Interleukin 27 (IL-27) Alleviates Bone Loss in Estrogen-deficient Conditions by Induction of Early Growth Response-2 Gene*§

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A growing understanding of the bone remodeling process suggests that inflammation significantly contributes to the pathogenesis of osteoporosis. T cells and various cytokines contribute majorly to the estrogen deficiency-induced bone loss. Recent studies have identified the IL-12 cytokine family as consisting of pro-inflammatory IL-12 and IL-23 and the anti-inflammatory IL-27 and IL-35 cytokines. IL-27 exerts protective effects in autoimmune diseases like experimental autoimmune encephalomyelitis; however, its role in the pathogenesis of osteoporosis remains to be determined. In this report, we study the effect of IL-27 supplementation on ovariectomized estrogen-deficient mice on various immune and skeletal parameters. IL-27 treatment in ovariectomized mice suppressed Th17 cell differentiation by inhibiting transcription factor RORyt. Supplementation of IL-27 activates Egr-2 to induce IL-10 producing Tr1 cells. IL-27 treatment prevented the loss of trabecular micro-architecture and preserved cortical bone parameters. IL-27 also inhibited osteoclastogenesis through increased Egr-2 expression, which induces anti-apoptotic factors like MCL-1. IL-27 suppressed osteoclastogenesis in an Egr-2-dependent manner that up-regulates Id2, the repressor of the receptor activator of nuclear factor-κB ligand-mediated osteoclastogenesis. Additionally, these results were corroborated in female osteoporotic subjects where we found decreased serum IL-27 levels along with reduced Egr-2 expression. Our study forms a strong basis for using humanized IL-27 toward the treatment of post-menopausal osteoporosis.

Bone is an active tissue and serves multiple functions, including mechanical support, protection, and storage (1). Bone is mainly composed of 60% crystalline hydroxyapatite and 30% organic matrix (2), and it is continuously maintained by the process of bone remodeling, a lifelong process where mature bone is removed by osteoclasts and new bone is formed by osteoblasts. Osteoblasts are of mesenchymal origin and are the bone-forming cells (1, 3). The main transcription factors regulating osteoblast function and differentiation include Runx-2/Cbfα-1 and osterix (Ovx)4 (4). Besides growth factors like TGF-β, bone morphogenetic proteins, Wnt (5–7), and cytokines IL-1, IL-6, and tumor necrosis factor-α (TNF-α) play an important role in osteoblast functions (1). Osteoblasts are incorporated into the bone matrix as osteocytes or remain at the bone surface as bone-lining cells at the end of the remodeling phase. In contrast, osteoclasts are the bone-resorbing cells derived from hematopoietic stem cells following stimulation by two essential factors: the macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor-κB (RANK) ligand (RANKL). The process of bone remodeling is a tightly coupled process where it is ensured that bone resorption does not exceed the process of bone formation. However, in diseases like osteoporosis, the balance between bone resorption and formation is disturbed (1).

Until recently, osteoporosis etiology was mainly attributed to various endocrine, metabolic, and mechanical factors (8). However, a growing understanding of the bone-remodeling process suggests inflammation significantly contributes to the etiopathogenesis of osteoporosis (8). Studies have shown that T cells are major contributors to estrogen deficiency-induced bone loss (9). In estrogen-deficient conditions, there is an up-regulation of TNF-producing T cells. The TNF produced by these activated T cells is sufficient to augment RANKL-induced osteoclastogenesis (9). Besides, activated T cells also produce RANKL,

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the main osteoclastogenic cytokine. Reports from our laboratory and other labs have shown that estrogen deficiency induces the production of interleukin-17 (IL-17)-secreting Th17 cells, the major osteoclastogenic subset that contributes to bone loss by augmenting the production of pro-osteoclastogenic cytokines (10). In fact, we have shown that functional block of IL-17 in Ovx mice prevents bone loss (10).

Recent studies have identified the IL-12 cytokine family, which includes four members. Although IL-12 and IL-23 are pro-inflammatory, IL-27 and IL-35 are anti-inflammatory cytokines (11). Among these four cytokines, IL-27 was particularly intriguing as earlier studies suggested it to have a pivotal role as both pro- and anti-inflammatory cytokine (12). However, later studies have established it mainly as an anti-inflammatory cytokine (13). IL-27 is a heterodimeric glycosylated protein consisting of p28 and EBI3 subunits (14). IL-27 is predominantly secreted by the antigen-presenting cells and signals through a receptor complex consisting of the common IL-6 receptor chain, gp130, and the IL-27 receptor α-chain, WSX-1. IL-27 suppresses Th1, Th2, and Th17 cell functions in vivo as IL-27−/− mice showed enhanced T cell functions (14). The anti-inflammatory action of IL-27 is via IL-10 induction from various T cell subsets like Tr1 (type 1 regulatory T cells) (15). IL-27 uses transcription factors like STAT-1, STAT-3, early growth response-2 (EGR-2), and B lymphocyte-induced maturation protein 1 (BLIMP-1) to induce IL-10 production (15).

Effect of IL-27 has also been studied on bone cell types. A study by Park et al. (16) has shown that IL-27-mediated suppression of osteoclastogenesis is IFN-γ-dependent. Kamiya et al. (17) in an earlier study reported that osteoclastogenesis from bone marrow cells induced by soluble RANKL was partially inhibited by IL-27 with a reduced number of functional osteoclasts. A report by Kalliolias et al. (18) has also shown the direct effect of IL-27 on osteoclastogenesis by suppressing responses of osteoclast precursors to RANKL. IL-27 inhibits human osteoclastogenesis by abrogating RANKL-mediated induction of NFATc1 and suppressing proximal RANK signaling (18). In osteoblasts, IL-27 stimulated STAT-3 activation but had no significant effect on alkaline phosphatase activity and proliferation of osteoblasts (17).

Studies have shown that IL-27Ra deletion in mice leads to excessive Th17 responses in experimental autoimmune encephalomyelitis (EAE) (19). IL-27 also has been reported to provide protection against collagen-induced arthritis and rheumatoid arthritis by suppressing Th17 cells and augmenting T regulatory cell differentiation (20). As Th17 differentiation is under negative regulation of estrogen and estrogen deficiency augments Th17 differentiation, it was interesting to study whether IL-27 can provide protection under estrogen deficiency-induced bone loss conditions.

Thus, in this study we investigate the role of IL-27 cytokine in estrogen deficiency-induced bone loss conditions where bone resorption exceeds the process of bone formation. We have determined the effect of IL-27 on T cell proliferation and Th17/Treg cell differentiation. It has also been determined whether IL-27 has an osteoprotective effect on Ovx-induced bone loss situations and what might be the possible mechanism involved. Finally, we have measured the levels of serum IL-27 in healthy, osteopnenic, and osteoporotic post-menopausal women to determine whether IL-27 level correlate with bone mineral density data.

**Results**

**Serum Level of IL-27 and mRNA Expression in PBMCs Decreased in Ovx Mice—**IL-27 has been shown to provide protection in collagen-induced arthritis; however, its effect in estrogen deficiency-induced bone loss is not studied. Thus, we sought to study the role of IL-27 in pathogenesis of post-menopausal osteoporosis. Hence, the first step was to determine the levels of IL-27 in mouse PBMC and serum after 1 month of Ovx. mRNA expression of IL-27 and serum levels was significantly reduced in Ovx animals compared with sham-operated (vehic)ole) animals (Fig. 1, A and B). This result formed a strong basis to determine the effect of IL-27 treatment to Ovx mice.

**Ovx Mice Treated with IL-27 Prevented Loss of Trabecular Microarchitecture—**As Ovx leads to deterioration in trabecular microarchitecture, IL-27 role in reversal of Ovx-induced loss of trabecular bones was studied. In gross observation by 3D-μCT, deterioration of the trabecular micro-architecture of femur bone was readily observed in the Ovx group compared with the sham group (Fig. 2A). Femoral response to various treatments was quantified. The Ovx group had reduced BV/TV (p < 0.001) (Fig. 2B), Tb.Th (p < 0.05) (Fig. 2C), and Tb.N (p < 0.001) (Fig. 2D) and increased Tb.Sp (p < 0.001) (Fig. 2E), SMI (p < 0.001) (Fig. 4F), and Tb.Pf (p < 0.001) (Fig. 2G) compared with sham group. Treatment of Ovx animals with IL-27 led to significant prevention of trabecular parameters as evident by increased BV/TV (p < 0.001) (Fig. 2B), Tb.Th (p < 0.05) (Fig. 2C), and Tb.N (p < 0.001) (Fig. 2D) and decreased Tb.Sp (p < 0.001) (Fig. 2E), SMI (p < 0.001) (Fig. 2F), and Tb.Pf (p < 0.001) (Fig. 2G). No difference was observed between sham control and Ovx group treated with IL-27 in all parameters (Fig. 2, B–G). A similar kind of pattern was observed in tibia bones (supplemental Fig. S1).

**Improved Cortical Bone Geometry and Bone Strength in Ovx Mice Treated with IL-27—**2D-μCT measurements at the site of femur mid-diaphysis showed that relative to the sham group,
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FIGURE 2. Ovx mice treated with IL-27 prevented the loss of femoral bone microarchitecture. A, representative images of femoral bones of different groups. B, BV/TV; C, Tb.Th; D, Tb.N; E, Tb.Sp; F, SMI; and G, Tb.Pf. Statistical analysis was performed by ANOVA method followed by the Newman-Keuls test of significance using Prism version 3.0 software. n = 8 mice/group; data are presented as mean ± S.E.; ***, p < 0.001 compared with Ovx group; **, p < 0.01 compared with Ovx group; *, p < 0.05 compared with Ovx group; †, p < 0.01 compared with Sham group; ‡, p < 0.001 compared with sham group; ‡‡, p < 0.05 compared with Sham + IL-27 group. No difference was observed between Sham and Sham control levels (Fig. 3, A–C). OPG/RANKL ratio in the BM B220+ B cells was also found to be increased in Ovx animals supplemented with IL-27 (Fig. 4D). OPG/RANKL ratio was also increased in osteoblast treated with IL-27 (supplemental Fig. S3). In addition, IL-27 treatment to Ovx animals led to a decrease in percentage of B220+ B cells, which was otherwise increased in the Ovx control group (Fig. 4, E and F). Sham group treated with IL-27 (Fig. 4, E and F) has a lower number of B cells than the sham control group.

Supplementation of IL-27 Initiates Egr-2 Activation That Induces IL-10 Producing Tr1 Cells—IL-27 cytokine facilitates the differentiation of CD49, LAG3-expressing Tr1 regulatory cells, which execute their suppressor functions by secreting IL-10 anti-inflammatory cytokine (21). Studies have shown that the early growth response gene-2 (Egr-2), whose activation is very low in Ovx animals, whereas there was a robust increase in the Ovx group had decreased cortical thickness (Cs.Th) (p < 0.001) and cortical bone area (B.Ar) (p < 0.05) (Fig. 3, A–C). Compared with the Ovx group, the IL-27-treated group showed greater Cs.Th (p < 0.05) and B.Ar (p < 0.05) (Fig. 5, B and C). Cortical thickness and cortical bone area were significantly higher in the sham group treated with IL-27 compared with sham alone (p < 0.001 each) (Fig. 3, B and C). In fact, both cortical thickness and bone area were better in the sham group treated with IL-27 compared with the Ovx group treated with IL-27 (p < 0.001 each) (Fig. 3, B and C).

In the 3-point bending test of femur, the Ovx group exhibited decreased stiffness (p < 0.001) (Fig. 3D), energy (p < 0.001) (Fig. 3F), and power (p < 0.001) (Fig. 3F) parameters compared with the sham group. IL-27 treatment to Ovx mice led to increased stiffness (p < 0.001), energy (p < 0.001), and power (p < 0.001) compared with the Ovx control group (Fig. 3, D–F). Power and stiffness of femoral bones were not different between sham and sham + IL-27 groups; however, higher energy was required to break the femoral bone in sham animals treated with IL-27 (p < 0.01) (Fig. 3, D–F).

IL-27 Treatment Inhibits Ovx-induced T Cell Proliferation and B Cell Lymphopoiesis—As Ovx leads to increased proliferation of effector T cells and B cells, it was therefore determined whether IL-27 cytokine alters this phenomenon. It was seen that Ovx-induced CD4+ T cell proliferation was significantly reduced in the IL-27-treated Ovx group and brought back to sham control levels (Fig. 4, B and C). No difference was observed in sham control and sham group treated with IL-27 (Fig. 4, B and C). OPG/RANKL ratio in the BM B220+ B cells was also found to be increased in Ovx animals supplemented with IL-27 (supplemental Fig. S3). In addition, IL-27 treatment to Ovx animals led to a decrease in percentage of B220+ B cells, which was otherwise increased in the Ovx control group (Fig. 4, E and F). Sham group treated with IL-27 (Fig. 4, E and F) has a lower number of B cells than the sham control group.

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FIGURE 3. **Improved cortical bone parameters and bone biomechanical properties in Ovx mice treated with IL-27.** A, representative 3D micro-CT images of cortical bone at the femoral diaphysis. B, Cts.Th was measured in all the groups. C, B.Ar was measured in all the groups. Three-point bending test of the femur was carried out to measure stiffness (D), energy (E) and power required to break femoral bone (F). n = 8 mice/group; data are presented as mean ± S.E.; ***, p < 0.001 compared with Ovx group; **, p < 0.01 compared with Ovx group; *, p < 0.05 compared with Ovx group; †, p < 0.001 compared with sham + IL-27 group; ‾, p < 0.001 compared with sham group.

FIGURE 4. **% CD4⁺ T cell populations in the BM of Sham, Sham + IL-27, Ovx, and Ovx + IL-27 groups were analyzed by flow cytometry.** A, quantitative representation of the FACS data. B, % CD4⁺ T cells in different groups. C, OPG/RANKL ratio was determined in B cells in BM of Sham, Sham + IL-27, Ovx, Ovx + IL-27 groups. D, representative images B220⁻ cells of the FACS data. E, % B cells in BM of Sham, Sham + IL-27, Ovx, and Ovx + IL-27 groups was analyzed by flow cytometry. n = 8 mice/group; data are presented mean ± S.E. and are representative of three independent experiments. ***, p < 0.001 compared with Ovx group; †, p < 0.001 compared with sham; ‾, p < 0.01 compared with sham; ‾, p < 0.001 compared with sham + IL-27 group; ‾, p < 0.001 compared with sham group.
Ovx animals treated with IL-27, and this was significantly higher than the sham control (Fig. 5A). Additionally, sham group animals treated with IL-27 exhibited significantly higher Egr-2 transcript levels than the sham group (Fig. 5A).

Once it was determined that Ovx animals treated with IL-27 expressed high levels of Egr-2 mRNA, it was investigated whether IL-27-mediated increased Egr-2 expression also induces Tr1 cell development. It was observed that although Ovx mice had a higher percentage of Tr1 cells than the sham group, treatment with IL-27 further led to a significant increase in % Tr1 cells (Fig. 5B and C). Also, sham group animals treated with IL-27 had a significantly higher percentage of Tr1 cells (Fig. 5B and C). As Tr1 cells exert their anti-inflammatory actions by secreting IL-10 cytokine, levels of IL-10 were determined in IL-27-treated CD4⁺ T cells. It was observed that IL-27 treatment significantly enhanced IL-10 production in CD4⁺ LAG3⁺ Tr1 cells (Fig. 5D–F). Furthermore, it was also observed that IL-6 can induce Tr1 cells, CD4⁺ T cells were treated with or without IL-6, and the percentage of Tr1 cells was determined. It was observed that IL-6 significantly induced the generation of Tr1 cells (Fig. 5G and H).

**IL-27 Suppresses Th17 Development and Differentiation**—Estrogen deficiency induces the differentiation of IL-17-secreting Th17 cells, whereas IL-27 has been reported to inhibit Th17 generation (23). Thus, it was determined whether IL-27 cytokine treatment can mitigate an increase in IL-17-secreting Th17 cells in Ovx-induced bone loss. It was observed that Ovx-induced increase in the percentage of Th17 cells in PBMC isolated from bone marrow was significantly reduced in IL-27-treated Ovx mice and was equal to sham control (Fig. 6A and B). Not much difference was observed between sham and the sham group treated with IL-27 cytokine. Transcription factor-like ROR-γt is crucial for Th17 differentiation (24), and its transcript level was determined in CD4⁺ T cells. As expected, the expression of ROR-γt was robustly enhanced in T cells harvested from Ovx animals, whereas treatment with IL-27 significantly reversed this effect and brought it back to the sham control and sham group treated with IL-27 (Fig. 6B). Transcript level of FoxP3, which is a negative regulator of Th17 development (24), was very low in T cells harvested from Ovx animals, whereas treatment with IL-27 significantly reversed this effect and brought it back to the sham control and sham group treated with IL-27 (Fig. 6B). Thus, IL-27 mitigates Ovx-induced Th17 generation and differentiation. A study by Liu and Rohowsky-Kochan (25) suggests that SOCS-3 (suppressor of cytokine signaling) participates in IL-27-mediated suppression of Th17 cell production; hence levels of SOCS-3 in Ovx and IL-27-treated Ovx mice were determined. It was observed that Ovx led to
robust down-regulation of SOCS-3, whereas treatment with IL-27 brought back the mRNA levels to sham control (Fig. 6E). Similar to FoxP3, a significant increase in SOCS-3 expression was observed in sham animals treated with IL-27 compared with sham (Fig. 6E). Thus, SOCS-3 participates in IL-27-mediated Th17 suppression in estrogen-deficient bone loss conditions.

**IL-27 Suppresses Ovx-induced Increase of Pro-inflammatory Cytokines**—Pro-inflammatory cytokines like TNF-α and IL-17 play a critical role in pathogenesis of osteoporosis (9). Therefore, we examined the effect of IL-27 treatment on the levels of major pro-inflammatory and anti-inflammatory cytokines. In the Ovx group, there was a significant increase in the levels of IL-2, IL-6, IL-17, IFN-γ, and TNFα and a decrease in IL-10 over the sham group (Table 1). However, treatment of Ovx animals with IL-27 significantly reduced the levels of these pro-inflammatory cytokines (Table 1). Especially, levels of pro-inflammatory cytokines like IL-17A and IL-6 in Ovx mice treated with IL-27 were even more reduced than the sham control group (Table 1). Even the sham group treated with IL-27 exhibited a significant decrease in IL-17A and IL-6 levels compared with sham control (Table 1). The most drastic decrease was observed in IL-6 levels in Ovx mice treated with IL-27, which was significantly better than Ovx, sham control, and sham group treated with IL-27 (Table 1). Anti-inflammatory cytokine IL-10 levels were quite low in Ovx animals; however, treatment with IL-27 significantly increased the IL-10 levels, which were even higher than that observed in sham control group and sham animals treated with IL-27 (Table 1).

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TABLE 1
Effect of IL-27 on expression of various cytokines

Cytometric bead array was used for the measurement of various levels of cytokines in different in vivo groups. Levels of IL-10, TNF-α, IL-17A, IFN-γ, IL-4, and IL-10 were measured in serum samples. Data are mean ± S.E. ***p < 0.001; **p < 0.01 compared with Ovx group; *p < 0.05 compared with sham group; †p < 0.001 compared with sham group; ‡p < 0.01 compared with sham + IL-27 group; and §p < 0.05 compared between sham group; †‡p < 0.01 compared between sham groups.

| Cytokines | Sham | Sham + IL-27 | Ovx | Ovx + IL-27 |
|-----------|------|--------------|-----|------------|
| IL-10     | 22.24 ± 1.78** | 24.04 ± 1.21** | 14.92 ± 1.08 | 31.1 ± 2.18**| |
| TNF-α     | 17.00 ± 0.75** | 15.41 ± 2.71*** | 25.28 ± 1.36 | 12.35 ± 1.04*** |
| IL-17A    | 15.13 ± 0.15*** | 11.49 ± 0.59*** | 26.66 ± 1.55 | 10.95 ± 0.50*** |
| IFN-γ     | 10.38 ± 1.31*** | 10.50 ± 0.07*** | 29.68 ± 0.89 | 11.93 ± 1.86*** |
| IL-4      | 18.3 ± 0.40     | 18.32 ± 0.05   | 12.70 ± 0.47 | 23.01 ± 3.56** |
| IL-6      | 17.48 ± 1.02*** | 14.24 ± 0.29***| 30.33 ± 1.64***| 9.83 ± 0.81*** |

A representative image and quantitative data of TUNEL staining (supplemental Fig. S2) indicate in vivo IL-27 treatment inhibits osteoblast apoptosis.

The data are presented as mean ± S.E. ***p < 0.001 compared with control group; **p < 0.01 compared with control group; *p < 0.01 compared with control group; †p < 0.001 compared with IL-17; ‡p < 0.01 compared with IL-17; †‡p < 0.01 compared with IL-17.

FIGURE 7. IL-27 inhibits mouse calvarial osteoblast apoptosis by enhancing Egr-2 expression and suppressing inhibitory effect of CD4+ T cells isolated from Ovx mice on osteoblast differentiation. A, representative images of apoptosis assay done by annexin and PI staining. B, percentage of apoptotic cells in each set. Transcript levels of Egr-2 (C) and MCL-1 (D) in each set (D). IL-27 prevented the decrease in osteoblast differentiation genes in mouse osteoblasts co-cultured with Ovx CD4+ T cells, which include BMP-2 (E), Runx-2 (F), and Wnt-10b (G). IL-27 prevents secretion of osteoclastogenic cytokines from osteoblasts. Data are presented as mean ± S.E. ***p < 0.001 compared with control group; **p < 0.01 compared with control group; *p < 0.01 compared with control group; †p < 0.001 compared with IL-17; ‡p < 0.01 compared with IL-17; †‡p < 0.01 compared with IL-17.

(26). Hence, expression of MCL-1 was also checked. MCL-1 expression was not different between control and IL-27-treated cells, but it was significantly reduced in IL-17-treated cells (p < 0.01 compared with control and IL-27-treated cells) (Fig. 7D). However, pre-treatment of IL-27 to osteoblast cells inhibited this effect of IL-17 and led to increased expression of MCL-1 over IL-17 alone treated cells (p < 0.01) (Fig. 7D). A representative image and quantitative data of TUNEL staining (supplemental Fig. S2) indicate in vivo IL-27 treatment inhibits osteoblast apoptosis.

To study the direct effect of immune cells on osteoblast differentiation and how IL-27 supplementation would affect this process, it was decided to study the effect of IL-27 in co-culture of osteoblast with CD4+ T cells isolated from Ovx mice. It was observed that expression levels of BMP-2, Runx-2, and Wnt-10b, the key osteoblast differentiation markers, were decreased in osteoblasts co-cultured with CD4+ T cells isolated from Ovx mice. However, treatment of IL-27 in the co-culture resulted in increased levels of these osteogenic gene markers, thus abolishing the inhibitory effect of CD4+ T cells isolated from Ovx mice (Fig. 7, E–G).

IL-27 Negatively Regulates Osteoclastogenesis through Egr-2-mediated Up-regulation of Id Protein—IL-27 is reported to inhibit RANKL-mediated osteoclastogenesis (18). In Ovx estrogen-deficient conditions, there is an increased rate of bone resorption. Histomorphometric analysis of decalcified femurs stained with TRAP showed increased TRAP staining in Ovx mice, which suggested more numbers of osteoclasts compared with sham group (Fig. 8A). However, in Ovx animals treated with IL-27, reduced TRAP staining was observed suggesting decreased osteoclast activity (Fig. 8A). Bone resorption marker CTX-1 was measured in serum samples of different groups, which validated TRAP staining data in different groups. CTX-1 level was enhanced in Ovx groups, whereas treatment with IL-27 prevented it (Fig. 8B).

Osteoclast number and osteoclast surface area were increased in Ovx groups, whereas treatment with IL-27 reduced osteoclast number and osteoclast surface area (p < 0.001) (Fig.
No difference was observed in sham control and sham group treated with IL-27 in both osteoclast number and surface (Fig. 8, B and C). Next, we sought to determine Egr-2 expression in bone marrow cells as there are studies to show that Egr-2 overexpression in bone marrow-derived macrophages suppresses osteoclastogenesis (27). Additionally, Egr-2 induces the expression of inhibitor of differentiation/DNA binding (IDs) helix-loop-helix proteins that repress RANKL-mediated osteoclastogenesis (27, 28). Thus, transcript levels of Egr-2 and ID2 were determined in bone marrow cells treated with RANKL and MCSF with or without IL-27. It was observed that Egr-2 levels were significantly enhanced in IL-27-treated cultures at days 2 and 4 compared with the control (Fig. 8D). ID2, which is enhanced by Egr-2, was also found to be expressed at high levels on days 4 and 6 (Fig. 8E). As Egr-2 suppresses osteoclastogenesis and also induces ID2, which also represses osteoclastogenesis, it was deemed important to determine the dependence of IL-27 on Egr-2 for its anti-osteoclastogenic effect. For this, Egr-2 was silenced in the osteoclast cells that were then cultured in the presence or absence of IL-27. Silencing of Egr-2 led to abrogation of Egr-2 mRNA expression. IL-27, which otherwise induced Egr-2 expression, failed to do so in Egr-2 siRNA-transfected bone marrow cells (Fig. 8F). Transfection of Egr-2 siRNA also abolished the inducing effect of IL-27 on ID2 expression (Fig. 8G). This result shows that IL-27-mediated increase in ID2 expression is mediated by Egr-2. These observations were further strengthened by analysis of TRAP expression in Egr-2 siRNA-transfected cells. It was observed that silencing of Egr-2 enhanced TRAP mRNA expression (Fig. 8H). IL-27 inhibited TRAP expression; however, in cells transfected with Egr-2 siRNA, the osteoclast-suppressing effect of IL-27 was inhibited (Fig. 8H). These results strongly support an important role of Egr-2 in the IL-27-mediated inhibitory effect on osteoclastogenesis.

Decreased Serum Level of IL-27 in Osteoporotic Patients and Correlation of BMD with Level of IL-27 in Post-menopausal Women—There have been no studies to determine IL-27 serum levels in human osteoporotic patients. As it is an anti-inflammatory cytokine that suppresses IL-17-secreting Th17 differentiation, we decided to determine IL-27 serum levels in healthy, osteopenic, and osteoporotic post-menopausal women. For this study, human blood was obtained from 45 post-menopausal women. Demographic data of the study population are shown in Fig. 9A. BMD of post-menopausal women was analyzed using dual-energy X-ray absorptiometry, and based on T-score value, they were divided in three groups, viz. normal, osteopenic, and osteoporotic. The mean age was 63.86 ± 2.68, 61.46 ± 1.99, and 64.23 ± 1.23 years of normal, osteopenic, and osteoporotic subjects, respectively. Serum levels of IL-27 were measured in all three groups. It was found that serum level of IL-27 was decreased in osteoporotic patients compared with healthy and osteopenic women. The serum level of IL-27 significantly correlated with BMD of post-menopausal women. These results strongly support an important role of IL-27 in the prevention of osteoporosis.
IL-27 was decreased significantly in the osteoporotic group in comparison with the normal group (Fig. 9B). Also, when the serum level of IL-27 in 45 post-menopausal women was correlated with BMD data, it showed a positive correlation (Fig. 9C). Additionally, transcript levels of EGR-2 were significantly down-regulated in PBMCs isolated from osteoporotic post-menopausal women compared with normal post-menopausal women. This observation was in corroboration with low IL-27 serum levels in osteoporotic patients (Fig. 9D).

**Discussion**

There is enough evidence to indicate that IL-27 alleviates the severity of autoimmune diseases like EAE and rheumatoid arthritis by suppressing Th17 cells and augmenting T regulatory cells (14, 19). Th17 cell differentiation is enhanced under estrogen deficiency, which leads to increased osteoclastogenesis and bone loss (10). In this study, we investigated whether IL-27 can alleviate estrogen deficiency-induced bone loss and what may be the possible mechanism involved. For this, BALB/c mice were ovariectomized, which rendered them estrogen-deficient. The estrogen-deficient mice were then supplemented with IL-27 cytokine exogenously, and various immune and skeletal parameters were studied to determine the action of IL-27.

Ovx leads to an up-regulation of TNF-α-producing T cells and B cell lymphopoiesis (9, 10). Increased production of TNF-α augments RANKL-induced osteoclastogenesis, which results in bone resorption (9). B cells are also an important source of RANKL, the major osteoclastogenic cytokine that binds to RANK on osteoclast precursors to initiate osteoclastogenesis (29). Increased T cell proliferation and B lymphopoiesis was observed in Ovx animals, whereas IL-27 treatment in Ovx mice reduced this phenomenon thus establishing that IL-27 suppresses T and B cell proliferation to inhibit the process of osteoclastogenesis. Mature B cells regulate osteoclastogenesis through the production of OPG and RANKL (30). An increased OPG/RANKL ratio was found in Ovx animals treated with IL-27 compared with an Ovx control group, which shows the anti-osteoclastogenic effect of IL-27.

One of the mechanisms by which IL-27 exerts its anti-inflammatory action is by inducing IL-10 production from FoxP3-negative Tr1 cells with aid from transcription factor-like Egr-2, which is essential for the differentiation of Tr1 cells (15). Studies by Li et al. (31) have shown that Egr2 and Egr3 transcription...
factors are essential for the control of inflammation and anti-
gen-induced proliferation of B and T cells. Thus, transcript lev-
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different groups. It was observed that Egr-2 levels were signifi-
cantly lower in Ovx animals, whereas treatment with IL-27 sig-
nificantly enhanced Egr-2 expression. Corroborating the data
were increased percentages of Tr1 cells in Ovx animals treated
with IL-27. As Tr1 cells exert their anti-inflammatory functions
by secretion of IL-10, the effect of IL-27 treatment was observed
on IL-10 secretion by CD49b+ LAG3+ Treg cells. Enhanced
IL-10 was generated by IL-27-treated Tr1 cells. Thus, IL-27
treatment to Ovx mice leads to induction of IL-10 secreting Tr1
anti-inflammatory cells with aid from transcription factors like
Egr-2. Intriguingly, %Tr1 cells were higher in Ovx animals com-
pared with sham. It might be possible that inflammation
induced as a result of Ovx may drive the cytokine balance to
yield an environment that promotes the generation of T regu-
latory cells. Moreover, there is a study by Jin et al. (32) that
shows that IL-6 induces the production of IL-10 generating Tr1
cells. As pro-inflammatory cytokines like IL-6 and TNF-α increase in estrogen-deficient conditions, they might induce
Tr1 production. To confirm this hypothesis, CD4+ T cells were
treated with or without IL-6, and % of Tr1 cells was determined.
It was observed that IL-6-treated CD4+ T cells had a high per-
centage of Tr1 cells. Thus, this might explain the increased
levels of Tr1 cells in Ovx mice. Importantly, Tr1 cells were
much higher in Ovx animals treated with IL-27.

Th17 cells are considered to be osteoclastogenic T helper
subsets as they augment the synthesis and secretion of RANKL,
which induces osteoclastogenesis (10, 24). IL-27 cytokine has
been reported to prevent Th17 development by inhibition of
the RORγt transcription factor that is essential for Th17 devel-
opment (33). These observations are supported by fact that
IL-27ra-/- mice are more susceptible to EAE due to increased
accumulation of Th17 cells (19). In our case, we observed that
IL-27 supplementation suppressed the Ovx-induced increase in the percentage of Th17 cells. Additionally, decreased RORγt
and increased transcript levels were observed in CD4+ T cells
harvested from IL-27-treated Ovx animals. Studies have shown
that SOCS-3 participates in IL-27-mediated suppression of
Th17 cell production (25); therefore, levels of SOCS-3 in Ovx
and IL-27-treated Ovx mice were determined. Reduced SOCS-
3 levels were found in Ovx mice, whereas IL-27 treatment
regained the SOCS-3 mRNA levels comparable with the sham
control group. The data indicate that SOCS-3 may have a role
to play in IL-27-mediated Th17 suppression in estrogen-defi-
cient bone loss conditions.

We next studied the skeletal effects of IL-27 treatment. It was
observed that Ovx-induced micro-architectural deterioration
was significantly prevented by IL-27 treatment in both femur
and tibia bones. Effect of IL-27 on cortical parameters, like cor-
tical thickness and bone area, was also determined because ana-
obolic agents, like parathyroid hormone, lead to an increase in
periosteal apposition (34). We observed increased cortical
thickness and cortical bone area in Ovx mice treated with IL-27.
More interestingly, sham group animals treated with IL-27 pre-
sented with better cortical thickness and bone area than the
sham control group, suggesting that IL-27 was quite effective
in maintaining cortical bones, which points toward its ana-
obolic potential. Femur biomechanical parameters showed that IL-27 treatment conferred greater stiffness, power, and
energy required for breaking force compared with Ovx-un-
treated mice.

The effect of IL-27 was also studied on various osteoblast and
osteoclast functional parameters. As pro-inflammatory cyto-
kines like TNF-α and IL-17 induce osteoblast apoptosis (24), we
determined the effect of IL-27 on IL-17-mediated osteoblast
apoptosis. It was observed that IL-17-induced increase in per-
cent apoptotic cells was significantly brought down by IL-27
treatment. Thus, IL-27 enhances osteoblast survival. Studies
have shown that epidermal growth factor receptor signaling
promotes the proliferation and survival of osteoprogenitors by
inducing Egr-2 response (26). As IL-27 initiates Egr-2 activa-
tion to induce IL-10 producing Tr1 cell development (15), we
hypothesized that in bone cells IL-27 might also be acting via
Egr-2 induction. We observed a significant increase in Egr-2
transcript levels in osteoblasts treated with IL-27. On the con-
trary, Egr-2 mRNA levels were significantly reduced in IL-17-
treated osteoblasts. However, pre-treatment of IL-27 with
osteoblasts abolished this effect of IL-17. Thus, IL-27 enhances
osteoblast survival via the up-regulation of Egr-2, which then
increases the anti-apoptotic genes, like MCL-1, as was corro-
borated by our data. Besides, IL-27 also alleviated the inhibitory
effect of T cells harvested from Ovx mice on osteoblast differ-
entiation in a CD4+ T cell-osteoblast co-culture.

Treatment of IL-27 to Ovx mice also led to significant reduc-
tion in osteoclast number and surface. As Egr-2 negatively
modulates osteoclast differentiation through up-regulation of
Id helix-loop-helix proteins (27), we therefore checked the
mRNA expression of both in IL-27-treated osteoclasts cells.
Increased transcript levels of Egr-2 and ID2 were found in bone
marrow cells treated with IL-27. These observations suggested
that Egr-2 might be important in the IL-27-mediated anti-os-
.teoclastogenic effect. This was proved by gene silencing exper-
iments where siRNA of Egr-2 abrogated the IL-27 inductor
effect on ID2 expression and also suppressed the IL-27 inhibi-
tory effect on osteoclastogenesis.

We also evaluated serum IL-27 levels in post-menopausal
women. To the best of our knowledge, IL-27 levels have never
been evaluated in post-menopausal osteoporotic women. Our
results show a significant decrease in serum IL-27 levels in post-
menopausal women with osteoporosis as compared with nor-
mal or osteopenic post-menopausal women. The decrease in
serum IL-27 levels correlated with BMD at lumbar spine (L1 to
L4) where patients with low BMD also exhibited decreased
IL-27 serum levels. Corroborating these observations was the
decreased Egr-2 mRNA expression in osteoporotic patients.
Thus, low serum IL-27 levels may be indicative of greater risk
of osteoporosis in post-menopausal women.

In conclusion, IL-27 alleviates bone loss in estrogen-deficient
conditions in an Egr-2-dependent manner. Exogenous supple-
mentation of IL-27 in Ovx estrogen-deficient mice leads to ac-
tivation of Egr-2, which induces the production of IL-10-secret-
ing Tr1 cells. Additionally, IL-27 treatment suppresses Th17
differentiation with reduction in transcription factors like
RORγt. This would suppress IL-17-induced osteoclastogenesis
IL-27 treatment also promotes osteoblast survival by activating EGF receptor-induced Egr-2 expression and suppression of IL-17-mediated osteoblast apoptosis. The overall effect leads to enhanced osteoblast proliferation and differentiation. IL-27 also directly suppresses osteoclast functions in an Egr-2-mediated Id2 up-regulation, which is a repressor of RANKL-induced osteoclastogenesis. The net result is reduced osteoclastogenesis and enhanced osteoblastogenesis, which inhibits Ovx-induced bone loss. This study forms a strong basis for using humanized IL-27 toward the treatment of post-menopausal osteoporosis.

**Materials and Methods**

Cell culture reagents like RPMI 1640 medium, α-MEM, FBS, non-essential amino acids, antibiotics, and primers were purchased from Sigma. Recombinant mouse IL-27 was purchased from R&D Systems. TRizol was procured from (Invitrogen). FACS antibodies were purchased from Biolegend (San Diego). MicroBeads and columns were purchased from Miltenyi Biotech (Singapore), and ELISA kits were procured from Boster Immunoleader (Pleasanton, CA). IL-27 and CTX-1 ELISA kits were purchased from Qayee-bio. siRNAs of EGR2 and negative controls were purchased from Thermo Fisher Scientific.

*In Vivo Study*—PBMCs were isolated for mRNA expression of IL-27 from 9- to 10-week-old BALB/c mice that were kept for 4 weeks after ovariectomy.

To check the *in vivo* effect of IL-27 in an ovx model, mice were randomized into four groups of eight animals each. 9- to 10-week-old female BALB/c mice were taken for the study and were housed at 25 °C in 12-h light/dark cycles and fed standard mouse chow and water *ad libitum*. The groups were sham-operated (ovary intact) mice, ovariectomized (Ovx), Ovx + IL-27 (0.04 mg/kg) and sham + IL-27 (0.04 mg/kg). Treatment was continued for a period of 4 weeks with subcutaneous injections twice weekly. After 4 weeks of the study, animals were autopsied. After autopsy, bones and spleen were collected for PBMC isolation. Femur and tibia bones were kept in 70% isopropyl alcohol for CT study. Serum was collected for cytokine bead array analysis. All experimental procedures were examined and approved by the Institutional Animal Ethics Committee, Central Drug Research Institute (CPSCEA registration no. 34/1999, dated November 3, 1999, extended to 2015; approval reference no. IAEC/2013/93/renew 01, dated December 03, 2014).

**Flow Cytometry**—To quantify the number of Tr1 cells, CD4 + T cells, macrophages, and B220 + B cells were flushed out from bone marrow, and PBMCs were isolated using Hisep (Himedia, Navi Mumbai, India). CD4 + T cells, macrophages, and B cells were isolated using columns, Macs separator, and Micro beads from Miltenyi Biotech (Singapore) by positive selection. All the steps were performed using the manufacturer’s instruction and as per as protocols in our previously published papers (10, 24, 35). After isolation the cells were collected in 1 ml of TRizol (Invitrogen) for RNA isolation.
FITC-conjugated anti-mouse CD4, aliphycocyanin-conjugated anti-LAG3 antibodies, PE-conjugated CD49b, and FITC-conjugated B220 and further incubated for 45 min at room temperature. After incubation, cells were washed twice with PBS and transferred to FACS tubes for analysis. FACSCalibur and FACSAria (BD Biosciences) were used to quantify percent of IL-17A-positive cells. Also, the percentage of CD4+ T cells and CD25+ Foxp3+ cells was measured by using FACSstar (BD Biosciences).

**Cytometric Bead Array (CBA) Flex**—Levels of IL-6, IFN-γ, TNF-α, IL-17A, IL-4, and IL-10 were assessed in serum samples by fluorescent bead-based technology using cytometric bead array (CBA) Flex sets according to manufacturer’s instructions (BD Biosciences). Fluorescent signals were read and analyzed on a FACSCalibur flow cytometer (BD Biosciences) with the help of BD FCPA Array version 1.0.1 software (BD Biosciences).

**Gene Expression Analysis by Real Time PCR**—Total RNA was extracted from isolated CD4 cells, B cells, and macrophages and PBMCs of all the in vivo groups using TRIzol (Invitrogen). 1 μg of total RNA was reverse-transcribed using the Revert Aid H minus First Strand cDNA synthesis kit (Thermo Fisher Scientific). For real time PCR, data acquisition and analyses were performed using the Light Cycler Real Time PCR system (Roche Diagnostics, Mannheim, Germany), and the relative levels of gene expression were normalized against GAPDH. Primers for RORγt, FoxP3, SOCS3, Egr-2, MCL-1, BMP-2, Runx-2, WNT10B, OPG, RANKL, and Id2 were designed using the Universal probe library (Roche Diagnostics). Primer sequences are given in Table 2.

**Micro-computed Tomography**—μCT determination of excised bones was carried out using Sky Scan 1076 CT scanner (Aartselaar, Belgium) using previously published protocol (10, 24, 36). Three samples were scanned per batch at nominal resolution pixel 18 μm. Reconstruction was carried out using a modified Feldkamp algorithm using the Sky Scan Nrecon Software, which facilitates network-distributed reconstruction carried on a personal computer running simultaneously. The X-ray source was set at 70 kV and 100 mA, with a pixel size of 18 μm. To analyze the trabecular region, the region of interest was

### Table 2

| Gene name | Primer sequence | Accession no. |
|-----------|-----------------|---------------|
| GAPDH | P1−5‘-AGCTGTTCTACAAAGGAGGAC-3’ | NM_008048.2 |
| IL-27 | P2−5‘-AGGCGGGATACCTCTCCTG-3’ | BC119402.1 |
| OPG | P1−5‘-GTTCTCCGAAAGCACAAT-3’ | U94331.1 |
| ID2 | P2−5‘-CCATCTCAATGGTCGTCGAGAA-3’ | NM_010496.3 |
| EGR2 | P2−5‘-CTAGGAGTGTCAGCACCA-3’ | NM_010118.3 |
| SOCS3 | P2−5‘-ATACACTGGGAGCACTAAGC-3’ | NM_007703.4 |
| EGR2 human | P2−5‘-TGGCTTCATGTTGAGTTCC-3’ | J04076.1 |
| RUNX2 | P1−5‘-CACACTTTTTCATGGCTTCC-3’ | AF053956.1 |
| ROR-γt | P1−5‘-CAGCTGGACTCTCACGAG-3’ | AF163668.1 |
| FOXP3 | P2−5‘-ATCCCTGGTCTCCATCTCTC-3’ | BC132333.1 |
| BMP2 | P2−5‘-ACACCTGGGAGGTCGTTG-3’ | NM_007553.2 |
| TNF-α | P1−5‘-TTCTCCGATTTTCCTGCTTC-3’ | NM_013693.2 |
| WNT10B | P1−5‘-GACAGGGATACCTGATAA-3’ | U61970.1 |
| IL-1β | P1−5‘-TGTGGTCTCCATCTCTCTC-3’ | M15131.1 |
| GAPDH human | P1−5‘-CCCTGCTTCCCTGCTTGAT-3’ | XM_508955 |

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drawn at a total of 100 slices in the region of secondary spongiosa situated at 1.5 mm from distal border of growth plate, excluding all primary spongiosa and cortical bone. For cortical bone analysis, 350 serial image slides were discarded from the growth plate to exclude any trabecular region, and 100 consecutive image slides were selected, and quantification was done using CTAN software.

Bone Strength Testing—Bone mechanical strength was examined by 3-point bending strength of femur mid-diaphysis using bone strength tester model TK252C (Muromachi Kikai Co. Ltd., Tokyo, Japan). The distance between the supports was kept constant at 1 cm. The load–displacement curves were used to calculate ultimate load (N), energy to fracture (N-mm), stiffness (N/mm).

Co-culture of Murine Osteoblast with Ovx CD4+/T Cells—For osteoblast-CD4+/T cell co-culture, PBMCs were harvested from bone marrow of 8–9-week-old ovariectomized BALB/c mice. CD4+/T cells were isolated from these PBMCs by using a magnetic cell separator and added to calvaria-derived osteoblasts. Osteoblastogenesis of murine calvarial cells was induced, as previously described calvarial cells were resuspended in α-MEM containing 10% fetal bovine serum and seeded in a T25 flask. After incubation for 2 days, cells were resuspended in an osteogenic medium (50 mg/ml ascorbic acid and 10 mM β-glycerophosphate) and seeded in 6-well plates. After attachment of osteoblast, cells were co cultured with ovx T cells at a ratio of 1:1. Co-cultures were treated with IL-27 (50 ng/ml) for 48 h. After 48 h, osteoblast cells were harvested for RNA isolation to check the expression of various genes like RUNX2, WNT-10b, and BMP-2.

Osteoclast Culture and TRAP Staining and siRNA Transfection—In vitro osteoclastogenesis was carried out using a standard protocol (37). Bone marrow was isolated from 8- to 9-week-old female BALB/c mice by flushing bones with α-MEM. Cells were seeded in a T25 flask for overnight in osteoclast medium (α-MEM, 10% FCS, antibiotic, Earle’s balanced salt solution, 10 ng/ml MCSF). After overnight incubation, non-adherent BMCs were seeded in 48-well plates at a density of 200,000 cells/well and cultured for 5–6 days in osteoclast medium with 50 ng/ml RANKL and 10 ng/ml MCSF and IL-27 at 50 ng/ml concentrations. Medium was replaced every 48 h. After 6 days of culture, cells were washed with PBS for RNA extraction by TRizol for analysis of TRAP mRNA levels by real time PCR. For ex vivo trap staining, deparaffinized and hydrated femoral epiphysis sections of different groups were used for staining of tartrate-resistant acid phosphatase using the standardized protocol. Histomorphometric analyses were conducted using Bioquant Image Analysis software (Bioquant, Nashville, TN) as reported earlier (24). siRNA of EGR2 and siRNA-negative control were transfected into mouse osteoclast cells at 60–70% confluence at 30 nm concentration with RNA Max (Invitrogen). Cells were harvested 48 h after transfection for measuring EGR2, ID2, and TRAP expression.

Bone Mineral Density Test in Post-menopausal Women—BMD of post-menopausal women was measured by dual energy X-ray absorptiometry. On the basis of BMD, women were divided into three groups: normal, osteopenic, and osteoporotic. Obtained results were presented as absolute values (g/cm²), T and Z score. Osteoporosis was defined as T score ≤–2.5; women with T score between –2.5 to –1 were defined as osteopenic, and those with T score between –1 to +1 were considered normal group.

Determination of IL-27 Concentrations in Post-menopausal Women and PBMC Isolation—Heparinized blood was collected from post-menopausal women for serum and PBMC isolation. Serum IL-27 was measured by sandwich enzyme-linked immunosorbent assay (ELISA). The absorbance was determined with an ELISA microplate reader at 405 nm. PBMCs obtained from healthy volunteers or osteoporotic patients were isolated from buffy coats using Hisep LSM 1084 (Himedia, Mumbai, India) by means of density (1.08460 0010 g/ml) gradient centrifugation technique. Human study was approved by Institutional Ethics committee, CSIR-CDRI and SGPGI Lucknow (approval no. CDRI/IEC/2015/A9). Written informed consent to participate in the study was obtained from all women before obtaining a blood sample.

Statistical Analysis—Data are expressed as mean ± S.E. The data obtained in the experiment were subjected to a one-way ANOVA followed by Newman-Keuls test of significance using Prism version 3.0 software. Qualitative observations have been represented following an assessment made by three individuals blinded to the experimental design. For human subjects, Kruskal-Wallis test was used for statistical analysis followed by a Dunn’s multiple comparison test. Data analysis was done using Graph Pad prism version 5.0 software. Student’s t test was used to compare between the two means. The correlation between variables was performed using Pearson’s correlation test. Values of p < 0.05 were considered significant.

Author Contributions—P. S. and M. N. M. contributed mainly in experimental work, analysis of data, and manuscript writing; M. K. and M. S. performed BMD of human subjects and data analysis; D. S. and S. K. G. did study design, data analysis, and manuscript writing.

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