 μg total RNA with SuperScript II Reverse Transcriptase (Invitrogen). The cDNA was amplified by PCR (denaturation: 1 min, 95 °C; annealing: 1 min, 60 °C; extension; 1 min, 72 °C; 23 cycles) using specific primers. PCR products were subjected to electrophoresis on 2% agarose gels and visualized with ethidium bromide.

Western Blot Analysis

Cells were lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1% Triton-X, 1 mM DTT, and protease inhibitors (Roche, Basel, Switzerland). The protein contents of the samples were measured using a protein assay kit (Pierce, Hercules, CA, USA). Protein samples (20 μg) were subjected to 5–20 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred electrophoretically to a polyvinylidene fluoride membrane at 100 V for 1 h at 4 °C. The membrane was incubated with primary antibodies against CCL2, CCL3 and CCL5 (sc-30032, sc-33203, sc-1410; Santa Cruz Biotech, Delaware, CA, USA) at 1:500 dilutions in 5% skim milk solution overnight at 4 °C. Blots were then incubated with horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG in 5% skim milk-TBST (10 mM Tris-HCl, 50 mM NaCl, 0.25% Tween-20) and developed using an enhanced chemiluminescence system (GE, Arlington Heights, IL, USA).

Enzyme-Linked Immunosorbent (ELISA) Assay

The amount of CCL3 secreted into the culture medium following stretching-induced MS of mouse PDL cells for 1–7 days was determined by ELISA assay (Bender Medsystems, New Jersey, NJ, USA) according to the manufacturer's recommended protocol.

Immunohistochemistry

Mice were sacrificed at 0, 4 and 7 d after bonding wire to the occlusal surface. The mandibles were dissected and immediately placed in a 4% paraformaldehyde solution for 24 h at 4 °C for fixation. After fixation, specimens were decalcified for 2 weeks in

10% EDTA solution. Specimens were dehydrated in 30% sucrose overnight, embedded in OCT compound and frozen in dry ice/isopentane. Bone sections (6 μm in thickness) were prepared using Frigocut CM3050S (Leica, Wetziar, Germany). Sections were incubated overnight at 4 °C with the same primary antibodies against CCL2, CCL3 and CCL5 as used for Western blotting (1:500 in phosphate-buffered saline solution). Bound antibody was visualized using Alexa Fluor-conjugated IgG secondary antibody (1 μg/ml, Molecular Probes, OR, USA) for 30 min at room temperature. Similar results were obtained in five independent experiments.

Statistical Analysis

Data are expressed as mean values with standard errors (± SE) from (n) number of cells. Differences were analyzed using paired one-way analysis of variance, where appropriate. Values of $P < 0.05$ were considered to be significant.

RESULTS

Stretching-induced MS causes compensatory CCL3 expression in CCL2- and CCR2-deficient PDL cells in an *in vitro* model of hyperocclusion

Three chemokines (CCL2, CCL3 and CCL5) are reportedly associated with osteoclast differentiation (Chae *et al*., 2002; Graves *et al*., 2002; Toh K, et al., 2004; Kim *et al.*, 2006). We have recently described how MS induced by intermittent stretching causes CCL2 expression in rat PDL tissues (Goto *et al.*, 2011). To clarify how the absence of CCL2/CCR2 signaling affects chemokine expression responses to stretching-induced MS, we examined the expression of CCL2, CCL3 and CCL5 at 0 and 4 days following stretching-induced MS in PDL cells derived from WT, $CCL2^{(-/-)}$ and $CCR2^{(-/-)}$ mice using conventional RT-PCR, real-time PCR, western blotting and ELISA methods (Fig. 1 and Appendix Fig). The expression of CCL2 mRNA and protein significantly increased in WT PDL cells, but was accompanied by only a small amount of CCL3 and negligible CCL5 expression four days after stretching-induced MS (Fig. 1). In contrast, CCL3 expression in $CCL2^{(-/-)}$ and $CCR2^{(-/-)}$ PDL cells was endogenously upregulated in the absence of any MS, apparently to compensate for the loss of CCL2/CCR2 signaling. Furthermore, the expression of CCL3 mRNA and protein was further increased four days after stretching-induced MS. To clarify the amount and time-course of CCL3 protein release from the three types of PDL cells, we examined MS-induced CCL3 secretion using ELISA. CCL3 protein secretion was temporally and quantitatively similar in all three cell types following stimulation (Appendix Figure).

Regulation of CCL3 expression in *in vivo* mouse hyperocclusion model using CCL2- and CCR2-deficient mice

We examined the effects of MS during hyperocclusion on the expression of chemokines in a mouse *in vivo* hyperocclusion model using WT, CCL2$^{(-/-)}$ and CCR2$^{(-/-)}$ mice (Figs. 2, 3 and 4). No remarkable inflammation was detected by hematoxylin-eosin staining until 7 days after hyperocclusal MS in any mouse genotype (Fig. 2, 3 and 4). Almost no TRAP-positive cells were observed before MS in WT mice, but these cells significantly increased in number in alveolar bone on day 4 of hyperocclusal MS, reaching a maximum on day 7 (Fig. 2). Similarly, few CCL2-positive cells were detected before MS in WT mice, but these were increased in PDL tissues after four days of MS and peaked at seven days (Fig.2 and 3B). The expression of CCL2 was especially localized to the periodontal tissue around the root furcation of the first molar after four and seven days of hyperocclusal MS stimulation. CCL3 expression was negligible in these WT tissues.

In contrast, in CCL2$^{(-/-)}$ and CCR2$^{(-/-)}$ mice, even in the absence of stimulation, TRAP-positive cells appeared in the in the alveolar bone and CCL3 expression was up-regulated in the PDL tissues (Figs. 3 and 4). Number of TRAP-positive cells in hyperocclusal MS loading side of PDL tissues were significantly increased after four days of hyperocclusion compared to unloading side, and peaked on day 7 in CCL2$^{(-/-)}$and CCR2$^{(-/-)}$mice (Fig. 3). Furthermore, TRAP-positive cells were localized in the alveolar bone on day 4, but on day 7 were found nearer to the PDL space. CCL3, increased gradually in PDL tissues toward a maximum on day 7 after hyperocclusal MS in both knockout mice (Fig. 4) but CCL5 was unchanged by this stimulation (data not shown). The expression of CCL2 was detected in CCR2 $^{(-/-)}$ PDL cells 4 and 7 days after MS.

DISCUSSION

We have recently reported that excessive MS during hyperocclusion induces predominantly CCL2 expression in PDL tissues, leading to MS-dependent osteoclastogenesis in alveolar bone (Goto *et al.*, 2011). In the present study, we investigated the effects of negating the CCL2/CCR2 signaling pathways on MS-induced chemokine expression. We found that knockout of CCL2/CCR2 signaling caused compensatory expression of CCL3 and up-regulation of TRAP-positive cells at seven days after MS. The MS-induced CCL3 secreted from PDL tissues may, through receptors such as CCR1 and CCR5, contribute to the differentiation and activation of osteoclasts in alveolar bone. These results suggest that the co-expression of CCL3 and CCL2 following hyperocclusal mechanical stress synergistically promotes the destruction of periodontal tissues and alveolar bone during occlusal traumatism.

It has recently been shown that CCL2, CCL3 and CCL5 each play important roles in osteoclast recruitment, differentiation, and bone resorption activity during bone remodeling (Okamatsu *et al.*, 2004; Masella and Meister, 2006; Toh *et al.*, 2004; Kim *et al.*, 2006; Tsubaki *et al.*, 2007). Osteoclast precursors are known to express CCR2 and CCR5 and to respond to chemokines such as CCL2, CCL3 and CXCL12 (Bendre *et al.*, 2003; Yu *et al.* 2004; Silva *et al.*, 2007). Furthermore, CCL2 and CCL3 expression levels are thought to be significantly higher in areas of alveolar bone that are under constant compression forces, which are characterized by high bone resorption activity during orthodontic tooth movement (Alhashimi *et al.*, 1999; Garlet *el al.*, 2007).

The expression of CCL2 and CCR2 have been reported to be stimulated by RANKL and NFκ-B (Cappellen *et al.* 2002; Miyamoto *et al.*, 2009), indicatting that CCL2 contributes to osteoclastogenesis and bone resorption. However, the expression of genes

associated with bone resorption, such as cathepsin K and MMP9, was minimal in CCL2-induced osteoclasts, resulting in the inhibition of bone resorption (Kim *et al.*, 2006). Although CCL2 levels are markedly increased in joints displaying rheumatoid arthritis with abnormal bone resorption (Koch *et al.*, 2005), CCR2 gene ablation aggravates collagen-induced arthritis in rats. $CCR2^{(-/-)}$ mice exhibit enhanced intra-articular expression of CCL3 (Quinones *et al.*, 2004), which is involved in osteoclast recruitment and differentiation (Okamatsu *et al.*, 2004; Kim *et al.*, 2006; Tsubaki *et al.*, 2007). *In vitro* studies have shown that CCL3 increases the expression of RANKL by osteoblasts and induces osteoclast-osteoblast interaction, increasing osteoclast differentiation and, consequently, accelerating bone resorption (Tsubaki *et al.*, 2007; Watanabe *et al.*, 2004). Furthermore, CCL3 is up-regulated in a multiple myeloma microenvironment and is correlated with osteolytic lesions and tumor burden, while inhibition of CCL3 significantly reduces bone destruction in a mouse model of multiple myeloma (Choi *et al.*; 2001). The expression levels of RANK and RANKL are decreased in $CCL3^{(-/-)}$ mice after mechanical loading, which is consistent with reduced bone resorption. Our current findings show that the *in vivo* and *in vitro* experimental models of MS used here also induce expression CCL3 in WT PDL cells, in addition to CCL2. More importantly, when CCL2/CCR2 signaling is knocked out, CCL3 expression is dramatically elevated. The expression of CCL3 in PDL tissues was followed, temporally and spatially, by an increase in TRAP-positive cells, suggesting that alveolar bone resorption during occlusal traumatism is invoked not only by CCL2 but also by CCL3.

Previous studies have shown that neutrophil infiltration was augmented in mice deficient in CCL2 and CCR2 (Montgomery *et al.*, 2007; Sawanobori et al., 2008).

CCL2$^{(-/-)}$ and CCR2$^{(-/-)}$ mice showed impaired recruitment of macrophages, expressed higher levels of inflammatory osteolytic factors, and exhibited an increased number of TRAP-positive cells (Garlet *et al.*, 2010; Chen *et al.*, 2011). In the present experiments, we found that CCL3 expression in PDL tissues and TRAP-positive cells in alveolar bone were both up-regulated in CCL2$^{(-/-)}$ and CCR2$^{(-/-)}$ mice, even in the absence of hyperocclusal MS, consistent with the findings of the previous study. The MS increased both CCL3 expression and the number of TRAP-positive cells after both four and seven days of MS stimulation. These results indicate that the expression and activation of CCL3 could be induced not only by inflammation of periodontal tissue, but also by MS during hyperocclusion in situations where CCL2/CCR2 signaling is deficient.

In summary, CCL2 and CCL3 both appear to have roles in osteoclast differentiation and thus alveolar bone resorption induced by hyperocclusal MS loading. The inhibition of CCL2 and CCL3 signaling that occurs following hyperocclusal MS may be a therapeutic strategy for treating alveolar bone resorption associated with occlusal traumatism.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (24592823) and the Strategic Study Base Formation Support Business (S1001059).

REFERENCES

Alhashimi N, Frithiof L, Brudvik P, Bakhiet M (1999) Chemokines are upregulated during orthodontic tooth movement. *J Interferon Cytokine Res*19:1047-1052.

Andrade Jr I, Silva TA, Silva GAB, Teixeira AL, Texeira MM (2007) The role of tumor necrosis factor receptor type 1 in orthodontic tooth movement. *J Dent Res*86: 1089-1094.

Bendre MS, Montague DC, Peery T, Akel NS, Gaddy D, Suva LJ (2003) Interleukin-8 stimulation of osteoclastogenesis and bone resorption is a mechanism for the increased osteolysis of metastatic bone disease.*Bone*33:28-37.

Cappellen D, Luong-Nguyen NH, Bongiovanni S, Grenet O, Wanke C, Susa M (2002) Transcriptional program of mouse osteoclast differentiation governed by the macrophage colony-stimulating factor and the ligand for the receptor activator of NFkappa B. *J Biol Chem* 277:21971-21982.

Chae P, Im M, Gibson F, Jiang Y, Graves DT (2002) Mice lacking monocyte chemoattractant protein 1 have enhanced susceptibility to an interstitial polymicrobial infection due to impaired monocyte recruitment. *Infect Immun*70:3164-3169.

Chen M, Forrester JV, Xu H (2011) Dysregulation in retinal para-inflammation and age-related retinal degeneration in CCL2 or CCR2 deficient mice. *PLos one* 6:e22818.

Choi SJ, Cruz JC, Craig F, Chung H, Devlin RD, Roodman GD, Alsina M (2000) Macrophage inflammatory protein 1-alpha is a potential osteoclast stimulatory factor in multiple myeloma. *Blood* 96:671-675.

Garlet TP, Coelho U, Silva JS, Garlet GP (2007) Cytokine expression pattern in

compression and tension sides of the periodontal ligament during orthodontic tooth movement in humans. *Eur. J. Oral Sci*115:355-362.

Garlet TP, Coelho U, Repeke CE, Silva JS, Cunha FQ, Garlet GP (2008) Differential expression of osteoblast and osteoclast chemmoatractants in compression and tension sides during orthodontic movement. *Cytokine* 42:330-335.

Garlet TP, Fukada SY, Saconato IF, Avila-Campos MJ, da Silva TA, Garlet GP, Cunha Fde Q (2010) CCR2 deficiency results in increased osteolysis in experimental periapical lesions in mice. *JOE* 36:244-250.

Gerard C, Rollins BJ (2001) Chemokines and disease. *Nat Immunol* 2:108-115.

Goto-TK, Kajiya H, Nemoto T, Tsustumi T, Tsuzuki T, Sato H, Okabe K (2011) Hyperocclusion stimulates osteoclastogenesis via CCL2. *J Dent Res* 90:793-798.

Graves DT, Alsulaimani F, Ding Y, Marks Jr SC (2002) Developmentally regulated monocyte recruitment and bone resorption are modulated by functional deletion of the monocytic chemoattractant protein-1 gene. *Bone* 31:282-287.

Kim MS, Day CJ, Selinger CI, Magno CL, Stephens SR, Morrison NA (2006) MCP-1 induced human osteoclast-like cells are tartrate-resistant acid phosphatase, NFATc1, and calcitonin receptor-positive but require receptor activator of NFkappaB ligand for bone resorption. *J Biol Chem* 281:1274-1285.

Koch AE (2005) Chemokines and their receptors in rheumatoid arthritis: future targets? Arthritis Rhem 52:710-21.

Masella RS, Meister M (2006) Current concepts in the biology of orthodontic tooth movement. *Am J Orthod Dentofacial Orthop*129:458-468.

Miyamoto K, Ninomiya K, Sonoda KH, Miyauchi Y, Hoshi H, Iwasaki R, Miyamoto H, Yoshida S, Sato Y, Morioka H, Chiba K, Egashira K, Suda T, Toyama Y, Miyamoto T (2009) MCP-1 expressed by osteoclasts stimulates osteoclastogenesis in an autocrine/paracrine manner. *Biochem Biophys Res Commun* 383:373-377.

Montgomery RR, Booth CJ, Wang X, Blaho VA, Malawista SE, Brown CR (2007) Recruitment of macrophages and polymorphonuclear leukocytes in L:yme carditis. *Infect Immun* 75:613-620.

Okamatsu Y, Kim D, Battaglino R, Sasaki H, Späte U, Stashenko P (2004) MIP-1 gamma promotes receptor-activator-of-NF-kappa-B-ligand-induced osteoclast formation and survival. *J Immunol* 173:2084-2090.

Quinones MP, Ahuja SK, Jimenez F, Schaefer J, Garavito E, Rao A, Chenaux G, Reddick RL, Kuziel WA, Ahuja SS (2004) Experimental arthritis in CC chemokine receptor 2-null mice closely mimics severe human rheumatoid arthritis. *J Clin Invest* 113:856-66.

Ross, Zlotnik A (2000) The biology of chemokines and their receptors. *Annu Rev Immunol* 18:217-242.

Sawanobori Y, Ueha S, Kurachi M, Shimaoka T, Talmadge JE, Abe J, Shono Y, Kitabatake M, Kakimi K, Mukaida N, Matsushima K (2008)

Chemokine-mediated rapid turnover of myeloid-derived suppressor cells in tumor-bearing mice. *Blood* 111:5457-5466.

Silva TA, Garlet GP, Fukuda SY, Silva JS, Cunha FQ (2007) Chemokines in oral inflammatory diseases: apical periodontitis and periodontal disease *J Dent Res* 86:306-319.

Toh K, Kukita T, Wu Z, Kukita A, Sandra F, Tang QY *et al.* (2004) Possible involvement of MIP-1a in the recruitment of osteoclast progenitors to the distal tibia in rats with adjuvant-induced arthritis. *Lab Invest* 84:1092-1102.

Tsubaki M, Kato C, Manno M, Ogaki M, Satou T, Itoh T, Kusunoki T, Tanimori Y, Fujiwara K, Matsuoka H, Nishida S (2007) Macrophage inflammatory protein-1alpha (MIP-1alpha) enhances a receptor activator of nuclear factor kappaB ligand (RANKL) expression in mouse bone marrow stromal cells and osteoblasts through MAPK and PI3K/Akt pathways. *Mol Cell Biochem* 304:53-60.

Yoshinaga Y, Ukai T, Abe Y, Hara Y (2007) Expression of receptor activator of nuclear factor kappa B ligand relates to inflammatory bone resorption, with or without occlusal trauma, in rats. *J Periodont Res* 42:402-409.

Yu X, Huang Y, Collin-Osdoby P, Osdoby P (2004) CCR1 chemokines promote the chemotactic recruitment, RANKL development, and motility of osteoclasts and are induced by inflammatory cytokines in osteoblasts. *J Bone Miner Res* 19:2065-77.

Watanabe T, Kukita T, Kukita A, Wada N, Toh K, Nagata K, Nomiyama H, Iijima T (2004) Direct stimulation of osteoclastogenesis by MIP-1alpha: evidence obtained from studies using RAW264 cell clone highly responsive to RANKL. J

Endocrinol 180:193-201.

Walker CG, Ito Y, Dangaria S, Luan X, Diekwisch TGH (2008) RANKL, osteopontin, and osteoclast homeostasis in a hyperocclusion mouse model. *Eur J Oral Sci* 116: 312-318.

FIGURE LEGENDS

Figure 1. Effects of stretch-induced mechanical stress on the expression of CCL2, CCL3 and CCL5 mRNA and protein in periodontal ligament (PDL) cells from wild type (WT), $CCL2^{(-/-)}$ and $CCR2^{(-/-)}$ mice. (A) PDL cells from the three different mouse genotypes were intermittently stretched for either 0 or 4 days. Expression of CCL chemokines and GAPDH mRNAs were analyzed using reverse transcriptase-polymerase chain reaction (RT-PCR). Results shown are representative of five independent experiments. (B) Expression of CCL2, CCL3, CCL5 and β-actin mRNAs were analyzed using real time-PCR. Data represent the mean±SEM from six culture wells from different passages cells. $*P<0.05$ and $**P<0.01$ *vs*. day 0 of MS. (C) The expression level of CCL2 and CCL3 proteins were detected by western blot analysis. Results shown are representative of three independent experiments.

Figure 2. Phenotype of alveolar bone sections from WT mice. Alveolar bone sections from WT mice following 0 (left column), 4 (middle column) or 7 (right column) days of hyperocclusal MS stimulus were stained with hematoxylin-eosin (HE; upper row), and for tartrate-resistant acid phosphatase (TRAP; upper-middle row), CCL2 (lower-middle row) and CCL3 (lower row). Results shown are representative of five independent experiments. Arrow heads indicate TRAP-positive cells. Bars indicate 100 μm.

Figure 3. TRAP staining of alveolar bone sections from WT, $CCL2^{(-/-)}$ and $CCR2^{(-/-)}$ mice. (A) Alveolar bone sections from three phenotypes of mice following 0 (left column), 4 (middle column) or 7 (right column) days with (loading side) or without (unloading side) hyperocclusal MS stimulus were stained for TRAP. Results shown are

representative of five independent experiments. Arrow heads indicate TRAP-positive cells. Bars indicate 100 μm. (B) Number of TRAP positive cells in three phenotype mice with hyperocclusal MS loading. Data represent the mean±SEM from seven sections in each phenotype of mice. *P<0.05 and **P<0.01 *vs*. day 0 of MS.

Figure 4. CCL3 immunostaining of alveolar bone sections from WT, $CCL2^{(-/-)}$ and $CCR2^{(-/-)}$ mice. Alveolar bone sections from three phenotypes of mice following 0 (left column), 4 (middle column) or 7 (right column) days with or without hyperocclusal MS stimulus were immunostained for CCL3. Results shown are representative of five independent experiments. Bars indicate 100 μm.