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MEETING REPORTS

Osteoimmunology in Santorini. Meeting Report from the 3rd International Conference on Osteoimmunology: Interactions of the Immune and Skeletal Systems

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From an anatomical perspective, a connection between the skeletal and immune systems has long been recognized. However, the depth of interconnectivity between these systems remains a mystery, though great strides are being made toward a more complete understanding of this link thanks to the proliferation of osteoimmunological research studies. An increasing number of experiments support the notion that multiple levels of interaction exist between bone and immune cells at both the cellular and molecular level. In order to foster a collaborative environment in which advancements are shared and new partnerships are formed among researchers in this field, the 3rd International Conference on Osteoimmunology: Interactions of the Immune and Skeletal Systems was held in Santorini, Greece from June 21-25, 2010 (http://www.aegeanconferences.org/).

More than thirty years have passed since the behaviors of osteoblasts and osteoclasts were shown to be influenced by cytokines, yet the roles these interactions play in the physiologic and pathologic function of bone cells remain unknown. Historically, scientists studying bone cells like osteoblasts, osteoclasts and osteocytes or immune cells like lymphocytes, macrophages and granulocytes concentrated exclusively on selected areas of their function. In contrast, osteoimmunology as a discipline is dedicated to fully understanding the mechanisms that govern these cells through a comprehensive examination of their interactions. The discovery of receptor activator NF-κB (RANKL) in 1998 led to a tidal wave of studies investigating these interactions. First identified as a T cell-derived immunomodulatory molecule and then found to be the master regulator of the differentiation of osteoclasts from bone marrow (BM) precursors, the discovery of RANKL and its receptors (RANK and OPG) facilitated the identification of many different cytokines that influence bone metabolism and helped reveal how this influence is expressed. A growing quantity of pathways and transcription factors, once considered the exclusive domain of the immune system, are now known to be utilized by bone cells. In addition, recent progress in delineating the niche for hematopoietic stem cells (HSCs) in adult bone has designated unique roles for osteoblasts in the evolution of the niche.

As in the past two meetings, three key subjects dominated the 3rd International Conference on Osteoimmunology: osteoblast lineage cells and their interaction with HSCs; the regulation of bone lineage

cell differentiation; and the interactions of bone cells with inflammatory cells. Some of the featured lectures are summarized below.

During the session dedicated to exploring how cells in the bone talk to HSCs or osteoclast precursors, *Takashi Nagasawa (Kyoto University, Japan)* updated the role that chemokines and BM niches play for HSCs. The nature of the molecular regulatory mechanisms for the hematopoietic niche in bone is not well-described. Chemokines, particularly CXCL12 (stromal-derived factor-1, SDF-1) and its receptor CXCR4, are essential for the colonization of BM by hematopoietic cells. Dr. Nagasawa and his group have identified non-hematopoietic cells that express high amounts of CXCL12 and have named these CXCL12-abundant reticular (CAR) cells. These cells are scattered throughout the BM and are closely associated with hematopoietic stem cells, early B cell precursors, end-stage B cells, plasma cells and plasmacytoid dendritic cells. These results suggest that CAR cells are critical elements of the hematopoietic niche in BM.

Moving on to osteoclast precursors and their movement in the body, *Masaru Ishii (Osaka University, Japan)* used an advanced imaging technique with intravital two-photon microscopy and visualized *in situ* behavior of osteoclasts and their precursors within intact bone tissues. He found that sphingosine-1-phosphate (S1P), a lipid mediator enriched in blood, controls the movement of osteoclast precursors between the blood and endosteum. Osteoclast precursor monocytes in bone tissues express functional S1P receptors, and potent S1P receptor agonists stimulated their mobilization *in vivo*. Because S1P concentration in blood is higher than in tissues, S1P-mediated chemotaxis of osteoclast precursors contributes to their recirculation from bone tissues to systemic blood flow. Further examination reveals the possible role of several bone-enriched chemokines as regulators of the migration of osteoclast precursors in bone tissues. The bulk of these results support the hypothesis that fine-tuning of osteoclast migration mediated by various chemokines and lipid mediators dynamically modulates bone homeostasis, suggesting a unique point of action on osteoclastogenesis that may be promising as a future therapeutic target.

As in the past two meetings, arthritis was a major topic of discussion. Using several arthritic models, *Georg Schett (University Erlangen-Nuremberg, Germany)* articulated the recent advances in understanding T cell regulation of bone mass. In contrast to inflammatory T cell subsets like TH17 and Th1 cells, regulatory T cells suppress osteoclast differentiation and inhibit bone resorption. He showed that regulatory T cells are effective at preventing bone loss in a variety of *in vivo* models such as local and systemic inflammatory bone loss and postmenopausal osteoporosis. Regulatory T cells bind directly to osteoclast precursors by engaging CD80/CD86 co-stimulation molecules on the surface of these monocytic cells, which prevents their further differentiation into bone-resorbing cells. Dr. Schett concluded that regulatory T cells appear to represent a bone-protection system, which allows the adaptive immune system to balance bone resorption. *Rik Lories (Katholieke University Leuven, Belgium)* continued his discussion of the relationship between inflammation, destruction and remodeling in chronic joint disease. The identification of therapeutic agents that target tumor necrosis factor (TNF) has dramatically improved clinical outcomes in individuals with various forms of inflammatory arthritis. TNF-directed therapies not only improve signs and symptoms, but are also effective in attenuating articular bone and joint destruction. The availability of data from longitudinal and cross-sectional studies of patients with different forms of inflammatory arthritis reveals that there are differential patterns of response in these conditions that are dependent on the specific form of inflammatory arthritis. This is the case with ankylosing spondylitis, in which the adverse effects of joint inflammation are compounded by the development of progressive spine and joint ankylosis. The ankylosis has been linked to activation of the BMP and Wnt signaling pathways and the results of ongoing clinical studies indicate that targeting TNF may not effectively

prevent the process of ankylosis. In part, the differential pattern of response may be linked to the initial site of inflammation in ankylosing spondylitis, which targets the anatomic site of the enthesis, unlike rheumatoid arthritis. An understanding of the mechanisms involved in joint ankylosis could lead to new therapeutic approaches for patients suffering from this condition.

Several speakers updated our understanding of the molecular mechanisms governing osteoblast and osteoclast differentiation. *Laurie Glimcher (Harvard School of Public Health, USA)* expounded on various aspects of osteoblast differentiation including the effects that the Schnurri family (Shn1, Shn2, and Shn3) of proteins have on osteoblast activity. In addition, she discussed how the p38 MAPK pathway is required for normal skeletogenesis, as mice with genetic deletions in *Mkk3*, *Mkk6*, *p38a*, or *p38b* display profoundly reduced bone mass secondary to defective osteoblast differentiation. Among the MAP3K family, she identified TAK1 (MAP3K7) as the critical upstream activator of p38 in osteoblasts. Osteoblast-specific deletion of Tak1 resulted in clavicular hypoplasia and delayed fontanelle fusion, a phenotype similar to the cleidocranial dysplasia of humans who are haploinsufficient for Runx2. Mechanistic analysis revealed that the TAK1, MKK3/6, p38 MAPK axis phosphorylates Runx2, promoting its association with the coactivator CBP, which is required to regulate osteoblast genetic programs. *Xu Cao (Johns Hopkins University, USA)* discussed the coupling of bone resorption to formation. He showed that the conditioned medium from an *in vitro* bone resorption system induced the migration of human STRO-1^+ BM mesenchymal stem cells (MSCs) and that active transforming growth factor (TGF)β1 released during bone resorption coordinates bone formation by inducing recruitment of MSCs to the bone resorptive sites in mouse models. The active TGFβ1 stimulates directed migration through the Smad pathway. The interruption of TGFβ1 signaling by the knockdown of Smad3, by knockout of Smad4, or by over-expression of Smad 7 in MSCs inhibited the migration induced by the conditioned medium. In addition, $CD29^+Sca1^+CD45^-CD11b^-$ MSCs were sorted from mouse BM, labeled with GFP, and injected directly into the medullary canal of immunodeficient *TGFβ1*(+/+)*Rag2*(-/-) and *TGFβ1*(-/-)*Rag2*(-/-) mice. GFP^+ cells were found on bone surfaces or in the bone matrix of the *TGFβ1*(+/+)*Rag2*(-/-) mice. In contrast, GFP^+ cells were in the BM and not on bone surfaces in the *TGFβ1*(-/-)*Rag2*(-/-) mice. These data indicate that TGFβ1 is essential for the migration of MSCs to bone surfaces and that TGFβ1 is activated during bone resorption and functions to couple bone resorption to bone formation. *Laurie McCauley (University of Michigan, USA)* presented her ongoing work investigating the increased bone formation and proliferation of HSCs in response to PTH. B6 mice received a sublethal (325 cGy) dose of x-rays and were then injected with PTH daily for 3-21 days. As expected, body and spleen weight were reduced as well as total BM cells in response to the irradiation. In contrast, $Lin^-Sca\text{-}1^+ckit^+$ (LSK) cells increased. Perhaps most significantly, cells lining bone surfaces and marrow adipocytes were retained. Irradiation also caused a reduction of calvarial cells and osteoblast/stromal cells *in vitro*. The bone anabolic effect was enhanced in irradiated mice as evidenced by a 30% increase in bone area, as well as by elevated circulating osteocalcin and P1NP. Irradiation increased the gene expression of FGF2, IGF1 and IL-6, all of which are associated with PTH action. These findings suggest that irradiation causes a permissive environment for the anabolic actions of PTH.

Jan Tuckermann (Leibniz Institute for Age Research, Germany) exhibited his data analyzing glucocorticoid-induced osteoporosis (GIO) and the anti-inflammatory effects of glucocorticoids (GCs) in rheumatoid arthritis (RA). RA and GIO models in GC receptor (GR)-deficient mice treated with GCs were used. He showed that dimerization of the GR is central to the anti-inflammatory effects, but not for GC-mediated bone loss. GCs are unable to suppress bone formation in mice lacking GR expression in osteoblasts. However, GCs still suppressed bone formation in mice expressing a dimerization-

defective GR. The inhibitory effect of GCs on osteoblasts can be explained by a novel mechanism involving suppression of specific cytokines through an interaction of monomeric GR with AP-1, but not NF-κB. *Anna Teti (University of L'Aquila, Italy)* spoke about classifying osteoclasts as immune cells since they are members of the myeloid lineage and share receptors for immune cell-derived cytokines including RANKL, interferon, and ITAMs. Thus, it is important to have agents that specifically target osteoclasts to avoid cross-reactions with other immune cells. She then presented data that showed osteoclast function could be inhibited using a matrix protein called PRELP, which is transported to the nucleus where it impairs NF-κB transcriptional activity. PRELP inhibits late stage osteoclast differentiation *in vitro* and *in vivo*, but does not affect osteoblast function *in vitro* or *in vivo*. This selectivity is dependent on the ability of the heparin-binding domain of the protein to interact with cell surface chondroitin sulfates and annexin II.

On the subject of osteoclast differentiation, several key signaling pathways were examined in detail. *Brendan Boyce (University of Rochester Medical Center, USA)* discussed the NF-κB pathway. NF-κB canonical (RelA/p50) and non-canonical (RelB/p52) signaling pathways are activated in immune responses by cytokines and other inducers to regulate numerous cellular functions and affect bone remodeling either positively or negatively. Signaling through both NF-κB pathways is required in osteoclast precursor cells (OCPs) for their differentiation into mature osteoclasts and is mediated by c-Fos and NFATc1. However, the roles of both pathways in hematopoietic and stromal cell functions remain poorly understood. These investigators showed that TNF limits RANKL and TNF-induced osteoclast formation *in vitro* and *in vivo* by increasing NF-κB p100 protein accumulation in OCPs. In contrast, TNF induced robust osteoclast formation *in vivo* in mice lacking RANKL or RANK when the mice also lacked NF-κB p100. Also, TNF transgenic mice (TNF-Tg) lacking NF-κB p100 had more severe joint erosion and inflammation than did TNF-Tg littermates with NF-κB p100. TNF, but not RANKL, increased OCP expression of TNF receptor-associated factor 3 (TRAF3), an adapter protein that regulates NF-κB p100 levels in B cells. TRAF3 siRNA prevented TNF-NF-κB p100 accumulation and inhibition of osteoclastogenesis. These findings suggest that pharmacologic manipulations that upregulate TRAF3 or NF-κB p100 expression or inhibit NF-κB p100 degradation in OCPs could limit bone destruction and inflammation-induced bone loss caused by bone diseases. *Deborah Novack (Washington University, USA)* continued the discussion of the NF-κB pathway. Her studies on the role of individual NF-κB subunits have demonstrated that p65 is important for OCP survival during a critical period of differentiation, but is not necessary for transcription of the OC differentiation program. In contrast, RelB is required for OC differentiation *in vitro* and for pathological bone loss *in vivo*. Thus, the alternative NF-κB pathway represents an interesting potential target for the control of bone loss in diseases such as inflammatory arthritis. In addition, Dr. Novack has shown that NIK-deficient mice are unable to process p100 to p52. This prevents nuclear translocation, and thus activation, of RelB. *NIK*(-/-) precursors are unable to form OCs *in vitro* with RANKL stimulation and are resistant to osteolysis in the serum transfer model of arthritis. Mutation of the TRAF3 binding domain of NIK prevents its rapid degradation in resting cells, leading to constitutive activation of the alternative NF-κB pathway. *In vitro,* expression of activated NIK allows osteoclastogenesis at lower doses of RANKL. Furthermore, transgenic expression of constitutively active NIK in the OC lineage causes osteoporosis and increased numbers of OCs *in vivo*. Therefore, both deletion and activation of NIK significantly modulate osteoclastogenesis.

Steven Teitelbaum (Washington University) presented his findings on the interaction between cytoskeletal organization and costimulatory signals during osteoclast differentiation. His data suggest that *Dap12*(-/-) and wild-type (WT) BM macrophages differentiate into equal numbers of osteoclasts when generated with

WT osteoblasts. In contrast, Dap12-deficient cells fail to spread and organize their cytoskeleton. Cytoskeletal dysfunction is further substantiated by the low yield of osteoclasts attached to plastic in these co-cultures treated with collagenase, indicating the inability of the mutant cells to transmigrate through the osteoblast layer. The capacity of retroviral-expressed Dap12, but not FcRγ, to liberate DKO osteoclasts to spread and transmigrate establishes Dap12 as the dominant co-stimulatory molecule involved in cytoskeletal organization. To determine if FcRγ mediates osteoclast function in the absence of Dap12, Dr. Teitelbaum and his team overexpressed a hybrid consisting of the FcRγ co-receptor, OSCAR, fused to FLAG (OSCAR-FLAG) in *Dap12*(-/-) osteoclasts. The construct is activated by culturing the transduced osteoclasts on anti-FLAG mAb. OSCAR-FLAG fails to normalize the actin cytoskeleton in cells resident on glass but induces formation of atypical actin rings on bone and the degradation of bone. On the other hand, OSCAR-FLAG overexpression fails to impact the abnormal cytoskeleton of *DAP12*(-/-) osteoclasts generated with osteoblasts. These findings show that cytoskeletal disorganization is the dominant consequence of Dap12 deficiency in osteoclasts. While activated, overexpressed OSCAR modifies the *DAP12*(-/-) cytoskeleton phenotype of bone. The failure of osteoblasts to do so indicates that functionally relevant quantities of OSCAR ligand do not reside in bone-forming cells.

Sakae Tanaka (University of Tokyo, Japan) discussed several molecular pathways controlling differentiation, functionality and survival of osteoclasts. TGFβ is a multimodal cytokine abundantly stored in bone that causes widespread proliferation and differentiation in many types of cells. Dr. Tanaka's group found that the deprivation of TGFβ signaling by a specific inhibitor or the introduction of a dominant negative mutant of type II TGFβ receptor markedly suppressed osteoclast differentiation, even in the presence of M-CSF and RANKL. This inhibitory effect is at least partly mediated by the direct molecular interaction between Smad3 and the TRAF6-TAB1-TAK1 complex. In addition, these investigators found that several TGFβ-regulating genes also play important roles in osteoclast development. In a separate study, they examined the mechanisms by which nitrogen-containing bisphosphonates (N-BPs) inhibit osteoclast function. N-BPs are known to act directly on osteoclasts to induce apoptosis and suppress bone-resorbing activity by targeting farnesyl pyrophosphate synthase and inhibiting posttranslational prenylation of small GTP-binding proteins. Dr. Tanaka found that apoptosis and the bone-resorbing activity of osteoclasts were reciprocally regulated through Erk and Akt pathways, which were both suppressed by N-BPs *in vitro*. These results provide insight into the mechanism that causes N-BPs to act on osteoclasts *in vivo*.

Finally, several talks branched out from the realm of traditional bone biology. *Hiroshi Takayanagi (Tokyo Medical and Dental University, Japan)* addressed the interconnectivity of the skeletal and adaptive immune systems through the concept of a shared axis. In particular, he explained the role of TH17 cells in bone loss. TH17 cells are the major cellular source of the proinflammatory cytokine interleukin (IL)-17 and produce potent inducers of osteoclastogenesis and bone loss. A thorough characterization of the molecular pathways leading to TH17 differentiation are important to understanding the exact regulation of T cell-driven bone loss. Differentiation of TH17 cells depends on particular transcription factors, such as the orphan nuclear receptors RORα and RORγ. However, ROR transcription factors alone are not sufficient to trigger full Th17 differentiation. The discovery of IkBζ, a transcriptional component of the NF-κB pathway, appears to have closed this gap in understanding the instigation of TH17 differentiation as it is essential for the process. *Shigekazu Nagata (Kyoto University, Japan)* discussed the role of DNA degradation in the pathogenesis of arthritis. He showed that mice without Dnase II, which lack the capacity to degrade DNA from apoptotic cells, develop inflammatory arthritis. Disease results from an accumulation of DNA in monocytes/macrophages that triggers the

production of interferon (IFN)-β and TNF, which, in turn, elicits an inflammatory response. Whereas IFN-β appears to be primarily responsible for the development of anemia, TNF seems to be the key mediator for inducing arthritis in this axis. Dealing with complicated interactions among many different cell types *in vivo*, *Roberto Pacifici (Emory University School of Medicine, USA)* presented data that showed T cells are required for the anabolic effects of intermittent PTH on bone. To further test this hypothesis, he treated mice, which lacked the T cell receptor β, and hence, were deficient in αβ T cells, with PTH. micro-CT, histomorphometric analysis, and mechanical testing were used to demonstrate that the mice exhibited a blunted response to PTH. This effect was attributed to a failure to increase both bone resorption and bone formation. The necessity of T cells was confirmed in additional strains of T cell-deficient mice, including nude MHC class I and class II double knockout mice and *RAG2*(-/-) mice. Mechanistic studies revealed that T cells express the PTH receptor and that PTH increases cAMP levels in these cells. Dr. Pacifici found that PTH increased Wnt10b in BM CD8 T cells, which, in turn, activated canonical Wnt signaling in pre-osteoblasts. The silencing of T cell-produced Wnt10b blocked the anabolic activity of PTH, confirming the pivotal role of this pathway. *Erwin Wagner (CNIO-Spanish National Cancer Research Center, Spain)* highlighted recent insights into the role of AP-1 proteins in bone homeostasis. Summarizing his latest work on the role the AP-1 family member Fra-2 plays in the skeleton, Dr. Wagner showed that Fra-2-deficient newborns develop giant osteoclasts due to impaired signaling through the leukemia inhibiting factor (LIF) pathway and hypoxia. LIF is a transcriptional target of Fra-2. Deficiency of LIF, LIF receptor or Fra-2 leads to hypoxic bones with giant osteoclasts. Moreover, he indicated novel genetic strategies to use tissue-specific expressions of Fos and Fra proteins as a tool to better define the role of these proteins in human diseases such as inflammation, bone loss and cancer.

Summary

The third meeting of the International Conference on Osteoimmunology featured increased participation from new attendees, many of them relatively young among practitioners of laboratory medicine, and provided further confirmation of the broadness of this developing field of study. Some general topics that were expanded upon this year that show the potential wide-ranging impact of osteoimmunology include the regulation of bone turnover in both healthy and pathologic physiology, the maturation of osteoclasts from a common myeloid precursor, which also gives rise to dendritic cells and macrophages, and the notion that immune and bone cells interact on multiple levels, including the development of the BM microenvironment. In addition, this meeting supplied clear evidence that the mechanisms controlling the fate of bone cells are not unique to the skeletal system. What's to come at the next meeting? One question we would like to see explored further is the extent to which adaptive immune responses control bone metabolism and vice versa. Another area would be to study other inflammatory diseases beyond arthritic bone diseases. The number of young investigators at this year's conference (about 30%) and the continued expansion of possible medical applications are promising signs for the future of osteoimmunology.

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