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Original Article

IL-34 Aggravates Steroid-Induced Osteonecrosis of the Femoral Head via Promoting Osteoclast Differentiation

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ABSTRACT

IL-34 can promote osteoclast differentiation and activation, which may contribute to steroid-induced osteonecrosis of the femoral head (ONFH). Animal model was constructed in both BALB/c and IL-34 deficient mice to detect the relative expression of inflammation cytokines. Micro-CT was utilized to reveal the internal structure. *In vitro* differentiated osteoclast was induced by culturing bone marrow-derived macrophages with IL-34 conditioned medium or M-CSF. The relative expression of pro-inflammation cytokines, osteoclast marker genes, and relevant pathways molecules was detected with quantitative real-time RT-PCR, ELISA, and Western blot. Up-regulated IL-34 expression could be detected in the serum of ONFH patients and femoral heads of ONFH mice. IL-34 deficient mice showed the resistance to ONFH induction with the up-regulated trabecular number, trabecular thickness, bone value fraction, and down-regulated trabecular separation. On the other hand, inflammatory cytokines, such as TNF-α, IFN-γ, IL-6, IL-12, IL-2, and IL-17A, showed diminished expression in IL-34 deficient ONFH induced mice. IL-34 alone or works in coordination with M-CSF to promote osteoclastogenesis and activate ERK, STAT3, and non-canonical NF-κB pathways. These data demonstrate that IL-34 can promote the differentiation of osteoclast through ERK, STAT3, and non-canonical NF-κB pathways to aggravate steroid-induced ONFH, and IL-34 can be considered as a treatment target.

Keywords: IL34; Osteonecrosis; Osteoclast; M-CSF; Inflammation

INTRODUCTION

As a progressive and degenerative disease, osteonecrosis of the femoral head (ONFH) can cause subchondral micro-fracture and subsequent femoral head collapse, eventually leading to hip joint dysfunction (1,2). ONFH usually affects young to middle-aged individuals and poses a huge socioeconomic burden. There are no effective preventive measures and therapeutics for steroid-induced ONFH. What makes it more serious is that nearly 67% of asymptomatic patients will progress to symptomatic stages within the first 2 years and require surgical intervention, even total hip arthroplasties (3,4).

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
BAP, bone-specific alkaline phosphatase; BGP, bone gla protein; BMC, bone mineral content; BMD, bone mineral density; BV/TV, bone value fraction; CSF1R, colony-stimulating factor 1 receptor; ONFH, osteonecrosis of the femoral head; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness.

Author Contributions
Conceptualization: Wang F, Wang F, Jiang C, Zong Y, Ma X, Lin Y, Zhou Z, Yu X; Data curation: Wang F, Wang F, Shan H, Yin F, Jiang C, Zong Y, Ma X, Lin Y, Zhou Z, Yu X; Formal analysis: Wang F, Shan H, Zong Y, Ma X, Zhou Z; Funding acquisition: Yu X; Investigation: Wang F, Yin F; Methodology: Lin Y; Supervision: Yu X; Validation: Wang F, Wang F, Shan H, Yin F, Jiang C, Zong Y, Ma X, Lin Y, Zhou Z, Yu X; Writing - original draft: Wang F, Wang F, Shan H, Yin F, Jiang C, Zong Y, Ma X, Lin Y, Zhou Z, Yu X; Writing - review & editing: Yu X.

The exact cause of ONFH is unknown, alcohol and tobacco abuse, chemotherapy, and coagulation abnormalities are considered as the main cause (5-7). On the other hand, intensification of therapy with steroid administration can lead to steroid-induced ONFH, which is a common iatrogenic complication in autoimmune disease and severe acute respiratory syndrome treatment (8-10). Enhanced reactive oxygen species production, altered mechanical stress, dysregulated autophagy, and increased inflammatory response are considered to contribute to the development of ONFH (4,11-13).

Although the pathologic mechanisms leading to steroid-induced ONFH are not fully understood, excessive osteoclast activity is widely accepted to mediate bone integrity loss, subchondral bone fracture, and femoral head collapse (14,15). IL-34 derived from multiple myeloma cells could promote the differentiation of human $CD14^+$ monocytes and mouse bone marrow cells into osteoclasts (16). On the other hand, IL-34 can function as an osteoclastogenic cytokine to enhance osteolysis. IL-34 activates the downstream MEK/ERK, JNK/c-Jun, and NF-κB pathway through the binding with colony-stimulating factor 1 receptor (CSF1R), which is a receptor tyrosine kinase that is activated upon binding to its ligands of IL-34 and M-CSF (17). All of these indicate that IL-34 may be targeted to maintain the integrity of the femoral head in osteonecrosis.

However, the alteration of IL-34 expression and the activity of osteoclast in steroid-induced ONFH remain unclear. Our investigation demonstrates that IL-34 induction following steroid administration may lead to the hyperactivity of osteoclast and the progression of subsequent ONFH.

MATERIALS AND METHODS

Patient samples

Twenty-four ONFH patients (male and female, 1:1, **Table 1**) were enrolled at Shanghai Jiao Tong University Affiliated Sixth People's Hospital from 2019 to 2020. These patients were diagnosed as Association Research Circulation Osseous classification stages II or III in accordance with the judgment of magnetic resonance imaging images as indicated in previous research (6). The diagnosis was also verified with preoperative X-ray radiographs. Peripheral blood-derived serum was taken at diagnosis. The characteristic information of patients involved in this study is provided in **Table 1**. The protocol was approved by the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital, and the consent form was signed by the participants.

Steroid-induced ONFH

Sixty 8 wk-old C57BL/6 mice were provided by the GemPharmatech (Nanjing, China). $IL34^{tm1e(EUCOMM)Wtsi}$ (2010004A03Rik) mice with B6 background were obtained from European Mouse Mutant Cell Repository. Methylprednisolone (Pfizer, New York, NY, USA) was injected into C57BL/6 mice and $IL34^{tm1e(EUCOMM)Wtsi}$ mice intramuscularly at the dose of 20 mg/kg/d on the first 3 days of every week, for the whole 3 wk. Six wk later, histological analyses and micro-CT were conducted to analyze the success of ONFH construction. Animal study was granted by Shanghai Jiao Tong University Affiliated Sixth People's Hospital.

Table 1. Patient characteristics

Healthy			ONFH		
Sample number	Sex	Age	Sample number	Sex	Age
1	Male	35	1	Male	25
2	Male	28	2	Male	53
3	Male	37	3	Male	57
4	Male	39	4	Male	52
5	Male	35	5	Male	31
6	Male	30	6	Male	75
7	Male	40	7	Male	75
8	Male	47	8	Male	69
9	Male	75	9	Male	22
10	Male	76	10	Male	54
11	Female	69	11	Male	54
12	Female	75	12	Male	28
13	Female	27	13	Male	48
14	Female	71	14	Male	39
15	Female	71	15	Female	40
16	Female	63	16	Female	21
17	Female	34	17	Female	47
18	Female	40	18	Female	60
19	Female	34	19	Female	65
20	Female	40	20	Female	61
21	Female	37	21	Female	76
22	Female	56	22	Female	39
23	Female	42	23	Female	63
24	Female	43	24	Female	27

Micro-CT analysis

Left femoral heads were dissected and fixed in 10% formalin overnight and scanned with SkyScan1178 (Bruker MicroCT; Bruker Belgium SA, Kontich, Belgium). The resolution was set as 9 µm per pixel. Sagittal sections of representative samples were constructed using DataViewer.

Histopathological assessment

Femoral heads were dissected and fixed with formalin (10%), then decalcified with 14% ethylenediaminetetraacetic acid for 2 wk, which were further embedded in paraffin. The samples were cross-sectioned into 3 µm slides for hematoxylin and eosin staining. Histopathological assessments were examined with a Nikon 80*i* microscope by 3 independent investigators. Diffuse pyknotic nuclei of trabecular bone cells surrounding bone marrow cell necrosis were utilized to verify the success of ONFH induction. Quantitative microstructural analysis, such as trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), trabecular number (Tb.N) and bone value fraction (BV/TV), was utilized to assess the osteonecrosis.

In vitro osteoclastogenesis

Bone mineral cells were flushed from tibia and femurs and cultured for 4 days in DMEM medium containing fetal bovine serum (20%) and M-CSF (50 ng/ml) to induce bone marrow-derived macrophages. The osteoclastogenesis was performed by culturing bone marrow-derived macrophages with M-CSF (50 ng/ml) or IL-34 (50 ng/ml) containing media for 3 days, then cultured with receptor activator of nuclear factor-kappa-B ligand (50 ng/ml) for another 3–4 days.

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Quantitative real-time RT-PCR

Total RNA from femoral heads and osteoclasts was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was reverse-transcribed with cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and amplificated with SYBR Green (Roche, Mannheim, Germany). The reaction was set as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 60 s. Primer sequences were listed: *Cstk*, forward, 5′-CCAGTGGGAGCTATGGAAGA-3′, reverse, 5′-AAGTGGTTCATGGCCAGTTC-3′; *Acp5*, forward, 5′-CGTCTCTGCACAGATTGCAT-3′, reverse, 5′-GTAGTCCTCCTTGGCTGCTG-3′; *Calcr*, forward, 5′-CGGACTTTGACACAGCAGAA-3′, reverse, 5′-GTCACCCTCTGGCAGCTAAG-3′; *Il-6*, forward, 5′-CTGATGCTGGTGACAACCAC-3′, reverse, 5′-CAGACTTGCCATTGCACAAC-3′; *Tnf*, forward, 5′-CATCTTCTCAAAATTCGAGTGACAA-3′, reverse, 5′-CCAGCTGCTCCTCCACTTG-3′; *Il-34*, forward, 5′-CAGGAGGTTCAGACATTGCTGG-3′, reverse, 5′-GCAGTTGTCCAGCAAGGCTTTG-3′. The relative interest genes expression was normalized to β-actin expression.

Western blot

Bone marrow cells-derived macrophages after incubation with IL-34 or IL-34 plus M-CSF were lysed with RIPA buffer (supplemented with EDTA-free protease and phosphatase inhibitors) and allowed to rotate at 4°C for an hour. The whole-cell lysates were spun down for 10 min at 14,000 g at 4°C, and supernatants were collected for western blot. For the preparation of nuclear and cytoplasmic/membrane fractions from whole-cell lysates, fractionation was carried out using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendation.

The cellular lysate was separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%), which was transferred onto polyvinylidene fluoride membrane, then further blocked with 5% nonfat dry milk. The membrane was incubated with corresponding primary interest antibodies against STAT3 (CST, 9132), p-STAT3 (CST, 9131), p-ERK1/2 (CST, 4370), ERK1/2 (CST, 4695), p100/p52 (TB4, NCI), RelB (Santa Cruz, D-4), and Lamin B (Santa Cruz, C-20) at 4°C overnight. The membrane was further incubated with peroxidase-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at room temperature. The signal was examined with an enhanced ECL system (GE Healthcare Life Sciences, Seoul, Korea). The relative expression was normalized to β-actin (C-4, Sigma) with NIH-Image J1.5 (National Institutes of Health, Bethesda, MD, USA).

ELISA

The serum levels of IL-34, IL-17A, TNF-α, IFN-γ, IL-2, IL-6, IL-12, bone gla protein (BGP), bone-specific alkaline phosphatase (BAP), and osteocalcin were measured with corresponding ELISA kits (eBioscience, San Diego, CA, USA) following the manufacturer's instructions. SpectraMax M5 microplate reader was utilized to measure the optical density values at a wavelength of 450 nm.

Statistical analysis

All tests were performed with the Student's *t*-test, one or 2-way ANOVA analysis and a *post hoc* test using GraphPad Prism. The significance level was set as p-value <0.05.

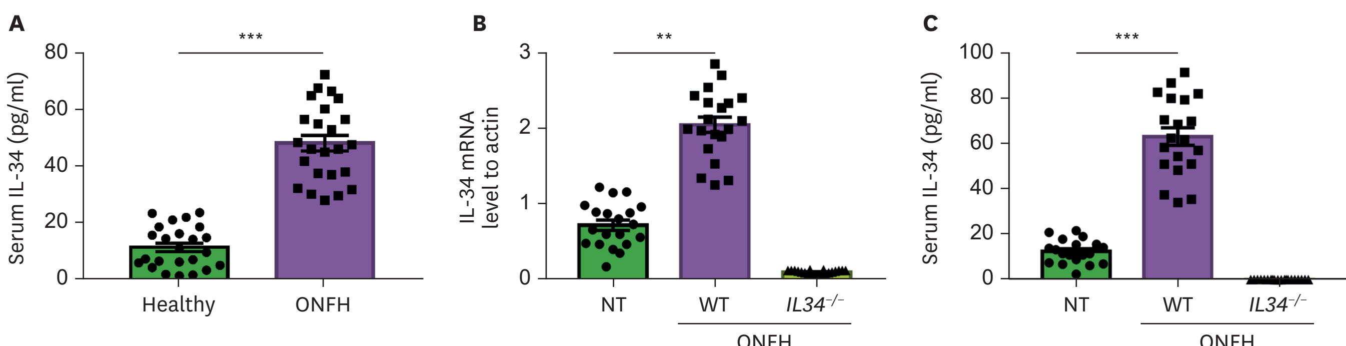

Figure 1. Upregulated IL-34 in steroid-induced ONFH mice. (A) IL-34 level in the serum of ONFH patients was detected by ELISA (n=24). (B)The mRNA level of IL-34 in the femoral heads of WT and *IL34*$^{-/-}$ mice were assessed by quantitative real-time PCR (n=20). (C) IL-34 level in the serum of steroid-induced ONFH WT and *IL34*$^{-/-}$ mice was detected by ELISA (n=20). All data are presented as fold relative to the Actb mRNA level. Data are presented as mean ± SD values. Student's *t*-test or one-way ANOVA followed by Tukey's test was applied for statistical comparison between groups.
NT, non-treatment group; WT, wild type.
**p<0.01; ***p<0.005.

RESULTS

Steroid administration induces IL-34 expression in ONFH

In ONFH patients, we detected a high level of serum IL-34 (**Fig. 1A**). IL-34 expression in the femoral head (**Fig. 1B**) and the secretion in the serum (**Fig. 1C**) were significantly higher in the steroid-induced ONFH mice than those in the normal (NT) mice. As expected, the relative IL-34 expression was not detected after steroid administration in IL-34 deficient mice (**Fig. 1B and C**). All of these indicated that high-level IL-34 might contribute to the development of ONFH.

IL-34 deficiency attenuates osteonecrosis in mice of steroid-induced ONFH with up-regulated osteoblast activity

Furthermore, the association of IL-34 and osteonecrosis phenotype was investigated in IL-34 deficient mice. Steroid-induced ONFH mice had significantly lower BV/TV, Tb.Th, and Tb.N and higher Tb.Sp when compared with NT mice, while IL-34 deficient can significantly restore the degenerative microstructure change induced by steroid administration (**Fig. 2**). We then used histopathological analysis to verify the association of IL-34 and osteonecrosis phenotype in steroid-induced ONFH. ONFH mice showed obvious osteonecrosis phenotypes, such as bone marrow cell necrosis and adipocyte occupation. However, IL-34 deficient mice showed less cavitation and adipocytes after ONFH induction (**Fig. 3A**). In addition, bone mineral density (BMD) and bone mineral content (BMC) of bone and femur in IL-34 deficient mice were significantly restored compared with the wide type mice after steroid administration (**Fig. 3B**). While the indicators for osteoblast activity, such as osteocalcin, BGP, and BAP, were significantly high in IL-34 deficient ONFH induced mice than wild type ONFH mice (**Fig. 3C**). Although reduced BMD and BMC were symptoms of ONFH and were not directly linked to IL-34 expression, our results indicated that IL-34 contributed to the development of steroid-induced ONFH with the diminished activity of osteoblast.

IL-34 deficiency ameliorates inflammatory response in steroid-induced ONFH mice

As expected, high levels of inflammation cytokines, such as IL-6, TNF-α, IL-12 (**Fig. 4A**), IFN-γ, IL-2, and IL-17A (**Fig. 4B**), were detected in the serum of steroid-induced ONFH mice. While in IL-34 deficient mice, such increase was reversed when compared with wide type

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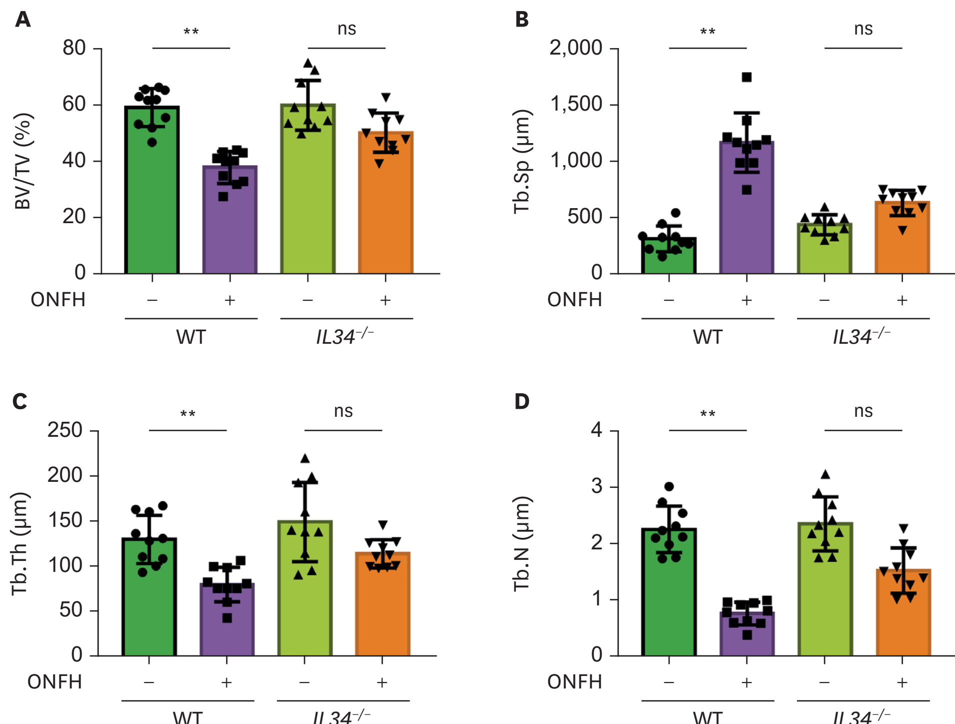

Figure 2. IL-34 deficient mice displayed resistance to ONFH induction. Statistical analysis was performed on BV/TV (A), Tb.Sp (B), Tb.Th (C), and Tb.N (D) (n=10) of WT and *IL34*$^{-/-}$ mice that were induced an ONFH model. Data are presented as mean ± SD values. One-way ANOVA followed by Tukey's test was applied for statistical comparison between groups.
ns, not significant difference; WT, wild type.
**p<0.01.

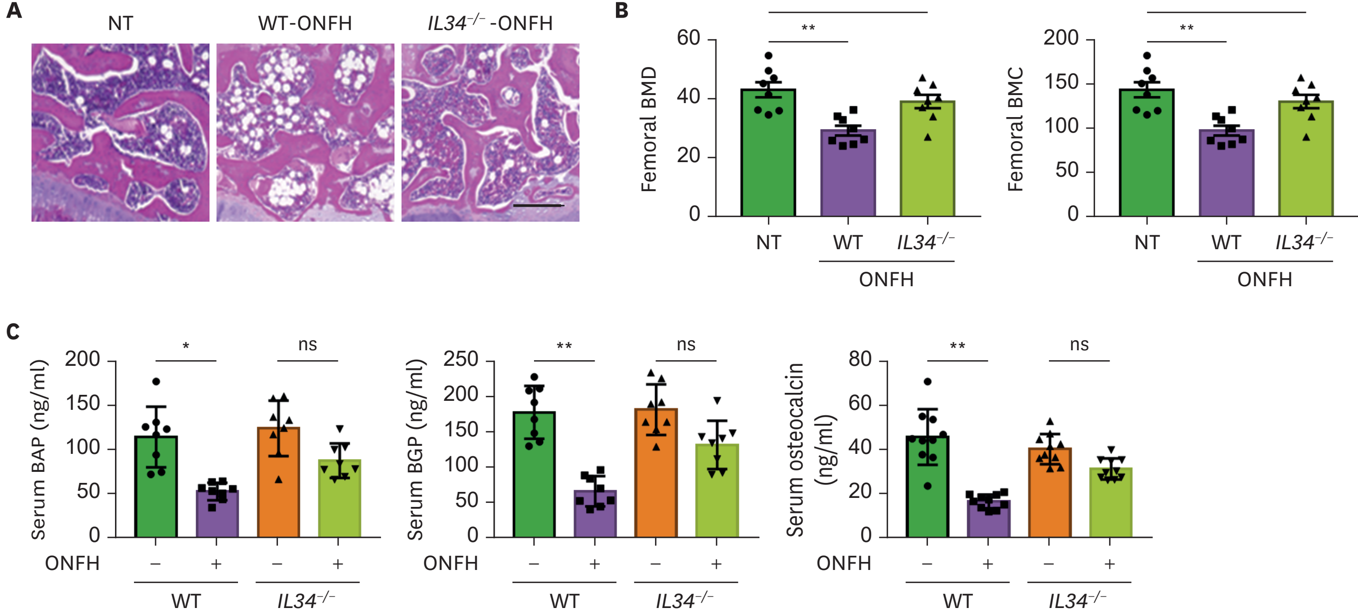

Figure 3. IL-34 deficiency attenuated osteonecrosis in steroid-induced ONFH mice. (A) Hematoxylin-Eosin staining of the femoral head. (B) BMD and BMC of bone and femur were restored in the WT and *IL34*$^{-/-}$ mice after steroid administration (n=8). (C) Serum osteocalcin, BGP, and BAP in the WT and *IL34*$^{-/-}$ mice were measured with ELISA (n=8). Data are presented as mean ± SD values. One-way ANOVA followed by Tukey's test was applied for statistical comparison between groups.
ns, not significant difference; WT, wild type.
*p<0.05; **p<0.01.

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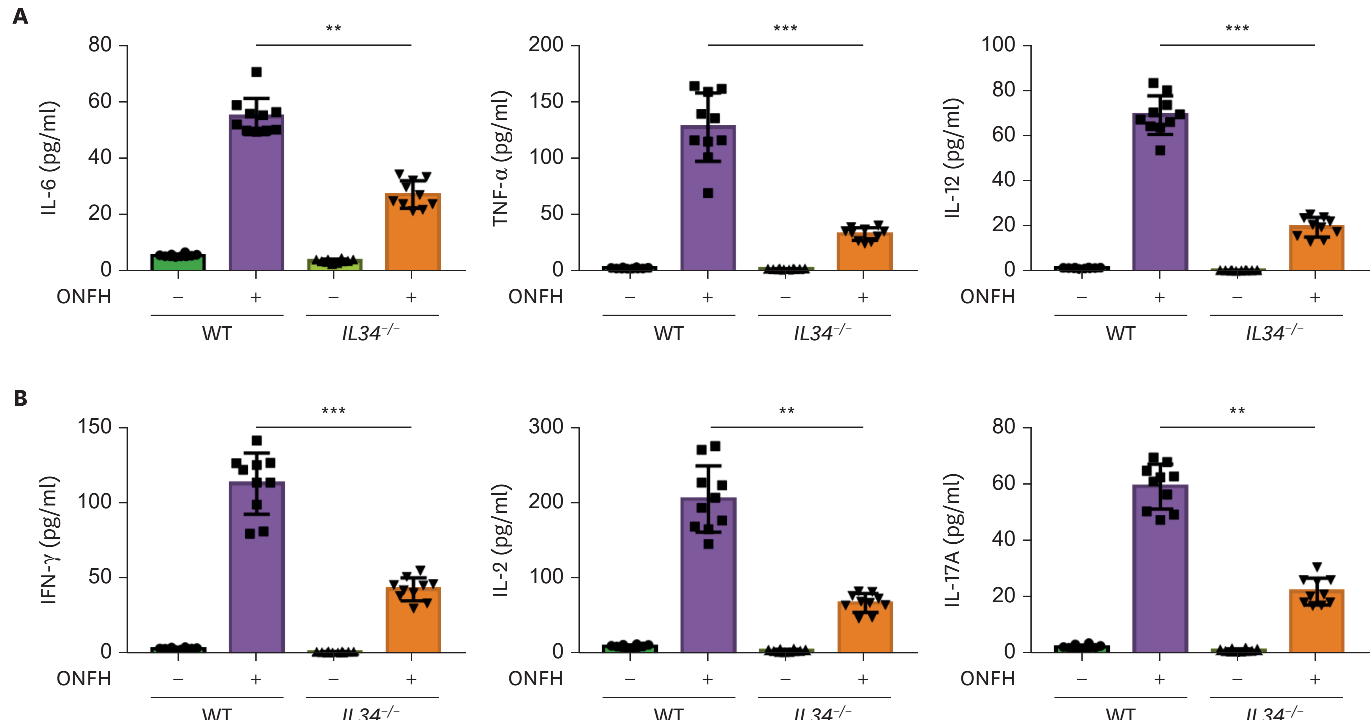

Figure 4. IL-34 deficiency ameliorated inflammatory response in steroid-induced ONFH mice. (A) The expression of innate immunity-related cytokines in steroid-induced WT and *IL34*$^{-/-}$ ONFH mice were measured by ELISA (n=10). (B) ELISA of the adaptive immunity-related cytokines in the serum of steroid-induced WT and *IL34*$^{-/-}$ ONFH mice (n=10). Data are presented as mean ± SD values. One-way ANOVA followed by Tukey's test was applied for statistical comparison between groups.
WT, wild type.
$^{**}p<0.01$; $^{***}p<0.005$.

mice after steroid administration (**Fig. 4**). Th17 related cytokines (IL-17A) and Th1 related cytokines (TNF-α, IL-12, and IFN-γ) may contribute to the development of osteonecrosis (18,19). These results suggested that IL-34 related inflammation cytokines may contribute to the development of ONFH.

IL-34 promotes osteoclastogenesis

As testified in previous research, M-CSF could induce the expression of osteoclast markers (20,21). Bone marrow-derived macrophages stimulated by IL-34 could also significantly induce the relative expression of osteoclast markers, such as Acp5, Calcr, and Cstk, in a concentration-dependent manner (**Fig. 5A**). In accordance with M-CSF, M-CSF to induce expression of osteoclast markers, such as Acp5, Calcr, and Cstk. Interestingly, pro-inflammation cytokines, such as IL-6 and TNF-α, did not show any differential expression between M-CSF or IL-34-induced osteoclast when stimulated with IL-1β (**Fig. 5B**), which indicated that IL-34 induced osteoclast possessed the similar inflammation stimulation activity with M-CSF induced osteoclast. These results demonstrated that IL-34 could promote osteoclastogenesis, and IL-34 induced osteoclast can react to inflammation stimulation.

IL-34 promotes the activation of multiple signal pathways

In order to decipher the main IL-34 affecting pathways contributing to osteoclastogenesis, the downstream signal pathway of CSF1R was detected. IL-34 could up-regulate the expression of p-ERK1/2 and p-STAT3 after 30 min treatment (**Fig. 6A**), and significantly induce the expression of non-canonical NF-κB pathway molecules (p52, RelB) after 24 h treatment (**Fig. 6B**). Previous research also testified that M-CSF could promote the activation

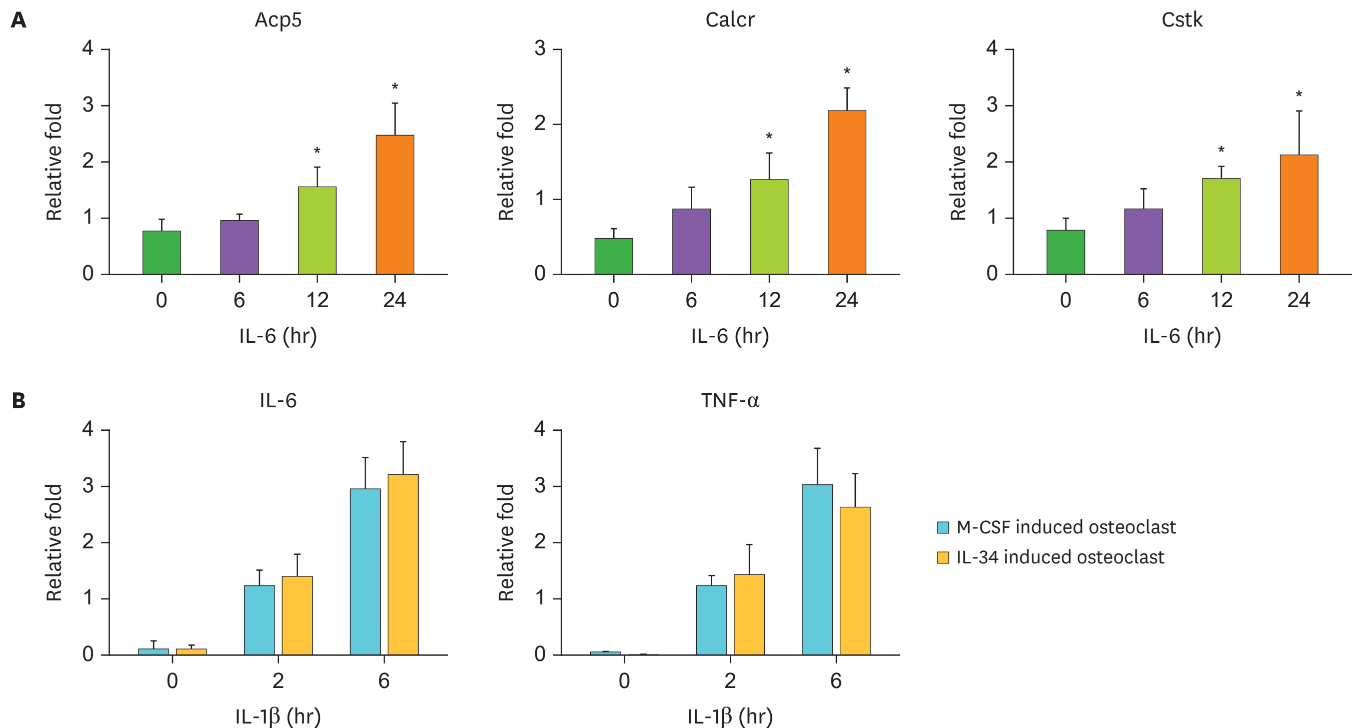

Figure 5. IL-34 promotes osteoclastogenesis and osteoclast-related gene expression. (A) Bone marrow-derived macrophages were cultured in IL-34 (50 ng/ml) containing medium for 3 days and RANKL (50 ng/ml) for the other 3 days. The relative expression of osteoclast-related genes such as Cstk, Acp5, and Calcr, was determined by qRT-PCR. (B) The relative expression of IL-6 and TNF-α in IL-1β (10 ng/ml) stimulated M-CSF or IL-34 induced osteoclast was measured by qRT-PCR. All data are presented as fold relative to the Actb mRNA level. Data are presented as mean ± SD. One-way ANOVA followed by Tukey's test or 2-way ANOVA followed by Bonferroni's test was applied for statistical comparison between groups.
RANKL, receptor activator of nuclear factor-kappa-B ligand; qRT-PCR, quantitative real-time RT-PCR.
*p<0.05.

of ERK, STAT3, and non-canonical NF-κB pathways (17,22,23). Our results further indicated that IL-34 and M-CSF combination had an excellent synergistic effect, and the presence of IL-34 and M-CSF could further increase the activation of ERK1/2, STAT3, and NF-κB non-canonical pathways.

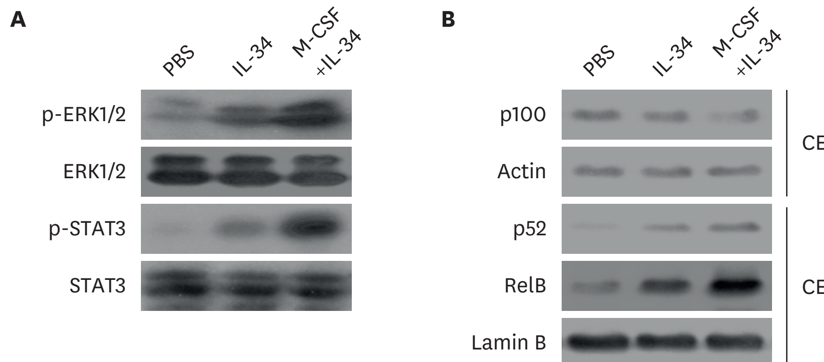

Figure 6. IL-34 promotes the activation of multiple signal pathways. The activation of ERK1/2 and STAT3 signal pathway (A) and noncanonical NF-κB signal pathway (B) in the bone marrow cells-derived macrophages after incubation with IL-34 or IL-34 plus M-CSF. Data are representative of at least 3 independent experiments.
CE, cytoplasmic extract; NE, nuclear extract.

DISCUSSION

In this investigation, we find that up-regulated IL-34 could be detected in steroid-induced ONFH mice and ONFH patients. IL-34 promotes the differentiation of osteoclasts *in vitro* with up-regulated inflammation cytokines expression. The differentiation and activation of osteoclasts in steroid-induced ONFH are mainly achieved by the activation of ERK, STAT3, and non-canonical NF-κB pathway, which can work in coordination with M-CSF. Our study further reveals that IL-34 induced by steroid administration may lead to the hyperactivity of osteoclast and the subsequent progression of ONFH.

As a novel cytokine identified in 2008, IL-34 is testified as monocytes and macrophages-specific CSF-1R ligand, and the interaction results in CSF-1R specific tyrosine residues autophosphorylation, which may lead to adaptor proteins and kinases recruitment to activate STAT3, ERK1/2, and NF-κB pathway (22,24). Such activation may promote IL-34-mediated differentiation and viability of monocytes and macrophages (25-27). We further testify that consistent with the function of M-CSF and IL-34 also could promote the differentiation of osteoclasts. IL-34 may function in coordination with M-CSF to activate the downstream signal pathway (17), which indicates that CSF-1R may work in concert to promote the osteoclastogenesis of macrophages.

IL-34 has also been testified to augment the inflammatory circle by up-regulating some pro-inflammatory cytokines expression in various cell types, such as monocytes, macrophages, and microglia (28,29). Additionally, IL-34 differentiated macrophages could constitutively express membrane-type IL-1α, which renders the differentiation of non-committed memory T cells into conventional Th17 cells (23,30). In this investigation, we also find steroid administration could up-regulate IL-34 expression with the increased expression of pro-inflammation cytokines and adaptive immune reaction-relevant cytokines such as IL-34, IL-17A, IL-2, IL-12, and IFN-γ. All of these results indicate that IL-34 stimulated bone marrow-derived macrophages can differentiate into osteoclasts and promote the switch of memory $CD4^+$ T into Th17 cells. In other words, IL-34 could also amplify the inflammation reaction mediated by osteoclasts to induce Th17 cell differentiation.

Some limitations should be indicated there. The cellular source of IL-34 may need further investigation to decipher the steroid-induced mechanism since the origin of IL-34 is unclear. For IL-34 transcripts are testified to be expressed by various tissues in the body, while at the protein level, IL-34 can only be detected in a tissue-specific manner (31). It is of great interest to decipher the cellular source and master the transcription mechanism related to IL-34. Another issue should be indicated here. Only a small number of stimuli are known to activate NF-κB via a non-canonical pathway, and the precise mechanisms involved may pave a new way for the understanding of IL-34.

Our data clearly show that IL-34 may contribute to the development of steroid-induced ONFH with the promotion of osteoclasts differentiation and up-regulated inflammation cytokine expression. The anti-IL-34 inhibitory antibody can have the same effect as observed in IL-34 deficient mice, and IL-34 blocking strategy may have clinical utility in the future.

In conclusion, IL-34 may function as an osteoclastogenic cytokine to promote steroid-induced ONFH via promoting osteoclasts differentiation through ERK, STAT3, and non-canonical NF-κB pathways, which could be considered as a therapeutic target in the future.

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