# Role of the Immune System in Postmenopausal Bone Loss

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Postmenopausal osteoporosis stems from estrogen deficiency. The mechanisms by which estrogen deficiency drives bone destruction are complex and poorly understood. Recent findings from animal models suggest that postmenopausal bone loss may stem in large measure from a pathologic upregulation of the adaptive immune response. While the role of activated T cells in the bone loss driven by inflammatory conditions such as rheumatoid arthritis has been well documented, only recently has the role of T cells in the bone destruction associated with estrogen deficiency begun to be appreciated. In vivo and in vitro models of postmenopausal osteoporosis demonstrate that the activation and expansion of tumor necrosis factor-α producing T cells is a key step in estrogen deficiency driven bone loss and is regulated by multiple interacting cytokines including transforming growth factor-β, interleukin-7, and interferon-γ, as well as by the process of antigen presentation. This paper presents recent findings pertaining to this new view of postmenopausal osteoporosis.

## Introduction

The osteoclast is the exclusive bone resorbing cell in the body. Postmenopausal osteoporosis stems from estrogen deficiency, causing an upregulation in osteoclastic bone resorption relative to formation. The mechanisms by which estrogen deficiency drives bone loss are complex and poorly understood. Most of the bone sparing activity exerted by sex steroids occurs through modulation of bone cell life-span and decreased cytokine-driven osteoclastogenesis. While it is now accepted that estrogen deficiency disrupts bone homeostasis by acting on multiple cytokine cascades and cell types, it is only recently that the depth of estrogen action and its intercalation into the immune response has begun to be appreciated.

Recent studies in animal models of estrogen deficiency (ovariectomized mice or rats) now suggest that bone loss may ultimately stem from a pathologic realignment of the adaptive immune response driving up the production of osteoclastogenic cytokines and stimulating bone resorption, while simultaneously limiting the magnitude of the compensatory increase in bone formation necessary to require bone homeostasis. This paper explores the most recent concepts that have emerged from in vivo and in vitro models of postmenopausal osteoporosis that support a clear role for pathologic immune activation in the mechanism of estrogen deficiency induced bone loss. The relevance of these concepts for human disease is still largely unknown, and is only now beginning to be investigated. However, a considerable body of circumstantial evidence now exists to support a role for immune activation in the bone destruction associated with postmenopausal osteoporosis. These tantalizing links between animal models and human disease will be discussed.

## Role of Tumor Necrosis Factor-α in Ovariectomy Induced Bone Loss

It has long been recognized that the cytokine tumor necrosis factor (TNF)-α plays a critical role in ovariectomy induced bone destruction in humans and animals. Early studies have found elevated levels of TNFα and interleukin (IL)-1 produced by mononuclear cells derived from ovariectomized women, but not from ovariectomized women receiving hormone replacement therapy, or from hysterectomized women with intact ovaries. These elevated levels of TNFα and IL-1 paralleled enhanced biochemical indices of in vivo bone resorption [1]. These findings in humans have been verified and replicated in numerous animal studies using the standard experimental model for postmenopausal osteoporosis, the ovariectomized mouse, or rat. In mice and rats, antagonists of TNFα and IL-1 prevent ovariectomy induced bone loss [2,3]. Consistent with these findings transgenic mice over-expressing a soluble TNFα decoy receptor that inhibits TNFα action in vivo, are protected from ovariectomy induced bone loss [4].

Although a role for TNFα in estrogen deficiency mediated bone destruction has long been suspected, the sources of TNFα and the mechanisms by which TNFα stimulates osteoclastogenesis have only recently begun to be clarified. The preponderance of evidence now suggests that TNFα potently synergizes with the key osteoclastogenic cytokine, receptor activator of nuclear factor kappa-B ligand

(RANKL) to augment RANKL induced osteoclastogenesis [5••,6,7,8••]. TNFα also stimulates the bone resorbing activity of mature osteoclasts [9] and acts in part by upregulating RANKL production by bone marrow osteoblasts [10]. Finally, TNFα has been reported to suppress bone formation in vitro [11] and in vivo [3] exacerbating bone loss.

Under physiologic conditions monocytes and macrophages represent a major cellular source of TNFα in the bone marrow micro-environment. However, monocytes and macrophages do not appear to be the basis of elevated TNFα concentrations after ovariectomy. Instead, T cells have been identified as the source of the enhanced levels of TNFα necessary to upregulate bone destruction. Interestingly, estrogen deficiency does not lead to an upregulation of T cell TNFα production per cell, but instead stimulates the expansion of the population of TNFα producing Th1 T cells [5••,12••].

How T cells are induced to proliferate after estrogen ablation, involves a complex cascade of interacting cytokines and suppression of any one of these cytokine cascades is capable of preventing ovariectomy induced bone loss in mice.

## Interleukin-7—A Key Player in Ovariectomy Induced Bone Loss

Much of the realignment of the adaptive immune response that occurs during ovariectomy appears to center around IL-7, a potent lymphopoietic cytokine that stimulates bone destruction when injected into mice in vivo [8••,13]. IL-7 was originally suggested to mediate bone loss through cells of the B lineage [13]. However, recent studies now demonstrate that it is predominantly the action of IL-7 on T cells that precipitates bone destruction [8••,14,15••]. In a human in vitro culture system IL-7 was shown to stimulate the secretion of soluble RANKL from T cells, and therefore promoted osteoclast formation [14]. In contrast, IL-7 may also be capable of suppressing osteoclast formation in vitro through inhibitory actions on early pluripotent osteoclast precursors [16]. Such negative feedback loops appear to be a common theme of cytokines involved in osteoclastogenesis and may be critical to limit pathologic bone destruction. However, the net balance of IL-7 action in vivo is strongly pro-osteoporotic [8••,13]. IL-7 potently stimulates bone destruction when injected into intact wild type mice, but fails to induce bone loss in T cell deficient nude mice. Reconstitution of nude mice with T cells, by means of adoptive transfer, rescues IL-7 induced bone destruction demonstrating that IL-7 dependent bone resorption is a T–cell-mediated phenomenon. In these studies IL-7 was demonstrated to stimulate bone resorption by a mechanism that involved T–cell-derived RANKL induced osteoclastogenesis, amplified by TNFα [8••].

The capacity of estrogen deficiency and IL-7 to mediate bone loss by stimulating RANKL and TNFα in T cells is more than just coincidental. In fact IL-7 is significantly upregulated after ovariectomy [15••]. Importantly, the in vivo neutralization of IL-7 completely prevents ovariectomy induced bone loss in mice [15••], by blocking the expansion of TNFα producing T cell populations [17]. In addition, suppressing the rise in IL-7 levels after ovariectomy also leads to upregulated bone formation [15••]. Together the data suggest that IL-7 is a key mediator of estrogen deficiency induced bone destruction acting by upregulation of osteoclastic bone resorption, through a mechanism involving T cells, and like TNFα, exacerbating bone loss by suppressing the magnitude of the compensatory increase in bone formation known to occur in response to estrogen deficiency.

The mechanism by which IL-7 drives T cell proliferation and activation is extremely complex. New studies suggest that IL-7 promotes proliferation of lymphoid hematopoietic stem cells in the bone marrow, T cell differentiation within the thymus, enhanced thymic output, and peripheral T cell expansion of naïve and memory T cells. Consequently, thymectomy is partially effective in blunting ovariectomy induced bone loss (Unpublished data), while IL-7 neutralization completely suppresses osteoclastogenesis and bone destruction [15••]. The finding that the thymus plays an important role in ovariectomy induced bone loss could be particularly relevant for the osteoporosis sustained by younger women undergoing surgical menopause. However, in the case of postmenopausal women the role of the thymus, if any, is presently unclear. Age-associated thymic atrophy results in a decline in T lymphocyte output concurrent with decreased IL-7 production [18], suggesting that IL-7 may play a role in the maintenance of the thymus. Consistent with this notion, administration of IL-7 has been shown to partially reverse age-associated declines in thymopoiesis [19]. It is consequently possible that during estrogen deficiency elevated levels of IL-7 may reactivate thymic function and contribute to the expansion of circulating T cells. Recent data suggest that the adult thymus can indeed contribute to T cell reconstitution as the increase in naïve T cell numbers in adult patients receiving anti-retroviral therapy for HIV/AIDS is largely derived from the thymus [20]. This provides direct evidence for the functional capacity of the adult thymus. It is also possible that an age-related decrease in thymic T cell output could mitigate the stimulatory effects of sex steroid deprivation on osteoclastogenesis, and may explain in part why the rate of bone loss in postmenopausal women diminishes with age [21]. For now however, a role for increased thymic output in postmenopausal bone loss in humans awaits further investigation.

In addition to the trophic effects of IL-7 on T cells, high levels of IL-7 also lead to a breakdown of tolerance [22] whereby normally tolorogenic physiologic self-antigens and foreign antigens such as peptide by-products of digestion and antigens of bacterial origin that are routinely absorbed in the gut, become potent stimulators of T-cell activation. This autoimmune like condition is greatly exacerbated by another action of IL-7, the promotion of a Th1 T cell phenotype that leads to the copious secretion of interferon (IFNγ) [23•].

## Interferonγ—A Potent Stimulator of Inducible Antigen Presentation and Bone Loss

Interferonγ is a potent stimulator of inducible antigen presentation and acts by upregulating the class II transactivator (CIITA), a key transcription factor necessary for major histocompatibility complex II expression by professional antigen-presenting cells (APCs) [24]. The engagement of antigen bearing major histocompatibility complex II molecules with T cell receptors is a major physiologic mechanism for the activation and expansion of naïve and memory T cells. Surprisingly, recent data demonstrate that a significant part of the T cell expansion process after ovariectomy is indeed driven by enhanced antigen presentation [25••]. Consequently, it is proposed that elevated levels of IL-7 after ovariectomy lead to induction of IFNγ, which stimulates CIITA and inducible antigen presentation in macrophages. These events cause an upregulation in antigen presentation to T cells whose tolerance to weak antigens may have already been degraded by enhanced concentrations of IL-7 after ovariectomy. This APC-driven T-cell activation and expansion further drives up additional IFNγ production that feeds back on APC activity sustaining the response. In line with this concept new unpublished studies from our laboratory demonstrate that in vivo neutralization of IL-7 after ovariectomy in mice completely prevents the upswing in IFNγ, CIITA, and antigen presentation after ovariectomy. These data are consistent with reports describing the pro-resorptive properties of IFNγ in vivo [25••,26]. Interestingly, like IL-7, IFNγ also has the capacity to mitigate its destructive actions on bone by suppressing osteoclastogenesis through direct inhibitory actions on osteoclast differentiation [27]. IFNγ action is extremely complex and recent in vitro studies demonstrate that preexposure of osteoclast precursors to RANKL renders them resistant to the suppressive effects of IFNγ [28]. Consequently, as levels of RANKL increase the more likely IFNγ is to overcome its direct suppressive effects on osteoclastogenesis, in favor of its indirect pro-osteoclastogenic effects mediated through enhanced APC activity.

## Transforming Growth Factor-β—A Potent Mediator of Estrogen Action in Bone and Immunity

The mechanisms by which estrogen suppresses IL-7 and how IL-7 is stimulated after ovariectomy are not presently known. However, one candidate cytokine is transforming growth factor-β (TGFβ). TGFβ is an estrogen regulated cytokine with anti-osteoclastogenic properties [29,30] and whose concentration declines after ovariectomy [31••]. TGFβ has been reported to antagonize IL-7 production [32], and IL-7 to antagonize that of TGFβ [33]. In addition TGFβ represses the production of IFNγ by directly targeting T cells and inhibiting their proliferation [34]. In addition to its effects on IFNγ production, TGFβ acts on bone marrow macrophages to decrease the responsiveness of the CIITA gene to IFNγ [35].

Consistent with this notion, mice with a T–cell-specific blockade of TGFβ signaling are completely insensitive to the bone-sparing effects of estrogen. This results from a failure of estrogen to repress IFNγ production, which, in turn, leads to increased T cell activation and T cell TNF production. Furthermore, overexpression of TGFβ in vivo prevents ovariectomy-induced bone loss [31••].

Because of its capacity to repress antigen presentation through modulation of CIITA expression, its direct suppressive effect on T cell proliferation and differentiation, and its ability to blunt the production of IL-7, TGFβ may be a pivotal upstream mediator of estrogens protective action in bone.

## Role of Interleukin-1 in Bone Loss

While the upregulation of IL-1 after ovariectomy in humans and mice has long been recognized [1], and suppression of IL-1 shown to be partially effective in preventing ovariectomy induced bone loss in mice and rats [2,3,36], the mechanism of IL-1 action is still not well understood. IL-1 has been reported to have the capacity to stimulate RANKL from osteoblastic cells [10] providing one potential mechanism to explain how IL-1 may promote bone loss. Recently, it has been reported that the capacity of TNFα to stimulate osteoblastic RANKL production is mediated by IL-1 and that IL-1 may directly stimulate the differentiation of osteoclast precursors [37]. This effect of IL-1 on osteoclast precursors is consistent with earlier studies showing that IL-1 synergizes with other cytokines including IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF) to promote the differentiation of human peripheral blood mobilized precursor cells into osteoclasts in vitro [38].

The proposed mechanism by which estrogen regulates immune functions involved in bone turnover is summarized in Figure 1.

## From Animal Models to Human Disease

Because of the inherent difficulties associated with human experimentation, the majority of studies presented in this paper represent investigations in animals. Consequently, their applicability to human disease, in particular postmenopausal osteoporosis, remains uncertain. However, a number of lines of evidence suggest that pro-osteoclastogenic immunologic perturbations are not uncommon in estroprevic humans and evidence is beginning to accumulate to suggest that T cells play a relevant role in regulating bone resorption in humans. It has recently been reported that RANKL-expression on lymphocytes and bone marrow stromal cells is significantly elevated during estrogen deficiency in humans and correlates directly with increases in bone resorption markers and inversely with serum estrogen levels [39]. The production of increased levels of TNFα and IL-1 in the conditioned media of peripheral blood

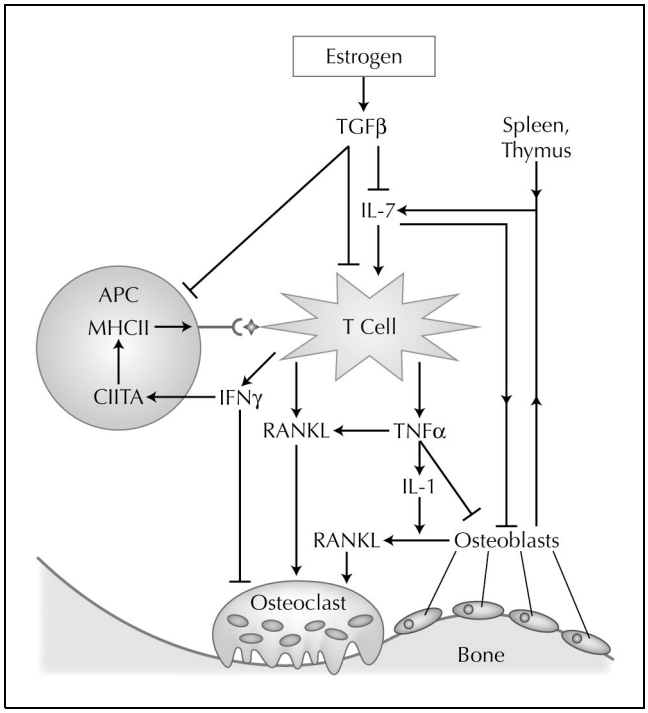


**Figure 1.** A model describing the role of the adaptive immune response in estrogen deficiency mediated bone loss. Estrogen is a potent inducer of the anti-inflammatory cytokine TGFβ which directly represses T cell activation, APC activity, and IL-7 production. IL-7 is a key cytokine in this cascade and acts to stimulate T cell expansion and activation leading to a predominantly Th1 T cell phenotype. Th1 cells are characterized by high levels of IFNγ, TNFα, and RANKL production. In addition IL-7 lowers the threshold of tolerance of T cells to prevailing antigens which together with an IFNγ driven upregulation in APC activity, further drives up T cell activation and antigen presentation. TNFα, through IL-1 further upregulates RANKL production by osteoblasts, while TNFα together with IL-7 concurrently represses the magnitude of the compensatory increase in bone formation, further exacerbating bone destruction. APC—antigen presenting cells; CIITA—class II transactivator; IFN—interferon; IL—interleukin; MHC—major histocompatibility complex; RANKL—receptor activator of nuclear factor kappa-B ligand; TGF—transforming growth factor; TNF—tumor necrosis factor.

cells derived from postmenopausal women is well established [1], while the number of CD3+ CD56+ T cells (a TNFα producing adherent T cell population) is reported to show a highly significant negative correlation with femoral and lumbar bone density in estroprevic postmenopausal women [40].

Furthermore, animal models of postmenopausal osteoporosis show striking similarities to autoimmune diseases. A significant body of evidence now suggests that estrogen is indeed a relevant player in autoimmune disease in humans. Firstly the majority of autoimmune diseases have a gender bias toward the female population. In particular Sjögren's syndrome, systemic lupus erythematosus, autoimmune thyroid disease (Hashimoto's thyroiditis as well as Graves' disease), and scleroderma, in which the patient population is greater than 80% women, and rheumatoid arthritis (RA), multiple sclerosis, and myasthenia gravis, in which the sex distribution is 60% to 75% women [41]. Secondly, puberty, menopause, and pregnancy all alter the incidence and the course of many autoimmune diseases further suggesting a role for sex hormones in autoimmunity. These modifications of disease activity by sex steroids have been suggested to involve the generation of a hormonal environment that favors a Th2 response. In multiple sclerosis and RA, this environment may suppress the ongoing Th1 responses to central nervous system and joint antigens whereas in systemic lupus erythematosus a Th2 environment would enhance antibody production and possibly exacerbate disease progression [41]. Recent evidence therefore implicates a role for sex hormones in modulating the incidence, course, and severity of autoimmune diseases.

## Bone Loss in Rheumatoid Arthritis

Some of the most compelling evidence to suggest that estrogen suppresses immunologic activation and bone loss driven by inflammatory cytokines in humans comes from human studies of RA. RA is a debilitating chronic inflammatory autoimmune disease [42] with complex etiology and uncertain cause. Among the major clinical manifestations of RA is local and systemic bone loss [43]. In many aspects this bone loss bears striking similarities to the bone loss mediated by estrogen deficiency. Indeed, several studies suggest that estrogen mitigates the clinical manifestations of RA suggesting a common mechanism. RA typically manifests at the time of menopause as estrogen levels decline [44]. RA typically remits during pregnancy, in parallel with increasing levels of corticosteroids, estrogens, and progesterone [45]. This is again consistent with sex steroid deficiency playing a role in the onset of disease. Oral contraceptives, which generate a condition of pseudo-pregnancy, also decrease the risk for RA [45]. In addition, RA is ameliorated by treatment with estrogen-containing oral contraceptives and by hormone therapy [46,47]. Most importantly, recent studies now suggest a common molecular basis for the systemic bone loss sustained in RA and the bone destruction mediated by estrogen deficiency. Studies in humans and animals have identified common cytokine cascades shared between these two disease states. The most important of these cytokines are RANKL, TNFα, IFNγ, and IL-7.

## The Role of Estrogen Regulated Cytokines IL-7, TNFα, and IFNγ in Rheumatoid Arthritis

Elevated levels of IL-7 have long been associated with RA and juvenile RA. Increased levels of circulating IL-7 have been identified in the plasma and synovial fluid of patients with juvenile RA [48] and increased IL-7 messenger RNA and protein secretion have been identified in human synoviocytes isolated from RA patients [49]. Increased levels of IL-7 not only increase antigen-driven proliferative responses

to high-affinity antigen but also, convert otherwise tolerogenic or non-immunogenic antigens into proliferative stimuli. In this regard, IL-7 has been found to be a critical modulator of low affinity peptide induced proliferation, a feature central to the homeostatic regulation of T cell populations [22]. This ability of IL-7 to increase the sensitivity of T cells to weak or non-immunogenic stimuli may be of great importance in the break down of self-tolerance, setting the stage for autoimmune diseases such as RA. IL-7 may therefore play an important role in the initiation of autoimmune disease, although this notion remains speculation.

Importantly, a recent clinical study has verified a significant five-fold increase in levels of IL-7 in vivo in the serum of 34 RA patients compared with 32 healthy controls. Importantly, as observed in the murine ovariectomy model [17], IL-7 was found to potently stimulate IFNγ, TNFα, and RANKL production by CD4+ T cells derived from RA patients [23•]. RANKL is the master regulator of osteoclast formation and IL-7 is known to stimulate T cells to secrete RANKL in vitro [14] and in vivo [8••]. In line with these data, studies conducted in vivo in a rat adjuvant arthritis model have demonstrated that RANKL production by activated T cells is central to the systemic bone loss associated with RA [50•].

Tumor necrosis factor-α is a factor that has now been definitively implicated in RA inflammation and whose neutralization in human patients by soluble decoy receptor (etanercept) or monoclonal antibody (infliximab) is now used clinically to control the progression and symptoms of RA and arrest bone destruction. Together the data suggests that IL-7 plays a critical role in sustaining the inflammatory levels of TNFα which are necessary for driving inflammation and bone loss in human patients with RA.

Taken together the available data now suggests that RA and postmenopausal osteoporosis are two sides of the same biologic coin. Although postmenopausal osteoporosis stems from estrogen deficiency and RA from autoimmunity, both diseases lead to bone destruction through an upregulation of T cells and appear to involve the same cascades of pro-inflammatory and pro-osteoclastogenic cytokines, including IL-7, TNFα, IFNγ, and RANKL.

## Conclusions

Current data now suggests that postmenopausal osteoporosis bears much of the hallmarks of a classic inflammatory autoimmune disease like RA. An interacting cascade of pro-inflammatory cytokines including IL-7, IFNγ, IL-1, RANKL, and TNFα appears to drive bone destruction by upregulating osteoclastic bone resorption and repressing compensatory responses in osteoblastic bone formation. The recognition of postmenopausal osteoporosis as a form of autoimmunity may lead to new perspectives on the treatment of this malady should translational studies presently underway in humans bear out the wealth of knowledge now assembled from animal studies. Future therapies for postmenopausal osteoporosis may need to also involve agents targeting the immune system to fully regulate disease progression in addition to agents that target the final mediators of bone turnover, osteoclasts, and osteoblasts.

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While T cells have long been associated with inflammation in RA this landmark study demonstrated that the bone destruction in an animal model of RA was also a consequence of T cell derived osteoclastogenic cytokines, in particular RANKL.