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RANKL downregulates cell surface CXCR6 expression through JAK2/STAT3 signaling pathway during osteoclastogenesis

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ABSTRACT

The receptor activator of nuclear factor-κB ligand (RANKL), as a member of the tumor necrosis factor (TNF) family, plays an essential role in osteoclast differentiation and function. Chemokines and their receptors have recently been shown to play critical roles in osteoclastogenesis, however, whether CXCL16–CXCR6 plays role in RANKL-mediated osteoclastogenesis is unknown. In this study, we first reported that RANKL decreased CXCR6 in a dose-dependent manner, which may be through deactivation of Akt and STAT3 signaling induced by CXCL16. Interestingly, RANKL-mediated CXCR6 reduction may be associated to the activation of STAT3 by phosphorylation. When STAT3 activation was blocked by JAK2/STAT3 inhibitor AG490, RANKL failed to shut down CXCR6 expression during osteoclastogenesis. However, CXCL16 alone did not augment RANKL-mediated osteoclast differentiation and did not alter RANKL-receptor RANK mRNA expression. These results demonstrate that reduction of CXCL16–CXCR6 is critical in RANKL-mediated osteoclastogenesis, which is mainly through the activation of JAK2/STAT3 signaling. CXCL16–CXCR6 axis may become a novel target for the therapeutic intervention of bone resorbing diseases such as rheumatoid arthritis and osteoporosis.

1. Introduction

Osteoclasts are multinucleated cells formed by the fusion of mononuclear progenitors of monocyte/macrophage lineage [1]. The receptor activator of NF-κB ligand (RANKL) produced by osteoblasts is a tumor necrosis factor (TNF) superfamily cytokine which plays a key role in osteoclast differentiation and activation [2]. During physiological or pathological process of bone remodeling, transcription factors, including NF-κB, AP-1 and Nfatc1, are activated by the binding of RANKL to its receptor RANK on the surface of osteoclast precursors, which subsequently stimulates the transcription of osteoclast associated genes and leads to the formation of multinucleated tartrate-resistant acid phosphatase (TRAP) positive giant cells [1,3]. In addition, chemokines and their receptors have recently been shown to play critical roles in osteoclastogenesis [4–6].

CXCL16, binding to its receptor CXCR6, is a membrane-bound chemokine expressed on dendritic cells (DCs), macrophages and endothelial cells [7–9]. CXCL16 shows similar structural characteristics to fractalkine, the other transmembrane-type chemokine with a chemokine domain fused to a mucin-like stalk [10]. Data from Shimaoka et al. [11] have confirmed that interaction of transmembrane CXCL16–CXCR6 strongly induces cell-to-cell contact in a manner without CXCR6-mediated signal transduction or integrin activation. However, upon cleavage by disintegrin-like metalloproteinase ADAM10 and ADAM17, CXCL16 extracellular domain is released as a soluble chemokine which acts as a chemoattractant of effector/memory T cells that express CXCR6 [7,9]. Recent advances in the function of CXCR6 and its ligand CXCL16 in regulating metastasis and invasion of cancer showed that CXCR6-positive prostate cancer cells were observed to migrate to CXCL16-positive osteocytes, and this results in increased bone metastasis [12,13]. But the role of CXCL16–CXCR6 interaction in osteoclastogenesis remains to be characterized.

Several types of cytokines could regulate the expression of CXCR6. For example, the addition of interleukin (IL)-12 up-regulates the expression of CXCR6 on T cells, while IL-4 shows inhibitory effect [14]. Further characterization of CXCR6 regulation is essential for understanding the physiological and pathological interaction of CXCL16–CXCR6. In the present study, we found that CXCR6 was down-regulated during RANKL-mediated osteoclastogenesis through JAK2/STAT3 pathway, while CXCL16 treatment alone failed to directly induce osteoclastogenesis in RAW264.7 cells.

2. Materials and methods

2.1. Antibodies and reagents

Phycoerythrin-conjugated rat monoclonal anti-mouse CXCR6 antibody was purchased from R&D systems (Minneapolis, MN,

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USA), and the isotypic antibody, phycoerythrin-conjugated rat IgG2b, was from eBioscience (San Diego, CA, USA). Anti-phospho-Akt (Ser-473), anti-Akt, anti-phospho-p44/42 ERK (Thr^{202}/Tyr^{204}), anti-ERK, anti-phospho-STAT3 Tyr^{705} and anti-STAT3 antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against mouse CXCR6, phospho STAT3 (Ser^{727}) and CXCL16 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Abcam (Cambridge, MA, USA), respectively. Recombinant mouse RANKL, CXCL16 and macrophage colony-stimulating factor (M-CSF) were obtained from Peprotech (Rocky Hill, NJ, USA). LY294002, PD98059 and AG490 were supplied by Selleck Chemicals (Houston, TX, USA). Dimethyl sulfoxide was obtained from Amresco (Solon, OH, USA).

2.2. Cell culture

A murine osteoclast precursor cell line RAW264.7 (Peking Union Medical College, Beijing, China) was cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, NY, USA) supplemented with 10% (v/v) inactivated fetal bovine serum (FBS, Gibco), 100 U/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco) at 37 °C in 5% CO_{2-} enriched atmosphere.

2.3. Western Blot analysis

RAW264.7 cells were incubated with or without RANKL (100 ng/ml). After incubation for 24 h, whole cell lysates were prepared in the lysis buffer and centrifuged at 14,000 rpm for 15 min. Protein concentration in the supernatant was measured using the Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). The protein samples were separated on 10% sodium dodecylsulfate polyacrylamide (SDS) gel electrophoresis and transferred to polyvinylidinedifluoride (PVDF) membrane (Immobilon-P; Millipore, Billerica, MA, USA). For Western blot, the membrane was blocked with 5% skim milk in 0.05% Tween 20 in Tris buffered saline (TBST) at room temperature for 2 h. The primary antibodies, diluted in 5% BSA-TBST, were added and incubated overnight at 4 °C. The membrane was washed three times with TBST, followed by addition of horseradish peroxidase-conjugated secondary antibody, and the membrane was then incubated for 1 h at room temperature. The blots were developed by using an enhanced chemiluminescence detection kit (Pierce, Rockford, IL, USA).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

For RT-PCR assays, total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Two micrograms of total RNA from each sample was reversely transcribed with Superscript II reverse transcriptase (Invitrogen). The reverse transcription reaction was carried out at 42 °C for 50 min, and then at 70 °C for 15 min. PCR amplification was performed by denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s by using TaKaRa Ex Taq (Takara Bio, Shiga, Japan). The amplified PCR products were separated on a 1.5% agarose gel. The primers used were as follows: for GAPDH, forward: 5′-AAATGGTGAAGGTCGGTGTG-3′, reverse: 5′-TGAAGGGGTCGTTGATGG-3′; TRAP, forward: 5′-TCCCCTGGTA TGTGCTGG-3′, reverse: 5′-GCATTTTGGGCTGCTGA-3′; Cathepsin-K, forward: 5′-GTTGTATGTATA ACGCCACGGC-3′, reverse: 5′-CTTTC TCGTTCCCCACAGGA-3′; RANK, forward: 5′-CGAGGAAGATTCCC-ACAGAG-3′, reverse: 5′-CAGTGAAGTCACAGCCCTCA-3′.

2.5. Flow cytometric analysis

RAW264.7 cells were treated with or without RANKL for 24 or 48 h and harvested in phosphate-buffered saline (PBS). The cells were washed and resuspended in PBS containing Phycoerythrin-conjugated rat monoclonal anti-mouse CXCR6 antibody for 45 min on ice. The cell suspension was washed and analyzed by the FACS Calibur system (BD Biosciences, San Jose, CA, USA), and the acquired data were analyzed using the FlowJo software (Tree Star, Ashland, OR,USA).

2.6. Migration assay

Transwell cell culture chambers (Costar, Cambridge, MA, USA) were used in the migration assay. RAW264.7 cells were treated with or without RANKL (100 ng/ml) for 48 h, harvested and then washed with FBS-free medium and resuspended in 0.1% BSA medium at a density of 1.0×10^6 cells/ml. Hundred microliters of aliquots of cell suspension were added to the upper compartment, while the lower compartments contained 600 μl medium with or without 100 or 200 ng/ml CXCL16. After incubation at 37 °C for 12 h, cells migrated to the lower surface were stained with hematoxylin for 4 min, washed in water and stained again with eosin for 1 min. For each filter, cells in five randomly chosen fields were counted and total numbers were calculated.

2.7. Cell adhesion assay

Cell adhesion to immobilized CXCL16 was measured as described previously [11]. Briefly, each well of the 96-well plate was precoated with recombinant mouse CXCL16 in PBS at 4 °C overnight. After washing with PBS, nonspecific binding sites were blocked with adhesion buffer (RPMI 1640 containing 1% BSA and 20 mM HEPES, pH 7.4). After co-incubation with RANKL (100 ng/ml) for 48 h, RAW264.7 cells were added to the wells (1×10^5 cells/well), and incubated at 37 °C for 1 h. After washing, adherent cells in each well were counted under the microscope. In some experiments, plates were preincubated for 1 h at room temperature with or without rabbit anti-mouse CXCL16 mAb before assay.

2.8. Osteoclast differentiation assay

Differentiation of osteoclasts *in vitro* was performed as described previously [15]. In brief, osteoclast precursors were suspended in DMEM supplemented with 10% (v/v) inactivated FBS and cultured in a 24-well culture plate at 2×10^5 cells per well. 12 h later, the culture medium was replaced with fresh culture medium in the presence of different combination of mouse recombinant soluble M-CSF (10 ng/ml), RANKL (100 ng/ml) and CXCL16 (100 ng/ml). The medium was changed on day 3. After 5 days, cells were fixed with 4% paraformaldehyde for 20 min at room temperature and then stained with Naphthol AS-BI phosphate and a tartrate solution for 30 min at 37 °C, followed by counterstaining with a hematoxylin solution. Osteoclasts were determined to be TRAP-positive staining multinuclear (three or more nuclei) cells and counted under light microscopy.

2.9. Statistical analysis

Each experiment was performed in triplicate and was repeated at least twice. All quantitative data were expressed as mean ± SD for each condition. For comparisons of multiple groups, a one-way analysis of variance (ANOVA) followed by a Scheffe's post hoc test was performed. Differences relative to a probability of two-tailed $P < 0.05$ were considered significant.

3. Results

3.1. RANKL down-regulates CXCR6 expression during osteoclastogenesisin RAW264.7 cells

To determine the effect of RANKL on CXCR6 expression during osteoclastogenesis, RAW264.7 cells were treated with or without different doses of RANKL for 24 h. Flow cytometry analysis demonstrated that expression of cell surface CXCR6 was significantly reduced by RANKL in a dose-dependent manner (Fig. 1A–a and B). Similar effect was observed in 48 h treatment groups (Fig. 1A–b). As expected, Western blot showed CXCR6 protein level in whole cellular pool was also decreased (Fig. 1C). Osteoclastogenesis-related genes, including TRAP and Cathepsin-K, were dose-dependently up-regulated by RANKL (10–100 ng/ml) (Fig. 1D). These results demonstrated that repression of CXCR6 was paralleled to RANKL-induced osteoclast differentiation in RAW264.7 cells.

3.2. RANKL blocks CXCL16-mediated kinase signaling

Several intracellular signaling pathways, including PI3K/Akt, MAPKs and JAK2/STAT3, were activated by RANKL binding to RANK on osteoclast precursors [16]. To investigate the functional significance and the potential signaling pathway influenced by the reduction of cell surface CXCR6, we analyzed the cellular response in RAW264.7 cells induced by recombinant CXCL16, the ligand for CXCR6. After 24 h incubation, CXCL16 (100 ng/ml) strongly activated Akt, Erk and STAT3 pathway by increase in phospho-AKT-Ser^{473}, phosphor-p44/42 ERK-Thr^{202}/Tyr^{204} and phosphor-STAT3-Ser^{727}, and the co-incubation with RANKL (100 ng/ml) suppressed activation of these kinase signaling, especially the phosphorylation of Akt and STAT3 (Fig. 2A). Interestingly, RANKL alone only activated STAT3 phosphorylation in RAW264.7 cells, which may be important for RANKL-mediated suppression of CXCL16–CXCR6 interaction and function. CXCL16 has been shown to have chemotactic effect [7,9]. Indeed, we found that CXCL16 protein showed a dose-dependent increase in migration cells (Fig. 2B). In consistent with the inhibition of Akt signaling, RANKL exhibited an obvious inhibition of cell migration mediated by CXCL16 (Fig. 2B). CXCL16 immobilization assay further confirmed RANKL significantly suppressed cell adhesion ($P < 0.0001$, Fig. 2C). As expected, when the CXCL16 immobilized plates were preincubated with anti-CXCL16 antibody, the cell adhesion was reduced ($P = 0.0211$, Fig. 2C) and RANKL further augmented this reduction ($P < 0.0001$, Fig. 2C). These results demonstrated that RANKL dramatically attenuated CXCL16 function in cell migration and adhesion activity mainly through suppression of Akt and STAT3 activation in RAW264.7 cells.

3.3. Inhibition of STAT3 activity prevents diminishment of cell surface CXCR6 mediated by RANKL

In order to further confirm which pathway (Akt or STAT3) plays more important role in RANKL-induced reduction of CXCR6

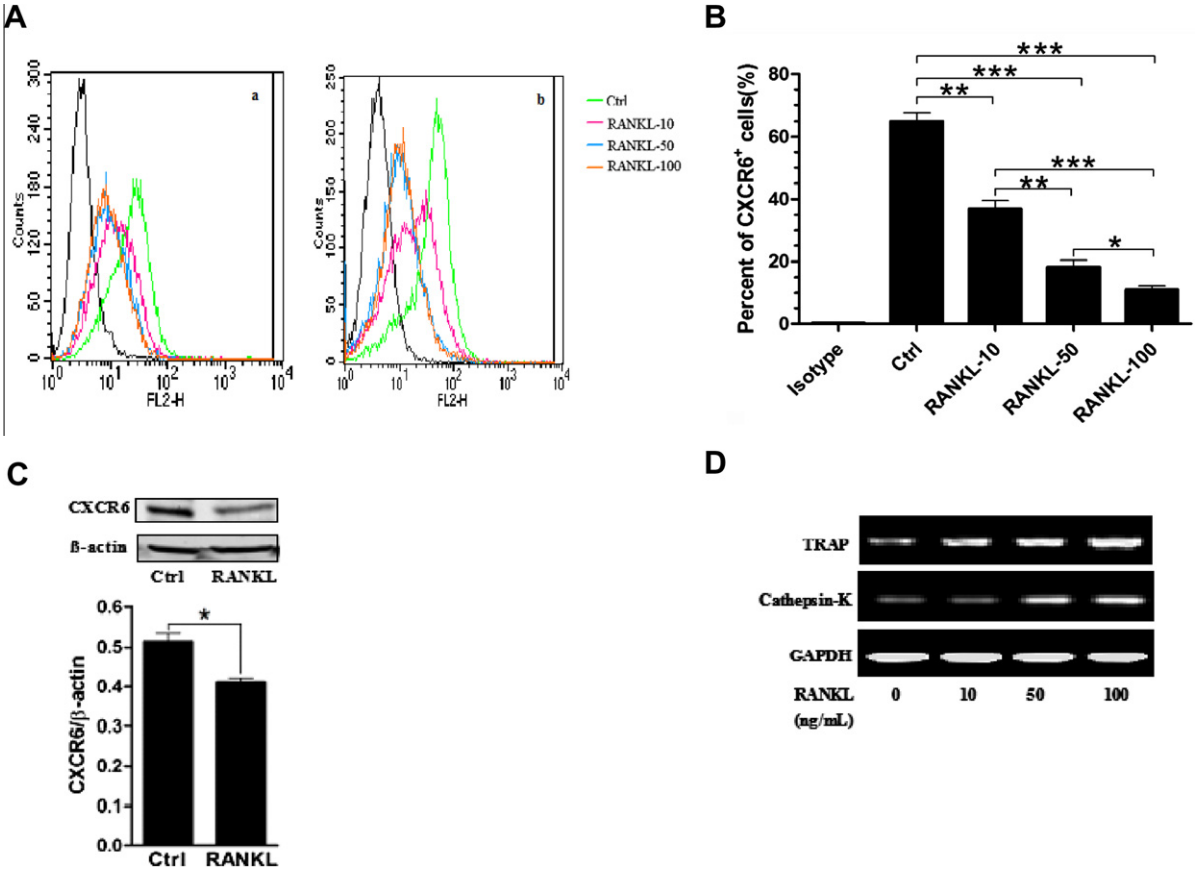

Fig. 1. RANKL downregulates CXCR6 expression in RAW264.7 cells. (A) RAW264.7 cells were cultured in the presence of medium alone (Isotype, Ctrl), 10, 50 or 100 ng/ml (RANKL −10 to −100) RANKL for 24 h (a) or 48 h (b). CXCR6-positive staining cells were analyzed by FACS by using anti-CXCR6 antibody. CXCR6 staining profiles are shown in colorful lines indicated on the figure and corresponding isotype control IgG2b profiles are superimposed, (B) RAW264.7 cells were treated as in (A–a), the percentage of CXCR6$^+$ cells by FACS was quantified, (C) total CXCR6 protein was reduced by RANKL. RAW264.7 cells were treated with RANKL (100 ng/ml) for 24 h. The cell lysates were used for analysis of CXCR6 protein expression by Western blot. 40 μg protein was loaded for each lane. ß-actin was used as loading control and (D) RANKL increased osteoclastogenesis-related gene expression in RAW264.7 cells by RT-PCR. GAPDH was used as loading control $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

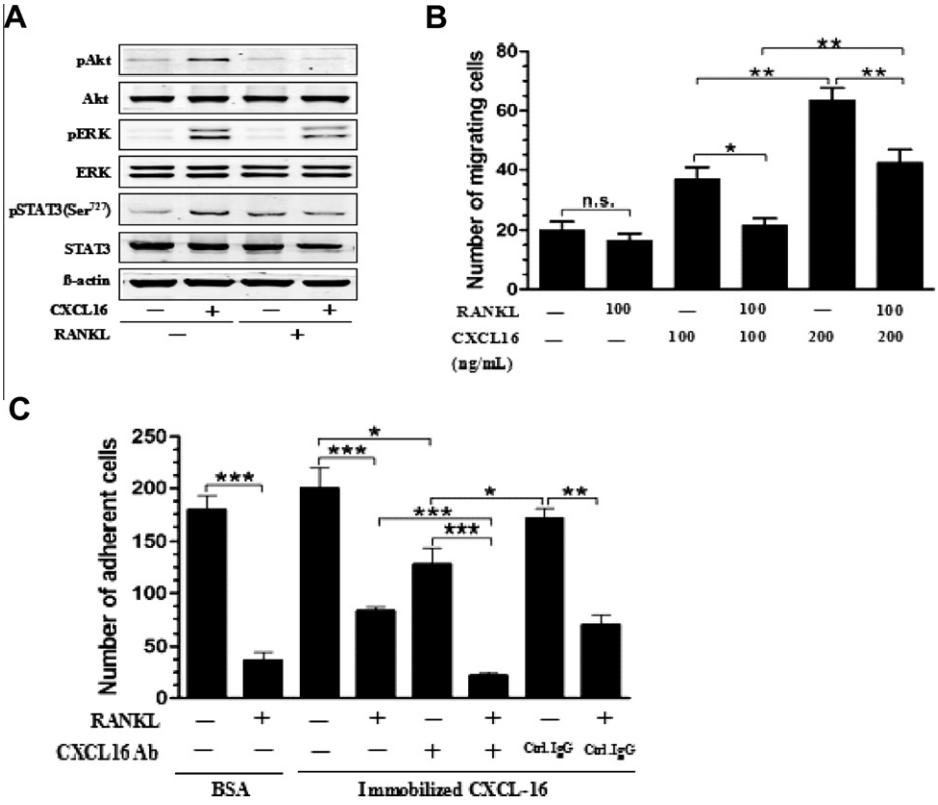

Fig. 2. RANKL disrupts CXCL16-mediated kinase signal activation. (A) RAW264.7 cells were treated with or without RANKL (100 ng/ml) for 24 h, followed by CXCL16 (100 ng/ml) for 30 min. Whole cell lysates were used for Western blot. 40 μg protein was loaded for each lane. ß-Actin was used as loading control, (B) RANKL inhibits CXCL16-mediated cell migration. RAW264.7 cells were cultured for 48 h with or without RANKL, 1.0×10^5 cells/100 μL were added in each upper compartment of Trans-well chamber. The lower compartments contained 600 μL medium with or without 100 or 200 ng/ml CXCL16. After incubation for 12 h, cells migrated to the lower surface were stained with hematoxylin and eosin and cell numbers were counted and (C) RAW264.7 cells were treated with or without RANKL as in (B). The cells were washed and added to the wells precoated with CXCL16 protein. 1 h later, bound cells were counted. The concentration for rat anti-CXCL16 mAb and control rat IgG were used 10 μg/ml. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. n.s., not significant.

expression in RAW264.7 cells, we treated the cells with different kinase inhibitors, including phosphatidylinositol 3-kinase (PI3K/Akt) inhibitor LY294002 (20 μM), MEK-1/MAPK inhibitor PD98059 (20 μM) and JAK2/STAT3 inhibitor AG490 (50 μM). Osteoclast precursor cells were preincubated for 1 h in the presence of the different signal inhibitors and stimulated with RANKL (100 ng/ml) for 24 h. Expression of cell surface CXCR6 was measured by flow cytometric analysis. As shown in Fig. 3A–a, b and B, inhibition of PI3K and MEK1 activities had no effect on RANKL-mediated reduction of CXCR6. In contrast, inhibition of the activity of JAK2/STAT3 pathway by AG490 almost completely restored CXCR6 level (Fig. 3A–c). The data from 48 h treatment showed the similar change (Fig. 3A, d–f). This is in line with the fact that RANKL-mediated CXCR6 reduction requires activation of STAT3 by phosphorylation (Fig. 2A). Western Blot analysis further confirmed the re-expression of CXCR6 by combination of RANKL and AG490, meanwhile treatment with RANKL alone only activated STAT3 phosphorylation at Ser^{727}, but not Tyr^{705} in RAW264.7 cells (Fig. 3C). These data indicate that RANKL-mediated downregulation of CXCR6 depends on JAK2/STAT3 activation.

3.4. CXCL16 did not augment RANKL-induced osteoclastogenesis

We have provided evidence that RANKL attenuates CXCL16 cellular response in RAW264.7 cells, but whether CXCL16 has direct effect on osteoclastogenesis or synergetic effect on RANKL-induced differentiation remains unclear. In the presence of M-CSF, RANKL strongly induced RAW264.7 cell differentiation to osteoclast precursors as measured by TRAP staining assay (Fig. 4A–b), while CXCL16 did not show any visible effect either used alone (Fig. 4A–c). Co-incubated CXCL16 with RANKL did not change RANKL-induced osteoclastogenesis (Fig. 4A–d). TRAP positive multinucleated cell counting further confirmed these observations (Fig. 4B). As expected, CXCL16 did not change RANRL-receptor RANK mRNA expression (Fig. 4C), which may explain, at least in part, the failure of induction of osteoclastogenesis by CXCL16.

4. Discussion

Chemokines activate receptors, members of a large family of seven transmembrane G-coupled proteins, and play primary roles in controlling the trafficking of leukocytes and other types of cells during inflammation. Up-regulation of chemokine receptors is an important inducible response, commonly observed under physiological and/or pathological conditions. For example, tumor microenvironment prompt the up-regulation of CXCR4 and CXCR6 in human prostate cancer cells, and then their respective ligands mediate the directional migration of cancer cells into specific organs, such as bone [17]. CXCR6 may also participate in the recruitment of memory T cells to the sites of inflammation [18]. However, little is known about the regulation of CXCR6 in osteoclastogenesis.

Previous study showed CXCR6 expression on T cells was declined by activation with monoclonal antibodies to CD3 and CD28 which mimics physiological activation via the TCR and co-stimulatory signals delivered by APCs [19]. Interestingly, the expression of CXCR6 on differentiating osteoclasts is down-regulated in a dose-dependent manner by RANKL, which is

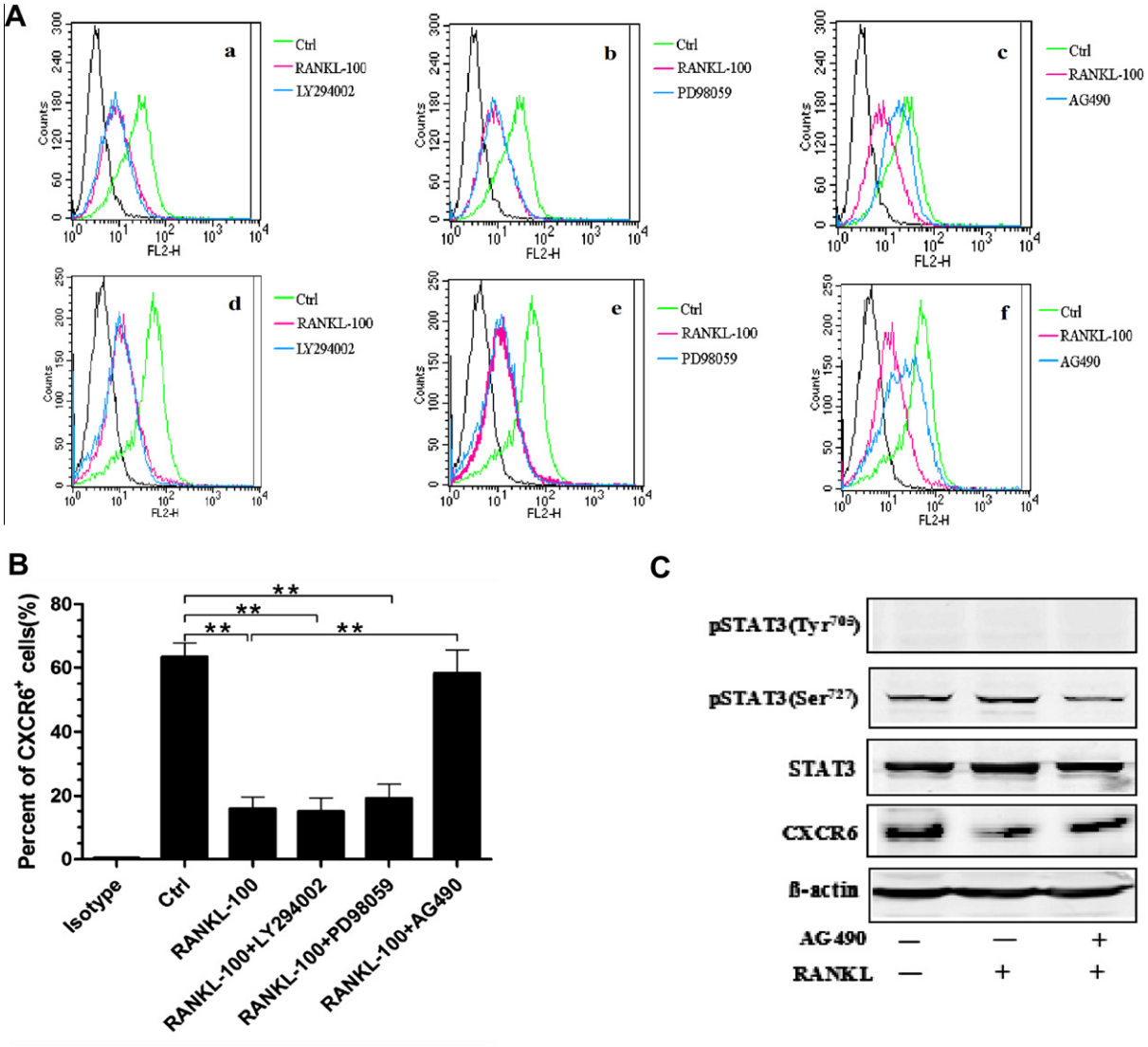

Fig. 3. Deactivation of JAK2/STAT3 signaling prevents RANKL-mediated CXCR6 reduction during osteoclastogenesis in RAW264.7 cells. (A) RAW264.7 cells were pretreated with PI3K/Akt inhibitor LY294002 (a, d), MAPK inhibitor PD98059 (b, e), or JAK2/STAT3 inhibitor AG490 (c, f) and Ctrl (dimethyl sulfoxide, DMSO) for 1 h, followed by RANKL (100 ng/ml) stimulation for 24 h (a, b, c) or 48 h (d, e, f). Membrane CXCR6-positive cells were quantified by flow cytometry analysis. The black line in the figure (A) denotes the isotype control, (B) bar graphs showed the proportion of $CXCR6^+$ cells when RAW264.7 cells were treated with different kinase inhibitors for 24 h and (C) RAW264.7 cells were cultured in the presence or absence of 50 μM AG490 for 1 h and followed by treatment of 100 ng/ml RANKL for 24 h. 40 μg of cell lysates were used for Western blot in analysis of CXCR6 protein level, phospho-STAT3-Ser^{727} and -Tyr^{705} and total STAT3. $^{**}P < 0.01$.

paralleled to high levels of TRAP and cathepsin-K expression during *in vitro* osteoclastogenesis in RAW264.7 cells. This is consistent with the data from another chemokine receptor CX3CR1 which was shown to decrease in expression by RANKL stimulation [20]. In general, the ligation of chemokine receptors by their cognate chemokine causes the rapid internalization of the receptor/ligand complex via endocytosis, which contributes to the reduction of cell surface receptor [21,22]. As we observed, total CXCR6 was also reduced after RANKL treatment, therefore, the decline of surface CXCR6 by RANKL should be a result of reduction in the total cellular CXCR6 pool rather than redistribution of CXCR6.

In order to figure out the potential functional significance of reduction of CXCR6 by RANKL, we tested the activation of some kinase signaling pathways, such as PI3k/Akt, MEK1, JAK2/STAT3, by CXCL16 and whether RANKL changes the activation. Despite MAP kinase signaling cascade is essential for maintenance the properties of multinucleated and matured osteoclasts, and consequent induction of osteoclastic bone resorption [23], our results showed that RANKL does not function through this pathway and the treatment of MEK1 inhibitor does not rescue the decline of CXCR6 by RANKL. Intriguingly, CXCL16 activates Akt and STAT3 pathways by increase in phosphorylation, and this effect was completely blocked by RANKL. It is noteworthy that RANKL increases Sphospho-STAT3 (Ser^{727}), but not phospho-Akt and phosphor-Erk1/2. The importance of this activation is further confirmed by using STAT3 inhibitor AG490, which prevents the reduction of CXCR6 expression induced by RANKL during osteoclastogenesis. We also found that RANKL selectively activates the phosphorylated form of STAT3 on $Serine^{727}$ but not Tyr^{705}-phosphorylated STAT3 (Fig. 3C). This result is in agreement with the previous study showing an increase in base level of Ser^{727}-phospho-STAT3 in osteoclast and AG490 completely abolished RANKL-induced osteoclastogenesis [24]. Our results clearly indicate the necessary reduction of CXCR6 during RANKL-mediated osteoclastogenesis.

Consistent with the above results, we found that RANKL stimulated RAW264.7 cells represses CXCL16-induced cellular responses. During osteoclast differentiation, osteoclast precursors, which existed in bone marrow or peripheral blood, should migrate

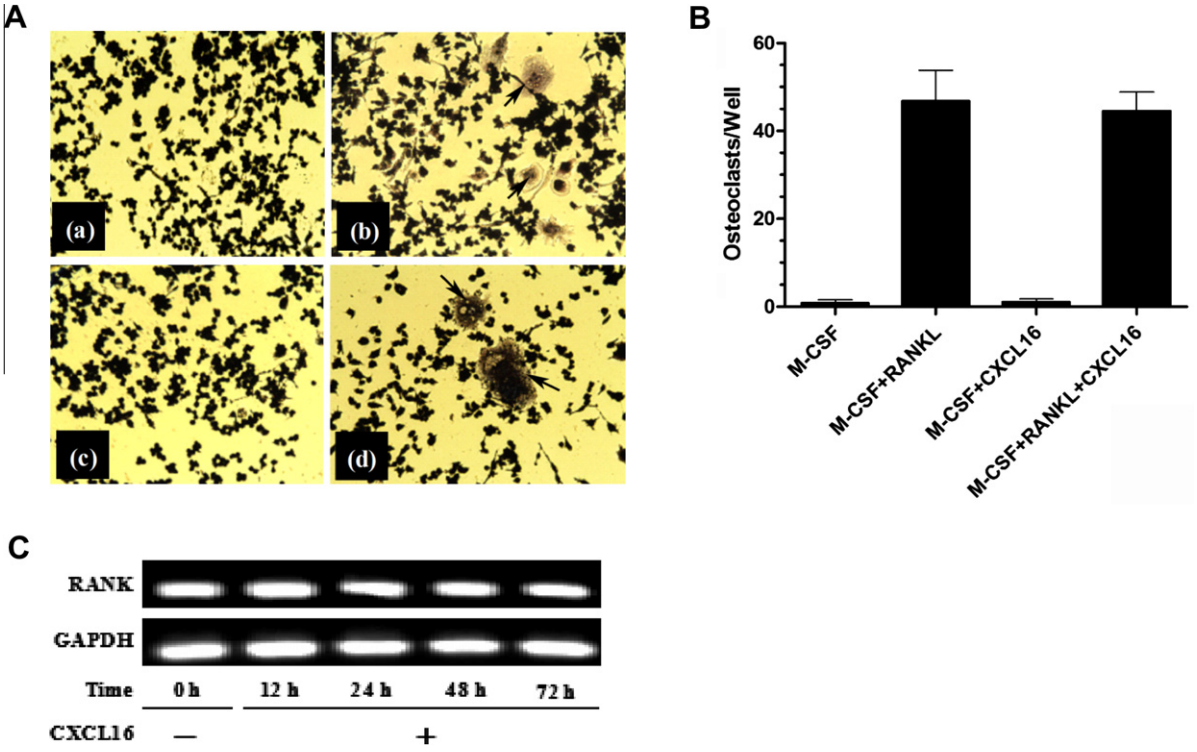

Fig. 4. CXCL16 does not augment RANKL-induced osteoclastogenesis in RAW264.7 cell. (A) RAW264.7 cells were cultured in the presence of recombinant M-CSF (10 ng/ml; a) with RANKL (100 ng/ml; b), CXCL16 (100 ng/ml; c), or M-CSF (10 ng/ml) + RANKL (100 ng/ml) + CXCL16 (100 ng/ml) (d) for 5 days. TRAP staining was performed. Arrows indicated TRAP positive osteoclasts. Experiments were performed independently at least three times (original magnification, ×100), (B) $TRAP^+$ multinucleated cells were counted under light microscope and (C) RANK mRNA expression was quantified by RT-PCR. RAW264.7 cells were cultured in the presence of recombinant CXCL16 for 0–72 h. GAPDH served as the loading control.

and closely adhere to osteoblasts which expresses RANKL. As shown here, although soluble CXCL16 drastically increased cell migration, osteoclast precursor cells were almost completely eliminated its migratory response by pretreatment with RANKL. Given that chemokine CXCL16 has dual functions in not only migration but also integrin-independent firm adhesion toward CXCR6-expressing cells [11], therefore, the RANKL-induced CXCR6 reduction in our study also mediates obvious decrease in the adhesive capacity of cells. In this context a better comprehension is that mature osteoclasts may need to reverse the adhesion to osteoblasts and to rustle in the bone matrix to accomplish bone resorption. Therefore, the RANKL-induced down-modulation of CXCR6 may imply a novel mechanism for releasing osteoclast from the osteoblast equipped with the firm adherent property through CXCL16. Obviously, further studies are necessary to elucidate the exact role of the CXCL16-CXCR6 axis in the osteoclast differentiation and bone resorption.

As it was unclear whether CXCL16 was indeed essential for osteoclast development *in vitro*, TRAP staining assay was performed to detect the effect of CXCL16 alone treatment or combined with RANKL in RAW264.7 cells. The results implied that the soluble CXCL16 did not directly induce differentiation of osteoclast precursors, and also did not enhance RANKL-induced osteoclastogenesis via regulating RANK expression. Thus, we proposed that chemokine, like the inflammatory cytokine, such as IL-6 or TNF-α, might mediate osteoclastogenesis in an indirect manner via up-regulating RANKL expression in osteoblasts. This potential effect of CXCL16 should be investigated further.

Taken together, our data indicated cell surface CXCR6 reduction was nearly reversed by inhibition of JAK2/STAT3 signaling pathway in osteoclast precursor cells. Thus, the decreased CXCL16–CXCR6 interaction may play a potential role in osteoclastogenesis.

Acknowledgments

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References

[1] W.J. Boyle, W.S. Simonet, D.L. Lacey, Osteoclast differentiation and activation, Nature 423 (2003) 337–342.
[2] T. Suda, N. Takahashi, N. Udagawa, E. Jimi, M.T. Gillespie, T.J. Martin, Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families, Endocrinol. Rev. 20 (1999) 345–357.
[3] M. Asagiri, H. Takayanagi, The molecular understanding of osteoclast differentiation, Bone 40 (2007) 251–264.
[4] S. Gronthos, A.C. Zannettino, The role of the chemokine CXCL12 in osteoclastogenesis, Trends Endocrinol Metab. 18 (2007) 108–113.
[5] M. Yang, G. Mailhot, C.A. MacKay, A. Mason-Savas, J. Aubin, P.R. Odgren, Chemokine and chemokine receptor expression during colony stimulating factor-1-induced osteoclast differentiation in the toothless osteopetrotic rat: a key role for CCL9 (MIP-1) in osteoclastogenesis *in vivo* and *in vitro*, Blood 107 (2006) 2262–2270.
[6] N. Ishida, K. Hayashi, A. Hattori, K. Yogo, T. Kimura, T. Takeya, CCR1 acts downstream of NFAT2 in osteoclastgenesis and enhances cell migration, J. Bone Miner. Res. 21 (2006) 48–57.
[7] M. Matloubian, A. David, S. Engel, J.E. Ryan, J.G. Cyster, A transmembrane CXC chemokine is a ligand for HIV-coreceptor bonzo, Nat. Immunol. 1 (2000) 298–304.
[8] T. Shimaoka, N. Kume, M. Minami, K. Hayashida, H. Kataoka, T. Kita, Molecular cloning of a novel scavenger receptor for oxidized low density lipoprotein SR-PSOX on macrophages, J. Biol. Chem. 275 (2000) 40663–40666.
[9] A. Wilbanks, S.C. Zondlo, K. Murphy, S. Mak, D. Soler, P. Langdon, Expression cloning of the STRL33/BONZO/TYMSTR ligand reveals elements of CC, CXC, and CX3C chemokines, J. Immunol. 166 (2001) 5145–5154.
[10] J.F. Bazan, K.B. Bacon, G. Hardiman, W. Wang, K. Soo, D. Rossi, D.R. Greaves, A. Zlotnik, T.J. Schall, A new class of membranebound chemokine with a CX3C motif, Nature 385 (1997) 640–644.

[11] T. Shimaoka, T. Nakayama, N. Fukumoto, N. Kume, S. Takahashi, J. Yamaguchi, Cell surface-anchored SR-PSOX/CXC chemokine ligand 16 mediates firm adhesion of CXC chemokine receptor six-expressing cells, J. Leukocyte Biol. 75 (2004) 267–274.
[12] L. Deng, N. Chen, Y. Li, H. Zheng, Q. Lei, CXCR6/CXCL16 functions as a regulator in metastasis and progression of cancer, Biochim. Biophys. Acta 2010 (1806) 42–49.
[13] W. Hu, X. Zhen, B. Xiong, B. Wang, W. Zhang, W. Zhou, CXCR6 is expressed in human prostate cancer *in vivo* and is involved in the in vitro invasion of PC3 and LNCap cells, Cancer Sci. 99 (2008) 1362–1369.
[14] M. Heydtmann, D.H. Adams, Understanding selective trafficking of lymphocyte subsets, Gut 50 (2002) 150–152.
[15] S.K. Kwok, M.L. Cho, M.K. Park, H.J. Oh, J.S. Park, Y.M. Her, S.Y. Lee, J. Youn, J.H. Ju, K.S. Park, S.I. Kim, H.Y. Kim, S.H. Park, Interleukin-21 promotes osteoclastogenesis in humans with rheumatoid arthritis and in mice with collagen-induced arthritis, Arthritis Rheum. 64 (2012) 740–751.
[16] H.B. Kwak, H.S. Kim, M.S. Lee, K.J. Kim, E.Y. Choi, M.K. Choi, J.J. Kim, H.J. Cho, J.W. Kim, J.M. Bae, Y.K. Kim, B.H. Park, H. Ha, C.H. Chun, J. Oh, Pyridone six, a pan-janus-activated kinase inhibitor suppresses osteoclast formation and bone resorption through down-regulation of receptor activator of nuclear factor-k B (NF-k B) ligand (RANKL)-induced c-Fos and nuclear factor of activated T cells (NFAT) c1 expression, Biol. Pharm. Bull. 32 (2009) 45–50.
[17] A. Müller, B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S.N. Wagner, J.L. Barrera, A. Mohar, E. Verástegui, A. Zlotnik, Involvement of chemokine receptors in breast cancer metastasis, Nature 410 (2001) 50–56.
[18] C.H. Kim, E.J. Kunkel, J. Boisvert, Bonzo/CXCR6 expression defines type 1-polarized T-cell subsets with extra lymphoid tissue homing potential, J. Clin. Invest. 107 (2001) 595–601.
[19] S. Koprak, S. Matheravidathu, M. Springer, S. Gould, F.J. Dumont, Down-regulation of cell surface CXCR6 expression during T cell activation is predominantly mediated by calcineurin, Cell Immunol. 223 (2003) 1–12.
[20] Y. Saitoh, K. Koizumi, H. Sakurai, T. Minami, I. Saiki, RANKL-induced down-regulation of CX3CR1 via PI3K/Akt signaling pathway suppresses Fractalkine/CX3CL1-induced cellular responses in RAW264.7 cells, Biochem. Biophys. Res. Commun. 364 (2007) 417–422.
[21] A. Mueller, E. Kelly, P.G. Strange, Pathways for internalization and recycling of the chemokine receptor CCR5, Blood 99 (2002) 785–791.
[22] A.Z. Fernandis, R.P. Cherla, R.D. Chernock, R.K. Ganju, CXCR4/CCR5 down-modulation and chemotaxis are regulated by the proteasome pathway, J. Biol. Chem. 277 (2002) 18111–18117.
[23] T. Miyazaki, H. Katagiri, Y. Kanegae, H. Takayanagi, Y. Sawada, A. Yamamoto, M.P. Pando, T. Asano, I.M. Verma, H. Oda, K. Nakamura, S. Tanaka, Reciprocal role of ERK and NF-kB pathway in survival and activation of osteoclasts, J. Cell Biol. 148 (2000) 333–342.
[24] L. Duplomb, M. Baud'huin, C. Charrier, M. Berreur, V. Trichet, F. Blanchard, Interleukin-6 inhibits receptor activator of nuclear factor B ligand-induced osteoclastogenesis by diverting cells into the macrophage lineage: key role of Serine 727 phosphorylation of signal transducer and activator of transcription 3, Endocrinology 149 (2008) 3688–3697.