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Follicle-stimulating hormone stimulates TNF production from immune cells to enhance osteoblast and osteoclast formation

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Declining estrogen production after menopause causes osteoporosis in which the resorption of bone exceeds the increase in bone formation. We recently found that mice deficient in the β -subunit of follicle-stimulating hormone (FSH β) are protected from bone loss despite severe estrogen deficiency. Here we show that FSH β -deficient mice have lowered TNF α levels. However, TNF α -deficient mice are resistant to hypogonadal bone loss despite having elevated FSH, suggesting that TNF α is critical to the effect of FSH on bone mass. We find that FSH directly stimulates TNF α production from bone marrow granulocytes and macrophages. We also explore how TNF α up-regulation induces bone loss. By modeling the known actions of TNF α , we attribute the high-turnover bone loss to an expanded osteoclast precursor pool, together with enhanced osteoblast formation. TNF α inhibits osteoblastogenesis in the presence of ascorbic acid in culture medium, but in its absence this effect becomes stimulatory; thus, ascorbic acid reverses the true action of TNF α . Likewise, ascorbic acid blunts the effects of TNF α in stimulating osteoclast formation. We propose that hypogonadal bone loss is caused, at least in part, by enhanced FSH secretion, which in turn increases TNF α production to expand the number of bone marrow osteoclast precursors. Ascorbic acid may prevent FSH-induced hypogonadal bone loss by modulating the catabolic actions of TNF α .

postmenopausal osteoporosis | TNF α | bone | ascorbic acid | hypogonadal

Postmenopausal osteoporosis is a leading cause of morbidity and mortality in the increasingly aging population, with fracture rates exceeding the combined incidence of breast cancer, stroke, and heart attacks in postmenopausal women (1). Traditionally, this bone loss has been attributed solely to declining estrogen levels. However, we recently showed that the pituitary hormone follicle-stimulating hormone (FSH), the secretion of which is under estrogenic feedback, directly enhances osteoclast formation and function. The deletion of its β -subunit (FSH β) protects against bone loss despite severe hypogonadism (2). This finding indicates that FSH is a requirement for hypogonadal bone loss and, although awaiting definitive proof, suggests strongly that elevated FSH contributes to the genesis of postmenopausal osteoporosis.

However, enhanced osteoclastogenesis, a consequence of the direct action of FSH on its G_T-coupled receptor on osteoclast precursors, does not fully explain hypogonadal bone loss. There are accompanying alterations in bone and bone marrow, notably enhanced bone formation, increased T lymphocyte production, and macrophage activation. The alterations in immune function have been attributed to an increase in TNF α production that is thought to arise solely from estrogen deficiency. However, because TNF inhibits osteoblast differentiation *in vitro*, the increased bone formation has not been attributed to TNF α . Thus, the genesis of enhanced bone formation, an essential component of the high-turnover bone loss, has remained unclear.

Ablation of the TNF α gene in mice abrogates gonadectomy-induced bone loss, osteoclastic and osteoblastic activation, and the

accompanying immune cell alterations (3). That gonadectomy elevates FSH levels in these animals suggests that TNF α is essential for, and downstream of, FSH action on bone (3). Consistent with this hypothesis, TNF α does not modulate FSH secretion (4). In fact, there is direct evidence in Sertoli cells and testicular macrophages, respectively, that FSH enhances TNF receptor and TNF α expression (5, 6). Together, these findings prompted us to explore whether FSH mediates the production of TNF α and whether the abrogation of bone loss in FSH β -deficient mice arises in part from decreased TNF α production.

We show that FSH β -deficient mice have low circulating TNF α , that FSH directly stimulates TNF α production from bone marrow granulocytes and macrophages, and that TNF α stimulates osteoclast precursor expansion and osteoblast differentiation. We also find that ascorbic acid, which is required for posttranslational modification of proline to hydroxyproline in collagen and is thus used as a differentiation inducer in culture studies, acts to reverse the stimulatory effects of TNF α on osteoblast and osteoclast formation. Confirmatory evidence that high-turnover bone loss is due to enhanced osteoclast precursor expansion, and that TNF α is proosteoblastic rather than antiosteoblastic, comes from further mathematical modeling of the known action of TNF α in TNF α -overexpressing mice. We propose that the effects of FSH on bone mass identified earlier (2) are, at least in part, exerted via the modulation of TNF α production by bone marrow macrophages and granulocytes.

Results

FSH Regulates TNF α Production. Although estrogen is known to suppress TNF α production from immune cells (7), whether FSH or its lack thereof alters TNF α expression has never been investigated. We find that, despite severe estrogen deficiency, FSH β -deficient mice have lower serum levels of TNF α compared with littermate controls (Fig. 1A). This finding suggests that impaired FSH signaling attenuates the otherwise stimulatory effect of estrogen deficiency on TNF α production. In parallel experiments, the exposure of bone marrow cultures to recombinant FSH caused an increase in supernatant TNF α levels measured using an ELISA (Fig. 1B).

Both macrophages, which together with granulocytes make up

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Abbreviations: OPG, osteoprotegerin; FSH, follicle-stimulating hormone; M-CSF, macrophage colony-stimulating factor.

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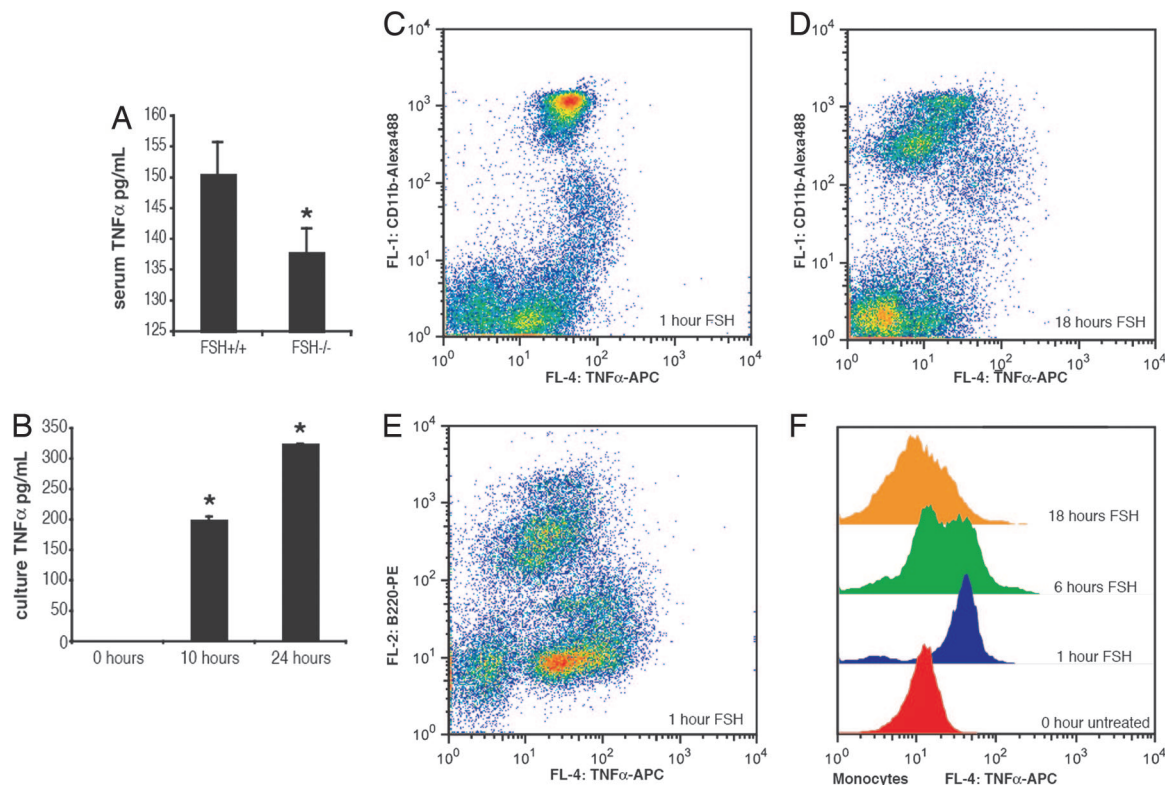


Fig. 1. FSH regulates TNF α production. (A) FSH $\beta^{-/-}$ mice display reduced levels of serum TNF α despite estrogen deficiency ($P = 0.03$; $n = 4$; sampled twice). (B) Secretion of TNF α into cell culture supernatants was analyzed at 10 and 24 h after FSH (100 ng/ml) addition ($P = 0.03$ for 10 h and 0.01 for 24 h). (C–E) Effect of FSH on TNF α production in primary CD11b⁺ macrophages/granulocytes or B220⁺ cells. Bone marrow from C57BL mice was flushed and resuspended in OPTI-MEM containing 5% FBS. After 2 h of incubation, FSH (100 ng/ml) was added, and the cells were sampled at 1 h (C and E), 6 h (data not shown), and 18 h (D) after addition. The cells were fixed in PhosphoFix, permeabilized in 90% MEHOH, and stained with either of two antibody combinations: CD11b-Alexa Fluor 488 and TNF α -allophycocyanin (C and D) or B220-phycoerythrin and TNF α -allophycocyanin (E). (F) A display of the time course of TNF α staining after the addition of FSH (100 ng/ml) in gated macrophages/granulocytes.

half of the cells present in bone marrow, and T lymphocytes, which account for 1–3% of cells in the bone marrow, have been suggested as sources of TNF α production in estrogen deficiency (8, 9). To elucidate which cell(s) were producing TNF α , we doubly stained cells from bone marrow with antibodies to TNF α , as well as to markers for macrophages/granulocytes (CD11b), B lymphocytes

(B220), and T lymphocytes (CD3). We found that macrophages and granulocytes expressed high levels of TNF α after FSH stimulation but that other cell types did not (Fig. 1 C–F). These results suggest that TNF α up-regulation is likely downstream of FSH and is, at least in part, mediated by granulocytes and macrophages present in the bone marrow.

Table 1. Bone changes in transgenic (Tg) TNF-overexpressing mice

Mouse	Osteoclast precursors, % of CD11b ^{hi} splenocytes	Total bone mineral density, mg/cm ³	Osteoclasts per surface/bone surface	Osteoblasts per surface/bone surface	Osteoblasts per paw after 4 weeks of treatment
Wild type	1.8–2.6 (11, 34)	452 (35)	3.75 (35)	5 (35)	
TNF-Tg	9.1–15.5 (11, 34)	330 (35)	6 (35)	12 (35)	
TNF-Tg + RANK-Fc injection	13.2 (34)				
TNF-Tg \times RANK ^{-/-}	20.7 (34)				
TNF-Tg + etanercept injection	3.2 (11)				
Wild type + OPG injection		535 (35)	2 (35)	1 (35)	
TNF-Tg + OPG injection		623 (35)	1.5 (35)	4 (35)	
TNF-Tg at 10 weeks of age					25 (36)
TNF-Tg at 14 weeks; untreated					65 (36)
TNF-Tg at 14 weeks; OPG injection					20 (36)
TNF-Tg at 14 weeks; OPG and etanercept					1 (36)
TNF-Tg at 14 weeks; etanercept injection					30 (36)

References are shown in parentheses.

TNF α Induces High-Turnover Bone Loss Through Osteoclast Precursor Expansion. We next sought to understand how elevations in TNF α contribute to the observed high-turnover bone loss seen in hypogonadal states. Prior investigations suggest that there are three major actions of TNF α on bone. TNF α strongly augments osteoclast differentiation and resorptive function by stimulating the same signal transduction pathways induced by RANK-L (10). TNF α also increases the number of CD11b⁺ osteoclast precursors by enhancing macrophage colony-stimulating factor (M-CSF)-mediated proliferation (11, 12). Additionally, TNF α potentially inhibits osteoblast differentiation through negatively regulating the lifespan of osteoblast progenitors (13). To determine which of these actions was critical in bringing about high-turnover bone loss, we adapted a mathematical model of bone metabolism to allow for the individual application of each of the three actions of TNF α (14). Specifically, we examined how each of these actions contributed to the bone loss phenotype observed in animals overexpressing TNF α (Table 1; see also Table 2, which is published as supporting information on the PNAS web site).

Using this model, we found that simulating TNF α -induced increases in the number of osteoclast precursors correctly accounted for most of the observed phenotype of TNF α -transgenic mice (Fig. 2A). Furthermore, this model suggested that, if TNF α served solely to augment osteoclast differentiation, the number of osteoclast precursors would fall, not rise as was observed *in vivo*. Moreover, if TNF α inhibited osteoblast differentiation (as has been shown *in vitro*; see ref. 13), there would be concomitant declines in osteoclast numbers, not increases as noted *in vivo*. In agreement with the *in silico* finding that TNF α 's main action is to augment osteoclast precursor numbers, TNF α added to osteoclast precursors 24 h before the addition of the differentiation-inducing cytokine RANK-L led to significantly greater increases in osteoclast formation than when TNF α and RANK-L were added simultaneously (Fig. 2B).

Ascorbic Acid Reverses the Effects of TNF α on Osteoblasts and Osteoclasts. Elevations in osteoblast function accompany the high-turnover bone loss induced by estrogen deficiency. However, FSH does not impact osteoblast differentiation (2), and TNF α is an established potent inhibitor of osteoblast differentiation *in vitro* (15). However, TNF α -overexpressing transgenic mice have elevated osteoblast activity (Table 1 and 2). This incongruence prompted us to reexamine the function of TNF α on osteoblast differentiation. Although changes in osteoclast precursor numbers led to reactive increases in osteoblast numbers in our mathematical model, we attempted to isolate the function of TNF α on osteoblast differentiation by recreating the phenotype of the TNF α -transgenic mouse treated with the RANK-L inhibitor osteoprotegerin (OPG), which blocks osteoclastogenesis. Interestingly, the bone mineral density of TNF α -transgenics treated with OPG is higher than that of wild-type mice treated with OPG (Table 1). Attempts to reproduce that *in vivo* finding with our *in silico* model suggested that TNF α does not inhibit osteoblast differentiation; in contrast, they suggested that TNF α may increase osteoblast formation or decrease osteoblast apoptosis (Fig. 5, which is published as supporting information on the PNAS web site).

Consistent with previous reports of TNF α action on osteoblast differentiation, we found that TNF α dose-dependently decreased the number of osteoblast-like colonies using the established protocol for CFU-F colony formation (Fig. 3B). The established osteoblast differentiation method uses ascorbic acid (vitamin C) to induce the expression of osteoblast-specific genes. Although ascorbic acid is believed to boost the immune system, ironically recent findings suggest that it may inhibit inflammation. Specifically, the ascorbic acid derivative dehydroascorbic acid (DHA) noncompetitively inhibits I κ B kinases and thus prevents the activation of the downstream transcription factor NF- κ B (16). When NF- κ B activation is blocked, TNF α signaling leads to either apoptosis or repli-

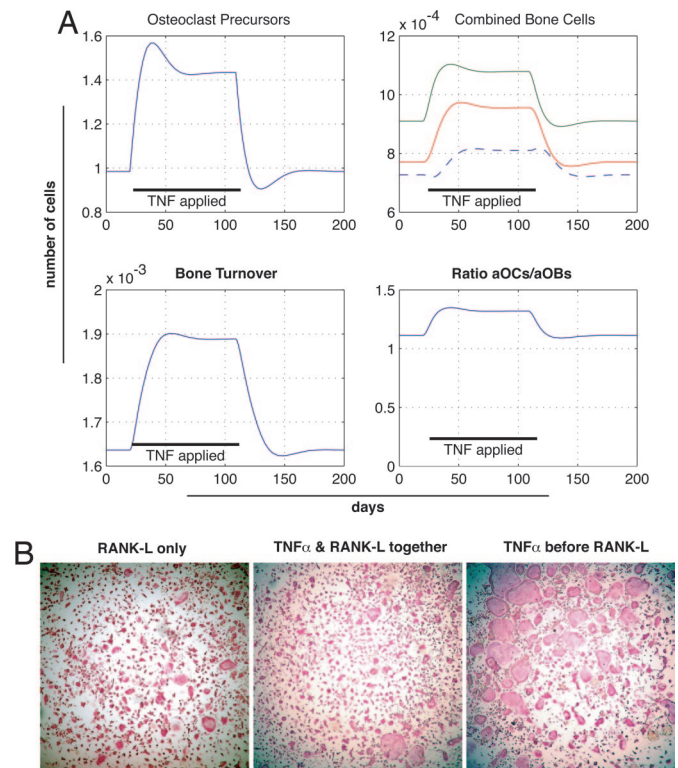


Fig. 2. Modeling of TNF α action suggests that it induces osteoclast precursor expansion. A mathematical model of bone metabolism (14) was adapted such that osteoclast differentiation proceeded from a pool of osteoclast precursors that could be varied independent of changes in the differentiation rate of osteoclasts. (A) The pool of osteoclast precursors was increased, as indicated by the black bar, and the effects on the number of osteoclast precursors (Upper Left), the number of osteoclasts, osteoblast precursors, and osteoblasts (Upper Right), the bone turnover (Lower Left), and net bone formation/loss (Lower Right) are shown. Note that disturbing the system by increasing the number of osteoclast precursors caused a rise in the number of active osteoclasts, responding osteoblasts, and active osteoblasts, as well as an increase in bone turnover and bone loss. (B) The effects of TNF α on expanding the osteoclast precursor pool were tested experimentally by stimulating osteoclast precursors with TNF α either 24 h before the addition of the differentiation signal RANK-L (Right) or by adding TNF α at the same time RANK-L was added (Center). The number of osteoclasts formed when TNF α was allowed to expand the osteoclast precursor pool was greater than when TNF α was given at the same time as the differentiation signal RANK-L. (Left) Control, RANK-L only.

cative senescence (17, 18). We hypothesized that ascorbic acid in the culture media might account for the inhibitory effects of TNF α on osteoblast differentiation. When we cultured total bone marrow without ascorbic acid, we found that TNF α dose-dependently increased the number of alkaline phosphatase-positive CFU-F colonies (Fig. 3A).

More nonalkaline phosphatase-positive cells, such as macrophages, were observed in TNF α -stimulated cultures without ascorbic acid compared with when it was added. We thus speculated that ascorbic acid might also inhibit TNF α -induced macrophage differentiation. To test this hypothesis, we analyzed by flow cytometry the number of CD11b⁺ cells in total bone marrow cultures after the addition of increasing amounts of TNF α . We found that TNF α application increased the number of CD11b⁺ cells but that the addition of ascorbic acid largely prevented this increase (Fig. 3C). Likewise, the percentage of cells expressing the TNF α -induced differentiation marker CD38 was attenuated by the inclusion of ascorbic acid (Fig. 3D). Because CD11b⁺ cells serve as osteoclast precursors, we examined whether the loss of their expansion in the

Serum ELISAs. Serum from FSH β -null mice and control littermates was taken as previously described (2). A commercial kit for murine TNF α (KMC3011; Invitrogen) was used according to the manufacturer's directions. Serum was diluted 1:1 in diluent provided by the manufacturer. Four animals for each group were assayed two times; Student's paired *t* tests were performed from the sum of all measurements for each group.

Mathematical Modeling. The mathematical model of bone metabolism first reported by Lemaire *et al.* (14) was adapted such that the one-component osteoclast system was divided to include a preosteoclast component and an osteoclast component. All other parameters were set as previously described (14). Calculations and

graph plotting were carried out by using MatLab (Release 14; MathWorks, Natick, MA) with a three-file program consisting of separate files for parameters, model, and execution/graphing.

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