Up-regulation of TNF-producing T cells in the bone marrow: A key mechanism by which estrogen deficiency induces bone loss *in vivo*

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Communicated by David M. Kipnis, Washington University School of Medicine, St. Louis, MO, October 9, 2001 (received for review May 30, 2001)

***In vivo* studies have shown T cells to be central to the mechanism by which estrogen deficiency induces bone loss, but the mechanism involved remains, in part, undefined. *In vitro*, T cells from ovariectomized mice produce increased amounts of tumor necrosis factor (TNF), which augments receptor activator of NF-κB ligand (RANKL)-induced osteoclastogenesis. However, both the mechanism and the relevance of this phenomenon *in vivo* remain to be established. In this study, we found that ovariectomy increased the number of bone marrow T cell-producing TNF without altering production of TNF per T cell. Attesting to the essential contribution of TNF, ovariectomy induced rapid bone loss in wild type (wt) mice but failed to do so in TNF-deficient (TNF$^{-/-}$) mice. Furthermore, ovariectomy induced bone loss, which was absent in T cell-deficient nude mice, was restored by adoptive transfer of wt T cells, but not by reconstitution with T cells from TNF$^{-/-}$ mice. These findings demonstrate the key causal role of T cell-produced TNF in the bone loss after estrogen withdrawal. Finally, ovariectomy caused bone loss in wt mice and in mice lacking p75 TNF receptor but failed to do so in mice lacking the p55 TNF receptor. These findings demonstrate that enhanced T cell production of TNF resulting from increased bone marrow T cell number is a key mechanism by which estrogen deficiency induces bone loss *in vivo*. The data also demonstrate that the bone-wasting effect of TNF *in vivo* is mediated by the p55 TNF receptor.**

ovariectomy | osteoporosis | mouse | pQCT

It is now recognized that one of the main mechanisms by which estrogen deficiency causes bone loss is by stimulating osteoclast formation (1), a process induced by the simultaneous stimulation of osteoclast precursors by macrophage colony-stimulating factor (M-CSF) and a tumor necrosis factor (TNF)-related factor known as receptor activator of NF-κB ligand (RANKL) (also known as OPGL, TRANCE, or ODF) (2–4).

In physiologic, unstimulated conditions, the differentiation of osteoclast precursors into mature osteoclasts in the bone marrow depends on the production of M-CSF by monocytes and stromal cells and RANKL by stromal cells and osteoblasts (5). However, in stimulated conditions, additional bone marrow cells contribute to regulating osteoclast formation by producing soluble and membrane-bound pro- and antiosteoclastogenic cytokines. Among them are naïve and activated T cells, which modulate osteoclast formation trough increased production of RANKL (6–8), osteoprotegerin (9), and IFN-γ (10).

During inflammation and autoimmune arthritis, activated T cell production of RANKL promotes bone resorption and bone loss (6) whereas release of IFN-γ limits T cell-induced bone wasting (10). Recent studies from our laboratory have disclosed that activated T cells play an essential causal role not only in inflammation-induced bone loss, but also in the bone wasting induced by estrogen deficiency (11). In fact, whereas ovariectomy (ovx) stimulated bone resorption and induced rapid bone loss in T cell-replete mice, athymic T cell-deficient mice were protected completely against the increase in bone resorption and the bone loss induced by ovx (11).

When cultured *in vitro*, T cells harvested from ovx mice secrete higher amounts of TNF than T cells from estrogen-replete mice. The amount of TNF produced by ovx T cells, but not that released from T cells from estrogen-replete mice, is sufficient to augment RANKL-induced osteoclastogenesis through engagement of the TNF receptor p55 expressed on the surface of bone marrow monocytes (11). Together, these observations suggest that the bone loss induced by ovx results, at least in part, from increased T cell production of TNF and by the potentiating effects of this cytokine on RANKL-induced osteoclastogenesis. However, the relevance of this mechanism *in vivo* remains to be demonstrated.

In this study, we have investigated the role of T cell-produced TNF and the p55 TNF receptor *in vivo*. We report that ovx increases T cell production of TNF in the bone marrow and that TNF-deficient (TNF$^{-/-}$) mice are completely protected against ovx-induced bone loss. Moreover, adoptive transfer of wild-type (wt) T cells into T cell-deficient mice restores the capacity of ovx to induce bone loss, whereas transfer of T cells lacking the capacity of producing TNF does not. We also show that ovx fails to induce bone loss in mice lacking the p55 TNF receptor, thus establishing that T cell-produced TNF and its interaction with the p55 receptor play a key causal role in the bone loss induced by estrogen deficiency *in vivo*.

Methods

Experimental Protocol. All animal procedures were approved by the Animal Care and Use Committee of Barnes–Jewish Hospital.

C57BL/6 homozygous (nu/nu) athymic nude mice (12) and wt C57BL/6 mice control littermates were obtained from Taconic Farms. C57BL/6 mice TNF$^{-/-}$ and wt control littermates of identical genetic background were kindly provided by M. Old (Ludwig Institute for Cancer Research, New York). C57BL/6 mice lacking either TNF receptor 1 (p55$^{-/-}$) or TNF receptor II (p75$^{-/-}$) mice and wt controls of the same genetic background were obtained from The Jackson Laboratory. Mice either were sham-operated or ovx at the age of 6 weeks and killed at the age of 10 weeks as described (11, 13). Additional groups of intact (nonoperated) mice were killed at 10 weeks of age to serve as control for the operated mice. At the time the mice were

Abbreviations: RANKL, receptor activator of NF-κB ligand; ovx, ovariectomy; wt, wild type; TNF, tumor necrosis factor; M-CSF, macrophage colony-stimulating factor; ns, not significant; BMD, bone mineral density.

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killed, tibiae were excised for measurements of bone density and femurs were used to harvest whole bone marrow. Uterus weight was determined at death to verify the successful removal of the ovaries.

T Cell Preparation and Adoptive Transfer. Spleen cell suspensions were prepared from C57BL/6 donors killed 2 weeks after either ovx or sham operation. T cells were purified from wt and $TNF^{-/-}$ mice by positive immunoselection by using MACS Microbeads (Miltenyi Biotech, Auburn, CA) coupled to anti-CD90 (Thy1.2) Ab, as described (11). T cells (1×10^7) then were injected i.v. into the lateral tail vein of allotype-congenic C57BL/6 nude recipients by using established methods (14, 15). On the same day, T cell-reconstituted mice were subjected to either ovx or sham operation. Four weeks after T cell transfer, the proportion of T cell in the spleen of injected mice was assessed by flow cytometry as described below. We found that, at 4 weeks, spleen T cells were 2.0 ± 0.2% in control nude mice and 8.6 ± 0.3% in T cell-reconstituted mice ($P < 0.05$). Similar numbers of spleen T cells have been found by others after T cell reconstitution (14, 15). Thus, the data establish that our procedure was effective in reconstituting T cells in nude mice.

Measurement of Bone Density. Trabecular bone mineral density (BMD) was measured in excised tibiae by pQCT (XCT-960M; Nordland Medical Systems, Fort Atkinson, WI) as described (11), using a modification of the method of Bucay *et al.* (16). Briefly, six cross sections (two cortical at the middiaphysis and four trabecular at the proximal epiphysis) were scanned and data for the four trabecular sites were analyzed by using a threshold value of 200 mg/cm^3 to select for bone and to exclude soft tissue. To analyze the same anatomical location in all mice, the machine was set to position the first slice to a distance from the knee joint space equal to 7% of the total length of the tibia. The length of the tibia was the same in sham and ovx mice of the same phenotype. Cortical and trabecular bones were separated by "concentric peel," with the inner core (45%) defined as trabecular bone. An inner bone density threshold of 400 mg/cm^3 was selected to eliminate any residual or infiltrating cortical bone. Trabecular bone density (mg/cm^3) was calculated by using the internal software program CALCBD (Nordland Medical Systems). The short term *in vitro* reproducibility of this technique is 2%.

Immunohistochemistry. The immunostaining was performed as reported (17). Whole bone marrow cells from tibiae of ovx and sham-operated wt C57BL/6 mice were resuspended in 100 μl of Aim-V (GIBCO/BRL) medium after lysis of red blood cells. Cells (10^6) in 10 μl were smeared onto glass slides, air-dried, and fixed in 10% neutral buffered formalin solution (Sigma) for 10 min at room temperature. After rinsing in 0.1 M PBS (pH 7.4) containing 0.3% Triton X-100 (PBST), the smeared cells were incubated for 30 min with PBST containing 10% normal donkey serum (Santa Cruz Biotechnology), 1% BSA, and 0.1% sodium azide, followed by incubation overnight at 4°C with a mixture of (1:50 dilution) goat anti-mouse TNF antibody (Santa Cruz Biotechnology) and (1:50 dilution) either the anti-mouse CD90 antibody G7 (PharMingen), which recognizes both Thy-1.1 and Thy-1.2 alloantigens, or the rat anti-mouse CD3 antibody 17A2 (PharMingen). The slides then were incubated for 3 h at room temperature with a mixture of FITC-conjugated anti-goat donkey IgG (10 μg/ml; Santa Cruz Biotechnology) and rhodamine-conjugated anti-mouse donkey IgG (10 μg/ml; Santa Cruz Biotechnology) secondary antibodies. Slides were washed three times for 5 min in PBST and observed under confocal microscopy (Nikon Eclipse E800; Nikon). Ten microscopy fields comprising an area of 512 μm × 512 μm from each slide were selected randomly, and CD90-positive cells, CD90, and TNF double-positive cells, and total bone marrow cells were counted by an investigator blind to the data set and analyzed by using the software program NE NORTHERN ECLIPSE (Empix Imaging, North Tonawanda, NY). Negative staining was assessed by omitting primary antibodies and by using an isotype-matched normal IgG in place of primary antibodies. Purified $CD90^+$ cells and $CD90^+$ cells stimulated with phorbol 12-myristate 13-acetate + ionomycin were used as positive controls for $CD90^+$ and TNF, respectively.

Flow Cytometry. Two-color flow cytometry was performed after permeabilization and fixation of cells by using a Cytofix/cytoperm kit (PharMingen). Briefly, whole bone marrow cells or purified T cells from sham or ovx-operated C57BL/6 mice were washed once in ice-cold PBS, suspended in α-MEM medium containing 10% FBS, and cultured overnight at 37°C in α-MEM containing 10% FBS in the presence of Golgistop (PharMingen) to prevent secretion of TNF from the cell. To reduce nonspecific binding, the cells were preincubated with 15% normal goat serum (Sigma) in PBS for 10 min at room temperature. After fixation and permeabilization in Cytofix/Cytoperm solution for 20 min at 4°C and two washes in 1× Perm/Wash solution, the cells were incubated for 30 min at 4°C in 1× Perm/Wash solution containing phycoerythrin-labeled anti-mouse TNF and either FITC-labeled anti-mouse CD90 (specific for both CD90.1 and CD90.2) or FITC-labeled anti-mouse CD3 (PharMingen) antibodies. After washing twice with 1× Perm/Wash solution, the cells were resuspended in 1% FBS and 0.09% sodium azide in PBS and analyzed by flow cytometry on a three-color Becton Dickinson flow cytometer (FACScan). A positive control was generated by treating purified T cells overnight with phorbol 12-myristate 13-acetate (5 ng/ml) and ionomycin (500 ng/ml). Nonspecific staining was assessed by using FITC and phycoerythrin-conjugated, isotype-matched normal IgG antibodies (PharMingen).

Statistical Analysis. Group mean values were compared by two-tailed Student's *t* test or one-way ANOVA and Fisher-protected LSD test, as appropriate.

Results

Ovx Increases T Cell TNF Production in the Bone Marrow. To determine the effects of ovx on T cell production of TNF *in vivo*, whole bone marrow was harvested 4 weeks after either ovx or sham operation, smeared on a glass slide, and analyzed by immunohistochemistry. In agreement with previous reports (18), triplicate experiments revealed (Table 1) ovx to cause a 2.5-fold increase in the total number of bone marrow cells. Ovx also resulted in an ≈5-fold increase in the number of T cells ($CD90^+$ cells). When the number of T cells was normalized for total bone marrow cell number and expressed as a percentage of total bone marrow cells, ovx was found to cause a 2-fold increase in this ratio, as compared with sham-operated mice.

Double staining with anti-CD90 and anti-TNF antibodies revealed that ovx increased the number of TNF producing T cells by ≈5-fold (when the data were expressed as average number of cells per field) and by 2-fold (when data were expressed as a percentage of total bone marrow cells). There was no difference between sham-operated and ovx mice with respect to the fraction of total T cells producing TNF, because TNF was found to be expressed in ≈50% of sham and ovx T cells. Immunostaining for CD3 and TNF confirmed that ovx increased both the percentage of $CD3^+$ cells and that of $CD3^+/TNF^+$ cells by 2-fold, thus establishing that ovx up-regulates T cell number (not shown).

Analysis of unfractionated spleen cells also revealed an increase in the number of total T cells and in that of TNF-producing T cells in samples from ovx mice that was of the same

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Table 1. Ovx increases T cell number in the bone marrow (mean ± SEM of 10 fields)

	Sham BM	Ovx BM	Fold increase	P
Total BM cells (number of cells per field)	85.4 ± 4.3	213.9 ± 14.2	2.50	<0.01
$CD90^+$ cells (number of cells per field)	6.4 ± 1.0	31.1 ± 2.8	4.86	<0.01
$CD90^+$ cells (% total BM cells)	7.3 ± 0.7	14.7 ± 1.2	2.01	<0.01
$CD90^+/TNF^+$ cells (number of cells per field)	3.2 ± 0.6	16.2 ± 1.3	5.06	<0.01
$CD90^+/TNF^+$ cells (% total BM cells)	3.1 ± 0.6	7.6 ± 0.4	2.45	<0.01
$CD90^+/TNF^+$ cells (% total $CD90^+$ cells)	48.2 ± 6.0	53.3 ± 6.1	1.10	NS

BM, bone marrow; NS, not significant.

magnitude as that observed in the bone marrow (not shown), suggesting that the regulatory effects of sex steroids are not limited to the bone marrow.

To confirm the results from immunohistochemistry, the expression of TNF in bone marrow was analyzed further by FACS. Triplicate experiments revealed that the percentage of TNF-producing T cells was ≈2-fold higher in ovx than in sham-operated mice (7.5 + 0.5% vs. 4.3 + 0.4.% of total bone marrow cells, $P < 0.05$, respectively). FACS analysis of purified $CD90^+$ cells also demonstrated (Fig. 1) that the amount of TNF produced by T cells from ovx mice was identical to that produced by an equal number of T cells from sham-operated mice. Together, the data demonstrate that ovx increases TNF levels in the bone marrow by increasing total T cell number without altering TNF production per T cell.

Ovx Does Not Induce Bone Loss in TNF-Deficient Mice. To investigate the relevance of TNF as a mediator of the effects of E2 deficiency on bone, $TNF^{-/-}$ mice and control littermates of identical genetic background were either ovx or sham-operated at the age of 6 weeks. The BMD of the trabecular compartment of the tibia was measured 4 weeks after surgery, a length of time sufficient to induce a detectable bone loss by bone densitometry (11, 13). BMD also was measured in a group of age-matched, intact wt and $TNF^{-/-}$ mice.

Intact 10-week-old wt and $TNF^{-/-}$ mice had similar BMD values. At 4 weeks from surgery, the BMD of wt sham-operated mice was ≈3% higher [P = not significant (ns)] than that of age-matched, intact mice (Fig. 2), whereas the BMD of wt ovx mice was 12.5% lower ($P < 0.05$) than that of age-matched, intact wt mice. As a result, at 4 weeks, the BMD of wt ovx mice was ≈15.5% lower ($P < 0.05$) than that of sham-operated wt mice. In contrast, 4 weeks after surgery, the BMD of $TNF^{-/-}$ ovx mice was only slightly lower than that of both sham-operated $TNF^{-/-}$ mice (≈1.8%, P = ns) and age-matched, intact $TNF^{-/-}$ mice (≈2.3%, P = ns). These data demonstrate that TNF-deficient mice are protected against the bone loss induced by ovx whereas wt mice of the same genetic background sustain a rapid bone loss after ovx.

T Cell Reconstitution with T Cells from $TNF^{-/-}$ Mice Does Not Restore ovx-Induced Bone Loss. To investigate the role of T cell-produced TNF in the pathogenesis of ovx-induced bone loss *in vivo*, we measured the BMD of the trabecular compartment of the tibia in euthymic and nude mice, as well as that of nude mice subjected to T cell reconstitution, both at baseline and 4 weeks after surgery.

Nude mice were reconstituted by using purified wt and $TNF^{-/-}$ spleen $CD90^+$ cells. FACS analysis revealed (Table 2) that >95% of both sham and ovx donor $CD90^+$ cells expressed the T cell marker CD3. T cell preparations from both sham and ovx donors contained ≈60% $CD4^+$ and 40% $CD8^+$ cells. TNF was produced by ≈40% of both $CD4^+$ and $CD8^+$ cells from wt sham donors and by ≈35% of both $CD4^+$ and $CD8^+$ cells from wt ovx donors. As expected, TNF was not detectable in T cells purified from $TNF^{-/-}$ sham and ovx donors.

To verify that T cell reconstitution led to engraftment of TNF-producing T cells, spleens were harvested from recipient mice and analyzed by FACS. We found that the majority of engrafted T cells were $CD4^+$ cells and that ovx increased the percentage of engrafted $CD4^+$ cells from ≈70% to ≈80% of total T cells (P = ns). Ovx also increased slightly the percentage of engrafted $CD4^+$ and $CD8^+$ cells producing TNF. In fact, TNF was produced by ≈50% of both engrafted $CD4^+$ and $CD8^+$ cells in sham recipients and by 49% of $CD4^+$ and 64% of $CD8^+$ cells in ovx recipients (P = ns). These findings demonstrate that a large fraction of reconstituted T cells are capable of producing TNF.

At baseline, the bone density of nude mice was similar to that

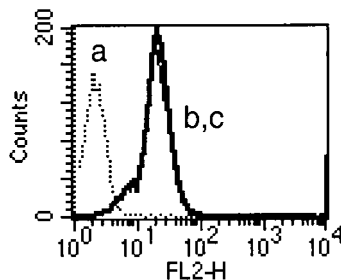

Fig. 1. Flow cytometry profiles of anti-TNF Ab staining of $CD90^+$ cells from ovx and sham-operated mice. Data are plotted as fluorescence intensity vs. cell number. Histograms are representative of results obtained in triplicate experiments. (*a*) Isotype control. (*b* and *c*) Sham and ovx cells. Data are representative of triplicate experiments. Variability among experiments was <10%.

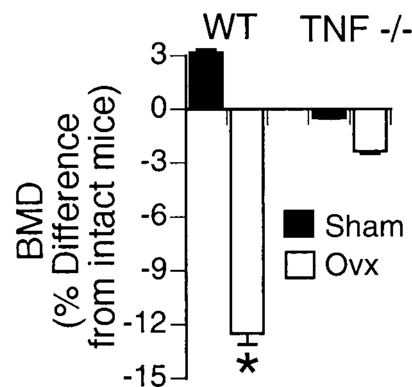

Fig. 2. $TNF^{-/-}$ mice are protected against ovx-induced bone loss. Trabecular BMD (mean ± SEM) of excised tibiae was measured by pQCT both in intact wt and $TNF^{-/-}$ mice of 10 weeks of age and in additional groups of 10-week-old wt and $TNF^{-/-}$ mice, 4 weeks after ovx or sham operation. Data (n = 6 per group) are expressed as percent difference from intact mice. *, $P < 0.05$ compared with all other groups.

Table 2. FACS analysis of donor T cell preparations and engrafted T cells (mean ± SEM)

	$CD90^+$ cells purified from sham donors	$CD90^+$ cells purified from ovx donors	Engrafted cells in sham recipients	Engrafted cells in ovx recipients
$CD3^+$ cells (% of total cells)	96 ± 9	97 ± 10	7.6 ± 8	9.7 ± 10
$CD4^+$ cells (% of total cells)	60 ± 5	58 ± 6	70 ± 8	81 ± 9
$CD8^+$ cells (% of total cells)	38 ± 4	38 ± 4	29 ± 3	18 ± 2
$TNF^+/CD4^+$ cells (% of total cells)	38 ± 3	38 ± 4	52 ± 3	49 ± 5
$TNF^+/CD8^+$ cells (% of total cells)	43 ± 3	33.9 ± 4	53 ± 4	64 ± 7

of wt controls of the same genotype. Sham operation did not cause significant bone loss in either wt (−0.5%, P = ns) or nude mice (−1.3%, P = ns) during the 4 weeks of follow-up (Fig. 3*A*). Ovx caused a significant decrease (−13.3%, $P < 0.05$) in the BMD of euthymic controls, whereas it failed to do so in nude mice (−2.3%, P = ns).

Injection of T cells from wt sham-operated mice into nude sham-operated mice was followed by a small decrease in BMD (−3.5%, P = ns), whereas injection of T cells from wt ovx mice into nude ovx mice caused a significant decrease in BMD (−17.9%, $P < 0.05$), a change slightly larger than that observed in wt ovx mice. These findings confirm our earlier report (11) that T cells mediate the effect of ovx on bone density. Interestingly, reconstitution of both sham nude with wt ovx T cells and of ovx nude mice with wt sham T cells also was followed by significant bone loss (−13.3% and −14.6%, respectively). This suggests that the lack of estrogen in either the donor or the recipient mouse enables T cells to acquire the capacity to induce bone loss.

Having determined that the largest bone loss takes place in ovx nude reconstituted with ovx T cells, we investigated whether the lack of T cell-produced TNF is sufficient to prevent bone loss in this group. We found that injection of T cells harvested from ovx $TNF^{-/-}$ mice into nude ovx mice (Fig. 3*B*) was not followed by significant bone loss (−2.8%, P = ns). There also was no bone loss observed in nude sham-operated mice injected with T cells from $TNF^{-/-}$ sham mice. Together, these findings demonstrate that the production of TNF by T cells is required for ovx to induce bone loss.

T Cell-Produced TNF Causes Bone Loss Through the p55 TNF Receptor. To determine whether T cell-produced TNF induces bone loss through the p55 TNF receptor, BMD was measured at baseline and 4 weeks after surgery in wt, $p55^{-/-}$, and $p75^{-/-}$ mice (19) of the same genetic background. We found (Fig. 4) that intact wt, $p75^{-/-}$, and $p55^{-/-}$ mice had similar BMD values. Sham operation did not induce significant changes in the BMD of wt (1.1%, P = ns), $p75^{-/-}$ (−1.3%, P = ns), and $p55^{-/-}$ (1.7%, P = ns) mice. In contrast, ovx led to a significant decrease in the BMD of both wt (−14.2%, $P < 0.05$) and $p75^{-/-}$ ovx (−13.5%, $P < 0.05$) mice, but not in that of $p55^{-/-}$ mice (−0.6%, P = ns). As a result, at 4 weeks from surgery, the BMD of ovx $p55^{-/-}$ mice was similar to that of both intact and sham-operated $p55^{-/-}$ mice. The finding that ovx did not induce bone loss in $p55^{-/-}$ mice demonstrates that *in vivo* the bone wasting effect of TNF is mediated exclusively by the p55 TNF receptor.

Discussion

Bone densitometry and bone histomorphometry are established techniques for the detection of the bone loss induced by withdrawal of sex steroids. Analysis of bone mass by densitometry and histomorphometry correlates closely (13, 20–23), although bone densitometry is not subjected to the sampling error that affects bone histomorphometry (24). Thus, in this study, the

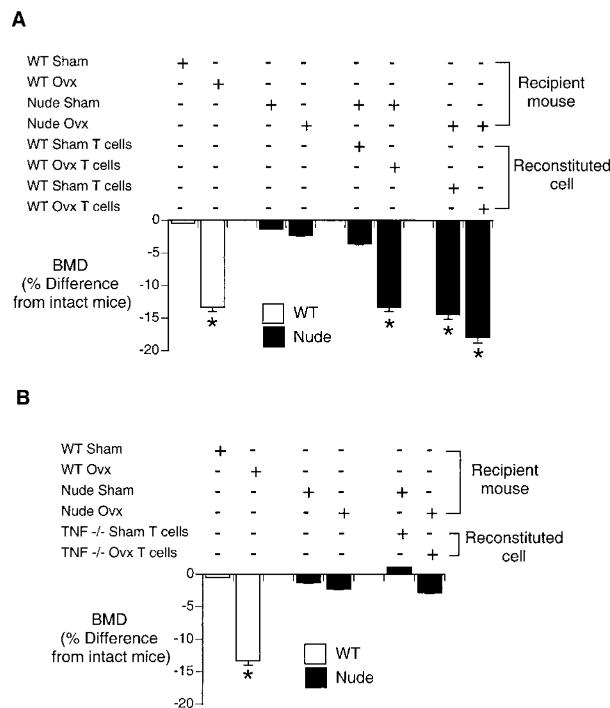

Fig. 3. Ovariectomy induces bone loss in nude mice injected with T cells from wt (*A*) but not $TNF^{-/-}$ (*B*) mice. Trabecular BMD (mean ± SEM) of excised tibiae was measured by pQCT both in intact mice at 10 weeks of age and in additional groups of mice of the same age, 4 weeks after ovx or sham operation. Data (n = 6 per group) are expressed as percent difference from intact mice. *, $P < 0.05$ compared with all other groups.

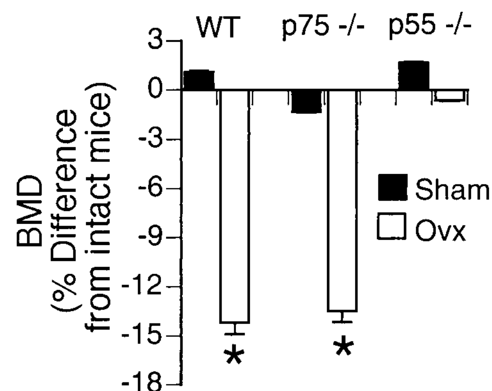

Fig. 4. Mice lacking the p55 TNF receptor are completely protected against ovx-induced bone loss. Trabecular BMD (mean ± SEM) of excised tibiae was measured by pQCT both in intact wt, $p75^{-/-}$, and $p55^{-/-}$ mice at 10 weeks of age and in additional groups of mice of the same age, 4 weeks after ovx or sham operation. Data (n = 6 per group) are expressed as percent difference from intact mice. *, $P < 0.05$ compared with all other groups except $p75^{-/-}$ ovx mice.

effects of ovx on bone density were evaluated by pQCT, a highly sensitive and precise technique that measures the density of the trabecular compartment of weight-bearing segments of the skeleton (13, 16). This method is particularly suitable for studying ovx mice, because estrogen withdrawal is known to induce mainly a loss of trabecular bone. As a result, pQCT measurements of BMD at 4 weeks from surgery yield a larger difference between sham-operated and ovx mice than other techniques that provide an integral measurement of cortical and trabecular bone (11, 13). Because of higher group separation, measurements of BMD by pQCT allowed us to determine whether ovx was followed by bone loss and whether T cell transfer was effective in restoring the capacity of ovx to induce bone loss.

Several lines of evidence implicate TNF in the pathogenesis of ovx-induced bone loss. Among them is the finding that cultures of bone marrow cells from ovx animals and humans produce increased amounts of TNF (25, 26). Although the source of TNF originally was ascribed to adherent cells of the monocytic lineage, recent investigations from our laboratory have revealed that ovx does not increase monocyte TNF production whereas it up-regulates the *in vitro* production of TNF by T cells present in cultures of adherent mononuclear cells (13). In this study, we found, by immunohistochemistry and FACS analysis, that ovx increases the total number of T cells without altering the fraction of T cells that release TNF. These changes result in a net increase in the number of TNF-producing T cells in the bone marrow. In addition, the data suggest that estrogen deficiency does not up-regulate the amount of TNF produced by each active T cell.

Reports by us also had revealed that mice treated with a TNF inhibitor (27) are completely protected against ovx-induced bone loss, although TNFbp has only a partial protective effect in the rat (28). Moreover, ovx does not induce bone loss in transgenic mice insensitive to TNF because of the overexpression of a soluble TNF receptor (29). In the current study, we have found that TNF-deficient mice, a strain exhibiting no remodeling abnormalities in unstimulated conditions, lost only a negligible amount of bone after estrogen withdrawal, thus conclusively demonstrating that TNF is required for ovx to induce bone loss.

We also used T cells from TNF$^{-/-}$ mice to reconstitute T cell function in nude mice. T cell adoptive transfer has been used previously to investigate the role of T cells in several pathological processes including alveolar bone resorption (14) and the regulatory effects of estrogen on delayed-type hypersensitivity (15). In earlier studies, recipient mice were injected with semipurified T cell preparations containing significant proportions of other bone marrow or spleen cells (14, 15). Although this was found not to be a confounding factor, we used T cells purified by positive selection to minimize transferring into nude mice cells other than T cells. Attesting to the success of the T cell transfer procedure, we found that at 4 weeks, nude mice injected with T cells had a number of spleen T cells ≈3-fold higher than control nude mice, a finding in agreement with those of previous T cell reconstitution studies (14). Confirming our recent observations (11), ovx failed to induce bone loss in T cell-deficient mice. However, T cell reconstitution via injection into nude mice of purified T cells from wt ovx mice was followed by rapid bone loss. The degree of bone loss observed in nude mice injected with wt ovx T cells was slightly higher than that observed in wt ovx mice of the same genetic background presumably because the reconstituted cells had been activated previously by the lack of estrogen in the donor microenvironment. In contrast, wt mice T cells become active some time after ovx (11). These findings confirm that T cells play a key role in mediating the bone-wasting effects of estrogen deficiency *in vivo*.

Importantly, injection of T cells harvested from TNF$^{-/-}$ ovx mice into nude ovx recipients induced only a small, insignificant, decrease in BMD, thus establishing that *in vivo* T cell-produced TNF is key for the bone loss caused by estrogen withdrawal. Nude mice were protected against ovx-induced bone loss even though they possess monocytes and other TNF-producing cells. Thus, it is unlikely that contaminating donor cells may account for the ability of T cell reconstitution to restore the capacity of ovx to induce bone loss.

In this study, T cells were purified by using a positive selection technique for CD90 cells. This marker was selected because a CD3 selection system for murine T cells is not commercially available. CD90 is expressed not only by T lymphocytes, but also in early hematopoietic progenitors and neurons (30). Thus, it could be argued that cells other than T lymphocytes may have contributed to restoring the capacity of ovx to induce bone loss in reconstituted mice. Against this hypothesis again is the lack of bone loss in ovx nude mice, a strain that possesses all CD90^{+} lineages except T cells.

Our findings also argue against a direct regulatory effect of ovx on the production of TNF by cells other than T cells and establish T cells as a critical source of E2-regulated TNF production. However, we cannot exclude the possibility that TNF produced by ovx T cells may stimulate the production of TNF by other bone marrow cells.

TNF is known to potentiate RANKL-induced osteoclastogenesis (11, 31), a phenomenon that results from the ability of the two cytokines to markedly potentiate each other's effects on NF-κB and JNK activities (31), two signaling pathways essential for osteoclast formation. Amounts of TNF sufficient to potentiate RANKL-induced osteoclast formation are produced *in vitro* by T cells from ovx mice but not by those from estrogen-replete animals (11). These findings, the results of the current study, and the lack of osteopetrosis in estrogen-replete mice lacking either TNF or its receptors add further support to the hypothesis that T cell-produced TNF is essential for the bone loss induced by estrogen deficiency but not for the control of physiologic, unstimulated bone remodeling.

Other cytokines have been implicated in the pathogenesis of ovx-induced bone loss, including IL-1 (32) and M-CSF (13, 33, 34). IL-1 stimulates OC activity through direct targeting of mature osteoclasts (5, 35) although it does not promote monocyte differentiation into osteoclasts (5). Thus, inhibition of TNF production accounts for the ability of estrogen to block osteoclast formation, whereas the inhibitory effects of sex steroids on IL-1 production contributes to explain how estrogen down-regulates osteoclast activity. A direct repressive effect of estrogen on IL-1 gene expression remains to be demonstrated. Moreover, studies have shown that TNF neutralization blocks IL-1 production whereas IL-1 neutralization does not block TNF (36). Thus, it is likely that the increased bone marrow levels of IL-1 observed in ovx mice are, at least in part, a result of increased TNF production. M-CSF is an essential osteoclastogenic cytokine produced by stromal cells in response to TNF stimulation (5). Although estrogen represses M-CSF production, this effect is not caused by direct estrogen targeting of stromal cells but, rather, by the ability of estrogen to block the production of IL-1 and TNF, factors that both induce M-CSF production in stromal cells (33, 34).

When taken together, the data suggest that TNF is central to the pathogenesis of ovx-induced bone loss not only because of its direct effects, but also for its regulatory effects on other relevant cytokines. We recognize, however, that the relevance of TNF in the pathogenesis of postmenopausal osteoporosis in humans remains to be determined.

Previous *in vitro* studies had shown that TNF augments RANKL-induced osteoclastogenesis mainly by means of engagement of the TNF receptor p55 expressed on osteoclast precursors (11, 37, 38). We now have used mice lacking p55 and/or p75 TNF receptors, which undergo normal development and exhibit a normal adult bone phenotype, to investigate the impact of estrogen deficiency on bone mass. These studies revealed that *in*

vivo the ability of TNF to induce bone loss in ovx mice is mediated exclusively by the p55 receptor.

When taken together, the available data demonstrate that T cell-produced TNF and the binding of this cytokine to the p55 TNF receptor play a key causal role in the bone involution characteristic of estrogen deficiency. Circumstantial evidence in favor of a role for T cells in the pathogenesis of postmenopausal osteoporosis also is emerging in human studies. For example, T cell subset alterations occur in women with postmenopausal osteoporosis (39–42), and a significant inverse correlation between bone density and number of CD3/CD56$^+$ cells (an estrogen-regulated T cell subset known to produce TNF) has been reported in postmenopausal women (43).

The results of the current investigation are relevant for the pathogenesis and the treatment of postmenopausal osteoporosis, because identification of the cellular and molecular targets of estrogen in bone may lead to the development of new therapeutic agents for postmenopausal osteoporosis.

This study was supported, in part, by grants from the National Institutes of Health (AR 41412 and DK55746), the Pharmacia/Washington University Biomedical Program, the Eastern Missouri Chapter of the Arthritis Foundation, and The Lilly Centre for Women's Health. M.N.W. is supported in part by grants from the Barnes–Jewish Hospital Foundation (2001–22) and the National Institutes of Health (AR46375).

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