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PC-PLC is involved in osteoclastogenesis induced by TNF-α through upregulating IP3R1 expression

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ABSTRACT

The precise mechanism of how TNF-α promotes osteoclast formation is not clear. Previous reports show TNF-α targets molecules that regulate calcium signaling. Inositol-1,4,5-trisphosphate receptors (IP3Rs) are important calcium channel responsible for evoking intracellular calcium oscillation. We found that TNF-α increased the expression of IP3R1 and promoted osteoclastogenesis in RANKL-induced mouse BMMs. Phosphatidylcholine-specific phospholipase C (PC-PLC) specific inhibitor D609 eliminated the upregulation of IP3R1 by TNF-α, and decreased the autoamplification of nuclear factor of activated T-cells 1 (NFATc1), thus resulted in less osteoclasts formation. However, D609 did not inhibit RANKL-induced osteoclastogenesis. Our data suggest TNF-α promotes RANKL-induced osteoclastogenesis, at least partially, through PC-PLC/IP3R1/NFATc1 pathway.
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1. Introduction

Osteoclast, derived from mononuclear progenitors of pluripotent hematopoietic stem cells, is multinucleated cell and unique in its ability to resorb mineralized tissues like bone. It has been well accepted that two factors are critically required for osteoclastogenesis: macrophage-colony stimulating factor (M-CSF) and ligand of receptor activator of NF-κB (RANKL). Two receptors, c-Fms and RANK, mediate the functions of M-CSF and RANKL, respectively [1]. Considerable progress has been made in our understanding of the signals downstream M-CSF and RANKL. The major breakthroughs include the identification of nuclear factor of activated T-cells c1 (NFATc1), a master osteoclastogenic transcription factor, and the co-stimulatory signaling essential for activation of NFATc1 through Ca^{2+} signaling activation [2,3].

Higher level of TNF-α have been found in many diseases, such as rheumatoid arthritis, periodontal disease and aseptic periprosthetic osteolysis. These diseases are characterized by bone loss around affected joints or teeth caused by increased osteoclastic bone resorption. The importance of TNF-α in the pathogenesis of various forms of bone loss is supported by both experimental and clinical evidences [4–9]. Much effort has been made in trying to clarify its mechanisms during osteoclastic differentiation and bone degradation [10–13]. Takayanagi et al. demonstrated that the effect of TNF-α was RANK-independent. Signaling molecules immediately activated by RANKL stimulation, such as NF-κB or TNF receptor associated factor 6 (TRAF6), were not involved in osteoclastogenesis induced by TNF-α, while prominent NFATc1 expression was evoked in the late stage of osteoclast differentiation [14–16]. Ivashkiv et al. identified delayed signaling responses induced by TNF-α in primary human macrophages and found a sustained wave of Ca^{2+} oscillations started after one-two day of TNF-stimulation. Their work further revealed that Ca^{2+} signaling culminated and caused induction and activation of NFATc1. Evoked NFATc1 mediated a gene expression program linked to cell fusion and osteoclast differentiation [17]. It was then concluded that TNF-α targets molecules which regulate calcium signaling.

Inositol-1,4,5-trisphosphate receptors (IP3Rs) are large tetrameric transmembrane proteins found primarily in the endoplasmic reticulum (ER), where they mediate release of intracellular calcium stores in response to extracellular signals [18]. The calcium-releasing function of IP3R channel is regulated by its principal ligands, such as inositol-1,4,5-trisphosphate, calcium ions and some interaction proteins including kineases for which IP3R is a substrate. There are three subtypes of IP3R: IP3R1, IP3R2, and

Abbreviations: NFATc1, nuclear factor of activated T-cells c1; NF-κB, nuclear factor-κB; RANKL, receptor activator of NF-κB ligand; TNF-α, tumor necrosis factor-alpha; IP3R, Inositol-1,4,5-trisphosphate receptor; PC-PLC, phosphatidylcholine-specific phospholipase C

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IP3R3. The three types are expressed in a tissue and development-specific manner. Different subtype of IP3R mediates different Ca^{2+} signaling. Among the IP3Rs, IP3R2 is known to be the most sensitive to IP3 and is required for long lasting Ca^{2+} oscillation [19], while IP3R1 mediates less regular Ca^{2+} oscillation and IP3R3 generates only monophasic Ca^{2+} transients. The three subtypes have been found expressing in mouse osteoclast precursor cells [20]. There are articles reported that over-expression of IP3R1 enhanced both IP3-binding activity and sensitivity, resulting in more active Ca^{2+}-releasing activity which exits in disease [21]. For example, elevated IP3R1 might be related to initiating or perpetuating diseases such as atrial fibrillation and asthma [22,23]; IP3R1 upregulation induced by TNF-α correlated with alterations in Ca^{2+} homeostasis and might be associated with Parkinson's disease [24]. Therefore, we hypothesize that increased IP3R1, induced by TNF-α, stimulates calcium oscillation and enhance the autoamplification of NFATc1, thus further promotes the RANKL-induced osteoclastogenesis in vitro.

2. Materials and methods

All use of experimental animals complied with the national guidelines in China and the experimental procedures were proved by the Shandong University Medical College, Institutional Animal Care and Use Committee.

2.1. Materials

Recombinant mouse Tumor necrosis factor-α (rmTNF-α, 410-MT-010), Receptor Activator for Nuclear Factor-κ B Ligand (rmRANKL, 462-TEC-010) and macrophage colony-stimulating factor (rmM-CSF, 416-ML-050) were from R&D Systems (Minneapolis, MN, USA). α-minimum essential medium (α-MEM, 12571), fetal bovin serum (FBS, 10099) and Hanks balanced salt solution (HBSS, 14025) were purchased from Gibco (USA). 2′,7′-dichlorofluorescein diacetate (DCFH-DA, D6883), Histopaque-1077 (Ficoll, 1077-1), PC-PLC specific inhibitor D609 (tricyclodecan-9-yl-xanthogenate, T8543) and tartrate-resistant acid phosphatase (TRAP) staining kit (387A-1KT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Micro BCA™ Protein Assay kit was from Pierce (23235, USA). Rabbit polyclonal antibody against IP3R1 was from Lifespan bioscience (LS-B2640, USA). Anti-NFATc1 antibody was from Santa Cruz Biotechnology (sc-13033, USA) and anti-β-actin antibody was from Abcam (ab1801, USA). RNeasy Mini Kit for total RNA isolation was from QIAGEN (74106, Valencia, CA, USA). Reverse transcription reagents including Oligo$(dT)_{12-18}$ Primer (18418-012), RNaseOUT™ Recombinant Ribonuclease Inhibitor (10777-019), Deoxyribonuclease I, Amplification Grade (18068-015), SuperScript III Reverse Transcriptase (18080-093, 18080-044, 18080-085) were purchased from Invitrogen (USA). Fluo-3AM calcium probe and Pluronic F127 were from Biotium (50013, USA).

2.2. Cell culture and differentiation

Six to eight weeks old male mice were sacrificed by decapitation under deep anesthesia with 10% Chloral hydrate. Bone marrow cells were isolated from tibiae and femurs. The cells were cultured in α-MEM containing 10% heat-inactivated FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37 °C overnight under 5% CO_2. Non-adherent cells were carefully layered onto a Ficoll (Histopaque-1077) density gradient solution and centrifuged at 400 g for 30 min at room temperature. Cells lying in the upper layer were harvested as bone marrow derived monocytes (BMMs) and washed with PBS. The cells were seeded into 6-well plate (5×10^5 cells/well) or 96-well-plate (1×10^4 cells/well) and exposed to the medium supplemented with 50 ng/ml M-CSF. 1 ng/ml RANKL and 3 ng/ml TNF-α were added at the start of induction. D609 (10 μM) pretreatment was performed 1 h before induction.

2.3. TRAP staining

To evaluate osteoclast differentiation cells were stained for tartrate-resistant acid phosphatase (TRAP). Cells were fixed with 4% formaldehyde for at least 15 min at room temperature and stained for TRAP using Acid Phosphatase, leukocyte kit according to manufacturer's protocol. Multinucleated TRAP-positive cells with at least 3 nuclei were scored as osteoclasts. Osteoclast cell number was counted under a light microscope at 20× magnification. The experiments, each with triplicates, were performed on at least three different occasions.

2.4. Assay of cell viability

Cells were seeded into 96-well plates and induced to differentiate. The viability of cells was determined according to the method of MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphe-nyltetrazolium) assay. The absorbance was measured at 490 nm with a microplate (Bio-tek Synergy™ HT, USA).

2.5. Assay of intracellular ROS

Cells cultured in 96-well plates were washed at certain time point with PBS and then incubated in α-MEM containing 10 μM DCFH-DA, 37 °C for 1 h under 5% CO_2. At the end of the incubation, the cells were washed in serum-free medium three times. 100 μl α-MEM was added to each experimental well. Culture medium without cells was used as negative control. The DCF (2′,7′-dichlorofluorescein) fluorescent absorbance was determined by measuring the fluorescence emission at 525 nm with a fluorescence microplate reader (Bio-tek Synergy™ HT, USA). The absorbance of negative control was subtracted from samples. Relative intracellular ROS production was calculated as following formula:

$$\text{Relative intracellular ROS production} = \frac{\text{DCF fluorance}}{\text{MTT 490 nm absorbance}}$$

2.6. Cytosolic Ca^{2+} measurement

Cells were seeded into confocal microscopy dishes and induced to differentiate for 48 h. They were washed three times with Hanks balanced salt solution (HBSS) and incubated for 30 min at 37 °C with 5 μM Fluo-3AM and 0.04% Pluronic F127. Wash the cells three times with HBSS again and intracellular Ca^{2+}-bound Fluo-3 fluorescence was recorded at 1s intervals for 120 s by a laser-scanning confocal microscopy.

2.7. Semiquantitative RT-PCR

Total RNA was extracted from cells cultured in 6-well plates by using RNeasy Mini Kit according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA using the SuperScript reverse transcriptase system in a total volume of 20 μl. 2 μl of the resulting cDNA products were subjected to PCR analysis using specific primers. Sequence and optimal annealing temperature for each primer are presented as Table 1.

PCR amplification was run according to the reaction profile: initial setup at 95 °C for 3 min,30 amplification cycles consisting of denaturation for 30 s at 95 °C and annealing for 30 s, final step is extension for 1 min at 72 °C. PCR products were separated by

Table 1
Primer sequences and optimal annealing temperatures.

IP3R1	Forward 5′-AACTGTGGGACCTTCACCAG-3′ Reverse 5′-AACTCTCGCCAGTTTCTGGA-3′	62 °C NM_010585.4
IP3R2	Forward 5′-GTTACAGGATGTCGTGGCCT-3′ Reverse 5′-ATTCGCCGTAATGTGCTACC-3′	62 °C NT_039361.6
IP3R3	Forward 5′-CAATGAGCACCACGAGAAGA-3′ Reverse 5′-AACTTGACAGGGGTCACCAG-3′	62 °C NW_001030605.1
NFATc1	Forward 5′-TTCCTTCAGCCAATCATCCCCCCAGTTAC-3′ Reverse 5′-CGATGTCTGTCTCCCCTTTCCTCAGCTC-3′	65 °C NW_001030635.1
GAPDH	Forward 5′-GTCGTGGAGTCTACTGGCGTC-3′ Reverse 5′-GAAGTCACAGGAGACAACCTGG-3′	58 °C XM_003085777.1

electrophoresis on a 1% agarose gel and visualized by staining the gel with ethidium bromide. The images and the intensities of bands were obtained using FluorChem (Alpha Innotech, USA).

2.8. Western blot analysis

Cells from 6-well culture plates were lysed in ice-cold RIPA lysis buffer [50 mM Tris(pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM Na_3VO_4, 0.5 μg/ml leupeptin], containing 1 mM Phenylmethanesulfonyl fluoride (PMSF). Cell debris was removed by centrifugation at 10,000 rpm for 15 min at 4 °C. Protein concentration was measured using Micro BCA™ Protein Assay kit. Protein was denatured by boiling for 3 min before electrophoresis. 30 μg of each protein sample was subjected to 6% SDS–PAGE, and transferred to Nitroate cellulose (NC) membrane. The membrane was blocked with 3% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h, and incubated with anti-IP3R1 or NFATc1 antibody at room temperature for 2 h, followed by washing with TBS-T for 5 min three times. After incubation with HRP-conjugated anti-rabbit IgG antibody at room temperature for 1 h, and washing with TBS-T for 5 min three times, signals were developed using Immubilon Western Chemiluminescent HRP substrate for 1 min. The images and the intensities of bands were obtained using FluorChem (Alpha Innotech).

2.9. Data analysis

Data are presented as means ± standard deviations (SD) from at least 3 different experiments. Student's t-test was used for

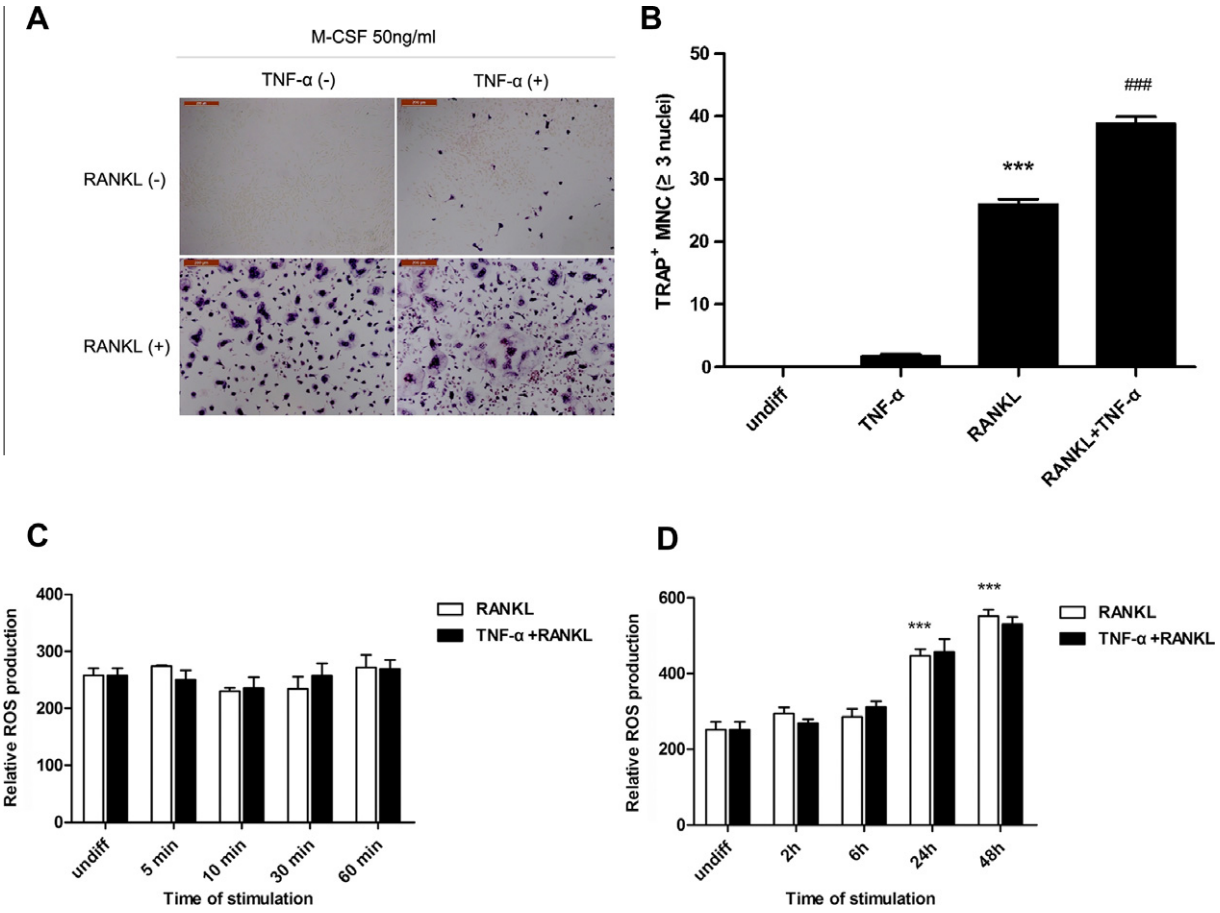

Fig. 1. TNF-α promotes RANKL-induced osteoclastogenesis not through ROS pathway. (A) TRAP staning. BMMs stimulated by RANKL with or without TNF-α for 72 h were fixed and stained for TRAP. (B) TRAP-positive multinucleated cells with three or more nuclei were counted. (C) Instant intracellular ROS production. (D) Late intracellular ROS production. The values are expressed as mean ± SD ($n = 4$). *Represents vs. undiff. Undiff is the abbreviation of undifferentiated group which represents cultured BMMs without RANKL and TNF-α stimulation.

statistical analysis. $P < 0.05$ (*) was considered statistically significant. **$P < 0.01$, ***$P < 0.005$.

3. Results

3.1. TNF-α promotes RANKL-induced osteoclastogenesis not through ROS pathway

It has been reported that long term ROS production induced by RANKL mediates the Ca^{2+} oscillations and regulates osteoclast differentiation [25]. TNF-α targets molecules which regulate calcium signaling. Thus we speculate that intracellular ROS level is involved in osteoclastogenesis induced by TNF-α.

BMMs were treated by RANKL and M-CSF. TNF-α was added to the cultures at the start of stimulation. After 72 h cells were stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive multinucleated cells (MNCs) with more than three nuclei were scored as osteoclasts and the number of osteoclasts was counted (Fig. 1A). Compared to RANKL- stimulation, adding TNF-α to RANKL treatment dramatically increased the number of TRAP-positive MNCs ($P < 0.005$, Fig. 1B). Relative intracellular ROS level was measured with a fluoresce probe DCFH-DA at different time point. There was no obvious change of ROS level detected until 24 h of RANKL stimulation. Addition of TNF-α did not alter intracellular ROS level after 24 h stimulation (Fig. 1 C and D).

3.2. PC-PLC inhibitor D609 doesn't inhibit osteoclastogenesis induced by RANKL but removes the promotional effect of TNF-α

Since there was no further promotion of intracellular ROS production by TNF-α, we checked other signaling molecules involved in TNF-α effect on osteoclastogenesis. PC-PLC, a molecule that produces DAG and phosphorylcholine from phosphatidylcholine (PC) hydrolysis, is reported to mediate TNF-α signaling in various cells [26–28]. Then we wondered whether the enzyme participates in stimulatory effect of TNF-α during osteoclast differentiation. To test this, PC-PLC specific inhibitor D609 was incubated with BMMs for 1 h before the cells were exposed to RANKL or RANKL plus TNF-α. The toxicity of D609 was excluded by measuring cellular viability with the method of MTT (Fig. 2A). Cells were stained for TRAP after 72 h of stimulation (Fig. 2B). D609 moderately increased the number of TRAP-positive MNCs induced by RANKL ($P < 0.05$, Fig. 2C), but remarkably decreased the number in the group of RANKL combining TNF-α ($P < 0.01$, Fig. 2C).

3.3. PC-PLC is involved in the regulation of intracellular Ca^{2+} signaling and NFATc1 autoamplification by TNF-α

NFATc1 is an important transcription factor during osteoclastogenesis both in vitro and in vivo. Previous reports showed that TNF-α promoted autoamplification of NFATc1 as a result of enhanced Ca^{2+} oscillations in primary human macrophages [17]. As the results showed above that PC-PLC was involved in the the promotion of TNF-α on osteoclast formation, we examined if PC-PLC participated in the upregulation of Ca^{2+} oscillations and NFATc1 by TNF-α. For NFATc1 examination, primary mouse BMMs were seeded into 6-well plates and total RNAs were extracted after 48-h stimulation by RANKL with or without TNF-α. PCR results were shown in Fig. 3A and B. The presence of TNF-α in RANKL-culture upregulated mRNA expression of NFATc1, the increasing effect was eliminated when the cells were pre-treated by D609. The

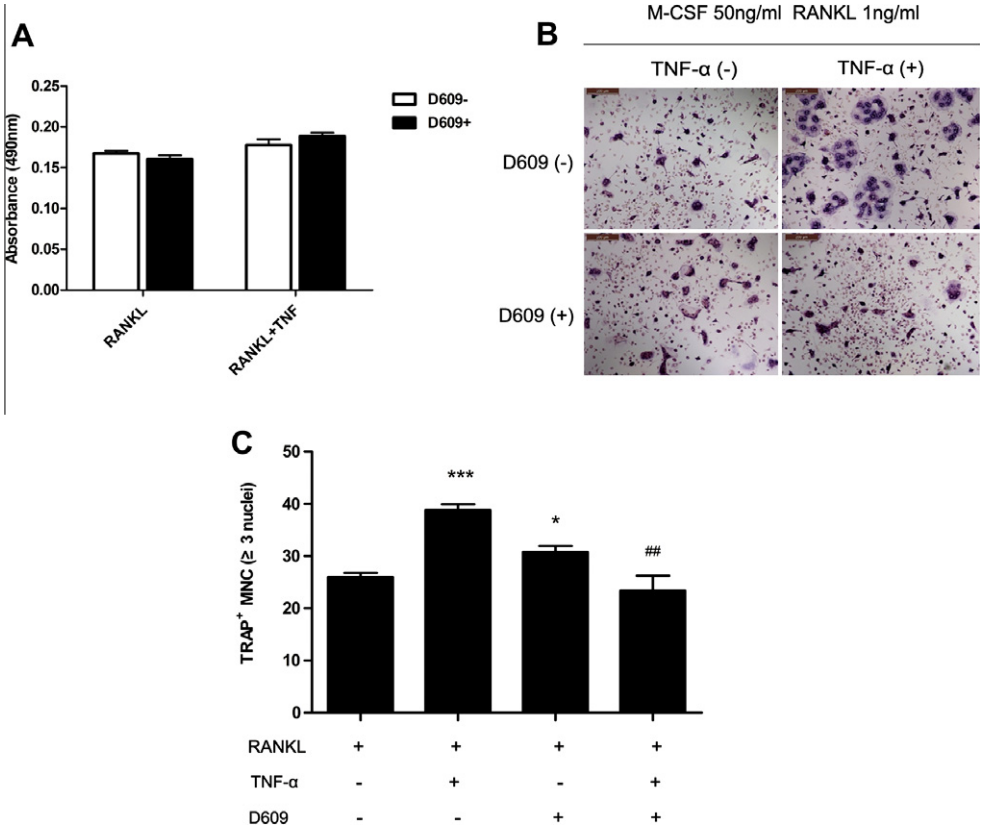

Fig. 2. PC-PLC specific inhibitor D609 reduces the promotional effect of TNF-α on RANKL-induced osteoclast formation (A) The toxicity of D609 was measured by the method of MTT. (B) BMMs stimulated by RANKL with or without TNF-α and D609 for 72 h were fixed and stained by TRAP staining kit. (C) TRAP-positive multinucleated cells with three or more nuclei were counted. The values are expressed as mean ± SD, $n = 4$. *represents vs. RANKL, # vs. RANKL plus TNF-α.

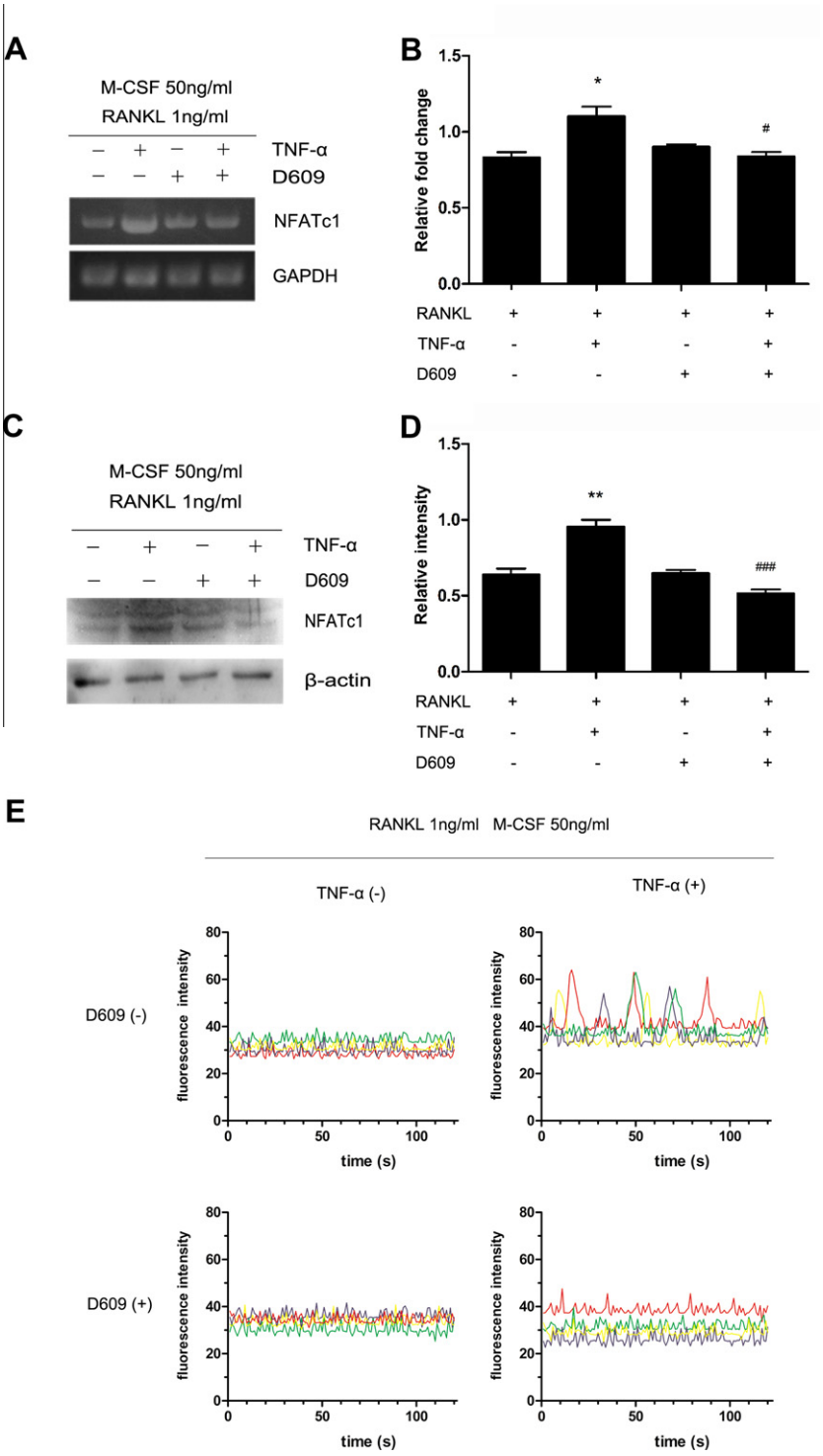

Fig. 3. PC-PLC is involved in the regulation of intracellular Ca^{2+} signaling and NFATc1 autoamplification by TNF-α. BMMs were stimulated for 48 h by RANKL with or without TNF-α, RT-PCR and Western blot were performed to determine mRNA (A and B) and protein (C and D) expression of NFATc1. Data was pooled from at least three different experiments. *represents vs. RANKL, # vs. RANKL plus TNF-α. (E) Intracellular Ca^{2+} oscillations were measured with confocal microscope at 1s intervals for 120 s. Each color indicates a different cell in the same field.

effect of D609 on NFATc1 mRNA expression was further investigated at protein level after 48 h stimulation. Western blot results were consistent with the mRNA changes (Fig. 3C and D). For measurement of Ca^{2+} oscillations, we examined the cells loaded by calcium probe with a confocal microscope. As shown in Fig. 3E, TNF-α promoted intracellular Ca^{2+} oscillations, and D609 inhibited the promotion effect induced by TNF-α.

3.4. TNF-αenhances Ca^{2+}-NFATc1 axis by upregulating the expression of IP3R1, but not IP3R2 or IP3R3

It is mentioned by other group that TNF-α increases the expression of IP3R1 at both mRNA and protein levels in human neural cells and mesangial cells [24,29], we investigated whether the factor shows the similar effect in mouse BMMs under induction

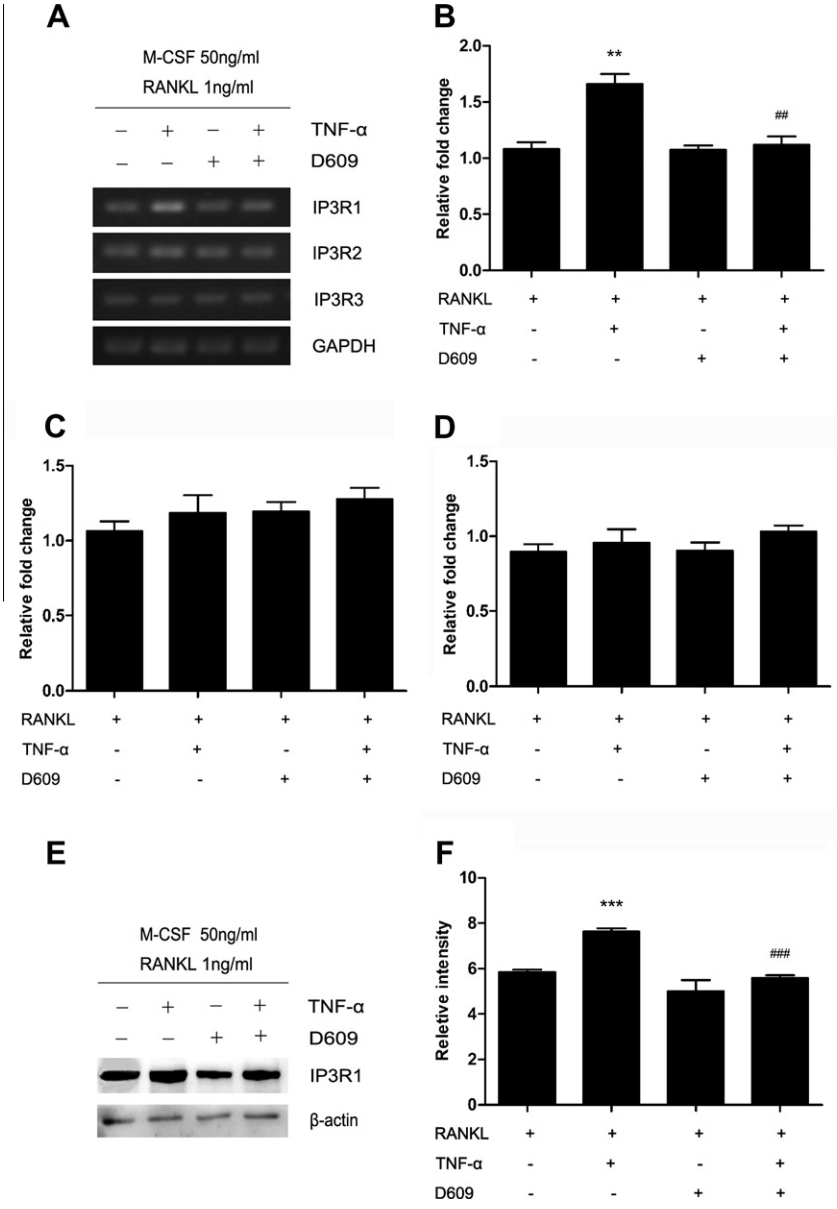

Fig. 4. TNF-α enhances Ca^{2+}-NFATc1 axis by increasing the expression of IP3R1, but not IP3R2 or IP3R3. BMMs were stimulated for 48 h by RANKL with or without TNF-α, (A) PT-PCR were performed to determine mRNA expression of IP3R1 (B), IP3R2 (C), and IP3R3 (D). Expression of IP3R1 protein level (E and F) was determined by western blot. Data are representative of 3 independent experiments. *represents vs. RANKL, # vs. RANKL plus TNF-α.

of RANKL. After BMMs were stimulated for 48 h, we detected mRNA expressions of all three types of IP3Rs. Expression of all the three receptors was detectable in RANKL-induced BMM, while addition of TNF-α caused different changes of the expression of the three subtypes. IP3R1 mRNA was raised by the addition of TNF-α consistent with its upregulation in autoamplification of NFATc1 (Fig. 4A and B). No obvious alterations of IP3R2 and IP3R3 mRNA expression were detected (Fig. 4A, C and D). We further investigated the role of PC-PLC in IP3R1 mRNA expression by using its specific inhibitor D609. D609 inhibited the promotional effect of TNF-α in RANKL-induced osteoclast differentiation without changing basal level of IP3R1 expression. As proved by Western blot enhanced IP3R1 at transcription level resulted in higher steady-state level of protein (Fig. 4E and F).

4. Discussion

Higher level of TNF-α have been detected in many diseases which are characterized by increased osteoclastic bone resorption, such as periodontitis and osteoporosis. It is universally accepted that TNF-α promotes RANKL-induced osteoclast formation both in vitro and in vivo. However, the molecular mechanism of the promotion has not been elucidated yet. Accumulating evidences indicate that TNF-α leashes this effect by direct acting on osteoclast precursor cells but not through supporting mesenchymal cells [5,30], and the direct effect plays a critical role under certain conditions in vivo [30]. TNF-α is found to affect osteoclast differentiation at late stage when sustained calcium oscillation and NFATc1 autoamplification are active [14–16].

However the relationship between TNF-α and calcium signaling in RANKL-induced osteoclastogenesis is not well studied. It has been recently found that TNF-α induces calcium oscillation after several days of culture [17]. Yip et al. have reported that Ca^{2+} –ATPase inhibitor Thapsigargin(TG) promoted RANKL-induced osteoclastogenesis and elevated intracellular ROS production[31]. They found that low concentration(10 nM) of TG which was not sufficient to raise basal level of intracellular calcium promote osteoclast formation and ROS production. However, the oscillations of calcium was not determined by their study. Long time lasting ROS production induced by RANKL is reported to mediate Ca^{2+} oscillations and essential for osteoclast precursor differentiation [25], thus intracellular ROS level is probably involved in the promotion of TNF-α on calcium oscillation. Production of ROS associates to signals mediated by TNF-α [32,33]. It is not known whether ROS contribute to TNF-α induced calcium oscillation, so we firstly tested intracellular ROS level from 5 min to 48 h after stimulation of RANKL with or without TNF-α. We observed that ROS level of BMMs was up-regulated by RANKL at 24 h and 48 h. Our data is consistent with the results reported by Kim et al. [25]. They found that ROS production as well as calcium oscillaton in mouse BMMs was elevated after the cells were stimulated for 24 h by RANKL. However, addition of TNF-α in our work did not obviously increase ROS level during the whole period of the culture. This result differs from that of other studies which reported that TNF-α stimulate ROS production [32,33]. The reason of such difference may due to different cell types which have been studied. Another possibility of the contradiction would be that RANKL activated NF-κB inhibits ROS production induced by TNF-α. Hiroyasu Nakano et al. [34] reported that TNF-α leaded to ROS accumulation in murine embryonic fibroblasts (MEFs) which were deficient in NF-κB p65 subunit, but not in wild-type MEFs. Another group demonstrated that NF-κB antagonizes apoptosis induced by TNF-α through suppressing the accumulation of ROS [35].

We further investigated another molecule involved in TNF-α signaling. PC-PLC, associated with cell proliferation, apoptosis and differentiation, is a molecule that produces DAG and phosphorylcholine from phosphatidylcholine (PC) hydrolysis. Many cytokines such as TNF-α, LPS and IFN-γ induce inflammatory response and apoptosis through activating PC-PLC in different types of cells [26–28]. It has been reported that TNF-α increases the production of IP3R1 through PC-PLC activation in human neural cells and mesangial cells, leading to higher calcium-releasing activity [24,29]. To examine the involvement of PC-PLC during osteoclast differentiation induced by TNF-α, we used a specific inhibitor of PC-PLC, D609. The toxicity of D609 was clarified by MTT assay. Cell viability was not affected by the chemical inhibitor when cultured for 48 h. TRAP staining revealed that D609 moderately increases the TRAP-positive MNCs number induced by RANKL for 72 h, while remarkably decreased the number in the group of RANKL combining TNF-α. These phenomena demonstrate that PC-PLC may participate in the promotional effect of TNF-α on osteoclastogenesis.

We next investigated how PC-PLC regulates NFATc1 autoamplification and calcium associating molecules in this process. Our results showed the amplitude of intracellular calcium oscillation and NFATc1 expression in primary murine BMMs induced by RANKL for 48 h were increased by addition of TNF-α. The IP3R1 mRNA expression was increased as well. However, the expression of IP3R2 and IP3R3 mRNA were not changed, suggesting that IP3R1 may be probably involved in the upregulation of calcium oscillation and NFATc1 autoamplification. Increased expression of IP3R1 protein implicates more ligand-binding sites for IP3, thus recruited more IP3 further leads to more sustainable calcium oscillation resulting in the enhancement of autoamplification of NFATc1 and osteoclast formation. Adding D609 significantly inhibited the regulation of IP3R1 expression caused by adding TNF-α, the inhibition conversely implicates a positive role of PC-PLC in signaling stimulated by TNF-α during RANKL-induced osteoclastogenesis.

In conclusion, we found that D609 removed the promotional effect of TNF-α on IP3R1 expression during osteoclast formation induced by RANKL. The reducing effect of D609 on IP3R1 expression correlated to the downregulation of NFATc1 expression. Exposing BMMs to D609 before the cells were conducted to differentiation decreased TRAP-positive MNC number in the culture of RANKL with TNF-α, while moderately increased the number in RANKL without TNF-α. Our data indicate that PC-PLC is involved in osteoclatogenesis induced by TNF-α probably through upregulating IP3R1 expression. The precise mechanism by which TNF-α stimulates osteoclastogenesis needs further discussion.

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