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Chi3l1 induction in response to LPS suppresses osteoblast apoptosis

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Abstract- Upregulation of Chitinase-3-like protein 1(Chi3l1), a member of glycohydrolase family 18, is frequently seen in diseases associated with inflammatory responses, such as atherosclerosis, meningitis and asthma. However, little is known about either its regulation or its functions in the physiological and pathological processes in bone and related cells. In the mouse model of osteomyelitis used in this study, Chi3l1 was induced in the infected area. In vitro stimulation of osteoblasts and mesenchymal stem cells (MSCs) by lipopolysaccharide (LPS) resulted in elevated Chi3l1 expression. Overexpression of Chi3l1 attenuated TNF**α**-induced osteoblast apoptosis and promoted cell survival. Furthermore, Chi3l1 induced phosphorylation of AKT in a time-dependent fashion, while an inhibitor of the AKT signaling pathway abolished both the pro-survival and the anti-apoptotic effects of Chi3l1. Therefore, Chi3l1 might play a protective role in infected or inflammatory bone tissues by suppressing osteoblast apoptosis via an AKT-dependent pathway.

Keywords: *AKT, apoptosis, chitinase-3-like protein 1, LPS, TNF**α**.*

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Chi3l1 induction in response to LPS suppresses osteoblast apoptosis

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Abstract- **Upregulation of Chitinase-3-like protein 1(Chi3l1), a member of glycohydrolase family 18, is frequently seen in diseases associated with inflammatory responses, such as atherosclerosis, meningitis and asthma. However, little is known about either its regulation or its functions in the physiological and pathological processes in bone and related cells. In the mouse model of osteomyelitis used in this study, Chi3l1 was induced in the infected area. In vitro stimulation of osteoblasts and mesenchymal stem cells (MSCs) by lipopolysaccharide (LPS) resulted in elevated Chi3l1 expression. Overexpression of Chi3l1 attenuated TNFα-induced osteoblast apoptosis and promoted cell survival. Furthermore, Chi3l1 induced phosphorylation of AKT in a time-dependent fashion, while an inhibitor of the AKT signaling pathway abolished both the pro-survival and the anti-apoptotic effects of Chi3l1. Therefore, Chi3l1 might play a protective role in infected or inflammatory bone tissues by suppressing osteoblast apoptosis via an AKT-dependent pathway.**

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I. Footnotes

The abbreviations used are: Chi3l1, chitinase 3-like protein 1; LPS, lipopolysaccharides; mesenchymal stem cells , MSCs; TNFα, tumor necrosis factor-α; DMEM, Dulbecco's modified Eagle's medium; α-MEM , alpha minimum essential medium; RT, reverse transcription; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; PI3K, phosphatidylinositol 3-kinase.

Osteomyelitis, caused by pathogenic bacterial infection [1, 2], is primarily mediated by lipopolysaccharide (LPS) in a manner similar to that described for other infectious bone diseases [3, 4]. In addition to stimulating bone resorption, LPS also exerts adverse effects on bone formation through the induction of cytokine production (e.g., TNFα) [5]. Apoptosis of osteoblasts induced by cytokines is an important contributing factor to bone destruction [6, 7]; therefore, the identification of molecules that could prevent infection- or inflammation-induced osteoblast apoptosis might provide an effective potential anti-osteomyelitis therapy.

Chitinase 3-like protein 1 (Chi3l1), a member of the mammalian chitinase family [8], is induced in many inflammatory diseases, including arthritis, atherosclerosis, and meningitis [9-11]. Since LPS can trigger inflammation in various tissues, it is reasonable to speculate that LPS might be responsible for the induction of Chi3l1 expression in infected bone and relevant cells.

Although some hypotheses about the functions of Chi3l1 have been proposed, its physiological or pathological role has not been elucidated. Some research indicated that Chi3l1 may have a protective function in response to some types of stress [12]. However, whether Chi3l1 could suppress cell apoptosis, or whether AKT activation mediates the anti-apoptotic effect of Chi3l1, is still unclear. Nonetheless, it has been widely accepted that the AKT pathway plays a critical role in the regulation of cell survival and apoptosis in various cells and tissues [13, 14]. In this study, therefore, we examined the expression patterns of Chi3l1 in bone tissues and related cells under infectious stimulation and tested the hypothesis that Chi3l1 may inhibit osteoblast apoptosis via the AKT pathway.

II. Materials and Methods

a) Generation of the mouse model of osteomyelitis

We generated osteomyelitis in mice using the method described by Yoshii, T. [15]. Briefly, a piece of cotton thread was immersed in an overnight culture of S. aureus for 1 h, then dried for 2 h. BALBc mice were anesthetized and incisions were made in the femurs to insert either this piece of cotton thread or a piece of sterile thread. The mice were sacrificed at the indicated post-infection time points. All experimental procedures were conducted in accordance with the institutional guidelines for the care and use of laboratory animals at Tsinghua University, and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals. The femurs were collected for real-time PCR and Western blotting analysis.

b) Cell culture

Primary mouse calvarial osteoblastic cells were isolated from 6-day old mice as described previously [16]. Briefly, calvaria was cleaned and finely minced and then digested by collagenase and trypsin. The released cells were allowed to grow for 48 h and were then subcultured in α-minimum Eagle's medium (α-MEM) supplemented with 10% FBS and replaced every 3 days. Mesenchymal stem cells (MSCs) were obtained from 4- to 6-week-old normal BALBc mice, sacrificed by cervical dislocation to isolate the femurs. The bone marrow was

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consequently harvested by flushing the bone with DMEM containing 10% FBS. The cells were seeded on a culture plate, after 3 days, the non-adherent cells were discarded by replacing the medium. Adherent cells were designated as the first generation MSCs. The medium was changed twice a week, after reaching 90% confluence, the MSCs were detached using trypsin to undergo the subculture process. The third generation cells were used in our experiments.

c) Quantitative Real-time PCR

Total RNA was isolated using Trizol (Invitrogen), and the first-strand cDNAs were synthesized using the SuperscriptTM First-Strand Synthesis System for RT-PCR kit (Invitrogen).

Real-time PCR was performed using the TaqMan Universal PCR Master Mix kit (?) and the MX-3000P Real-time PCR Instrument (Stratagen). PCR assays were performed using the primers shown in Table 1. The PCR conditions were 95 °C for 10 min (initial denaturation), followed by 40 cycles at 95 °C for 15 sec, and 58 °C for 1 min.

d) Expression of Chi3l1 protein

The full length coding region of mouse Chi3l1 was cloned into pRSET A and protein expression was induced by IPTG in E. coli. This protein was used to generate polyclonal antibodies against Chi3l1.

The coding region of the Chi3l1 gene was also cloned into pcDNA3.1(+). The expression vector or empty vector was transfected into cos7 cells using lipofectamine 2000 (Invitrogen). After 72 h, the medium was collected and centrifuged. The supernatants were stored at -80°C for later experiments.

e) Cell viability assay

Primary osteoblastic cells were seeded in a 96-well plate. 24 h later, the Chi3l1 expression supernatant or the control supernatant collected from cos7 cells were added into the culture medium and then the cells were exposed to TNFα (20 ng/ml, Peprotech). After another 48 h, the culture medium was discarded and 180 μl α-MEM and 20 μl MTT (5 mg/ml) were added. The incubation was continued for 4 h. Finally, the solution was removed and 150 μl DMSO was added. Cell viability was assessed by measuring the absorbance at 492 nm, with a reference wavelength at 650 nm.

f) Cell cycle analysis by flow cytometry

Primary osteoblastic cells were seeded in a 10-cm culture plate. The Chi3l1 expression supernatant or the control supernatant collected from cos7 cells was added into the culture medium. After 48 h, the cells were collected by trypsin treatment, rinsed in PBS, fixed with ice-cold 70% ethanol for 20 min. Fixed cells were incubated with 50 μg/ml propidium iodide (PI) for 15 min, analyzed using a BD Biosciences FACScan flow cytometer.

g) Analysis of apoptosis

Primary osteoblastic cells were incubated with Chi3l1 expression or the control supernatant. Apoptosis was stimulated by TNFα, then the cells were incubated with FITC-conjugated Annexin V and PI (BD Biosciences) for 15 min. Cell apoptosis was analyzed using a BD Biosciences FACScan flow cytometer.

To measure caspase-3 activity, a caspase-3 activity assay kit (Nanjing KeyGen) was used. Briefly, after treated with Chi3l1 expression supernatant or control supernatant, the cells were stimulated with TNFα for 8 h. Then, the cells were lysed and 100 μg of protein in 100 μl volume was mixed with caspase-3 substrate for 5 h at 37℃. The caspase-3 activity was evaluated by measuring the absorbance at 405 nm.

h) Detection of AKT activation by Western blotting

Primary osteoblastic cells were serum-starved overnight, subsequently treated with Chi3l1expression or the control supernatant collected from cos7 cells for 0～60 min. The cells were then rinsed with ice cold PBS and lysed with RIPA buffer. Total protein concentrations were determined by a Protein Assay kit (Biorad). This was followed by Western blot assays using antibodies against AKT, or Ser473-phosphorylated AKT (Cell Signaling Technology).

i) Statistical analysis

The results were expressed as means ± S.D. A student's t-test was used to determine statistical significance. $P < 0.05$ was considered significant.

III. Results

a) Chi3l1 was up-regulated in osteomyelitis

We examined Chi3l1 expression in a mouse model of osteomyelitis. Real-time PCR analysis revealed a significant increase in Chi3l1 expression the second day after infection, compared with the sham-operated and the contra-lateral control (Figure 1A). Western blot assay showed similar results: as early as the second day, there was an increase in Chi3l1 proteins levels; upon reaching the fifth day, the increase became even more pronounced (Figure 1B).

b) LPS induced Chi3l1 expression in osteoblasts and MSCs

Primary osteoblastic cells were stimulated with LPS. Real-time PCR and Western blotting showed an obvious promotion of Chi3l1 expression at both the RNA and protein levels (Figure 2A and B).

MSCs were isolated from the bone marrow of mice and they could be induced to differentiate into osteoblasts both in vivo and in vitro. The cells were characterized by fluorescence-activated cell sorting (FACS) analysis. MSCs were negative for CD34 and

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CD31, but positive for CD29 and CD44 (Supplementary Figure 1). We stimulated MSCs with LPS and observed similar stimulating effects on Chi3l1 expression level as in osteoblasts (Figure 2A and C).

c) *Chi3l1 promoted osteoblast survival without affecting the cell cycle*

MTT assays demonstrated that treatment with TNFα resulted in decreased viability of primary osteoblastic cells. However, incubation with the Chi3l1 expression supernatant partially reversed this phenomenon (Figure 3A). Cell cycle analysis were performed to investigate whether this increase in survival was due to the increase of cell proliferation. After incubation with Chi3l1 for the indicated time period, cells were stained by PI and flow cytometry data showed that no obvious difference in cell growth was observed (Figure 3B, C). When the cells were stimulated by Chi3l1 in the absence of FBS, the percentage of cells in S phrase changed from 4.68%±1.7% to 5.3%±2.1%. And in the presence of FBS, the percentage changed from 20.2%±3.8% to 24.6%±5.1%.

In order to identify the concentration of Chi3l1 in the supernatant, Western blotting was performed and protein concentration was quantified by gray intensity analysis using software (Figure 3D). The estimated concentration of Chi3l1 in the supernatant was about 1000 ng/ml.

d) *Chi3l1 suppressed TNFα-induced osteoblast apoptosis*

Since Chi3l1 had no obvious effect on cell cycle, it probably possessed the activity of repressing apoptosis. We performed Annexin V and PI staining to scan cell apoptosis. The results showed that the percentage of apoptotic cells decreased from 58.2±4.6% to 40.1±3.2% (Figure 4A, B) after treatment with Chi3l1 supernatant. Moreover, Chi3l1 also suppressed the increase of caspase-3 activity stimulated by TNFα (Figure 4C). These data suggested that Chi3l1 had no effect on osteoblast growth, instead, it promoted cell survival at least partly mediated by preventing apoptosis

e) *AKT activation mediated the pro-survival and anti-apoptotic effects of Chi3l1*

The PI3K/AKT signaling pathway is considered to be a critical regulator of cell survival and apoptosis. An activated AKT is in the downstream region of PI3K. Our data showed that AKT phosphorylation could be observed in a time-dependent manner after the addition of Chi3l1 (Figure 5A). Furthermore, incubation with 1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3O-octadecylcarbonate (HIMO, Calbiochem), an inhibitor of the AKT signaling pathway, blocked both the phosphorylation of AKT and the pro-survival effect of Chi3l1 (Figure 5B, C). Meanwhile, the same concentration of HIMO had no obvious effect on cell survival. These data indicated that Chi3l1 played a protective role in osteoblasts via the activation of the AKT signaling pathway. Furthermore, Chi3l1 also showed anti-apoptotic effects on osteoblasts (Figure 5D, E). Therefore the pro-survival effect of Chi3l1 was at least partly mediated by the suppression of apoptosis.

IV. Discussion

Chi3l1, secreted by chondrocytes, macrophages and a number of other cells, is a member of the mammalian chitinase family [9, 17, 18]. In this research, we demonstrated that its expression was induced as a part of the disease symptoms of osteomyelitis. Previous studies have reported that the secretion of Chi3l1 is often elevated in serum or tissues of patients suffering from inflammatory stress, the results of our study supported these observations.

To identify the cells that may contribute to the Chi3l1 expression in osteomyelitis, we performed an in vitro study, which showed that primary osteoblastic cells and MSCs may be the sources of Chi3l1 elevation. The administration of LPS has been widely used to simulate the influence of bacterial infection on organisms. Our data showed that LPS treatment resulted in Chi3l1 up-regulation in both osteoblasts and MSCs.

A recent report has demonstrated that serum Chi3l1 concentration was elevated in multiple myeloma (MM) patients [19]. MM is associated with inflammatory stress and increased secretion of cytokines, which are essential for the progress of MM [20, 21], our results are in line with these observations. In osteomyelitis tissues, cytokines are induced shortly after infection [22]; we also found that TNFα could up-regulate Chi3l1 in MSCs and osteoblasts (our unpublished data). Thus, it is reasonable to propose that the induction of Chi3l1 is a response of bone tissue to an unfavorable environment, such as inflammation, both in MM and in osteomyelitis. However, whether the expression level of Chi3l1 in osteomyelitis reflects the severity of the disease, as is seen in the case of MM or rheumatoid arthritis, is still unknown. Clinical research with relevant patients may enable us to address this problem.

TNFα, as a primary inflammatory cytokine induced by LPS, has been shown to enhance osteoblast apoptosis [23]. Excessive apoptosis decreases the osteoblast population and has a negative impact on bone formation, indicating that osteoblast apoptosis induced by cytokines contributes to inflammatory bone loss [7, 24, 25]. Our findings have demonstrated that Chi3l1 promoted osteoblast survival and blocked TNFα-induced apoptosis. Meanwhile, it had no obvious effect on osteoblast proliferation. Therefore, the increase of cell viability is due to decrease of apoptosis rather than promotion of cell growth. In our opinion, promotion of cell survival by suppression of cytokine-induced apoptosis is an important mechanism by which this

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protein protects inflammation-stressed bone. The Chi3l1 concentration we used falls into the pathological concentration range in human beings [11, 26, 27], Therefore, it is reasonable to speculate that, by down-regulating osteoblast apoptosis, Chi3l1 might reduce the risk of bone destruction associated with deficient bone formation in vivo.

Some studies have shown that the PI3K signaling pathway is involved in cell apoptosis regulation. PI3K/AKT mediates the anti-apoptotic effect of Wnt in MC3T3-E1 cells [28] and AKT signaling is essential for survival of acute lymphoblastic leukemia cells, osteoblasts and endothelial cells [29-31]. Thus, we hypothesized that Chi3l1 promoted osteoblast survival and reduced apoptosis by activation of the AKT signaling pathway. Our data illustrated that Chi3l1 had an obvious inducing effect on AKT phosphorylation. Moreover, an inhibitor of the AKT pathway abrogated the pro-survival and anti-apoptotic effects of Chi3l1.

The association of Chi3l1 expression with both normal and pathological tissue turnover has been reported [12, 32] and a protective role in these situations has been proposed. The skeleton is constantly resorbed by osteoclasts and replaced by osteoblasts in the tissue turnover process, so osteoblasts are essential for maintaining the integrity of bone. However, any decrease in osteoblast viability as a result of infectious or inflammatory skeletal diseases disrupts the balance between bone formation and resorption. The discovery of the enhancement of cell survival and the suppression of apoptosis by Chi3l1 thus suggests a potential therapeutic opportunity for controlling bone loss in relevant pathological processes.

V. Acknowledgements

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Figure legends

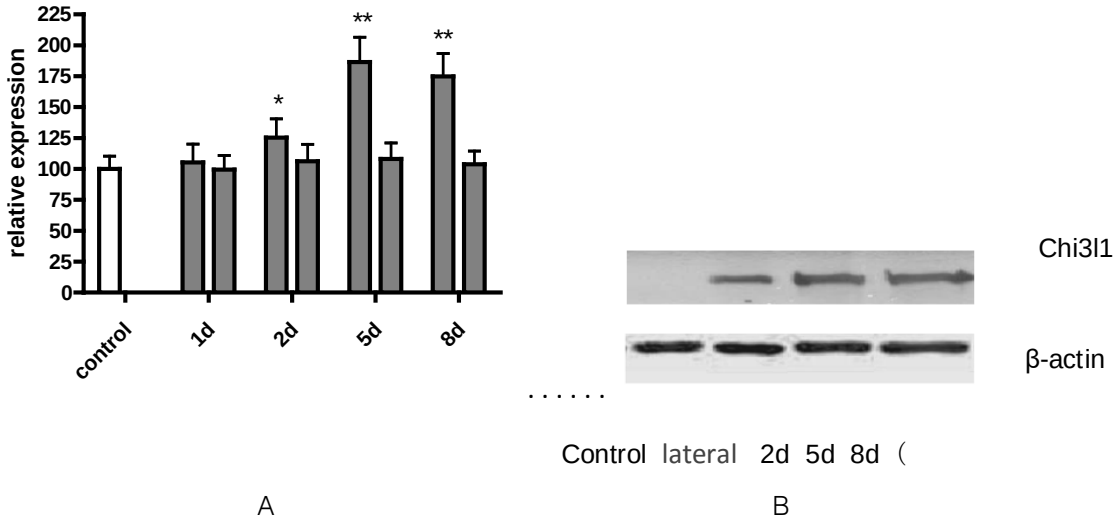
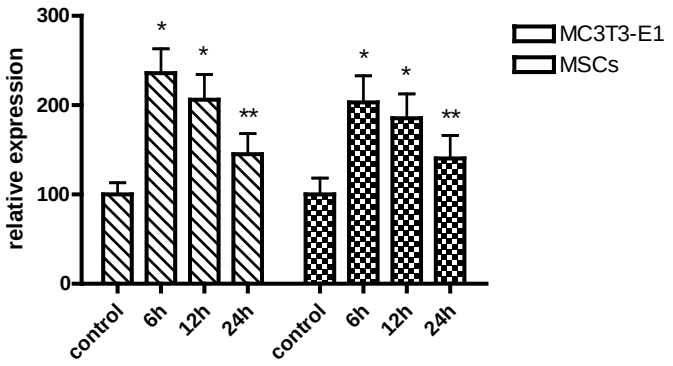


Fig. 1: Changes of Chi3l1 expression in osteomyelitis. (A) At different time points after infection, the femur tissues around the incisions (0.3cm upwards and downwards from the incisions) were collected and cleared of surrounding tissues. The contra-lateral femur and the one infected by bacteria was isolated at the same time. The infected and lateral femur were represented by the left bar and right bar in each group (grey bars) respectively. The bone tissues were immediately minced with shears, then ground in liquid nitrogen. Trizol was added to the tissue powder and the mixture was homogenized to ensure sufficient yield of RNA. Then real-time PCR assays were performed. The expression levels of Chi3l1 were normalized to GADPH levels. The results are expressed as the copy numbers relative to GADPH. *, $P < 0.05$; **, $P < 0.01$ vs control. (B) The femurs were isolated and homogenized as described above. After homogenization, the total proteins were extracted by Trizol from bone according to the protocol. Then Western blot assays were performed. β-actin was used as an internal control.



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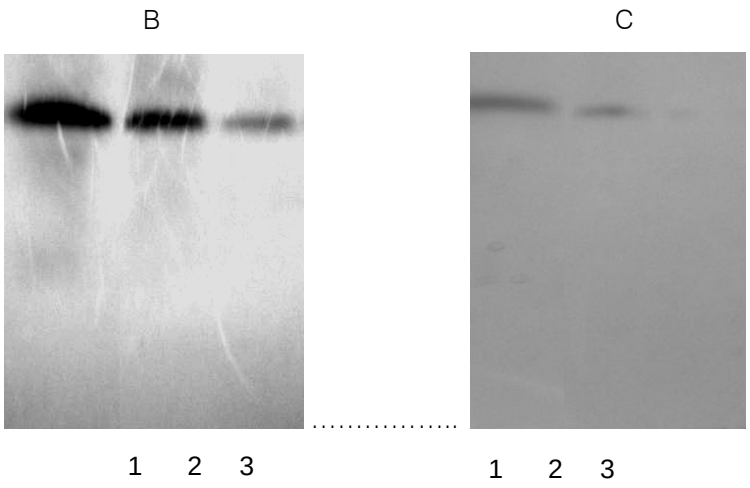
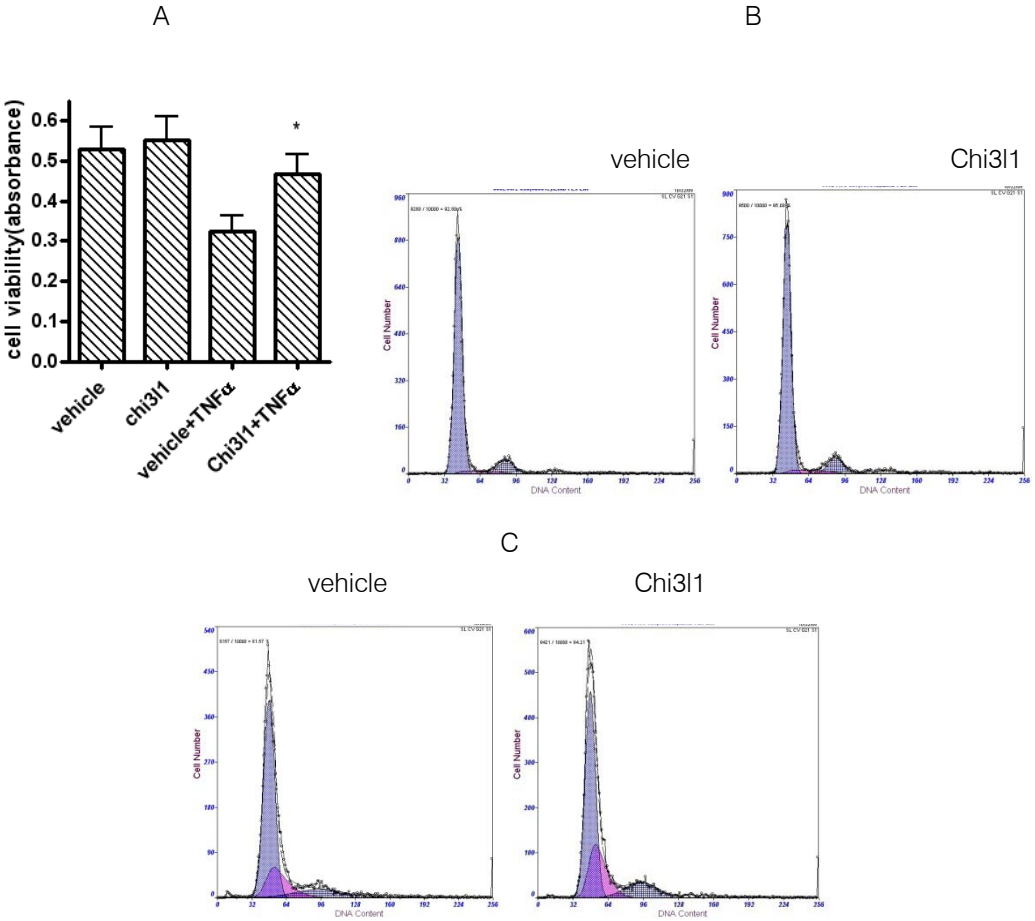


Fig. 2: LPS-stimulated Chi3l1 expression. (A) Primary osteoblastic cells were stimulated by LPS (1ug/ml) for 6 h, 12h or 24 h; then the mRNA expression levels were analyzed by real-time PCR. GADPH was used as an internal control. * , P <0.01;**, P < 0.05 vs control. (B) Primary osteoblastic cells were treated with LPS (1ug/ml); culture media were collected after 48 h and proteins were precipitated by the addition of 3 volumes of ice-cold ethanol. Western blotting was performed to confirm the results of real-time PCR. (C) MSCs cells were stimulated with LPS in the same way as primary osteoblastic cells. Western blot assays were performed to examine the changes of Chi3l1 expression at protein level. 1: The recombinant protein purified from E.coli. was used as a positive control . 2. The cells treated with PBS were used as a negative control. 3. The cells were treated with LPS



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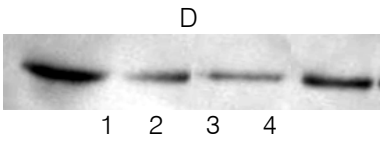
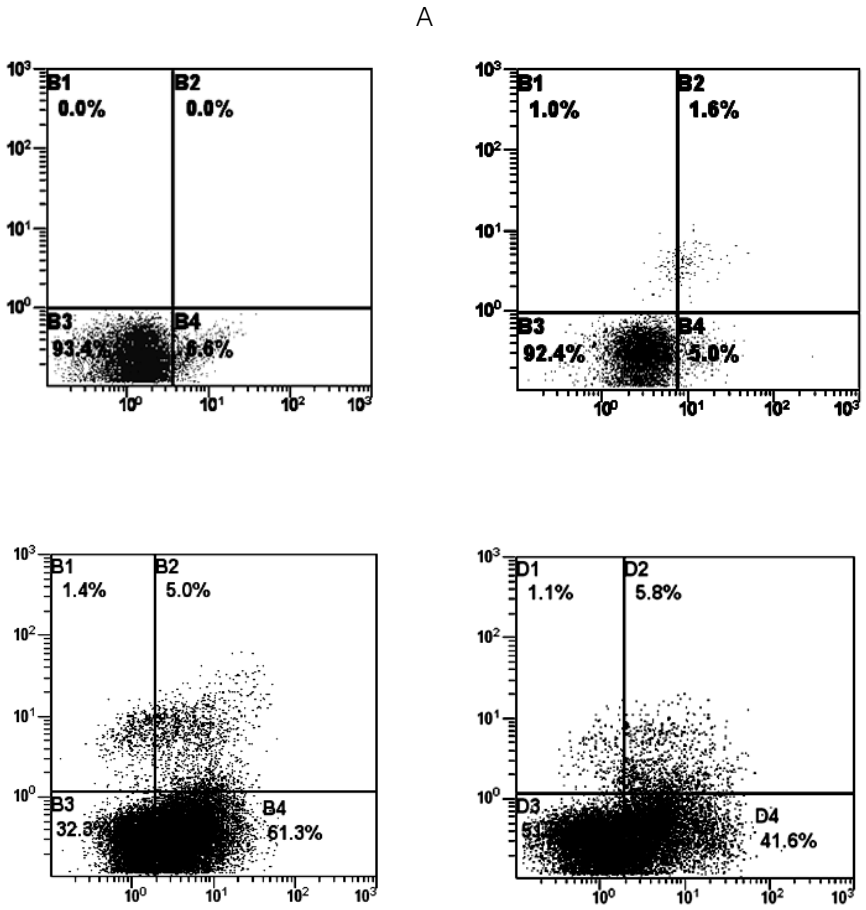


Fig. 3: Chi3l1 promoted survival and did not influence cell growth in osteoblasts. (A) Cos7 cells were transfected with the expression vector of Chi3l1 or the empty vector and the supernatant was collected after 72h. The primary osteoblastic cells were cultured with the expression supernatant or the control supernatant with or without TNFα (20ng/ml) for 48h. MTT assays were performed to evaluate cell viability. The results are expressed as the absolute absorbance values. *, P <0.05 vs the vehicle+TNFα group. (B) Primary osteoblastic cells were cultured in serum –free medium for 16h, then treated with Chi3l1 expression supernatant or the control supernatant for 48h. The cells were collected and stained by PI. Flow cytometry assays were performed to analyse the cell cycle. (C) The primary osteoblastic cells were cultured in medium containing 10% FBS, and then incubated with the expression supernatant or the control supernatant for 48h. Flow cytometry assays were performed to analyze cell cycle. (D) Cos7 cells were transfected with the expression vector of Chi3l1 and 72h later, supernatant was collected. Western blotting was performed with expression supernatant and a series of concentrations of Chi3l1 protein purified from E.coli. 1, 2, 3: The recombinant protein of Chi3l1 from E.coli. The concentrations of these were 400, 800, 3000ng/ml, respectively. 4: The expression supernatant collected from cos7 cells



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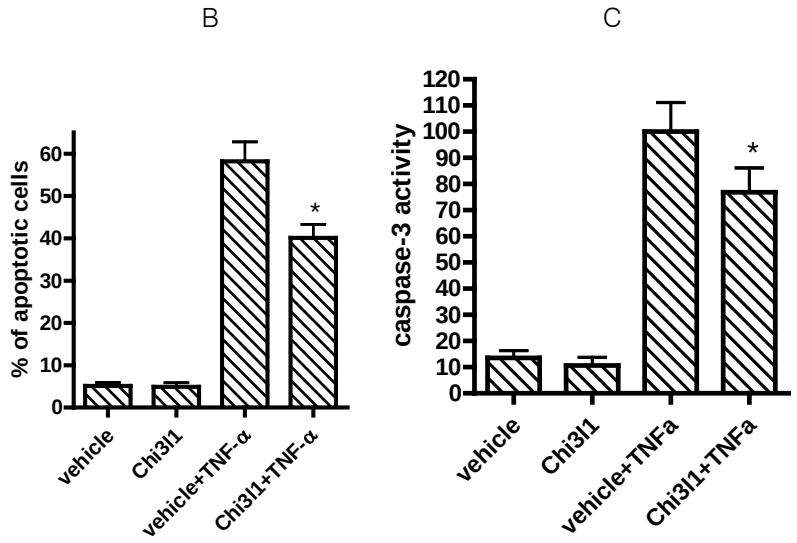
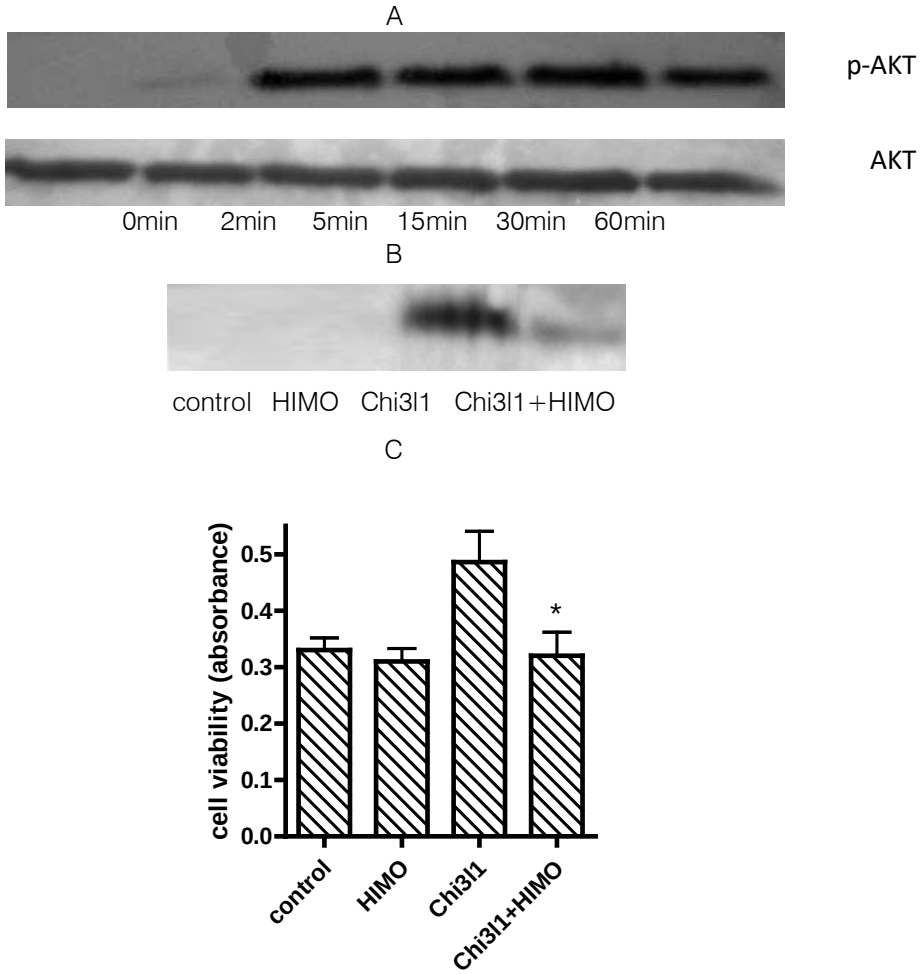


Fig. 4: Chi3l1 inhibited apoptosis in osteoblasts. (A) Primary osteoblastic cells were cultured with Chi3l1 expression supernatant or the control supernatant with or without TNFα (20ng/ml). 24h later, Annexin V and PI staining was performed to assess cell apoptosis. Representative figures of flow cytometry analysis were shown.The numbers in the figures indicate the percentage of cells within respective subpopulations. (B) The statistic analysis of the flow cytometry data. (C) Primary osteoblastic cells were cultured with the expression supernatant or the control supernatant with or without TNFα (20ng/ml) for 8h. Then cell lysates were prepared and relative caspase-3 activity was evaluated by a commercial available kit according to the protocol. *, $P < 0.05$; **, $P < 0.01$ vs the vehicle+TNFα group



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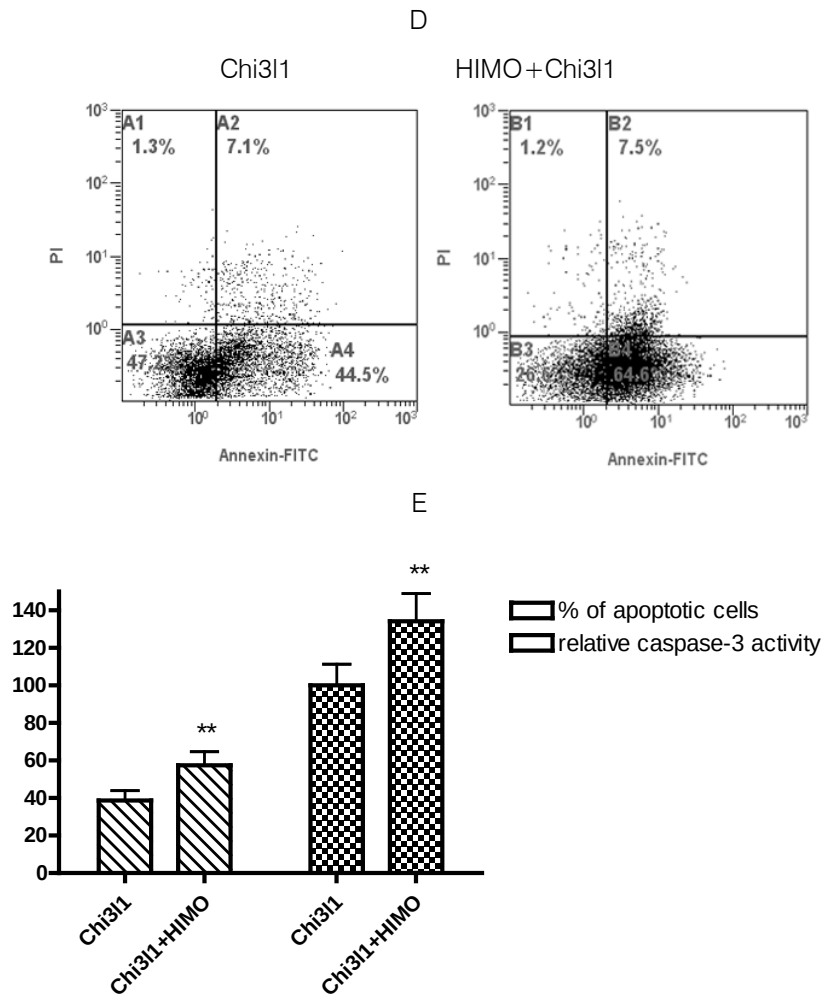

Fig. 5: The AKT pathway mediated the protective effect of Chi3l1. (A) Primary osteoblastic cells were cultured in serum-free medium overnight, then cells were incubated with Chi3l1 expression supernatant collected from cos7 cells for 0~60min (with a volume ratio of 1:1). At the end of the indicated period, cells lysates were prepared and the phosphorylated AKT was detected by Western blotting. (B) Effect of HIMO on AKT phosphorylation. Primary osteoblastic cells cells were incubated with control or Chi3l1 expression supernatant for 30min with or 10μM without HIMO. (C), (D) and (E) Primary osteoblastic cells cells were incubated with Chi3l1 expression supernatant collected from cos7 cells with or without 10μM HIMO. The cells were incubated with TNFα (20ng/ml) for 48, 24 or 8 h, respectively. MTT, Annexin V and PI staining, as well as relative caspase-3 activity assays, were performed. *, $P < 0.01$; **, $P < 0.05$ vs the Chi3l1+ TNFα group

Table 1: Oligo-deoxyribonucleotide primers and probes sequences used in real-time PCR

Gene	Primer(5'-3')	Probe(5'-3')
GADPH	F: CTGCCAAATATGATGACA	AGGTGGTGAAGCAGG
	R: CCCAGGATGCCCTTGA	CGTCG
Chi3l1	F: TCCAGCCAGGCAGAGAGAA	TCCTGCTCAGCGCAGC
	R: TGTCAATGGCCACCTTTCCT	TTTGTCA

The reporter dye of probe used on the 5' end was FAM and quencher dye on the 3' end was DABCYL.

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