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Role of CCL4 and CCR5 during osteoclastogenesis of murine bone marrow-derived monocytes

마우스 골수 유래 단핵세포로부터 파골세포로의 분화 과정에서 케모카인 CCL4와 수용체 CCR5 역할

2017년 2월

서울대학교 대학원
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Abstract

Role of CCL4 and CCR5 during osteoclastogenesis of murine bone marrow-derived monocytes

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Chemokine CCL4 (MIP-1β) is released from osteoblast cells to restore the homeostasis of hematopoietic stem cells during the activation of bone marrow. In this study, the function of CCL4 during osteoclastogenesis was investigated. CCL4 promoted the migration and viability of pre-osteoclast cells. However, CCL4 had no direct effect on the receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis in mouse pre-osteoclast cells. During osteoclastogenesis, the expression of CCR5, the CCL4 receptor, was rapidly reduced by RANKL treatment. CCR5 down-regulation by RANKL was mediated by MEK and JNK in pre-osteoclast cells and played a role in osteoclastogenesis.

These results suggest that the chemoattractant effect and viability of CCL4 is involved in recruiting pre-osteoclasts but is diminished later its effect on osteoclastogenesis by the reduction of CCR5 when RANKL is prevalent.

Keyword: Chemokine CCL4, Chemokine receptor CCR5, RANKL, osteoclastogenesis, osteoblastic niche

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Abbreviations

Abbreviations	Full name
CCL4	Chemokine (C-C motif) ligand 4
MIP-1β	Macrophage inflammatory protein-1β
CCR5	C-C Chemokine receptor type 5
M-CSF	Macrophage colony-stimulating factor
RANKL	Receptor activator of nuclear factor kappa-B ligand
IFN-γ	Interferon gamma
TRAP	Tartrate-resistant acid phosphatase
CTSK	Cathepsin K
MAPK	Mitogen-activated protein kinases
ERK	Extracellular signal-regulated kinases
JNK	c-Jun N-terminal kinases
NFATc1	Nuclear factor of activated T-cells, cytoplasmic 1

Introduction

The stem cell niche is a microenvironment where stem cells interact with themselves, other cells, secreted factors, immunological factors, the extracellular matrix, and physical factors (Lane, Williams et al. 2014). Above all, the hematopoietic stem cell niche is important in hematopoiesis, inflammation and bone homeostasis. Hematopoietic stem cells (HSC) are precursor cells of blood and immune cells, including monocytes/macrophages that arise from osteoclast progenitor cells (Baldridge, King et al. 2011). There are two forms of hematopoietic stem cell niche: one is an endosteal niche (osteoblastic niche), and the other is a sinusoidal vascular niche. Among them, the osteoblastic niche contributes to the maintenance of the HSC balance, quiescence and activation (Yoshihara, Arai et al. 2007).

Bone marrow is a niche where HSCs are maintained and hematopoiesis and osteoclastogenesis occur. Bone marrow is suppressed by diverse sources of stress condition, such as oxidation, anemia, hypoxia, radiation, cytotoxic chemotherapy and inflammation (Wilson, Laurenti et al. 2008, Essers, Offner et al. 2009, Cao, Wu et al. 2011, Lucas, Scheiermann et al. 2013). Suppression of the bone marrow leads to a reduction in the function of bone marrow, including osteoclastogenesis. At that time, various inflammatory factors, such as cytokines and chemokines, are

secreted from the diverse niche cells (Avecilla, Hattori et al. 2004, Sugiyama, Kohara et al. 2006).

Previous studies in our lab have investigated the expression of genes encoding secreted proteins that enforce HSC niche restoration after 5-FU treatment. After administration of a sub-lethal dose of 5-FU, HSC niche-related gene expression in Osterix (Osx)-positive osteoprogenitor cells isolated from Osx-GFP::Cre transgenic mouse femurs and tibiae was examined by RNA-seq analysis. Among the regulated genes, the genes encoding secreted proteins and cellular membrane proteins were further analyzed. Genes that were shown to be up-regulated in 5-FU-activated Osx-positive cells in the RNA-seq analysis were selected (Preliminary our data. Table 1). To validate the expression of these candidate genes from the RNA-seq analysis in 5-FU-activated Osx-positive cells, real-time RT-PCR was performed (Preliminary our data. Fig 1), and the results revealed that the expression of CCL4 was dramatically increased in activated bone marrow niche cells in the 5-FU treatment group. Based on these previous results, CCL4 was chosen as a reinforcing factor of endosteal niche function.

CCL4, also known as macrophage inflammatory protein-1β (MIP-1β), is known to play a role in chemotactic activity in the immune cells (Menten, Wuyts et al. 2002). CCL4 can activate chemokine receptor CCR5, which is involved in diverse immune

responses (Ren, Guo et al. 2010). Among the diverse chemokines, CCL4 and its receptor CCR5 have been a focus of research on HIV (He, Chen et al. 1997, Guan, Wang et al. 2002, Dong, Wigmore et al. 2005, Fatkenheuer, Pozniak et al. 2005). CCL4 also is secreted by CD8+ T cells (Cocchi, DeVico et al. 1995, Wang, Patterson et al. 2013) and attracts monocytes, natural killer cells and other immune cells (Bystry, Aluvihare et al. 2001). CCR5 is mainly expressed on T cells, macrophages, dendritic cells, eosinophils and microglia (Barmania and Pepper 2013). In addition, CCR5 has been demonstrated to be involved in cancer (Che, Shao et al. 2016). For example, breast cancer metastasis is promoted by CCL5-CCR5 activation (Velasco-Velázquez and Pestell 2013). The tumor suppressor miRNA-107 has also been reported to directly target CCR5 and antagonizes the proliferation and invasion of cervical cells (Che, Shao et al. 2016).

Osteoclasts are developed by the fusion of macrophage cells of hematopoietic lineage. Receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) from the osteoblast and stromal cells are essential for osteoclastogenesis (Suda, Kobayashi et al. 2001). The monocytes/macrophages stimulated by M-CSF and RANKL induce the expression of osteoclast-related genes such as cathepsin K (CTSK), tartrate-resistant acid phosphatase (TRAP), calcitonin receptor and the β3-integrin. As a result, the mature osteoclasts

play a role in bone resorption (Boyle, Simonet et al. 2003). There are several chemokines, such as CCL20, CX3CL1 and CCL9, and their receptors that are related to osteoclastogenesis (Yang, Mailhot et al. 2006, Lisignoli, Piacentini et al. 2007, Koizumi, Saitoh et al. 2009), but the relationship between CCL4 and osteoclast differentiation is still unknown. Interestingly, several chemokine receptors, including CX3CR1 and CXCR6, are also known to be down-regulated by RANKL (Saitoh, Koizumi et al. 2007, Li, Zhao et al. 2012). Based on previous studies, the hypothesis that CCL4 and its receptor CCR5 inhibit osteoclast differentiation by RNAKL was established.

In this study, the functions of CCL4 and its receptor CCR5 were investigated in pre-osteoclast cells from mouse bone marrow to better understand the mechanisms of this chemokine and its receptor in osteoclastogenesis.

Materials and Methods

Reagents

The AKT inhibitor (LY294002) and MEK inhibitor (U-0126) were purchased from Enzo Life Sciences (Plymouth Meeting, PA). The NF-κB inhibitor (BAY11-7082) and p38 inhibitor (SB203580) were purchased from Calbiochem (Ellisville, MO). The phospho-p44/p42 MAPK (Erk1/2) antibody was purchased from Cell Signaling Technology (Cell Signaling, Danvers, MA). PE anti-mouse CD195 (CCR5) and PE Armenian Hamster IgG Isotype control were purchased from Biolegend (Biolegned, San Diego, CA). Recombinant murine MIP-1β (CCL4) and recombinant murine IFN-γ were purchased from Peprotech (Peprotech, Rocky Hill, NJ). Recombinant mouse RANKL and recombinant mouse M-CSF were purchased from R&D System (R&D System, Minneapolis, MN).

Isolation and osteoclast differentiation of murine bone marrow-derived monocytes

Mouse bone marrow cells were isolated by flushing femurs and tibias from 8-9-week old BALB/c mice and incubated for 24 h in MEM-α modification (1x) with 10% FBS and 1% penicillin/streptomycin. After 24 hr, only non-adherent cells were collected and used for differentiation (Weischenfeldt and Porse

2008). To initiate differentiation, non-adherent cells were cultured in MEM-α modification with 30 ng/ml recombinant mouse M-CSF (R&D, MN, USA). Then, the mouse bone marrow cells were differentiated to macrophages. The macrophage cultures in MEM-α modification with 20 ng/ml recombinant mouse M-CSF and 50 ng/ml recombinant mouse RANKL (R&D, MN, USA) were treated daily for 3 days to generate osteoclasts (Boyle, Simonet et al. 2003). RANKL-treated pre-osteoclast cells had a multinuclear cell phenotype as determined by tartrate-resistant acid phosphatase (TRAP) staining (SIGMA-ALDRICH, MO, USA). Osteoclasts were counted as TRAP-positive cells under light microscopy.

MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide) assay

The MTT assay was done as an indicator of cell survival and/or growth. The assay determines the presence of live cells with functional mitochondria. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) assay to determine relative cell growth (Riss, Moravec et al. 2015). Pre-osteoclast cells were placed into 48-well plates that were treated with varying concentrations (0, 0.25, 0.5, 1, 2.5, 5, 10, 50 and 100 ng/ml) of CCL4 for 24 hr. MTT (AMRESCO, Solon, OH) was added to each well and incubated for an additional 4 hr at 37 ℃. Following removal of the culture medium, the remaining crystals

were dissolved in 200 μl DMSO, and absorbance at 570 nm was measured.

Annexin V/PI staining

For assays measuring apoptosis, the pre-osteoclast cells were cultured at a concentration of 3×10^6 cells in a 60-mm Petri dish and treated as described. For measuring apoptosis, cells were harvested and stained with 5 μl of FITC Annexin V and 1 μl of the 100 μg/ml PI working solution for 15 min at room temperature. After the incubation period, 400 μl of 1X Annexin-binding buffer was added, and the samples were mixed gently and maintained on ice before analysis with flow cytometry.

Migration assay

Monocyte chemotactic activity was tested using 5.0-μm-pore polycarbonate membrane insert Transwell cell culture chambers (Corning, USA). Monocytes were washed with FBS-free medium and resuspended in 0.1% BSA medium at a density of $1x10^6$ cells per well in 48-well plates. Two hundred microliters of aliquots of cell suspension were added to the upper compartment, while the lower compartments contained 600 μl medium with or without 10 ng/ml CCL4 and with or without 50 ng/ml RANKL. After incubation for 2 hr at 37 ℃, cells that had migrated to the lower surface were counted.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time RT-PCR analysis

Total RNAs from pre-osteoclast cells were isolated using TRIzol reagent (Ambion, Carlsbad, CA) according to the manufacturer's recommendations. Then 500 ng to 1 μg of total RNA from each sample were reverse transcribed with an Omniscript Reverse Transcription kit (QIAGEN, Hilden, Germany). The reverse transcription reaction was carried out at 37 ℃ for 1 hr. To measure the expression of genes, fluorescence-based real-time RT-PCR was performed with the Thermocycler (Bio-Rad, Hercules, CA). SYBR green and Go-Taq ® Flexi DNA polymerase (Promega, Madison, WI) were used for DNA amplification and detection. Relative mRNA expression levels were determined by △△Ct calculation (Livak and Schmittgen 2001).

Western blot analysis

Pre-osteoclast cells were incubated with or without CCL4 (10 ng/ml) for 0, 10, 30 and 60 min. Then, whole cell lysates were prepared in a RIPA buffer (25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) (Thermo Scientific, Bellefonte, PA) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). Lysates were incubated in ice for 30 min and centrifuged at 14,000 rpm for 20 min. Protein concentrations were determined using the Bradford protein assay

kit (Bio-Rad, Hercules, CA, USA). Equal amounts of cell lysate protein (30 μg/lane) were subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% BSA in 0.05% Tween 20 in Tris buffered saline (TBST). The membranes were incubated with a p-Erk antibody (Cell Signaling Technology, Danvers, MA) overnight at 4 ℃, The membrane was washed three times with TBST and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. The blots were developed using a chemiluminescence detection system (ATTO corporation, Tokyo, Japan) and exposed to an X-ray film.

Flow cytometry

The expression of CCR5 on the surface of monocytes was assessed with PE anti-mouse CD195 (CCR5) (Biolegend, San Diego, CA). In brief, 3x105 treated cells were washed twice with PBS, labelled for 30 min at 4 ℃ with PE anti-mouse CD195 (CCR5), and then washed twice with cold PBS. Finally, cells were washed and resuspended in PBS containing 3% FBS before flow cytometry analysis with a FACS-can flow cytometer (Becton-Dickinson, San Jose, CA).

Statistical analyses

Data are presented as the mean ± S.D. GraphPad Prism software

(Version 7.01, GraphPad software, Inc., CA, USA) was used for statistical analyses. Comparisons were performed using a Student's t-test or one-way ANOVA followed by the Tukey-Kramer multiple comparisons tests. The cell survival data were analyzed by two-way ANOVA followed by Sidak multiple comparisons tests.

Results

CCL4 does not enhance RANKL-induced osteoclastogenesis in mouse

Whether CCL4 had a direct effect on osteoclast differentiation was investigated. In the presence of M-CSF and RANKL, pre-osteoclast cells were well differentiated into osteoclasts, and CCL4 treatment did not show any effect on differentiation (Fig 2A-a, A-c). Even CCL4 treatment with RANKL did not alter RANKL-mediated osteoclast differentiation (Fig 2A-d). Similarly, RANKL-mediated osteoclastogenesis induced expression of MMP-9 and cathepsin K, but these osteoclast-related genes were not expressed with CCL4 treatment without RANKL (Fig 2C, D). Osteoclasts were identified by TRAP staining, and multinucleated (≥3 nuclei) cells were counted under a microscope (Fig 2B). Osteoclast cells were induced by treatment with M-CSF + RANKL, but the differentiated cells were not altered by the addition of CCL4 treatment. These results suggested that CCL4 is not directly involved in osteoclastogenesis.

Name	Description	PBS(FPKM)	5FU(FPKM)
Camk4	calcium/calmodulin-dependent protein kinase IV	0.0670814	19.6714
Seh1l	SEH1-like (S. cerevisiae)	0.696726	119.672
Pam16	presequence translocase-asssociated motor 16 homolog (S. cerevisiae)	6.59643	2163.56
Fam131a	family with sequence similarity 131, member A	0.430057	343.756
Slc38a1	solute carrier family 38, member 1	0.06162	31.8122
Dcaf5	DDB1 and CUL4 associated factor 5	0.260236	117.372
Ylpm1	YLP motif containing 1	0.199516	68.4796
Rmi1	RMI1, RecQ mediated genome instability 1, homolog (S. cerevisiae)	0	46.3982
Lcp2	lymphocyte cytosolic protein 2	0.214236	118.854
pik3r1	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	0.833747	474.689
Nle1	notchless homolog 1 (Drosophila)	1.09874	1010.91
Ccl4	chemokine (C-C motif) ligand 4	1.54332	6555.11
Synrg	synergin, gamma	0.270098	37.9267
Aoc2	amine oxidase, copper containing 2 (retina-specific)	0	14.2641
Anapc13	anaphase promoting complex subunit 13	0.577137	626.949
Ctsc	cathepsin C	0.376766	93.1973
Ndufab1	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1	1.32182	327.225
Kntc1	kinetochore associated 1	0.028104	14.9707
Flt1	FMS-like tyrosine kinase 1	0.0320189	36.0988
Lrrcc1	leucine rich repeat and coiled-coil domain containing 1	0	9.06284
Igbp1	immunoglobulin (CD79A) binding protein 1	0.682015	438.012
Rasgrp1	RAS guanyl releasing protein 1	0	130.479
Klhl20	kelch-like 20	0	17.7368

Table 1. Up-regulated genes in Osx-positive osteoprogenitor cells by RNA-seq after 5FU treatment.

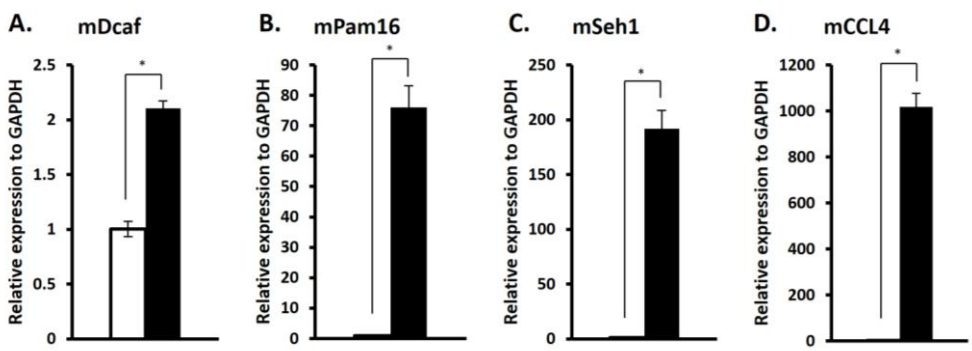

Figure 1. Expression of candidate genes from the RNA-seq analysis in 5FU-activated Osx-positive cells validated by real-time PCR.

(A) Expression of mDcaf; (B) expression of mPam16; (C) expression of mSeh1; (D) expression of CCL4. The data are presented as the mean ± S.D. (n=3; *P-value < 0.05, Student's t-test)

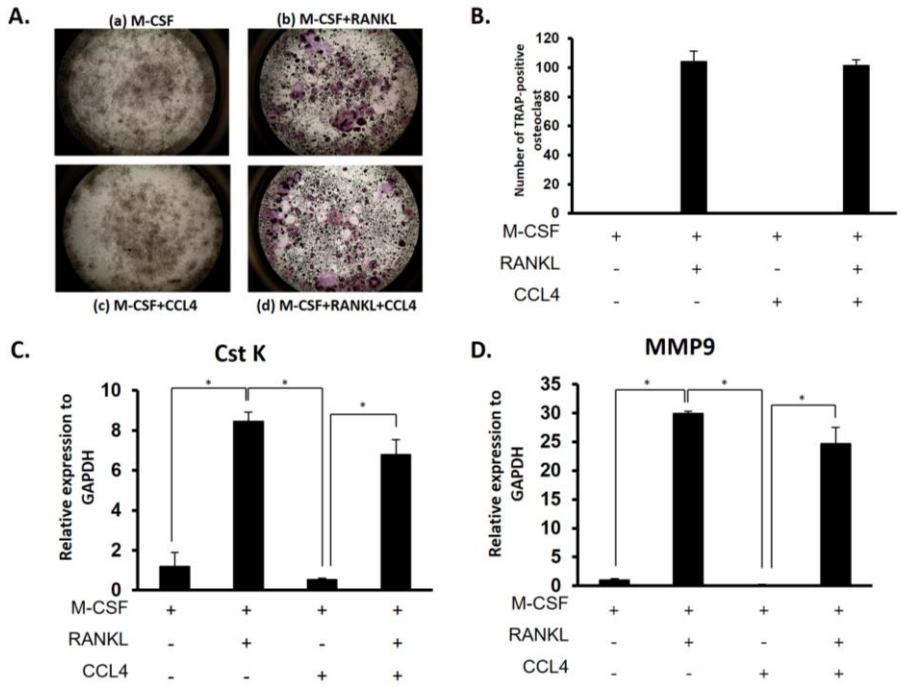

Figure 2. CCL4 does not augment RANKL-induced osteoclastogenesis.

(A) Mouse monocytes were differentiated in the presence of recombinant (a) M-CSF (30 ng/ml), (b) M-CSF (30 ng/ml) + RANKL (50 ng/ml), (c) M-CSF (30 ng/ml) + CCL4 (10 ng/ml) or (d) M-CSF (30 ng/ml) + RANKL (50 ng/ml) + CCL4 (10 ng/ml) for 6 days. Osteoclast cells were induced by M-CSF + RANKL treatment, but the differentiated cells were not altered by the addition of CCL4 treatment. The cells were stained for TRAP. (B) TRAP-positive cells were counted as osteoclasts (≥3 nuclei). Gene expression profiles related to osteoclasts were determined

by real-time PCR. (C) Expression of cathepsin K; (D) expression of MMP9. The data are presented as the mean ± S.D. (n=3; *P-value < 0.05, One-way ANOVA)

CCL4 promotes the migration and viability of pre-osteoclast cells

The chemotaxis of pre-osteoclast cells was investigated to determine the function of CCL4 in pre-osteoclast cells and was assessed using a transwell migration assay. Pre-osteoclast cells were placed in the upper chamber and induced to migrate through the pores. CCL4 induced pre-osteoclast cell migration when the lower chamber contained media including CCL4 (Fig 3A). However, RANKL treatment resulted in an evident inhibition of cell chemotaxis mediated by CCL4 (Fig 3A). CCL4 activated phosphorylation of Erk (Fig 3B), suggesting that enhanced cell migration by CCL4 might be mediated by p-Erk. Another function of CCL4 was to improve the viability of the pre-osteoclast cells, as measured by the MTT assay. Pre-osteoclast cells were treated with or without various concentrations of CCL4 for 3 days along with M-CSF (30 ng/ml). Compared to the cell viability exerted M-CSF treatment alone, CCL4 treatment at a concentration of 250 pg to 100 ng improved overall cell viability (Fig 3C). Moreover, in addition to cell viability, CCL4 treatment resulted in an anti-apoptotic effect. To determine the anti-apoptotic function of CCL4 on pre-osteoclast cells, Annexin V/PI staining was followed by FACS. When CCL4 was added, the percentage of viable cells negative for Annexin V/PI (Annexin V-/PI-) was increased from 51.7% to 59.2%, and percentage of Annexin V+/PI+ late apoptotic

cells was decreased from 44.3% to 34.6% (Fig 3D, E). The percentage of live or apoptotic pre-osteoclast cells is shown in the bar diagram as the mean ± S.D from three mice (Fig 3F, G). These results showed that CCL4 increased the viability of pre-osteoclast cells and protected the cells from apoptosis.

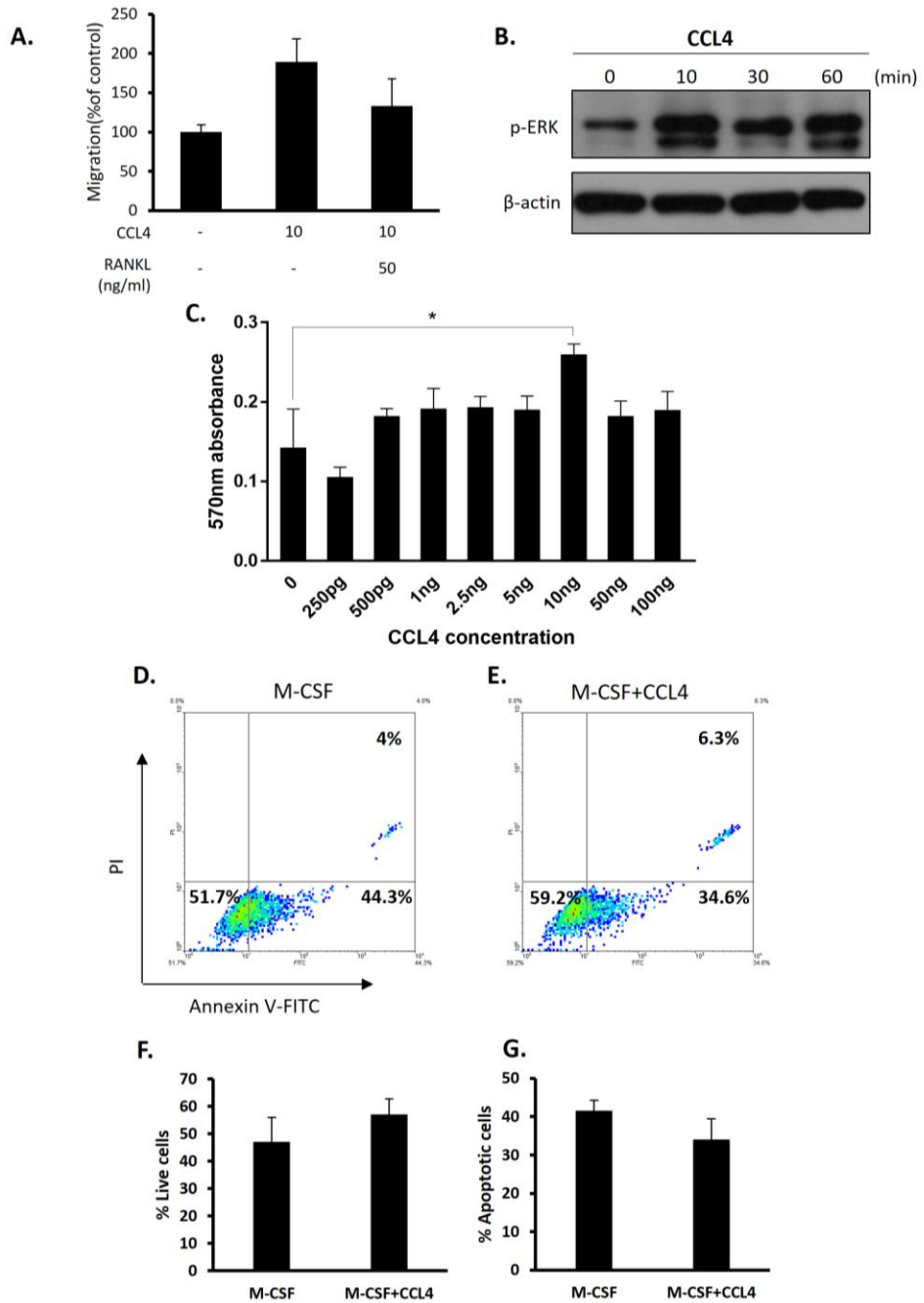

Figure 3. CCL4 promotes migration and viability of pre-osteoclast cells.

(A) Migration assay. Pre-osteoclast cells were resuspended in serum-free cell culture media in each upper Trans-well chamber. After incubation for 2 hr, cells that had migrated to the lower chamber were counted. The data are presented as the mean ± S.D. **(B)** Cells were treated with 10 ng/ml CCL4 for 0-60 min. Whole cells were lysed with RIPA buffer, resolved by SDS-PAGE and immunoblotted with the p-Erk and β-Actin antibody. **(C)** Effects of CCL4 on the survival of pre-osteoclast cells *in vitro*. Cell viability was measured by MTT assay. Cell were treated with various concentrations (0-100 ng) of CCL4. The data are presented as the mean ± S.D. (n=3; *P-value < 0.05, Student's One-way ANOVA). **(D, E)** Pre-osteoclast cells were incubated in medium with or without 10 ng/ml CCL4 for 3 days. To determine the apoptotic cell population, cells were stained with Annexin V/PI staining and analyzed by flow cytometry (FACS) analysis. In each panel, the lower left quadrant (Annexin V-/PI-) shows cells that are viable cell populations, the lower right (Annexin V+/PI-) shows cells that are in the early stage of apoptosis, and the upper right (Annexin V+/PI+) shows cells that are in the late stage of apoptosis. **(F)** The percentage of live cells shown in the bar diagram is presented as the mean (n=3) ± S.D. **(G)** The percentage of apoptotic cells shown in the bar diagram is presented as the mean (n=3) ± S.D.

RANKL down-regulates CCR5 expression during osteoclastogenesis in mouse pre-osteoclast cells

During osteoclastogenesis, the expression levels of CCR5 mRNA were investigated in pre-osteoclast cells treated with or without RANKL (50 ng/ml) for 3 days. CCR5 expression was decreased with RNAKL treatment regardless of the presence of absence of CCL4 (Fig 4A-B). In addition, the CCR5 mRNA level was investigated for 24 hr and was shown to slowly decline within 24 hr. When the primary cells were treated with CCL4 with M-CSF to differentiate into macrophages, CCR5 levels were maintained up to 6 hr after RANKL treatment (Fig 4C). RANKL-induced reduction of CCR5 was also examined by flow cytometry (FACS) analysis. This analysis demonstrated that the number of cells expressing the CCR5 protein was significantly reduced by RANKL (Fig 4D-c). Likewise, the number of cells expressing the CCR5 protein was increased by M-CSF and CCL4 treatment (Fig 4D-a, b). These results demonstrated that inhibition of CCR5 occurred in parallel to RANKL-mediated osteoclastogenesis in pre-osteoclast cells.

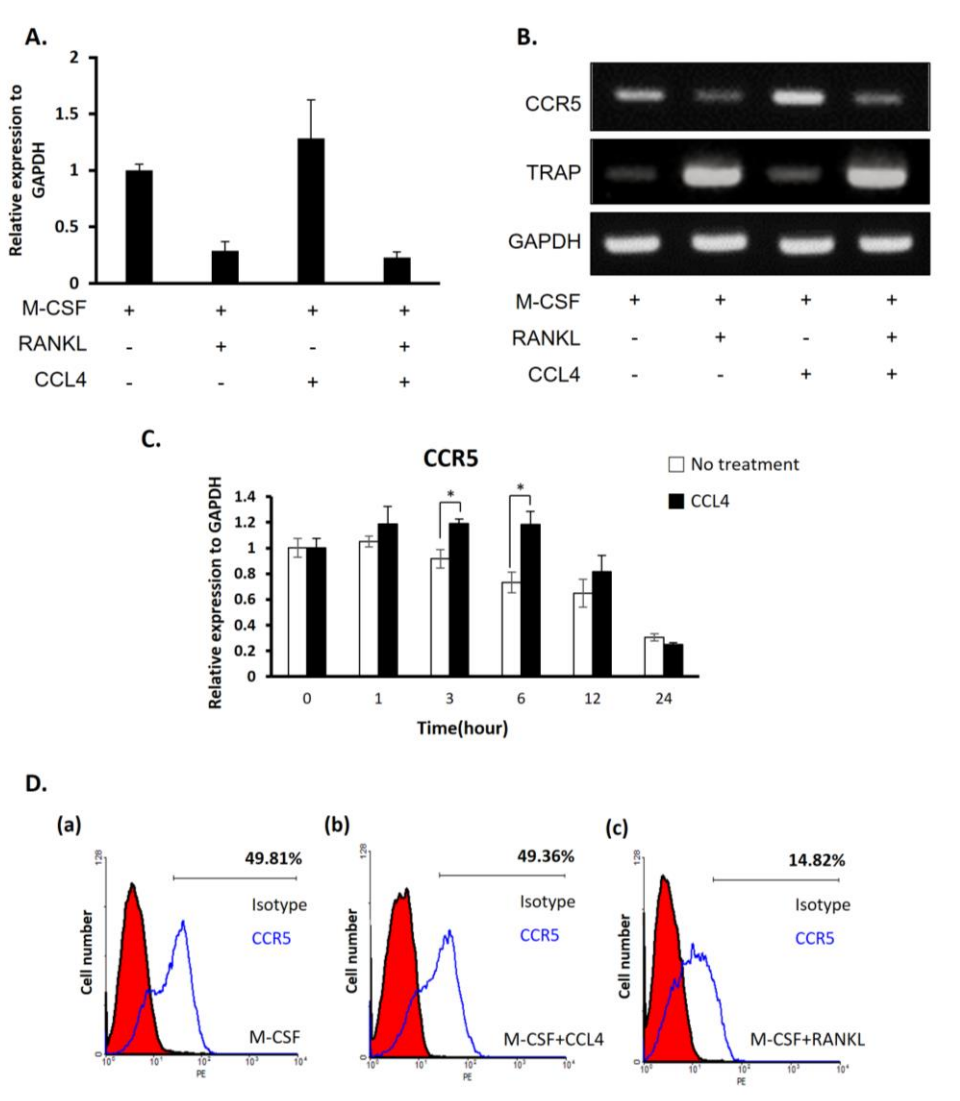

Figure 4. RANKL down-regulated CCR5 expression in pre-osteoclast cells.

(A) Pre-osteoclast cells were cultured for 4 days in the presence of recombinant M-CSF (30 ng/ml) with RANKL (50 ng/ml) and CCL4 (10 ng/ml). Total RNA was extracted and converted to cDNA, which was used for real-time RT-PCR and (B) conventional RT-

PCR. (C) Cells were stimulated with RANKL alone (50 ng/ml) or RANKL with CCL4 (10 ng/ml) for 0-24 hr. The expression levels of CCR5 mRNA were measured by real-time RT-PCR and normalized to GAPDH. The data are presented as the mean ± S.D. (n=3; * P-value < 0.05, Two-way ANOVA) (D) FACS analysis. Pre-osteoclast cells were pretreated with (a) M-CSF (30 ng/ml), (b) M-CSF (30 ng/ml) + CCL4 (10 ng/ml) or (c) M-CSF (30 ng/ml) + RANKL (50 ng/ml) for 48 hr.

CCR5 levels decreased by RANKL were restored by blocking MEK and JNK signal

Binding of RANKL to its receptor RANK induces several intracellular signaling molecules in pre-osteoclast cells, including MAPKs, Src kinase and NF-κB (Wada, Nakashima et al. 2006). To verify the signaling pathways causing the RANKL-induced reduction in CCR5 expression, the effects of multiple kinase inhibitors were examined, including a MEK inhibitor (U0126), p38 inhibitor (SB20380), JNK inhibitor (SP600125), NF-κB inhibitor (BAY 11-7082) and PI3K inhibitor (LY294002) (Fig 5). As shown in Figure 5A, treatment with 1 to 20 μM of the MEK inhibitor (U0126) restored the expression of CCR5 reduced by RANKL. In contrast, treatment with 5 to 30 μM of the p38 inhibitor (SB20380) did not affect RANKL-induced suppression of CCR5 (Fig 5B). Additionally, the JNK inhibitor, NF-κB inhibitor and PI3K inhibitor did not significantly restore the levels of CCR5 (Fig 5C-F). Thus, CCR5 expression was significantly down-regulated by RANKL.

Figure 6A shows that inhibition of MEK and JNK restored CCR5 mRNA levels. Additionally, when the MEK inhibitor was given with the JNK inhibitor, the levels of CCR5 were completely restored (Fig 6B). To confirm these data, the expression of cell surface CCR5 was measured by flow cytometry (FACS) analysis (Fig 6C). These results implied that RANKL-mediated down-regulation of CCR5 expression was associated with the MEK and JNK signaling.

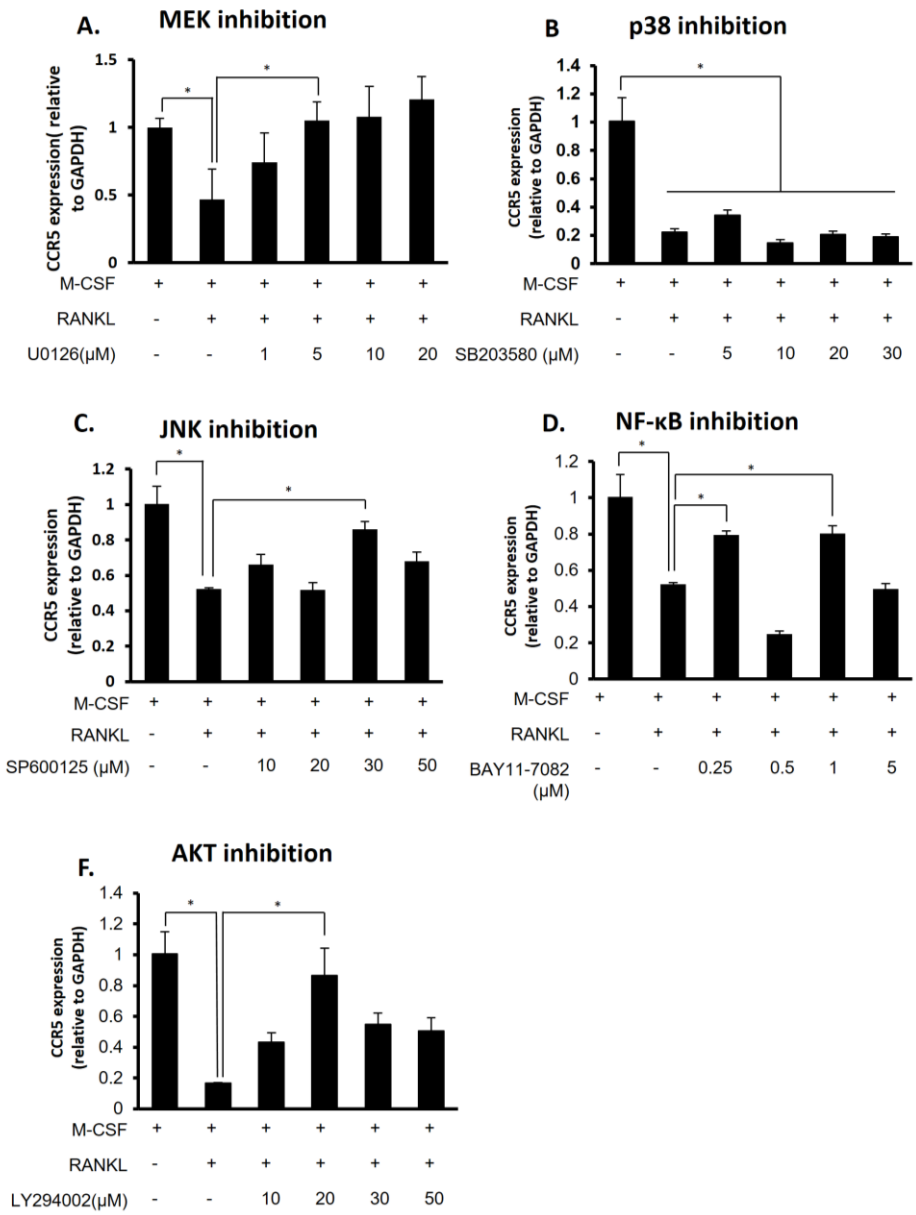

Figure 5. Inhibition of the RANKL-induced reduction of CCR5 mRNA by several inhibitors.

Pre-osteoclast cells were treated with or without (A) U0126, (B) SB203580, (C) SP600125, (D) BAY11-7082 and (E) LY294002 at

different concentrations for 30 min, followed by RANKL (50 ng/ml) for 24 hr. Expression levels of CCR5 mRNA were measured by real-time RT-PCR and normalized to GAPDH. The data are presented as the mean ± S.D. (n=3; * P-value < 0.05 One-way ANOVA)

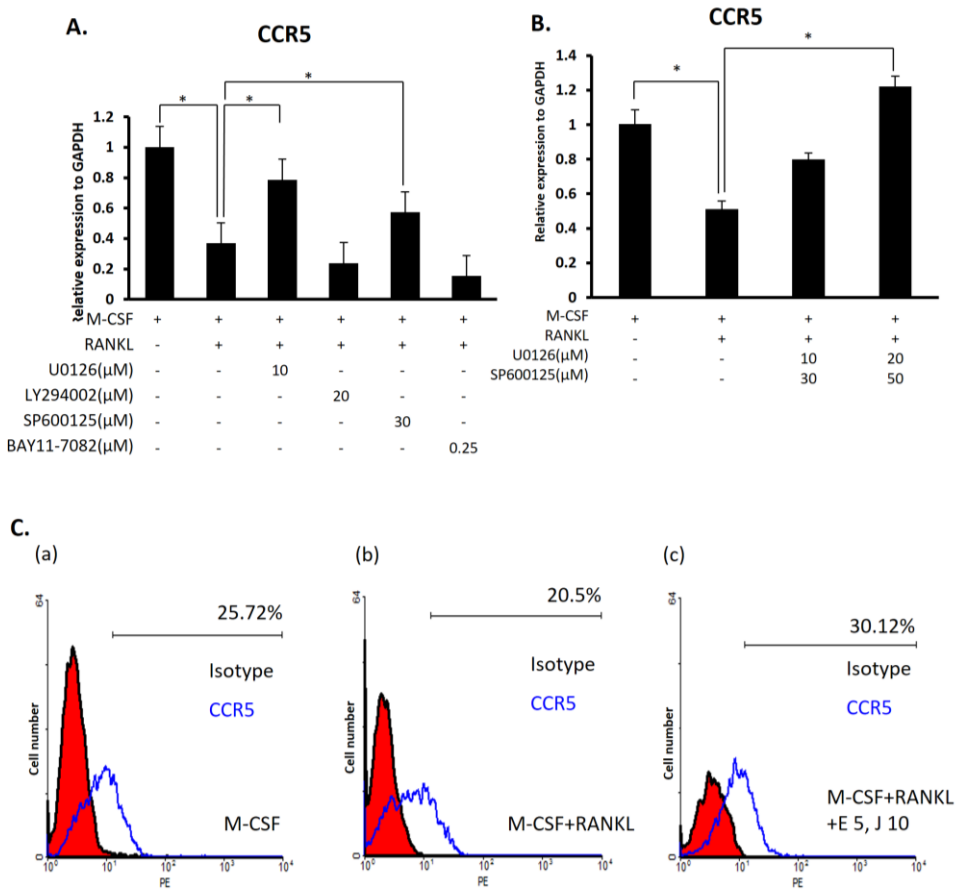

Figure 6. Inhibition of the RANKL-induced reduction of CCR5 mRNA. (A) U0126 (10 μM), LY294002 (20 μM), SP600125 (30 μM) and BAY11-7082 (0.25 μM). Expression levels of CCR5 mRNA were measured by real-time RT-PCR and normalized to GAPDH. The data are presented as the mean ± S.D. (n=3; * P-value < 0.05 One-way ANOVA) (B) Pre-osteoclast cells were pretreated with U0126 (10 μM) and SP600125 (30 μM) for 30 min, followed by RANKL (50 ng/ml) stimulation for 24 hr. Expression levels of CCR5 mRNA were measured by real-time RT-PCR and normalized to

GAPDH. The data are presented as the mean ± S.D. (n=3; * P-value < 0.05 One-way ANOVA) (C) Expression levels of CCR5 were also detected by the anti CCR5 antibody using flow cytometry analysis.

CCR5 inhibits differentiation to osteoclasts

To determine the biological meaning of CCR5 reduction during RANKL-mediated osteoclast differentiation, osteoclastogenesis was next performed in the presence of IFN-γ. IFN-γ, LPS and H_2O_2 are known to up-regulate CCR5 expression in T-cells, monocytes and other immune cells (Zella, Barabitskaja et al. 1998, Hariharan, Douglas et al. 1999, Juffermans, Paxton et al. 2000, Lehoux, Le Gouill et al. 2003). These factors are involved in osteoclast differentiation. In particular, IFN-γ is known to inhibit osteoclast differentiation (Takayanagi, Ogasawara et al. 2000). Considering these two findings, CCR5 was hypothesized to inhibit osteoclast differentiation. Treatment with M-CSF plus IFN-γ induced the expression of CCR5 approximately 30% more than treatment with M-CSF plus CCL4 (Fig 7A-c).

As shown in Figure 5A-b, treatment with M-CSF plus RANKL decreased CCR5 levels. However, CCR5 levels were increased when IFN-γ was added even in the presence of RANKL plus M-CSF (Fig 7A-d). In order to prove our hypothesis that a high level of CCR5 inhibits osteoclast differentiation, osteoclast differentiation was investigated in the presence of IFN-γ. When the pre-osteoclast cells were treated with IFN-γ, unlike with RANKL treatment (Fig 7B-b), the cells were not well differentiated (Fig 7B-b). However, blocking CCR5 with its antibody in the presence of IFN-γ resulted in better osteoclast differentiation than when

CCR5 was not blocked (Fig 7B-c, d). Osteoclastogenesis was measured by TRAP staining, and the expression levels of osteoclast markers TRAP and cathepsin K were higher in the cells in which CCR5 was blocked with its antibody in the presence of IFN-γ than in cells where CCR5 was not blocked (Fig 7C). Combined all together, these results implied that CCR5 inhibits osteoclast differentiation.

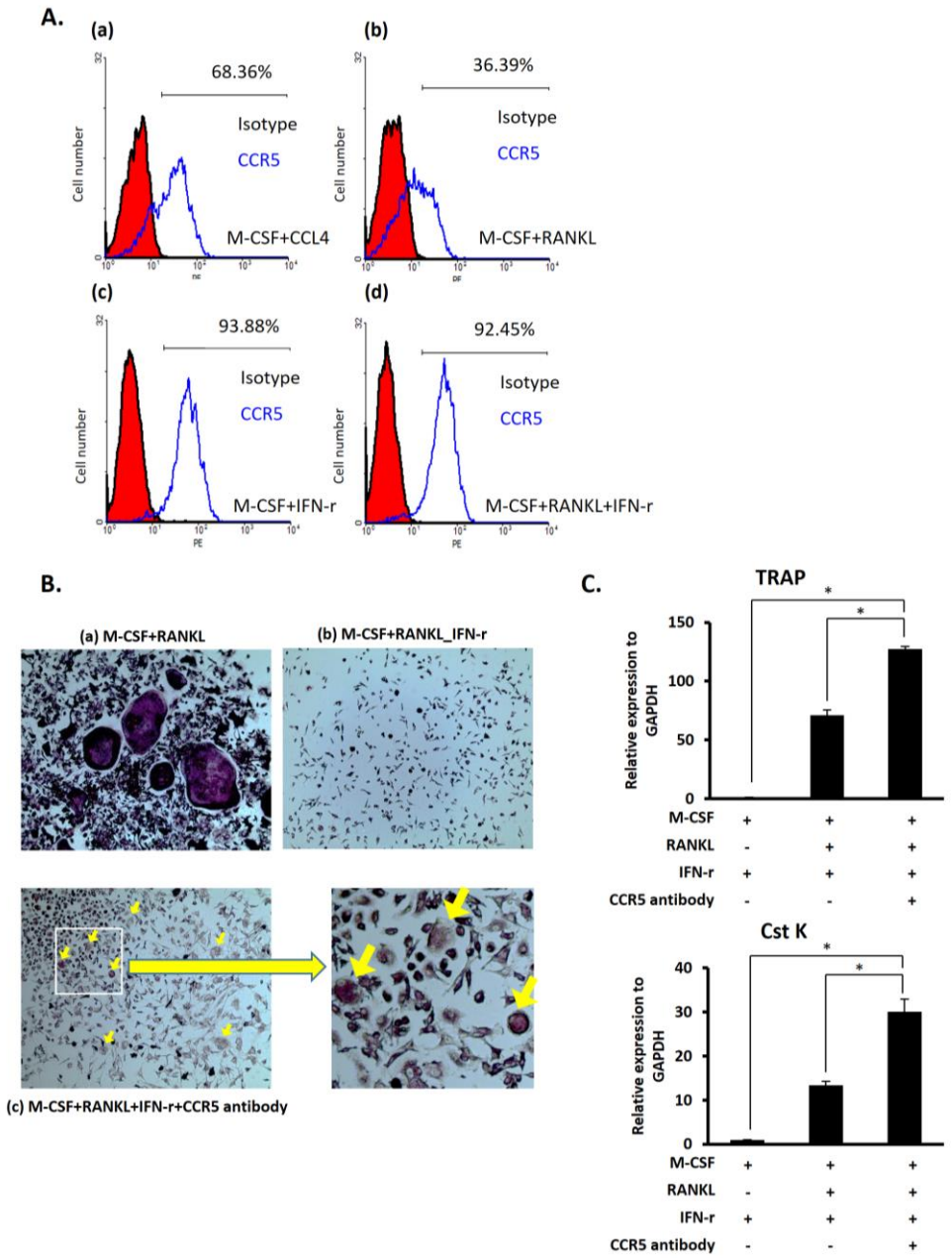

Figure 7. CCR5 inhibits differentiation of osteoclasts.

(A) FACS analysis. Pre-osteoclast cells were pretreated with (a) M-CSF (30 ng/ml) + CCL4 (10 ng/ml), (b) M-CSF (30 ng/ml) +

RANKL (50 ng/ml), (c) M-CSF (30 ng/ml) + IFN-γ (100 U/ml) or (d) M-CSF (30 ng/ml) + RANKL (50 ng/ml) + IFN-γ (100 U/ml) for 48 hr. (B) Mouse monocytes were differentiated in the presence of recombinant (a) M-CSF (30 ng/ml) + RANKL (50 ng/ml), (b) M-CSF (30 ng/ml) + RANKL (50 ng/ml) + IFN-γ (100 U/ml), (c) M-CSF (30 ng/ml) + RANKL (50 ng/ml) + IFN-γ (100 U/ml) + CCR5 antibody for 6 days, and (d) was enlarged from (c). (C) The expression of TRAP and cathepsin K, osteoclast-related genes, was determined by real-time PCR. The data are presented as the mean ± S.D. (n=3; * P-value < 0.05, One-way ANOVA)

Discussion

Activation of chemokine expression has been known to occur in several stress conditions (Lu, Zhu et al. 2012). A previous RNA sequencing analysis showed that CCL4 was significantly elevated in Osx-positive cells when acute bone marrow damage was induced by 5-FU treatment (Table 1, Fig 1). In this study, the functions of CCL4 secreted from the osteoblastic niche and its receptor CCR5 were investigated in the effector pre-osteoclast cells.

First, osteoclast differentiation was performed to determine whether CCL4 promoted osteoclastogenesis. Osteoclasts are known to resorb bone tissue and release TGF-b, which recruits pre-osteoblast cells to remodel bone and enhances the overall osteoblastic niche condition (Tang and Alliston 2013). However, our results revealed that CCL4 was not essential for osteoclast differentiation. TRAP staining showed that there was no significant difference in the differentiation of pre-osteoclast cells between cells treated with CCL4 and RANKL and cells only treated with RANKL (Fig 2). This result indicated that CCL4 was not directly involved in the osteoclastogenetic process.

Hence, CCL4 functions in recruiting pre-osteoclast cells and improving the osteoblastic niche were investigated. In the migration assay, CCL4 promoted pre-osteoclast cell migration (Fig 3A). On

the other hand, pre-osteoclast migration was reduced upon RANKL treatment. During osteoclastogenesis, the expression of the CCL4 receptor, CCR5, which plays a significant role in osteoclast differentiation, was repeatedly observed to be inhibited by RANKL (Fig 4). These results demonstrated that RANKL inhibited cell migration stimulated by CCL4 (Fig 3A) through down-regulation of its receptor CCR5 (Fig 4A, B).

Another function of CCL4 was promoting cell viability. Some chemokines are known to improve cell viability and enhance resistance to apoptosis (Ticchioni, Essafi et al. 2007). Our data also showed that CCL4 enhanced cell viability (Fig 3C) and prevented apoptosis in pre-osteoclast cells (Fig 3D-G). These results suggest that CCL4 plays a role in recruiting pre-osteoclast cells in good condition during the early osteoclast differentiation process and induces differentiation into mature osteoclasts.

During osteoclastogenesis, RANKL decreases the expression of several chemokine receptors, and the signaling pathways that are involved in the down-regulation of chemokine receptors by RANKL have been studied (Saitoh, Koizumi et al. 2007, Li, Zhao et al. 2012). Our data showed that CCR5 was down-regulated by RANKL (Fig 4). Next, the mechanism of RANKL-mediated down-regulation of CCR5 during osteoclast differentiation was investigated. Comparatively little is known about the mechanism and physiological role of down-regulation of chemokine receptors, in

contrast to up-regulation of their expression.

The inhibition of CCR5 during osteoclastogenesis further extended the idea that high levels of CCR5 would hinder osteoclast differentiation. Thus, osteoclast differentiation was carried out in the presence of IFN-γ to maintain high levels of CCR5. IFN-γ has been known to induce the expression of chemokine receptors including CCR5 and CCR3 (Zella, Barabitskaja et al. 1998). In the presence of IFN-γ, the CCR5 level was elevated (Fig 7A), and osteoclastogenesis was shown to not occur in our results (Fig 7B). In addition, blocking CCR5 increased the expression of TRAP and cathepsin K, both osteoclast markers, compared to that in the IFN-γ only treatment group (Fig 7C), although the levels were lower than that of the RANKL-treated group. Obviously, our data showed that the increase in CCR5 expression inhibited osteoclast formation. Blocking CCR5 may not have rescued osteoclast differentiation due to other chemokine receptors such as CCR3, which may be similarly up-regulated by IFN-γ (Zella, Barabitskaja et al. 1998)and down-regulated by RANKL treatment (Lean, Murphy et al. 2002). In our experiment, only CCR5 was blocked by an antibody, and CCR3 would be maintained at a high level in the presence of IFN-γ; thus, the increased level of CCR3 might inhibit osteoclastogenesis.

The RANKL-mediated signals, MEK and JNK, were identified to be involved in down-regulation of CCR5 expression. Binding of RANKL to RANK initiates downstream signaling pathways that are

related to osteoclast differentiation. ERK, JNK, p38, PI3K/AKT and IKK1/2 and transcription factors NFAT, AP-1, c-Fos and NF-κB are involved in osteoclast differentiation (Boyle, Simonet et al. 2003, Saitoh, Koizumi et al. 2007). Figure 6 shows that the RANKL-induced reduction of CCR5 expression was controlled via two downstream signals of RANKL, MEK and JNK. RANKL activates nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) through MEK and JNK signaling (Zhao, Shao et al. 2007). NFATc1 is a master transcription factor of osteoclastogenesis is known to regulate transcription of CCR5 as it binds to the CCR5 promoter (LIU, ZHAO et al. 1998, Wierda and Van Den Elsen 2012). Interestingly, a similar mechanism was found in the transcriptional regulation of chemokine receptor CX3CR1; NFAT1 regulates the CX3CR1 promoter in natural killer cells (Saitoh, Koizumi et al. 2007). Based on the results that suggest that CCR5 is down-regulated by RANKL through activation of MEK and JNK and NFAT is activated by RANKL through the MEK and JNK signals, NFAT might regulate CCR5 expression. Further studies are necessary to investigate the association between CCR5 expression and NFATc1 in osteoclast differentiation. RANKL-mediated chemokine reduction in osteoclast differentiation is important to study to obtain a better understanding of the osteoblastic niche.

In conclusion, CCL4 was shown to affect the osteoblastic niche, just as chemokines have been shown to play roles, such as

recruiting progenitor cells and maintaining viability, in the osteoblastic niche (Mendelson and Frenette 2014). Although the CCL4 chemokine did not directly promote later osteoclast differentiation, our study revealed that CCL4 recruited viable pre-osteoclast cells for osteoclastogenesis.

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초 록

마우스 골수 유래 단핵세포로부터 파골세포로의 분화 과정에서 MEK과 JNK 신호에 의한 CCR5 수용체 조절 및 역할

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줄기세포 니치는 줄기세포를 둘러싼 모든 환경을 말하며 그것에 의하여 줄기세포의 분화와 항상성이 적절하게 이루어지고 있다. 니치는 여러 경로에 의해 손상을 입게 되면 환경을 극복하기 위해 많은 인자들을 분비한다. 이 연구에서는 그 골수에 손상을 입었을 때 골수줄기세포 니치세포 중 조골세포에서 분비되는 케모카인 CCL4와 수용체 CCR5의 파골세포 분화과정에서의 역할에 대하여 조사하였다. CCL4는 직접 파골세포로의 분화를 향상시키기 보다는 파골전구세포의 이동과 생존능력을 향상시키는 결과를 얻었다. 흥미롭게도 파골세포로의 분화시 필수적인 요소인 사이토카인 RANKL에 의해서 CCL4 수용체인 CCR5의 발현이 감소하는 것을 확인하였는데 이것은 RANKL에 의해서 활성화 되는 신호인 MEK과 JNK에 의해 억제되는 것을 검증 하였다. 또한 CCR5이 높게 발현될 때는 파골세포로의 분화가 되지 않은 결과로

보아 CCR5는 파골세포의 분화를 억제하는 것을 시사한다. 따라서 본 연구에서는 골수에 손상이 일어났을 때 조골세포로부터 케모카인 CCL4가 분비되어 단핵세포의 이동과 생존능을 향상시켜주어 결론적으로 파골세포로의 분화를 돕는 것을 검증하였다. 또한 파골세포 분화시에는 수용체 CCR5가 분화를 억제하기 때문에 발현이 감소하는 것임을 알 수 있다.

주요어 : 케모카인 수용체, CCL4-CCR5, 파골세포, 조골모세포 미세환경

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