Biochemical and Biophysical Research Communications 435 (2013) 533–539

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

AG490 inhibits NFATc1 expression and STAT3 activation during RANKL induced osteoclastogenesis

Chang-hong Li, Jin-xia Zhao, Lin Sun, Zhong-qiang Yao, Xiao-li Deng, Rui Liu, Xiang-yuan Liu *

Department of Rheumatology and Immunology, Peking University Third Hospital, Beijing 100191, PR China

ARTICLE INFO

Article history:
Received 16 April 2013
Available online 7 May 2013

Keywords:
Osteoclastogenesis
AG490
RANKL
NFATc1
JAK2/STAT3

ABSTRACT

Commonly, JAK/STAT relays cytokine signals for cell activation and proliferation, and recent studies have shown that the elevated expression of JAK/STAT is associated with the immune rejection of allografts and the inflammatory processes of autoimmune disease. However, the role which JAK2/STAT3 signaling plays in the receptor activator of nuclear factor-κB ligand (RANKL)-mediated osteoclastogenesis is unknown. In this study, we investigated the effects of AG490, specific JAK2 inhibitor, on osteoclast differentiation *in vitro*. AG490 significantly inhibited osteoclastogenesis in murine osteoclast precursor cell line RAW264.7 induced by RANKL. AG490 suppressed cell proliferation and delayed the G1 to S cell cycle transition. Furthermore, AG490 also suppressed the expression of nuclear factor of activated T cells (NFAT) c1 but not c-Fos in RAW264.7. Subsequently, we investigated various intracellular signaling components associated with osteoclastogenesis. AG490 had no effects on RANKL-induced activation of Akt, ERK1/2. Interestingly, AG490 partly inhibited RANKL-induced phosphorylation of Ser^{727} in STAT3. Additionally, down-regulation of STAT3 using siRNA resulted in suppression of TRAP, RANK and NFATc1 expression. In conclusion, we demonstrated that AG490 inhibited RANKL-induced osteoclastogenesis by suppressing NFATc1 production and cell proliferation via the STAT3 pathway. These results suggest that inhibition of JAK2 may be useful for the treatment of bone diseases characterized by excessive osteoclastogenesis.

1. Introduction

In life, bone remodeling continuously regulates the skeletal mass and quality through spatially coordinated balance between bone-synthesizing osteoblasts and bone-absorbing osteoclasts. The perturbation of this balance between the two cell types usually leads to bone diseases, such as rheumatoid arthritis and osteoporosis, which are characterized by excessive activity of osteoclasts [1,2]. Multinucleated mature osteoclasts are derived from hematopoietic mononuclear progenitors following a sequential process which includes cell proliferation, differentiation, fusion and activation [2,3]. Two essential cytokines, macrophage-colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) produced by osteoblasts, could control osteoclast formation from monocyte/macrophage lineage [4]. Binding of RANKL to its receptor RANK on the surface of osteoclast precursor cells activates NF-κB, nuclear factor of activated T cells (NFAT) c1, c-Fos, and several other transcription factors, which are required for osteoclast differentiation, activation and survival [5,6]. Ectopic expression of NFATc1, a key modulator of osteoclastogenesis, can cause precursor cells to undergo efficient differentiation in the absence of RANKL signaling, and induces the up-regulation of diverse osteoclast associated genes, such as tartrate-resistant acid phosphatase (TRAP) [7].

The Janus kinases (JAKs) family is composed of four members (JAK1, JAK2, JAK3 and Tyk2) that transduce signals from multiple cytokine receptors and mediate various cell responses. Upon ligation of cytokine receptors, JAK kinases phosphorylate signal transducers and activators of transcription (STAT) proteins, which then translocate to the nucleus and activate numerous genes transcription [8–10]. Recently, the elevated activation of JAK and STAT, which results in abnormal cell proliferation, has been found in diverse malignant diseases [11,12]. And there are increasing evidences indicating that switching off the JAK/STAT signaling may be a potential therapy target for certain malignancies. In addition, inhibitor of the JAK/STAT pathway has also been used as a novel immunosuppressant for the treatment of autoimmune diseases, including rheumatoid arthritis and graft versus host disease [13,14]. However, the role that JAK/STAT plays in RANKL-induced osteoclastogenesis remains to be characterized.

AG490, also known as tyrphostin B42, has been reported to be a specific JAK2 kinase inhibitor [15,16]. And it has been widely studied in cancer and autoimmune diseases, but not investigation in the setting of RANKL-mediated osteoclast differentiation.

* Corresponding author.
E-mail address: liu-xiangyuan@263.net (X.-y. Liu).

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http://dx.doi.org/10.1016/j.bbrc.2013.04.084

Therefore, the influence of switching off JAK2/STAT3 signaling by using AG490 on osteoclasts need to be further investigated.

In this study, we investigated the effects of a JAK2 inhibitor, AG490, in osteoclast differentiation and addressed the molecular mechanisms involved in an effort to better understand how the JAK2/STAT3 pathway can be utilized in the treatment of bone diseases with excessive osteoclastogenesis.

2. Materials and methods

2.1. Antibodies and reagents

Phycoerythrin-conjugated rat monoclonal anti-mouse RANK antibody and the isotypic antibody, phycoerythrin-conjugated rat IgG2b, were 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). Antibody against mouse phospho STAT3 (Ser^{727}) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant mouse RANKL and macrophage colony-stimulating factor (M-CSF) were obtained from Peprotech (Rocky Hill, NJ, USA). AG490 was supplied by Selleck Chemicals (Houston, TX, USA). Dimethyl sulfoxide was obtained from Amresco (Solon, OH, USA).

2.2. Cell culture

RAW264.7, a murine macrophage cell line, was obtained from Peking Union Medical College (Beijing, China). Cells were 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) in a 37 °C incubator containing 5% CO_2-enriched atmosphere.

2.3. TRAP Staining

RAW264.7 cells were plated at a density of 2×10^4 cells/well in 24-well plates for 12 h and then treated with the indicated compounds for an additional 72 h. The supernatant was removed, and the cells were washed twice with PBS. 4% paraformaldehyde was added to the cells for 20 min at room temperature and then thoroughly removed with deionized water. Naphthol AS-BI phosphate and a tartrate solution were added to the cells for 30 min at 37 °C, followed by counterstaining with a hematoxylin solution.

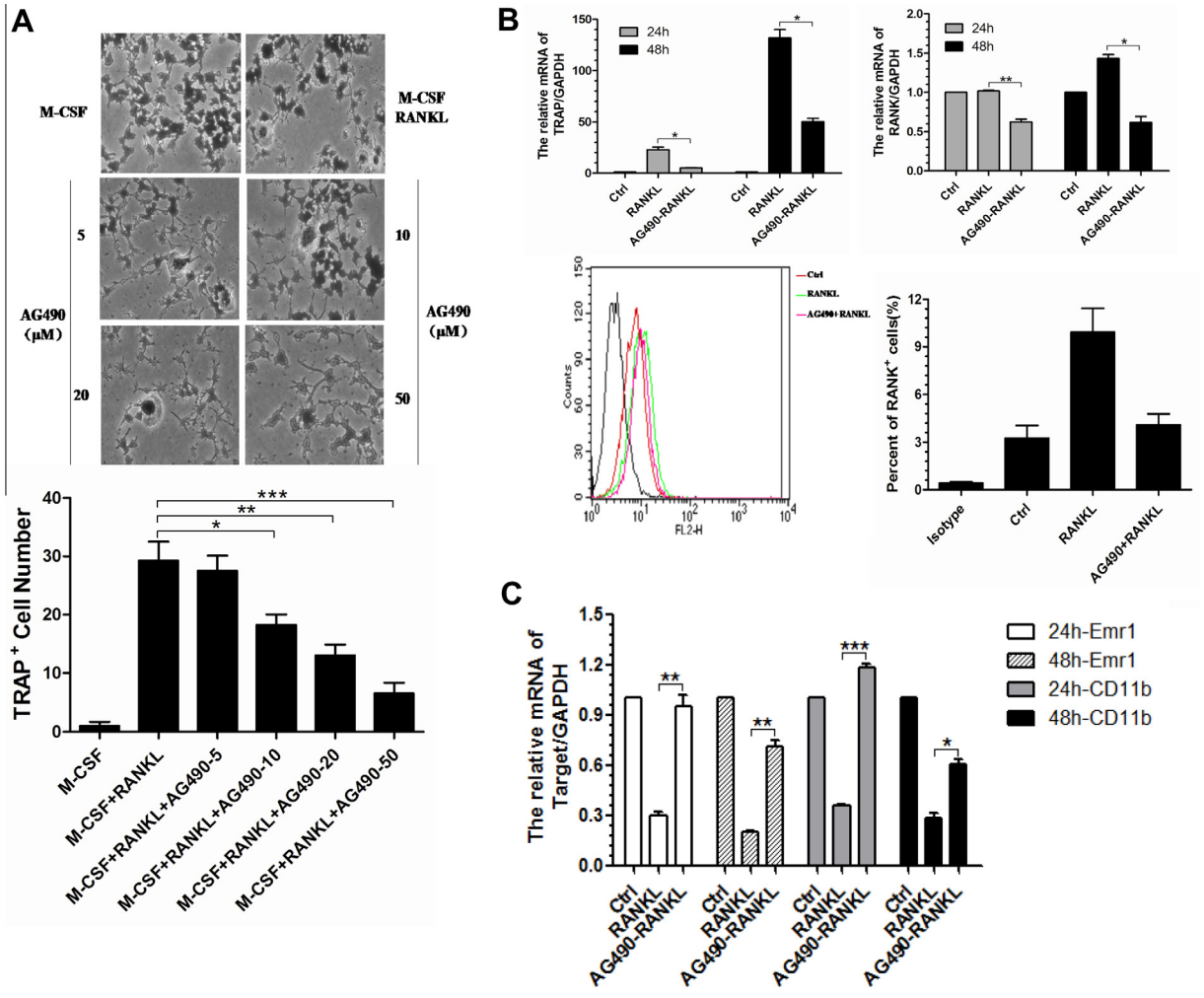

Fig. 1. AG490 inhibits RANKL-induced osteoclast formation. (A) RAW264.7 cells were cultured in the presence of recombinant M-CSF (10 ng/mL) and RANKL (100 ng/mL) and increasing concentrations of AG490. After 3 days, the cells were stained for TRAP activity (top, original magnification, ×100), and TRAP positive cells were counted as osteoclasts (bottom). (B) RAW 264.7 cells were treated in the presence of RANKL (100 ng/mL) with or without AG490 (50 μM) for 24 h or 48 h. The expression of TRAP, RANK mRNA was determined by real-time ploymerase chain reaction (PCR). Membrane RANK-positive cells were analyzed by FACS. The black line in the figure denotes the isotype control. And the percentage of RANK$^+$ cells by FACS was quantified. (C) RAW264.7 cells were treated as in (B), the expression of CD11b and Emr1 was analyzed by real-time PCR. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Osteoclasts were determined to be TRAP-positive staining multinuclear (three or more nuclei) cells and counted under light microscopy.

2.4. Real-time ploymerase chain reaction (PCR)

Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. 2 μg 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 on a Bio-Rad iQ5 Real-Time PCR system using SYBR Green dye (Promega). The melting curve temperatures of each PCR gene were all 60 °C. The primers used were as follows: for GAPDH, forward: 5′-AAATGGTGAAGGTCGGTGTG-3′, reverse: 5′-TGAAGGGGTCGTTGATGG-3′; TRAP, forward: 5′-CCAATGCCAAAGAGATCGCC-3′, reverse: 5′-TCTGTGCAGAGACGTTG CCAAG-3′; NFATc1, forward: 5′-CCGTTGCTTCCAGAAAATAACA-3′, reverse: 5′-TGTGGGATGTGAACT CGGAA-3′; c-Fos, forward: 5′-CGCAGAGCATCGGCAGAAGG-3′, reverse: 5'-TCTTGCAGGCAGGTCGGTGG-3'; CD11b, forward: 5′-GGC ACGCAGACAGGAAGT-3′, reverse: 5′-CCCAGCAAGGGACCATTA–3′; Emr1, forward: 5′-TCCTCCTTGCCTGGACACT-3′, reverse: 5′-GCCTT GAAGGTCAGCAACC-3′; RANK, forward: 5′-CGAGGAAGATTCCC-ACAGAG-3′, reverse: 5′-CAGTGAAGTCACAGCCCTCA-3′. The fold change compared to control was calculated by the formula $2^{-\Delta\Delta Ct}$.

2.5. Flow cytometric analysis

RAW264.7 cells were cultured in 60 mm dish for 24 h after RANKL treatment with or without AG490 and then harvested in phosphate-buffered saline (PBS). The cells were washed and resuspended in PBS containing Phycoerythrin-conjugated rat monoclonal anti-mouse RANK 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. Cell viability assay

RAW264.7 cells (5×10^3 cells/well) were cultured in 96-well plates for 48 h after RANKL treatment with or without AG490. The cell proliferation was assessed using the Cell Counting Kit-8(CCK-8; Promega, USA) according to the manufacturer's protocol. The absorbance was measured at 450 nm using a microplate reader (Thermo Scientific).

2.7. Western blot analysis

RAW264.7 cells were lysed in protein extraction solution. The lysates were centrifuged at 12,000 rpm for 15 min at 4 °C to remove the cell debris. Protein concentration of the extract was determined by using the BCA protein assay kit (ComWin Biotech, Beijing, China). Equal amounts of protein were separated on 10% sodium dodecylsulfate polyacrylamide (SDS) gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore, Billerica, MA, USA) and blocked with 5% skim milk in 0.05% Tween 20 in Tris buffered saline (TBST) at room temperature for 2 h. The membranes were then incubated with various primary antibodies, which were diluted in 5% BSA–TBST at 4 °C with gentle shaking overnight. After washing, the membrane was incubated with horseradish peroxidase–conjugated secondary antibody at room temperature for 1 h. The blots were

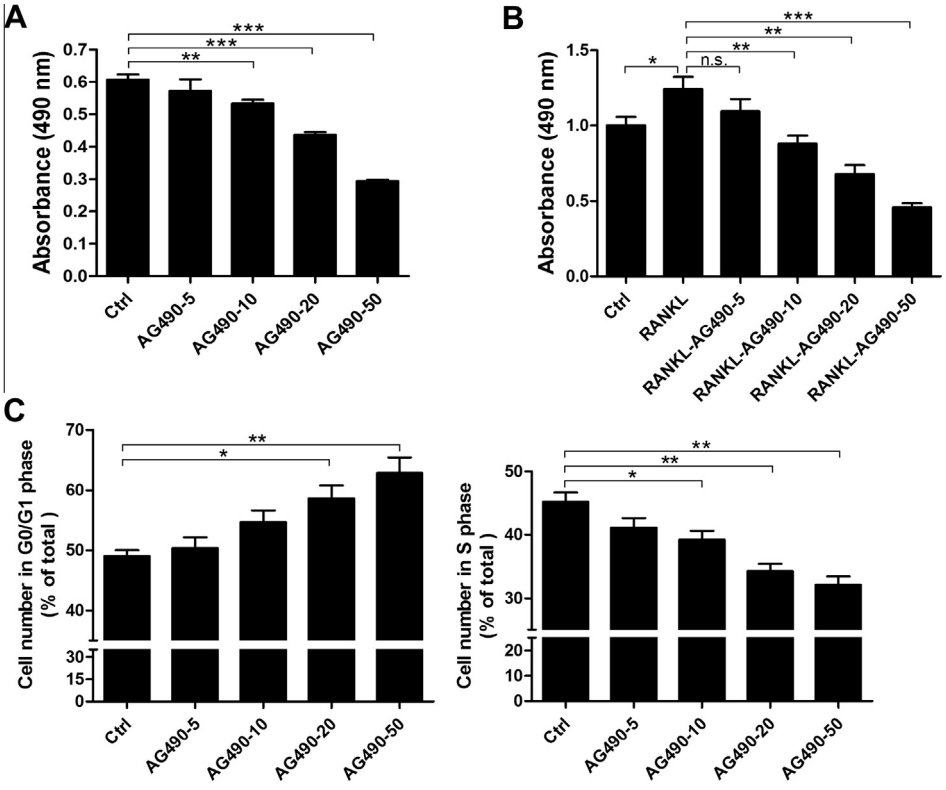

Fig. 2. AG490 reduces cell proliferation and causes cell cycle arrest. (A and B) Inhibitory effect of AG490 at the indicated concentrations on the growth of RAW264.7 cells treated with or without RANKL (100 ng/mL) for 48 h was determined using CCK-8 assay. (C) Cell cycle arrest at the G1/S boundary. The percentage of cells in each of the cell cycle phase was analyzed using flow cytometry at 48 h after AG490 treatment. $^{*}p < 0.05$, $^{**}p < 0.01$,$^{***}p < 0.001$. n.s., not significant.

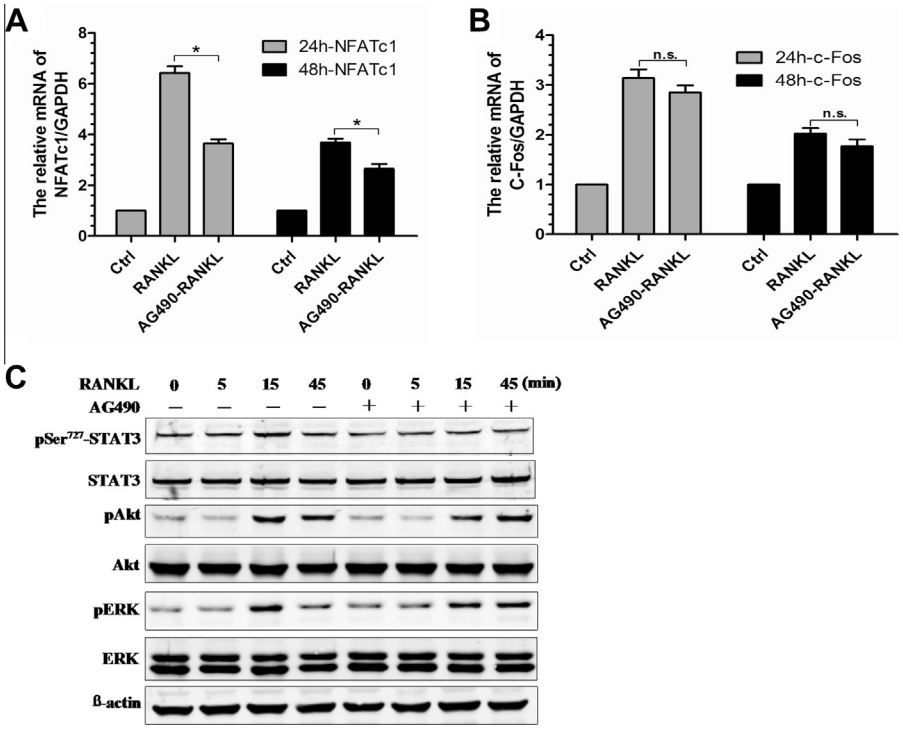

Fig. 3. AG490 disrupts RANKL-induced NFATc1 expression and JAK2/STAT3 signal activation. (A) RAW264.7 cells were treated in the presence of RANKL (100 ng/mL) with or without AG490 (50 μM) for 24 h or 48 h. The levels of NFATc1 and c-Fos mRNA were quantified by real-time ploymerase chain reaction (PCR). (B) RAW264.7 cells were pretreated with AG490 (50 μM) for 1 h before treatment with RANKL (100 ng/ml) for the indicated time. Whole cell lysates were used for Western blot. 40 μg protein was loaded for each lane. β-actin was used as loading control. $^{*}p < 0.05$. n.s., not significant.

developed by using an enhanced chemiluminescence detection kit (Pierce, Rockford, IL, USA) .

2.8. Small interference RNA (siRNA) for STAT3

siRNAs targeting STAT3 were designed and synthesised by Ribobio (Guangzhou, China). One hundred nanomolar siRNA duplex was transiently transfected to RAW264.7 cells by using Lipofectamine RNAimax (CA, USA), and the transfected cells were harvested at the indicated times for real-time PCR and Western blot analysis. STAT3 siRNA sequence 1: sense, CUGGAUAACUUCAUUAGCA, antisense, GACCUAUUGAAGUAAUCGU; sequence 2: sense, CCAACGACCUGCAGCAAUA, antisense, GGUUGCUGGACGUCGUUAU.

2.9. Statistical analysis

The statistical analyses were performed using the SPSS 12.0 (SPSS Inc, Chicago, IL) statistical software package and GraphPad Prism 5.0 (GraphPad, San Diego, CA). 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. *P* values <0.05 were considered statistically significant.

3. Results

3.1. AG490 suppresses RANKL-induced osteoclastogenesis in RAW264.7 cells

To test if AG490 affected RANKL-mediated osteoclastogenesis, we treated RAW264.7 cells with various concentrations of JAK2 specific inhibitor AG490 in the presence of RANKL and M-CSF. RAW264.7 cells treated by M-CSF and RANKL efficiently differentiated into multinuclear osteoclasts, while AG490 reduced the formation of TRAP-positive multinucleated cells in a dose-dependent manner and inhibited osteoclast formation by 6%, 38%, 56% and 78% at 5, 10, 20 and 50 μM, respectively (Fig. 1A). Then we investigated the mRNA expression of some osteoclast and macrophage markers in RAW264.7 cells cultured in the presence of 100 ng/mL RANKL with or without 50 μM AG490 for 24 and 48 h. Two specific osteoclast markers (TRAP, RANK) and two macrophage markers (Emr1 and CD11b) were analyzed by real-time PCR. As expected, RANKL alone treatment significantly increased osteoclastic markers expression at 48 h: 2.6 times for TRAP, and 2.3 times for RANK compared with in the presence of AG490 (Fig. 1B). And the flow cytometry analysis also demonstrated the expression level of cell surface RANK was significantly reduced by AG490 (Fig. 1B). As shown in Fig. 1C, RAW264.7 cells expressed a basal level of two macrophagic markers, which were decreased during RANKL induced osteoclast differentiation. When AG490 was present in the culture medium, the mRNA expression of the macrophagic markers increased even in the presence of RANKL, confirming that AG490 induced a macrophage phenotype instead of an osteoclastic one (Fig. 1C). These data indicated that AG490 exerted an inhibitory effect on RANKL-mediated osteoclastogenesis.

3.2. AG490 affects cell proliferation and cell cycle distribution

In order to confirm which factor contributed to the inhibitory effect of AG490, we determined the influence of AG490 on cell viability and cell cycle progression. The treatment of RAW264.7 with 5, 10, 20, 50 μM AG490 for 48 h resulted in the inhibition of cell proliferation (Fig. 2A). The inhibition of cell growth was dose-dependent. Furthermore, as shown in Fig. 2B, RANKL alone treatment enhanced cell proliferation, while combination with AG490

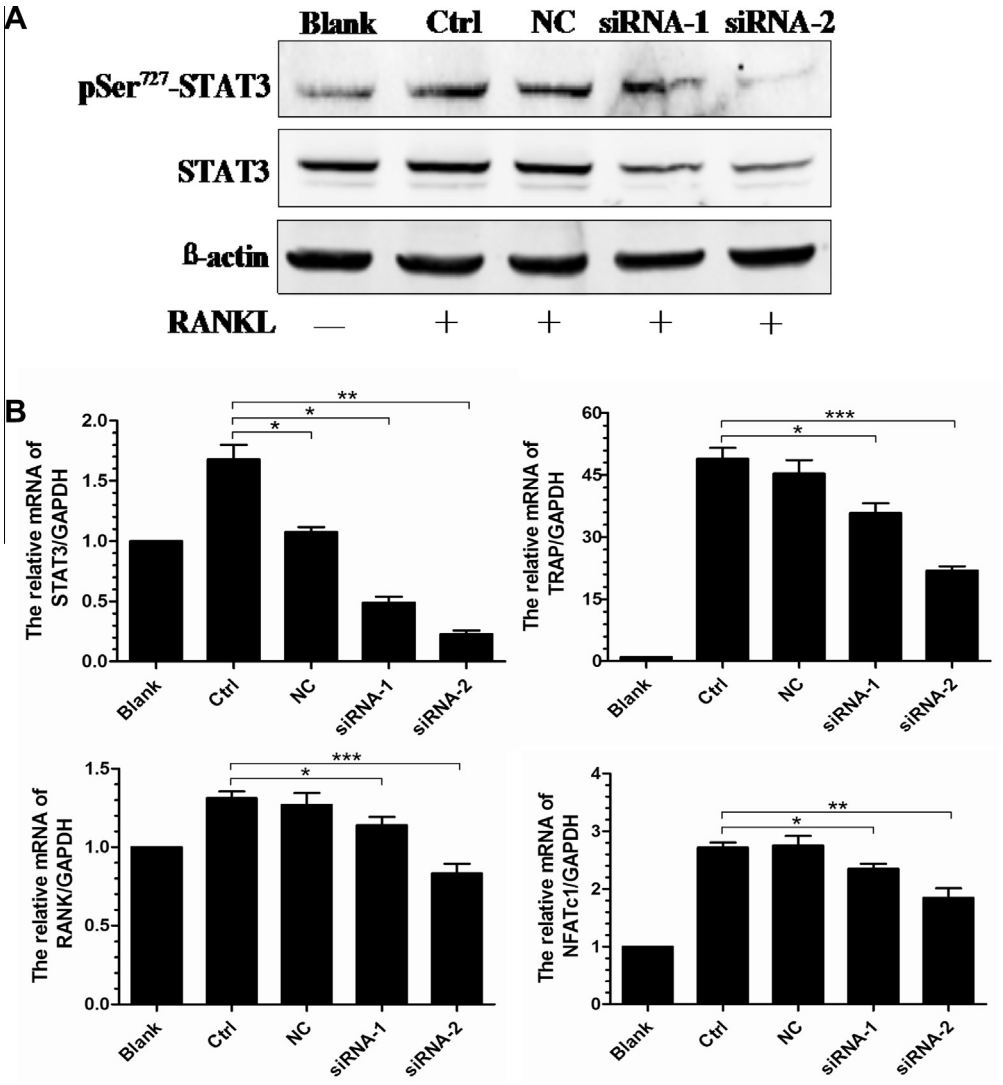

Fig. 4. Down-regulation of TRAP, RANK and NFATc1 expression after STAT3 siRNA transfection. (A) RAW264.7 cells were transiently transfected with 100 nM STAT3 siRNA (siRNA1; siRNA2) or scramble siRNA (negative control, NC) for 48 h and then stimulated with RANKL (100 ng/mL) for 15 min, and whole cell lysates were prepared for Western blot. Expression levels of STAT3, p-STAT3 (Ser 727) were determined by Western blot analysis. 40 μg protein was loaded for each lane. β-actin was used as loading control. Data represent a typical result from three independent experiments. (B) RAW264.7 cells were transiently transfected as in (A) and then stimulated with RANKL (100 ng/mL) for 24 h, and total RNA was isolated using Trizol reagent. Real-time PCR analysis was performed using primers specific for mouse STAT3, TRAP, RANK and NFATc1. GAPDH served as the control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

suppressed RANKL-induced cell growth effect in a dose-dependent manner. To distinguish between alterations in cell proliferation and cell viability, we used cycle-phase fractionation through flow cytometry, which provided more analytical details of cell cycle distribution among AG490 treated cells. As shown in Fig. 2C, AG490 caused an arrest of RAW264.7 cells at the G0/G1 phase of the cell cycle, and prevented their entry into the S and G2/M phase.

3.3. AG490 inhibits RANKL-induced NFATc1 expression and phosphorylation of Ser^{727} STAT3

Previous studies have shown that various intracellular signaling pathways, including MAPKs, such as JNK, ERK, and P38 and transcription factors, such as NF-kB, NFATc1, and STAT, are activated during RANKL induced osteoclastogenesis [17,18]. In order to determine the molecular mechanism of AG490 involved in RANKL-modulated osteoclast formation, we measured expression of above transcription factor and phosphorylation of various signaling molecules by Real-time PCR and Western blot analysis, respectively. As shown in Fig. 3A and B, AG490 obviously dampened RANKL-induced NFATc1 expression, but not c-Fos. Subsequently, we treated cells with RANKL for 0, 5, 15 and 45 min in the absence or presence of AG490, after which activation of Akt, ERK1/2 and STAT3 was determined. As expected, AG490 mildly inhibited RANKL-induced phosphorylation of Ser^{727} STAT3, but had no effect on the activation of Akt and ERK1/2 (Fig. 3C). How AG490 inhibits RANKL-induced STAT3 phosphorylation still remains to be further investigated. These results indicated that AG490 suppressed RANKL-mediated osteoclastogenesis via the STAT3 pathway in RAW264.7 cells (Fig. 3C).

3.4. Phosphorylation of Ser^{727} STAT3 is involved in RANKL induced TRAP, RANK and NFATc1 expression in RAW264.7 cells

In order to further confirm the role which STAT3 pathway played in RANKL-induced osteoclastogenesis, we determined the effects of STAT3 knockdown using STAT3 siRNA transfection. As shown in Fig. 4A, STAT3 knockdown markedly decreased not only

STAT3 expression, but also RANKL-induced Ser^{727} STAT3 phosphorylation. Moreover, gene expression of TRAP, RANK and NFATc1 was inhibited when treated with STAT3 siRNA (Fig. 4B). This result indicated that STAT3 pathway played a pivotal role in RANKL-stimulated osteoclast formation in RAW264.7 cells.

4. Discussion

The JAK/STAT signaling pathway was initially identified in the process of erythropoietin (EPO)-mediated haematopoiesis, where it was known to play a key role in the regulation of important cytokine genes during the conversion of bone marrow cells to red blood cells [19]. However, it has now become increasingly clear that this signaling pathway is also active in a number of other distinct cell and tissue types and plays a much broader role in the pathogenesis of different diseases than was initially appreciated. These additional roles include the regulation of lymphocyte differentiation, cancer cells growth and proliferation, immune rejection of allografts, and even the inflammatory processes of autoimmune diseases [16,20]. Now our current data extends to osteoclasts, which take advantage of the JAK2/STAT3 signaling pathway as a means of regulating cell fate choices and cellular functions.

In our original studies, we used specific inhibitor of JAK2 kinase, AG490, together with the well established RAW264.7 monocyte-macrophage-like/pre-osteoclast cell line to determine the potential role of JAK2/STAT3 signaling in the RANKL-mediated osteoclastogenesis. As a result of these studies, we found that AG490 potently blocked RANKL-induced differentiation of RAW264.7 cells into mature TRAP-positive, multinucleated osteoclasts in a dose-dependent manner. Consistent with this result, we further found AG490 suppressed the gene expression of osteoclast markers, such as TRAP and RANK, while the mRNA expression of macrophage makers, CD11b and Emr1, was increased even in the presence of RANKL. In general, the observation that AG490 act to block RANKL-induced osteoclast differentiation, provides a compelling evidence to implicate JAK2 as an essential downstream effector of the RANKL induced signal transduction pathway leading towards the induction of terminal osteoclast differentiation, and thereby helps define a novel role for JAK2 signaling in the regulation of osteoclastogenesis.

In order to figure out the possible mechanisms involved in the inhibitory effect of AG490, first we tested the influence of AG490 on viability and cell cycle transition of osteoclast precursor cells. The results showed that AG490 reduced RAW264.7 cell proliferation whether in the presence of RANKL or not. Furthermore, AG490 affected cell cycle progression and induced G1-S phase arrest. These data preliminarily suggest that AG490 exerts an inhibitory effect on osteoclastogenesis mediated by RNAKL via reducing the proliferation of RAW264.7 cells.

Previous studies showed two transcription factors, c-Fos and NFATc1, played an essential role in initiating RANKL-induced osteoclast differentiation and the expression of genes required to give rise to osteoclasts [21,22]. Therefore, osteoclastogenesis might be partly blocked through down-modulation of c-Fos and/or NFATc1. Intriguingly, AG490 significantly inhibited NFATc1 expression but did not affect c-Fos in RAW264.7 treated with RANKL. However, it was reported that c-Fos could bind to the promoter sequence of NFATc1 gene [23], suggesting that c-Fos might directly regulate NFATc1 expression. In this context a better comprehension is that AG490 may need other signaling pathways to influence the expression of NFATc1. Therefore, we tested the activation of some kinase signaling pathways, such as PI3k/Akt, MEK1, JAK2/STAT3, by RANKL and whether AG490 changes the activation. Despite PI3 kinase and MAP kinase signaling cascades are both essential for maintenance the survival and function of multinucleated and matured osteoclasts, and consequent induction of osteoclastic bone resorption [24], our results showed that AG490 does not function through these pathways. It is noteworthy that AG490 at least in part decreases RANKL activated phospho-STAT3 (Ser^{727}), but not phospho-Akt and phospho-Erk1/2. We also found that RANKL selectively activates the phosphorylated form of STAT3 on Ser^{727} but not Tyr^{705}-phosphorylated STAT3 (data not shown) in RAW264.7 cells. This result is in agreement with the work of Liu et al. which demonstrated a base level of phospho-STAT3 (Ser^{727}) without any detection of Tyr^{705} phosphorylation in a macrophage cell line [25]. Our results clearly indicate the reduction of NFATc1 was mediated through AG490 acting on JAK2/STAT3 signaling pathway.

In addition, we further observed here that STAT3 knockdown with siRNA reduced the expression of RANKL-induced osteoclast associated genes. However, RANKL-induced TRAP, RANK and NFATc1 expression was not significantly reduced compared with the decrease of STAT3 phosphorylation. This can be explained by the fact that STAT3 is not the only transcription factor, which modulates osteoclast associated genes expression during RANKL-mediated osteoclastogenesis in RAW264.7 cells. This result at least in part indicates that activation of STAT3 pathway plays a role in osteoclastogenesis stimulated by RANKL.

In conclusion, we demonstrated the inhibitory effects of AG490 on RANKL-induced osteoclastogenesis by targeting cell viability and NFATc1 expression through JAK2/STAT3 signaling pathway. These data can provide the basis for future understandings of the JAK2/STAT3 in osteoclastogenesis, and possibly go onto use AG490 as a novel therapeutic agent for treating various diseases with inflammatory bone destruction, such as rheumatoid arthritis and osteoporosis.

Acknowledgments

This work was supported by National Natural Science Foundation of China (No. 81072474), National Young Scholars Foundation of China (No. 81102255) and Beijing Municipal Natural Science Foundation (No. 7112143).

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