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Interleukin-35 stimulates tumor necrosis factor-α activated osteoblasts differentiation through Wnt/β-catenin signaling pathway in rheumatoid arthritis

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ABSTRACT

Interleukin (IL)-35 plays an important role in the pathogenesis of rheumatoid arthritis (RA), which is characterized by tumor necrosis factor (TNF)-α activated bone loss beginning early and persisting over time. The aim of this study was to explore the effects and signaling pathway of IL-35 on osteoblasts differentiation in MC3T3E1 cells and TNF-α activated MC3T3E1 cells. A microenvironment was established with low concentration and short-term treatment of TNF-α to mimic inflammatory activated osteoblasts of RA in vitro. The role of IL-35 on osteoblasts proliferation and apoptosis were assessed using cell counting kit (CCK)-8 assay and flow cytometry, respectively. Alkaline phosphatase (ALP) activity was measured by *p*-nitrophenyl phosphate assay. Extracellular matrix mineralization was measured by Alizarin red S staining. Osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) in response to IL-35 were investigated using real-time polymerase chain reaction and western blot analysis. Wnt/β-catenin signaling pathway in osteoblasts was investigated. In basal and TNF-α activated osteoblasts, IL-35 promoted proliferation and inhibited apoptosis. Basal and TNF-α activated ALP activity and mineralization in vitro was increased stimulated by IL-35. Furthermore, IL-35 increased the basal and TNF-α activated OPG expression and decreased basal and TNF-α activated RANKL expression. Blocking Wnt/β-catenin signaling pathway with Dickkopf (Dkk)-1 inhibited the osteogenic effects of IL-35. IL-35 stimulates basal and TNF-α activated osteoblasts differentiation through the Wnt/β-catenin signaling pathway, thus highlighting the IL-35 for pharmaceutical and medicinal applications for treating RA bone loss.

1. Introduction

The impact of rheumatoid arthritis (RA) on bone is well established both in terms of systemic bone loss including osteoporosis and fracture risk, and local bone loss including bone erosions and peri-articular bone loss. In inflammatory bone microenvironment, the balance will be broken between osteoclasts-related bone resorption and osteoblasts-related bone formation. Therefore, ideal therapeutics goal is to explore those chemicals that can inhibit bone resorption and stimulate bone formation for bone loss treatment. The wingless-type MMTV integration site family (Wnt) signaling pathway plays an important role, and its regulation is necessary for bone development [1,2]. In the bone, Wnt signaling regulates osteoblast differentiation by stimulating the Wnt/low density lipoprotein receptor-related protein 5/β-catenin/lymphoid enhancer-binding factor-T-cell factor/Runx2 signaling cascade [3]. Furthermore, Wnt/β-catenin signaling pathway can also modulate bone development by changing the osteoprotegerin (OPG)/Receptor Activator for Nuclear Factor-k B Ligand (RANKL) ratio, which can trigger osteoclastogenesis. Dickkopf (Dkk)-1 has been discovered as a secretory protein which can specifically inhibit the Wnt/β-catenin signaling pathway. In RA mice models, the treatment with Dkk-1 antibodies has shown protective effects against bone loss, indicating that it is associated with the suppression of the negative modulation of osteoblasts activity and maturation [4]. Additionally, previous studies have confirmed that multiple signaling pathways such as extracellular signal-regulated kinase/mitogen-activated protein kinases, nuclear factor κB and their crosstalk in bone are also important in the osteoblasts differentiation [5,6]. However, Wnt signaling pathway in osteoblasts, especially in TNF-α-activated osteoblasts has not been fully understood.

Tumor necrosis factor (TNF)-α is considered one of the main mediators of bone loss in RA. Several lines of evidence have demonstrated that TNF-α decreases bone formation through the promotion of

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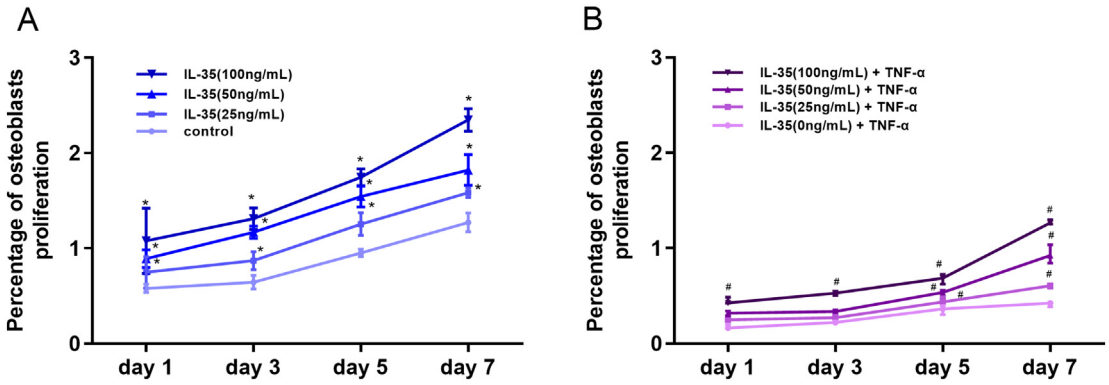

Fig. 1. The effects of IL-35 at different concentration (0, 25, 50, 100 ng/mL) for 1, 3, 5 and 7 days on the percentage of osteoblasts proliferation without and with induction of TNF-α (10 ng/mL, 6 h)..*$p < 0.05$ vs. IL-35(0 ng/mL)/TNF-α (−); #p < 0.05 vs. IL-35(0 ng/mL)/TNF-α (+). *P*-values were calculated using one-way ANOVA, followed by a Tukey's multiple comparisons post-test.

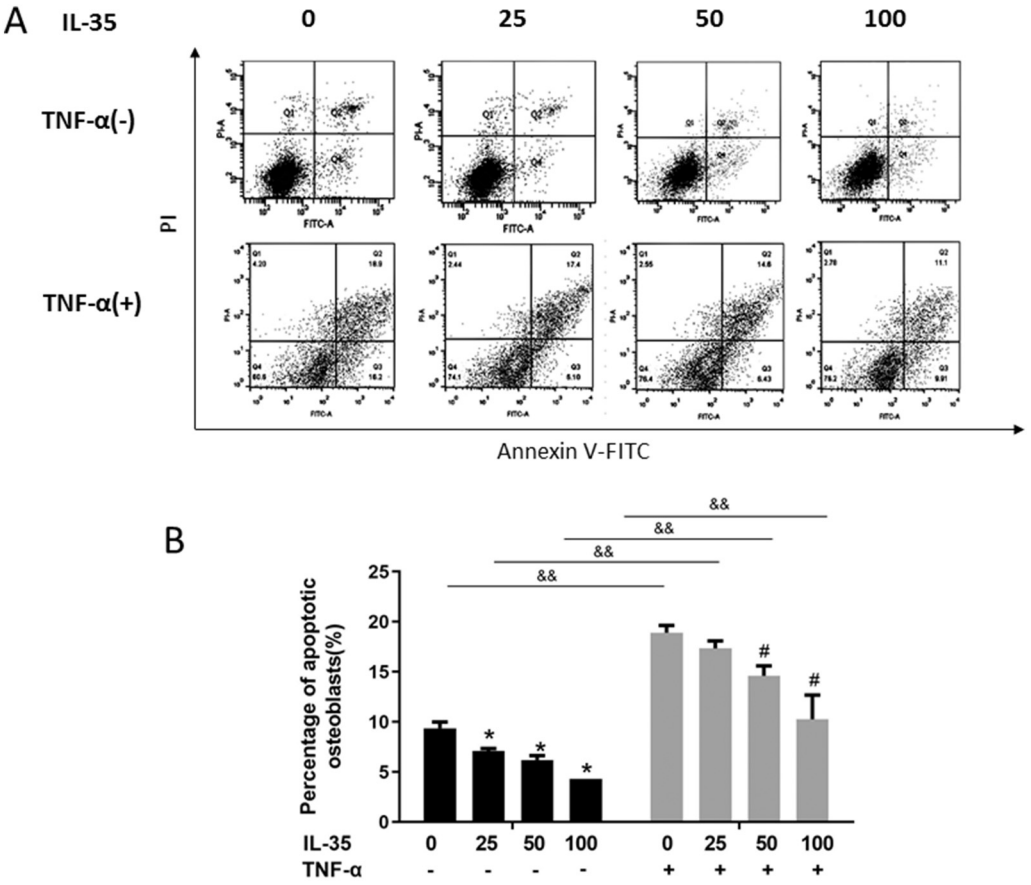

Fig. 2. IL-35 inhibits osteoblasts apoptosis. (A, B) Percentages of apoptotic osteoblasts after IL-35 treatment without and with TNF-α activation (10 ng/mL,6 h). *$p < 0.05$ vs. IL-35(0 ng/mL)/TNF-α (−); #p < 0.05 vs. IL-35(0 ng/mL)/TNF-α (+). && $p < 0.01$. *P*-values were calculated using one-way ANOVA, followed by a Tukey's multiple comparisons post-test.

osteoblasts apoptosis, the inhibition of osteoblasts differentiation and the suppression of the matrix protein expression such as osteocalcin by mature osteoblasts [7,8]. However, contradictory findings indicated that TNF-α can also activate osteoblastogenesis. In in vitro models, low concentration or short treatment of TNF-α increased osteogenic differentiation via promoting expression of ALP, Runx2 and osteocalcin [9–12]. The paradoxical role of TNF-α on osteoblasts appears to be dependent on the concentration and the exposure time as well as the differentiation state of the cell type studied.

Interleukin (IL)-35, is a heterodimer composed of Epstein Barr virus Induced gene 3 (EBI3) and IL-12p35 chains, which is found by Collison et al. in 2007 [13]. Together with IL-12, IL-23 and IL-27, IL-35 belongs to the IL-12 cytokine family. It has been substantiated that IL-35 is primarily produced by regulatory T cells (Tregs) [14]. And the subset of Tregs induced by IL-35 are termed as iTr35 cells [15]. Additionally, Tregs can induce $CD4^+Foxp3^-$ effector T cells to transform into iTr35 cells in a manner of secreting IL-35 and IL-10 [16]. Therefore, iTr35 cells exert immunosuppressive functions via IL-35, independent of IL-10 and transforming growth factor-β. An large body of literature indicated that IL-35 could inhibit the development of diverse inflammatory manifestations in experimental models such as rheumatoid arthritis, ulcerative colitis and encephalomyelitis [17–21]. In addition, IL-35 was found to inhibit TNF-α-induced osteoclastogenesis, indicating IL-35 can prevent bone loss regarding the osteoclasts [22].

However, it has not yet fully been explored whether IL-35 have an impact on basal and TNF-α activated osteoblasts. In present study, we established a microenvironment model with low concentration and short-term treatment of TNF-α which mimics inflammatory activated

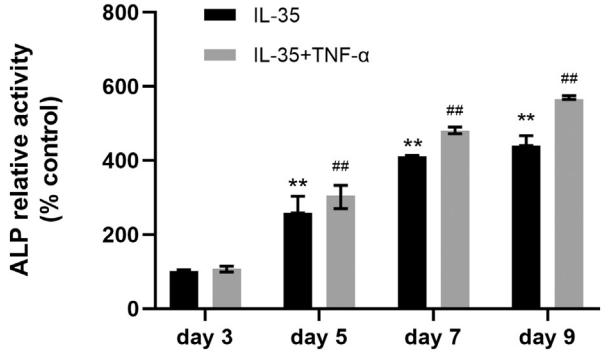

Fig. 3. ALP activity in osteoblasts after IL-35 treatment (50 ng/mL) without and with TNF-α (10 ng/mL, 6 h) activation. $^{**}p < 0.01$ vs. IL-35(0 ng/mL)/TNF-α (−); $^{\#\#}p < 0.01$ vs. IL-35(50 ng/mL)/TNF-α (+). *P*-values were calculated using one-way ANOVA, followed by a Tukey's multiple comparisons post-test.

osteoblasts of RA in vitro. We demonstrated that IL-35 could stimulate basal and TNF-α activated osteoblasts differentiation via Wnt/β-catenin signaling pathway.

2. Methods

2.1. Chemicals and reagents

IL-35 (mouse) was purchased from Sigma Company (USA). α-Modified minimal essential medium (α-MEM) was purchased from Gibco (USA). Cell Counting Kit (CCK)-8 was purchased from Dojindo Molecular Technologies (Japan). Fetal bovine serum (FBS) was purchased from Clark Bioscience Company (USA). Recombinant mouse Dkk-1 was purchased from R&D systems (USA). Alkaline phosphatase (ALP) staining kit was obtained from Beyotime Institute of Biotechnology (China). Alizarin red S solution was purchased from Beijing Solarbio Science & Technology Co., Ltd. (China). TRizol reagents and real-time polymerase chain reaction (PCR) kits were obtained from Takara Biotechnology Company (Japan) and Promega

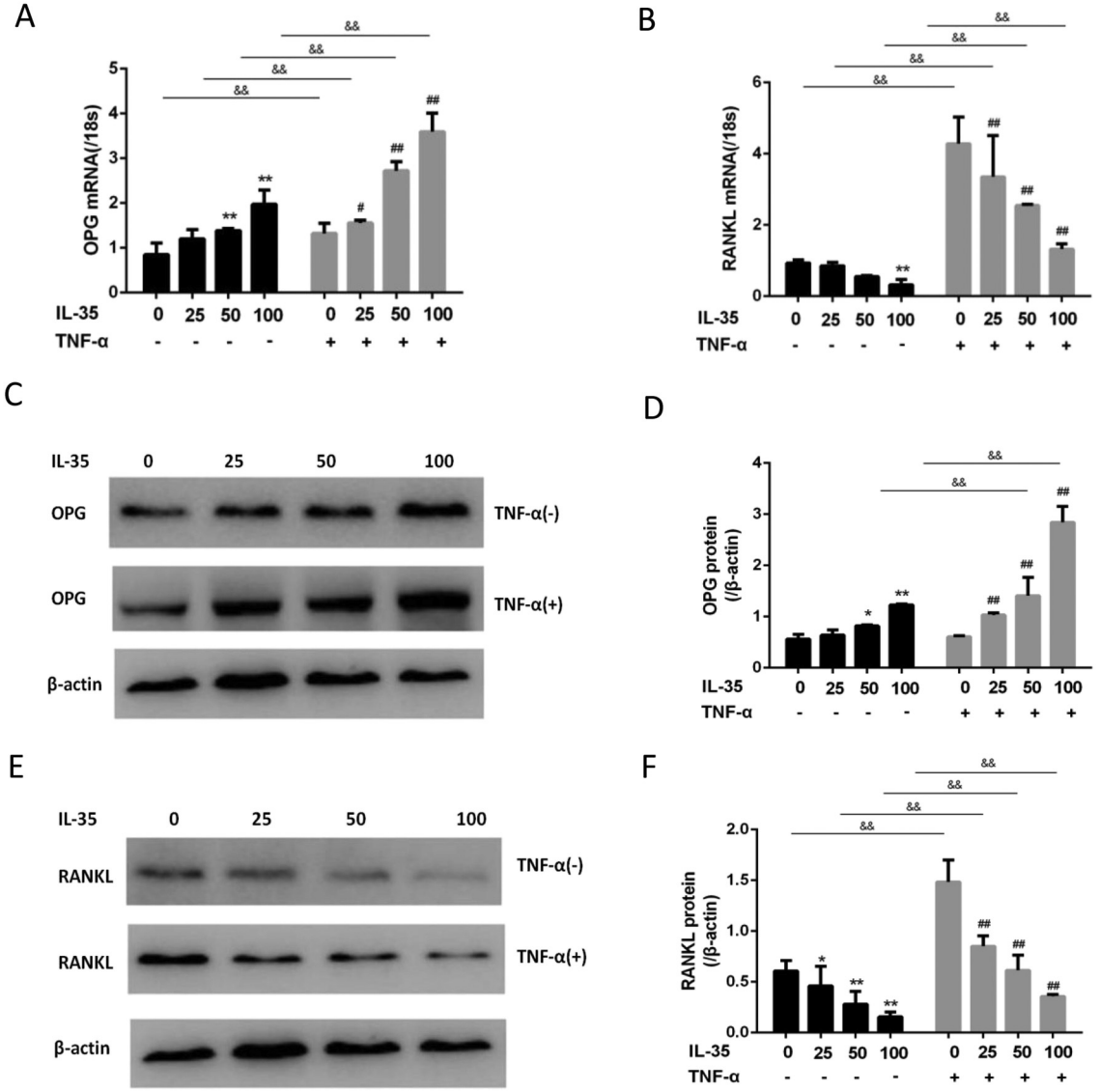

Fig. 4. (A, B) OPG and RANKL mRNA expression in osteoblasts after IL-35 treatment at different concentrations (0, 25, 50 and 100 ng/mL) without and with TNF-α activation (10 ng/mL, 6 h). (C, D, E, F) OPG and RANKL protein expression in osteoblasts after IL-35 treatment at different concentrations (0, 25, 50 and 100 ng/mL) without and with TNF-α activation (10 ng/mL, 6 h). $^{**}p < 0.01$ vs. IL-35(0 ng/mL)/TNF-α (−); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$vs. IL-35(0 ng/mL)/TNF-α (+). $^{\&\&}p < 0.01$. Comparison between two groups was conducted by Student *t*-test. *P*-values were calculated using one-way ANOVA, followed by a Tukey's multiple comparisons post-test.

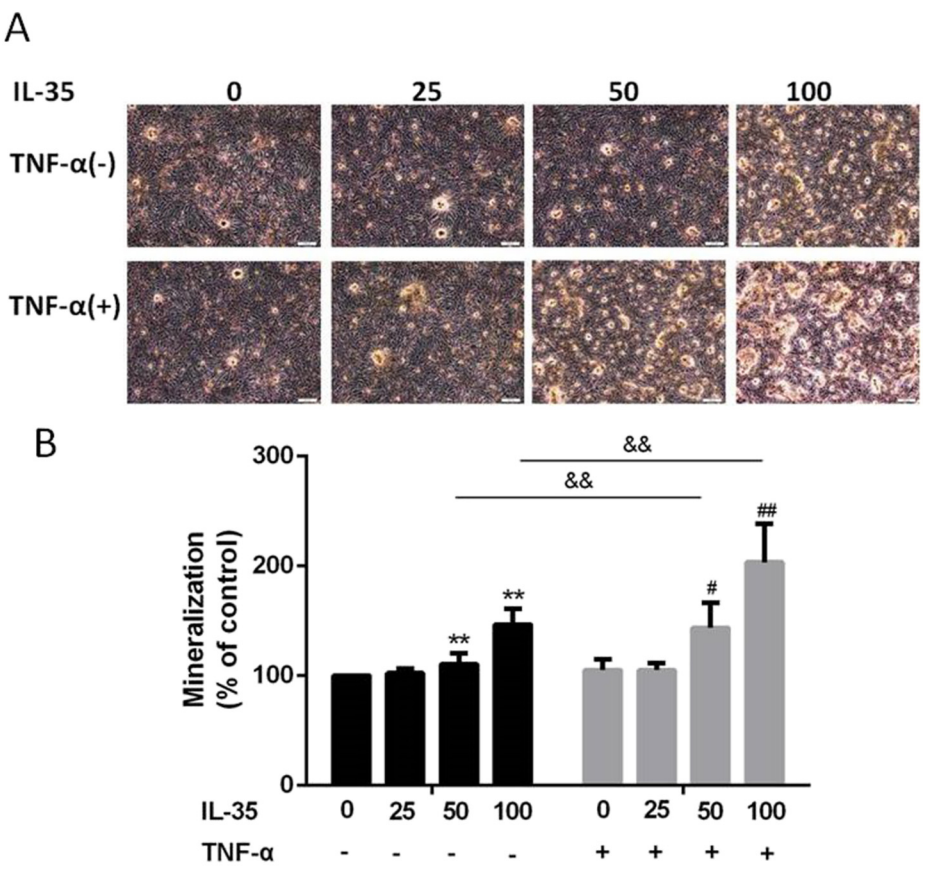

Fig. 5. Effect of IL-35 on osteoblasts mineralization. (A) With the extension of experimental time, IL-35 increased the area and the density of mineralized nodule in basal and TNF-α activated osteoblasts (magnification, ×40); (B) Evaluation of osteoblasts matrix mineralization by Alizarin Red S staining using image J software. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. IL-35(0 ng/mL)/TNF-α (−); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. IL-35(0 ng/mL)/TNF-α (+). $^{\&\&}p < 0.01$. Comparison between two groups was conducted by Student *t*-test. *P*-values were calculated using one-way ANOVA, followed by a Tukey's multiple comparisons post-test.

(USA), goat anti-mouse OPG monoclonal antibodies, goat anti-mouse RANKL monoclonal antibodies, rabbit anti-goat horseradish peroxidase (HRP)-conjugated secondary antibodies total protein extraction kit were obtained from Abcam Company (UK).

2.2. Cell culture

MC3T3E1 cells were seeded at a density of 1×10^5 cells/mL and cultured in α-MEM containing 10% FBS, 1% penicillin and streptomycin, 50 mg/mL ascorbic acid and 5 mM β-glycerophosphate in a humidified 5% CO_2 incubator at 37 °C. Without and with activation of TNF-α (10 ng/mL) for 6 h, the medium was replaced with fresh medium containing different concentrations of IL-35 (0, 25, 50 and 100 ng/mL).

2.3. CCK-8 assay to assess cell proliferation

Effect of IL-35 on cell proliferation was measured by CCK-8 assay. Briefly, osteoblasts were seeded in 96-well plates at a density of 1×10^4 cells per well. Then, cells were treated with IL-35 at various concentrations (0, 25, 50 and 100 ng/mL) for 1, 3, 5, 7 days without or with TNF-α activation. Then 10ul CCK-8 was added to each well and the mixture was incubated for 4 h at 37 °C. After shaking for 15 min, absorbance was measured on microplate reader at 450 nm.

Flow cytometry to assess apoptosis frequency.

Flow cytometry was used to detect apoptosis frequency in osteoblasts. Briefly, 1×10^6 osteoblasts were seeded into 6 well plates. Briefly, after incubation with IL-35 at various concentrations (0, 25, 50 and 100 ng/mL) for 5 days without or with TNF-α activation, cells were collected by trypsin digestion. Discard the supernatant and gently resuspend the cells by adding 195 μL of Annexin V-FITC binding solution. Then 5 μL Annexin V-FITC and 10 μL propidium iodide (PI) staining solution were added to each well and mix gently 15 min in darkness at room. The osteoblasts apoptosis frequency was analyzed by flow cytometer.

2.4. Measurement of ALP activity

Osteoblasts (1×10^4/well) were seeded in 96 well plates and then were incubated with IL-35 at different concentrations (0, 25, 50, 100 ng/mL) for 3, 5, 7 and 9 days without or with TNF-α activation. Briefly, after lysing in 0.1% Triton X-100 buffer, the ALP activity in the lysate was measured using *p*-nitrophenyl phosphate (pNPP) as the substrate, and the absorbance was measured at 405 nm. The lysate (150 μL) was incubated with 200 μL of the substrate solution containing 0.5 mM pNPP for 20 min at 37 °C. The reaction was stopped by adding 100 μL of reaction stop solution to each well.

2.5. Real time-PCR and Western blot to assess the expression of OPG and RANKL

2.5.1. Real time-PCR to assess the expression of mRNA

After incubation with IL-35 (0, 25, 50 and 100 ng/mL) for 48 h without or with TNF-α activation, total RNA in osteoblasts was then extracted using the TRIzol reagent according to the manufacturer's instructions and reverse transcribed to cDNA using SuperScriptTM III Reverse Transcriptase. The cDNA was then amplified using GoTaq® DNA Polymerase and the SYBR Green PCR Master Mix. The following primers were used: OPG (forward: 5′-TGG AGA TCG AAT TCT GCT TG-3′; reverse: 5′-ACA TCG GGA AGC GTA CCT ACA-3′) and RANKL(forward: 5′-ACA TCG GGA AGC GTA CCT ACA-3′; reverse: 5′-GCT CCC TCC TTT CAT CAG GTT-3′). 18S was included as an internal control. The relative expression of the target genes was calculated using the $^{\Delta\Delta}$CT method. All the assays were performed in triplicate.

2.5.2. Western blot to assess the expression of protein

After incubation with IL-35(0, 25, 50 and 100 ng/mL) for 48 h

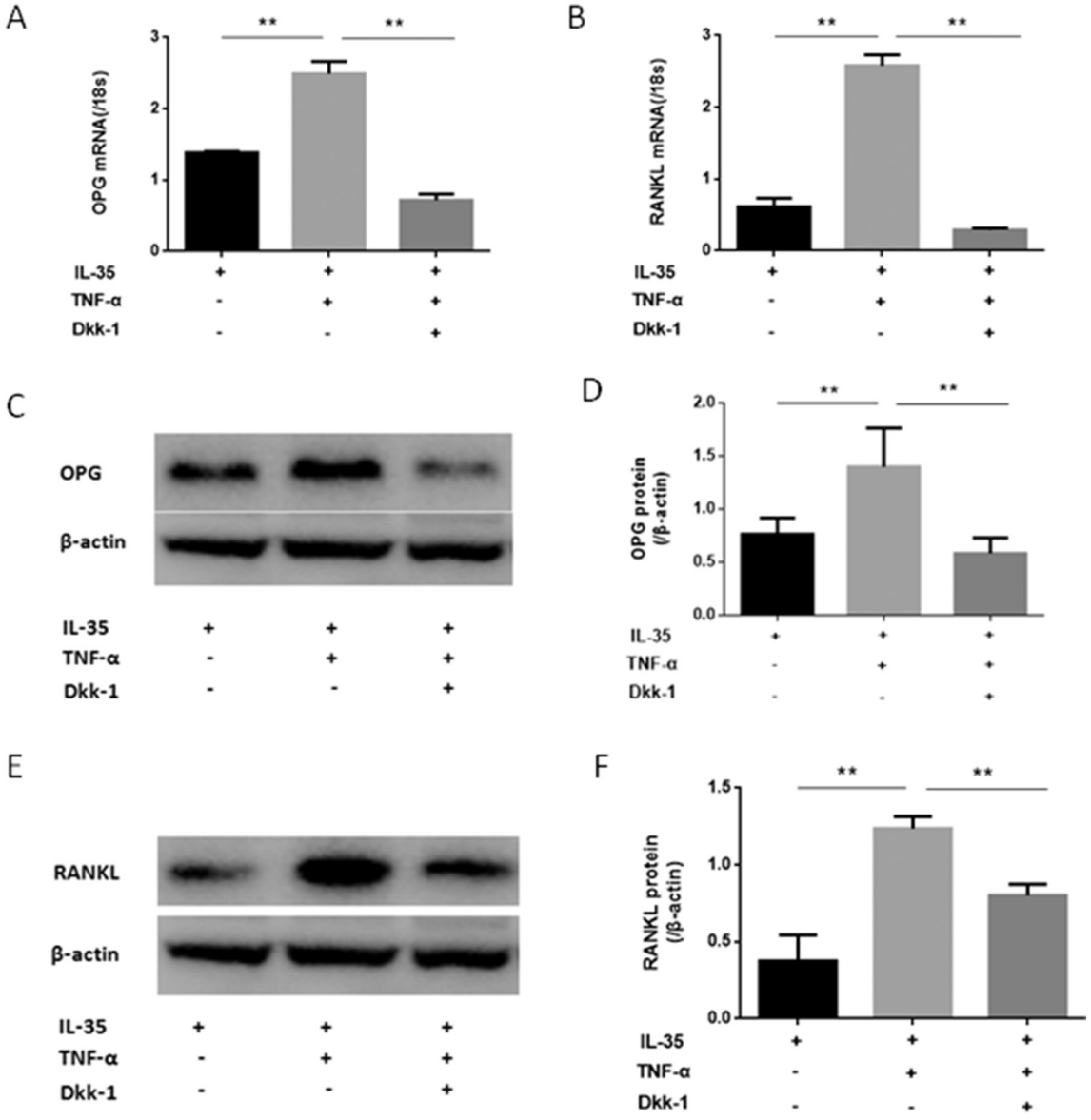

Fig. 6. Osteoblasts were pretreated with TNF-α (10 ng/mL), Dkk-1(0.1 μg/mL) and IL-35(50 ng/mL) either alone or in combination as indicated. (A, B) Dkk-1 blocks IL-35-induced OPG and RANKL mRNA in basal and TNF-α activated osteoblasts. (C, D, E, F) Dkk-1 blocks IL-35-induced OPG and RANKL protein levels in basal and TNF-α activated osteoblasts. Comparison between two groups was conducted by Student *t*-test. $^{**}p < 0.01$.

without or with TNF-α activation, the cells were washed twice with ice-cold PBS, and protein was then extracted with RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride and a 1% protease inhibitor cocktail. The total protein concentration was measured through a BCA protein assay, and the protein was stored at −20 °C; 45-75 mg of protein was subjected to SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to polyvinylidine fluoride membranes. The membranes were blotted with 5% fat-free milk for 1 h at room temperature and then incubated with the following primary antibodies: (i) anti-OPG, (ii) anti-RANKL. Then, the blots were washed with tris-buffered saline with tween (10 mM Tris-HCl, 50 mM NaCl, and 0.25% Tween 20), incubated with an HRP-conjugated secondary antibody, visualized with enhanced chemiluminescence and exposed to photographic film. β-actin was used as a loading control.

2.5.3. Alizarin red S staining to assess mineralization

Subsequently, we analyzed osteoblasts-mediated mineralization, calcium deposits which were determined by Alizarin red S staining. The cells were seeded in 6 well plates and incubated with IL-35 at different concentrations (0, 25, 50 or 100 ng/mL) without or with TNF-α activation. Briefly, at the day of differentiation (21 days later), the cells were washed three time with PBS, fixed with 4% paraformaldehyde for 30 min, washed with deionized water three time, stained with 0.2% Alizarin red S solution (pH 4.2) for 1 h at 37 °C and rinsed twice with PBS. Images of the stained calcium deposits were captured with a digital camera.

2.6. Osteogenic analysis after blockage with Dkk-1

The osteoblasts (1×10^5 cells/well in a six-well plate) were maintained in growth media for 24 h at 37 °C in 5% CO_2. After blockage with 0.1 μg/mL Dkk-1 for 24 h, osteoblasts were treated with 50 ng/mL IL-35 without or with induction of TNF-α. The experimental procedures for real time-PCR and western blot on OPG and RANKL expression were conducted as previously described.

2.7. Statistical analyses

All analyses were performed by using SPSS 17.0 (SPSS Inc., Chicago, IL) and GraphPad Prism 6 software. Differences of $P < 0.05$ were considered significant. All values were expressed as the mean ± standard deviation (SD) from at least three independent experiments. Differences between groups were assessed by Student's *t*-tests and One-way analysis of variance (ANOVA) followed by a Tukey's multiple comparisons post-test. P values < 0.05 were considered to be significantly different.

3. Results

3.1. IL-35 promoted basal and TNF-α activated osteoblasts proliferation

To identify the effect of IL-35 on TNF-α activated osteoblasts growth, we tested the cell viability rate using CCK-8 assay. As shown in Fig. 1, the results of CCK-8 assay showed that IL-35 promoted the proliferation of osteoblasts and TNF-α activated osteoblasts in a time and a dose dependent manner ($^{*}p < 0.05$ vs. IL-35(0 ng/mL)/TNF-α (−); $^{\#}p < 0.05$ vs. IL-35(50 ng/mL)/TNF-α(+).

3.2. IL-35 inhibited basal and TNF-α activated osteoblasts apoptosis

As shown in Fig. 2, after co-culturing the osteoblasts with IL-35, the apoptotic rate was decreased in osteoblasts. With TNF-α activation, the apoptotic rate was decreased as well, suggesting that IL-35 could inhibit osteoblasts apoptosis under normal condition and TNF-α activated inflammatory condition.

IL-35 increased basal and TNF-α activated ALP activity.

As shown in Figs. 1 and 2, the effect on TNF-α induced osteoblasts started from concentrations as low as 25 ng/mL, was noticeable at higher concentration (50 ng/mL) ($p < 0.05$). Therefore, IL-35 concentration of 50 ng/mL was chosen for follow-up experiment condition. IL-35 (50 ng/mL) on the ALP activity were measured using pNPP assay. The results showed that IL-35 increased ALP activity in osteoblasts. With TNF-α activation, ALP activity was remarkably elevated as well (Fig. 3).

3.3. IL-35 promoted the expression of OPG and inhibited the expression of RANKL in basal and TNF-α activated osteoblasts

OPG and RANKL were chose to investigate the effect of IL-35 on osteoblasts differentiation. The real-time PCR results showed that IL-35 significantly promoted the basal mRNA expression of OPG and inhibited the basal mRNA expression of RANKL in osteoblasts. Compared with control group (0 ng/mL IL-35), TNF-α significantly increased mRNA expression of OPG and inhibited the mRNA expression of RANKL; these effect of TNF-α were significantly enhanced by IL-35 in a dose dependently manner (Fig. 4A, B). Consistent with real time-PCR results, western blot analysis showed that IL-35 significantly promoted the basal protein expression of OPG and inhibited the basal protein expression of RANKL in osteoblasts. Compared with control group (0 ng/mL IL-35), TNF-α significantly increased protein expression of OPG and inhibited the protein expression of RANKL; these effects of TNF-α were significantly enhanced by IL-35 in a dose dependently manner (Fig. 4C,D,E,F).

3.4. IL-35 promoted basal and TNF-α activated mineralization

Last, we explored whether IL-35 treatment could stimulate matrix mineralization in osteoblasts. After IL-35 treatment for 21 days, IL-35 dose-dependently increased the area and the density of mineralized nodules. TNF-α significantly increased area and the density of mineralized nodules; this effect of TNF-α was significantly enhanced by IL-35 in a dose dependent manner (Fig. 5). Therefore, IL-35 can directly stimulate both basal and TNF-α activated matrix mineralization in vitro.

IL-35 promoted osteoblastic differentiation via the Wnt/β-catenin signaling pathway.

To confirm the involvement of the Wnt/β-catenin signaling pathway in the osteoblastic differentiation of IL-35, we tested the effects of exogenous Dkk-1 that specifically blocks the Wnt/β-catenin signaling pathway. After blocking with Dkk-1 (0.1 μg/mL) for 24 h, expression of OPG and RANKL was significantly reduced by Dkk-1 in TNF-α activated osteoblasts (Fig. 6). These results demonstrated Dkk1 inhibited the osteoblastic differentiation of IL-35, confirming that IL-35 exerts its osteoblastic differentiation through Wnt/β-catenin signaling pathway.

4. Discussion

RA is described by increased bone resorption and impaired bone formation under pathological conditions. This imbalanced bone remodeling has been observed both in the subchondral and periarticular bone of joints, where it contributes to erosions and periarticular osteopenia, and in the axial and appendicular skeleton, where it contributes to a generalized bone loss [23]. In the present study, the relationship between IL-35 and MC3T3E1 cells line was probed, a type of pre-osteoblasts cells line. During differentiation the MC3T3E1 cells line maintains its unique biological properties of osteoblasts such as ALP activity, matrix calcification [24]. Therefore, it is a common cell model for bone remolding research.

In present study, IL-35 promoted basal and TNF-α activated MC3T3E1 cells proliferation and inhibited basal and TNF-α activated MC3T3E1 cells apoptosis. We speculated that IL-35 stimulated bone formation through an increase in osteoblasts cell number and further an increase in bone mass in vitro. Previous studies have shown that differentiation of MC3T3E1 cells line occurs via two phases: an early phase and a late phase [25]. The early phase, which is replication without mineralization, occurs between days 1 and 9 and a late phase (from day 16 onwards) involves growth arrest, extracellular matrix formation and mineralization. ALP is considered the most ample glycoprotein in the extracellular matrix, and it is secreted by osteoblasts at the early stage of differentiation [26]. In current study, ALP activity was significantly increased in response to IL-35. This result indicated IL-35 could promote osteoblasts differentiation at an early stage. Furthermore, in the late stage, the matrix becomes mineralized. And calcium deposits formation are indicators of matrix mineralization. To estimate calcium deposits for bone nodule formation in the extracellular matrix, the cellular matrix was stained with Alizarin red S dye, which combines with calcium in the matrix [27]. In our study, IL-35 significantly stimulated matrix mineralization, indicating IL-35 stimulated osteoblasts differentiation at a late stage.

RANKL and OPG are key factors in the process of bone differentiation. RANKL regulates osteoclastogenesis by binding receptor activator of NF-κB (RANK) secreted by osteoclasts, whereas OPG serves on a decoy receptor of RANKL to restrain osteoclastogenesis [28]. Osteoclastogenesis hinges on the ratio of OPG to RANKL secreted by osteoblasts [29]. In our study, expressions of TNF-α activated RANKL and OPG are increased compared with basal expressions, suggesting that there is high bone remodeling activity in inflammatory conditions. In response to IL-35, the OPG expression was significantly increased and RANKL expression was significantly decreased, revealing an osteoblastic effect of IL-35 on osteoblasts under basal conditions or with TNF-α activation, suggesting that IL-35 can promote RA osteoblasts differentiation via changing OPG/RANKL ratio.

Evidence suggests that Wnt/β-catenin signaling pathway plays a central role in osteoblasts differentiation [30]. Meanwhile, Dkk-1 could specifically inhibit the Wnt/β-catenin pathway [31]. Mice over-expressing Dkk-1 in osteoblasts develop osteopenia due to impaired bone formation, suggesting that Dkk-1 could negatively modulate bone formation [32]. In present study, IL-35 significantly increased OPG and

inhibited RANKL expression; this effect of IL-35 was significantly impaired by Dkk-1. Previous studies have shown β-catenin could prevent osteoclastic bone resorption by influencing OPG and RANKL expression in osteoblasts [33]. The crosstalk between Wnt/β-catenin signaling pathway and OPG/RANKL likely engages the interplay in RA bone loss. The precise mechanisms involved need further investigation. Our findings in this study suggested that IL-35 might attenuate RA bone loss progression by stimulating bone differentiation via regulation of the Wnt/β-catenin signaling pathway, suggesting a potential treatment target for RA bone loss in the future.

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Yuxuan Li, Hui Shen, Liping Xia and Jing Lu designed the study and prepared the first draft of the paper. Jing Lu is guarantor. Yuxuan Li contributed to the experimental work. Lin Yuan, Shenyi Jiang and Siyan Liu were responsible for statistical analysis of the data. All authors revised the paper critically for intellectual content and approved the final version. All authors agree to be accountable for the work and to ensure that any questions relating to the accuracy and integrity of the paper are investigated and properly resolved.

Declaration of competing interest

None.

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