齒醫科學博士學位論文

The Roles of CCL19 and CCL21 Chemokines in Osteoclast Migration and Bone Resorption

파골세포 이동과 골흡수에 대한 CCL19 및 CCL21 케모카인의 역할

2017 년 2 월

서울대학교 대학원
치의과학과 세포및발생생물학 전공
이 지 연

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by

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Advisor:

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A Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

February, 2017

Division of Cell and Developmental Biology
Department of Dental Science, School of Dentistry
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이 논문을 치의과학박사학위논문으로 제출함

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ABSTRACT

The Roles of CCL19 and CCL21 Chemokines in Osteoclast Migration and Bone Resorption

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Department of Dental Science

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(Directed by Prof. Hong-Hee Kim, Ph.D)

OBJECTIVE: Bone resorption is a severe problem in inflammatory diseases such as periodontitis and rheumatoid arthritis (RA). Osteoclasts are responsible for bone resorption and CCL19 and CCL21 act as chemokines for several cell types. The purpose of this study was to investigate the role of CCL19 and CCL21 in bone resorption by osteoclasts.

METHODS: The levels of CCL19, CCL21 and CCR7 in RA and osteoarthritis patient samples were measured using ELISA. Data deposited to gene expression omnibus was also analyzed. The expression levels of these molecules and differentiation markers of osteoclasts were measured by real time polymerase

chain reaction (PCR), western blotting or flow cytometry. Osteoclast differentiation was evaluated by tartate-resistant acid phosphatase staining and osteoclast migration was assessed with transwell assay and Oris migration assay kits. Resorption activity was performed with calcium phosphate-coated dishes or dentin slices, which were analyzed by von Kossa staining or confocal microscopy, respectively. Involvement of CCR7 was demonstrated with the small interference RNA system and contribution of GTPase Rho was investigated by the Rho pull-down assay. Collagen transplantation model was used to examine the in vivo effects of these chemokines.

RESULTS: The expression levels of CCL19, CCL21 and CCR7 were higher in RA patient samples compared to OA or normal samples. Bone marrow macrophages and osteoclasts expressed more CCR7 in response to the stimulation of TNFα, IL-1β and LPS. CCL19 and CCL21 promoted the migration and resorption activity of both bone marrow macrophages and osteoclasts. Knock-down of CCR7 significantly reduced the CCL19- and CCL21-induced migration. Moreover, CCL19 and CCL21 stimulated small GTPase Rho and its downstream molecule, ROCK. Rho inhibitors suppressed both the migration and resorption activity of BMMs and osteoclasts. It demonstrates that the increase of migration and bone resorption by CCL19 and

CCL21 was mediated by the CCR7/Rho axis. Collagen transplantation study showed that CCL19 and CCL21 can promote bone resorption in vivo.

CONCLUSION: This study indicates that under inflammatory conditions, osteoclast precursors increase the CCR7 expression. CCL19 and CCL21 are up-regulated in RA patients and enhance the osteoclast migration and resorption activity, resulting in severe bone destruction. This study suggests that neutralizing antibody for CCL19 and CCL21 or CCR7 knock-down are potential therapeutic strategies for periodontitis and RA.

Keywords : Osteoclast, Migration, CCL19, CCL21, CCR7, Rheumatoid arthritis

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CONTENTS

ABSTRACT i

CONTENTS iv

LIST OF FIGURES viii

LIST OF TABLES x

ABBREVIATIONS xi

I. Introduction 1

1. Roles of osteoblasts and osteoclasts in bone remodeling 1
2. Rheumatoid arithritis and osteoclasts 5
3. Osteoclast migration and bone resorption 8
4. Roles of CCL19/CCL21 and CCR7 in rheumatoid arithritis ...13
5. Purpose of this study 17

II. Materials and Methods 19

1. Reagents 19
2. Bone marrow-derived macrophage (BMM) generation 19

3. Osteoclast differentiation 20
4. Enzyme-linked immunosorbent assay (ELISA) 20
5. Quantification of mRNA 21
6. Western blotting analysis 21
7. TRAP staining 22
8. Cytotoxicity assay 22
9. Flow cytometry 23
10. Small interference RNA transfection 23
11. Cell migration assay 24
12. Rho pull-down assay 25
13. von Kossa staining 25
14. Dentin resorption and confocal microscopy 25
15. Confocal fluorescence microscopy 26
16. In vivo mouse calvariae resorption assay 26
17. Statistical analysis 27
18. Institutional Review Board (IRB) 27

III. Results 28

1. Increased expression of CCR7 increased in synovial macrophages of RA patients 28

2. Expression levels of CCL19, CCL21 and CCR7 increased in synovial tissues of RA patients 31
3. Increased mRNA expression level of CCR7 in response to inflammatory stimuli 34
4. Increased protein expression level of CCR7 in response to inflammatory stimuli 37
5. Effects of CCL19 and CCL21 on osteoclast differentiation 40
6. Regulation of osteoclast migration activity by CCL19 and CCL21 44
7. CCL19 and CCL21 increase osteoclast migration through CCR7 47
8. CCL19 and CCL21 increase osteoclast resorption activity 49
9. CCL19 and CCL21 increase osteoclast actin ring density 53
10. CCL19 and CCL21 stimulate osteoclasts via Rho 55
11. Rho inhibitors suppress CCL19- and CCL21-induced osteoclast migration and resorption activity 58
12. CCL19 and CCL21 increase mouse calvariae resorption 61
13. LPS accentuates the CCL19-induced calvariae resorption 64

IV. Discussion 66

V. References 75

국문초록 89

LIST OF FIGURES

Figure 1. Roles of osteoblasts and osteoclasts in bone remodeling 4

Figure 2. Osteoclasts in rheumatoid arthritis 7

Figure 3. Several chemoattractants control the migration of osteoclast precursors 11

Figure 4. Actions of CCL19 and CCL21 and their receptor CCR7 16

Figure 5. Schematic diagram of the relationship between CCL19/CCL21 and bone 18

Figure 6. Expression levels of CCR family members in synovial macrophages of RA patients 29

Figure 7. Up-regulation of CCL19, CCL21 and CCR7 in synovial tissues of RA patients 32

Figure 8. Up-regulation of CCL19 and CCL21 in RA patients 33

Figure 9. CCR7 mRNA expression level increased in osteoclasts by inflammatory stimuli 35

Figure 10. CCR7 mRNA expression level increased in bone marrow macrophages by LPS 36

Figure 11. CCR7 protein expression level increased in bone marrow macrophages by LPS 38

Figure 12. CCR7 surface protein expression level increased in bone marrow macrophages by inflammatory stimuli 39
Figure 13. No cytotoxicity of recombinant CCL19 and CCL21 41
Figure 14. CCL19 does not influence osteoclast differentiation 42
Figure 15. CCL21 does not influence osteoclast differentiation 43
Figure 16. CCL19 and CCL21 increase the migration of osteoclast precursors 45
Figure 17. CCL19 and CCL21 increase the migration of osteoclasts 46
Figure 18. CCL19 and CCL21 increase BMM migration through CCR7 48
Figure 19. CCL19 and CCL21 increase the bone resorption activity of osteoclasts on calcium phosphate-coated plates 50
Figure 20. CCL19 and CCL21 increase the bone resorption by osteoclasts on dentin slices 51
Figure 21. CCL19 and CCL21 increase the actin ring density in osteoclasts 54
Figure 22. CCL19 and CCL21 activate Rho in BMMs 56
Figure 23. CCL19 and CCL21 activate ROCK in BMMs 57
Figure 24. Rho inhibitors decrease CCL19- and CCL21-induced osteoclast migration 59
Figure 25. Rho inhibitors suppress CCL19- and CCL21-induced osteoclast resorption activity 60
Figure 26. CCL19 increases mouse calvariae resorption 62

Figure 27. CCL21 increases mouse calvariae resorption 63

Figure 28. LPS accentuates the CCL19-induced calvariae resorption 65

Figure 29. Schematic diagram for the CCL19/CCL21 and CCR7 signal transduction axis in osteoclasts 74

LIST OF TABLES

Table 1. Possible chemoattractants and repellents for osteoclast precursors ...12

ABBREVIATIONS

RA	Rheumatoid athritis
RANK	Receptor activator of nuclear factor κB (NF-κB)
RANKL	RANK ligand
M-CSF	Macrophage-colony stimulating factor
BMMs	Bone marrow-derived macrophages
NFATc1	Nuclear factor of activated T cell c1
TRAP	Tartrate-resistant acid phosphatase
MNCs	Multinucleated cells
ATP6v0d2	v-ATPase subunit d2
DC-STAMP	Dendritic cell-specific transmembrane protein
RT-PCR	Reverse transcriptase polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay
siRNA	Small interfering ribonucleic acid
μ-CT	micro-computed tomography
BMPs	Bone morphogenetic proteins
Runx2	Runt-related transcription factor 2
HSCs	Hematopoietic stem cells

MSCs	Mesenchymal stem cells
CCL	CC chemokine ligand
CCR	CC chemokine receptor
GEO	Gene expression omnibus
MAPK	Mitogen-activated protein kinases
ROCK	Rho-associated protein kinase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
IFN	Interferons
IL	Interleukin
TNF-α	Tumor necrosis factor-α
TGFβ	Transforming growth factorβ
LPS	Lipopolysaccharides

I. Introduction

1. Roles of osteoblasts and osteoclasts in bone remodeling

Bone is a dynamic tissue which is consistently regulated by both osteoblasts and osteoclasts (Weilbaecher et al., 2011) (Fig. 1). Osteoblasts are differentiated from mesenchymal stem cells (MSCs) which give rise to bone, cartilage, muscle and fat (Jiang et al., 2002). Osteoblast differentiation is regulated by various cytokines such as bone morphogenetic proteins (BMPs) (Chen et al., 2004), TGFβ (Janssens et al., 2005) and WNTs (Day et al., 2005; Hill et al., 2005). During this process, various osteoblastic genes are activated including alkaline phosphatase (ALP), type 1 collagen (Col1), bone sialoprotein, osteopontin and osteocalcin (Schroeder et al., 2005). Runx2 is well known as a master transcriptional factor that induces expression of these genes. Deficiency of Runx2 causes complete lack of osteoblast development in mouse model (Otto et al., 1997). Cleidocranial dysplasia is a human skeletal disorder which has a mutation in Runx2 region. It demonstrates the importance of Runx2 in osteoblast differentiation (Mundlos, 1999). Osterix up-regulated by Runx2 is another important transcriptional factor that ultimately activates the expression of osteocalcin, osteopontin and Col1. Studies of osterix-lacking mice have revealed that osterix is also an important mediator for osteoblast differentiation (Nakashima et al., 2002).

Osteoclasts derived from hematopoietic stem cells (HSCs) are bone resorbing cells and become multinucleated during differentiation (Suda et al., 1999). Differentiation of osteoclasts from bone marrow macrophages (BMMs) is critically dependent upon two important cytokines, macrophage-colony stimulating factor (M-CSF) (Pixley and Stanley, 2004) and receptor activator of nuclear factor κB ligand (RANKL) (Boyle et al., 2003). RANKL is responsible for osteoclast differentiation, while M-CSF has important roles in osteoclast survival, proliferation and migration. For osteoclast differentiation, c-Fos (Wagner and Eferl, 2005) and nuclear factor of activated T cells c1 (NFATc1) (Takayanagi et al., 2002) are important transcription factors. These transcription factors ultimately activate the expression of osteoclast marker genes such as tartrate-resistant acid phosphatase (TRAP) (Suter et al., 2001), v-ATPase subunit d2 (ATP6v0d2) (Lee et al., 2006), cathepsin K (Gelb et al., 1996) and dendritic cell-specific transmembrane protein (DC-STAMP) (Zhang et al., 2014a). Notably, NFATc1 transcription factor has a distinct feature of positive feedback called auto-amplification (Asagiri et al., 2005).

The activities of osteoblasts and osteoclasts are finely regulated in normal condition. However, once an imbalance between their activities occurs, various bone related disorders arise. Among them, osteolytic disorders such as osteoporosis (Boyce et al., 2012), rheumatoid arthritis (RA) (Kuratani et al., 1998;

Romas et al., 2000), and Paget's disease (Roodman and Windle, 2005) are mainly initiated by an increase in osteoclast activity.

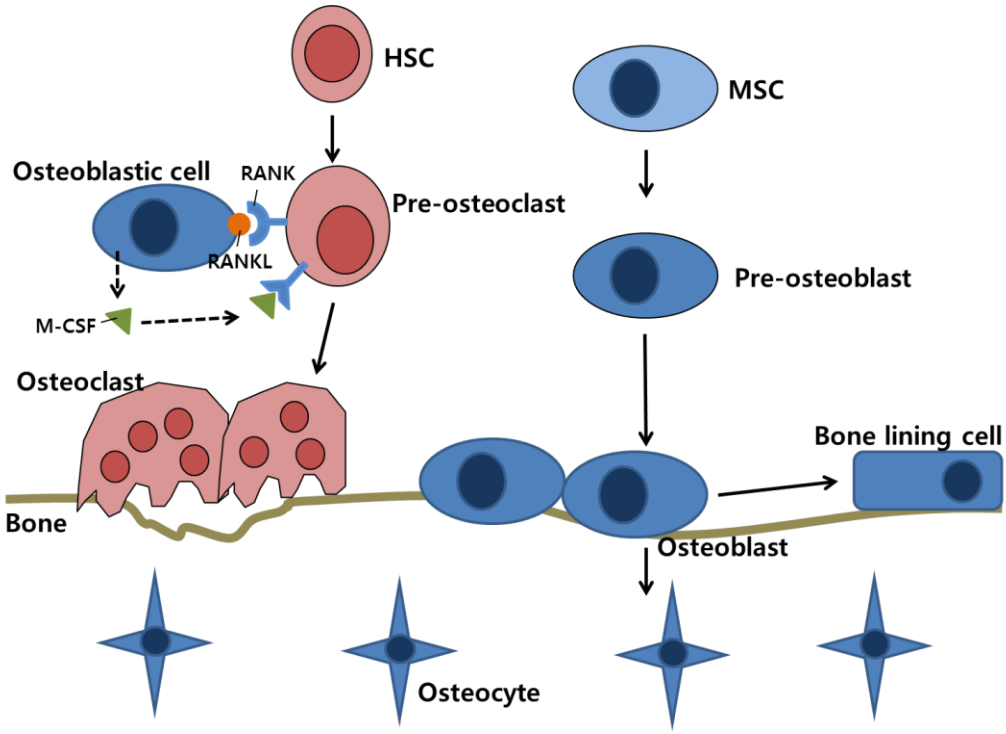

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Figure 1. Roles of osteoblasts and osteoclasts in bone remodeling.

Bone is a dynamic structure which is formed by MSC-derived osteoblasts and resorbed by HSC-derived osteoclasts. Osteoblast differentiation is regulated by various cytokines such as BMPs, TGFβ and WNTs and osteoblasts terminally differentiate into osteocytes or bone lining cells. Mature osteoclasts are generated from HSC in response to M-CSF and RANKL. There are also many cytokines that have an effect on osteoclast differentiation.

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2. Rheumatoid arthritis and osteoclasts

RA is a systemic autoimmune disease which causes inflammation and destruction of articular or periarticular structures. In physiological condition, synovial membrane surrounds the synovial cavity, so synovial fluid can provide lubrication and nutrition for articular cartilages. In RA condition, the synovium contains a variety of inflammatory cytokines, which later cause destruction of joint area (Okamoto and Takayanagi, 2011; Schett, 2007).

RA patients have four major distinct pathologic skeletal features including focal joint marginal erosions, subchondral bone erosions, periarticular osteopenia and systemic osteoporosis. Many studies have shown that bone surfaces near the inflamed synovial tissues have resorption lacunae containing plenty of osteoclasts (Bromley and Woolley, 1984a; Bromley and Woolley, 1984b; Gravallese et al., 1998). Another investigation has proven that osteoclasts are responsible for the RA induced bone destruction (Redlich et al., 2002).

Several studies have reported that RA patients have increased bone resorption based on the measurements of urinary C-terminal cross linking telopeptide of type 1 collagen (CTX-1) level (Garnero et al., 2002; Gough et al., 1998). It was recently shown that suppression of synovial inflammation reduced the bone resorbing markers (Seriolo et al., 2006; Vis et al., 2006). In addition, it

was shown that bone destruction becomes severe as the disease progresses (Sharp et al., 1991).

In mouse arthritis model, RANKL deficient mice did not develop the bone erosion phenotype (Pettit et al., 2001). Furthermore, the RANKL inhibitor denosumab was shown to reduce the progression of bone erosions in RA patients (Cohen et al., 2008). It implies that blockade of osteoclast differentiation is a reasonable therapeutic strategy for the reduction of articular bone destruction.

Inflamed synovium contains various kinds of substances such as RANKL, IL-1, IL-6, M-CSF, TNF-α and PTH-related peptide which can recruit osteoclast precursors (monocytes and macrophages) and facilitate osteoclast differentiation and activation (Gravallese et al., 2000; Walsh et al., 2005) (Fig. 2). Once osteoclast precursors are recruited to the joint area, they rapidly differentiate into mature osteoclasts and initiate bone resorbing activity. Therefore, migration of these cells to joint area is an important step in the bone destruction associated with pathogenesis of RA.

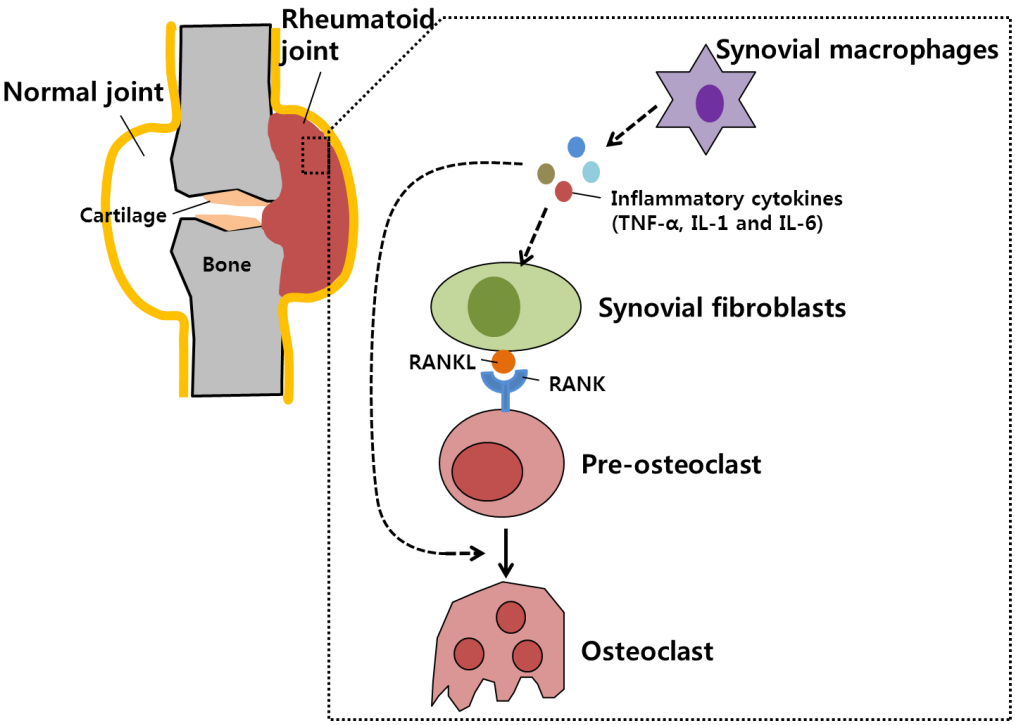

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Figure 2. Osteoclasts in rheumatoid arthritis.

Osteoclasts play an important role in the pathogenesis of RA. Osteoclast differentiation is accelerated by M-CSF, RANKL and several proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-1, and IL-6 which are up-regulated in the synovial joint area of RA patients.

3. Osteoclast migration and bone resorption

Bone resorbing function of osteoclasts is achieved by three steps, osteoclast precursor migration, differentiation and resorption (Kikuta and Ishii, 2013). The mechanisms for osteoclast differentiation and bone resorption have been the subject of investigations in the field of osteoclast biology (Alsina et al., 1996; Jilka et al., 1992; Roodman, 2001).

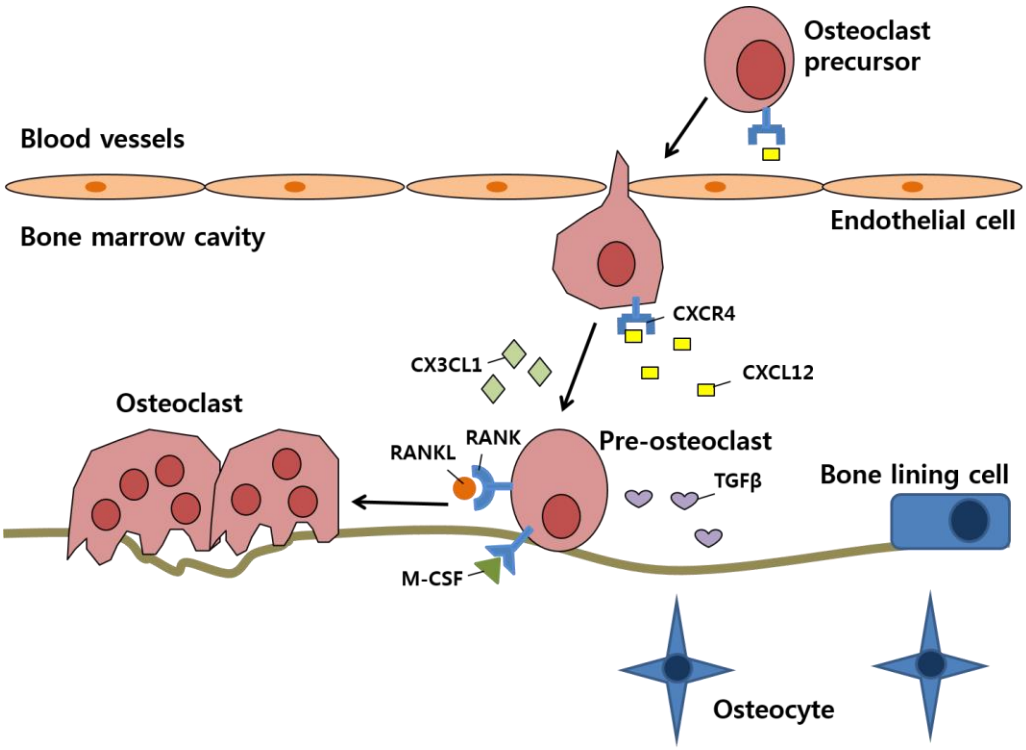
Recently, osteoclast precursor recruitment to the bone remodeling area has drawn attention as an interesting subject of research. Several factors such as CXCL12 (SDF-1), TGFβ and CX3CL1 (fractalkine) have been reported to promote osteoclast migration (Koizumi et al., 2009; Pilkington et al., 2001; Yu et al., 2003) (Fig. 3). These factors enhance the recruitment of osteoclast precursors to the bone surface and also in some cases increase osteoclastogenesis directly or indirectly. In addition, cytokine receptors such as CXCR4, CCR1 and CCR2 have been known for their ability to increase osteoclast precursor migration (Binder et al., 2009; Lean et al., 2002; Wright et al., 2005). Chemoattractants and their receptors are likely to play important roles in bone remodeling by making osteoclast precursors to exist bone marrow in appropriate time and to migrate to appropriate sites of bone surface (Table 1).

Bone resorption is the last step, but the most important step representing

the function of osteoclasts. During the resorption, osteoclasts express cathepsin K, matrix metalloproteases-9 (MMP-9) and v-type ATPase which are induced by RANKL stimulation (Corisdeo et al., 2001; Teitelbaum et al., 1995; Zaidi et al., 1993; Zaidi et al., 2001). Cathepsin K and MMP-9 are for digestion of matrix protein and v-type ATPase is for dissolution of hydroxy apatite by secreting proton. For these processes, osteoclasts are required to be polarized and build a special resorptive lacuna between bone surface and themselves. This unique structure is made of two distinct structures, ruffled border and sealing zone. Ruffled border is characterized by its villous structure and abundant proton pumps that make the resorption lacuna acidified easily. Sealing zone which is also called actin ring seals off the lacuna space and indirectly represents the resorption activity of osteoclasts (Vaananen et al., 2000).

For both osteoclast migration and resorption, small GTPases play important roles. Small GTPases have been well characterized for their functions in actin cytoskeleton rearrangement, which is related to the cellular migration and actin ring formation (Jaffe and Hall, 2005). Among several small GTPases, such as Rho, Rac and Cdc42, Rho is involved in the regulation of osteoclast activity. It has been reported that Rho can stimulates the formation of actin ring, osteoclast migration and bone resorption (Chellaiah et al., 2000; Touaitahuata et al., 2014). Therefore, regulation of the recruitment of osteoclast precursors or that of the

resorption activity of osteoclasts might be a good therapeutic strategy for bone lytic disorders.



Modified from IBMS BoneKEy, Vol.7: 279–286, 2010

Figure 3. Several chemoattractants control the migration of osteoclast precursors.

Osteoclast precursors need to be attracted to the bone surface from bloodstream in order to differentiate into mature osteoclasts. Several molecules such as CXCL12, TGFβ and CX3CL1 recruit osteoclast precursors to the bone surface where they resorb bone.

Table 1.

Possible chemoattractants and repellents for osteoclast precursors

Ligand	Synonym	Receptor	Function
CCL2	MCP-1	CCR2	Homing
CCL3	MIP-1a	CCR1/CCR5	Homing
CCL4	MIP-1b	CCR1/CCR5	Homing
CCL5	RANTES	CCR1/CCR5	Homing
CCL7	MCP-3	CCR2	Homing
CCL9/10	MIP-1c	CCR1	Homing
CCL12	MCP-5	CCR5	?
CCL19	ELC	CCR7	?
CCL21	SLC	CCR7	?
CCL22	MDC	CCR4	?
CCL25	TECK	CCR9	?
CCL27	CTARK	CCR10	?
CCL28	MEC	CCR10	?

Modified from IBMS BoneKEy, Vol.7:279–286, 2010

4. Roles of CCL19/CCL21 and CCR7 in rheumatoid arthritis

Chemokines refer the substances that are able to attract specific cells toward them. Chemokines are classified into several groups: mainly C, CC, CXC, and CX3C. This classification is generated based on the cysteine motif. The migration of osteoclast precursors is also induced by several chemokines.

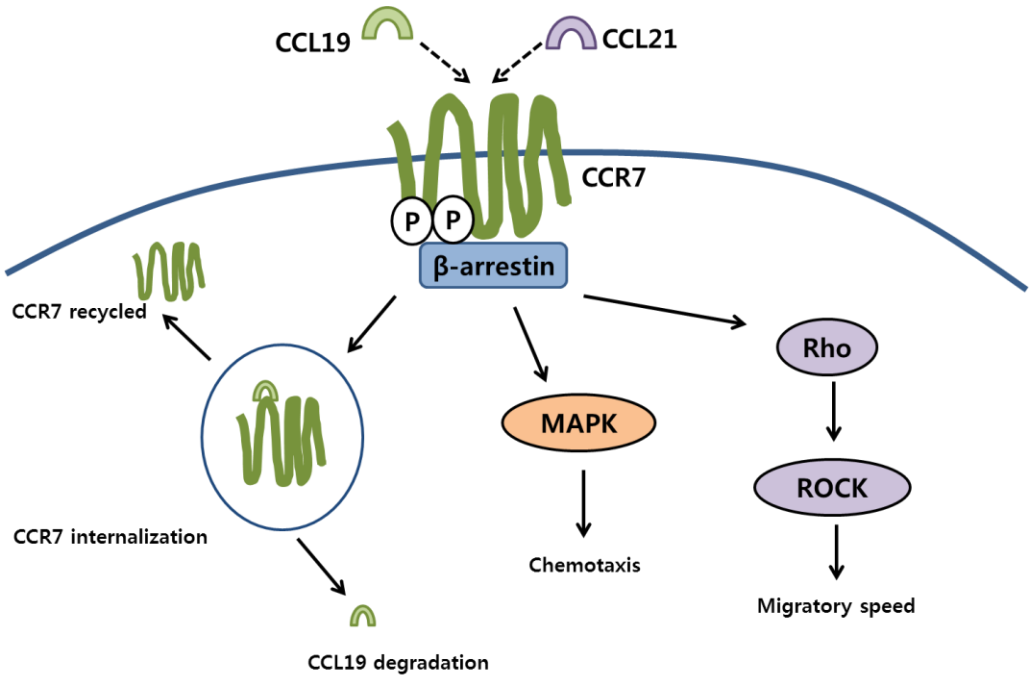
CC chemokine ligand 19 (CCL19) and CC chemokine ligand 21 (CCL21) belonging to the CC chemokine family have common function of recruitment of various cell types including dendritic cells (Seth et al., 2011), T lymphocytes (Ueno et al., 2002), neutrophils (Beauvillain et al., 2011) and certain tumor cells (Cabioglu et al., 2005; Koizumi et al., 2007; Peng et al., 2014). Both CCL19 and CCL21 have been shown to promote migration and maturation of dendritic cells in vitro (Britschgi et al., 2010; Scandella et al., 2002) and deficiency of these cytokine genes led to loss of migration ability of dendritic cells in mouse model. Dendritic cells share their precursors with osteoclasts and macrophages. In addition, CCL19 and CCL21 regulate the localization of macrophages in marginal zone of the spleen (Ato et al., 2004). These reports may infer that CCL19 and CCL21 have some roles in the migration and/or activation of osteoclasts that are differentiated from bone marrow macrophages.

Both CCL19 and CCL21 can bind to CC chemokine receptor 7 (CCR7) which is a G-protein linked receptor (Fig. 4). CCR7 is expressed in various hematopoietic cells like T cells, B cells, dendritic cells, macrophages and neutrophils (Comerford et al., 2013). The common function of CCR7 in these cells is promotion of migration. A recent study reported that CCR7 and its ligand CCL19 stimulates the migration of bone marrow mesenchymal stem cells that can differentiate into osteoblasts (Zhang et al., 2014b). However, the role of CCR7 in osteoclast migration has not been well studied yet.

Recent studies have shown that, in RA patients, CCL19 and CCL21 expression is elevated in synovial tissue (Pickens et al., 2011). Another study reported that the levels of plasma CCL19 and CCR7 expression on monocytes increased in early RA conditions, which were decreased with 1- and 5- year RA therapy (Ellingsen et al., 2014). These results suggest that these cytokines and their receptor have a crucial role in RA conditions. Another study has shown that CCL21 promoted angiogenesis in RA through CCR7 (Pickens et al., 2012).

There are several studies describing a relationship between CCR7 and the small GTPase Rho. Rho is responsible for CCR7-dependent migration of monocytes (Allaire and Dumais, 2012). In addition, CCR7-mediated chemotaxis and polarization were shown to require Rho kinase (ROCK), a downstream target of Rho, in T cells (Bardi et al., 2003). On the other hand, the involvement of Rho

(Chellaiah et al., 2000), Rac (Fukuda et al., 2005) and cdc42 (Ito et al., 2010) small GTPases in stimulation of osteoclast cytoskeleton rearrangement and migration and upregulation of bone resorption has been well documented. However, the association between CCR7 and osteoclasts has not been investigated.



Modified from Journal of Neuroinflammation, Vol.9:77, 2012

Figure 4. Actions of CCL19 and CCL21 and their receptor CCR7

Both CCL19 and CCL21 can bind and activate the G-protein coupled receptor CCR7. Ligand binding to CCR7 results in phosphorylation of CCR7, which ultimately recruit scaffold protein, β-arrestin. The phosphorylation of CCR7 leads to CCR7 internalization and desensitization by receptor endocytosis and CCL19 degradation in lysosome. Moreover, CCR7 activates Rho and mitogen-activated protein kinases (MAPK) signaling pathways and results in increase of migration in response to CCL19 and CCL21.

5. Purpose of this study

Previous studies have reported that inflammation is the first step of RA progression. RA patients experience severe problems from osteolysis by osteoclasts that are profoundly influenced by inflammatory factors. Bone homeostasis is closely related to the immunity reflecting the term ‘osteoimmunology’. Therefore, inflammation, bone homeostasis and RA are inter-related each other. It has been shown that CCR7 and its ligands CCL19/CCL21 are involved in the inflammation response and are increased in RA patient samples. Taken these together, I reasoned that CCR7 and CCL19/CCL21 might be closely related to the bone homeostasis, especially to osteoclasts under conditions involving inflammation (Fig. 5). The purpose of this study was to investigate effects of CCR7 and its ligands CCL19/CCL21 on osteoclast differentiation and activity.

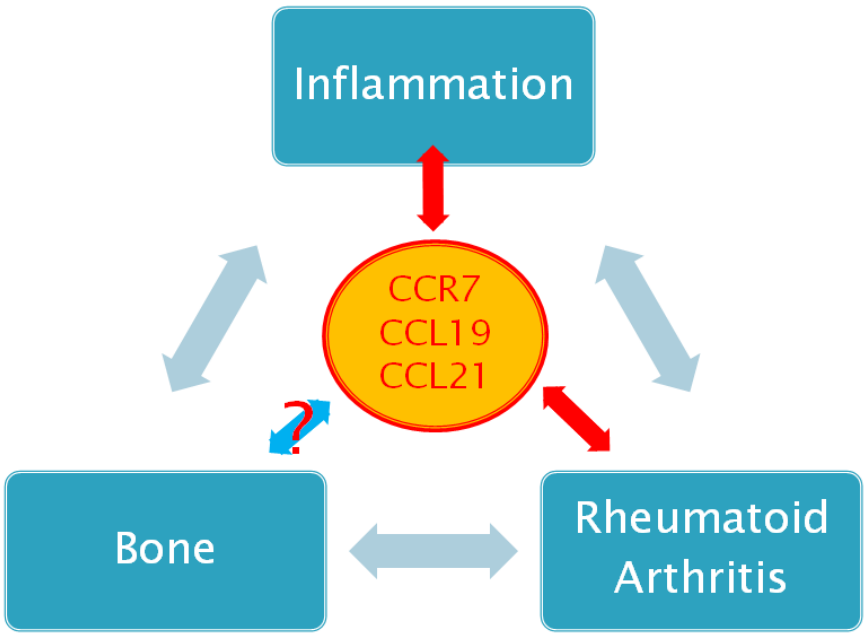


Figure 5. Schematic diagram of the relationship between CCL19/CCL21 and bone.

Inflammation, RA and bone homeostasis are inter-related each other. CCR7 and its ligands CCL19/CCL21 have been shown to be related to inflammation as well as RA. I hypothesized that CCR7 and its ligands CCL19/CCL21 can affect the osteoclast activity and bone homeostasis under inflammatory conditions.

II. Materials and methods

1. Reagents

Recombinant CCL19 and CCL21 were purchased from Prospec (East Brunswick, NJ, USA). Primary antibodies for CCR7, phosphoSer19 MLC, and β-actin were obtained from ABcam (Cambridge, Cambridgeshire, UK), Millipore (Temecula, CA, USA) and Sigma (St Louis, MO, USA), respectively. Anti-mouse and anti-rabbit secondary antibodies were purchased from Sigma. Rho Activation Assay kit was purchased from Millipore. Rho inhibitor simvastatin and Y27632 were purchased from Sigma. CCR7 siRNA was provided by Santa Cruz (Santa Cruz, CA, USA). Small interference RNA (siRNA) transfection reagent HiPerFect was from Qiagen (Valencia, CA, USA).

2. Bone marrow-derived macrophage (BMM) generation

Bone marrow cells were extracted from the femur and tibia of 5-week-old female ICR mice by flushing. Red blood cells were lysed with hypotonic solution and remaining cells were incubated on a culture dish (Nunc, Tewksbury, MA, USA) for 1 day with α-MEM (Welgene, Daegu, Korea) containing 10% FBS (Gibco, Grand Island, NY, USA). The non-adherent cells were incubated further on petri dish for 3 days with M-CSF (30 ng/ml). Adherent cells were collected and considered as bone marrow-derived macrophages (BMMs).

3. Osteoclast differentiation

BMMs were cultured in α -MEM containing 10% FBS with M-CSF (30 ng/ml) for survival and M-CSF (30 ng/ml) plus RANKL (100 ng/ml) for osteoclast differentiation. Usually, BMMs were seeded on plates one day before RANKL stimulation.

4. Enzyme-linked immunosorbent assay (ELISA)

CCL19 and CCL21 levels were measured by using CCL19 and CCL21 ELISA kit (R&D Systems, Minneapolis, MN, USA). To prepare ELISA plate, capture antibodies were diluted in PBS in working concentration, 100 μl of antibodies were added to 96 well plates and plates were incubated overnight at room temperature. Each well was aspirated and washed with wash buffer 3 times. Plates were blocked by 300 μl of reagent diluents, incubated 1 h at room temperature and washed again. 100 μl of standards or samples diluted in reagent diluent were prepared. Samples were added to each well and incubated for 2 h at room temperature. Plates were washed with wash buffer. After adding 100 μl of detection antibodies to each well, plates were incubated 2 h at room temperature and washed with wash buffer again. Next, 100 μl of streptavidin-HRP was added and incubated for 20 minutes at room temperature without direct light. After washing 3 times, 100 μl of substrate solution was added to each well and

incubated for 20 minutes at room temperature without any direct light. Finally, 50 μl of stop solution was added and optical density of each well was measured immediately at 450 nm.

5. Quantification of mRNA

Messenger RNA was obtained by using TRIZOL (Invitrogen, Carlsbad, CA, USA) and was reverse-transcribed into complementary DNA by using SuperScript II reverse transcriptase (Invitrogen). Complementary DNA was amplified with primers by polymerase chain reaction. Amplified DNA was measured by gel electrophoresis using 1.5% agarose gel or with a quantitative PCR equipment, ABI7500 (Life Technologies, Carlsbad, CA, USA). The mRNA expression level was normalized to the level of 18s rRNA.

6. Western blotting analysis

Whole cell lysates or cytoplasmic lysates were prepared with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin). Protein samples of 30 μg quantified with a protein assay kit (Biorad, Hercules, CA, USA) were loaded to polyacrylamide gels and separated proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk followed by incubation with primary antibody (1:1000) for overnight at 4℃ and subsequently with secondary antibody

(1:10000) for 1 h at room temperature.

7. TRAP staining

BMMs were seeded in 48 well plates at 2 x 10^4 cells per well and incubated for 24 h in survival condition. Cells were treated with RANKL in the absence or presence of CCL19 and CCL21. After 4 days of incubation, cells were fixed with 3.7% formaldehyde for 15 minutes and permeabilized with 0.1% Triton X-100 for 1 minute. They were treated with TRAP staining solution of Leukocyte Acid Phosphatase kit (Sigma). TRAP staining solution was freshly prepared for each experiment. For 10 ml TRAP staining solution, 100 μl of Fast Garnet GBC Base solution and 100 μl of sodium nitrite solution were mixed and incubated for 2 minutes. 100 μl naphthol AS-Bl phosphate solution, 400 μl acetate solution, 200 μl tartrate solution, 9 ml distilled water and a mixture of Fast Garnet GBC Base solution and sodium nitrite solution were mixed. After 15 minutes of incubation at 37℃, plates were washed with tap water and TRAP positive cells were counted under the Olympus23 light microscope (Tokyo, Japan).

8. Cytotoxicity assay

BMMs were incubated in 96 well plates under the survival condition for 24 h in the absence or presence of CCL19 and CCL21. After 24 h incubation, CCK solution (Itsbio, Seoul, Korea) at 10% volume of cell culture medium was

added to each well and incubated for 50 minutes at 37℃. After incubation, optical density was measured at 450 nm.

9. Flow cytometry

BMMs were incubated with LPS (5 ng/ml), TNF-α (5 ng/ml) or IL-1β (5 ng/ml) for 1 day under the survival condition. Cells were collected by a scraper, washed by 5 minute centrifugation at 400 *g* for 3 times in PBS containing 2% FBS, and incubated in CCR7 antibody (1:40) for 30 minutes at 4℃. After washing 3 times, cells were treated with secondary antibody (1:1000) (Invitrogen) for 20 minutes and washed 3 times again. Immediately, cells were analyzed with FACSCaliber (BD Science, Franklin Lakes, NJ, USA).

10. Small interference RNA transfection

The siRNA duplexes that target the CCR7 gene were used for knock down analysis. They were transfected to BMMs with HiPerFect transfection reagent following the manufacturer's instruction. $2x10^5$ cells were seeded in 6-well culture plates and incubated for 24 h in survival condition. Mixtures of 20 nM of siRNA, HiPerFect reagent and medium were prepared prior to transfection. In this step, the medium without any serum and antibiotics was used. Solutions were mixed gently and incubated for 10 minutes at room temperature to allow the formation of transfection complexes. 100 μl of transfection mixture was added to the plates and incubated for 24 h at 37℃ in a CO2 incubator. After 24

h incubation, medium containing transfection reagent was aspirated and incubated in a medium containing serum and antibiotics for various times. After 24 h, cells were detached by using a cell scraper and seeded in the upper chamber of transwell plates (Corning, Corning, NY, USA) for migration assay.

11. Cell migration assay

BMM migration was measured with transwell migration assay kit. BMMs ($1x10^5$) were seeded in the upper chamber and CCL19 or CCL21 was added to the lower chamber. For the Rho inhibitor treatment, BMMs were pre-treated with simvastatin or Y27632 for 1 h prior to the treatment of CCL19 or CCL21. After incubating for 18 h at 37℃, cells were fixed with 3.7% formaldehyde for 30 minutes and stained with crystal violet for 10 minutes. For osteoclast migration, Oris Cell Migration Assay kit (Platypus Technologies, Madison, WI, USA) was used following the manufacturer′s instruction. BMMs ($1x10^5$) were seeded in Oris Cell Migration Assay plate with stopper and they are treated with RANKL (100 ng/ml). After 2 days of incubation, stoppers were removed to allow cells to migrate into the detection zone. After 16 h incubation at 37℃, cells were fixed with 3.7% formaldehyde for 30 minutes and stained with crystal violet for 10 minutes. Migrated cells were counted under the Olympus23 light microscope.

12. Rho pull-down assay

$2x10^6$ BMM cells were cultured in a 100 mm culture dish and stimulated by CCL19 or CCL21 for different time periods. They were harvested in the lysis buffer (125 mM HEPES, pH 7.5, 570 mM NaCl, 5% lgepal CA-630, 50 mM $MgCl_2$, 5 mM EDTA and 10% glycerol) contained in the Rho Activation Assay kit. Protein samples were obtained from cell extracts by centrifugation and incubated with 20 μl of Rho Assay Reagent slurry (bead) for 1 h at 4 ℃. Beads were washed 3 times using the lysis buffer and the reducing sample buffer was added. After boiling, samples were subjected to Western blotting.

13. von Kossa staining

BMMs were seeded on calcium phosphate-coated plates and cultured with M-CSF and RANKL in the presence or absence of CCL19 or CCL21 for 7 days. After washing out cells with distilled water, plates were treated with 5% silver nitrate solution for 1 h, washed with distilled water, and treated with sodium carbonate-formaldehyde solution (5 g Na_2CO_3 in 75 ml distilled water plus 25 ml of 37% formaldehyde) for 2 minutes. Resorbed area was measured under the Olympus23 light microscope.

14. Dentin resorption and confocal microscopy

Dentin slices were placed in 48 well plates and incubated under UV light for sterilization. BMMs were seeded on each well and cultured with M-CSF and

RANKL in the presence or absence of CCL19 or CCL21 for 7 days. After washing out cells with distilled water, dentin slices were fixed onto a slide. Dentin surface was scanned with LSM 5 Confocal Laser Scanning Microscope (Carl Zeiss Microimaging, GmbH, Goettingen, Germany). After scanning, dentin slices were stained with trypan blue solution and resorbed area was quantified under the Olympus23 light microscope.

15. Confocal fluorescence microscopy

BMMs were cultured with M-CSF, RANKL and with or without CCL19 and 21 for 6 days. Cells were fixed in 3.7% formaldehyde for 30 minutes, blocked in 1% bovine serum albumin for 2 h, and treated with phalloidine rhodamin (Invitrogen) for 2 h. Cells were washed with PBS containing 1% BSA 3 times after each step. Zeiss LSM 7 PASCAL laser-scanning microscope (Carl Zeiss Microimaging) was used for observation and quantification of actin ring.

16. In vivo mouse calvariae resorption assay

Collagen sponges were soaked with 2 μg of CCL19, CCL21 or PBS and transplanted to 5-week-old ICR mouse calvariae subcutaneously. Mice were sacrificed after 7 days of transplantation. In LPS injection experiments, LPS was administered 5-week-old female ICR mice intra-peritoneally 2 times with a 4-day interval. The first injection was done on the day before transplantation. Mice were sacrificed after 8 days of the first injection. Calvariae were extracted, fixed

with 4% paraformaldehyde for 1 day and analyzed by micro-computed tomography (μ-CT) using Skyscan1072 (Skyscan, Aartselaar, Belgium) following the manufacturer's instructions. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Seoul National University.

17. Statistical analysis

All values represent mean ± SD. Statistical significance was tested using the Student t test (two-tailed distribution with two-sample equal variance) and analysis of variance (ANOVA) for in vitro and in vivo studies. For judgment, P values less than 0.05 were considered to be statistically significant.

18. Institutional Review Board (IRB)

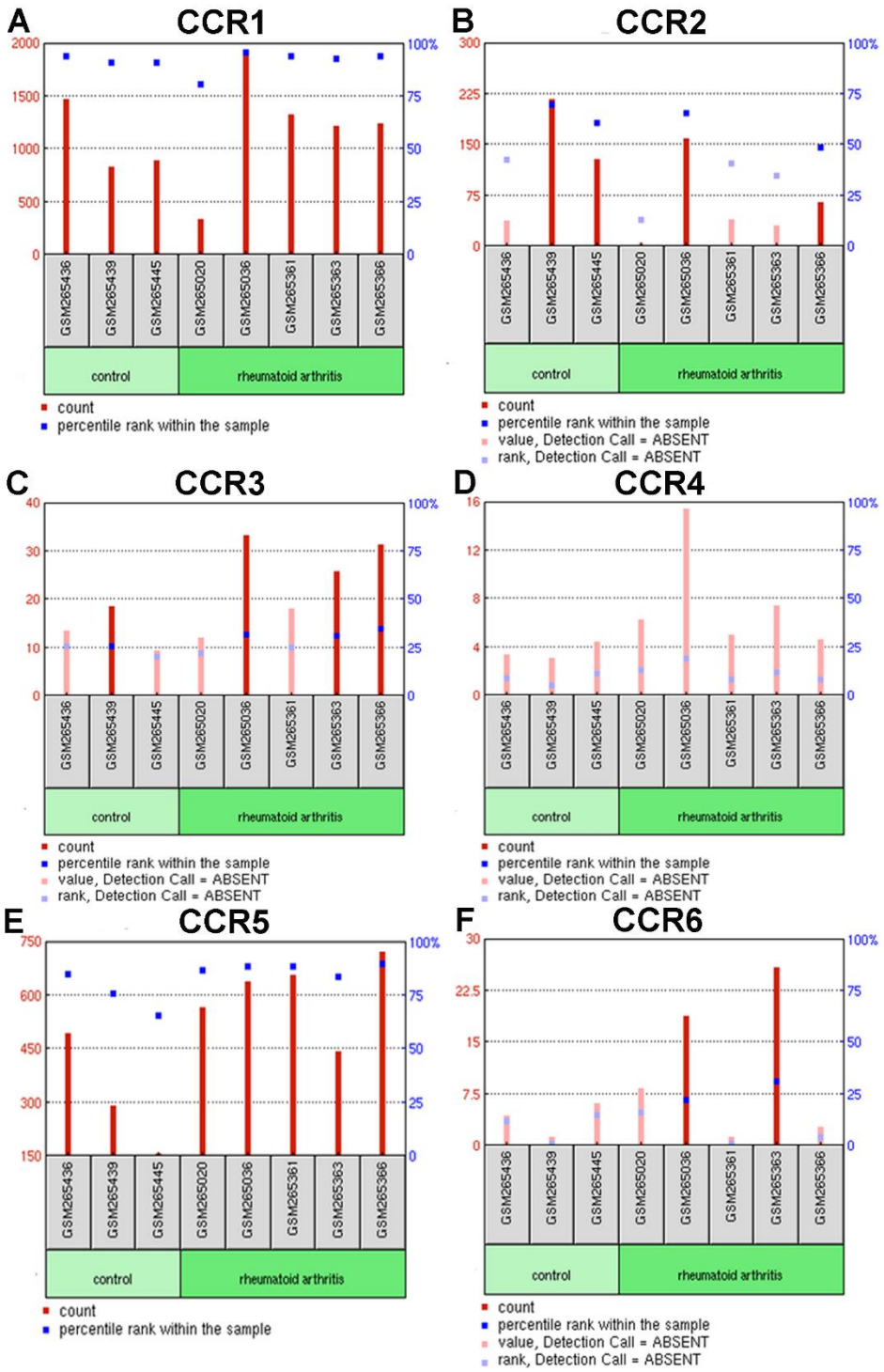
Serum and synovial fluid were obtained from osteoarthritis (n=15) and rheumatoid arthritis (n=24) patients. Patient samples were generously provided by Dr. Yeongwook Song (Division of Rheumatology, Seoul National University Hospital) following the rules of Institutional Review Board of Seoul National University Hospital.

III. Results

1. Increased expression of CCR7 in synovial macrophages of RA patients.

The number of osteoclasts is dramatically increased in synovial joints of RA patients. It is possible that specific chemokines are responsible for this recruitment of osteoclasts to the joint area. Therefore, I sought to find chemokines and chemokine receptors that are up-regulated in RA patients. To this end, I analyzed microarray data deposited to gene expression omnibus (GEO, http://www.ncbi.nlm.nih.gov/gds). Using a dataset obtained from synovial macrophages of normal donor or RA patients (GPS8300:GSE10500), I compared levels of some CCR family members, CCR1 to CCR9 (Fig. 6). CCR4, CCR8 and CCR9 expression levels were almost undetectable in this array system. CCR1, CCR2, CCR3, CCR5 and CCR6 did not show any significant differences between normal (GSM 265436, GSM265439 and GSM265445) and RA patient (GSM265020, GSM265036, GSM265361, GMS265363 and GSM 265366) groups.

In case of CCR7, synovial macrophages from RA patients expressed more CCR7 than those from normal donors (Fig. 6G). This result suggests that CCR7 may have an important role in RA conditions for macrophages and that macrophage responses to CCL19 and CCL21 might be accentuated in RA conditions.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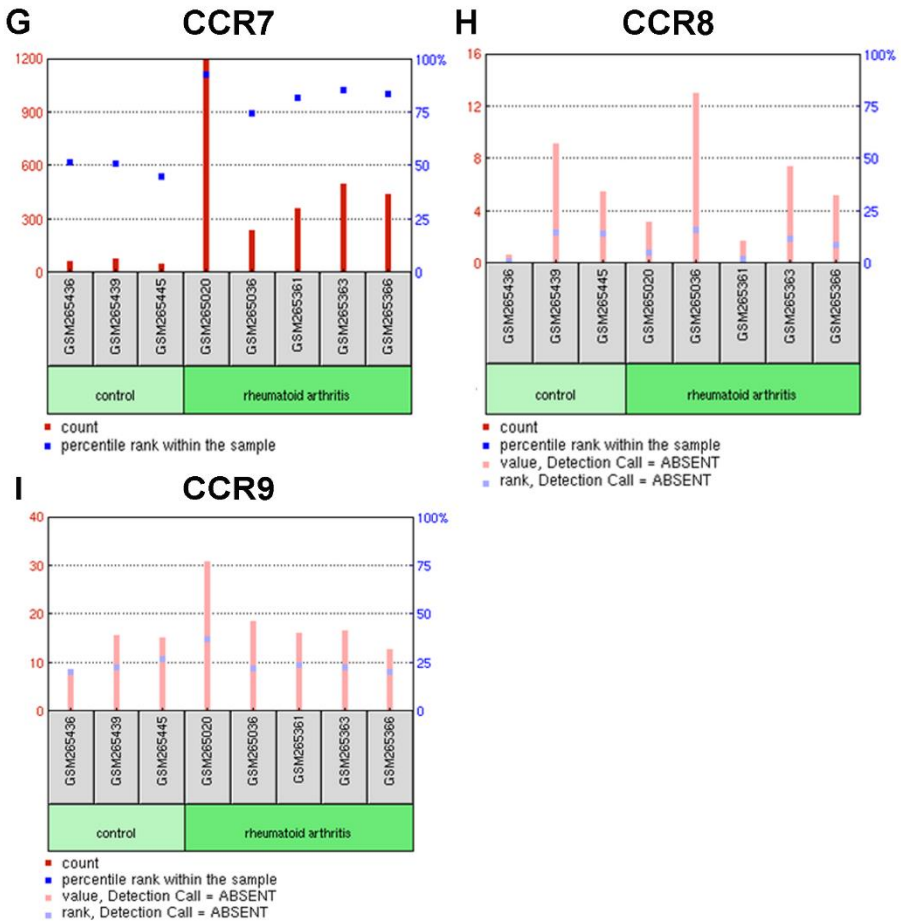

Figure 6. Expression levels of CCR family members in synovial macrophages of RA patients.

Expression levels of CCR1 (A), CCR2 (B), CCR3 (C), CCR4 (D), CCR5 (E), CCR6 (F), CCR7 (G), CCR8 (H) and CCR9 (I) in synovial macrophages of RA patients were analyzed with microarray data deposited to GEO (GPS8300:GSE10500). Only CCR7 was differentially expressed between normal donors and rheumatoid arthritis patients. Normal donor (n=3): GSM 265436, GSM265439 and GSM265445. RA patients (n=5): GSM265020, GSM265036, GSM265361, GMS265363 and GSM 265366.

2. Expression levels of CCL19, CCL21 and CCR7 increased in synovial tissues of RA patients.

In addition to CCR7, CCL19 and CCL21 expression levels are known to be elevated in RA condition so that I decided to evaluate the expression levels of CCL19 and CCL21 in synovial tissues. CCL19, CCL21 and CCR7 mRNA expression levels between RA and osteoarthritis patients or normal donors were analyzed with data deposited to GEO. Analysis of a GEO dataset (GPL91: GSE1919) revealed that CCL19, CCL21 and CCR7 mRNA expression levels increased in synovial tissues of RA patients compared to those of osteoarthritis patients or normal donors (Fig. 7).

I next examined protein levels of CCL19 and CCL21 with synovial fluid and serum of RA and osteoarthritis patients by ELISA. Both CCL19 and CCL21 levels in synovial fluid and serum from RA patients were significantly higher than those from osteoarthritis patients (Fig. 8).

These results are consistent with previous reports. It has been shown that the levels of CCL19 and CCL21, and their receptor CCR7 increased in synovial tissues of RA patients by mRNA, ELISA and immunohistochemical analyses and in synovial fluid of RA patients by ELISA (Pickens et al., 2011).

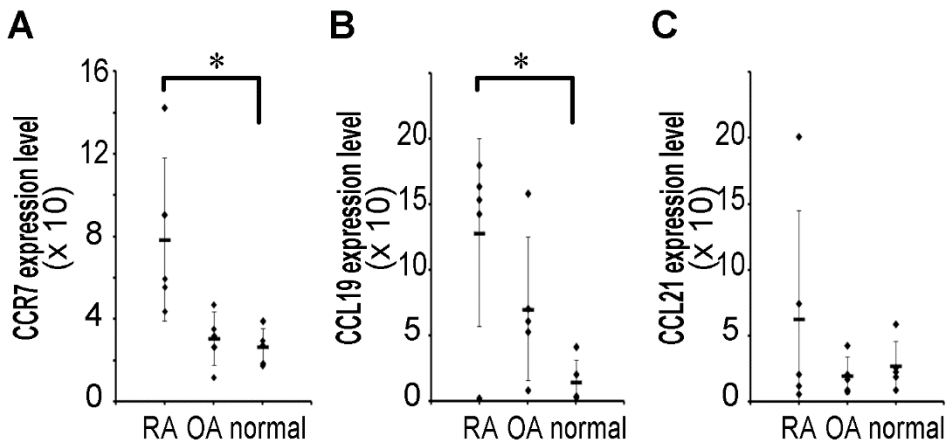

Figure 7. Up-regulation of CCL19, CCL21 and CCR7 in synovial tissues of RA patients.

Analyses of mRNA levels of CCR7 (A), CCL19 (B) and CCL21 (C) in synovial tissues of RA (n=5) and osteoarthritis (n=5) patients and normal donors (n=5) by using data deposited to GEO (GPL91:GSE1919) revealed higher expression of the genes in RA samples. *, $p < 0.05$. OA, osteoarthritis. Normal, normal donor.

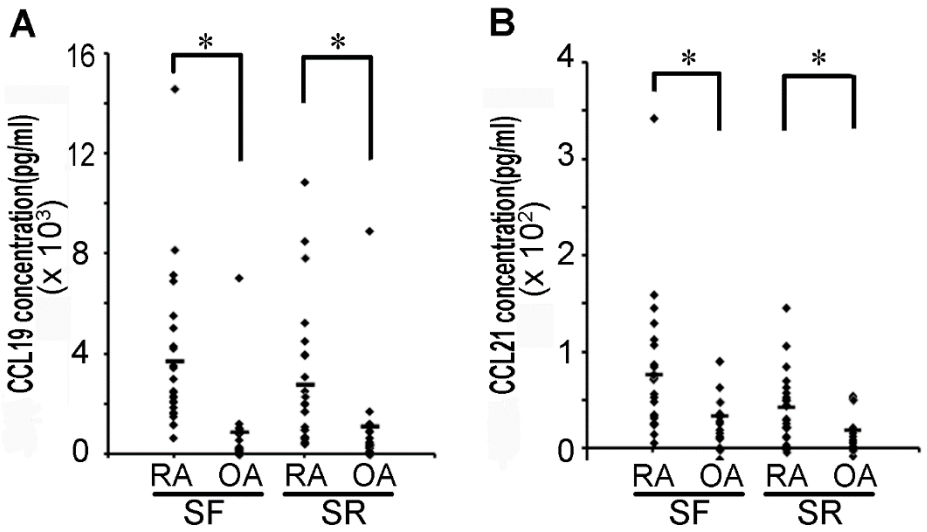

Figure 8. Up-regulation of CCL19 and CCL21 in RA patients.

(A) CCL19 levels of serum and synovial fluid of RA and osteoarthritis patients. CCL19 levels were measured with a human CCL19 ELISA kit. (B) CCL21 levels of serum and synovial fluid of RA (n=24) and osteoarthritis (n=15) patients. CCL21 was assayed with a human CCL21 ELISA kit. *, $p < 0.05$. OA, osteoarthritis. SF, synovial fluid. SR, serum.

3. Increased mRNA expression level of CCR7 in response to inflammatory stimuli.

RA is a chronic inflammatory condition in which TNF-α and IL-1β are constitutively increased. A previous study showed that TNF-α, IL-1β and LPS increased CCL19 expression and only IL-1β increased CCL21 expression in synovial fibroblasts (Pickens et al., 2011). Therefore, I hypothesized that inflammatory stimuli might increase the expression levels of CCL19, CCL21 and CCR7 in osteoclasts.

To examine whether these inflammatory stimuli have any effect on CCL19 and CCL21 expression during osteoclast differentiation, the expression levels of mRNA of these molecules were measured by real time PCR. CCL19 and CCL21 mRNA levels were undetectable in osteoclast precursor cells either untreated or treated with TNF-α, IL-1β or LPS during differentiation (data not shown). In contrast, CCR7 expression level was clearly detectable. Osteoclasts expressed higher level of CCR7 mRNA under the stimulation with TNF-α, IL-1β and LPS. TNF-α and IL-1β increased the CCR7 expression more than 4 times greater than controls (Fig. 9A and 9B). LPS increased the CCR7 expression about 15 times in the presence of RANKL and 50 times in the absence of RANKL (Fig. 9C and 10).

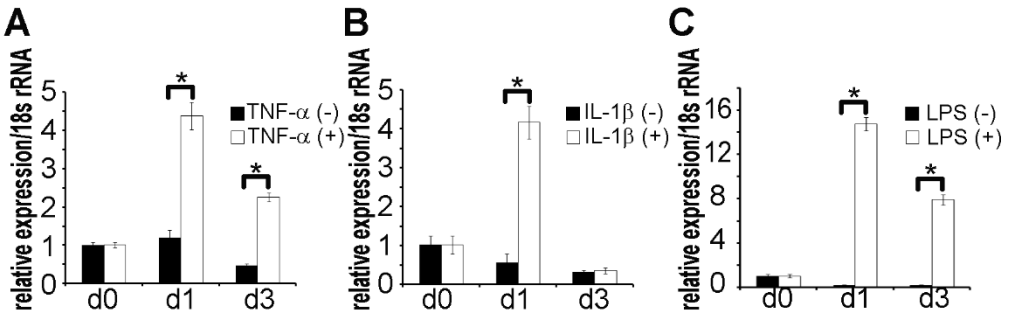

Figure 9. CCR7 mRNA expression level increased in osteoclasts by inflammatory stimuli.

BMMs were plated in 6-well plate and treated with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 1 day or 3 days. Cells were treated with (A) TNF-α (5 ng/ml), (B) IL-1β (5 ng/ml) and (C) LPS (5 ng/ml) for 1 day or 3 days. Messenger RNA was reverse transcribed to cDNA and levels of cDNA were measured by real time PCR. 18s rRNA level was used as a control. *, $p < 0.05$ versus stimulation with RANKL alone. All data are expressed as mean ± SD.

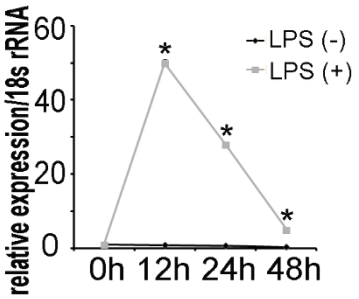

Figure 10. CCR7 mRNA expression level increased in bone marrow macrophages by LPS.

BMMs were plated in 6-well plate and treated with M-CSF (30 ng/ml). Cells were treated with LPS (5 ng/ml) for 12, 24 and 48 h. Messenger RNA was reverse transcribed to cDNA and levels of cDNA were measured by real time PCR. 18s rRNA level was used as a control. *, $p < 0.05$. All data are expressed as mean ± SD.

4. Increased protein expression level of CCR7 in response to inflammatory stimuli.

In addition to mRNA expression level, the protein expression level of CCR7 was examined in osteoclasts by both Western blotting and FACS analysis. CCR7 protein level showed little difference between the stimulated group and the non-stimulated group by Western blotting analyses of cytoplasmic fraction (Fig. 11B). However, the same method using whole lysates showed increased CCR7 protein level in stimulated group at 12 and 24 h, even though CCR7 protein decreased at 48 h (Fig. 11A). It infers that CCR7 stored in cytoplasmic fraction may translocalize to the cell membrane after stimulation. Accordingly, surface CCR7 level measured after 24 h stimulation by flow cytometry was higher in the stimulated group. Especially, LPS-treated group showed more than 10 times increase (Fig. 12).

Collectively, TNF-α, IL-1β and LPS increased CCR7 expression at both mRNA and protein levels.

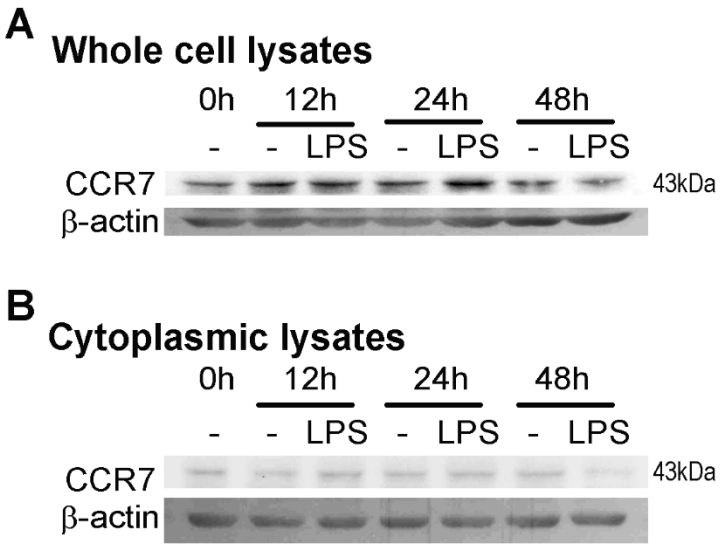

Figure 11. CCR7 protein expression level increased in bone marrow macrophages by LPS.

BMMs were plated in 6-well plates and treated with M-CSF (30 ng/ml). (A) For whole cell lysates preparation, cells were lysed with 6x boiling SDS sample buffer. (B) For cytoplasmic fraction, cells were lysed with RIPA buffer and cytoplasmic fraction was acquired by centrifugation. Both whole cell lysates and cytoplasmic lysates were subjected to Western blotting.

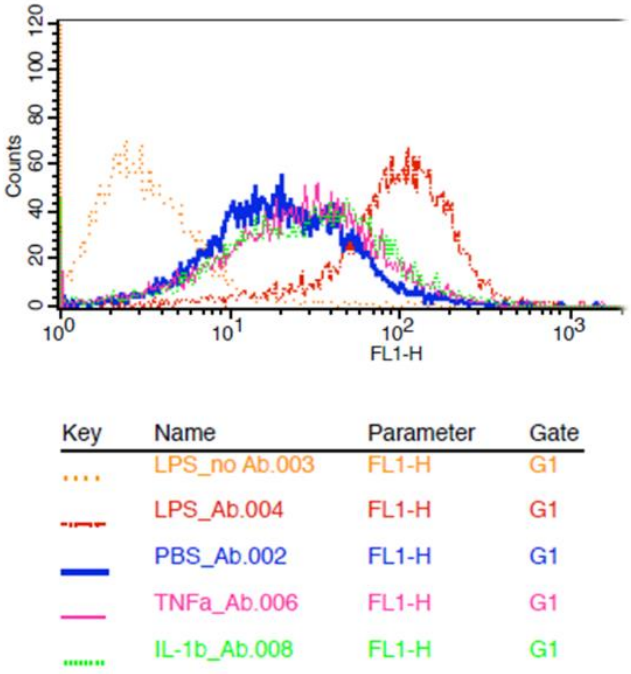

Figure 12. CCR7 surface protein expression level increased in bone marrow macrophages by inflammatory stimuli.

BMMs were treated with TNF-α (5 ng/ml), IL-1β (5 ng/ml) and LPS (5 ng/ml) for 24 h. Cells were scrapped and incubated with CCR7 antibody for 1 h and fluorescence tagged secondary antibody for 30 minutes before flow cytometry (FACS). The data represent at least three independent experiments. LPS_no Ab, LPS-treated cells stained without CCR7 antibody. LPS_Ab, LPS-treated cells stained with CCR7 antibody. PBS_Ab, PBS-treated cells stained with CCR7 antibody. TNFa_Ab, TNF-α-treated cells stained with CCR7 antibody. IL-1b_Ab, IL-1β-treated cells stained with CCR7 antibody.

5. Effects of CCL19 and CCL21 on osteoclast differentiation.

Bone resorption activity is a result of three steps: migration, differentiation and bone resorption of osteoclasts. To understand the effects of CCL19 and CCL21 on osteoclasts, I examined each step in the presence of these cytokines.

First of all, I tested the cytotoxicity of recombinant CCL19 and CCL21. They did not show any cytotoxicity for BMMs (Fig. 13).

I next evaluated the effect of CCL19 and CCL21 on differentiation of osteoclasts. To test the effects of CCL19 on osteoclastogenesis, cells were cultured with CCL19 in osteoclastogenic medium and subjected to TRAP staining. The number of TRAP positive multinuclear cells (osteoclasts) was not changed between the cytokine-treated group and -untreated group (Fig. 14A and 14B). In addition, results obtained from real time PCR analyses showed no differences in expression levels of differentiation marker genes such as c-Fos, NFATc1 and DC-STAMP between cytokine-treated group and -untreated group (Fig. 14C). CCL21 also showed no effects on the number of TRAP positive osteoclasts, and on the mRNA levels of differentiation markers (Fig. 15).

Based on these results, I conclude that CCL19 and CCL21 do not affect the differentiation of osteoclasts.

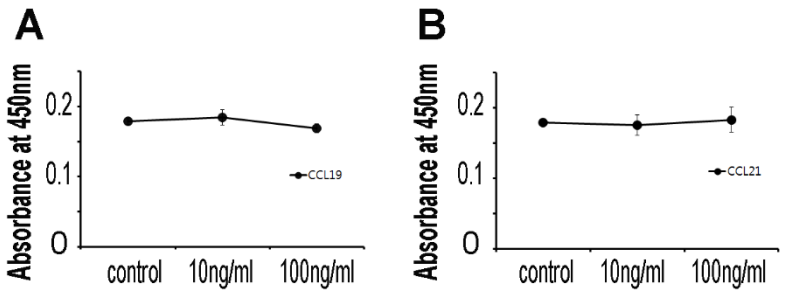

Figure 13. No cytotoxicity of recombinant CCL19 and CCL21.

BMMs were incubated in 96 well plates under the survival condition for 24 h in the absence or presence of CCL19 (A) and CCL21 (B). After 24 h incubation, cells were incubated for 50 minutes with CCK solution. After incubation, optical density was measured at 450 nm. The data represent the means of at least three independent experiments performed in triplicates. *, $p < 0.05$. All data are expressed as mean ± SD.

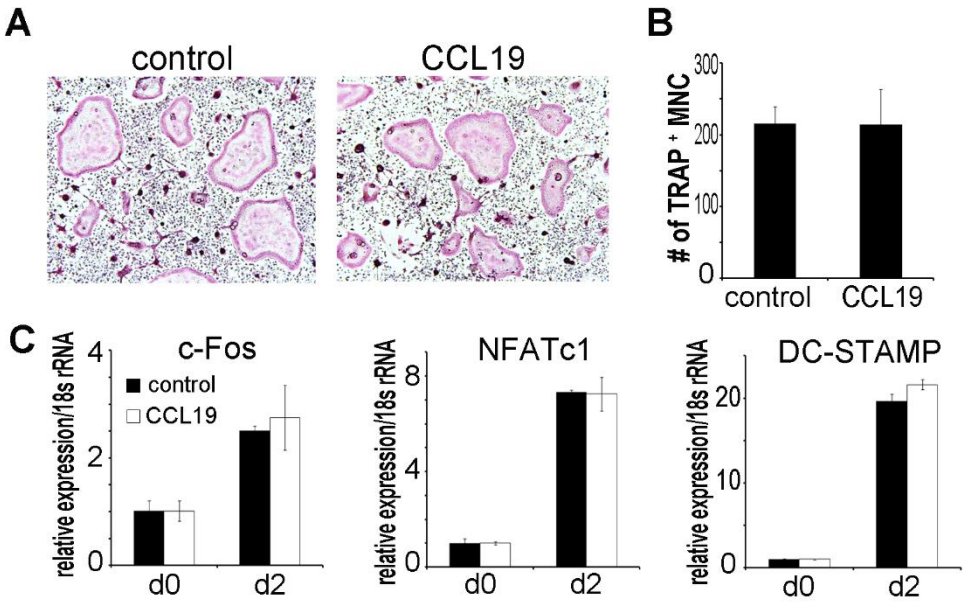

Figure 14. CCL19 does not influence osteoclast differentiation.

(A) Impact of CCL19 on osteoclast differentiation was assessed. BMMs were incubated with M-CSF (30 ng/ml) and RANKL (100 ng/ml) and with or without CCL19 (10 ng/ml). After 4 days, cells were fixed with formaldehyde and stained for tartrate-resistant acid phosphatase activity. (B) TRAP positive multinuclear cells were counted. (C) The osteoclast differentiation markers c-Fos, NFATc1 and DC-STAMP were quantified by real time PCR with 2 day cultures. The data represent the means of at least three independent experiments performed in triplicates. *, $p < 0.05$. All data are expressed as mean ± SD.

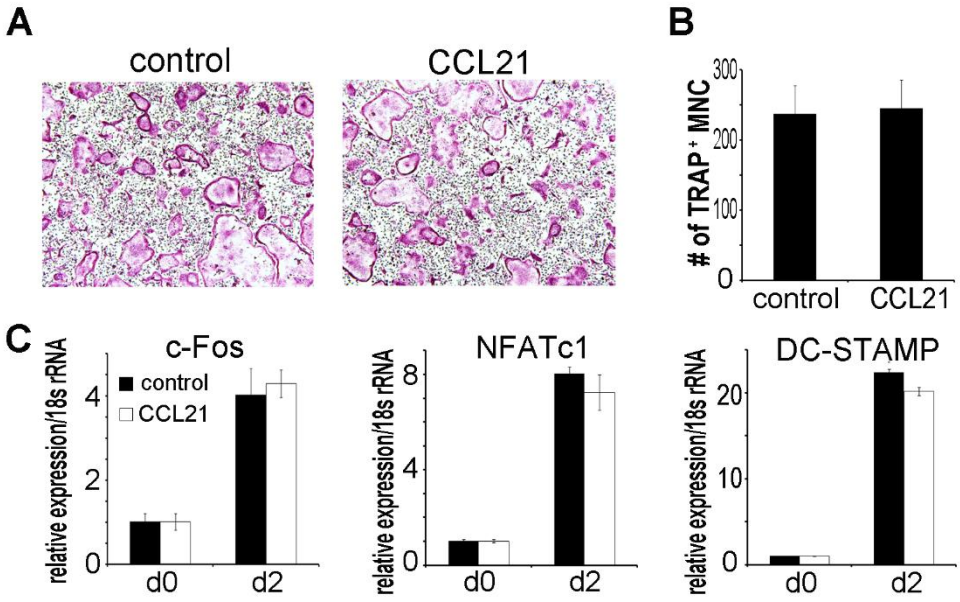

Figure 15. CCL21 does not influence to osteoclast differentiation.

(A) Impact of CCL21 on osteoclast differentiation was examined. BMMs were incubated with M-CSF (30 ng/ml) and RANKL (100 ng/ml) and with or without CCL21 (10 ng/ml). After 4 days, cells were fixed with formaldehyde and stained for tartrate-resistant acid phosphatase activity. (B) TRAP positive multinuclear cells were counted. (C) The osteoclast differentiation markers c-Fos, NFATc1 and DC-STAMP were quantified by real time PCR with 2 day cultures. The data represent the means of at least three independent experiments performed in triplicates. *, $p < 0.05$. All data are expressed as mean ± SD.

6. Regulation of osteoclast migration activity by CCL19 and CCL21.

Since CCL19 and CCL21 are known as chemokines which can attract immune cells, it is possible that osteoclasts can migrate easily with these cytokines. In the transwell assay, BMMs which are precursor of osteoclasts showed remarkably stronger migration in CCL19-treated group (Fig. 16A). There is also dramatic increase of migration in CCL21-treated group (Fig. 16B). CCL19 and CCL21 had similar effects on BMM migration at both 10 ng/ml and 100 ng/ml, which means that even small quantity of chemokines is enough to attract cells to move toward the chemoattractants.

Oris migration assay kit was used in order to measure the migration of osteoclasts. The transwell assay is only valid for transient time period which is insufficient for the generation of osteoclasts from BMMs. Consistent with the result of the transwell assay, osteoclast migration areas were larger when they were treated with CCL19 or CCL21 than control groups. It means that cytokine-treated groups showed more migration (Fig. 17).

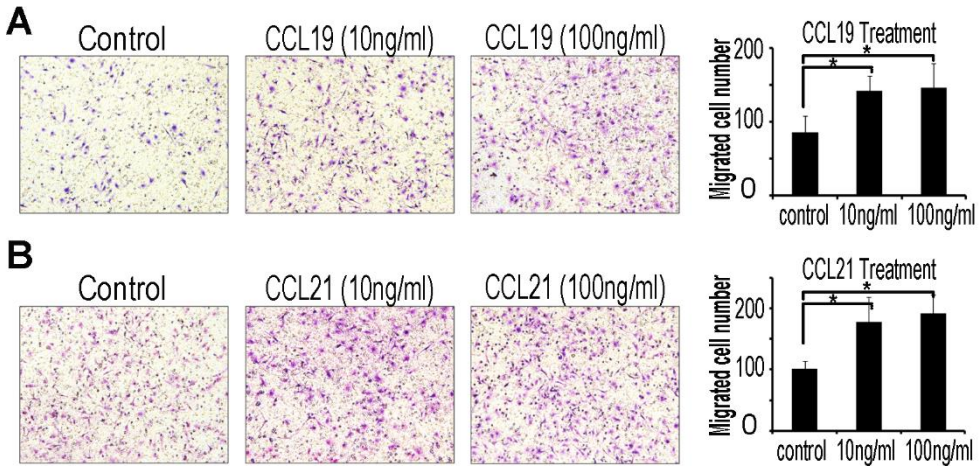

Figure 16. CCL19 and CCL21 increase the migration of osteoclast precursors.

BMMs were loaded in the upper chamber of transwell plates. CCL19 (A) or CCL21 (B) were added to the media in the lower chamber. After 16 h, cells were fixed and stained with crystal violet. Migrated BMMs were counted. The data shown are representative of three independent experiments performed in triplicates. *, $p < 0.05$. All data are expressed as mean ± SD.

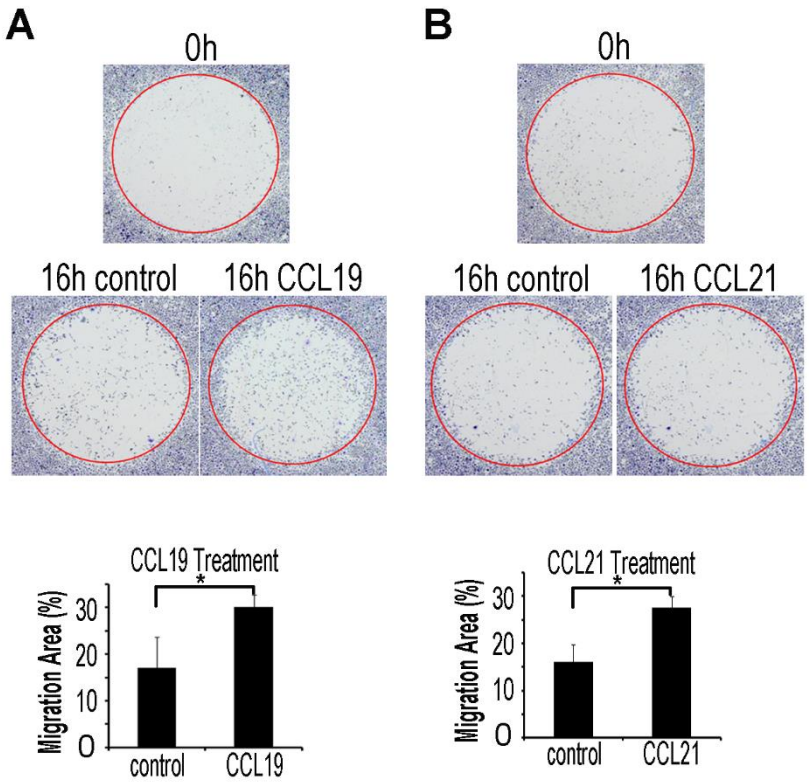

Figure 17. CCL19 and CCL21 increase the migration of osteoclasts.

BMMs were plated in Oris cell migration assay plates. Cells were incubated with M-CSF (30 ng/ml) and RANKL (100 ng/ml). After 2 days, stoppers were removed by Oris stopper tool and washed twice with fresh medium. Cells were incubated with or without CCL19 (10 ng/ml) (A) or CCL21 (10 ng/ml) (B). After 16 h, cells were fixed and stained with crystal violet and cell migration area was quantified. The data shown are representative of three independent experiments performed in triplicates. *, $p < 0.05$. All data are expressed as mean ± SD.

7. CCL19 and CCL21 increase osteoclast precursor migration through CCR7.

To examine whether CCR7 is responsible for osteoclast migration in response to the CCL19 and CCL21, CCR7 expression was knock-downed using siRNA technique. Reduced CCR7 levels were confirmed at both mRNA and protein levels (Fig. 18A and 18B). When receptor expression level was knock-downed, BMMs did not show any increase in migration when they were treated with CCL19 or CCL21 compared to the control group (Fig. 18C and 18D). This result implies that CCR7 allows osteoclast precursors to respond to CCL19 and CCL21.

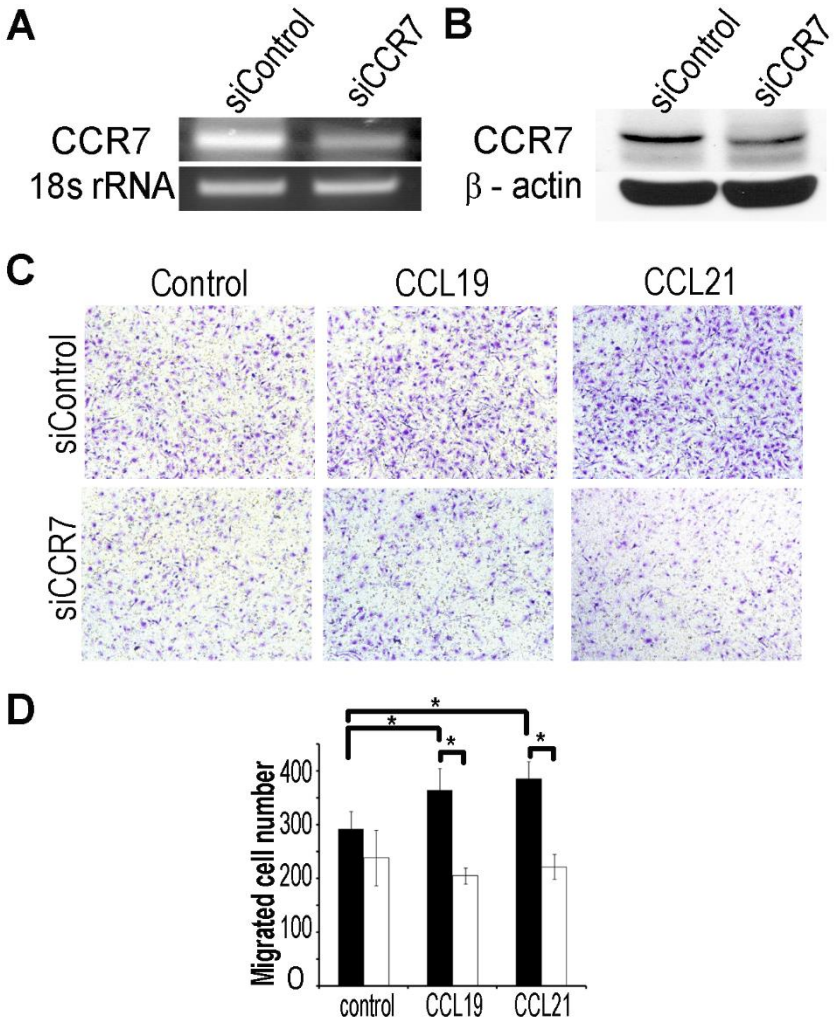

Figure 18. CCL19 and CCL21 increase BMM migration through CCR7.

BMMs were transfected with either control or CCR7 siRNA using Hiperfect for 12 h. Knock-down of CCR7 was confirmed by PCR (A) and Western blotting (B). Either CCR7 or control siRNA transfected BMMs were loaded in the upper chamber of transwell plates. CCL19 or CCL21 were added to the media in the lower chamber. After 16 h, cells were fixed and stained with crystal violet (C). Migrated BMMs were counted (D). *, $P < 0.05$. All data are expressed as mean ± SD.

8. CCL19 and CCL21 increase osteoclast resorption activity.

Since migration is controlled by the actin cytoskeleton and another important osteoclast function, resorption activity, is related to the actin cytoskeleton, the effects of CCL19 and CCL21 on osteoclast resorption activity were measured. To see the osteoclast function under the treatment with CCL19 and CCL21, osteoclasts were cultured in calcium phosphate-coated plates. Both cytokines increased the resorption activity and CCL21 showed slightly more increase in the resorption activity (Fig. 19).

The resorption activity of osteoclasts was also assessed with dentine slices. Eroded surfaces are distinguished by the indentation of dentin surfaces. Confocal microscopy scanned surface of dentine slices and evaluated the numerical values of resorption pits. The graph obtained from confocal analyses can visualize the depth of resorption pits (Fig. 20A) and 2-D images of scanned surface can show the width of resorption pits (Fig 20B). Dentin slices of cytokine-treated groups showed deeper and wider resorption pits. After confocal analysis, dentin slices were stained with trypan blue dye (Fig. 20C). The areas of stained pits were significantly larger in CCL19- or CCL21-treated group than the control group (Fig. 20D).

These results show that CCL19 and CCL21 up-regulate osteoclast resorption activity.

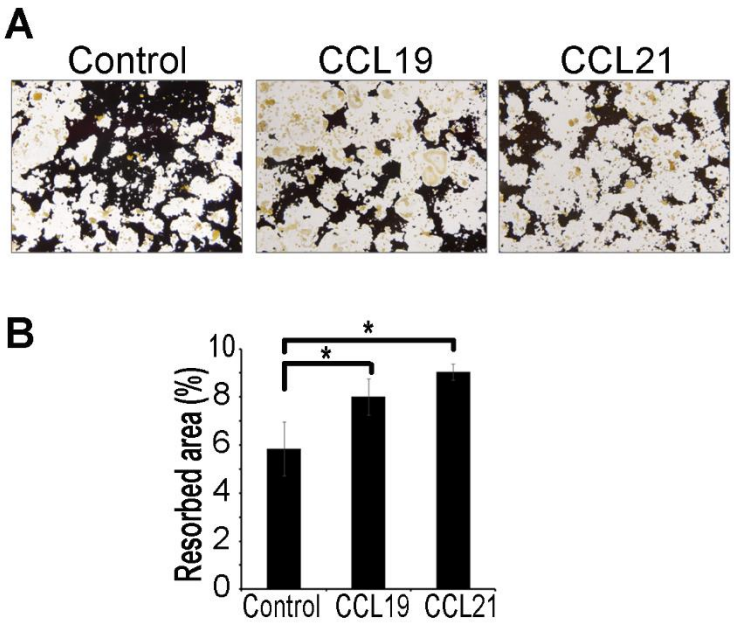

Figure 19. CCL19 and CCL21 increase the bone resorption activity of osteoclasts on calcium phosphate-coated plates.

(A) BMMs were placed on calcium phosphate-coated plates and cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) with CCL19 (10 ng/ml) or CCL21 (10 ng/ml). After 6 days of incubation, remained calcium phosphate (black) was stained with von Kossa reagents. (B) Resorbed area was quantified. *, $P < 0.05$. All data are expressed as mean ± SD.

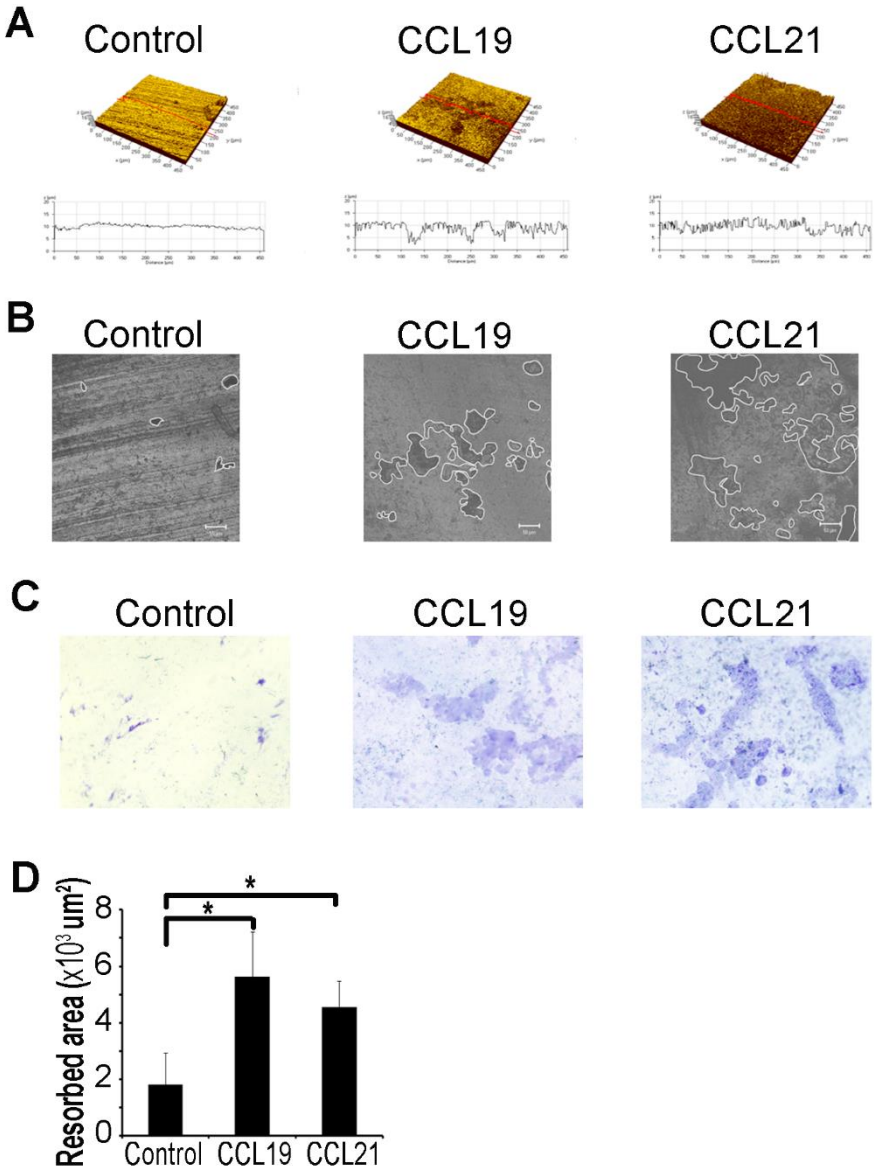
A
Control
CCL19
CCL21
B
Control
CCL19
CCL21
C
Control
CCL19
CCL21
D
Resorbed area (x10³um²)
0
2
4
6
8
*
*
Control
CCL19
CCL21

Figure 20. CCL19 and CCL21 increase the bone resorption by osteoclasts on dentin slices.

BMMs were placed on the dentin slice and cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) with CCL19 (10 ng/ml) or CCL21 (10 ng/ml). After 6 days of incubation, the surface of dentin slice was measured by confocal microscopy. Results obtained from confocal microscopy show the depth (A) and width (B) of dentin slices. Resorption pits were outlined with white line. (C) Dentin slices were stained with trypan blue. (D) The stained area was quantified. The data shown are representative of three independent experiments performed in triplicate. *, $P < 0.05$. All data are expressed as mean ± SD.

9. CCL19 and CCL21 increase osteoclast actin ring density.

To visualize the actin ring formation of osteoclasts, actin was stained with rhodamin-conjugated phalloidine and visualized by confocal microscopy. CCL19- and CCL21-treated groups showed dense and prominent actin ring formation (Fig. 21A). Osteoclasts with a strong actin ring density and definite actin ring boundary are classified as the high actin ring intensity group. Osteoclasts having a weak actin ring density and vague actin ring boundary is defined as low actin ring intensity group. The numbers of osteoclasts in each group were counted as well as the total number of osteoclasts. The number of osteoclasts showing high actin ring intensity was increased while the number of those having low actin ring intensity was decreased in the cytokine-treated groups (Fig. 21B). This result implies that osteoclasts form thicker and prominent actin rings in response to CCL19 or CCL21.

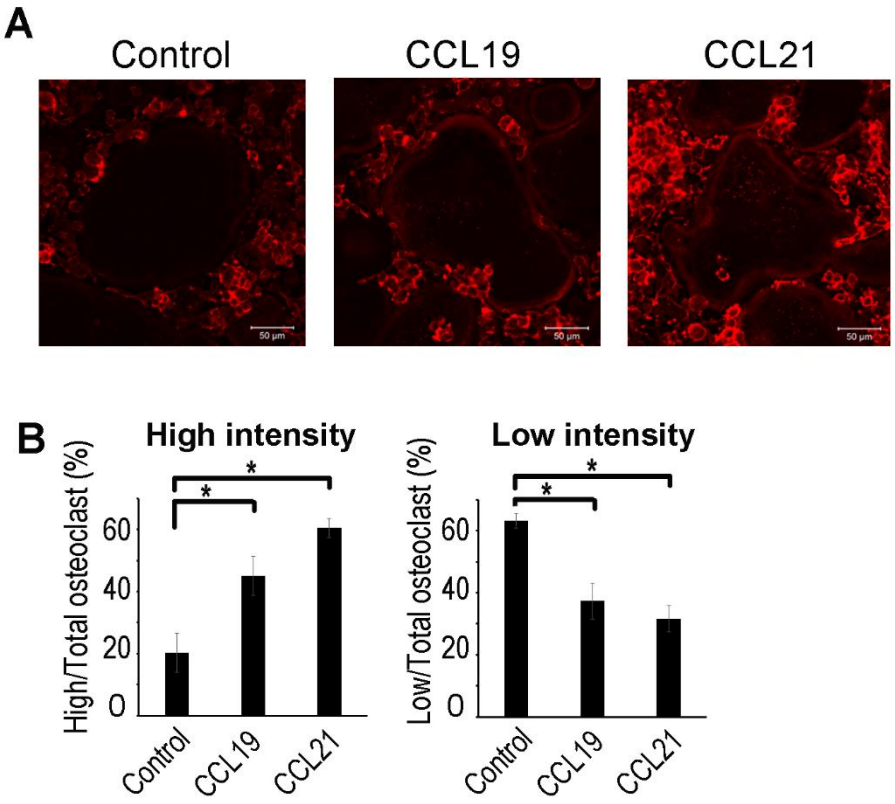

Figure 21. CCL19 and CCL21 increase the actin ring density in osteoclasts.

(A) BMMs were cultured on the cover glass and cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) with CCL19 (10 ng/ml) or CCL21 (10 ng/ml). After 6 days of incubation, cells were fixed and incubated with rhodamin-conjugated phalloidine. Actin ring (red) fluorescence were measured by confocal microscopy. (B) The number of osteoclasts were counted depending on their levels of actin ring intensity. The data shown are representative of three independent experiments. *, $P < 0.05$. All data are expressed as mean ± SD. High, the number of osteoclasts with high actin ring intensity. Low, the number of osteoclasts with low actin ring intensity.

10. CCL19 and CCL21 stimulate osteoclasts via Rho.

CCL19 and CCL21 increased osteoclast migration in my study. It has been reported that active Rho GTPase is involved in migration of cells. In addition, CCR7 is known for activating Rho GTPase. To verify the role of Rho in CCL19 and CCL21 stimulation, active Rho levels were measured by Rho pull-down assay in different conditions. Both CCL19 and CCL21 increased the active Rho in 15 minutes after stimulation (Fig. 22).

ROCK is a major downstream target of activated Rho. To evaluate the ROCK activity, phosphorylated myosin light chain (p-MLC) level was measured. The level of p-MLC increased after 15 minutes of stimulation (Fig. 23).

Other signaling pathways such as mitogen-activated protein kinases (MAPK) and NFκB, which have been shown for their activation upon CCL19 and CCL21 stimulation, were not activated by CCL19 and CCL21 in BMMs (data not shown).

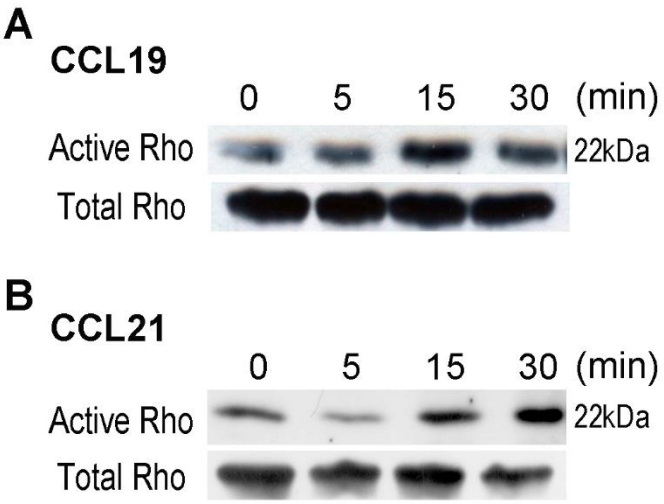

Figure 22. CCL19 and CCL21 activate Rho in BMMs.

BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) with CCL19 (10 ng/ml) (A) or CCL21 (10 ng/ml) (B). After 0, 5, 15 and 30 minutes, cells were lysed and GTP bound active Rho was measured by Rho pull-down assay. The data shown are representative of three independent experiments.

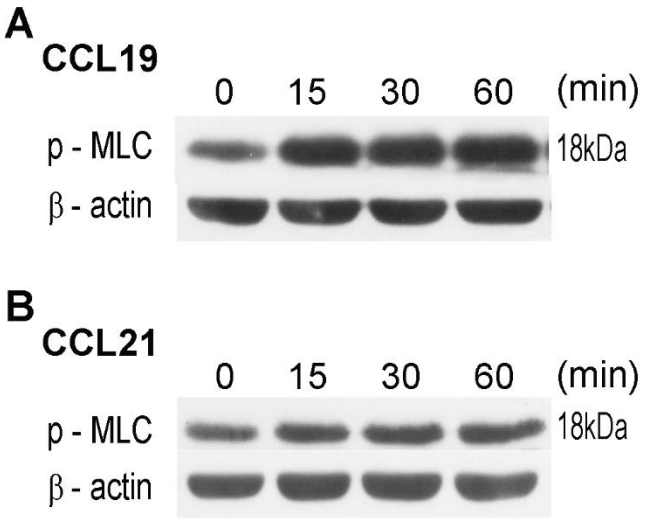

Figure 23. CCL19 and CCL21 activate ROCK in BMMs.

BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) with CCL19 (10 ng/ml) (A) or CCL21 (10 ng/ml) (B). After 0, 15, 30 and 60 minutes, cells were lysed and pMLC (18kDa) was measured by Western blotting. The data shown are representative of three independent experiments.

11. Rho inhibitors suppress CCL19- and CCL21-induced osteoclast migration and resorption activity.

To test whether Rho is responsible for the regulation of osteoclast migration and resorption activity by CCL19 and CCL21, Rho inhibitors simvastatin and Y27632 were used.

Osteoclast migration was decreased in the Rho inhibitor-treated groups compared to the group treated with CCL19 or CCL21 only (Fig. 24). Osteoclast resorption activity was also decreased in the Rho inhibitor-treated groups compare to the cytokine-treated control group (Fig. 25).

These results suggest that CCL19 and CCL21 increase osteoclast migration and bone resorption through the Rho signaling pathway.

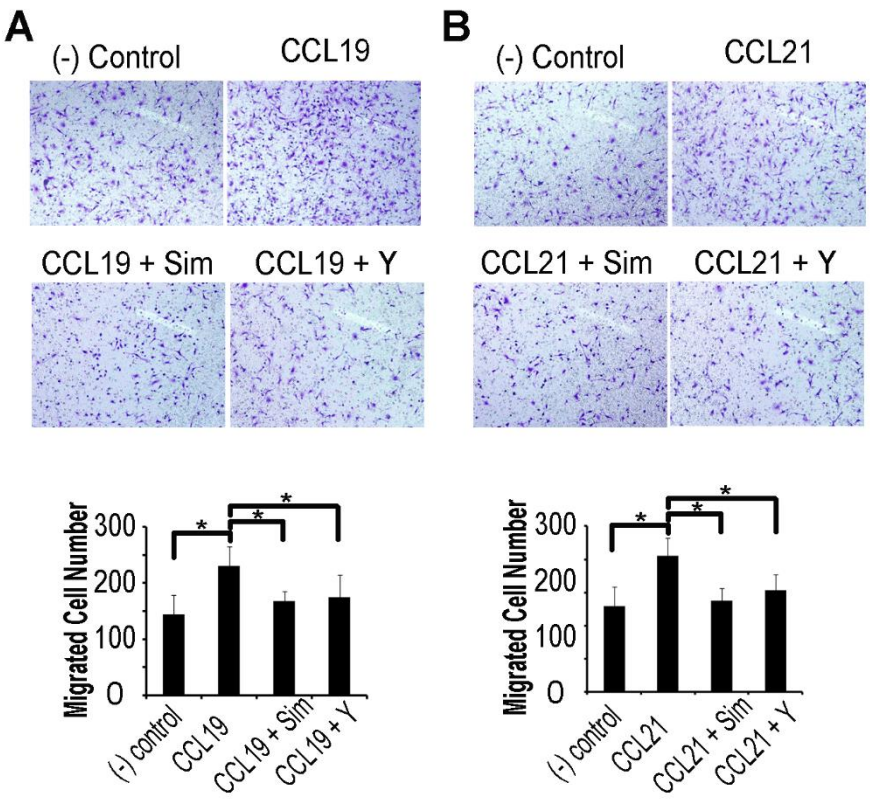

Figure 24. Rho inhibitors decrease CCL19- and CCL21-induced osteoclast migration.

BMMs were loaded to the upper chamber of transwell plates. Cells were pre-treated with Rho inhibitors (simvastatin and Y27632) for 1 h and treated with CCL19 (10 ng/ml) (A) or CCL21 (10 ng/ml) (B) for 16 h. Cells were fixed and stained with crystal violet and migrated cells were counted. The data shown are representative of three independent experiments performed in triplicates. *, $P < 0.05$. All data are expressed as mean ± SD. Sim, simvastatin. Y, Y27632.

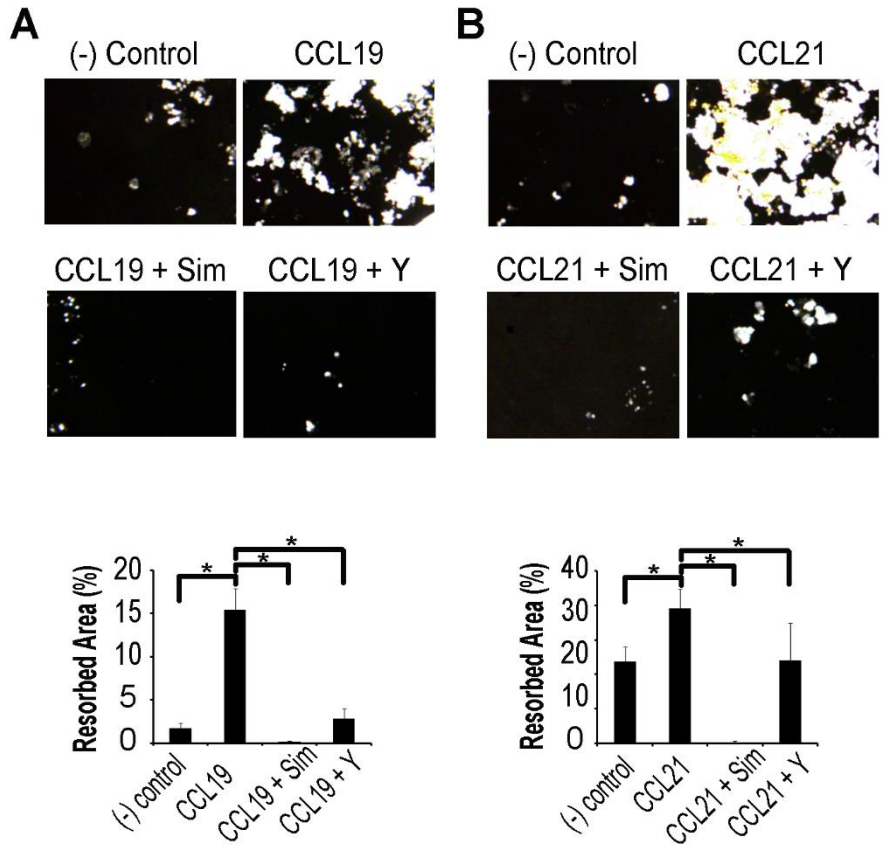

Figure 25. Rho inhibitors suppress CCL19- and CCL21-induced osteoclast resorption activity.

BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) on calcium phosphate-coated plates. After 4 days, cells were treated with Rho inhibitors (simvastatin and Y27632) in addition to CCL19 (10 ng/ml) (A) or CCL21 (10 ng/ml) (B). After 2 days, cells were lysed and remained calcium phosphate (black) was stained with von Kossa reagents. Resorbed area was quantified. The data shown are representative of three independent experiments performed in triplicates. *, $P < 0.05$. All data are expressed as mean ± SD. Sim, simvastatin. Y, Y27632.

12. CCL19 and CCL21 increase mouse calvariae resorption.

To examine the effects of CCL19 on bone resorption in vivo, CCL19-soaked collagen sponges were transplanted to mouse calvariae. After 7 days of transplantation, calvariae were analyzed by μ-CT. CCL19-treated calvariae showed significantly more resorbed area compared to the PBS-treated group. PBS-treated group showed 1.79 times higher bone volume than CCL19-treated group (Fig. 26).

CCL21 also increased the resorption in calvariae, and PBS-treated group showed 1.35 times higher bone volume than CCL21-treated group (Fig. 27).

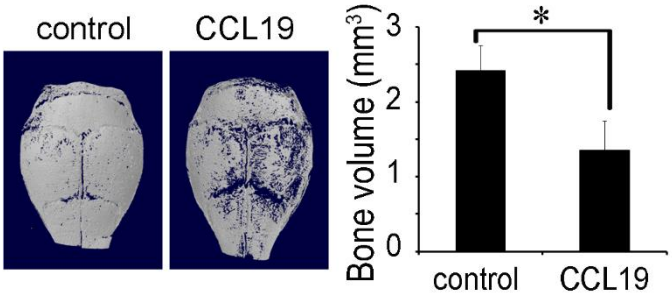

Figure 26. CCL19 increases mouse calvariae resorption.

Collagen sponges were soaked with 2 μg of CCL19 or PBS before transplantation to 5-week-old ICR mouse calvariae subcutaneously. Mice were sacrificed after 7 days of transplantation. Calvariae was extracted, fixed with 4% paraformaldehyde for 1 day and analyzed by μ-CT. *, $P < 0.05$. All data are expressed as mean ± SD.

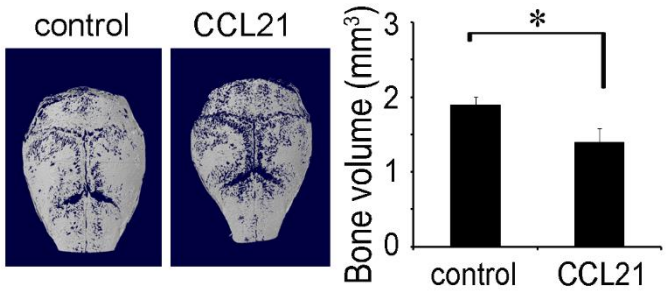

Figure 27. CCL21 increases mouse calvariae resorption.

Collagen sponges were soaked with 2 μg of CCL21 or PBS and transplanted to 5-week-old ICR mouse calvariae subcutaneously. Mice were sacrificed after 7 days of transplantation. Calvariae was extracted, fixed with 4% paraformaldehyde for 1 day and analyzed by μ-CT. *, $P < 0.05$. All data are expressed as mean ± SD.

13. LPS accentuates the CCL19-induced calvariae resorption.

Since LPS or inflammatory cytokines increased the expression level of CCR7, it is possible that the response to CCL19 or CCL21 may be more sensitive in inflammatory conditions. To test this possibility, LPS was injected to the mouse intra-peritoneally in the day before cytokine-soaked collagen transplantation. CCL19 increased the bone resorption and PBS-treated group showed 2.55 times higher bone volume than CCL19-treated group (Fig. 28). This result suggests that LPS might have increased the sensitivity to CCL19 by up-regulating CCR7 expression.

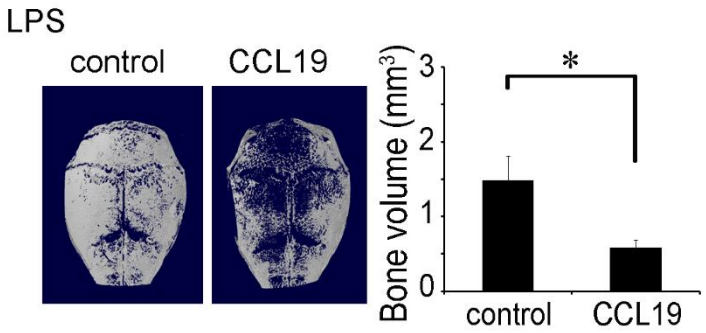

Figure 28. LPS accentuates the CCL19-induced calvariae resorption

LPS was injected 5-week-old female ICR mice intra-peritoneally 2 times with a 4-day interval. The first injection was done at the day before transplantation. Collagen sponges with 2 μg of CCL19 were transplanted to the mouse calvariae. Mice were sacrificed after 8 days of the first injection. Calvariae were extracted, fixed with 4% paraformaldehyde for 1 day and analyzed by μ-CT. *, $P < 0.05$. All data are expressed as mean ± SD.

IV. Discussion

In summary, the levels of CCL19, CCL21 and CCR7 were elevated in RA patients. There were dramatic increases in CCR7 expression of osteoclasts under inflammatory conditions. While CCL19 and CCL21 did not affect the differentiation of osteoclasts, these chemokines increased osteoclast migration and resorptive activity via the CCR7/Rho axis (Fig. 29).

In this study, CCL19, CCL21 and CCR7 expression levels of RA patient samples were measured by using ELISA and GEO dataset of NCBI. CCL19 and CCL21 expression levels measured by ELISA were higher in both serum and synovial fluid of RA patients than in those of osteoarthritis patients. Analyses of the data obtained from GEO revealed that CCR7 expression in synovial macrophages and CCL19 and CCR7 expressions in synovial tissues were significantly elevated in RA patients compared to osteoarthritis patients or normal donors. A previous study reported that the levels of CCL19 and CCL21 increased in RA patients (Pickens et al., 2011). Their common receptor CCR7 on monocytes and plasma CCL19 level also increased in RA patients (Ellingsen et al., 2014). In the same study, disease-modifying anti-rheumatic drug therapy was shown to significantly reduce CCR7 and CCL19 levels. It implies that CCL19, CCL21 and their receptor CCR7 may be related to RA progression. The sources of CCL19 and CCL21, which are up-regulated in RA conditions, needs to be investigated in future studies. In addition, the expression levels of CCR family

members CCR1 to CCR9 on synovial macrophages were compared using GEO data in my study. Apart from other receptors, only CCR7 showed the significant differences in expression levels between control and RA patient groups. It refers that CCR7 might be the important mediator of RA compared to the other CCR types.

It has been shown that dendritic cells increase CCR7 expression levels in response to TNF-α (Geissmann et al., 2002) and anti-TNF-α therapy reduced the CCR7 expression level in dendritic cells (van Lieshout et al., 2005). Also, CCR7 expression level was shown to be increased in response to LPS in astrocytes (Gomez-Nicola et al., 2010) and dendritic cells (Yen et al., 2010). In this study, osteoclasts also expressed higher level of CCR7 under inflammatory conditions such as treating with TNF-α, IL-1β and LPS. Therefore, this result is consistent with the previous reports studied in dendritic cells and astrocytes. CCR7 expression has been shown to be elevated by several transcription factors such as Foxo1, NF-κB, peroxisome proliferator activated receptor γ, Runx3, liver X receptor and IFN regulatory factor 4 (Angeli et al., 2003; Bajana et al., 2012; Fainaru et al., 2005; Hopken et al., 2002; Kerdiles et al., 2009; Villablanca et al., 2010). TNF-α, IL-1β and LPS are potential activators for these transcription factors to induce CCR7 expression in osteoclasts. This up-regulation of CCR7 seems to make osteoclasts become more sensitive to CCL19 and CCL21. In fact, previous studies showed that up-regulation of CCR7 levels increased the responsiveness to its ligands. IFN-induced monocyte-derived dendritic cells

express more CCR7 than other types of dendritic cells and have more potential to be attracted to its ligands (Parlato et al., 2001). In addition, another study demonstrated that immune complexes induce the expression of CCR7 and increase directional migration to CCL19 gradients in dendritic cells (Clatworthy et al., 2014). On the other hand, decreased level of CCR7 by gamma ray, triptolide and IFN-β reduce the dendritic cell migration induced by CCL19 (Liu et al., 2011; Liu et al., 2007; Yen et al., 2010).

In this study, the CCR7 mRNA and surface expression levels were slightly increased in TNF-α and IL-1β-treated groups and dramatically increased in LPS-treated group. LPS seems to have a higher potential to stimulate osteoclasts to express CCR7. However, the cytoplasmic fraction of protein did not change much while the amount of CCR7 in whole lysates and surface area increased. The inconsistency of the amount of surface and total protein has also been reported by other groups (Scandella et al., 2002). In addition, it has been shown that both M1 and M2 types of macrophages express CCR7 and M1 express CCR7 on cell surface but M2 in the cytosol. As the final outcome, only the M1 type responds to the CCL19/CCL21, resulting in an increase in their migration (Xuan et al., 2014). It implies that the expression levels of surface CCR7 is important to its responsiveness to its ligands. It seems that CCR7 is expressed constitutively and stored in cytoplasmic fraction, most likely endosome, in normal condition. As soon as stimulated by the inflammatory cytokine or LPS, CCR7 translocalize to the cell surface. There is one study regarding constitutive

ubiquitylation of CCR7, which suggested ubiquitylation is responsible for the CCR7 recycling (Schaeuble et al., 2012). As ubiquitylation is related to not only the recycling but also degradation, precise dynamics of CCR7 about synthesis, expression, ubiquitylation, recycling and internalization should be elucidated.

Unlike dendritic cells which express both CCL19 and CCL21 ligands and the CCR7 receptor, macrophages seldom express CCL19 and CCL21 (data not shown). There is a difference in the ligand expression between them, even though dendritic cells and macrophages differentiate from same precursor monocytes. Dendritic cells, T lymphocytes and lymph nodes are known as the sources of CCL19 and CCL21 (Carlsen et al., 2005; Link et al., 2007; Luther et al., 2000; Willimann et al., 1998). In addition, the expression levels of CCL19 and CCL21 are affected by inflammatory circumstances. CCL19 was increased by LPS, TNF-α and IFNγ in bone marrow stromal cells (Kim et al., 1998). A recent study showed that synovial fibroblasts express more CCL19 upon TNF-α, IL-1β and LPS stimulation, and CCL21 upon IL-1β stimulation (Pickens et al., 2011). The exact source and stimulant of CCL19 and CCL21 expression in joint area need to be elucidated.

Some types of migration-inducing cytokines such as TGFβ (Pilkington et al., 2001), CX3CL1 (Koizumi et al., 2009), and SDF1 (CXCL12) (Yu et al., 2003) are known to affect the differentiation directly and indirectly. There are other types of factors which can reduce the migration. Macrophage migration inhibitory factor (MIF) is one of them and it inhibits osteoclastogenesis (Jacquin

et al., 2009). The migration-related cytokines seem to affect the differentiation in a positive or negative fashion. However, CCL19 and CCL21 do not affect the differentiation of osteoclasts, in other words they do not affect signaling mechanisms involved in differentiation. They only affect the actin cytoskeleton related responses, migration and resorption. Furthermore, mature dendritic cells have stronger chemotactic movement towards CCL19 rather than CXCR4 ligand CXCL12 (Humrich et al., 2006). Thus, this needs to be elucidated whether macrophages or osteoclast progenitors have the same characteristic. It is possible that CCL19 and CCL21 are good therapeutic targets which only affect the migration and resorption activity of osteoclasts and moreover these molecules have the possibility of a strong chemotactic potency than any other chemokines. Besides, CCL19 and CCL21 are known to promote the migration of several cell types such as B lymphocytes, T lymphocytes, dendritic cells and monocytes (Cote et al., 2009; Randolph, 2001; Randolph et al., 2008; Reif et al., 2002; Sallusto et al., 1999; Szanya et al., 2002). The results of my study support these studies because both bone marrow macrophages and osteoclasts were recruited by CCL19 and CCL21. Osteoclast recruitment to the joint area is important in RA pathologic process since bone destruction is caused by resorptive function of osteoclasts. It is significant to study about the factors inducing osteoclast migration because osteoclast migration is the first step to be carried out for the resorption function.

The most important activity of osteoclast function is resorption which

consists of several steps. At first, osteoclasts reorganize their cytoskeleton and acidify the lacunar space, then secrete the matrix degrading enzymes to remove both mineral components and organic proteins. Among them, actin cytoskeleton reorganization is responsible for forming podosome and sealing zone (Kikuta and Ishii, 2013). Bone resorption is controlled by several small GTPases. RhoA is responsible for osteoclast podosome organization (Chellaiah, 2006), motility, bone resorption (Chellaiah et al., 2000) and actin ring formation (Chellaiah, 2005). Deficiency of other GTPase family member, Rac2 cause defects in osteoclast chemotaxis and resorptive activity in mouse model (Itokowa et al., 2011). The downstream of RhoA, Rho kinase (ROCK), activates WASP, myosin light chain and Erzrin (Julian and Olson, 2014; Matsui et al., 1998). All these molecules were reported to enhance bone resorption. It is consistent with my results that RhoA was stimulated by CCL19 and CCL21, enhanced the actin ring formation and bone resorption, which were all inhibited by Rho inhibitors. The mechanism about how CCR7 activates Rho is also a subject of further investigation. In vivo mouse calvaria collagen transplantation showed that excessive CCL19 and CCL21 increase bone resorption, which may be caused by the increased recruitment of osteoclasts. This phenomenon is accentuated in LPS-treated condition under which CCR7 expression was increased. It suggests that the responses to either CCL19 or CCL21 become more sensitive in inflammatory conditions owing to increased level of CCR7.

RA conditions, which up-regulate inflammatory cytokines, increase the

expression levels of CCL19 and CCL21. At the same time, CCR7 is increased on osteoclasts upon treatment with the inflammatory cytokines and LPS, which amplifies the response to the cytokines. Therefore, it will be interesting to examine effects of neutralizing antibodies for CCR7 and its ligands. In addition to anti-resorptives such as cathepsin K inhibitor or denosumab (Cohen et al., 2008; Sharp et al., 2010; Teng et al., 2007), CCL19, CCL21 and CCR7 can be targets for a new therapeutic strategy. Neutralizing antibody for these cytokines or reducing receptor activity can be novel therapeutic strategy to minimize bone destruction by reducing both the migration and resorptive activity of osteoclasts. A recent study pointed out that anti-CCR7 monoclonal antibody inhibited migration of mantle cells in human lymphoma (Somovilla-Crespo et al., 2013). Another study showed that dendritic cells express CCR7 differentially depending on their lineage types. This phenomenon is caused by the epigenetic modification such as histone methylation. Repressive histone methylation H3K27me3 reduce the CCR7 transcriptional levels (Moran et al., 2014). The relevant mechanisms of histone methylation need to be studied to control the CCR7 expression at the transcriptional level. Recent studies have shown that miRNA can affect the expression levels of CCR7. Micro RNA 7a and miRNA 21 regulate the expression levels of CCR7 in breast cancer cells and T cells, respectively (Kim et al., 2012; Smigielska-Czepiel et al., 2013). These studies give us new inspiration to develop new therapeutic strategies such as histone methylation-induced gene silencing and miRNA-induced mRNA interference in osteoclasts.

There is one study demonstrating that treating immunosuppressive drugs modulated the expression levels of CCR7. For example, dexamethasone inhibited the migration of mouse dendritic cells in vivo and of human dendritic cells in vitro to CCL19 by inhibiting the CCR7 expression. On the other hand, rapamycin increased the migration of dendritic cells to CCL19 in vitro and in vivo by up-regulating the CCR7 expression level. This implies that immunosuppressive drugs differentially regulate the expression levels of CCR7 and regulate the potential of migration to CCL19 (Sordi et al., 2006). Consequently, these immunosuppressive drugs are potential agents to regulate the migration and resorption of osteoclasts. These various therapeutic strategies such as neutralizing antibodies, knock down system, histone methylation, miRNA and immunosuppressive drugs are potential therapeutic methods for anti-rheumatoid progression by reducing the osteoclast migration and bone resorption. These therapies will contribute to reducing and delaying the bone destruction caused by periodontitis or RA.

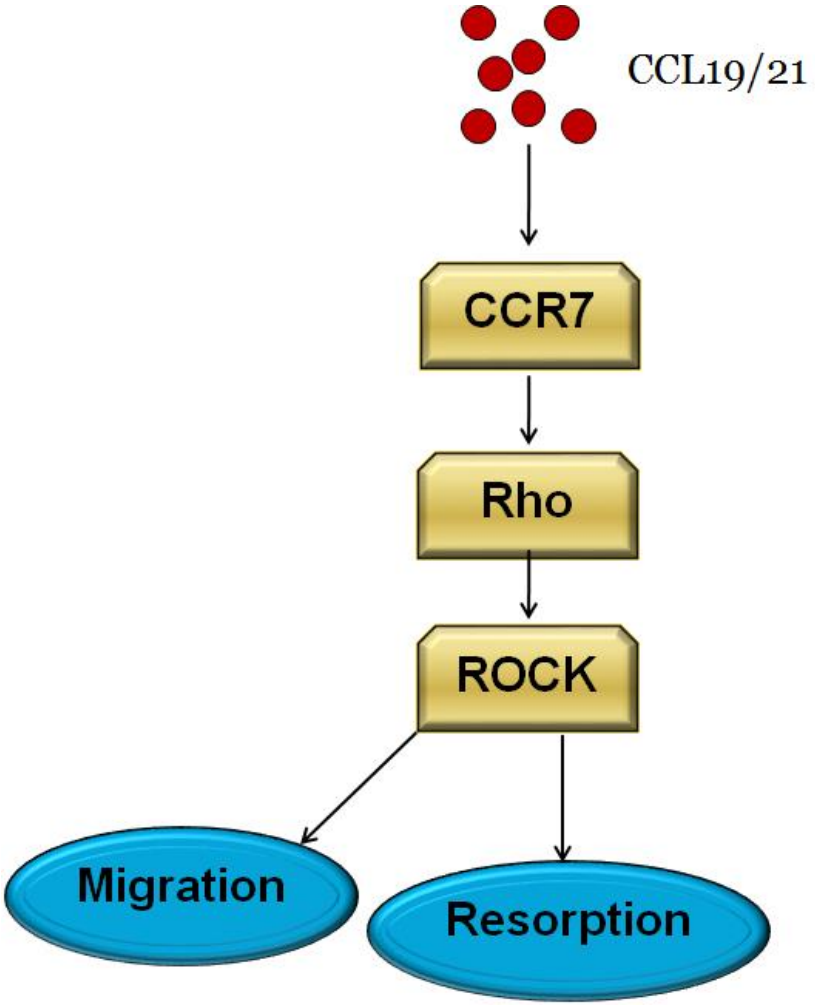

Figure 29. Schematic diagram for the CCL19/CCL21 and CCR7 signal transduction axis in osteoclasts.

CCL19 and CCL21 bind to the receptor CCR7, which increases the GTPase Rho activity and its downstream ROCK activity. This pathway finally increases osteoclast migration and resorptive activity.

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국문초록

파골세포 이동과 골흡수에 대한 CCL19 및 CCL21 케모카인의 역할

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이 지 연

치주질환자나 류마티스성 관절염 환자들은 심각한 골흡수를 수반하며 이 과정에서 파골세포가 중요한 역할을 한다는 사실이 잘 알려져 있다. CCL19와 CCL21은 몇 가지 세포에서 세포의 이동을 유도하는 케모카인으로 작용한다는 것이 알려져 있으며 이들의 파골세포에 대한 영향은 아직 알려진 바가 없다. 이 연구의 목적은 골흡수의 직접적인 원인인 파골세포에 대한 CCL19 및 CCL21의 역할을 밝혀내는 것이다.

NCBI의 gene expression omnibus 프로그램과 효소결합면역흡착분석법(ELISA)을 이용해 류마티스성 관절염과 퇴행성 관절염 환자에서의 CCL19, CCL21 및 CCR7의 양을 분석한 결과, 류마티스성 관절염 환자에서 이들의 발현량이 증가한 사실을 확인하였다. 또한 파골세포는 염증성 사이토카인이나 지질다당류(LPS)에 반응하여

CCR7의 발현량을 증가시켰다. CCL19 및 CCL21은 파골세포의 분화 자체에는 영향을 미치지 않았지만 파골세포의 이동이나 골흡수 능력을 향상시킨다는 사실을 tartate-resistant acid phosphatase(TRAP) 염색, transwell assay 및 Oris migration assay kit, 그리고 칼슘포스페이트가 흡착된 세포배양판이나 상아질을 이용한 실험으로 확인할 수 있었다. 또한 이러한 일련의 과정이 CCR7/Rho의 신호전달경로를 통해서 일어나는 것을 small interference RNA 기법, Rho pull-down assay 그리고 Rho 억제제를 사용한 실험을 통해 확인하였다. 쥐의 두개골에 콜라겐을 이식하는 동물 실험을 통하여 CCL19와 CCL21이 실제로 골흡수를 증가시킬 수 있다는 사실을 확인하였다.

본 실험은 염증 상황에서 파골세포들이 CCR7 발현량을 증가시키며, 류마티스성 관절염 환자의 염증성 관절 부위에서 CCL19 및 CCL21의 발현량이 증가되어 있음을 보여주었다. 그 결과, 파골세포가 CCL19와 CCL21이 분비되는 염증성 관절 주위로 더 많이 모이게 되고 결국 관절 주위의 심각한 골흡수를 초래하게 된다. 본 연구는 CCL19이나 CCL21을 중화시키는 항체를 사용하거나 CCR7의 발현량을 낮춰주는 small interference RNA 치료법을 사용하면 치주질환자나 류마티스성 관절염 환자에서 골흡수를 억제할 수 있을 것이라는 것을 제시한다.

주요어 : 파골세포, 세포 이동, CCR7, CCL19, CCL21, 류마티스성 관절염

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