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T CELLS: CRITICAL BONE REGULATORS IN HEALTH AND DISEASE

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Abstract

Postmenopausal osteoporosis and hyperparathyroidism are to two common forms of bone loss caused primarily by an expansion of the osteoclastic pool only partially compensated by a stimulation of bone formation. The intimate mechanisms by which estrogen deficiency and excessive production of PTH cause bone loss remain to be determined in part because in vitro studies do not provide the means to adequately reproduce the effects of ovx and PTH overproduction observed in vivo. This article examines the connection between T cells and bone in health and disease and reviews the evidence in favor of the hypothesis that T cells play an unexpected critical role in the mechanism of action of estrogen and PTH in bone.

INTRODUCTION

The association between inflammation and bone loss has long been recognized, leading to the assumption the cells of the immune system may participate in the control of bone remodeling. The discovery that the RANKL/RANK system play a pivotal role in adaptative immunity and osteoclastogenesis has provided the molecular evidence that has firmly established the link between the immune system and bone. A seminal work by Kong at al (1) demonstrating that T cell produced RANKL plays a pivotal role in the bone loss observed adjuvant arthritis has later provided the first incontrovertible evidence about the capacity of T cells to cause bone loss. It is now clear that T cells regulate bone homeostasis via direct interactions with bone marrow (BM) stromal cells (SCs) and osteoblasts (OBs) and by releasing osteoclastogenic cytokines and Wnt ligands. This article focuses on the role of T cells in the mechanism by which two essential calciotrophic hormones, estrogen and PTH, regulate bone homeostasis.

DEVELOPMENT, HOMING, AND FUNCTIONS OF BONE MARROW T CELLS

T cells are critical mediators of the adaptive immune response. These lymphocytes may be subdivided into major classes according to the subunits which form the T cell receptor (TCR). T cells express either an αβ or γδ TCR on the cell surface, and these receptors are responsible for recognizing a diverse range of antigens (2). Most T lymphocytes are αβ T

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cells, a lineage which express either the CD4 or CD8 marker. By contrast, the majority of γδ T cells lack expression of CD4 and CD8 and their function remains largely unknown (3). Another small subset of T cells (< 1 % total T cells in the mouse) is known as natural killer T (NKT) cells, which can be defined based on the expression of both the NK marker NK1.1 and the αβTCR (4). Although small in number, NKT cells can produce large amounts of cytokines, and have been implicated in a variety of immune responses including autoimmunity, graft rejection, and responses to pathogens (4). CD4+ T cells can also be subdivided based on the types of cytokines produced and broadly grouped into the Th1 (IFNγ), Th2 (IL-4), and Th17 (IL-17) subsets. Of these, Th17 T cells have been suggested to be the osteoclastogenic T cells. Th17 are produced when naïve T cells are activated by TGFβ and IL-6 in the mouse, or TGFβ and inflammatory stimuli in humans. The resulting clonal memory T-cell population will be instructed to produce the Th17 signature cytokines IL-17A, IL-17F, IL-22, and IL-26 (5). IL-17A, a cytokine which induces RANKL expression in SCs and OBs, is the only Th17 signature cytokine currently known to influence the biology of osteoclasts. However, Th17 produce additional non-signature cytokines relevant for bone, including RANKL and TNF (6). A subset of Th17 cells also produce small amounts of IFNγ, which in vitro moderate the osteoclastogenic activity of Th17 (7). The issue of whether IFNγ producing Th17 are more or less osteoclastogenic than non- IFNγ producing Th17 in vivo remains to be determined. The cytokine repertoires of specific Th17 subsets depend on master differentiation factors present in the microenvironment during initial antigen recognition (5)].

Another type of CD4 positive T cell can be defined based on expression of CD25 and the transcription factor FoxP3. These cells are known as regulatory T cells (Tregs), and are critical in the prevention of autoimmune disease. There are a number of mechanism by which Tregs dampen the immune response, including some requiring cell to cell contact.

Like all blood cells, T cells derive from hematopoietic stem cells (HSCs) that reside in the BM. HSCs can self-renew or differentiate into multipotent progenitors (MPPs), which in turn will give rise to lymphoid-primed multipotent progenitors (LMPPs), which then produce common lymphoid progenitors (CLPs). These progenitors mobilize from the BM into the blood, and from there into the thymus. Upon thymic entry, thymic settling progenitors differentiate into the earliest defined T-cell precursors in the thymus, tthe early T progenitors, which ultimately produce naïve T cells that go on to populate the periphery. BM T cells arrive from the blood route and, after homing to the BM, can move back to the blood and then migrate to other lymphoid organs. T cells can exit from the BM only through the blood route, as there are no lymphatic vessels that drain this organ (8). T-cell colonization of the BM is a competitive process between the incoming T cells and the resident T cells, which already inhabit the same saturable niche (9).

The BM serves as a site for the initiation of naïve T cells responses and as a reservoir of CD8 T cells (8). T cells represent ~5–8 % of the total mononuclear BM cells. The percentage of activated T cells is much higher in the BM than in other secondary lymphoid organs and this feature is both cytokine and antigen (Ag) driven (8). As a result, the BM is the lymphoid organ with the highest percentage and number of proliferating T cells, apart from the thymus. A distinctive feature of BM T cells is the high proportion of memory T cells. The BM is a crucial organ in mature T-cell traffic and contributes greatly to the homeostatic regulation of peripheral CD8 T cell number. The BM can prime naive T cells and recruit effector T cells, but also that it serves as a site of preferential proliferation for CD4 and CD8 T cells (8).

CROSS TALK BETWEEN T CELLS AND STROMAL CELLS

Within the BM, cells of hematopoietic origin are embedded in a heterogeneous mixture of SCs of mesenchymal origin, which includes fibroblasts, reticular cells and adipocytes. It is well recognized that BM SCs support hematopoiesis by establishing appropriate anatomical niches and secreting specific cytokines and growth factors (10). BM SCs support also the maintenance of mature B lymphocytes, long-lived plasma cells, and possibly naive and memory T cells (11).

Importantly, SCs exert an important suppressive effect on T cells (12). The involved mechanisms have not been completely elucidated. However, SCs have been found to inhibit antigen specific T cell proliferation, and to induce the generation of Tregs (12). Additional mechanisms involve the generation of nitric oxide, which is a potent suppressor of T cell responsiveness (13). Attesting to the potency of SCs as immunosuppressants, SCs have been successfully used to for the treatment of severe graft versus host disease (14) and to blunt skin graft rejection (12). Relevant for bone biology is the capacity of SCs to block T cell activation upon exposure to IFNγ or TNF (13), a finding which suggest that SCs may contribute to moderate T cell dependent bone loss.

The cross talk between SCs and T cells is bidirectional because while SCs suppress T cell function, T cells regulate SC activity. For example T cells produce IL-17, an inflammatory and osteoclastogenic cytokine that regulate SC proliferation, survival and function (15). Another important T cell/SC interaction relevant for bone is the capacity of activated T cells induce SC apoptosis via the Fas/Fas ligand pathway, thus blunting the compensatory increase in bone formation that limits bone loss in ovx mice (16)

Another mediator of T cell-SC crosstalk is the T cell costimulatory molecule CD40L (17). This surface molecule, also known as CD154, exerts its effects by binding to CD40 (18) and several integrins (19,20). CD40 is expressed on antigen presenting cells, hemopoietic progenitors and cells of the osteoblastic lineage (21). The CD40/CD40L system is crucial for many additional functions of the immune system and myelopoiesis. Recently, CD40L has been linked to post natal skeletal maturation because T cells, through the CD40L/CD40 system, promote production of the anti-osteoclastogenic factor OPG by B-cells (22). Consequently, CD40L deficient mice attain a reduced peak bone volume due to stimulated bone resorption (22). Low bone density has also been found in children affected by X-linked hyper-IgM syndrome, a condition in which CD40L production is impaired due to a mutation of the CD40L gene (23). However, mice lacking T cell expressed CD40L are protected against parathyroid hormone (PTH) induced bone loss (24), raising the possibility that CD40L may exert anti resorptive activities in unstimulated conditions, while promoting bone resorption under conditions of bone stress. .

T CELLS IN THE MAINTENANCE OF BASELINE BONE HOMEOSTASIS

It is well established that Infection and inflammation lead to T cell activation, and T cell production of osteoclastogenic cytokines such as RANKL and TNFα (TNF). Indeed activated T cells have been implicated in the bone loss in inflammation, autoimmune disorders (1), periodontitis (25), and in animal models of postmenopausal osteoporosis (26). However, under basal conditions T cells are not considered a significant source of RANKL, and T cell deficient nude mice do not show evidence of diminished RANKL mRNA in their BM (22). Moreover, resting T cells have been shown to blunt OC formation in vitro (27) and may contribute to dampen bone resorption in vivo (22). Indeed depletion of $CD4^+$ and $CD8^+$ T lymphocytes in mice in vivo enhances OC formation by a mechanism involving the complete suppression of osteoprotegerin production (28). Providing further support to this hypothesis others have found that 1,25 dihydroxyvitamin D3 was a more potent inducer of

OC formation in cultures of BM from T cell depleted mice than from control mice (28). The bone protective role of resting T cells was however clearly demonstrated by the finding that T cell deficient mice have a significant increased in basal OC number and reduced bone density as compared to controls (29).

As discussed above the mechanisms by which T cells promote basal skeletal integrity appear to involve the regulation of OPG production by B cells by T cells through CD40L/CD40 interactions (22). In vivo, the dominant source of the ligand for CD40 (CD40L) is T cells. Consistent with this notion, animals deficient in CD40 and CD40L expression (CD40 and CD40L KO mice) recapitulate the same bone phenotype as T cell and B cell null mice, displaying diminished BMD and mass, elevated bone resorption and an elevated RANKL/OPG ratio, due to diminished B cell OPG production (22).

ROLE OF T CELLS IN THE MECHANISM OF ACTION OF PTH IN BONE

1. Effects of PTH on bone

PTH plays a critical regulatory role in calcium metabolism. Secreted in response to small decrements in serum ionized calcium, this hormone defends against hypocalcemia, in part by stimulating bone resorption and thereby the release of calcium from the skeleton. In addition to its role in regulating the level of serum calcium, sustained overproduction, or in vivo continuous infusion of PTH (cPTH), is a cause of bone disease. Secondary hyperparathyroidism has been implicated in the pathogenesis of senile osteoporosis (30), and primary hyperparathyroidism, is associated with accelerated bone loss and osteopenia (31). However, when injected daily in humans and animals at low dose, a regimen known as intermittent PTH (iPTH) treatment, the hormone stimulates trabecular and cortical bone formation, leading to marked increases in bone volume and strength. Attesting to potency, iPTH, has been shown to decrease the risk of fractures in humans, and is an FDA approved treatment modality for postmenopausal women and men with osteoporosis (32).

PTH exerts its anabolic activity by binding to the PPR receptor (also known as PTH-1R), a G protein coupled receptor expressed on OBs, osteocytes and SCs (33). Ligand binding to PPR activates the cyclic AMP–dependent protein kinase A, and calcium-dependent protein kinase C signaling pathways. Mice in which the PPR has been ablated by homologous recombination have decreased trabecular bone and increased thickness of cortical bone during fetal development (34). Conversely, osteoblastic expression of the constitutively active PPR increases osteoblastic function in the trabecular and endosteal compartments, and decreases OB activity in the periosteum (35). The net effect of these actions is a substantial increase in trabecular bone volume and a decrease in cortical bone thickness of the long bones (35). Transgenic mice expressing a constitutively active PTH receptor exclusively in osteocytes also exhibit increased bone mass and bone remodeling, as well as reduced expression of the osteocyte-derived Wnt antagonist sclerostin, increased Wnt signaling, increased OC and OB number, and decreased OB apoptosis (36). PTH receptor signaling in osteocytes has been shown to increase bone mass and the rate of bone remodeling through LRP5 dependent and independent mechanisms, respectively (36).

PTH promotes bone formation by increasing the number of OBs (37,38) through multiple effects, including activation of quiescent lining cells (39), increased OB proliferation (40) and differentiation (41), attenuation of pre-OB and OB apoptosis (42,43), and signaling in osteocytes (36). However, the specific contribution of each of these effects to the overall anabolic activity of PTH remains controversial.

PTH signaling has been observed to intersect Wnt pathways in OBs. Wnts are secreted signaling proteins which influence diverse developmental processes. Activation of Wnt

signaling induces OB proliferation (44) and differentiation (45), prevents pre-OB and OB apoptosis (46), and augments OB production of OPG (47). Wnt proteins initiate a canonical signaling cascade by binding to receptors of the Frizzled family together with coreceptors, members of the low-density lipoprotein receptor-related protein (LRP) family, LRP5 and LRP6, which results in the stabilization of cytosolic β-catenin. Recently a new member of this family LRP4, has been discovered on OBs and implicated in the mechanism of action of PTH (48). Lrp4 is expressed in bone and cultured osteoblasts and binds Dkk1 and sclerostin in vitro (48). A nuclear complex of beta-catenin and the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors then interacts with DNA to regulate the transcription of Wnt target genes (49). Wnt proteins also signal through non-canonical pathways which involve the Src/ERK and Pi3K/Akt cascades (46).

PTH is a canonical Wnt signaling agonist which increases β catenin levels in osteoblastic cells (50). PTH, once bound to PPR, is also capable of forming a complex with LRP6 which results in LRP6 signaling and β catenin activation (51). Thus, PTH activates Wnt signaling in osteoblastic cells through both Wnt ligands dependent and Wnt ligands independent mechanisms. Moreover, PTH down regulates the production of sclerostin, an osteocyte derived Wnt antagonist which blocks Wnt signaling by binding to LRP5 and LRP6 (52,53). Recently, convincing evidence has emerged that PTH receptor signaling in osteocytes and the resulting direct regulation of sclerostin production play a particularly relevant role in the anabolic activity of PTH (36). PTH also regulates Dickkopf-1, a soluble LRP5 and LRP6 signaling inhibitor (50), and Sfrp-4, a factor which binds Wnt proteins thus antagonizing both canonical and non-canonical Wnt signaling (54). Uncertainty remains with regard to the identity and the source of Wnt ligands which activate Wnt signaling in response to PTH treatment are not completely understood.

2. T cells and PTH induced bone loss

T lymphocytes, expresses functional PPR (55), responds to PTH (56), and stimulates OB differentiation (57). Yet a role for T cells in the mechanism of action of PTH has remained undetected for a long period of time in part because transplantation of tumors producing PTH and/or PTHrP in nude mice is known to cause hypercalcemia and increased bone resorption in spite of the absence of T cells in the host (58). Hory et al (59), were the first to report that transplantation of human parathyroid gland fragments from patients with primary and secondary hyperparathyroidism into nude mice fails to stimulate OC formation and bone resorption. The apparent discrepancy between these reports is explained by the higher levels of circulating PTH/PTHrP attained by transplanting PTH/PTHrP producing tumors, as compared to those obtained by transplanting hyperplastic parathyroid tissues.

These observations prompted more in depth investigations on the role of T cells as mediators of the pro-resorptive effect of cPTH treatment. We found that an infusion of cPTH that mimics hyperparathyroidism fails to induce OC formation, bone resorption and cortical bone loss in mice lacking T cells (24). By contrast, cPTH equally stimulated bone formation in T cell replete and T cell deficient mice. These studies further revealed the existence of a cross-talk between T cells and SCs mediated by the CD40L/CD40 signaling system (Figure 1). T cells provide proliferative and survival cues to SCs and sensitize SCs to PTH through CD40L, a surface molecule of activated T cells that induces CD40 signaling in SCs. An important element of this regulatory loop is the capacity of PTH to upregulate the expression of CD40 in SC from T cell replete mice but not from T cell deficient mice (24). Thus T cells contribute to the CD40L/CD40 mediated exchange of information between T cells and SCs in two ways. Firstly by providing CD40L. Secondly, by upregulating the expression of CD40 on SCs. As a result, mice lacking T cells or T cell expressed CD40L have lower number of SCs. Furthermore, these SCs produce lower amount of RANKL and have an even smaller suppression of OPG secretion in response to PTH. Therefore, SCs

from T cell deficient mice have a lower capacity to support OC formation in vivo and in vitro. The alteration in SC function is the ultimate reason why deletion of T cells or T cell expressed CD40L blunts the bone catabolic activity of PTH (24).

Preliminary studies have also shown that cPTH increases the T cell production of TNF. Attesting to the relevance of this cytokine, mice lacking the production of TNF by T cells are protected against the bone loss induced by cPTH treatment. If confirmed, these observation would suggest the existence of a second regulatory mechanism by which T cells potentiate the bone catabolic activity of cPTH.

In spite of a smaller SC pool and a blunted SC osteoclastogenic activity, T cell deficient mice possess a normal anabolic response to cPTH. This may suggest that the SCs which produce osteoclastogenic factors and support OC formation are not the same cells that differentiate into OBs, the cells that form osteoid and mineralize it, a hypothesis recently proposed by O'Brien et al (60). Indeed osteoclastogenic SCs express intercellular adhesion molecule-1 (ICAM-1) which is necessary for binding to OC precursors (61), while matrix producing osteoblastic cells are ICAM-1 negative (62). However, it is also possible that a defective stimulation of bone formation might emerge in T cell deficient mice in response to long-term cPTH treatment.

Antigen activated Th2 cells have been shown to produce PTH, which contributes to maintain the anabolic activity of OBs under inflammatory conditions (63). However mice lacking T cells are not protected against the catabolic activity of cPTH because of the absence of Th2 cell produced PTH. In fact, activated Th2 cells mitigate bone resorption by lowering the RANKL/OPG ratio largely through PTH independent mechanisms (63).

Cortical bone represents about 80 % of the entire skeletal mass and cortical volume and thickness are major predictors of bone strength and fracture risk (64). Thus, the T cell dependent bone effects of PTH are relevant for the risk of long bone fractures associated with primary and secondary hyperparathyroidism.

PTH does not increase antigen presentation and T cell activation. However, since the expression of CD40L is a feature of activated T cells, it is likely that baseline Ag presentation, which leads to spontaneous activation of T cells in the BM, may be required for PTH to induce its catabolic effect. Indeed the BM contains a relative large number of memory T cells which have increased reactivity to self peptides and foreign Antigens. T cell activation takes place in the presence of several signals. The first is the presentation to the T cell receptor (TCR) of Ag derived peptides bound to MHC molecules which are expressed on the surface of Ag presenting cells (APCs). A second set of signals is provided by the interaction of the costimulatory molecules on APCs with the T cell expressed counter receptors such as CD28 and CD40L. A pharmacological approach to test the role of costimulation is provided by Abatacept, an agent approved for the treatment of Rheumatoid Arthritis which also blocks the bone loss induced by ovariectomy (65). We found that PTH induced bone loss is prevented using this inhibitor of costimulation. In the same study we found that arrest of Ag presentation through silencing of class I and class II MHC-TCR interactions prevents the capacity of cPTH to induce cortical bone loss. These findings provide further evidence of a novel regulatory link between the immune system and the mechanism of action of PTH.

3. Role of T cells in the anabolic activity of intermittent PTH treatment

The hypothesis that T cells may play a role in the anabolic response to iPTH was first proposed by Pettway et al (66), who investigated the effects of daily injections of PTH for up to 7 weeks on the growth of ectopic "ossicles" implanted in nude mice. This investigation

disclosed that iPTH increased the bone content of the implanted WT ossicles, a structure which contains normal BM, but failed to induce vertebral bone growth in host nude mice, a strain devoid of T cells (66).

These observations were followed by our investigation on whether T cells contribute to the anabolic response to iPTH. Studies conducted in four strains of T cell deficient mice (TCRβ −/−, RAG2 −/−, class I and II MHC double KO mice, and nude mice) revealed that mice lacking T cells, exhibit a blunted increase in bone formation and trabecular bone volume in response to iPTH (55). Furthermore, adoptive transfer of T cells into T cell deficient mice restored a normal response to iPTH. T cells were found to augment the capacity of iPTH to improve architecture in trabecular but not in cortical bone. Although the reason of this selectivity is unknown, a lack of access of T cells to cortical surfaces is not a likely explanation, as T cells reach endosteal and periosteal bone surface through blood vessels and recirculate in and out of the BM (67).

The effects of iPTH on bone volume and the indices of bone strength measurable by μCT are blunted but not abolished in the absence of T cells. By contrast, direct measurements of bone strength by 4-point bending revealed that the capacity of iPTH to improve bone strength is completely dependent on the presence of T cells. Although the reason for this discrepancy is unknown, is possible that T cells might be required to improve the material property of bone. It should also be noted that while the stimulation of bone formation induced by iPTH was severely blunted in T cell deficient mice, T cells did not improve the capacity of cPTH to stimulate bone formation. The reason for this critical difference remains to be determined.

With regard of the mechanism by which T cells potentiate the bone anabolic activity of iPTH, studies have disclosed that in the absence of T cells iPTH is unable to increase the commitment of SCs to the osteoblastic lineage, induce OB proliferation and differentiation, and mitigate OB apoptosis. All of these actions of PTH were found to hinge on the capacity of T cells to activate Wnt signaling in osteoblastic cells (55). Although it is well established that Wnt activation is a key mechanism by which iPTH expands the osteoblastic pool, little information is available on the nature and the source of the Wnt ligand required to activate Wnt signaling in OBs. We have found that PTH stimulates BM CD8+ T cells to produce large amounts of Wnt10b (55), a Wnt protein which activates Wnt signaling in SCs and OBs, thus increasing OB proliferation, differentiation and life-span Treatment with iPTH also caused a small increase in the production of Wnt10b by BM CD4+ cells which was associated with a slightly diminished anabolic response in class II MHC −/− mice, suggesting that production of Wnt10b by CD4+ cells contributes, in small part, to the anabolic activity of iPTH. The relevance of CD8+ cells was demonstrated by the inability of iPTH to promote bone anabolism in class I MHC −/− mice, a strain that lacks CD8+ cells (55). Additional studies revealed that iPTH does not improve bone architecture in T cell deficient mice reconstituted with CD4+ cells, while it does so in mice adoptively transferred with CD8+ cells (55). The pivotal role of T cell produced Wnt10b was revealed by the hampered effect of iPTH on bone volume in TCRβ −/− mice reconstituted with T cells from Wnt10b −/− mice (Figure 2). It is likely that iPTH directly targets CD8+ T cells and stimulates their production of Wnt10b. This hypothesis is supported by the strong expression of PPR in CD8+ T cells, and the capacity of in vitro PTH treatment to promote cAMP production and Wnt10b expression in CD8+ murine and human lymphocytes.

Together the data indicate that CD8+ T cells potentiates the anabolic activity of PTH by providing Wnt10b, which is a critical Wnt ligand required for activating Wnt signaling in osteoblastic cells Therefore in the absence of CD8+ cells, stimulation of osteoblastic cells by PTH is not sufficient to elicit maximal Wnt activation due to the lack of a critical Wnt ligand

(Figure 3). The residual bone anabolic activity of PTH observed in T cell deficient mice is presumably due to ligand independent activation of LRP6 (51), and suppressed production of sclerostin (36,52,53).

While in vitro PTH treatment increased Wnt10b production by all T cells, iPTH upregulated Wnt10b production only by BM T cells. This diversity might be explained by the different dose and time of exposure to PTH. However, since adoptive transfer of spleen T cells into TCRβ −/− mice was followed by a restoration of a full responsiveness to iPTH, the data suggest that the capacity of T cells to upregulate their production of Wnt10b in response to iPTH is not an intrinsic feature of T cells, but rather is induced by environmental cues.

The anabolic activity of iPTH is not identical in all strains of T cell deficient mice. In fact, while some strains had no increase in bone volume in response to PTH, other exhibited a blunted but not a completely absent response. Osteoblastic cells produce several bone anabolic Wnt ligands including Wnt10b, Wnt7a and Wnt3b (68,69). These factors are likely to contribute to the T cell independent anabolic activity of iPTH, and quantitative differences in their production may account, in part, to the strain dependent variability in the response to iPTH observed herein. Furthermore, the magnitude of the anabolic response to iPTH in T cell null mice may be related to a strain and age dependent capacity of iPTH to inhibit the bone cells production of Wnt inhibitors such as sclerostin, (52,53), Dickkopf-1 (50) and Sfrp-4 (54). These factors have been shown to contribute to the anabolic activity of iPTH through T cell independent mechanisms. The enhancement of bone formation induced by iPTH is accompanied by a stimulation of bone resorption which is driven by increased production of RANKL and decreased release of OPG in the bone microenvironment. The direct effects of PTH on RANKL/OPG production are mitigated, in part, by the iPTH induced activation of β catenin in OBs, as this transcriptional regulator stimulates their production of OPG (47) and represses that of RANKL (70). The latter is one of the mechanisms which prevent bone resorption from offsetting the anabolic activity of iPTH.

Osteoblastic cells from WT mice treated with iPTH in vivo exhibited increased commitment to the osteoblastic lineage, proliferation, differentiation and life span in vitro, as compared to the corresponding cells from T cell deficient mice. Thus, T cells, like PTH, affect all aspects of OB life cycle. Remarkably, these differences were demonstrated in OBs purified from BM cultured for 7 days without the addition of PTH, suggesting that in vivo the hormone regulates early commitment steps of SCs and their osteoblastic progeny through T cell produced Wnt10b, and that these steps are not reversed by the absence of PTH and T cells in vitro. This model is consistent with the capacity of Wnt signaling to guide cell fate determination (71). A similar paradigm has been described in ovariectomized mice, a model where estrogen withdrawal in vivo leads to the formation of SCs which exhibit an increased osteoclastogenic activity which persists in vitro for 4 weeks (72).

T CELLS AND THE BONE LOSS INDUCED BY MENOPAUSE

1. Mechanisms by which menopause causes bone loss

The decline of ovarian function at menopause results in decreased production of estrogen and a parallel increase in FSH levels. The combined effects of estrogen deprivation and raising FSH production cause a marked stimulation of bone resorption (73) and a period of rapid bone loss which is central for the onset of postmenopausal osteoporosis. In mice the acute effects of estrogen deprivation and FSH elevation are modeled by ovariectomy (ovx) which, like natural menopause in humans, stimulates bone resorption by increasing osteoclast (OC) formation (26) and lifespan (74,75). The net bone loss caused by ovx is limited, in part, by an increase in bone formation resulting from stimulated osteoblastogenesis (76). This compensation is fueled by an expansion of the pool of BM

SCs, increased commitment of such pluripotent precursors toward the osteoblastic lineage (76), and enhanced proliferation of early OB precursors (77). The capacity of estrogen deficiency to stimulate OB apoptosis (78,79) and to extend OC lifespan (74,75) is the likely reason why after ovx bone formation does not increase as much as resorption. The stimulatory effect of ovx on SCs is equally relevant for osteoclastogenesis as one of the consequences of estrogen deprivation is the formation of osteoblastic cells with an increased osteoclastogenic activity (72), that is the capacity to support OC formation.

It is well established that estrogen withdrawal leads to two distinct forms of bone loss (80). The first is a rapid loss of trabecular and cortical bone due to stimulation of bone resorption which occurs in the first few weeks after ovx, driven by increased osteoclastogenesis and decreased OC apoptosis (26,81). This phase is followed by a slower but more prolonged loss of mainly cortical bone, which is due to incomplete refilling of the resorption cavities, which is in turn secondary to insufficient OB activity and lifespan (81). An expansion of the osteoclastic pool is therefore the key mechanism responsible for the bone loss which occurs early after ovx. One cytokine responsible for augmented osteoclastogenesis during estrogen deficiency is TNF, and its relevance has been demonstrated in multiple animal models. For example, ovariectomy (ovx) fails to induce bone loss in TNF knockout mice and in mice lacking the p55 TNF receptor (82). Likewise, transgenic mice insensitive to TNF due to the overexpression of a soluble TNF receptor (83), and mice treated with the TNF inhibitor TNF binding protein (84) are protected from ovx induced bone loss. The presence of increased levels of TNF in the BM of ovx animals and in the conditioned media of peripheral blood cells of postmenopausal women is well documented (85–87).

Attesting to the relevance of T cells in estrogen deficiency induced bone loss in vivo, are reports from our laboratory that ovx fails to induce trabecular and cortical bone loss in nude mice (82,88–90), WT mice deleted of T cells by injection of anti T cells antibodies (91), and WT mice treated with Abatacept (65) an agent which blocks T cell costimulation and induces T cell anergy and apoptosis (92,93). An independent confirmation of the role of T cells in ovx induced bone loss was provided by Yamaza et al who reported that ovx fails to induce bone loss in nude mice, and in WT mice in which T cell activation was blocked by aspirin (16). Studies have also shown that menopause increases T cell activations and T cell production of TNF and RANKL in humans (94). By contrast, Lee et al (95) showed that nude mice are protected against the loss of cortical, but not trabecular bone induced by ovx. In the same study other strains of T cell, and T and B cell deficient mice were found to lose either trabecular or cortical bone after ovx (95). The discrepancy between our reports (65,82,88–91) and that of Lee at al (95) is likely explained by differences in the experimental design, the lack of B cells in some models, and compensation mechanisms.

T cells are key inducers of bone-wasting because ovx increases T cell TNF production to a level sufficient to augment RANKL-induced osteoclastogenesis (88). The specific relevance of T cell TNF production in vivo was demonstrated by the finding that while reconstitution of nude recipient mice with T cells from wild type (WT) mice restores the capacity of ovx to induce bone loss, reconstitution with T cells from TNF deficient mice does not (82). T cell produced TNF may further augment bone loss by stimulating T cell RANKL production.

2. Mechanisms of estrogen regulation of T cell TNF production

Ovx upregulates T cell TNF production primarily by increasing the number of TNF producing T cells (82). This is the result of a complex pathway which involves the thymus and the BM. The mechanisms by which estrogen deficiency expands the pool of TNF producing T cells are summarized in Figure 4. In the BM, ovx promotes T cell activation, resulting in increased T cell proliferation and life span through antigen presentation by macrophages and dendritic cells (DCs) (96,97). This process is due to the ability of estrogen

deficiency to upregulate their expression of MHCII (96–98). The question thus arises as to the nature of the antigens. Estrogen deficiency is likely to increase T cell reactivity to a pool of self and foreign antigens physiologically present in healthy animals and humans. This is consistent with the fact that T cell clones expressing TCRs directed against self antigens not expressed in the thymus, survive negative selection during T cell maturation (99). Such clones ("autoreactive" or "self-reactive" T cells) reside in peripheral lymphatic organs of adult individuals. In addition, foreign antigens of bacterial origin are physiologically absorbed in the gut. As these peptides come into contact with immune cells locally and systemically, they induce a low grade T cell activation. Thus, a moderate immune response is constantly in place in healthy humans and rodents due to presentation by MHCII and MHCI molecules of both self and foreign peptides to $CD4^+$ and $CD8^+$ T cells (100). This autoreactive response is thought to be essential for immune cell survival and renewal (101).

The effects of ovx on antigen presentation and the resulting changes in T cell activation, proliferation and lifespan are explained by a stimulatory effect of ovx on the expression of the gene encoding Class II Transactivator (*CIITA*). The product of *CIITA* is a non-DNA binding factor induced by IFNγ that functions as a transcriptional coactivator at the MHC II promoter (102). Increased *CIITA* expression in macrophages results from ovx-mediated increases in both T cell IFNγ production and the responsiveness of *CIITA* to IFNγ (96). This factor was initially described as an anti-osteoclastogenic cytokine because is a potent inhibitor of osteoclastogenesis in vitro (103). The notion that IFNγ is an inhibitor of bone resorption was reinforced by the finding that silencing of IFNγR−/− signaling leads to a more rapid onset of collagen induced arthritis and bone resorption (104) as compared to WT controls, and by the report that IFNγ decreases serum calcium and osteoclastic bone resorption in nude mice (105). However, observations in humans and in experimental models of disease indicate that IFNγ promotes bone resorption and causes bone loss in a variety of conditions. Studies with IFN −/− and IFNR −/− mice have revealed that among these conditions are estrogen deficiency and endotoxin-induced bone disease (90,96). Mice lacking IFNγ production are also protected against infection induced alveolar bone loss (106), while in erosive tubercoloid leprosy and psoriatic arthritis IFNγ production correlates positively with tissue destruction (107). In addition, randomized controlled trials have shown that IFNγ does not prevent bone loss in patients with RA (108), nor the bone wasting effect of cyclosporin A (109). Furthermore, IFNγ has been reported to be efficacious in the treatment of osteopetrosis through restoration of bone resorption, both in humans (110) and rodents (111). These latter findings conclusively demonstrate that in some conditions, including estrogen deficiency, the net effect of IFNγ in vivo is that of stimulating osteoclastic bone resorption.

The complex effects of IFNγ can be explained by the fact that IFNγ influences OC formation both via direct and indirect effects (90). IFNγ directly blocks OC formation through targeting of maturing OC (112). This effect is best observed in vitro (103). However, IFNγ is also a potent inducer of antigen presentation and thus of T cell activation. Therefore, when IFNγ levels are increased in vivo, activated T cells secrete pro-osteoclastogenic factors and this activity offsets the anti-osteoclastogenic effect of IFNγ.

One mechanism by which estrogen deficiency upregulates the production of IFNγ is through TGFβ. Estrogen has a direct stimulatory effect on the production of this factor which is mediated through direct binding of estrogen/ER complex to an ERE region in the TGFβ promoter (113). TGFβ is recognized a powerful repressor of T cell activation. Indeed, TGFβ exerts strong immunosuppressive effects by inhibiting the activation and the proliferation of T cells and their production of proinflammatory cytokines, including IFNγ. Studies in a transgenic mouse which expresses a dominant negative form of the TGFβ receptor exclusively in T cells have allowed the significance of the repressive effects of this cytokine

on T cell function in the bone loss associated with estrogen deficiency to be established (89). This strain, known as CD4dnTGFβRII, is severely osteopenic due to increased bone resorption. More importantly, mice with T cell-specific blockade of TGFβ signaling are completely resistant to the bone sparing effects of estrogen (89). This phenotype results from a failure of estrogen to repress IFNγ production which, in turn, leads to increased T cell activation and T cell TNF production. Gain of function experiments confirmed that elevation of the systemic levels of TGFβ prevents ovx-induced bone loss and bone turnover (89).

Another mechanism by which estrogen regulates IFNγ and TNF production is by repressing the production of IL-7, a potent lymphopoietic cytokine and inducer of bone destruction in vivo (114). Importantly levels of IL-7 are significantly elevated following ovx (115–117) and in vivo IL- 7 blockade, using neutralizing antibodies, is effective in preventing ovx induced bone destruction (115) by suppressing T cell expansion and TNF and IFNγ production (116). The relevance of IL-7 in the mechanism of ovx induced bone loss has been confirmed, in part, by another investigation showing that ovx does not induce cortical bone loss in IL-7 KO mice (118). Indeed, the elevated BM levels of IL-7 contribute to the expansion of the T cell population in peripheral lymphoid organs through several mechanisms. Firstly, IL-7 directly stimulates T cell proliferation by lowering tolerance to weak self antigens. Secondly, IL-7 increases antigen presentation by upregulating the production of IFNγ. Thirdly, IL-7 and TGFβ inversely regulate each other's production (119,120). The reduction in TGFβ signaling, characteristic of estrogen deficiency may serve to further stimulate IL-7 production, thus driving the cycle of osteoclastogenic cytokine production and bone wasting. In estrogen deficiency IL-7 compounds bone loss by suppressing bone formation thus uncoupling bone formation from resorption.

3. Estrogen, oxidative stress and T cell dependent bone loss

Reactive oxygen species (ROS) play an important role in postmenopausal bone loss by generating a more oxidized bone microenvironment (121,122). In vivo support of this hypothesis is found from experiments in which ovx induces oxidative stress and impairs the expression of antioxidants (65,81,123,124). Furthermore, administration of antioxidants prevent ovx induced bone loss (65,123,125), while depletion of glutathione by buthionine sulfoximine (BSO), which inhibits glutathione synthesis, enhances bone loss (123). Bone loss caused by BSO has significant similarities to bone loss induced by estrogen deficiency, as both processes are TNF-dependent (126). Moreover, soluble TNF receptors prevent both bone loss and the rise in thiol-based antioxidants characteristic of estrogen deficiency (126). The negative impact of oxidative stress is offset, in part, by oxidative defense mechanisms. Among them is the retention of FoxO transcription factors into the nucleus and the induction of FoxO mediate transcription of antioxidant enzymes, and genes involved in cell cycle, DNA repair and apoptosis (81). The bone wasting effects of ROS has indeed been linked to its capacity to activate the association of FoxO to β-catenin and promote FoxO mediate transcription at the expenses of β-catenin/T cell specific transcription factor mediated transcription (81,127). This matter has been addressed and reviewed extensively elsewhere (81,125,127–130) and will not be discussed herein.

An alternative explanation to link oxidative to ovx induced bone loss is through immune mediated mechanisms. ROS are indeed important stimulators of antigen presentation by DCs, and DC induced T cell activation. Conversely, anti-oxidants potently inhibit DC differentiation and their ability to activate T cells (131,132), in part by suppressing expression of MHC class II and costimulatory molecules in response to antigen (133). N-Acetyl-Cystein (NAC), which acts as an intracellular scavenger by restoring intracellular concentration of glutathione, can block DC maturation (134) and DC-mediated T cell activation (135). ROS are also generated upon DC interaction with T cells (136) and can

reduce T cell lifespan by stimulating T cell apoptosis (137). Interestingly, NAC treatment has been shown to protect against ovx-induced bone loss (65,123,128). These data are consistent with studies demonstrating that NAC treatment blunts ovx-induced DC activation in the BM, decreases antigen presentation and expression of costimulatory molecules, and prevents T cell activation and TNF production (65).

We have found that ovx increases the production of ROS in the BM, which lead to expansion of mature DCs which express the costimulatory molecule CD80, and increased DC mediated Ag presentation (65). This effect is tissue specific because ovx does not increase oxidative stress outside the BM. Attesting to the relevance of oxidative stress we have found that treatment with antioxidants blocks the stimulatory effects of ovx on DC and T cell activation and, and prevents ovx induced bone loss (65). Multiple enzymatic pathways regulate the intracellular redox state through modulation of ROS levels (138). Ovx blunts the BM levels of GSH, a critical ROS scavenger, and those of APE1/Ref-1 and Prx-1, proteins which contributes to limit the production of intracellular ROS (139). Like the activation of DCs, the effects of ovx on the redox state are spatially limited to the BM a phenomenon previously noted by Lean et al (123).

The finding that ovx increases the expression of the costimulatory molecule CD80 on DCs, suggest that the process of costimulation plays an important role in ovx induced bone loss. This was confirmed by the finding that ovx induced bone loss is completely blocked by CTLA4-Ig (Abatacept) (65) an agent which blocks T cell costimulation and induces T cell anergy and apoptosis by binding to CD80 (and CD86) and blocking their interaction with CD28, (92,93). Abatacept also blocked the increase in T cell activation, proliferation and TNF production resulting from E withdrawal. Taken together, these data suggest a model for ovx-induced bone loss in which estrogen deficiency lowers antioxidant levels, thereby increasing ROS, which in turn induces TNF expression by OCs directly, as well as by stimulating the APC-induced expansion of TNF-producing T cells central to bone destruction.

CONCLUSIONS

Remarkable progress has been made in understanding how T cells participate in the regulation of bone remodeling in health and disease. In parallel with exciting discoveries about molecular and signaling pathways by which T cells and their products control OC and OB development and function, there has a been the recognition of specialized function of distinct T cell populations such as Tregs and Th17. Progress has been made in recognizing that T cells play an unexpected role in the function of major calciotrophic and reproductive hormones such as PTH and estrogen, and therefore in common and clinically relevant forms of bone loss such postmenopausal osteoporosis and hyperparathyroidism. Much remains to be done especially in translating observations accrued in experimental animals into studies in humans. Strategies to utilize T cells/bone cells interactions as the bases of new therapies also remain to be developed.

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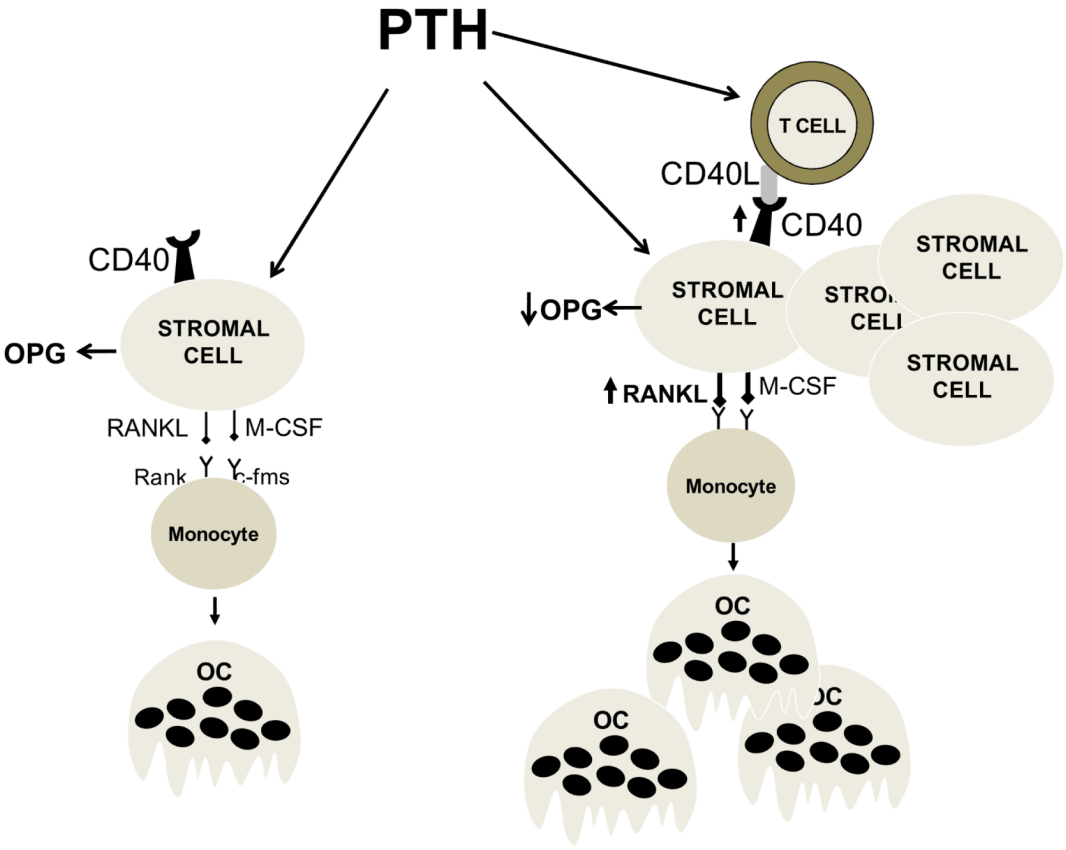

Figure 1.

Schematic representation of the role of T cells in the mechanism by which continuous PTH stimulates OC formation. In the absence of T cells (left panel) BM SCs are fewer in number (not shown), and exhibit a blunted capacity to support PTH induced OC formation due to diminished production of RANKL and continuous production of the RANKL decoy receptor OPG. In the presence of T cell expressed CD40L (right panel) the pool of SCs in the BM is 2-fold larger. Furthermore, CD40L/CD40 signaling increases the osteoclastogenic activity of SCs by augmenting the SC production of RANKL and blunting their secretion of OPG. The result is a potentiation of the capacity of PTH to stimulate the formation of OCs.

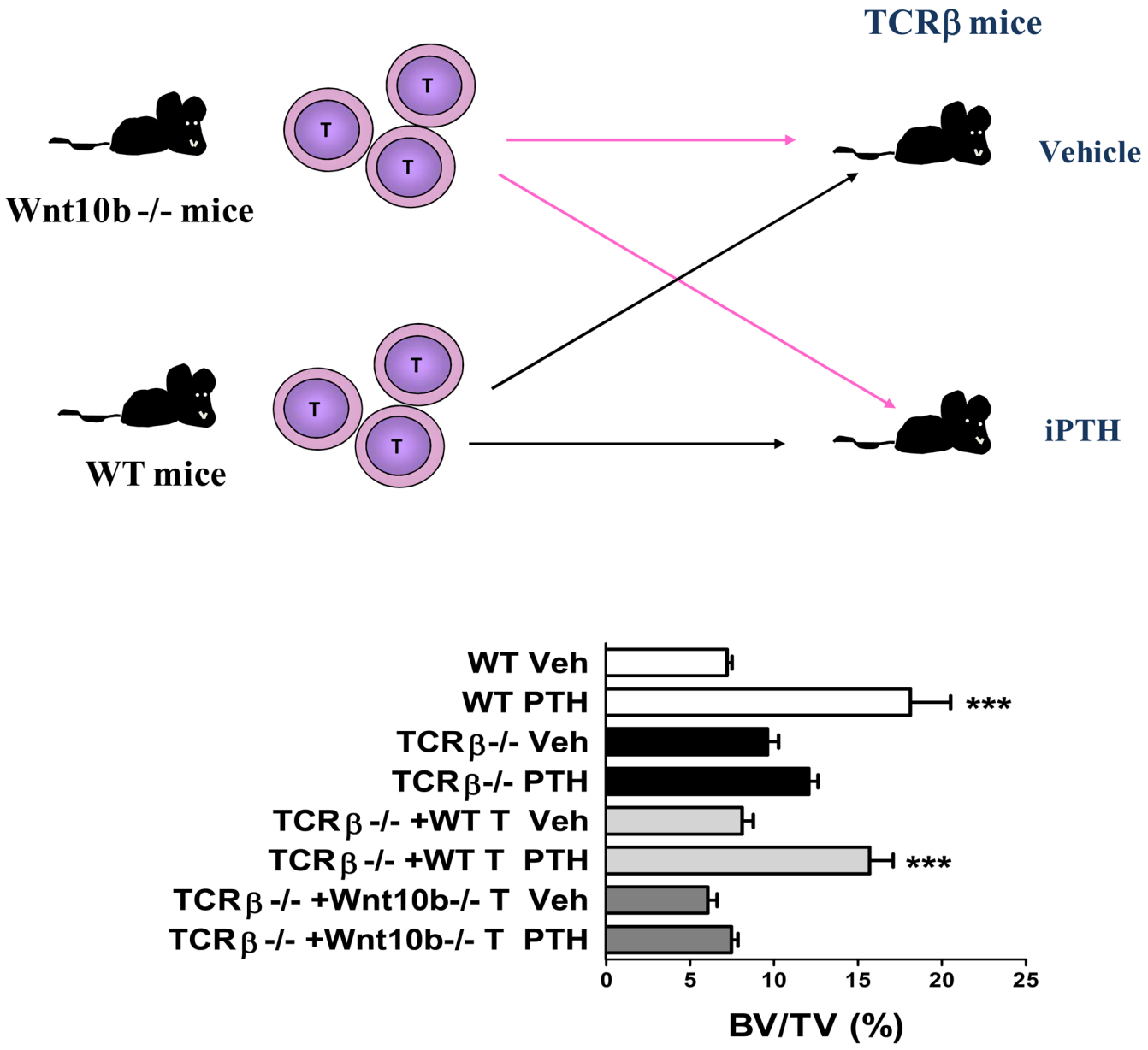

Figure 2.

Analysis of the effects of iPTH treatment in WT mice, TCRβ −/− mice, and TCRβ −/− mice subjected to adoptive transfer of T cells derived from WT mice and Wnt10b −/− mice 1 week before initiation of iPTH. The upper panel shows a schematic representation of the experimental design. The bottom panel shows trabecular bone volume (BV/TV) as measured by μCT. *** = $p<0.001$ compared to the corresponding vehicle treated group.

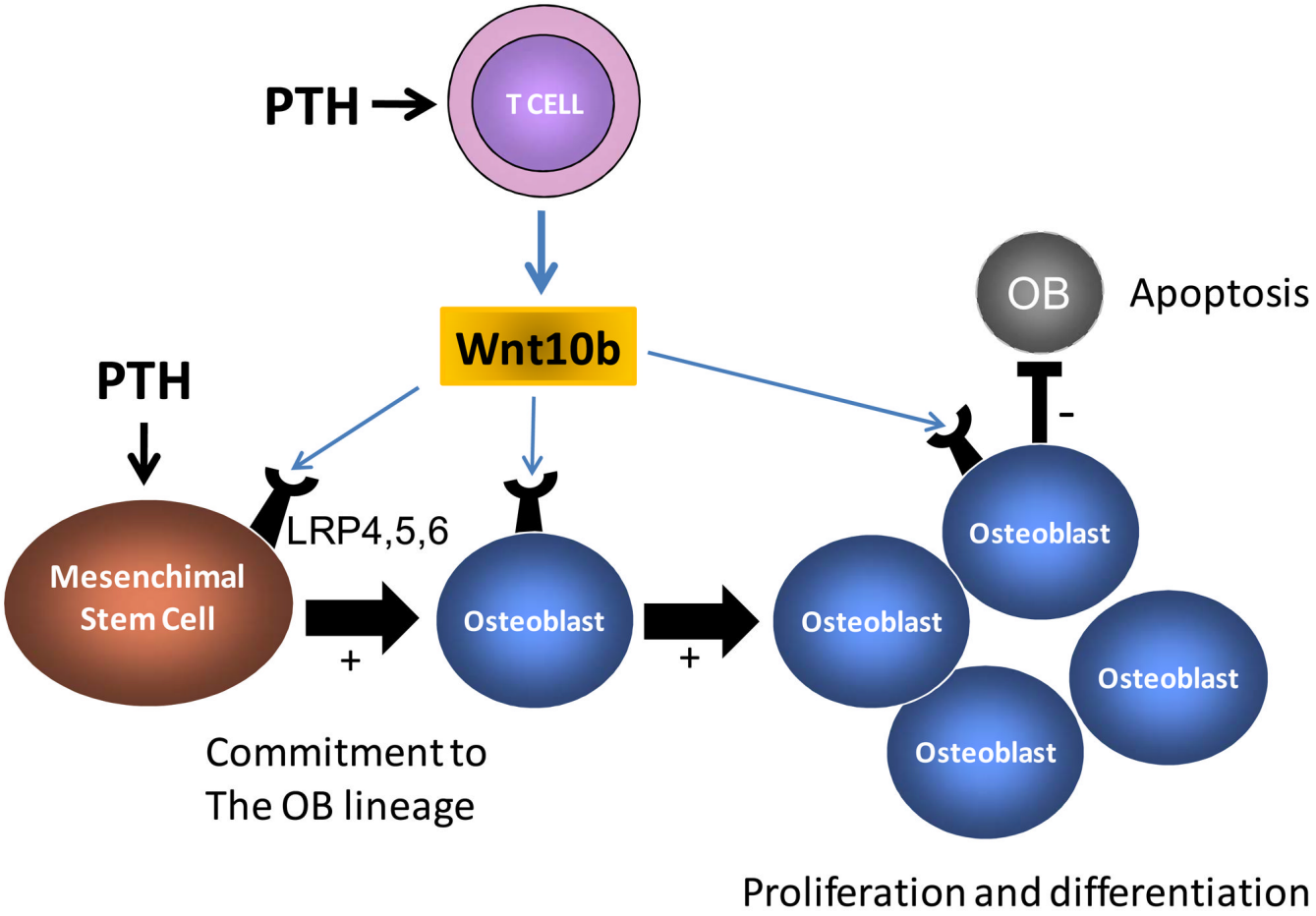

Figure 3.

Schematic representation of the role of T cells in the mechanism by which intermittent PTH treatment stimulates bone formation. PTH stimulates T cells to secrete Wnt10b, a Wnt ligand required to activate Wnt signaling in SCs and OBs. In the presence of T cell produced Wnt10b, stimulation of osteoblastic cells by PTH result in the activation of the Wnt signaling pathway. This event leads to increased commitment of mesenchimal stem cells to the osteoblastic lineage, increased osteoblast proliferation and differentiation, and decreased osteoblast apoptosis.

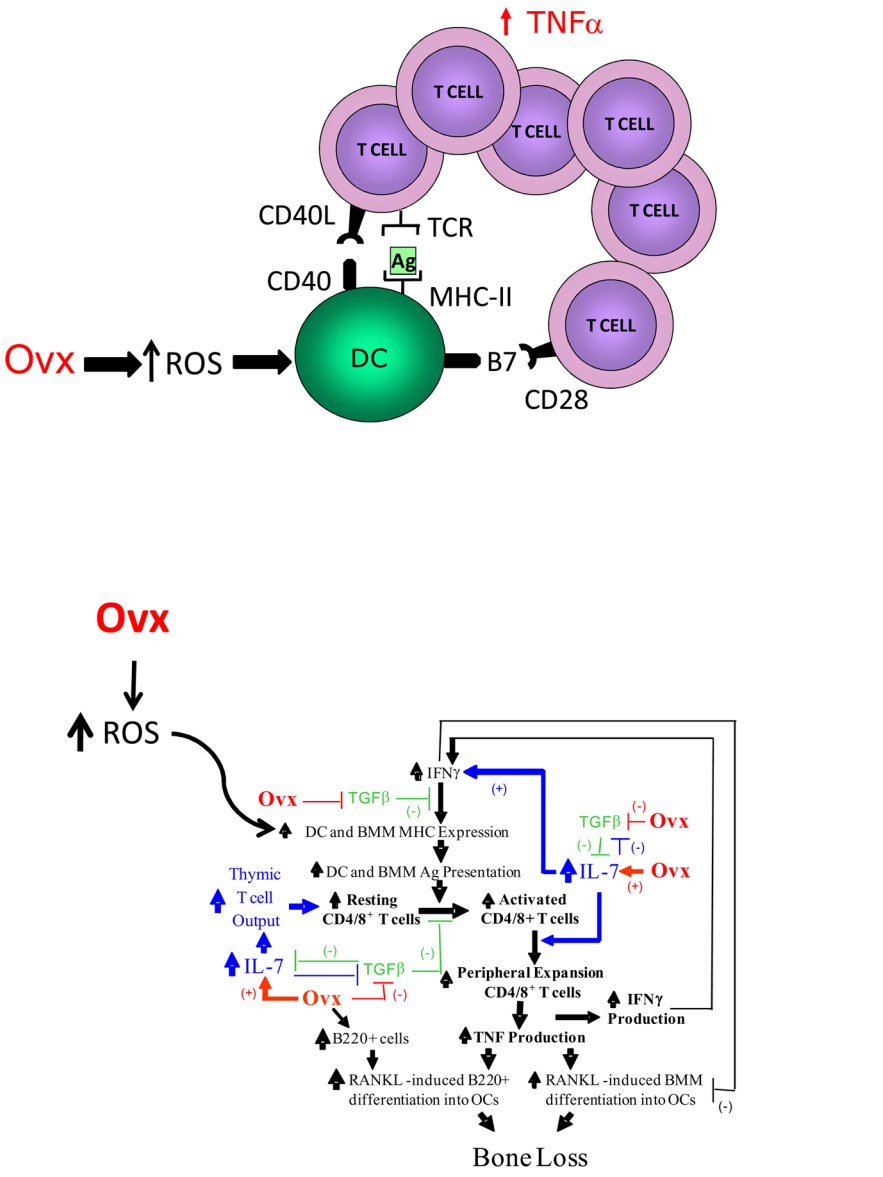

Figure 4.

Schematic representation of the mechanism by which ovx increases antigen presentation through ROS. The upper diagram shows that ovx increases the activity of DCs through ROS generation, leading to increased T cell activation, T cell proliferation and secretion of TNF through a mechanism involving the T cell receptor and costimulatory molecules. The bottom diagram shows in detail the cells and the cytokines by which ovx leads to T cell production of TNF and bone loss.