Original Article
IL-17A promotes osteoclast formation via inducing RANKL in synovial cells

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Abstract: Rheumatoid arthritis (RA) can cause the destruction of articular cartilage and bone tissue. As a kind of important support cells in the process of osteoclast differentiation, human fibroblast-like synoviocytes (HFLS) inflammation degree and regulatory cytokine expressions directly affect osteoclast differentiation formation and absorption. This study explored the role of IL-17 in affecting HFLS and osteoclasts. Articular cavity fluid was collected from RA and osteoarthritis patients. IL-17A content was tested by enzyme-linked immunosorbent assay (ELISA). Th17 cell ratio was determined by flow cytometry. HFLS-osteoclast co-culture system was treated by 5 ng/ml IL-17A. Ki-67 expression in HFLS was evaluated by flow cytometry. Osteoclast cell number and bone resorptive pits were calculated. The co-culture system was divided into control, IL-17A group, and IL-17A + antibody group. RANKL mRNA and protein expressions, nuclear NFATc1 expression, osteoclasts formation, and bone resorption were compared. IL-17A content and Th17 cell ratio in the synovial fluid were significantly higher in RA patients compared with control. IL-17A obviously upregulated Ki-67 expression, promoted osteoclast formation, and increased bone resorptive pits in HFLS. IL-17A markedly upregulated RANKL mRNA and protein expressions in co-cultured HFLS, while IL-17 antibody treatment significantly declined RANKL mRNA and protein levels in HFLS, suppressed HFLS proliferation, reduced NFATc1 expression in the nucleus of osteoclast, decreased osteoclast formation, and attenuated bone absorption. IL-17A content and Th17 cell ratio significantly increased in the synovial fluid of RA patients. IL-17A promoted osteoclast differentiation and enhanced bone absorption via inducing RANKL expression in HFLS.

Keywords: RA, osteoclast, HFLS, IL-17A

Introduction
Rheumatoid arthritis (RA) is a kind of systemic disease with complex pathogenesis and featured as chronic synovitis. It may involve small joints on hands and feet, and characterized as multiple joints, symmetry, and invasive. Severe cases can result in joint deformity and loss of function, which seriously influence work, life ability, and quality of life [1, 2]. Female is a high-risk group for RA, whose incidence is 2~3 times higher than male [3]. Bone destruction is an important reason to induce joint structure and function disorder in RA patients. Bone destruction refers to multiple processes including Bone destruction in the process of RA pathogenesis involves the destruction of articular cartilage destruction, subchondral bone destruction, para-articular osteopenia and synthetic bone loss, of which osteoclast function enhancement play a critical role in promoting bone absorption and inducing bone loss [4, 5]. Interleukin-17 (IL-17) is a recently discovered pro-inflammatory cytokine that mainly expressed and secreted by Th17 cells [6]. Numerous studies suggested that IL-17 played a key role in promoting inflammation and mediating autoimmune, which is closely associated with various autoimmune diseases, such as RA [7], multiple sclerosis [8], and systemic lupus erythematosus [9].

As an inflammatory cytokine, IL-17 participates in the development of RA through inducing granulocytes and macrophages accumulation, infiltration, and inflammatory factors release, suppressing chondrocytes matrix synthesis, and inducing cartilage matrix degradation [10].
Recent study revealed that IL-17 was also involved in regulating osteoclasts differentiation and affecting osteoclasts function in RA [11]. Receptor activator of NF-κB ligand (RANKL) is an important activator in the differentiation process of osteoclasts function by acting on receptor activator of NF-κB (RANK) receptor to activate downstream NF-κB signaling pathway and promote osteoclasts differentiation and maturation [12]. Fibrous synovial cell is the major secretory cell of RANKL. It can interact with osteoclasts to regulate osteoclast differentiation [13]. As an important support cell in the process of osteoclast differentiation, the inflammation degree and osteoclast differentiation regulatory factor expression in synovial cells directly affects osteoclasts formation and absorption. This study investigated the role of IL-17 in affecting RANKL expression and regulating osteoclasts formation in synovial cells.

**Materials and methods**

**Main reagents and materials**

DMEM and fetal bovine serum (FBS) were purchased from Hyclone (USA). TRAP staining kit was got from Sigma (USA). Recombinant Human IL-17A Protein and Human IL-17A Antibody were bought from R&D Systems (USA). IL-17 ELISA kit was obtained from RayBiotech (USA). Recombinant human M-CSF was acquired from Sino Biological Inc (Beijing, China). PCR primers were synthetized by Sangon (Shanghai, China). Realtime PCR kit and SYBR Green dye were purchased from Toyobo (Japan). RANKL antibody and Lamin B antibody were obtained from Cell Signaling Technology (USA). Vimentin antibody was bought from Abcam (USA). NFATc1 antibody was provided by Santa Cruz (USA). Secondary antibody was purchased from Jackson ImmunoResearch (USA).

**Clinical sample collection**

A total of 39 cases of RA patients received arthroscopic synovectomy between Oct 2015 and Aug 2016 in our hospital were enrolled, including 14 males and 25 females with mean age at 56.3 ± 13.1 (42-67) years old. The resected synovial tissues and synovial fluid were collected. No patient received vitamin D, corticosteroids, or immunosuppressant drugs treatment before surgery. Another 21 cases of osteoarthritis (OA) patients were selected as control to extract synovial fluid. The bone marrow used in this study was obtained from the patients received iliac grafting in our hospital. All the enrolled subjects had signed informed consent and the study was approved by the ethics committee.

**Human fibroblast-like synoviocytes (HFLS) isolation and cultivation**

The adipose on the surface of synovial tissue was removed. Next, the tissue was cut into pieces and digested by 0.1% collagenase type II at 37°C for 3 h. Then the tissue was further digested by 0.05% Trypsin-EDTA for 10 min and stopped by DMEM containing 10% FBS. After filtered by strainer, the mixture was centrifuged at 1200 rpm for 10 min. Then the cells were resuspended in DMEM containing 10% FBS and 1% penicillin-streptomycin. After cultured in 37°C and 5% CO₂, the cells were passaged at 1:4. The cells in the fourth generation were used for identification and the following experiment.

**Immunofluorescence detection**

The synovial cells were washed by PBS for twice and fixed in 4% paraformaldehyde for 15 min. After washed by PBS for three times, the cells were treated by 0.1% Triton® X-100 at room temperature for 30 min and blocked by BSA at room temperature for 60 min. Next, the cells were incubated in mouse anti human Vimentin antibody (1:200) at 4°C overnight and Alexa Flour 594 labeled secondary antibody (1:200) at room temperature for 60 min. At last, the slice was stained by 0.1% DAPI for 1 min and observed under the fluorescent microscope.

**ELISA**

A total of 100 μl diluted IL-17 standard substance or sample were added to the 96-well ELISA plate coated by IL-17 antibody and incubated at room temperature for 2.5 h. After washed by 1× Wash Solution for four times, the plate was incubated in 100 μl biotin labeled secondary antibody at room temperature for 60 min. Next, the plate was treated by 100 μl streptavidin solution at room temperature for 45 min. Then the plate was added with 100 μl TMB One-Step substrate reagent at room temperature for 30 min. At last, the plate was treated by 50 μ stop solution and measured at 450 nm.
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Calf cortical bone preparation

The fresh calf limb cortical bone was fixed in 5% glutaraldehyde for 3 h, and then dehydrated by gradient ethanol. After ultrasonic washed by 0.25 mol/L weak aqua ammonia, the bone was irradiated by γ-ray. At last, the tissue was stored at PBS containing 2% penicillin-streptomycin.

Osteoclast induction

The bone marrow was diluted by DMEM at 1:2 and resuspended in DMEM containing 10% FBS, 20 ng/ml M-CSF, and 1% penicillin-streptomycin after centrifugation. Next, the cells were seeded in 24-well plate with cortical bone to test bone resorptive pits. The other cells were seeded in 24-well plate without cortical bone to calculate osteoclast number.

Synovial cells-osteoclasts co-culture

Transwell chamber was put into the abovementioned 24-well plate seeded by osteoclasts with or without cortical bone. The synovial cells in the fourth generation were seeded into the upper chamber. After cultured for 72 h, the cells were collected to detect the related index.

Osteoclasts and bone resorptive pits observation

After cultured in 24-well plate without cortical bone for 7 days, the cortical bone was ultrasonic washed by weak aqua ammonia, stained by toluidine blue o, differentiated by hydrochloric acid alcohol, and dehydrated by acetone to calculate the bone resorptive pits number.

qRT-PCR

Total RNA was extracted and reverse transcribed to cDNA using random primer or oligoDT primer. The cDNA was used as template to perform PCR amplification. The primer sequences used were as follows. RANKL: forward, 5’-CAA-CATATCGTGGATCACAGCA-3’; reverse, 5’-GAG-AGACTCATTATGGGAAAC-3’; β-actin: forward, 5’-GAACTCATTATGGGAAAC-3’; reverse, 5’-TGTCACGCAGATTCC-3’. The total reaction system contained 5 μl 2× SYBR Green Mixture 5.0 μl, 0.5 μl positive and reverse primers, 1 μl cDNA, and 3.0 μl ddH2O. The reaction was performed at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min on ABI 7500.

Western blot

Total protein was extracted and separated by SDS-PAGE. Then the protein was transferred to PVDF membrane and blocked by 5% skim milk at room temperature for 60 min. Next, the membrane was incubated in primary antibody (RANKL at 1:300, NFATc1 at 1:200, Lamin B at 1:300, β-actin at 1:600) at 4°C for 12-14 h and horse radish peroxidase labeled secondary antibody at room temperature for 60 min. At last, the membrane was developed by ECL and scanned.
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Statistical analysis
All data analyses were performed on SPSS 18.0 software. The measurement data were presented as mean ± standard deviation and compared by t test or ANOVA. P < 0.05 was considered as statistical significance.

Results
IL-17 overexpressed in the synovial fluid of RA patients
ELISA assay demonstrated that compared with control, IL-17 content significantly increased in synovial fluid from RA patients (Figure 1A). Flow cytometry revealed that Th17 cell percentage in synovial fluid from RA patients was obviously higher than the control (Figure 1B). It suggested that IL-17 participated in the pathogenesis of RA.

IL-17 induced HFLS proliferation, promoted osteoclasts formation and bone absorption
Immunofluorescence detection showed strong Vimentin positive reaction in the isolated cells from the fourth generation, confirming HFLS (Figure 2A). Flow cytometry exhibited that Ki-67 expression in HFLS treated by 5 ng/ml IL-17A was obviously higher than the control, indicating that IL-17 induced HFLS proliferation (Figure 2B). Co-culture system demonstrated that IL-17 markedly promoted osteoclasts formation and increased bone resorptive pits number in the cortical bone (Figure 2C).

IL-17A blockage suppressed HFLS proliferation, inhibited osteoclasts formation, and restrained bone absorption
Flow cytometry revealed that IL-17 antibody significantly downregulated Ki-67 expression in HFLS treated by IL-17A (Figure 3A). Osteoclasts formation and bone absorption ability obviously declined after IL-17 antibody treatment (Figure 3B). qRT-PCR detection demonstrated that RANKL mRNA expression was upregulated in co-cultured HFLS by IL-17A, while it was reduced by IL-17 antibody (Figure 3C). Western blot showed that RANKL protein expression was enhanced in co-cultured HFLS by IL-17A, while it was decreased by IL-17 antibody (Figure 3D).

Discussion
The maintenance of healthy bone shape and normal bone mass relies on the dynamic balance of osteoblast and osteoclast. Bone absorption and damage occurred when some pathological factors increase osteoclasts number and enhance function over the compensatory capacity of osteoblasts. As the precursor cell of osteoclast, monocyte/macrophage can differentiate and fuse to a huge multinuclear macrophage, namely osteoclast, under the effect of M-CSF and other factors. Osteoclasts mainly degrade bone matrix through acid decalcification and protein hydrolysate to play an important role in bone destruction and bone resorption [14]. It was showed that RANKL was the key activator of precursor osteoclasts in differentiating into mature osteoclasts. RANKL could be expressed and secreted by various cells, such as fibroblast-like synoviocytes, osteoblasts, macrophages, and T cells [13]. RANK is an important receptor on the precursor osteoclasts. After binding with RANK, RANKL can promote iκB phosphorylation degradation through TRAF6 to activate NF-κB p65 nuclear translocation. It further activates NF-κB signaling pathway to enhance c-Fos expression, leading to the upregulation of NFATc1 to promote osteoclasts differentiation [12]. Osteoprotege-
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Figure 3. IL-17A blockage suppressed HFLS proliferation, inhibited osteoclasts formation, and restrained bone absorption. A. Flow cytometry detection of Ki-67 expression. B. Osteoclasts and bone resorptive pits counting. C. qRT-PCR detection of RANKL mRNA expression in HFLS. D. Western blot detection of protein expression. *P < 0.05, compared with control. #P < 0.05, compared with IL-17A + antibody.

OPG (OPG) is a false receptor of RANKL, which can competitive inhibit the binding between RANKL and RANK, thus to block the differentiation and maturation of osteoclast and induce osteoclast apoptosis [13]. RANKL/RANK/OPG mechanism axle is the most important signaling pathway in regulating osteoclast differentiation, maturation, and apoptosis. RANKL overexpression and/or OPG deficiency may lead to the excessive activation of osteoclasts, resulting in bone destruction and absorption. Proliferative synovial inflammation is the pathological basis of cartilage and bone destruction in RA patients. As an important support cell in osteoclast differentiation, HFLS proliferation and inflammation play a critical role in RA pathogenesis, and bone and cartilage destruction.

IL-17 is mainly expressed and secreted by Th17 cells. It also can be produced by innate immune cells, including CD8+ T cells, NKT cells, mast cells, and neutrophils. IL-17A is a major member in IL-17 family. It was exhibited that IL-17 plays a key role in various autoimmune diseases [8, 9]. IL-17 participates in the pathogenesis of RA through inducing inflammatory cells accumulation and inflammatory factors release, inhibiting osteoclasts matrix synthesis, and inducing cartilage matrix degradation [7]. Recent studies demonstrated that the regulation of osteoclasts differentiation and function is also the pathogenesis of IL-17 in RA [11]. As an important support cell in osteoclast differentiation process, synovial cell inflammation and osteoclast differentiation regulatory factor expression directly affect the osteoclast formation and absorption. This study explored the impact of IL-17 in synovial cells and osteoclasts.

Our results showed that IL-17 content significantly increased in synovial fluid from RA patients, while Th17 cell percentage also increased. It suggested that IL-17 participated in the pathogenesis of RA. Tsai et al. [15] reported that IL-17 content and Th17 cell expression obviously elevated in the synovial fluid from RA patients. Miao et al. [16] demon-
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strated that the ratio of IL-17 secreted cells in peripheral blood from RA patients was markedly higher than the healthy control, and was closely associated with the disease activity. The cell proportion in high activity cases was obviously higher than that in low activity patients. It could be treated as the specific indicator to distinguish the disease activity. Pavlovic et al. [17] also observed IL-17A content elevation in the peripheral blood of RA patients. Hwang et al. [18] indicated that synovial fluid mononuclear cells in synovial fluid from RA patients highly expressed IL-17A. This study found IL-17 content upregulated in synovial fluid from RA patients, which was in accordance with Tsai [15] and Miao [16]. Flow cytometry revealed that IL-17A markedly induced HFLS proliferation. Lee et al. [19] reported that IL-17 promoted HFLS survival and proliferation through activating STAT3 to upregulate Bcl-2 expression. Hashizume et al. [20] also found that IL-17 stimulus enhanced FLS cell proliferation. This study observed HFLS proliferation increased after IL-17 treatment, which was in accordance with Lee [19] and Hashizume [20]. Uluckan et al. [21] discovered that IL-17A can facilitate bone absorption and loss through downregulating the activity of Wnt signaling pathway. Kotake et al. [22] found that IL-17 induced osteoclasts differentiation and maturation in osteoblasts-osteoclasts co-culture system, thus to play its role in the pathogenesis of RA. Moon et al. [11] suggested that IL-17 can synergistically act with IL-32 to induce osteoclasts differentiation and maturation, and promoted bone absorption and destruction. This study observed IL-17A apparently upregulated RANKL mRNA and protein expressions in co-cultured HFLS, while IL-17 antibody obviously declined RANKL mRNA and protein levels, suppressed HFLS proliferation, reduced osteoclasts formation, and attenuated bone absorption. In the investigation of IL-17 and osteoclasts differentiation, Kotake et al. [22] revealed that IL-17 promoted osteoclasts differentiation via enhancing osteoclast differentiation factor formation. On the contrary, osteoclastogenesis inhibitory factor antagonized osteoclasts production induced by IL-17. In addition to influence osteoclast differentiation and function, IL-17 also can regulate bone cell function and differentiation to affect bone reconstruction. Uluckan et al. [21] showed that IL-17A inhibited osteoblasts formation through downregulating Wnt signaling pathway, whereas IL-17 antagonism facilitated osteoblast function and delay the process of bone resorption and bone loss. Kim et al. [23] also found that IL-17 significantly suppressed the differentiation of precursor osteoblasts to mature osteoblasts in vitro and inhibited the mice skull defect repair process in vivo. This study revealed that IL-17 played its role in RA by promoting HFLS cell proliferation, increasing RANKL expression, and facilitating osteoclasts differentiation and maturation. Moon et al. [11] reported that IL-17 can directly induce osteoclast differentiation and maturation, and promote bone absorption and destruction. Therefore, this study cannot exclude the possibility of IL-17 directly affects osteoclast differentiation. However, it cannot be neglected that IL-17 may affect osteoclasts differentiation by regulating RANKL expression in HFLS.

Conclusion

IL-17A content and Th17 cell proportion significantly increased in the synovial fluid of RA patients. IL-17A promoted osteoclast differentiation and enhanced bone absorption via inducing RANKL expression in HFLS.

Disclosure of conflict of interest

None.

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