IL-6 Enhances Osteocyte-Mediated Osteoclastogenesis by Promoting JAK2 and RANKL Activity *In Vitro*

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**Key Words**
Surgery first • Interleukin-6 • IL-6 receptor • Osteocyte • Receptor activator of nuclear factor-κB ligand • AG490 • Janus kinase • Signal transducer and activator of transcription • Osteoclastogenesis

**Abstract**
**Background/Aims:** Evidence suggests that IL-6 affects bone mass by modulating osteocyte communication towards osteoclasts. However, the mechanism by which IL-6 enhances osteocyte-mediated osteoclastogenesis is unclear. We aimed to investigate the inflammatory factors in serum after orthodontic surgery and their relationship between osteocytes and osteoclasts.

**Methods:** Serum was obtained from 10 orthodontic surgery patients, and inflammatory factors were detected by ELISA. We treated the osteocyte-like cell line MLO-Y4 with recombinant mouse IL-6 and IL-6 receptor (IL-6R), and used quantitative RT-PCR and Western blotting to explore Receptor activator of nuclear factor-κB ligand (RANKL) expression at both the mRNA and protein level. MLO-Y4 cells were co-cultured with osteoclast precursor cells, and the formation of osteoclasts was detected by tartrate-resistant acid phosphatase (TRAP) staining. To explore the role of JAK2 in the osteocyte-mediated osteoclastogenesis, AG490, a JAK2 inhibitor, was used to inhibit the JAK2-STAT3 pathway in osteocytes.

**Results:** In our study, we found that IL-6 and RANKL were stimulated in serum 3-7 days after orthognathic surgery. Therefore, IL-6 and IL-6 receptor enhanced the expression of RANKL at both the mRNA and protein level in MLO-Y4. Furthermore, when MLO-Y4 cells were co-cultured with osteoclast precursor cells, it significantly stimulated osteoclastic differentiation and the formation of TRAP-positive multinucleated cells after stimulation with IL-6 and IL-6R. Our results also indicated that treatment with IL-6 and IL-6R increased RANKL mRNA expression and the RANKL/OPG expression ratio. Meanwhile, the phosphorylation of Janus kinase 2 (JAK2) and Signal transducer and activator of transcription (STAT3) also correlated with RANKL levels. Furthermore, we investigated the effects of a specific JAK2 inhibitor, AG490, on the expression of RANKL in osteocyte-like MLO-Y4 cells and osteocyte-mediated osteoclastogenesis. The results showed that AG490 inhibited (p)-JAK2 and RANKL expression. Osteoclastic differentiation was...
decreased after pretreatment in MLO-Y4 with mouse IL-6/IL-6R and AG490; therefore, we concluded that IL-6 increased osteocyte-mediated osteoclastic differentiation by activating JAK2 and RANKL. **Conclusion:** The effects of IL-6/IL-6R and AG490 on osteocyte-mediated osteoclastogenesis contribute to our understanding of the role of inflammatory factors in the interaction between osteocytes and osteoclast precursors. IL-6 and RANKL are key factors for bone remodelling after the orthodontic surgery, and their roles in bone remodelling may be fundamental mechanisms accelerating tooth movement by orthodontic surgery.

**Introduction**

A previous study confirmed that IL-6, TNF-α (Tumour Necrosis Factor α), and IL-1β (Interleukin-1β) contribute to bone remodelling in the early stage during fracture healing, and activated bone remodelling also appears after fracture surgery [1]. Bone remodelling is maintained by osteoblasts, osteocytes, and osteoclasts, which are regulated by inflammatory factors and hormones [2]. IL-6 has been shown to stimulate RANKL production in osteoblastic cells with the activation of STAT3 being required [3], and IL-4 can inhibit RANKL expression on macrophages [4]. Among the inflammatory factors, the IL-6 family is closely related with osteocytes, osteoblasts, and osteoclasts. Therefore, we aimed to investigate the inflammatory factors in serum after orthodontic surgery, and their relationship with osteocytes.

Bone metabolism is regulated by osteoclasts, osteoblasts, and osteocytes, and osteocytes comprise approximately 90–95% of the cells in bone tissue [5]. In addition, osteoblasts regulate bone formation and can differentiate into osteocytes, which can secrete Macrophage colony-stimulating factor (M-CSF), RANKL, and osteoprotegerin (OPG) in the bone matrix, regulating osteoclast formation and influencing bone resorption [6]. In recent years, osteocytes were shown as one source of RANKL, and they play an important role in osteoclast formation. Osteocytes regulate osteoblast and osteoclast activity and have been found to act as a key factor of bone remodeling [5, 7, 8]. IL-6 is thought to be the ‘classic’ bone-resorbing pro-inflammatory cytokine. Researchers found that IL-6 could stimulate osteoclast formation not only by contacting osteoclast precursors but also by stimulating osteoblast lineage to express RANKL to enhance osteoclastogenesis [9]. However, limited information is available on the effect of IL-6 to osteocytes. Thus, osteocytes and their osteoclastogenic role under the effect of IL-6 require further study.

IL-6 could induce bone resorption and directly increase osteoclast formation in periodontal disease and rheumatoid arthritis [10, 11]. The combination of IL-6/IL-6R and gp130 can activate Janus activated kinase (JAK). JAK induces the phosphorylation of signal transducer and activator of transcription 3 (STAT3), and RANKL induced by IL-6/IL-6R requires the activation of STAT3 [12]. IL-6R is involved in many important cellular functions and has been reported to utilize “trans-signalling”. Membrane-bound IL-6Rs are expressed on leukocytes and hepatocytes, but they are present at low expression levels on osteoblasts and fibroblasts [13]. Furthermore, previous study shows that RANKL expression is increased upon the activation of JAK2 and STAT3 in fibroblast-like synoviocytes [14].

AG490 is a JAK2 inhibitor, but it has not been investigated in osteocyte-mediated osteoclast differentiation [15]. AG490 is a derivative of benzylidene malononitrile and inhibits the activity of JAK. Therefore, by using AG490, we investigated whether IL-6 affects osteocytes through JAK2 and RANKL activation to improve our understanding of the role of the JAK2/STAT3 pathway in modulating the expression of RANKL and osteocyte-mediated osteoclastogenesis.

In our study, we hypothesized that IL-6 affects osteocytes and osteocyte-mediated osteoclastogenesis through the JAK2-STAT3 signalling pathway. Additionally, by down-regulating (p)-JAK2 with AG490, we aimed to understand the mechanism by which IL-6 interacts with osteocytes and osteoclast precursors. The results of this study may be beneficial to our interpretation that orthognathic surgery accelerates tooth movement.
Materials and Methods

Patients
Serum was obtained from 10 orthognathic surgery patients. The experimental protocol was approved by the human ethics committees of the School of Stomatology and Tongji University Hospital (No. 2016004). Informed consent was obtained from the patients at the time of study enrolment. All human material was obtained with informed written consent from the donor, as required and approved by the ethics committees.

Reagents
Rat tail collagen type I was purchased from Gibco BRL (Grand Island, NY, USA); FBS and calf serum (CS) were purchased from HyClone Laboratories, Inc. (Logan, UT, USA). Anti-RANKL, JAK2, (p)-JAK2, STAT3, (p)-STAT3, anti-actin antibodies, an ELISA kit for mouse RANKL, and IL-6 were purchased from Abcam (Cambridge, UK). Recombinant mouse (rm) IL-6 and rmIL-6R were purchased from R&D Systems (Minneapolis, MN, USA). AG490 were obtained from Calbiochem (Schwalbach, Germany).

Cell culture
MLO-Y4 cells served as the cell model for osteocytes and were provided by Dr. Lynda Bonewald (University of Missouri-Kansas City, KS). MLO-Y4 cells were cultured on rat-tail collagen coated glass plates in α-MEM supplemented with 2.5% foetal bovine serum, 2.5% calf serum, and 1% penicillin/streptomycin. RAW264.7 monocyte cells were purchased from the Cell Bank of the Chinese Academy of Medical Sciences and served as the cell model for the osteoclast precursors and were cultured in DMEM supplemented with 10% FBS and 1% P/S. Cells were sub-cultured when they reached 80-90% confluence.

RT-PCR
MLO-Y4 cells were treated with 0, 10, 30, 50, or 100 ng/ml recombinant mouse IL-6 and IL-6R for 24 h or 0, 30, 50, and 100 μM AG490. TRIzol reagent was used for RNA extraction. The cDNA was synthesized using a Transcriptor First Strand cDNA Synthesis Kit and stored at -20°C until the real-time PCR analysis. The expression of RANKL mRNA was quantified by RT-PCR. PCR reactions were repeated three times. GAPDH was used for normalization, and mRNA expression was calculated using the 2-ΔΔCt method.

The primer sequences were as follows: RANKL: forward, 5'-GTCGCTTCGGCCAGTGTG-3', reverse, 5’-GGAAAGGCAAGTCCAGAGGTG-3'; OPG forward, 5'-GAGGAGTCTGGTAGTGGTTCC-3', reverse, 5’-GGGCGTTTCGTTGAATATGCG-3'; and GAPDH: forward, 5’-TGGCACCCAGCACAATGAA-3', reverse, 5’-CTAAGTCATAGTCCGCCTAGAAGCA-3’.

Western blot analysis
MLO-Y4 cells were treated as above. The cells were washed twice with PBS and then harvested in RIPA buffer on ice. The supernatants were collected and stored at -80°C after centrifugation at 12,000 rpm at 4°C for 10 min. Protein contents were detected using a BCA protein assay kit (Thermo, USA). Then, samples containing 25 μg of protein were separated on 8% and 12% SDS-polyacrylamide gels and transferred onto PVDF membranes (Sigma, USA). The PVDF membranes were incubated for 1 h with BSA to block non-specific binding. Then, the membranes were incubated at 4°C overnight with antibodies against phosphorylated (p)-JAK2 (Tyr1007), or JAK2, and phosphorylated (p)-STAT3 (Y705), or STAT3, RANKL, SOCS3, and β-actin. The membranes were washed three times with TBST for 10 min, incubated with the secondary antibody against rabbit at 37°C for 1 h, and then washed three times with TBST for 10 min. Each sample was also probed with an anti-β-actin antibody. The band intensity was analysed using the SmartChem™ Image Analysis System (Sagecreation, China). The results were reproduced in three independent experiments using different samples.

ELISA
For IL-6, RANKL analysis, serum samples were obtained from 10 orthognathic surgery patients and stored at -80°C; standards were assayed in duplicate. The values were normalized to the total protein content.
Co-culture and TRAP staining

MLO-Y4 cells were pre-incubated with 0, 10, 30, 50, or 100 ng/ml recombinant mouse IL-6 and IL-6R for 24 h or with 0, 30, 50, or 100 μM AG490. Subsequently, RAW264.7 cells were seeded on top of the rmIL-6/rmIL-6R-treated osteocytes. The cells were plated at 2000 cells/well in 24-well plates in α-MEM containing 10% foetal bovine serum (FBS). The medium was replenished every 2 days. After 7 days, the cells were stained for tartrate-resistant acid phosphatase (TRAP) using a TRAP staining kit (387A, SIGMA, USA). TRAP-positive multinuclear cells that possessed more than 3 nuclei were counted as osteoclasts. In each section, five fields near the bone crest region were randomly selected at 200X magnification, and the TRAP-positive cells in these fields were counted by two blinded observers.

Statistical analysis

Statistical analyses were performed using SPSS 20.0 and GraphPad Prism 6.0. All quantitative data were expressed as the mean ± SD for each condition. For ELISA data, variance analysis of single factor repeated measures was performed. For comparisons between groups, one-way analysis of variance (ANOVA) was performed. Western blots were quantified by Image J. P values < 0.05 were considered statistically significant.

Results

The expression of IL-6 and RANKL were significantly increased 3-7 days after orthognathic surgery

The patient clinical characteristics were as follows: age 20-30 years, no potential systematic diseases, and underwent bimaxillary surgery. To examine changes of inflammatory factors in serum after orthognathic surgery, the expression of IL-6 and RANKL were measured by ELISA. The results indicated that the expression of IL-6 and RANKL reach their peak after 3-7 days and then decreased gradually (p<0.01, Fig. 1).

IL-6 enhances osteoclastogenesis through the upregulation of RANKL expression in MLO-Y4 cells

RAW264.7 cells can differentiate into TRAP-positive multinucleated OCs in the presence of RANKL [16]. RAW264.7 cells were co-cultured with MLO-Y4 cells pre-treated with recombinant mouse IL-6 and IL-6R (rmIL-6 and rmIL-6R), and TRAP-positive multinucleated cells were counted after 10 days. To explore the effect of IL-6 on osteocytes, we used increasing concentrations of recombinant mouse IL-6 and IL-6R (0, 10, 30, 50, and 100 ng/ml) and observed that MLO-Y4 cells promoted osteoclast differentiation in a dose-dependent manner. The results showed that the MLO-Y4 cells pre-treated with 50 ng/ml and 100 ng/ml of rmIL-6 and rmIL-6R could efficiently stimulate the differentiation of osteoclast precursors (Fig. 2A, 2B)

Fig. 1. The expression of IL-6 and RANKL after orthognathic surgery in patient serum. A, B) The levels of IL-6 and RANKL in patient serum after orthognathic surgery; preoperative, 3d, 7d, 14d, 28d were observed.
To understand the mechanisms by which IL-6/IL-6R promoted osteoclast differentiation in the co-culture conditions with MLO-Y4 cells, we used different methods to test RANKL expression and activity. Fig. 2 shows the effect of IL-6/IL-6R on the differentiation of osteoclasts in co-cultures with MLO-Y4 cells. A) Co-cultures of RAW264.7 and MLO-Y4 cells pre-stimulated with IL-6/IL-6R to induce OC differentiation. MLO-Y4 cells were seeded onto type I collagen coated culture wells and cultured for 24 h in the presence of 10, 30, 50, or 100 ng/ml IL-6 and IL-6R. The cells were then co-cultured with RAW264.7 cells for 7 days. TRAP-positive multinucleated cells, indicated by red arrows, were counted. The results are presented as the mean and standard deviation of three separate experiments (a. blank: RAW264.7 cells cultured alone; b. Con group: MLO-Y4/RAW264.7 + no pretreatment with IL-6 and IL-6R; c. MLO-Y4 pre-treated with 10 ng/ml IL-6 + 10 ng/ml IL-6R; d. 30 ng/ml IL-6 + 30 ng/ml IL-6R; e. 50 ng/ml IL-6 + 50 ng/ml IL-6R; f. 100 ng/ml IL-6 + 100 ng/ml IL-6R). B) TRAP-positive multinuclear cells that possessed more than 3 nuclei were counted as osteoclasts. Data are represented as the mean ± SEM of three independent experiments (bars ± SD, #p>0.05, compared to Con groups, *p<0.05, compared to Con groups, **p<0.01, compared to Con groups; scale bar: 50 μm).

Fig. 3. The effect of IL-6 on RANKL mRNA and protein expression in MLO-Y4 cells. Cells were pre-treated with 10, 30, 50, or 100 ng/ml IL-6 and IL-6R for 24 hours. A, B, C) RANKL, OPG mRNA expression and ratio was detected by RT-PCR. The data represent the mean and standard deviation of three separate experiments. D) IL-6/IL-6R activates the expression of RANKL in MLO-Y4 cells, and the activated form of RANKL was determined by Western blot analysis. The data represent one of three independent experiments. E, G) Semi-quantification of the Western blots by Image J, n=3. F) IL-6/IL-6R activate the phosphorylation of JAK2/STAT3 in MLO-Y4 cells, as determined by Western blot analysis. The data represent one of three independent experiments (bars ± SD, *p<0.05 compared to Con group, **p<0.01 compared to Con group).
expression as follows: real-time PCR and Western blot. We determined that RANKL was a key target of the IL-6/IL-6R treatment, and different concentrations of IL-6/IL-6R could stimulate the secretion of RANKL at different levels. After treating MLO-Y4 cells with 50 ng/ml and 100 ng/ml of IL-6/IL-6R, RANKL mRNA and protein increased in a dose-dependent manner (Fig. 3A, 3D, 3E). In addition, the increased expression of OPG and the RANKL:OPG expression ratio after treatment with rmIL-6/rmIL-6R was also shown by real-time PCR (Fig. 3B, 3C). It obviously shows that the expression of OPG had no significant changes, and the RANKL:OPG expression ratio shows similar trends with RANKL. This observation suggested the existence of a relationship between RANKL expression and IL-6/IL-6R.

**IL-6/IL-6R induced RANKL expression via JAK2 and STAT3 activation in MLO-Y4 cells**

The phosphorylation of STAT3 and JAK2 was detected by a Western blot analysis of MLO-Y4 cells treated with rmIL-6/rmIL-6R. Our study indicated that rmIL-6/rmIL-6R activated the phosphorylation of STAT3 and JAK2 in MLO-Y4 cells, and there were no significant differences in JAK2 and STAT3 protein levels. (Fig. 3F, 3G).

In addition, we incubated MLO-Y4 cells with different concentrations (10, 30, 50, and 100 μM) of AG490 and 30 ng/ml of rmIL-6/rmIL-6R for 24 hours. First, the cytotoxicity of AG490 was assessed by the CCK-8 assay, and the results indicated that AG490 at the tested concentrations (10, 30, 50, and 100 μM) had a lesser influence on the MLO-Y4 cells. Second, the expression of RANKL was determined by real-time PCR and a Western blot analysis, and RANKL mRNA and protein expression was decreased after inhibiting JAK2 activity. The expression of OPG has no significant changes, and the RANKL:OPG expression ratio shows similar trends with RANKL (Fig. 4A, 4B, 4C).

![Fig. 4.](image-url)
AG490 inhibits the expression of P-STAT3/P-JAK2/RANKL and decreases the differentiation of osteoclasts co-cultured with MLO-Y4 cells

AG490, a specific JAK2 kinase inhibitor, can counteract the effect of rmIL-6/rmIL-6R and decrease the secretion of RANKL, which could result in the suppression of osteoclastogenesis. After inhibiting JAK2 with AG490, Western blot analysis showed that the expression of RANKL was not increased by the rmIL-6/rmIL-6R treatment (Fig. 4D, 4E). In addition, MLO-Y4 cells were pre-treated with different concentrations of AG490 and 30 ng/mL of rmIL-6/rmIL-6R for 24 hours. Then, RAW264.7 cells were seeded onto the chamber slides. The number of mature osteoclasts was inhibited in the presence of AG490, and this number decreased in a dose-dependent manner (Fig. 5).

Discussion

Our results indicated that the expression of IL-6 and RANKL increased after orthodontic surgery, and subsequently, our study demonstrated that IL-6 enhanced osteocyte-mediated osteoclastogenesis and JAK2 activation in osteocyte-like MLO-Y4 cells and showed the negative effects of AG490 on the expression of RANKL and the osteocyte-mediated osteoclastogenesis. IL-6 stimulates bone remodelling between osteocyte and osteoclast precursors, and it may be one of the fundamental mechanisms accelerating tooth movement by orthodontic surgery.

Increasing evidence indicates that osteocytes are important determinants of bone remodelling, and the activity of IL-6 has recently been identified as a key factor for orthodontic tooth movement and periodontal disease [17]. Some evidence suggests that IL-6 is a mediator of osteoblastic differentiation in PDL (periodontal ligament) cells, as it acts as an osteolytic factor [18]. Furthermore, it has been reported that IL-6 could affect bone mass by influencing osteocyte communication towards osteoblasts [17]. However, a delayed callus formation and reduced osteoclast density appeared in IL-6 knockout mice [19]. IL-6 stimulates bone resorption and osteoclast activity by increasing osteoblast interactions with osteoclasts [20].
Moreover, RANKL plays a key role in bone growth and maintenance, and it is expressed in a variety of cell types, including fibroblasts, lymphocytes and osteocytes [21]. Carbon monoxide suppresses osteoclast differentiation by inhibiting the RANKL-induced activation of PPAR-γ [22]. IL-6 and TNF-α are associated with the expression of RANKL mRNA and protein levels [23]. Importantly, osteocytes express higher amounts of RANKL to support osteoclastogenesis than osteoblasts and bone marrow cells [7]. Therefore, we propose that the effects related to IL-6 are key events for the functionality of osteocyte communication with osteoclasts. In addition, osteoblasts express gp130 and IL-6 receptor-α, and the receptor complex is activated by IL-6 [24]. However, MLO-Y4 osteocyte-like cells express low amounts of IL-6R mRNA, and soluble IL-6R is present in the serum, where it acts as a stimulant to activate IL-6 signalling. Therefore, we supplied IL-6 in the presence of IL-6R for the treatment of MLO-Y4 cells. We confirmed that the treatment with IL-6 and IL-6R increased the expression of RANKL mRNA and protein. Moreover, the downstream effectors of the IL-6 pathway, including P-JAK2 and P-STAT3, also correlated with the RANKL levels. Co-culturing MLO-Y4 cells pre-treated with IL-6/IL-6R and RAW264.7 cells indicated that IL-6/IL-6R promoted osteoclastogenesis through the MLO-Y4 osteocyte-like cells.

In our study, we speculate that the MLO-Y4 cells could replace the osteocytes, and RAW264.7 cells could replace pre-osteoclasts. We used different concentrations of IL-6 and IL-6R for treating MLO-Y4 cells, which produced different levels of RANKL. Co-culture of pre-treated MLO-Y4 cells with RAW264.7 cells promoted osteoclastogenesis in a dose-dependent manner. In vitro reports indicated that RANKL derived from IL-1-stimulated MLO-Y4 cells could enhance MLO-Y4-mediated osteoclastogenesis [25]. Furthermore, there is some evidence that MLO-Y4 cells could secrete large amounts of M-CSF and express RANKL on their surface [26]. However, it has been reported that IL-6 stimulates osteoclastogenesis only by increasing the expression of RANKL by osteoblasts [20]. Moreover, treatment of MLO-Y4 cells with 100 pg/ml IL-6 does not indicate that IL-6 affects osteocyte-to-osteoclast communication [17]. Evidence suggested that membrane-bound RANKL has a crucial role in osteoclastogenesis in the co-culture with osteocytes [6]. Therefore, we tentatively concluded that these observations may be because IL-6 and the expression of RANKL were not sufficient to stimulate osteoclastogenesis.

JAK and STAT play a critical part in bone metabolism. In addition, compared to the other STATs, STAT3 has a broad impact on bone homeostasis. First, to study the role of the JAK2-STAT3 signalling pathway in the expression of RANKL, we used different concentrations of rmIL-6 and rmIL-6R to treat MLO-Y4 cells, and we observed that IL-6 stimulates the expression of (p)-STAT3, (p)-JAK2, and RANKL, which are the downstream effectors of the IL-6-JAK2-STAT3 pathway. Second, we used different concentrations of AG490 to treat the MLO-Y4 cell line. In the presence of AG490, the secretion of RANKL and P-JAK2 levels were reduced. Third, we found that AG490 effectively decreased the formation of multinucleated osteoclasts in co-culture conditions with MLO-Y4 cells in a dose-dependent manner. Furthermore, the expression of OPG has no significant changes, and the RANKL:OPG expression ratio shows similar trends with RANKL. This implies that OPG expression is not associated with IL-6-JAK2. Co-culturing osteocytes with osteoclasts does not increase osteoclast differentiation because the RANKL secreted by the osteocytes is insufficient to stimulate osteoclast differentiation [6]. However, pre-treating MLO-Y4 cells with IL-6 increased the secretion of RANKL, and in contact with the RAW264.7 cells, it strongly increased the number of osteoclasts.

In summary, our study indicated that IL-6 enhanced osteocyte-mediated osteoclastogenesis and JAK2 activation in osteocyte-like MLO-Y4 cells and that AG490 had a negative effect on the expression of RANKL. AG490 inhibits RANKL expression by suppressing JAK2 in MLO-Y4 cells pre-treated with IL-6/IL-6R. At the same time, RANKL expression in MLO-Y4 cells could induce osteoclast differentiation and also suggests a relationship between osteocytes and IL-6. In conclusion, our findings contribute to our understanding of bone remodelling and provide a background for orthodontic treatment.
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Disclosure Statement

No conflict of interest exits in the submission of this manuscript.

References

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