Review

Osteoimmunology — Bidirectional dialogue and inevitable union of the fields of bone and immunity —

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Abstract: Bone is a critically important part of the skeletal system that is essential for body support and locomotion. The immune system protects against pathogens and is active in host defense. These two seemingly distinct systems in fact interact with each other, share molecules and create a collaborative regulatory system called the “osteoinmune system”. The most representative osteoimmune molecule is receptor activator of NF-κB ligand (RANKL), which plays multiple roles in the osteoinmune system under both physiological and pathological conditions such as rheumatoid arthritis and cancer metastasis to bone. Based on accumulating evidence for such mutual dependence, it is concluded that the relationship between bone and the immune system did not develop by accident but as a necessary consequence of evolution. Here I describe the history of and recent advances in osteoimmunology, providing a perspective in the contexts of both science and medicine.

Keywords: osteoimmunology, bone metabolism, immunity, rheumatoid arthritis, bone marrow microenvironment, inter-organ crosstalk

Introduction

Bone is composed of hard extracellular matrix along with a small number of crucially important bone cells. The extracellular matrix is mainly made up with type I collagen and hydroxypapatite (calcium phosphate crystal). Bone cells are comprised of bone-forming osteoblasts and bone-resorbing osteoclasts, both of which are effector cells that act on the surface of bone.1,2) Osteocytes are embedded in the bone matrix and are terminally differentiated cells of osteoblast lineage, functioning as a command cell for osteoblasts and osteoclasts in response to stimuli such as mechanical stress and hormones.1,2) Old and damaged bone, which is detected by poorly understood mechanisms, is resorbed by osteoclasts, after which osteoblasts make an equivalent amount of resorbed bone. If the balance between resorption and formation is shifted to resorption, the bone mass decreases, leading to osteoporosis. If osteoclastic bone resorption is impaired, the bone mass increases (osteopetrosis) but the bones become susceptible to fracture.1,2) Thus, bone should be constantly renewed by osteoclasts and osteoblasts in a proper balance in this remodeling process. In addition to osteoporosis, excessive osteoclastic bone resorption beyond osteoblastic bone formation is seen in various pathological conditions such as rheumatoid arthritis, periodontitis and congenital bone diseases as well as primary and metastatic bone tumors.1,2) Thus, understanding the mechanism of differentiation and activation of osteoclasts and osteoblasts is vitally important for developing future therapeutic strategies for bone and joint diseases.

The immune system detects and eradicates non-self entities such as pathogens for the purpose of host defense. The invasion of pathogens induces the activation of the innate immune system followed by the antigen-specific response of the acquired immune system. The bone marrow is one of the two primary lymphoid organs. It is the primary site of hematopoiesis, harboring hematopoietic stem cells (HSCs) and myeloid and lymphoid progenitors, as well as mature
immune cells, including B cells, neutrophils, macrophages and T cells.\textsuperscript{1,2} Therefore, bone and immune cells share the same microenvironment in the bone marrow. It is highly likely that bone and immune cells interact with each other and elicit mutual influence.\textsuperscript{1,2} However, until fairly recently, little attention has been paid to the interaction and shared molecular mechanisms of the bone and immune systems.

**Discovery of osteoclast differentiation factor**

Osteoclasts are formed in co-cultures of osteoblastic cells and osteoclast precursor cells of the monocyte/macrophage lineage. Since direct contact with osteoblastic cells is required, it was expected that the osteoclast differentiation factor should be expressed by osteoclastogenesis-supporting cells, such as osteoblastic cells in a membrane-bound form.\textsuperscript{3} Osteoprotegerin (OPG) was first identified as a factor that inhibits osteoclastogenesis.\textsuperscript{4,5} Subsequently, osteoclast differentiation factor was discovered as the ligand of OPG in 1998.\textsuperscript{6,7} Surprisingly, osteoclast differentiation factor was found to be identical to the receptor activator of NF-κB (RANKL) already reported by immunologists as a factor expressed by T cells.\textsuperscript{8,9} The essential role of RANKL in osteoclast differentiation was proven by the finding that RANKL-deficient mice lack osteoclasts completely.\textsuperscript{10} RANKL-deficient mice exhibit osteopetrosis with no tooth eruption and a defect in the immune system: no lymph node formation.\textsuperscript{10} RANKL was found independently by bone biologists and immunologists, and plays crucial roles in both the bone and immune systems, and is thus regarded as one of the most representative osteoimmune molecules. In humans, loss-of-function mutations of RANKL are reported to cause autosomal recessive osteopetrosis.\textsuperscript{11} Anti-RANKL antibodies have thus been developed that target osteoclasts in the treatment of metastatic bone tumors and osteoporosis, with fairly successful clinical outcomes.\textsuperscript{12,13}

**The mechanism of bone destruction in rheumatoid arthritis**

Rheumatoid arthritis is a prevalent autoimmune disease characterized by the inflammation of multiple synovial joints followed by joint destruction and deformity. In the 1990s it was not well understood why bone is affected by autoimmunity. Certain researchers proposed that bone damage can be caused by the proteinases produced by inflammatory cells, but from the standpoint of bone biology, it was difficult to construe such severe bone loss occurring without the involvement of osteoclasts.\textsuperscript{14} We accordingly undertook an investigation of the mechanism of the bone destruction that occurs in arthritis by focusing on the contribution of osteoclasts.\textsuperscript{14} Numerous osteoclasts were observed at the interface of the inflammatory synovium and bone. Furthermore, it was found that osteoclasts were formed in cultures of the synovial cells derived from rheumatoid arthritis patients.\textsuperscript{14} We attributed the bone destruction to pathological synovial fibroblasts, since these cells have the ability to support osteoclastogenesis similar to osteoblastic cells in bone.\textsuperscript{14} After RANKL was cloned, we tested the expression of RANKL in rheumatoid arthritis patients and found that synovial fibroblasts express RANKL and induce osteoclastogenesis.\textsuperscript{15}

Because T cells also produce RANKL, there was a possibility that T cells infiltrate into the synovium and induce osteoclasts by expressing RANKL.\textsuperscript{16} We thus explored the effect of T cells on osteoclastogenesis in greater detail. Despite surface expression of RANKL, activated cells that produce interferon-γ suppressed osteoclastogenesis.\textsuperscript{17} We found that T cells produce various membrane-bound and soluble factors that influence osteoclastogenesis, and the effect of T cells on osteoclastogenesis is dependent on the balance between and among cytokines that T cells produce.\textsuperscript{17} This study explicitly dealt with the molecular mechanisms underlying the interaction between immune and bone cells.\textsuperscript{17} Dr. Yongwon Choi (Howard Hughes Medical Institute, Rockefeller University, currently University of Pennsylvania) coined the term “osteoinmunology” to describe this interdisciplinary research.\textsuperscript{18} If interferon-γ-producing T cells suppress osteoclastogenesis, which T cells are osteoclastogenic? We examined the effect of helper T cell subsets on osteoclasts and revealed interleukin-17 (IL-17) producing Th17 cells to be the exclusive osteoclastogenic subset.\textsuperscript{19} In arthritis, Th17 cells accumulate in the synovium and produce IL-17A, IL-17F and IL-21. IL-17A and IL-17F stimulate RANKL expression on synovial fibroblasts and at the same time make synovial macrophages secrete inflammatory cytokines such as tumor necrosis factor (TNF) and IL-6.\textsuperscript{19,20} Inflammatory cytokines further increase RANKL expression and stimulate osteoclast precursor cells. As a result, RANKL-induced osteoclastogenesis is powerfully stimulated, leading to excessive osteoclastic bone resorption and bone damage (Fig. 1). Osteoblastic bone formation was shown to be suppressed by TNF
and TNF target molecules, such as dickkopf-1 (DKK1).21)

Biologic disease-modifying antirheumatic drugs such as anti-TNF and anti-IL-6R antibodies are widely used in the clinic and brought about epoch-making change in the prognosis of rheumatoid arthritis.1), 2) TNF or IL-6 blockage is considered to inhibit not only local inflammation but also the induction of RANKL and the osteoclast precursor cell activation, exerting a powerful suppressive effect on bone destruction.1), 2) Anti-RANKL antibodies, the most direct method to suppress bone destruction, were approved for rheumatoid arthritis in Japan in 2017. It is worth noting that all drugs for rheumatoid arthritis other than anti-RANKL antibodies were developed to inhibit inflammation. Initiating the targeting of osteoclasts in the treatment of rheumatoid arthritis is one of the important fruits of osteoimmunology.

The origin of bone-damaging T cells

Regulatory T (Treg) cells suppress unnecessary, excessive and/or prolonged immune responses. We examined the fate of Treg cells in arthritis and found that a certain population of Tregs with a low expression of CD25 convert to Th17 cells in the inflammatory synovium.22) The Treg cells that lose the expression of the hallmark transcription factor Foxp3 and produce IL-17 are called exFoxp3Th17 cells.22) exFoxp3Th17 cells are more highly pathogenic than naïve-T cell-derived Th17 cells in terms of both inflammatory and osteoclastogenic capability, playing a critical role in the acceleration of arthritis (Fig. 1).

In arthritis, the conversion of Treg cells to Th17 cells is exclusively pathogenic, but we also considered the role of exFoxp3Th17 cells in periodontitis, since Th17 cells are important for the host defense against bacteria in mucosal immunity.23) exFoxp3Th17 cells are more highly pathogenic than naïve-T cell-derived Th17 cells in terms of both inflammatory and osteoclastogenic capability. Moreover, systemic bacterial dis-
semification from periodontal tissue is suppressed by the removal of infected teeth.\(^2\) This is a rare observation of a beneficial effect of bone and tooth loss on host defense. We infer that the bone loss mechanism exerted by Th17 cells might be due to a primitive host defense against pathogens that arose in polyphyodont vertebrates.\(^2\)

**Osteoclastogenic signal transduction**

Before the discovery of RANKL, the knowledge about the molecules involved in osteoclast differentiation was obtained from naturally occurring or genetically modified mice that develop osteopetrosis.\(^1\),\(^2\) NF-κB, tumor necrosis factor receptor-associated factor 6 (TRAF6) and c-Fos were among the essential mediators of osteoclastogenic signaling, but none of these factors represented the specific master regulator of osteoclast differentiation.\(^1\),\(^2\) We performed a genome-wide screening of RANKL-inducible genes and identified nuclear factor of activated T cells c1 (NFATc1) as the master transcription factor for osteoclastogenesis.\(^24\) NFATc1 is markedly and specifically induced by RANKL in osteoclast precursor cells and is necessary and sufficient for osteoclast fate commitment.\(^24\) NFATc1 activation requires calcium signaling, which is dependent on the immunoreceptor tyrosine-based activation motif (ITAM) signaling mediated by Fc receptor γ-chain (FcγR) and DNAX-activating protein of 12 kDa (DAP12).\(^25\) These adaptor molecules associate with immunoglobulin-like receptors such as FcγRIII, osteoclast-associated receptor (OSCAR), paired immunoglobulin-like receptor A (PIR-A), signal-regulatory protein β-1 (SIRPβ1), sialic acid binding immunoglobulin-like lectin 15 (Siglec-15) and triggering receptor expressed on myeloid cells 2 (TREM-2) (Fig. 2). These receptors convey costimulatory signals for RANKL. The ligands of the costimulatory receptors are not yet identified, but include immunoglobulin G (IgG) immune complex, collagen and major histocompatibility complex (MHC) class I molecules.\(^1\),\(^2\),\(^26\) RANKL binding to its receptor RANK results in the recruitment and trimerization of TRAF6, leading to the activation of NF-κB and the induction of NFATc1 (Fig. 2). NFATc1 is persistently stimulated by the calcium signaling activated by Tec family kinases and phospholipase Cγ (PLCγ), which are phosphorylated downstream of RANK and ITAM signaling.\(^27\),\(^28\) NFATc1 and c-Fos cooperatively bind the NFATc1 promoter and this induces the autoamplification of NFATc1, which is a hallmark event of osteoclast differentiation (Fig. 2).\(^29\) It is notable that most of the molecules utilized in osteoclast signal transduction were originally identified and have been closely studied in the immunology field, highlighting the importance of the molecules and regulatory mechanisms shared by the bone and the immune systems.

**Which cells express RANKL?**

Even after the anti-RANKL antibodies had been introduced into the clinic, it remained unclear which cells were the major source of RANKL under physiologic and pathological conditions. *In vitro* experiments suggested that osteoblastic cells support osteoclastogenesis, but the *in vivo* source remained obscure, since osteoblastic cells include mesenchymal progenitor cells, osteoblasts and osteocytes. We and others provided *in vivo* evidence for osteocyte regulation of osteoclastogenesis through RANKL expression in postnatal bone remodeling.\(^30\),\(^31\) It was also shown that osteoblasts and chondrocytes are the major source of RANKL during embryonic development. RANKL flox mice also helped determine the RANKL-expressing cells in arthritis. Bone destruc-
tion was ameliorated in mice lacking RANKL in synovial fibroblasts but not in mice lacking RANKL in T cells, indicating that it is the synovial fibroblasts that are the main source of RANKL in arthritis.\textsuperscript{32} Although T cells are deeply involved in the inflammatory responses and the initiation of bone damage, T cells do not induce osteoclastogenesis by expressing RANKL, but rather stimulate the surrounding synovial fibroblasts to express RANKL and indirectly induce bone damage. Consistent with this, a recent report on single cell analysis of arthritic fibroblasts indicated that RANKL-expressing synovial fibroblasts are the fibroblast subset responsible for bone destruction.\textsuperscript{33,34} In periodontitis, RANKL was shown to be expressed by periodontal ligament cells and osteoblasts rather than T cells or B cells.\textsuperscript{23}

**Immunological function of RANKL**

As mentioned above, RANKL is regarded as a key osteoimmune molecule and drug target in bone metastasis, osteoporosis and rheumatoid arthritis. RANKL was first identified in T cells and suggested to regulate dendritic cells, but the function of RANKL in acute immune response has not been emphasized in the literature, partly because of the redundant function of CD40.\textsuperscript{1,2} When T cell-specific RANKL conditional knockout mice were examined in experimental autoimmune encephalomyelitis, we noticed that Th17 migration to the central nervous system was reduced in spite of a normal accumulation of Th17 in the periphery.\textsuperscript{35} RANKL expressed on T cells was involved in the T cell-astrocyte interaction and induction of chemokine (C-C motif) ligand (CCL)-20 required for Th17 migration across the blood brain barrier, suggesting RANKL blockade may have beneficial effect on diseases characterized by inflammation of the central nervous system, such as multiple sclerosis.\textsuperscript{35}

RANKL expressed by CD4 T cells is important for the establishment of central immune tolerance by fostering the thymic medullary microenvironment.\textsuperscript{36–38} It is interesting that RANKL also critically contributes to the development of another primary lymphoid organ, the thymus. RANKL together with CD40 regulates the differentiation of medullary thymic epithelial cells expressing auto-immune regulator (AIRE). AIRE is one of the factors that regulate the expression of tissue-restricted antigens that are required for the negative selection of autoreactive T cells.\textsuperscript{36,37}

RANKL has also been shown to be indispensable for the differentiation of microfold (M) cells, which are mainly found in gut-associated lymphoid tissue (GALT). M cells are specialized epithelial cells which take up and transport bacterial antigens.\textsuperscript{39} We found that RANKL-expressing mesenchymal cells (called M cell-inducer cells) directly interact with the gut epithelium to control CCL20 expression and M cell differentiation.\textsuperscript{39} The deletion of mesenchymal RANKL impairs M cell-dependent antigen sampling and B cell-dendritic cell interaction, which results in a reduction in IgA production and a decrease in microbial diversity. RANKL and OPG have emerged as critical regulators of the immune response in the gut mucosa.\textsuperscript{39}

**RANKL biology beyond the bone and immune systems**

RANKL has also been shown to be indispensable for mammary gland formation during pregnancy and involved in the central nervous system control of fever and female body temperature.\textsuperscript{40,41} Anti-RANKL antibodies have been applied to control the skeletal related events in metastatic bone tumors, mainly because tumor cells and osteoclasts form a vicious circle: metastasized tumor cells produce parathyroid hormone-related protein (PTHrP) and IL-6, inducing RANKL, which stimulates osteoclastic bone resorption, leading to the release of growth factors such as transforming growth factor (TGF)-β and insulin-like growth factor-1 (IGF-1), thus promoting tumor growth (Fig. 3).\textsuperscript{1,2} If RANKL function was limited to the activation of osteoclasts, anti-RANKL treatment would be effective in only the osteolytic tumors that stimulate osteoclasts. In fact, anti-RANKL therapy suppresses non-osteolytic tumors in models of bone metastasis. This shows that the role of RANKL is not limited to osteoclast activation in the process of bone metastasis.\textsuperscript{1,2} It has been demonstrated that RANKL functions as a chemotactic factor which recruits RANK-expressing tumors to bone.\textsuperscript{42} It has also been shown that tumors that favor bone metastasis frequently express RANK.\textsuperscript{42}

The RANKL expressed in the membrane-bound form is cleaved by proteinases into the soluble form. It has been suggested that the membrane-bound form functions more efficiently than the soluble form, but there was no clear in vivo data to support this difference.\textsuperscript{3,44} To address this question, we generated mice in which the cleavage site of RANKL was deleted.\textsuperscript{39,45} The mice had no detectable soluble form of RANKL but a normal level of membrane-bound RANKL.\textsuperscript{39,45} Surprisingly, the soluble RANKL-deficient mice exhibited no abnormalities
in bone development, osteoclastogenesis, lymph node organogenesis, thymic epithelial cells, M cells or mammary gland development.\textsuperscript{45}) We examined certain disease models including ovariectomy-induced osteoporosis and periodontitis, but there was no significant difference in the severity of the symptoms.\textsuperscript{23,45}) The only difference found was in bone metastasis cancer models.\textsuperscript{45}) Bone metastasis of B16F10 melanoma cells injected into the heart was markedly reduced in cases of soluble RANKL deficiency.\textsuperscript{45}) Therefore, soluble RANKL is physiologically dispensable but involved in the acceleration of tumor metastasis to bone (Fig. 3).\textsuperscript{45}) In humans, the serum level of soluble RANKL has been shown to correlate with the prognosis of bone metastasis. RANKL has also been shown to be involved in the acceleration of osteoclasts into the heart and in the regulation of anti-tumor immunity.\textsuperscript{1,2}) Thus, RANKL has attracted considerable attention from researchers in a variety of physiological and pathological conditions, which together might be called “RANKL biology”, extending outward from its initial role in the osteoimmune system.\textsuperscript{1,2})

\textbf{Regulation of immune cells by bone}

Since immune cells are able to infiltrate into any organ and exert effects in all tissues, the special relationship between bone and the immune system is not complete without a demonstration of bidirectional regulation. The regulation of immune cells by bone is particularly important in this context. HSCs are known to reside in the bone marrow after birth and the mesenchymal osteoprogenitor cells that express CXC chemokine ligand (CXCL)-12 and leptin receptors function as the HSC niche.\textsuperscript{1,2)} Mature osteoblasts mainly support lymphoid progenitor cells via IL-7 production.\textsuperscript{1,2,46)} In addition to physiological maintenance of hematopoietic progenitors, bone cells play a role in restraining HSCs from transformation into malignancy. The function of osteoclasts in HSC regulation is still controversial and needs further study, but osteogenic cells play a crucial role in the regulation of HSCs, shedding light on the bidirectional dialogue between the bone and immune systems (Fig. 4).\textsuperscript{1,2)} Bone functions as a part of the skeletal system, a calcium reservoir and lymphoid organ. In light of the fact that all of the cells in the bone marrow share the same microenvironment, the time has come to recognize that all bone functions take place in the context of the osteoimmune system.

\textbf{Bone cell communication factors}

If all of the cells in the bone marrow interact with each other and regulate bone function in cooperation,
it is of critical importance to identify the molecular mechanisms involved in bone cell communication. Since bone is formed in a magnitude that coordinated with the level of bone resorption, it has long been expected that this coupling process (i.e., the transition from resorption to formation) is linked by certain molecules called “coupling factors”. IGF-1 and TGF-β are among the classical coupling factors that are released by bone resorption and increase bone formation. More recently, factors such as sphingosine-1-phosphate (S1P), ephrins, semaphorins and Wnt family proteins have been suggested to play a role in bone cell communication, but the interacting effect is not always the same as classical coupling mechanisms. For example, the semaphorin (Sema)-4D produced by osteoclasts suppresses bone formation, suggesting that this molecule is working in the bone resorption phase (Fig. 5).

OPG, one of the earliest identified bone cell communication factors, was thought to explain the inhibitory effect of osteoblast conditioned medium on osteoclast formation. We found such inhibitory activity even in the conditioned medium of OPG-deficient osteoblasts and purified the molecule related to this activity. Sema3A was thus shown to be a potent osteoproteective factor, inhibiting osteoclast differentiation and promoting osteoblastic bone formation. Since Sema3A is produced by neuronal cells and acts as a repulsive factor for neurite growth, we investigated the in vivo source of Sema3A using Sema3A conditional knockout mice. In conclusion, Sema3A produced by osteocytes plays a major role in bone homeostasis in the postnatal stage by autoregulation of osteocyte survival in addition to osteoclast suppression and osteoblast stimulation (Fig. 5).

**Evolutionary insight into the interplay of the bone and the immune systems**

Bone has apparently unrelated functions: locomotion, calcium reservoir and hematopoiesis. During the course of vertebrate evolution, the skeletal system needed to be strengthened when aquatic vertebrates moved onto land and had to cope with the resistance of gravity in the new environment. At the same time, the calcium which had previously been readily...
taken up from sea water now needed to be stored in the body.\footnote{1} The development of the acquired immune system also accelerated after exposure to the complexity of microorganisms in the terrestrial conditions.\footnote{1} Furthermore, the exposure to a high level of ultraviolet light necessitated the housing of HSCs in a shielded place.\footnote{1} The reason why so many different functions came to center on bone can be speculatively attributed to the simultaneous requirements of a strong skeleton, calcium storage, an advanced immune system and a hiding place for HSCs (Fig. 6).

**Future perspectives**

Twenty years have passed since the term osteoimmunology was first coined. Since osteoimmunology deals with subjects beyond the scope of traditional immunology or bone research, it was at first not readily accepted as a discipline. However, the last two decades have witnessed the emergence of certain critically important insights from the osteoimmunological approach that have made it difficult for either immunologists or bone biologists to adequately understand their own fields without osteoimmunological input. The osteoimmune system is now one of the critical concepts in biology. Ultimately, it was human beings who separated the bone and the immune systems into distinct fields of inquiry, which nature did not. The time has come to go beyond the conventional paradigm and investigate biological systems from the viewpoint of multi-organ linkages. The clinical application of anti-osteoclast therapy to rheumatoid arthritis has been one of the important achievements of the osteoimmunological approach. The future discovery of bone cell communication factors would be expected to provide valuable new therapeutic targets in bone and immune diseases. Thus, the dynamic field of osteoimmunology will provide a thought-provoking paradigm for understanding vertebrate biology as a whole as well as providing a molecular basis for critically needed new treatments.

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Fig. 6. (Color online) Origin of the osteoimmune system in evolution. The bone and immune systems became linked during the course of vertebrate evolution. When aquatic vertebrates moved onto land, the skeletal system adapted to the need to support locomotive activity in the terrestrial environment. At the same time, the immune system rapidly evolved so as to cope with the plethora of terrestrial pathogens, and the location of hematopoiesis moved to the bone marrow to protect HSCs against the higher level of ultraviolet (UV) light exposure on land. Thus, the bone and immune systems developed synchronously, ultimately comprising an osteoimmune system, functioning as a locomotor organ and mineral reservoir as well as a primary lymphoid organ.

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Profile

Hiroshi Takayanagi was born in Tokyo in 1965. He graduated from The University of Tokyo and became an orthopaedic surgeon and rheumatologist in the Department of Orthopaedic Surgery, Graduate School of Medicine, The University of Tokyo Hospital. After serving as a surgeon for 7 years, he started his basic research career in the Department of Orthopaedic Surgery. He studied the mechanism of bone destruction in rheumatoid arthritis, with a specific focus on the RANKL protein. He then moved to the Department of Immunology in the same university. After being awarded his Ph.D. degree in 2001, he became an Assistant Professor in the Department of Immunology. He explored the role of immune molecules such as interferons in the regulation of bone metabolism. He revealed that the key factors NFATc1 and immunoglobulin-like receptors in osteoclast development and pioneered a new interdisciplinary field called “osteoimmunology.” From 2003 through 2012, he was a Professor at the Tokyo Medical and Dental University. Since 2012, he has served as a Professor, Department of Immunology, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo, focusing on osteoimmunology and the pathogenesis of autoimmune disease as well as the mechanism of immune tolerance established in the thymus. He has been President of the Japanese Society of Osteoimmunology since 2014. He was awarded the Japan Academy Prize in 2019.