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Ovariectomy-induced bone loss in TNFα and IL6 gene knockout mice is regulated by different mechanisms

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Abstract

We examined the effects of tumor necrosis factor-α (TNFα) and interleukin-6 (IL6) gene knockout in preserving the bone loss induced by ovariectomy (OVX) and the mechanisms involved in bone metabolism. Twenty female wild-type (WT), TNFα-knockout (TNFα−/−) or IL6-knockout (IL6−/−) mice aged 12 weeks were sham-operated (SHAM) or subjected to OVX and killed after 4 weeks. Bone mass and skeletal microarchitecture were determined using micro-CT. Bone marrow stromal cells (BMSCs) from all three groups (WT, TNFα−/− and IL6−/−) were induced to differentiate into osteoblasts or osteoclasts and treated with 17β-estradiol. Bone metabolism was assessed by histological analysis, serum analyses and qRT-PCR. OVX successfully induced a high turnover in all mice, but a repair effect was observed in TNFα−/− and IL6−/− mice. The ratio of femoral trabecular bone volume to tissue volume, trabecular number and trabecular thickness were significantly decreased in WT mice subjected to OVX, but increased in TNFα−/− mice (1.62, 1.34, 0.27-fold respectively; P < 0.01) and IL6−/− mice (1.34, 0.80, 0.22-fold respectively; P < 0.01). Furthermore, we observed a 29.6% increase in the trabecular number in TNFα−/− mice when compared to the IL6−/− mice. Both, TNFα−/− and IL6−/− BMSCs exhibited decreased numbers of TRAP-positive cells and an increase in ALP-positive cells, with or without E2 treatment (P < 0.05). While the knockout of TNFα or IL6 significantly upregulated mRNA expressions of osteoblast-related genes (Runx2 and Col1a1) and downregulated osteoclast-related mRNA for TRAP, MMP9 and CTSK in vivo and in vitro, TNFα knockout appeared to have roles beyond IL6 knockout in upregulating Col1a1 mRNA expression and downregulating mRNA expressions of WNT-related genes (DKK1 and Sost) and TNF-related activation-induced genes (TRAF6). TNFα seemed to be more potentially invasive in inhibiting bone formation and enhancing TRAF6-mediated osteoclastogenesis than IL6, implying that the regulatory mechanisms of TNFα and IL6 in bone metabolism may be different.
Introduction

Sex steroids play a regulatory role in bone metabolism. After menopause, declining estrogen levels combined with a parallel increase in follicle-stimulating hormone (FSH) levels disrupt the orchestrated balance between bone formation and bone resorption, often resulting in postmenopausal osteoporosis (Zaidi 2007). In postmenopausal osteoporosis, the bone-resorbing activity outpaces the bone-forming activity, which leads to a rapid loss of bone strength and an increased risk of fracture (Camacho et al. 2016).

Estrogen regulates bone homeostasis through several potential mechanisms like direct effects on bone cells and indirect effects on the immune system (Weitzmann & Pacifici 2006). Like natural menopause, ovariectomy (OVX) stimulates bone resorption by enhancing osteoclast formation and life span (Sun et al. 2006, Weitzmann & Pacifici 2006, Nakamura et al. 2007). This phenomenon is fueled by an expansion of hematopoietic and mesenchymal stem cell (HSCs/MSCs) pool in the bone marrow (BM) and their increased commitment toward osteoclastic and osteoblastic cells respectively (Jilka et al. 1998). While bone anabolic activity is enhanced after OVX, it is not enough to match increased bone desorption (Novack 2007). Subsequent net bone loss is induced by escalated apoptosis of osteoblasts (Kousteni et al. 2001, 2003) and extended survival of osteoclasts (Nakamura et al. 2007, Krum et al. 2008). In addition, the proliferation and differentiation of these two cell types is influenced by the bone marrow, which is a microenvironment rich in diverse cytokines. Immune cells producing inflammatory cytokines have a critical role in the mechanism of OVX-induced bone loss (Pacifici 2012, Redlich & Smolen 2012). The stimulatory effect of OVX on BM-HSCs/MSCs is relevant to an increase in pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα), interleukin 6 (IL6) and IL1, which may enhance the activity of osteoclasts and suppress bone formation. However, the regulatory role of estrogen in the cross-talk between the immune system and skeletal metabolism is largely unknown.

TNFα, a pivotal and multifunctional cytokine chiefly produced by macrophages, has been demonstrated to profoundly affect the differentiation and activity of osteoblasts and osteoclasts during chronic inflammatory conditions such as rheumatoid arthritis (Firestein 2003) and menopause (Pfeilschifter et al. 2002). The primary role of increased TNFα production after estrogen deficiency is to promote osteoclastogenesis (Teitelbaum 2000). The loss of function studies in vivo indicate that TNF-deficient and -null p55 TNF receptor can prevent bone loss induced by OVX (Roggia et al. 2001). Furthermore, in vitro studies show that TNFα may inhibit osteoblastic differentiation via p55 TNF receptor and the ubiquitination of runt-related transcription factor 2 (Runx2), mediated by Smurf1 and Smurf2 (Gilbert et al. 2002, 2005, Kaneki et al. 2006). The possible mechanism of regulating bone homeostasis may be associated with enhanced NFκB activity and its ligand, the receptor activator of nuclear factor kappa-B ligand (RANKL) (Lam et al. 2000).

IL6, another critical cytokine mainly secreted by a variety of cells (e.g. macrophages, T-cells, osteoblastic cells and stromal cells), may exert both pro- and anti-inflammatory effects in bone metabolism (Steeve et al. 2004, Karsenty & Olson 2016). IL6, defined as a resorptive agent, stimulates the activity of bone-resorbing osteoclasts. It is worth noting that OVX fails to induce bone loss in IL6-deficient mice (Poli et al. 1994). Several indirect mechanisms might be responsible for the stimulatory effects of IL6 on osteoclastogenesis. These include cytokines such as IL1 and TNFα, increasing osteoblastic RANKL production or independent activation by conditions such as stress (Ikejima et al. 1990, Devlin et al. 1998, Yokota et al. 2014). On the other hand, physiological levels of IL6 have no effect on stimulating bone resorption and osteoclastogenesis, unless abundant levels of IL6 or other cytokines is present (Murakami et al. 1993, De La Mata et al. 1995, Kishimoto 2010). Interestingly, certain threshold levels of IL6 are known to enhance the commitment of BM-MSCs toward the osteoblastic lineage and subsequent differentiation (Taguchi et al. 1997, Erices et al. 2002). However, IL6 also has been reported to inhibit osteoblastic differentiation in vivo and in vitro (Peruzzi et al. 2012). Thus, the effects of IL6 in bone homeostasis are contradictory.

While the blockade of single cytokines (TNFα or IL6) prevents bone loss following ovariectomy, their controversial effects reported in literature and different roles in inducing bone loss under conditions of estrogen deficiency need further investigation. In the present study, we aimed to better understand the dominant acute effects of TNFα and IL6 on WT and OVX mice with respect to bone loss.

Materials and methods

Animals and intervention

All animal experiments were performed in line with the guide for the care and use of laboratory animals and...
were approved by the Institutional Animal Care and Use Committee of West China Hospital, Sichuan University. Female C57BL/6j (wild type (WT)) mice, TNFa or IL6-knockout (TNFa-/- and IL6-/-, C57BL/6j background) mice were obtained from the Jackson Laboratory. All mice were maintained in ventilated racks with pathogen-free barrier conditions at a constant temperature (22°C) under a 12-h light/darkness cycle, and normal chow diet and water were provided ad libitum. At 12 weeks, the mice were randomly divided into six groups of 10 animals each: SHAM-operated (ovary intact) mice from each genotype and ovariectomized (OVX) mice from each genotype. Mice were weighed weekly and killed after 4 weeks. Blood was collected from eyes prior to killing. The uterus was carefully dissected and weighed for each mouse to evaluate the estrogen agonistic activity.

Serum parameters for bone metabolism

Serum bone formation biomarker procollagen type I N-terminal propeptide (PINP; Elabscience, China) and bone resorption biomarker C-terminal telopeptides of type I collagen (CTX; Elabscience) were used to evaluate bone turnover.

Micro-CT for bone mass and microstructure

Distal ends of left femora were fixed with 4% paraformaldehyde for 24h and scanned on the Inveon Multi-Modality micro-CT (Siemens). Femurs were scanned at the energy of 80 kV and intensity of 500μA. As previously described (Zhou et al. 2014, Zhang et al. 2015, Chen et al. 2016, Wang et al. 2016), trabecular analysis for 100 contiguous slices started at 0.6mm proximal to the growth plate and extended proximally 1.5mm. Inveon Research Workplace, version 4.0 was used for three-dimensional (3D) reconstruction in the region of interest (ROI). Bone microstructure parameters included bone volume to tissue volume ratio (BV/TV), trabecular number (Tb. N), trabecular thickness (Tb. Th), total trabecular bone mineral density (BMD) and trabecular thickness separation (Tb. Sp).

Bone histological analysis

The left femur was dissected from soft tissue, fixed in the 4% paraformaldehyde solution for 1–2 days and then decalcified in 20% ethylene diamine tetra acetic acid (EDTA) buffer at 37°C. The buffer was changed every 3–4 days until complete decalcification. The samples were then dehydrated and embedded in paraffin wax. Longitudinal sections from the middle of femur (5μm) were stained with hematoxylin-eosin (H&E) and tartrate-resistant acid phosphatase (TRAP; Sigma-Aldrich, kit 387-A) for histological analysis. TRAP-positive multinucleated cells (three or more nuclei as osteoclasts) were observed in the regions 2mm beneath of the growth plate and counted in six different visual fields (magnified 10×40) via Axio Scope Light Microscope (Zeiss). The total red areas of TRAP-positive osteoclasts were calculated using digital planimetry software (Image-Pro Plus 6.0, Media Cybernetics, Inc., USA).

Real-time qRT-PCR for bone-related gene expression

After the removal of bone marrow, total RNA was extracted from the distal metaphyses of right femurs using TRIzol reagent, according to protocol provided by the manufacturer (Invitrogen). RNA quality was evaluated by A260/A280 ratio. Total 1μg RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio). Real-time quantitative PCR was performed with 2μL cDNA with the SYBR Premix Ex Taq II kit (Takara Bio) in CFX Real-Time PCR Detection System (Bio-Rad). The sequence of oligonucleotide primers is listed in Table 1. Each RNA quantification was carried out in triplicate, and every experiment was repeated independently at least three times. The relative mRNA expression levels were normalized to the GAPDH in the same sample and analyzed with the 2−ΔΔCT method as our previous report (Zhou et al. 2014, Zhang et al. 2015, Chen et al. 2016, Wang et al. 2016).

Comparison of osteoblast and osteoclast differentiation between WT and gene knockout mice in vitro

Bone marrow stromal cell (BMSCs) cultures

Femurs and tibias from WT, TNFa-/- and IL6-/- mice at 6–8 weeks of age were used for BMSC culture, as previously described (Zhou et al. 2014, Zhang et al. 2015, Chen et al. 2016, Wang et al. 2016). Briefly, bone marrow cells were flushed out with a 3 cc syringe and 25G needle. BMSCs were plated on 24-well plates at a density of 1×10^3/well and cultured in regular medium (containing α-MEM, Hyclone, USA), 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin (Invitrogen) and 1% beta-glutamine (Invitrogen) at 37°C. The culture medium was changed every other day. The day of plating was counted
osteoclastic differentiation medium, consisting of regular medium, 25 ng/mL of human macrophage-colony stimulating factor (M-CSF) (R&D Systems) and 30 ng/mL RANKL (R&D Systems). Estrogen treatment maintained the same as above in osteoblasts cultures. Medium was changed every 3 days. At day 7, TRAP staining kit (Sigma-Aldrich, kit 387-A) and real-time PCR of osteoclast-related genes were performed to determine osteoclast formation. Cells with three or more nuclei were identified as TRAP-positive osteoclasts. The methods evaluating osteoclast formation in six different visual fields (magnified 10× 10, 10× 20, respectively) were identical to those in osteoblasts evaluation.

### Statistical analysis

All values were analyzed using SPSS 22.0 software and presented as mean ± S.E.M. Statistically significant differences were assessed by one-way analysis of variance (ANOVA) followed by Fisher's PLSD post hoc test for comparison between groups and two-way analysis for the interactions between genotypes and treatment. A P value of <0.05 was considered significant.

### Results

#### OVX-induced abnormal metabolic parameters are repaired in TNFα−/− and IL6−/− mice

No significant differences in body weight were found in SHAM WT, TNFα−/− and IL6−/− mice at all time points tested. OVX significantly increased body weight in WT mice by 11.2% (22.92 ± 0.67 vs 20.60 ± 0.39, P<0.05; Fig. 1A) and 11.4% (23.31 ± 0.76 vs 20.92 ± 0.45, P<0.05) compared with that of SHAM WT mice at 3 and 4 weeks, respectively. However, TNFα−/− and IL6−/− mice showed an inhibitory effect on OVX-induced body weight gain when compared to the WT mice. Conversely, body weight of OVX TNFα−/− mice exhibited a 10.9% growth in comparison to the OVX WT mice at 4 weeks (23.31 ± 0.76 vs 21.01 ± 1.12, P<0.05; Fig. 1A). Furthermore, OVX significantly lowered uterine weight in all three genotypes compared to respective SHAM group (P<0.01; Fig. 1D), which indicated that OVX worked successfully as a model of estrogen withdrawal. The success of OVX was further confirmed by high turnover in all mice, although a repair effect was observed in TNFα−/− and IL6−/− mice. The growth of CTX induced by OVX witnessed a 1.35-fold change in WT mice, but 1.24- and 1.22-fold (P<0.05; Fig. 1B), respectively, in IL6−/− and TNFα−/− mice. While

### Osteoblast differentiation and estrogen treatment

At day 7, BMSC culture medium was switched to differentiation medium containing regular medium plus 50 μg/mL ascorbic acid and 10 mM glycerophosphate. 17β-Estradiol (E2; Sigma-Aldrich, E2758) was used for the treatment and prepared according to manufacturer’s instructions. Cells were treated with 10−7 M E2 in absolute ethanol or absolute ethanol alone at the start of osteogenic differentiation for 7 days. Alkaline phosphatase (ALP) staining was performed using ta kit (Sigma-Aldrich, 86R-1KT), and real-time PCR for osteoblast-related genes was performed to determine the osteogenic differentiation. Image-Pro Plus 6.0 (Media Cybernetics, Inc.) was used for cells count and ALP activity in six different visual fields (magnified 10× 10).

### Osteoclast differentiation and estrogen treatment

Bone marrow cells were collected and induced to differentiate into osteoclasts. Cells were cultured in

### Table 1 Oligonucleotide primers for RT-PCR.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5′–3′)</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>F: TGCACCCACCAACTGGTAC&lt;br&gt;R: GGATGCAGGGATATGTTT</td>
</tr>
<tr>
<td>Runx2</td>
<td>F: GGTACTTTGCTGACATCTTGC&lt;br&gt;R: GCTTCCGTACCGTCAACA</td>
</tr>
<tr>
<td>Col1a1</td>
<td>F: CGCAAGCCAAAGCTGCA&lt;br&gt;R: CTGGTGGTGTGGATGAC</td>
</tr>
<tr>
<td>DKK1</td>
<td>F: GATATCCCAAGAATCACCAG&lt;br&gt;R: GGACCAAGAAGTTGCTGCA</td>
</tr>
<tr>
<td>Sost</td>
<td>F: GCCGGACCTAATACAGCAG&lt;br&gt;R: CACGTAGCCCAACATCAC</td>
</tr>
<tr>
<td>CTSK</td>
<td>F: GAAGAAGACTCAGCAG&lt;br&gt;R: TCCAGGTTATGGCCAGAGTT</td>
</tr>
<tr>
<td>MMP9</td>
<td>F: CTGGACAGCCAGACATTAAG&lt;br&gt;R: CTGGCCGCAAAGTCTGCA</td>
</tr>
<tr>
<td>TRAP</td>
<td>F: CAGCACGTCCATGGAAGAGG&lt;br&gt;R: CTGGAAACCTTCTGCGCTGG</td>
</tr>
<tr>
<td>TRAF6</td>
<td>F: AGTGGCAGCTGGAATGAAA&lt;br&gt;R: CACTTATCCGCAGGAAAGAT</td>
</tr>
<tr>
<td>NFATc1</td>
<td>F: GCTCACACGTGTTACCTGGAAGATGA&lt;br&gt;R: ACTTTGGTGGTGGACAGATGAT</td>
</tr>
<tr>
<td>c-Fos</td>
<td>F: AGGGGCAAACTAGAAGCTA&lt;br&gt;R: CAATCTCAGTCTGCAAGCA</td>
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Col1a1, collagen type I alpha 1; CTSK, cathepsin K; DKK1, dickkopf-related protein 1; MMP9, matrix metallopeptidase 9; NFATc1, nuclear factor of activated T cells, cytoplasmic 1; Runx2, runt-related transcription factor 2; Sost, sclerostin; TRAP, tartrate-resistant acid phosphatase; TRAF6, TNF receptor-associated factor 6; F, Forward; R, reverse.

as day 0. When the cells became confluent around day 5–6, the culture medium was changed to osteoblastic or osteoclastic differentiation medium.
OVX increased higher levels of PINP (Fig. 1C) in all mice than that of SHAM group, no significant difference was detected between WT and gene knockout mice.

**OVX fails to induce femoral trabecular bone loss in TNFα−/− or IL6−/− mice**

To ensure the homogeneity of analyses, selected ROI volume of distal femoral metaphysis was maintained equally in all groups (P>0.05; Fig. 2A). In SHAM groups, no significant differences were observed for analyzed parameters among three genotypes. After OVX operation, WT mice showed 55.7% reduction in BV/TV (P<0.01; Fig. 2D), 50.8% reduction in Tb. N (P<0.01; Fig. 2E), 20.7% reduction in Tb. Th (P<0.01; Fig. 2B), 42.3% reduction in BMD (P<0.01; Fig. 2F) and 148% growth in Tb. Sp (P<0.01; Fig. 2C) in comparison to the SHAM WT mice. OVX failed to alter these parameters in TNFα−/− and IL6−/− mice. TNFα−/− mice showed 1.62-, 1.34-, 0.27- and 1-fold higher values for BV/TV, Tb. N, Tb. Th, and BMD, but 2.07-fold lower value of Tb. Sp in comparison to OVX WT mice (P<0.01). Similar fold changes in IL6−/− mice were observed (1.34, 0.80, 0.22, 0.74 and 1.19-fold respectively, P<0.01). Furthermore, TNFα−/− and IL6−/− mice showed similar parameters after OVX, except a slight increase of Tb. N to 29.6% in TNFα−/− mice (P<0.05; Fig. 2E).

Figure 1
Changes in metabolic parameters after OVX. OVX induced greater weight gain only in WT mice, and gene knockout mice could correct the effect of OVX on body weight (A). The high bone turnover was confirmed in all mice, although a repair effect was observed in gene-knockout mice (B and C). OVX significantly lowered wet uterine weight (D) in all genotypes and resulted in uterus atrophy, reflected by representative uterus sample (E). Data are expressed as mean ± s.e.m. *P<0.05, **P<0.01, compared to the same genotype. *P<0.05, **P<0.01, compared to WT mice subjected to similar treatment; n=9. A full colour version of this figure is available at https://doi.org/10.1530/JME-17-0218.

Image from 3D reconstruction also indicates that OVX-induced trabecular bone loss only in WT mice (Fig. 2G).

Gene knockout prevents OVX-induced bone structure deterioration by inhibiting osteoclastogenesis

Representative H&E staining images for trabecular microarchitecture under the epiphyseal growth plate of left femora displayed a notable reduction in the trabecular number only in OVX WT mice (Fig. 3A, B, C, D, E and F), which further verified that knockout of TNFα and IL6 played a critical role in preventing bone loss induced by OVX. TRAP staining showed that deletion of TNFα and IL6 in SHAM-operated mice decreased the number and size of TRAP-positive multinucleated cells compared to SHAM WT mice, but only the differences between TNFα−/− and WT mice was obvious (P<0.05; Fig. 4B and C). Moreover, OVX significantly increased the number and size of TRAP-positive multinucleated cells in WT mice (P<0.01). In contrast, the number and size of TRAP-positive multinucleated cells in TNFα−/− mice were less than that of OVX WT mice approximately by 86.3% and 85.2% respectively (P<0.01), and the percent changes in IL6−/− mice reached to 92.4% and 84% respectively (P<0.01). The results suggested OVX stimulated osteoclast formation...
only in WT mice while in TNFα and IL6 knockout, OVX acted as an inhibitor in osteoclastogenesis.

**TNFα and IL6 knockout differently reversed the bone-wasting effect of OVX**

The mRNA expressions of related genes in bone formation were detected by real-time RT-PCR. In SHAM groups, mRNA levels of Runx2 and Col1a1 in TNFα−/− mice were 2.04- and 2.79-fold higher than those in WT mice (P<0.01; Fig. 5A and B). Similarly, the fold changes of Runx2 and Col1a1 in IL6−/− mice were 1.95 and 2.31 respectively. OVX significantly downregulated mRNA expressions of Runx2 and Col1a1 in three genotypes (P<0.01), whereas TNFα−/− mice showed higher mRNA levels by 3.57- and 12.5-fold respectively and that of IL6−/− mice was 3.42- and 10.45-fold (P<0.01). Moreover, mRNA expressions of Col1a1 in TNFα−/− mice in SHAM and OVX were 1.21- and 1.16-fold higher than those of IL6−/− mice. TNFα and IL6 knockout in SHAM groups distinctly inhibited mRNA expressions of DKK1 and Sost by threefold and twofold compared to WT mice (P<0.01; Fig. 5C and D). After OVX, WT mice exhibited a significant increase in the mRNA levels of DKK1 and Sost (3.30- and 2.34-fold), while OVX failed to upregulate mRNA expressions of DKK1 and Sost in gene-knockout mice (P<0.01). On the contrary, a slight decrease of Sost mRNA by 17.8% was found in OVX TNFα−/− mice compared to OVX IL6−/− mice (P<0.05).

We further investigated the bone resorption markers and analyzed the mRNA expression of TRAP, MMP9, CTSK and TRAF6. After SHAM operation, mRNA expressions of TRAP, MMP9, CTSK and TRAF6 were downregulated by 4.76-, 1.43-, 2.38- and 2.56-fold respectively in TNFα−/− mice (P<0.01; Fig. 5E, F, G and H), and IL6−/− mice exhibited a similar decrease of these mRNA expressions by 3.57-, 1.64-, 2.27- and 1.67-fold respectively (P<0.01). All three genotypes showed upregulation in mRNA levels of TRAP, MMP9, CTSK and TRAF6 after OVX, whereas only the fold changes in WT mice were significant: 2.69, 2.01, 2.34 and 1.89 respectively (P<0.01). No obvious differences were found in the upregulation of these genes expressions in TNFα−/− and IL6−/− mice except the mRNA levels of MMP9 and TRAF6 in IL6−/− mice (P<0.01). Furthermore, TNFα−/− mice under SHAM and OVX exhibited 1.54- and 1.48-fold decrease in the mRNA expression of TRAF6 than that of IL6−/− mice respectively.
TNFα and IL6 knockout antagonized trabecular architectural deterioration. Representative hematoxylin eosin (H&E) images for bone microarchitecture of the distal femora in WT, TNFα−/− and IL6−/− mice after SHAM (A, B and C) and OVX (D, E and F) respectively. The first left and right panel show the original magnification x10, scale bar = 100 μm. Images besides show equivalent staining at 5x and 20x magnification respectively, scale bar = 200 and 50 μm. A full colour version of this figure is available at https://doi.org/10.1530/JME-17-0218.

**TNFα and IL6 knockout potentially exert different effects on osteoblast and osteoclast differentiation in vitro**

TNFα- and IL6-knockout mice exhibited higher osteogenic differentiation evidenced by increasing osteoblasts number, size and ALP activity (P<0.01, Fig. 6A, B, C and D) under both vehicle and E2 treatment in comparison to the WT mice. E2 treatment increased osteoblasts number by 62.3% (P<0.01) only in WT mice, osteoblasts size by 52.7, 48.6 and 46.9% (P<0.01) and ALP activity by 60.3, 60.2 and 63.9% (P<0.01), respectively, in WT, IL6−/− and TNFα−/− mice. No obvious differences were observed between TNFα−/− and IL6−/− mice except a higher growth of ALP activity in TNFα−/− mice (P<0.01). Real-time PCR showed higher mRNA expression levels for Runx2 and Col1a1 and lower mRNA levels for DKK1 and Sost in osteoblasts from TNFα−/− and IL6−/− mice under basal conditions (P<0.01; Fig. 7A, B, C and D). Moreover, TNFα−/− osteoblasts showed 1.15-fold higher Runx2 expression and 2.09-fold lower DKK1 expression in comparison to IL6−/− osteoblasts (P<0.05). E2 exposure not only upregulated Col1a1 expression in WT and TNFα−/− osteoblasts by 1.84 (P<0.01) and 2.25-fold (P<0.05) respectively, but also downregulated DKK1 expression in WT and IL6−/− osteoblasts by 2.04- (P<0.01) and 4.16-fold (P<0.05) and Sost expressions in WT and TNFα−/− osteoblasts by 1.42- (P<0.01) and 2.56-fold (P<0.05) respectively. E2-treated TNFα−/− osteoblasts displayed significantly higher (1.21-fold) Col1a1 and 1.43-fold lower Sost expression than that of IL6−/− osteoblasts (P<0.01).

On the other hand, osteoclast differentiation cultures in vitro shows that the number and size of TRAP-positive osteoclasts from TNFα−/− and IL6−/− mice were lower than that for WT mice with or without E2 treatment (Fig. 6F, G). On E2 treatment, the number of TRAP-positive cells from WT, TNFα−/− and IL6−/− mice decreased by 56.4, 33.1 and 28.3% respectively when compared to their corresponding controls (P<0.01). E2 treatment significantly reduced the size of WT osteoclasts by 84.6% (P<0.01). Osteoclast-related gene analysis showed that in TNFα and IL6 knockout, there was a downregulation in the mRNA expressions of TRAP, MMP9, CTSK, TRAF6, c-Fos and NFATc1 with or without E2 treatment. After treatment with E2, the mRNA levels of TRAP, MMP9,
lower uterus weight in WT mice. However, OVX failed to drastically increase the body weight in TNFα−/− and IL6−/− mice and did not cause a significant change in the uterus weight. It is worth noting that OVX in mature female mice with adipocyte augmentation and adipose tissue inflammation is associated with upregulation of peroxisome proliferator-activated receptor-γ (PPARγ) (Rogers et al. 2009, Cho et al. 2011). Furthermore, our early studies showed that TNFα (Zhang et al. 2015) and IL6 (Wang et al. 2016) gene knockout can inhibit body weight gain induced by high-fat diet via the downregulation of PPARγ. On the other hand, the present study demonstrated no significant differences in BV/TV, Tb. N, Tb. Th, BMD and Tb. Sp between gene knockout and WT mice after SHAM operation, indicating that TNFα and IL6 knockout did not affect normal skeletal phenotype, consistent with other reports (Jilka et al. 1992, Roggia et al. 2001, Franchimont et al. 2005). While we previously reported that TNFα−/− and IL6−/− mice exhibit higher Tb. N and Tb. Th respectively under basal condition (Zhang et al. 2015, Wang et al. 2016), gender differences might account for the variations. Consistent with a role for OVX in inducing bone loss (Weitzmann & Pacifici 2006), we observed that OVX distinctly decreased BV/TV, Tb. N, Tb. Th and BMD and increased Tb. Sp. Moreover, our findings show that TNFα−/− and IL6−/− mice were protected from the deterioration of bone microarchitecture, as evidenced by increased BV/TV, Tb. N, Tb. Th and BMD and decreased Tb. Sp, revealing a pivotal role of TNFα and IL6 in bridging estrogen deficiency and bone loss (Poli et al. 1994, Cenci et al. 2000, Roggia et al. 2001, Naka et al. 2002).

OVX-induced bone loss is relevant to the imbalance in bone remodeling, resulting in the expansion and extension of osteoclasts (Nakamura et al. 2007, Krum et al. 2008). The proliferation and differentiation of osteoclasts and its precursors (HSCs) are cytokine driven (Teitelbaum 2000). Estrogen deficiency stimulates the secretion of circulating and local inflammatory cytokines such as TNFα and IL6 to enhance osteoclastogenesis in both human (Abrahamsen et al. 1997, Rachon et al. 2002, D’Amelio et al. 2008) and animals (Weitzmann & Pacifici 2005). In the present study, OVX significantly promoted bone resorption and osteoclast formation, evidenced by the increase in the growth of serum CTX levels, the number and size of femoral TRAP-positive cells in WT mice and simultaneous upregulation of TRAP, MMP9 and CTSK expressions. In contrast, the deletions of TNFα and IL6 attenuated the formation of over-activated osteoclasts induced by OVX. At the same time, the decrease in the mRNA levels of TRAP, MMP9 and CTSK in gene knockout

### Discussion

Decreased energy expenditure and loss of gonad function as a result of OVX is evidenced by previous studies (Rogers et al. 2009, Zhao et al. 2012). Our observations reveal that OVX certainly enhanced body weight and decreased

Figure 4

Evaluation of osteoclastogenesis. OVX significantly increased the number and size of TRAP-positive multinucleated cells (indicated by red arrows), whereas TNFα and IL6 knockout acted reduced osteoclastogenesis (A, B and C). Original magnification x40, scale bar = 20 μm. Data are expressed as mean ± s.e.m. *P<0.05, **P<0.01, compared to the same genotype. *P<0.05, **P<0.01, compared to WT mice with the same treatment, n=6. A full colour version of this figure is available at https://doi.org/10.1530/JME-17-0218.

CTSK, TRAF6, c-Fos and NFATc1 in WT mice decreased by 1.43 (P<0.05; 7E), 1.66-, 2.50-, 1.88-, 2.63- and 3.12-fold (P<0.01; Fig. 7F, G, H, I and J) respectively. E2 treatment also significantly reduced TRAF6 expression in TNFα−/− and IL6−/− osteoclasts by 1.61- and 1.31-fold (P<0.01) respectively. Furthermore, TNFα−/− osteoclasts treated with vehicle alone or E2 exhibited 1.41- and 1.15-fold lower mRNA expression of TRAF6 than that of IL6−/− osteoclasts (P<0.01) respectively.
mice was observed after OVX, which further supported the inhibitory roles of TNFα and IL6 knockout in osteoclasts formation and function (Zhang et al. 2015, Wang et al. 2016). Similar trends with respect to gene expression were also observed in vitro. Furthermore, we observed OVX significantly overactivated TRAF6 expression in WT mice. Through downregulation of TRAF6 mRNA expression, TNFα and IL6 might regulate osteoclastogenesis via the induction of TRAF6. In the context of osteoclast differentiation, activating adaptor proteins such as TNF-receptor-associated factor (TRAF) family is identified as a central step in the signaling (Wong et al. 1998). Loss-of-function studies report that TRAF6, which activates NF-κB and MAPK signaling, acts on the differentiation and function of osteoclasts (Kobayashi et al. 2001, Kadono et al. 2005). In vitro, TNFα and IL6 knockout and E2 treatment downregulated the mRNA expression of TRAF6, c-Fos and NFATc1, which further confirmed the regulatory role of TRAF6 in the signaling mechanisms regulating osteoclast formation. More importantly, the downstream activation of AP1 component c-Fos (Wagner & Eferl 2005) and nuclear factor of activated T cells cytoplasmic 1 (NFATc1) (Takayanagi et al. 2002), the master transcription factor of osteoclast differentiation, have been further described to induce the maturation of osteoclasts and enhance bone resorption (Takayanagi 2007). The TRAF6-NF-κB axis and c-Fos signaling are essential to induce NFATc1, which regulates the expression of a number of osteoclast-specific genes, including CTSK, TRAP and MMP9 (Takayanagi et al. 2002, Takayanagi 2007). Taken together, our results demonstrate that TNFα and IL6 knockout are able to suppress the proliferation and differentiation of osteoclasts induced by OVX by inhibiting NF-κB and AP1 activity.

Bone loss induced by estrogen deficiency can also inhibit osteoblast function. Phenotypic studies have found that treatment with estrogen enhances osteoblast function and life span in order to maintain bone formation (Kousteni et al. 2001, Khosla et al. 2012). However, osteoblast function is affected following OVX due to elevated levels of pro-inflammatory cytokines such as TNFα and IL6 (Krum et al. 2010, Redlich & Smolen 2012).
In the present study, histological analyses observed that TNFα- and IL6-knockout mice were protected from the deterioration of femoral trabecular microarchitecture induced by OVX. Consistent with these phenotypic results, OVX induced decrease in mRNA expression of Runx2, and Col1a1 was inhibited in the TNFα and IL6 knockout revealing the negative regulatory role of TNFα and IL6 in osteoblast formation. Interestingly, a contradiction between a slightly decrease in bone formation in vivo and a classic high turnover evidenced by the increased serum PINP was observed in the present study. It is possible that the OVX-induced bone loss in the present study was mediated primarily by the changes in signaling patterns in the bone marrow, which were flushed away during killing. Previous data analyzing the distal femoral metaphysis without bone marrow support our present findings in bone turnover (Uno et al. 2011, de Castro et al. 2012). In vitro, BMSCs induced osteoblasts in the gene knockout mice under both settings showed increased osteoblasts number, size and higher ALP activity along with Runx2 and Col1a1 expressions compared to the WT mice. These results further support the role of OVX in decreased functionality of osteoblasts. In addition, it is well documented that the activation of canonical WNT signaling acts a crucial role in bone formation (Baron & Kneissel 2013). So it is worth noting that pro-inflammatory cytokines may exert negative effects on osteoblasts by inducing the inhibitors of WNT signaling i.e. DKK1 and Sost (Diarra et al. 2007, Mason & Williams 2010). Consistent with the inhibition of DKK1 and Sost in rescuing OVX-induced bone loss (de Castro et al. 2012, Jastrzebski et al. 2013, Artsi et al. 2014), our study found that OVX-induced upregulation of DKK1 and Sost expressions were not seen in TNFα−/− and IL6−/− mice subjected to OVX. We also found decreased osteoblast mRNA levels for DKK1 and Sost in gene knockout and E2 treatment. Our findings suggest that impaired bone formation induced by OVX can be corrected by the knockout of TNFα and IL6 possibly through reactivation of WNT signaling.

Figure 6
Effects of estrogen, TNFα and IL6 on osteoblast and osteoclast differentiation in vitro. E2 treatment and knockout of TNFα and IL6 enhanced osteoblast differentiation by increasing osteoblasts number, size and ALP activity (A, B, C and D), and inhibited osteoclast differentiation by decreasing the number and size of TRAP-positive multinucleated cells (E, F and G). Original magnification ×20. Data are expressed as mean±S.E.M. *P<0.05, **P<0.01, compared to the same genotype. #P<0.05, ##P<0.01, compared to WT mice with the same treatment, n=6. A full colour version of this figure is available at https://doi.org/10.1530/JME-17-0218.
Abundant evidence indicates that both TNFα and IL6 are important cytokines involved in osteopenia after estrogen withdrawal, although there is a lack of know-how on which cytokine plays a more important role. Kimble and coworkers have reported that OVX-induced bone loss and high bone turnover can be suppressed by the functional blockage of circulating TNFα but not IL6 (Kimble et al. 1997). While this finding supported that TNFα possibly plays a more critical role in mediating bone loss induced by OVX, at least in part, neutralization of rTNFα and rIL6 in serum was insufficient to explain the differences. We also found subtle differences in bone mass and bone formation between TNFα and IL6 knockout. After OVX, TNFα knockout exhibited higher Tb. N than that of IL6−/− mice. Similarly, the mRNA levels of Col1a1 were also higher in TNFα−/− mice. Furthermore, TNFα−/− BMSCs exhibited higher levels of ALP activity in E2 treatment, Runx2 and Col1a1 respectively in vehicle and E2 treatment compared to IL6−/− BMSCs. These results suggest that TNFα has a more important role than IL6 in abnormal bone formation induced by OVX. In addition, lower Sost and Dkk1 mRNA expressions were observed in TNFα−/− mice and BMSCs, which further supports that the inhibitory effect of TNFα on WNT signaling-mediated bone formation was more obvious than that of IL6. In inflammatory arthritis, TNFα has been proved as a key inducer of DKK-1 in the formation of osteophytes, both in mice.
and humans (Diarra et al. 2007). Furthermore, the induction of Sost in osteoblastic cells is dependent on the increase in RANKL (Tu et al. 2015), a TNF-related activation-induced cytokine, implying that IL6 and other cytokines may play a secondary role in inhibiting WNT signaling pathway compared to TNFa. On the other hand, it is worth noting in the present study that TNFa knockout significantly downregulated mRNA expression of TRAF6, both in vivo and in vitro, compared to that of IL6 knockout, which indicated that TNFαs and IL6 might regulate osteoclast formation via separate mechanisms. Generally, TNFα and IL6 synergistically stimulate osteoclast differentiation by inducing the secretion of RANKL and M-CSF derived from bone marrow (Ragab et al. 2002). However, in vitro studies in human and animals confirm that TNFα can directly induce osteoclastogenesis independent of the increase in RANKL (Azuma et al. 2000, Kudo et al. 2002). Mouse genetic models demonstrate that TNFα-induced osteoclastogenesis involves sequential induction of TRAF6-mediated signaling (Kaji et al. 2001). In contrast, IL6 has been suggested to act strongly in combination with RANKL and other pro-inflammatory cytokines to induce osteoclastogenesis (Kurihara et al. 1990, Devlin et al. 1998, Steeve et al. 2004). The dominant action of IL6 in osteoclastic trans-signaling is associated with the activation of MAPK family components, which reveals that the mechanism between IL6- and TRAF6-mediated osteoclastogenesis is indirect (Steeve et al. 2004). In light of these findings, we suggest that TNFα and IL6 might have a different regulatory role in TRAF6-mediated osteoclastogenesis, and TNFα has a stronger effect.

In conclusion, our findings demonstrate that OVX lowered femoral bone mass in mice by increasing osteoclastogenesis and reducing osteoblastogenesis. The TNFα- and IL6-knockout mice restored the bone-wasting effect of OVX and preserved osteoporotic bone mass via the adaptive inhibiting NF-kB and AP1 activity and the de-repressing WNT signaling. While TNFα and IL6 both had an important role in mediating OVX-induced bone loss, TNFα had a stronger effect than IL6 potentially in inhibiting bone formation and enhancing TRAF6-mediated osteoclastogenesis, suggesting the role of different regulatory mechanisms governing TNFα and IL6 action on bone metabolism (Fig. 8). Future investigations are required to elucidate the regulatory network of TNFα and IL6 in OVX-induced bone loss in order to develop their potential therapeutic targets against estrogen deficiency induced bone loss.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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