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# Lenalidomide regulates osteocytes fate and related osteoclastogenesis via IL-1β/NF-κB/RANKL signaling

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ABSTRACT

Osteolytic diseases are closely associated with osteocyte fate, indicating a more efficient and crucial role of osteocyte-targeting strategy in inhibiting osteoclastogenesis. Here, we investigated the effects of lenalidomide (Lena) on osteocyte fate in order to regulate osteoclastogenesis via effective cascade-controlling response. Our data revealed that lenalidomide treatment notably rescued IL-1β induced loss of osteocyte viability by inhibiting osteocyte apoptosis with decreased osteoclast-related factors, RANKL and Sclerostin, as demonstrated by the restricted osteoclast formation and reduced bone resorption. Additionally, iTRAQ assay revealed that IL-1β induced activation of NF-κB inhibitor α/β were remarkably downregulated by lenalidomide, showing that lenalidomide impaired NF-κB signaling in osteocytes for inhibiting the expression of osteoclast specific genes in osteoclasts, which was further confirmed by KEGG pathway analysis and Western blot. More interestingly, the *in vivo* analysis of osteocyte apoptosis and osteoclastogenesis in osteoarthritis mice model indicated a role of lenalidomide in the regulation of osteocyte fate and the consequent inhibition of RANKL-induced osteoclastogenesis. Together, these results suggest that lenalidomide regulates osteocyte fate by attenuating IL-1β/NF-κB signaling, thereby inhibiting RANKL expression for the attenuated osteoclastogenesis both *in vitro* and *vivo*, indicating a more efficient remedy among future anti-osteoclastogenesis approaches.



## 1. Introduction

Osteolytic lesions are a characteristic feature of a number of bone diseases including multiple myeloma, breast cancer bone metastasis, osteoporosis, and osteoarthritis [1–4]. Bone destruction triggered by increased activation of osteoclasts contributes to the impaired bone physiology and skeletal functionality. Therefore, it is crucial to explore the novel approaches that mediate the inhibition of osteoclast activation to improve the quality and span of life of afflicted patients efficiently.

The formation of osteoclasts from hematopoietic progenitors is activated by various stimulating cytokines, such as receptor activator for nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (MCSF) [5]. Since RANKL is indispensable for osteoclastogenesis, numerous studies have described the utilization of RANKL-inhibitory strategies in attenuating the survival and differentiation of osteoclast precursors [6,7]. Osteocytes, the most abundant cells in the bone tissues [8,9], secrete RANKL *in vivo* and thus maintain a balance between osteoclastogenesis and osteoblastogenesis [10,11]. Previously, our group found that targeting osteocytes could attenuate $1,25(OH)_2D_3$-induced osteoclast formation in breast cancer bone metastasis nude mice and it could serve as an effective anti-osteoclastogenesis strategy [12]. In addition to hormonal stimulus, apoptosis also increases the expression of RANKL in osteocytes [13]. Hence, targeting apoptotic osteocytes may be a promising approach for the treatment of osteoclast-associated osteolytic diseases.

Lenalidomide (Lena) has shown potential application as an anti-cancer and anti-inflammatory drug [14,15]. Lenalidomide has been shown to reduce abnormal bone formation and neovascularization in an osteoarthritis model by inhibiting tumor necrosis factor α (TNF-α) [16]. Despite the extensive applications of lenalidomide in pre-clinical translational researches, its role in regulating the fate of osteocytes remains largely unexplored. In addition, numerous reports [17–20] mention that osteocyte apoptosis can be studied in

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the subchondral bone region of an anterior cruciate ligament transection (ACLT) osteoarthritis (OA) mouse model that displays significant osteolysis. Therefore, we employed this model to establish the microenvironment of apoptotic osteocytes *in vivo*. In a nutshell, we induced osteocyte apoptosis and investigated the effects of lenalidomide on apoptotic osteocytes and subsequent osteoclastogenesis *in vitro* and *in vivo*, thereby, aiming at gaining novel insights into the application of lenalidomide in osteolytic disorders.

## 2. Materials and methods

### 2.1. Cell culture

Osteocytic MLO-Y4 cells were cultured on dishes pre-coated with rat-tail collagen I in α-Minimum Essential Meidum (α-MEM) supplemented with 5% calf bovine serum and 5% fetal bovine serum. Murine bone marrow monocytes (BMMs) were harvested from the tibiae and femurs of C57BL/6 mice and cultured in α-MEM with 30 ng/ml MCSF and 10% FBS. Cells were incubated at 37 °C in the presence of 5% $CO_2$.

### 2.2. Cell viability

The viability of MLO-Y4 cells was evaluated with the Cell Counting Kit-8 (CCK-8) method [21]. Osteocytes were treated with various concentrations of IL-1β and lenalidomide for different time-points. Optical density at 450 nm ($OD_{450}$) was measured.

### 2.3. Flow cytometry

MLO-Y4 apoptosis was assessed by flow cytometry following Annexin V/PI staining [22]. MLOY-4 cells were treated with IL-1β and lenalidomide and the proportion of apoptotic cells (%) was presented by the addition of events in the upper right ($FITC^+/PI^+$) and lower right ($FITC^+/PI^-$) quadrants.

### 2.4. Enzyme-linked immunosorbent assay (ELISA)

The supernatant and animal serum was collected to determine the expression of RANKL, sclerostin and CTX via the ELISA kits following the manufacturers' instructions.

### 2.5. Apoptotic osteocyte-induced osteoclastogenesis

We used two approaches to investigate the relationship between apoptotic osteocytes and osteoclasts. For osteocyte CM-induced osteoclastogenesis [23], we collected the CM from osteocytes following treatment with 50 ng/ml IL-1β and varying concentrations of lenalidomide. Next, the osteocyte CM was supplemented with 10 ng/ml RANKL and 50 ng/ml MCSF for subsequent osteoclast stimulation. The medium was changed after every 48 h for 7 consecutive days. To establish the co-culture system [24], MLO-Y4 cells were first stimulated with 50 ng/ml IL-1β to induce apoptosis, followed by the addition of BMMs after 24 h in the same well. Exogenous RANKL was not added since apoptotic osteocytes alone are supposed to support osteoclastogenesis via enhanced release of endogenous RANKL by osteocytes. The medium in the co-culture system supplemented with 30 ng/ml MCSF and varying concentrations of lenalidomide was changed for 7 consecutive days.

### 2.6. Osteoclast staining in vitro

Tartrate-resistant acid phosphatase (TRAP) staining was used to analyze the mature multinucleated osteoclast [25]. TRAP-positive multinucleated cells with at least three nuclei were classified as mature osteoclasts.

### 2.7. Bone resorption pit formation

BMMs were firstly seeded on bovine bone slices. After osteocyte co-culture and CM stimulation, cells were removed by mechanical agitation and sonication. Bone resorption pits were observed under scanning electron microscope (SEM).

### 2.8. iTRAQ labeling and KEGG pathway analysis

To determine the possible pathway in osteocytes after lenalidomide, we isolated proteins from osteocytes for LC-ESI-MS/MS analysis [26,27]. The KEGG pathway analysis was subsequently performed [28].

### 2.9. Quantitative real-time PCR

After osteocyte-CM stimulations, BMMs were harvested to extract total RNA using the Qiagen RNeasy Mini Kit. Primers used are listed in Table S1.

### 2.10. Western blotting

MLO-Y4 cells treated with IL-1β and lenalidomide were harvested for assessment of NF-κB signaling in osteocytes. Cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors. The total protein extract (30 μg) was separated on 10% SDS-PAGE and transferred to a PVDF membrane to visualize the protein expression levels under Odyssey Infrared Imaging System.

### 2.11. Osteoarthritis mice evaluation

Twenty-four 6-week-old C57BL/6 mice were obtained from Shanghai Slac Laboratory Animal Company and kept under specific pathogen-free conditions. After the anesthetization of mice (n = 18), the stifle knee joint of the right hind limb was shaved and disinfected. A medial parapatellar skin incision was made to provide access to the joint space. The ACL was transected, after which an anterior drawer test was performed to ensure complete disruption of the ligament. The incision was then closed and mice were transported to warm conditions for recovery. No fatalities were observed due to surgery.

Three days later, the ACLT mice were divided into three groups randomly: OA group, low-dose lenalidomide group (L-Lena) and high-dose lenalidomide group (H-Lena) (n = 6 for each). The non-ACLT mice were used as control (n = 6). The control and OA group mice received 500 μL of sterile PBS injection intraperitoneally 3 times a week, while L-Lena and H-Lena mice were treated with 500 μL lenalidomide of 1 mg/ml and 2 mg/ml per Kg body-weight intraperitoneally 3 times a week. Mice were injected and observed for 8 weeks.

### 2.12. μCT, histological and immunohistochemical analyses

8 weeks after drug administration, mice were sacrificed and the right hind limbs were harvested for μCT scan [12]. Subchondral bone of knee joint was paraffin-embedded and sectioned for hematoxylin/eosin (HE), caspase-3, RANKL, and TRAP staining following protocols [12].

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