IL-17A regulates the autophagic activity of osteoclast precursors through RANKL-JNK1 signaling during osteoclastogenesis in vitro

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Interleukin-17A (IL-17A), a proinflammatory cytokine, may have effects on osteoclastic resorption in inflammation-mediated bone loss, including postmenopausal osteoporosis. IL-17A could alter autophagic activity among other tissues and cells, thereby causing corresponding lesions. The aim of this study was to clarify how IL-17A influenced osteoclastogenesis by regulating autophagy. The present study showed that IL-17A could facilitate osteoclast precursors (OCPs) autophagy and osteoclastogenesis at a low concentration. Furthermore, suppression of autophagy with chloroquine (CQ) or 3-MA could significantly attenuate the enhanced osteoclastogenesis by a low level of IL-17A. It was also found that a low level of IL-17A couldn’t up-regulate OCPs autophagy after removal of RANKL (Receptor Activator for Nuclear Factor-κB Ligand), and JNK (c-Jun N-terminal kinase) inhibitor only inhibited autophagy at a low level of IL-17A. These results suggest that a low concentration of IL-17A is likely to promote autophagic activity via activating RANKL-JNK pathway during osteoclastogenesis.

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1. Introduction

Interleukin-17 (IL-17) is distinguishingly produced by T helper 17 cells (Th17). Like other pro-inflammatory cytokines derived from activated T cells, IL-17 can influence osteoclastogenesis and subsequent bone resorption in many inflammation-mediated bone diseases, such as postmenopausal osteoporosis (PMO) [1]. The IL-17 cytokine family includes at least six members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F, while IL-17 is usually referred to IL-17A. It is important to note that the role of IL-17 in PMO remains controversial so far. Some studies suggested that IL-17 contributed to the development of PMO [1,2], while others believed that IL-17 had protective effects against osteoporosis induced by estrogen deficiency [3]. Consistently, the studies of osteoclastogenesis under direct interference of IL-17A showed different results. Based on the previous studies [4–6], the following rules can be revealed: a low concentration of IL-17A stimulates the differentiation and activity of osteoclasts, which are inhibited by a high concentration of IL-17A. Nevertheless, the underlying mechanisms remain unclarified.

Previous studies have revealed that a series of autophagy proteins, including ATG5, ATG7, LC3 and ATG4B, are involved in polarization of osteoclasts, and associated with the formation of osteoclast ruffled border, H+ secretion by lysosomes, and protein hydrolysis [7]. In addition, both ATG7 and Beclin1 play a crucial role in osteoclastogenesis as well as the following activity of bone absorption [8]. Besides, during the osteoclastogenesis induced by RANKL, knockout of P62 can also lead to inhibition of osteoclastogenesis [9]. The studies above disclose that protective autophagy is involved in multiple stages of osteoclast proliferation and differentiation, and bone absorption activity of osteoclasts. In addition, the prior research has shown that IL-17A could promote the process of pulmonary fibrosis via the change of autophagic activity [10]. Similarly, the changes of autophagic activity induced by IL-17A are also reported in relevant studies about other system lesions [11–13]. However, it remains unclear whether or how IL-17A alters...
the autophagic activity of osteoclast precursors (OCPs) in osteoclastogenesis.

It has been expounded that there is a synergistic effect between IL-17A and RANKL in promoting osteoclast formation, based on the reality that compared with treatment of RANKL only, combination of the two cytokines could result in an obviously increased maturation of osteoclast [5]. Moreover, It has been reported that RANKL plays a significant role in autophagy activation during osteoclastogenesis [9,14–16]. In addition, JNK, a downstream signaling molecule of RANKL-mediated osteoclastogenesis [17–21], can also activate autophagy in the active form [22–24]. Our team has demonstrated that RANKL could activate autophagy through JNK signaling, thereby promoting osteoclast formation (unpublished results). Collectively, it was presumed that IL-17A might enhance autophagic activity of OCPs via RANKL-JNK pathway during osteoclastogenesis, which deserves confirmation as well.

The present study disclosed the effect of autophagy on IL-17A-regulated osteoclast formation under different cytokine levels. In addition, it was clarified that JNK signal was likely to mediate OCPs autophagy induced by a low level of IL-17A.

2. Materials and methods

2.1. Cell culture

RAW264.7 macrophage cell line’s incubations were performed in Dulbecco’s modified Eagle’s medium (DMEM; HyClone; Logan, Utah, USA) with 10% Fetal bovine serum (FBS; Gibco; Grand Island, NY, USA), 100IU/ml penicillin, and 100 µg/ml streptomycin. The cells were kept under 37 °C and 5% CO2. Before all trials, RAW264.7 cells were added into α-MEM along with 10% FBS, antibiotics and M-CSF (20 ng/ml in all experiments; Peprotech; Rocky Hill, NJ, USA) for 2 days to induce the OCPs.

2.2. Small interfering RNA (siRNA) transfection

The control siRNA or siRNA targeting mouse TRAF3 was designed from Invitrogen (Carlsbad, California, USA). The target sequences were: 5′-GCAAAGGCTCAGTTTA-3′ (control); 5′-GCAATGCTGCTTAACTGCA-3′ (TRAF3). Following treatment of M-CSF, siRNAs were transfected into OCPs using RNAiMAX reagent (Invitrogen) for 24 Hours (h) according to the manufacturer’s instructions. Then, endogenous TRAF3 levels were observed by western-blot

2.3. Osteoclast differentiation assay

RAW264.7 (2 × 10⁵) cells were seeded into 24-well plates. After the pretreatment process, OCPs were cultured in α-MEM (HyClone) complete medium containing 0, 0.5, 5, or 50ng/ml recombinant IL-17A (Peprotech) together with soluble RANKL (60 ng/ml in all experiments; Peprotech) plus M-CSF for 4–5 days (d). Tartrate resistant acid phosphatase (TRAP) staining-positive cells with more than three nuclei were observed by light microscopy, and counted in all visual fields of each well.

2.4. Western-blot analysis

RAW264.7 (1.5 × 10⁶) cells were cultured in 6-well plates. Following pretreated with M-CSF, whole-cell proteins from OCPs with indicated treatment were extracted with lysis buffer (Beyotime Biotechnology; Shanghai, China) supplemented with a cocktail of protease inhibitors (Roche; Basel, Switzerland). Insoluble material was removed by centrifugation and the supernatant was subjected to 10% SDS-PAGE gels. The lysate was electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane. Then the proteins were probed with antibodies specific to LC3, Atg5, Atg7, TRAF3 (Cell Signaling; Beverly, MA, USA), or to phosphorylated JNK (p-JNK) and β-actin (Bioworld; Harrogate, UK). After incubated with the Horseradish peroxidase-linked secondary antibodies, the signals were visualized with an ECL kit.

2.5. Quantitative real-time PCR

Total RNA of OCPs was isolated using Trizol reagent according to the manufacturer’s protocol (Invitrogen). The designed primers sequences for real-time quantitative PCR (qPCR) were as follows: TRAF3 F-5′-CCCAAGAAGCATCATAAAAGAC-3′ and TRAF3 R-5′-CTATTCGAGACGTAGACCTGAA-3′; Cyclophilin A (housekeeping control) F-5′-CGAGCTGTGACGACTGGAGA-3′ and Cyclophilin A R-5′-TGAGGTAAAAGTCACCAAAC-3′. The reaction was performed using ABI7500 PCR machine (Applied Biosystems; Waltham, MA, USA) and SYBR Premix Ex TaqTM kit (TakaRa; Tokyo, Japan).

2.6. Electron microscopy

RAW264.7 (1.5 × 10⁶) were cultured on 6-cm dishes, provided with the indicated treatments, centrifuged to tube bottom, fixed, wrapped, and post-fixed in 1% buffered osmium tetroxide at 20 °C for 2 h. Then Samples were dehydrated through graded ethanolos to 100%, infiltrated with EMbed 812 overnight, baked in 60 °C oven and cut into ultrathin sections. Subsequently, The sections were stained, and observed using hitachi 7700 Transmission EM (Tokyo, Japan).

2.7. Statistical analysis

The data are presented as the mean ± SEM. Statistical differences among groups were evaluated with a two-way ANOVA analysis. The P value < 0.05 was considered statistically significant.

3. Results

3.1. A low concentration of IL-17A could enhance OCPs autophagy, whereas a high level of IL-17A is contrary

The autophagic activity is generally observed by the LC3 conversion rate (LC3II/I) [25]. The administration of IL-17A at a concentration of 0.5 ng/ml enhanced the LC3 conversion. However, with the increase of IL-17A levels, the LC3 conversion decreased gradually, and was inhibited to the greatest extent at 50 ng/ml (Fig. 1A and 1B). The detection of autolysosome under TEM is another approach to assess autophagic activity. Consequently, autolysosomes were significantly increased in 0.5 ng/ml IL-17A group compared to untreated group, while autolysosomes in 50 ng/ml cytokines group were obviously reduced (Fig. 1F and 1G). Meanwhile, along with the changes in LC3 conversion and autolysosomes, the protein levels of Beclin1 were increased in 0.5 ng/ml IL-17A group, whereas Beclin1, Atg5 and Atg7 levels were reduced in two other groups (Fig. 1D and 1E).

3.2. Autophagy inhibitor can attenuate IL-17A-promoted osteoclastogenesis

After 5-day differentiation induction of OCPs, compared to the untreated group, a combination of RANKL, M-CSF and 0.5 ng/ml IL-17A led to a significant increase of mature osteoclasts, while both other two groups suppressed maturation of osteoclasts (Fig. 2A and 2B). Meanwhile, the increased osteoclastogenesis due to 0.5 ng/ml IL-17A was evidently inhibited by both of the pharmacological
inhibitor of autophagy, CQ or 3-MA, which did not affect differentiation of 50 ng/ml group (Fig. 2A and 2B).

3.3. JNK signal mediates IL-17A-promoted OCPs autophagy

In the absence of RANKL, it was found that 0.5 ng/ml of IL-17A couldn’t enhance LC3 conversion compared with the untreated group (Fig. 3A). It was noted that the conversion could still be down-regulated when the concentration was 50 ng/ml (Fig. 3A). In addition, the protein expression of p-JNK was significantly up-regulated with 0.5 ng/ml of IL-17A plus RANKL (Fig. 3B). It was also found that LC3 conversion was obviously reduced by SP600125 in the 0.5 ng/ml cytokine group. Nevertheless, the other two concentration groups were not influenced by SP600125 addition (Fig. 1A and C). Besides, SP600125 inhibited osteoclasts differentiation in the untreated group and IL-17A-treated group (Fig. 2A and 2B).
3.4. IL-17A has an effect on the TRAF3 degradation during osteoclastogenesis

Previous studies have reported that TRAF3 could prevent OC differentiation [15,26], and TRAF3 degradation is triggered by autophagy activation, subsequently promoting osteoclastogenesis [15]. To further investigate the precise mechanisms of IL-17A regulating OCPs autophagy, IL-17A at different concentrations was added into OCPs to observe the degree of TRAF3 degradation. Besides, OCPs differentiation was detected after the intervention of IL-17A and TRAF3 silencing with siRNA. As a result, an obvious decrease of TRAF3 proteins was observed after the addition of RANKL, which were further decreased by an addition of 0.5 ng/ml IL-17A. Nonetheless, accompanied by the elevation of cytokines, TRAF3 levels increased gradually, subsequently reaching the peak at 50 ng/ml (Fig. 4A and 4B). In addition, there was no significant difference in mRNA levels of TRAF3 between those groups (Fig. 4C).

4. Discussion

Our study indicated that IL-17A could promote OCPs autophagic activity and osteoclastogenesis at a low concentration, and the promoting effect was gradually changed to an inhibiting effect in response to increased IL-17A levels, which reached the minimum level at the peak concentration. The result above is consistent with the study by Satoshi et al. [4]. They found that IL-17A enhanced osteoclastogenesis only at the lowest concentration, while IL-17A gradually exerted an increasingly powerful inhibition of osteoclastogenesis as the IL-17A concentration rose. In addition, the similar viewpoints have also been elucidated by Adamopoulos et al. [5] and Toru et al. [6].

Furthermore, knockdown of TRAF3 by siRNA could promote osteoclasts maturation with a high level of IL-17A (Fig. 4E and 4F).
autophagy could promote the proliferation and differentiation of osteoclasts as well as the activity of bone absorption [7–9]. Besides, the effect of IL-17A on autophagy has been elucidated by several studies in spite of inconsistent effects. For instance, Shi et al. [11] demonstrated that IL-17A could activate microglia autophagy, while IL-17A-induced autophagy was also involved in the removal of pathogenic microorganisms by macrophages [12]. Nevertheless, IL-17A was considered to prevent starvation-induced autophagy in hepatocellular carcinoma [13]. Liu et al. [10] also reported that IL-17A was able to promote the progression of pulmonary fibrosis by inhibiting autophagy. There were inconsistent results in different studies, and various factors, including cytokine concentrations, cell types, experimental conditions, were responsible for the results. Our study unmasked the role of concentration in IL-17A on OCPs autophagy. Collectively, IL-17A is likely to regulate osteoclastogenesis by influencing autophagic activity. Moreover, suppression of autophagy with CQ or 3-MA obviously prevented the enhanced osteoclastogenesis by a low-concentration of IL-17A, which further emphasized the effect of IL-17A regulated autophagy on osteoclastogenesis.

Adamopoulos et al. [5] demonstrated that a combination of IL-17A and RANKL contributed to a significantly greater osteoclastogenesis compared to administration of RANKL only, indicating that IL-17A might promote osteoclastogenesis via RANKL. In our study, after the removal of RANKL, LC3 conversion was not up-regulated by the low-concentration of IL-17A, while it was still inhibited by the high level cytokine. Considering the effect of IL-17A on OCPs autophagy in the presence of RANKL, we speculate that RANKL is involved in mediating the autophagy activation, which is caused by the low-concentration of IL-17A. The binding of RANKL to its membranal receptor, RANK, can activate the downstream signaling pathway to promote osteoclast formation. Notably, JNK has been verified to be a crucial signal of the above process [17–21]. The positive effect of JNK on osteoclastogenesis has been verified by JNK signal mediated IL-17A-enhanced OCPs autophagy. (A) OCPs were treated with 0, 0.5, 5, or 50 ng/mL IL-17A without RANKL for 8 h, and the ratio of LC3-II/I was observed by western-blot analyses. (B) OCPs were treated with 0, 0.5, 5, or 50 ng/mL IL-17A plus RANKL for 8 h, and the protein levels of p-JNK were detected by western-blot analyses. The experiments were replicated for at least three times. Data are presented as the mean ± SEM from three independent experiments. *P < 0.05. R, RANKL; ns, nonsignificant. (C) The diagram of a working model about our results.
through inhibition of JNK by pharmacological inhibitor and gene silencing [18,20,21]. Meanwhile, activation of JNK can induce the autophagy protein Beclin1 into autophagy flux, subsequently activating autophagy [22], which has also been confirmed in osteoclastogenesis by our team (unpublished results). In the present study, p-JNK was significantly increased after addition of the low-concentration of IL-17A. In addition, JNK inhibitor could significantly suppress the increased LC3 conversion mediated by the low-concentration of IL-17A without any effect on other two level cytokines. Therefore, the activation of RANKL-JNK-autophagy pathway might exist in IL-17A enhanced osteoclastogenesis. JNK inhibitor reduced osteoclastogenesis in IL-17A treated OCPs. Our findings also supported the above viewpoint. However, the underlying mechanisms of the inhibition of osteoclast autophagy and formation under the high concentration of IL-17A require further investigation.

Fig. 4. IL-17A had an effect on the TRAF3 degradation during osteoclastogenesis. (A-C) Following pretreatment with M-CSF, proteins (A-B) or mRNA levels (C) of TRAF3 in the OCPs treated with RANKL plus the indicated concentrations of IL-17A for 12 h. (D) The proteins of TRAF3 in the OCPs transfected with control-siRNA (si-CONT) or TRAF3-siRNA (si-TRAF3). (E) Representative pictures of TRAP+ multinucleated cells derived from OCPs treated with M-CSF plus RANKL along with 0.5, 50 ng/mL IL-17A for 4 days after transfected with siRNA. (F) Quantity of TRAP+ multinucleated cells (E). The experiments were replicated for at least three times. Data are presented as the mean ± SEM from three independent experiments. *P < 0.05. R, RANKL; ns, no-significant; si, siRNA.
TRAFA3 could be degraded by autolysosome after autophagy activation [15]. In our study, it was found that the low concentration of IL-17A enhanced the TRAF3 degradation, while the high concentration of IL-17A was contrary. Moreover, silencing of TRAF3 could also reverse the reduced osteoclastogenesis attributed from the high concentration of IL-17A. Taken together, these findings further implicate that IL-17A can affect the autophagy activity during osteoclastogenesis. At present, monoclonal antibody targeting IL-17 can better improve the histomorphometry, cortical bone parameters and bone biomechanical properties, compared to antibodies targeting RANKL and TNF-α [1]. Considering the controversy over the effect of IL-17A on osteoclastogenesis, it is extremely essential to elucidate the autophagic activity changes induced by IL-17A during osteoclastogenesis, and more explorations on one hand, provide more pharmacological targets for the clinical treatment of inflammation-mediated bone diseases, which are mainly characterized by the increased bone absorption, and on the other hand, rationalize the administration of IL-17 monocular antibody in the treatment of inflammatory bone diseases.

Conflicts of interest

All authors have no conflicts of interest.

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