NEWS

Osteoimmunology: T Cells, RANKL, and Beyond

A recent IBMS BoneKEy webinar focused on the contribution of T cells to estrogen deficiency-induced bone loss

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In a seminal paper published in Nature in 1999, a team of researchers including Josef Penninger and colleagues offered evidence showing that activated T cells, by making the osteoclastogenic cytokine receptor activator of NF-κB ligand (RANKL), could induce the production of osteoclasts, and cause bone loss, in mice genetically engineered to have spontaneous activation of these immune system cells. "To our knowledge," the investigators of the study concluded, "our results provide the first definitive linkage between activated T cells and bone homeostasis and [show] that systemic or local activation of T cells triggers bone loss through expression of [RANKL]." Furthermore, they found that the bone loss associated with a T cell-dependent model of rheumatoid arthritis in rats could be prevented by blocking RANKL.

During Osteoimmunology: T Cells, RANKL, and Beyond, a webinar hosted by IBMS BoneKEy in early December of last year, webinar presenter and osteoimmunology expert Roberto Pacifici made the case that a similar state of affairs, where T cells drive bone loss through effects on RANKL and in a variety of other ways, also exists for another pathologic condition: estrogen deficiency-induced bone loss. Dr. Pacifici, a professor of medicine, and director of the Division of Endocrinology, Metabolism and Lipids at Emory University in Atlanta, Georgia marshaled evidence from his own preclinical studies as well as animal and clinical data from other groups to support this contention, with a particular focus on the cellular and molecular mechanisms mediating T cell effects on bone. A distinguished group of panelists discussed the strengths and limitations of this argument, including how it affects our understanding of both postmenopausal osteoporosis (PMO) and of the potential impact of RANKL inhibition on the immune system. While these experts agreed that the evidence is compelling for a role of T cells in estrogen deficiency-induced bone loss, they cautioned that several important issues need further clarification before the bone field can confidently accept a key implication of this osteoimmunological perspective: that PMO is rightly viewed as an inflammatory disorder of bone.

Evidence of a Role for T Cells in the Bone Loss Characteristic of Estrogen Deficiency

In the first part of his talk, Dr. Pacifici noted that the Nature findings from the rheumatoid arthritis model led him to ask whether T cells might also play a role in estrogen deficiency-induced bone loss. To test this theory, he conducted initial studies, published in the Journal of Clinical Investigation in 2000, comparing the bone density of wild-type mice to nude mice, the latter a strain of mice lacking T cells. Results showed that while ovariectomy (OVX), as expected, resulted in decreased bone mineral density (BMD) as assessed by peripheral quantitative computed tomography (pQCT) in wild-type mice to nude mice, the latter a strain of mice lacking T cells. Results showed that while ovariectomy (OVX), as expected, resulted in decreased bone mineral density (BMD) as assessed by peripheral quantitative computed tomography (pQCT) in wild-type mice, nude mice were protected from OVX-induced bone loss, a finding confirmed in 2008 by another research group. Experiments measuring BMD by μCT confirmed these findings, and studies in nude mice whose T cells were restored by adoptive transfer showed that these animals now exhibited the bone loss expected with

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O VX. “These studies were the first to directly link estrogen deficiency and the bone loss induced by this condition to T cells,” Dr. Pacifici said.

To overcome the limitation that compensatory mechanisms may come into play in the genetic models of T cell deficiency employed in these early studies, Dr. Pacifici also described research that made use of a non-genetic model of T cell deficiency, consisting of animals treated with anti-CD4 and anti-CD8 antibodies, in order to replicate the initial findings. Here, too, results implicated T cells in OVX-induced bone loss: while mice treated with an irrelevant antibody exhibited the expected bone loss in response to OVX, mice who received the anti-CD4 and anti-CD8 antibodies were spared from this outcome. Additional studies have revealed that in response to OVX, T cells are activated and proliferate in the bone marrow, where they produce tumor necrosis factor (TNF)-α, a cytokine that acts in a synergistic fashion with RANKL to stimulate osteoclastogenesis.

Continuing to build the case for a link between T cells and OVX-induced bone loss, Dr. Pacifici also described imaging experiments comparing numbers of osteoclasts and T cells, as well as the location of each cell type in relation to the other, in sham-operated mice and OVX mice as seen in longitudinal sections of bone. While the former exhibit a small number of osteoclasts, as well as a small number of T cells that were distributed in a homogenous fashion throughout the bone marrow, the latter show not only increased numbers of T cells and osteoclasts, but also a striking pattern of distribution. “The interesting phenomenon is that the T cells tend to localize preferentially to the endosteal surface, and in particular near the osteoclasts,” Dr. Pacifici emphasized. The unique spatial distribution of these two cell types begs the intriguing question of how it arises in the first place, according to Serge Ferrari, IBMS BoneKEy editor-in-chief and moderator of the webinar. “The co-localization of T cells and osteoclasts in the bone environment raises the issue of whether osteoclasts and bone-resorbing activity release factors or products that would act as T cell chemoattractants,” Dr. Ferrari said. Preliminary data suggest that T cells accumulate in the bone marrow before osteoclasts are formed there. “These data are not conclusive, but the fact that T cells arrive there first suggests that their role is in preparing the groundwork for the formation of osteoclasts rather than being recruited in response to the formation of osteoclasts,” Dr. Pacifici explained.

Moving from rodents to humans, Dr. Pacifici described several lines of clinical evidence supporting a role for T cells, and the cytokines they produce, in estrogen deficiency-induced bone loss. For instance, studies performed by panelist Sundeep Khosla and colleagues have shown that blocking TNF-α with the biologic disease-modifying anti-rheumatic drug (DMARD) etanercept, or blocking interleukin-1 (IL-1) with anakinra, another biologic DMARD that is an antagonist of the IL-1 receptor, blunts the increase in markers of bone resorption seen in early postmenopausal women after estrogen treatment is discontinued, compared to control subjects receiving a saline injection. “This was one of the first direct demonstrations that these cytokines play a pivotal role in increasing bone resorption in women as a result of estrogen deficiency,” Dr. Pacifici stated. Additional clinical evidence showing that T cell TNF-α production increases at the time of natural menopause, and is associated with BMD in women with osteoporosis; that estrogen deficiency, through effects on T cells, increases osteoporosis; and, finally, that there are increased levels of RANKL produced not only by bone marrow stromal cells, but also by T and B cells, in postmenopausal women compared to premenopausal women and postmenopausal women on estrogen, all bolster the argument for a crucial role for T cells in the bone loss of estrogen deficiency.

If T cells are so crucial for mediating this process, then it appears inconsistent that other diseases where T cells are adversely affected would feature bone loss instead of bone gain, a question to which Dr. Ferrari
drew the webinar audience's attention. "Are there not some contradictory results in the clinical setting when we consider bone loss in situations of immune deficiency such as in HIV, or in the post-transplantation situation, where there is accelerated bone loss?" Dr. Ferrari asked the panelists. In the case of HIV/AIDS, Dr. Pacifici noted that he isn't surprised that patients with this disease exhibit bone loss even in the absence of T cells. "HIV/AIDS patients have extremely elevated levels of TNF-α induced through the incredibly complex inflammatory state that is characteristic of this condition, and I suspect that the bone loss is TNF-α-driven," he said, adding that it would be of great interest to assess the effects of TNF-α blockers in such patients.

**Do T Cells Share the Stage with Other Cell Types?**

If the role of T cells in the bone loss resulting from decreased levels of estrogen is a large one, what are the implications for the role of bone cell types in this condition – in particular, does estrogen have direct effects on bone-resorbing osteoclasts, and on bone-forming osteoblasts? Dr. Khosla, a professor of medicine and physiology at Mayo Clinic College of Medicine in Rochester, Minnesota noted that his research on the bone effects of blockers of T cell-produced cytokines makes this a tenable hypothesis. "When we gave the TNF-α or IL-1 blockers over 3 weeks, we found that, compared to untreated women, either blocker could reduce the increase in bone resorption by about 50%," Dr. Khosla said. While he noted that, because of toxicity concerns that made it impractical to administer both agents at the same time, it is uncertain whether both cytokine blockers given together would have completely eliminated the increase in bone resorption seen with estrogen deficiency, still, the absence of a complete elimination of that increase when either drug was given alone leaves the possibility open that direct effects of estrogen on osteoclasts and osteoblasts may be important.

In fact, regarding osteoclasts, Dr. Khosla pointed to mouse data in support of this idea, noting also that the findings observed in mice likely have relevance in humans. As far as osteoblasts are concerned, Dr. Khosla noted that, at least in humans, estrogen deficiency results initially in decreases in bone formation markers measured 3-4 weeks after estrogen withdrawal, a time period when bone resorption increases, but these markers subsequently increase as the length of time of estrogen deficiency increases, presumably because a coupling mechanism between osteoblasts and osteoclasts comes into play then. Though Dr. Khosla noted that he has not been able to replicate this finding in mice, Dr. Pacifici added that he has in fact been able to document a decrease in bone formation measured by histomorphometric methods in mice sacrificed 2 weeks after OVX. Considering all of these lines of evidence together, Dr Khosla concluded that T cells are likely not the sole players. "I suspect there are direct effects on osteoclasts, and at least in humans, I do believe there are important direct effects on osteoblasts that can contribute to the gap between resorption and formation that we see with estrogen deficiency."

Illustrative of the complex causes of bone loss witnessed with estrogen deficiency, the panel discussion focused on yet another cell type – this time not a bone cell but rather another cellular component of the immune system, namely the B cell. "This is an issue that people have been looking at for a long time, but, unfortunately, we still don't have a clear idea as to the role of B cells in estrogen deficiency," according to panelist Neale Weitzmann, an assistant professor of medicine, as well as a frequent collaborator of Dr. Pacifici's, at Emory. Dr. Weitzmann noted that, historically, focus in this area has been on a type of B cell precursor, known as a B220 cell, present in increased numbers during estrogen deficiency. However, while Dr. Weitzmann and other groups have confirmed the existence of such cells, their precise role remains unclear. "The B220s don't seem to be sufficient in and of themselves to induce bone loss in the OVX model, and we really don't know what role they are playing, if any, in OVX-induced bone loss," Dr. Weitzmann said. Noting the
research regarding the production of RANKL by B cells in postmenopausal women, Dr. Weitzmann said that mature B cells don’t seem to play a role in OVX-induced bone loss and described experiments he has performed in B cell knockout mice pointing to that conclusion. “We found that there doesn't seem to be any net effect of B cells on OVX-induced bone loss, as the magnitude of the bone loss was identical in wild-type and B cell-null animals after correcting for baseline differences,” he said.

There is evidence for the involvement of other antigen-presenting cells (APCs), such as dendritic cells, in the T cell response to estrogen deficiency. The importance of cells that make antigen available to T cells raises an obvious question. “The core of [Dr. Pacifici's] hypothesis is that T cell activation in the context of osteoporosis is mediated by antigen presentation, and so the question in hand is, what is the antigen that is mediating this event?” asked panelist Steven Teitelbaum. Dr. Teitelbaum, a professor of pathology and immunology at Washington University School of Medicine in St. Louis, Missouri noted that more clarity on the nature of the antigens (along with more data from human studies) would help pin down the case for viewing PMO as an inflammatory condition where T cells, and the cytokines they produce, play a central role.

Regarding the identify of the antigen required to induce T cell activation, Dr. Weitzmann pointed to research he has performed in this area, noting that initial work made use of a mouse strain in which antigen presentation is eliminated unless an exogenous antigen is administered. These early studies showed that such mice, in the absence of an exogenous antigen, were protected from the bone loss of OVX, but when they were given an exogenous antigen, then OVX did indeed induce bone loss. “From these data we concluded that there is definitely a requirement for antigen presentation and an antigen in OVX-induced bone loss. This system also suggested to us that no specific kind of antigen is needed,” Dr. Weitzmann said. To explain what might be occurring during estrogen deficiency as it relates to antigen presentation, Dr. Weitzmann pointed to an interesting feature of normal immune function. “It's now recognized that even in healthy animals and in humans, there is always a low-grade immunological response in place that is mediated by a host of antigenic stimuli that are present as the consequence of chronic exposure to self antigens and foreign antigens, and together these antigens all conspire to sustain a weak, basal antigenic activity that is proposed to constitute a necessary component of normal immune function and renewal,” Dr. Weitzmann explained. He noted that during estrogen deficiency these ever-present antigens – examples of which include foreign antigens inhaled in the lungs, and bacterial antigens absorbed in the gut – may simply come to play a more prominent role. “There seems to be an overall increase in sensitivity of the immune system to these prevailing endogenous antigens,” Dr. Weitzmann said.

**Molecular Mechanisms Leading to T Cell Activation**

Much of *Osteoimmunology: T Cells, RANKL, and Beyond* focused on the sequence of molecular events that follow after antigen presentation, whatever that antigen might be, to T cells. The key idea is that T cell activation requires a number of “co-stimulatory” interactions, of a highly coordinated nature, between multiple ligands and receptors expressed by APCs and T cells. A particularly important interaction of this kind that Dr. Pacifici highlighted is that between CD40 ligand (CD40L) and its receptor, CD40. During estrogen deficiency, APCs present to T cells antigen fragments that are recognized by receptors on the surface of T cells. In response, T cells express the co-stimulatory molecule CD40L, which signals back to APCs by binding to CD40 present on APCs. Consequently, APCs express another co-stimulatory molecule, B7, which signals back to T cells by binding to yet another costimulatory molecule, CD28, present on T cells. “It's the final engagement of CD28 that leads to T cell activation, proliferation, and production of cytokines,” Dr. Pacifici said.
A notable prediction follows from this intricate system: if co-stimulatory interactions are necessary to activate T cells in response to OVX, then blocking those interactions should prevent T cell activation and consequent bone loss. To test this hypothesis, Dr. Pacifici described studies he has conducted using abatacept, an antibody approved for the treatment of rheumatoid arthritis; by binding to B7 on APCs, abatacept blocks the interaction between CD28 and B7, causing T cell apoptosis. What Dr. Pacifici’s group found is that mice that received abatacept were in fact protected from the bone loss usually seen with OVX. Further evidence underscoring the importance of the CD40/CD40L system comes from experiments examining the effects of knocking out CD40L in mice. In these studies, the increase in both T cell and whole bone marrow production of TNF-α seen as the result of OVX in wild-type mice was prevented in CD40L knockout animals. In addition, these studies showed that unlike wild-type mice, CD40L knockout mice are also protected from the bone loss normally seen with OVX. Finally, Dr. Pacifici stressed that CD40/CD40L signaling also occurs between T cells and stromal cells, since the latter express CD40, and ultimately increase their production of RANKL and another osteoclastogenic cytokine, macrophage colony-stimulating factor (M-CSF), in response to that signaling.

**RANKL Inhibition and Potential Adverse Effects on the Immune System**

Dr. Pacifici also devoted part of his talk to another co-stimulatory interaction, one with particular implications for our understanding of potential immune system side effects of therapies based on RANKL inhibition (see [here](http://www.bonekey-ibms.org/cgi/content/full/ibmske;8/3/135) for past coverage of this issue by IBMS BoneKEy). Specifically, while the CD40/CD40L interaction is essential to the cross-talk between APCs and T cells, RANK-RANKL signaling also plays a co-stimulatory role during this process by helping to maintain the survival of dendritic cells; any interference with the RANK/RANKL system could thus potentially have adverse consequences for the immune response. In this regard, data presented during the webinar supported the key idea that when RANK/RANKL signaling is inhibited, CD40/CD40L signaling is sufficient to ensure dendritic cell survival (whereas if CD40/CD40L signaling is blocked, the RANK/RANKL interaction becomes more important). “There is no evidence or rationale to suspect an inhibition of RANKL might lead to systemic immune alterations because whatever RANKL normally does would be carried out by CD40L,” Dr. Pacifici said. Dr. Ferrari noted that while this would explain why blocking RANKL might not adversely affect adaptive immunity, he wondered whether there are other situations where this may not be so. “In conditions like senescence,” he said, “we know that there is a decrease in immune function, so is there concern that in elderly, partially immunodeficient people, perhaps RANKL inhibition would further compromise adaptive immunity?” While the potential does exist for this outcome, Dr. Pacifici noted that it has not been documented thus far, though the field should continue to be alert to this issue.

Interestingly, RANKL inhibition may in fact have immune system effects in one particular place: the skin. Indeed, Dr. Pacifici noted that one immune mechanism operative in this organ involves RANK/RANKL signaling between keratinocytes, skin cells that express RANKL, and Langerhans cells, the dendritic cells of the skin, which express RANK. This signaling is activated upon exposure of keratinocytes to ultraviolet (UV) light, resulting in the production of regulatory T cells (Tregs) leading to UV light-mediated immunosuppression, decreased contact hypersensitivity, and decreased systemic autoimmunity. Thus, because denosumab works by inhibiting RANKL, it is possible that the consequence of this activity—a decrease in the generation of skin Tregs—may explain some of the side effects seen in clinical trials of this agent. “There is the possibility that the skin reactions and eczema reported in patients treated with denosumab may indeed be a consequence of the decreased production of regulatory T cells in the skin due to the blockade of the RANK/RANKL interaction,” Dr. Pacifici said.
Interfering with the RANK/RANKL system could potentially also have implications for another function of the immune system, namely the immune surveillance of cancer. “It will be important to look at the effect of RANKL inhibition on the immune system of cancer patients treated with denosumab,” according to panelist Pierrick Fournier. Dr. Fournier, an assistant research professor at Indiana University School of Medicine in Indianapolis also noted that, with regard to bone metastases or multiple myeloma, the immune surveillance of cancer would not depend solely on effects of RANKL inhibition, but also on other factors in the bone microenvironment, such as TGF-β and hypoxia.

A Complex State of Affairs

The field of osteoimmunology has been receiving increasing attention in recent years (click here for the IBMS BoneKEy collection of articles on this topic), as researchers continue to document important connections between the skeletal and immune systems. If Osteoimmunology: T Cells, RANKL, and Beyond is any guide, the complexity of these links will keep researchers who study them occupied for a long time, with a need for more data, particularly from clinical studies, to cinch the case for viewing PMO as an inflammatory condition of bone. With the requirement of multiple cell types, ligands and receptors, whose coordinated activity affects the levels of several cytokines that go on to influence bone, the T cell response to estrogen deficiency is a multifaceted phenomenon whose complete understanding will take quite some time to achieve.