### Interaction between the immune system and bone metabolism: an emerging field of osteoimmunology

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Abstract: The interaction between the immune and bone systems has long been appreciated, but recent research into arthritis as well as various bone phenotypes found in immune-related knockout mice has highlighted the importance of the interplay and the interdisciplinary field called osteoimmunology. In rheumatoid arthritis, IL-17-producing helper T cells ( $T_H 17$ ) induces receptor activator of NF- $\kappa$ B ligand (RANKL), which stimulates osteoclast differentiation through nuclear factor of activated T cells (NFAT)c1. Accumulating evidence suggests that the immune and skeletal systems share cytokines, signaling molecules, transcription factors and membrane receptors. In addition, the immune cells are maintained in the bone marrow, which provides a space for mutual interaction. Thus, bone turns out to be a dynamic tissue that is constantly renewed, where the immune system participates to a hitherto unexpected extent. This emerging field of osteoimmunology will be of great importance not only to the better understanding of the two systems but also to the development of new treatment for rheumatic diseases.

Keywords: osteoimmunology, osteoclast, RANKL, T<sub>H</sub>17, NFATc1

### Introduction

Bone is a multifunctional organ, functioning as a vital component of the locomotor system, serving as a reservoir of calcium and providing a site for hematopoiesis.<sup>1)</sup> Although bone appears to be inert, it is actually a dynamic tissue that is constantly remodeled by bone-resorbing osteoclasts and bone-forming osteoblasts.<sup>2)</sup> This balance must be tightly controlled by various regulatory systems. Excessive activity of osteoclasts leads to pathological bone resorption, as seen in a variety of osteopenic diseases: autoimmune arthritis, periodontitis, postmenopausal osteoporosis, Paget's disease and bone tumors. Therefore, elucidating regulatory mechanisms of osteoclast differentiation is critical for an understanding of the health and disease of the skeletal system.<sup>3)</sup>

The endocrine system has been thought to be one of the most important regulatory systems of bone classically. However, the immune system has emerged as a novel crucial regulator of this system recently.<sup>1), 4)</sup> Immune and skeletal systems have a variety of regulatory molecules such as cytokines, in common. Furthermore, immune cells form in the bone marrow, interacting with bone cells. Consequently, the physiology and pathology of one system may very well affect the other: abnormal activation of the immune system leads to bone destruction in diseases like rheumatoid arthritis.<sup>5), 6)</sup> More recently, animal models deficient in immunomodulatory molecules have been found to frequently develop an unexpected skeletal phenotype.<sup>1)</sup> Thus, the crosstalk between the immune and skeletal systems and the interdisciplinary field called osteoimmunology have attracted much attention in recent years.

### Discovery of osteoclast differentiation factor

Since 1970's, it has been known that stimulated immune cells produce soluble factors that ac-

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tivate osteoclastic bone resorption.<sup>7</sup>) One of these factors was identified to be interleukin  $(IL)-1.^{8}$ This is one of the earliest observations in the immune regulation of osteoclasts. In the late 1980's, an *in vitro* osteoclast formation system was established by culturing bone marrow-derived cells of monocyte/macrophage lineage with "supporting cells" such as osteoblasts.<sup>9)</sup> These osteoclastogenesissupporting mesenchymal cells provide factors that are necessary for osteoclast differentiation. Analysis of op/op mice, a naturally-occurring strain with osteopetrosis, revealed one of the essential factors to be macrophage colony-stimulating factor (M-CSF).<sup>10)</sup> Since M-CSF is also critical for macrophage development, this is another important molecule in the immune regulation of osteoclastogenesis. However, M-CSF stimulation alone does not induce the differentiation of osteoclasts. Forced expression of antiapoptotic molecule Bcl-2 partially rescues the osteopetrotic phenotype of the op/op mice mice,<sup>11)</sup> suggesting M-CSF is a survival factor for osteoclast precursor cells, and the "osteoclast differentiation factor (ODF)" was yet to be identified.<sup>12)</sup>

The long-sought ODF was cloned in 1998 by two groups independently.<sup>13), 14</sup> Interestingly, this cytokine, which belongs to the tumor necrosis factor (TNF) family, is identical to receptor activator of NF- $\kappa$ B ligand (RANKL)<sup>15</sup> and TNF-related activation-induced cytokine (TRANCE),<sup>16</sup> which had been cloned in the previous year in the immune system. The cloning and subsequent functional analyses of ODF (RANKL, hereafter) have clearly indicated the intimate relationship between the immune and bone systems.<sup>17</sup>

Further insights into the molecular mechanism of osteoclast differentiation and function have been provided by osteoclast-lacking or -deficient osteopetrosis including mi/mi (a naturally-occurring strain carrying mutation in the *MITF* gene), c-Srcdeficient, c-Fos-deficient, NF- $\kappa$ B p50/p52-deficient and TNF receptor-associated factor 6 (TRAF6)deficient mice.<sup>3), 18)</sup> TRAF6 is an adaptor protein essential for osteoclastogenesis, which is recruited to RANK and activates downstream molecules such as NF- $\kappa$ B and mitogen-activated protein kinases (MAPKs).<sup>19)</sup> Analyses of these mice revealed that c-Fos<sup>20)</sup> and microphthalmia transcription factor (MITF)<sup>21)</sup> are among the essential transcription factors for differentiation, whereas c-Src is crucial for the bone-resorbing activity of osteoclasts.<sup>22)</sup> These molecules have profound significance in the immune system, suggesting further the shared mechanism between immune and bone systems.

### Essential role of osteoclasts in bone destruction associated with inflammation

The bone destruction observed in the joints of rheumatoid arthritis (RA) patients presents a challenging clinical problem.<sup>5), 6)</sup> Osteoclasts are now known to play a pivotal role in the pathogenesis of bone destruction in RA, but it was not until RANKL was cloned that the importance of osteoclasts came into general acceptance. We previously demonstrated efficient osteoclast formation in synovial cell cultures obtained from RA patients.<sup>23)</sup> Moreover, the expression of RANKL was detected specifically in the synovium of RA patients but not in patient synovium of other bone diseases.<sup>24), 25)</sup> Recent studies have provided further direct genetic evidence: RANKLdeficient mice, which lack osteoclasts, were protected from bone destruction in an arthritis model induced by serum transfer of K/BxN mice.<sup>26)</sup> Bone erosion was not observed in osteopetrotic  $Fos^{-/-}$ mice, even when they were crossed with TNF- $\alpha$ transgenic mice that develop erosive arthritis spontaneously.<sup>27)</sup> In both cases, bone destruction did not occur despite a similar level of inflammation, indicating that RANKL and osteoclasts are indispensable for the inflammatory bone loss. Consistent with this, anti-RANKL and anti-osteoclast therapies have been shown to be beneficial in the treatment of an animal model of arthritis.<sup>17), 28)</sup> Although other inflammatory cytokines such as TNF- $\alpha$ , IL-1 and IL-6 may not be requisite for inflammatory bone loss, they are still important accelerators of the bone destruction in RA. TNF- $\alpha$  is considered especially important because anti-TNF therapy reduces bone erosion as well as inflammation.<sup>29)</sup> TNF- $\alpha$  induces RANKL and M-CSF in stromal cells and also stimulates osteoclast precursor cells to synergize with RANKL signaling.<sup>30)</sup> Despite the well-accepted importance of TNF- $\alpha$  in the acceleration of RANKL signaling, it remains controversial whether TNF- $\alpha$  induces osteoclast differentiation RANKL-independently.<sup>31)</sup>

### Interplay between T cells and osteoclasts

As RANKL is expressed in activated T cells, it is of vital importance to determine whether T cells have the capacity to induce osteoclast differentiation. This question is particularly important

in understanding the pathogenesis of RA joints, in which activated  $CD4^+$  (helper) T cells are infiltrated. Indeed, Kong et al. showed that RANKL expressed on activated T cells directly acts on osteoclast precursor cells and induces osteoclastogenesis in vitro.<sup>32)</sup> Horwood et al. also reported that in vitro osteoclastogenesis could be induced by activated T cells.<sup>33)</sup> Note, however, that the T cells were fixed by formaldehyde and could not release any humoral factors in the former paper.<sup>32)</sup> Therefore, the effects of membrane-bound factors including RANKL were selectively evaluated in this system. In the latter paper,<sup>33)</sup> the T cells and osteoclast precursor cells were derived from different species, suggesting the effect of cytokines such as IFN- $\gamma$  would be much lower than that on cells of the same species. To fully understand the effects of T cells on osteoclastogenesis, it is absolutely required to include the effects of various cytokines T cells produce. The question then arises as to how T cell cytokines other than RANKL affect osteoclast differentiation.

Helper T  $(T_H)$  cells are divided into two main subsets according to the cytokines they produce, namely,  $T_{\rm H}1$  and  $T_{\rm H}2$ .  $T_{\rm H}1$  cells produce mainly IFN- $\gamma$  and IL-2 and are involved in cellular immunity, whereas  $T_{\rm H}2$  cells mainly produce IL-4, IL-5, and IL-10 and are involved in humoral immunity. Some researchers consider RA to be a disease in which the  $T_{\rm H}1/T_{\rm H}2$  balance is skewed toward  $T_{\rm H}1$ . However, IFN- $\gamma$  and IL-2, the key cytokines produced by Th1 cells, are not highly expressed in RA joints.<sup>34)</sup> It is worth noting that IFN- $\gamma$  strongly inhibits osteoclastogenesis even at minute concentrations, suggesting normal  $T_{\rm H}1$  cells inhibit osteoclastogenesis and bone loss.<sup>35)</sup> Consistent with the protective function by IFN- $\gamma$ , IFN- $\gamma$ -receptor deficient mice exhibit more severe bone destruction in a collagen-induced arthritis mode.<sup>36), 37)</sup> Cytokines that induce  $T_{\rm H}1$  differentiation, namely IL-12 and IL-18, are also inhibitory to osteoclastogenesis.<sup>1), 6)</sup> Interestingly, IL-4 and IL-10, both of which are classic T<sub>H</sub>2-type cytokines, also inhibit osteoclastogenesis.<sup>1), 6)</sup> Therefore, the positive effect of T cells on osteoclastogenesis can be observed under strictly limited conditions.

# $T_{\rm H}17$ cells exclusively function as an osteoclastogenic helper T cell subset

What is the pathologically important  $T_H$  cell subset responsible for abnormal bone resorption? We defined this subset as osteoclastogenic  $T_{\rm H}$  ( $T_{\rm H}Oc$ ) cells and have long worked on the identification of this population.<sup>1), 6), 35)</sup> Our previous investigations showed the osteoclastogenic T cells (i.e.,  $T_HOc$  cells) in RA joints fulfill the characteristics as described below. First, T<sub>H</sub>Oc cells do not produce a large amount of IFN $\gamma$ . Second, T<sub>H</sub>Oc cells trigger local inflammation and production of inflammatory cytokines, including  $TNF\alpha$ , that induce RANKL expression on synovial fibroblasts. Third, T<sub>H</sub>Oc cells express RANKL and might directly participate in accelerated osteoclastogenesis. Because  $T_HOc$  cells have such osteoclastogenic characteristics, they can tip the balance in favour of osteoclastogenesis in various aspects. Although autoimmune arthritis has been traditionally categorized as a T<sub>H</sub>1-type disease,  $T_{\rm H}1$  cells do not have such characteristics, indicating that the  $T_HOc$  cells might belong to an as-yet unknown subset. We explored the effects of various CD4<sup>+</sup> T cell subsets on osteoclast differentiation, and identified IL-17-producing T cells ( $T_{\rm H}17$ cells) as the exclusive osteoclastogenic T cell subset  $(T_HOc)$  among the known CD4<sup>+</sup> T cell lineages, whereas  $T_{\rm H}1$  or  $T_{\rm H}2$  cells have marked antiosteoclastogenic effects.<sup>38)</sup> It has been already reported that IL-17 expression is increased in RA joints.<sup>39)</sup> IL-17 is well known to induce local inflammation in autoimmune diseases through inflammatory cytokine production.<sup>40)</sup> In addition, IL-17 induces RANKL on mesenchymal cells.<sup>39)</sup> We also showed that  $T_{\rm H}17$  cells express RANKL stronger than  $T_H1$  or  $T_H2$  subsets.<sup>38)</sup> Therefore,  $T_H17$  cells represent the long-sought  $T_HOc$  subset fulfilling all the criteria mentioned above and link the abnormal T-cell response to bone damage in arthritis (Fig. 1). Since osteoclast-mediated bone destruction in the LPS-induced bone loss model is abolished in mice deficient in IL-17 or IL-23, it is strongly suggested that  $T_H 17$  cells function as the  $T_H Oc$  subset in bone destruction associated with inflammation.<sup>38)</sup> It will be an important issue in the near future to determine the subset of T cells in the RA joints.  $T_{\rm H}17$ cells are essential for the onset phase of autoimmune arthritis,<sup>41)</sup> but these results show that they are also critical for bone destruction phase. Thus, pathogenesis of autoimmune arthritis should be reconsidered in the context of a  $T_{\rm H}$ 17-type disease. Clearly, this subset will be an auspicious target of future therapy.



#### Fig. 1. Mechanism of bone destruction in autoimmune arthritis.

In rheumatoid arthritis, inflammatory synovium invades and destroys bone, which is mediated by osteoclasts induced by receptor activator of nuclear factor- $\kappa$ B (NF- $\kappa$ B) ligand (RANKL). CD4<sup>+</sup> T-cell infiltration, a hallmark of the pathogenesis of arthritis, link the abnormal immune responses to the activation of osteoclastic bone resorption. Interleukin (IL)-17-producing helper T (T<sub>H</sub>17) cells are the only osteoclastogenic T<sub>H</sub>-cell (T<sub>H</sub>Oc) subset characterized so far. T<sub>H</sub>17 cells do not produce interferon (IFN)- $\gamma$ , which suppresses RANKL signaling, but secrete a huge amount of IL-17 that induces RANKL on synovial fibroblasts. IL-17 also stimulates the local inflammation and activates synovial macrophages to secrete pro-inflammatory cytokines such as tumour necrosis factor (TNF)- $\alpha$ , IL-1, and IL-6. These cytokines activate osteoclastogenesis by either directly acting on osteoclast precursor cells or inducing RANKL on synovial fibroblasts. T<sub>H</sub>17 cells also express RANKL on their membrane, which partly contributes to the enhanced osteoclastogenesis.





RANKL binding to RANK results in the recruitment of TRAF6, which activates NF- $\kappa$ B and MAPKs. The induction of NFATc1, a key transcription factor for osteoclastogenesis, is dependent on the transcription factors AP-1 (containing c-Fos) and NF- $\kappa$ B. Costimulatory signals for RANK: immunoreceptors associated with ITAM-harboring adaptors stimulate calcium (Ca<sup>2+</sup>) signaling. NFATc1 is localized to the nucleus after the dephosphorylation by calcineurin that is activated by Ca<sup>2+</sup> signaling. Ca<sup>2+</sup>/calmodulin kinases IV is a main kinase that activates cAMP response element-binding protein (CREB), which is also important for osteoclast differentiation. Induction of c-Fos is partly mediated by CREB. In the nucleus, NFATc1 works together with other transcription factors such as AP-1, PU.1, MITF and CREB to induce various osteoclast-specific genes.

# NFATc1-the master transcription factor for osteoclastogenesis

In the course of genomewide screening of RANKL-inducible genes, we found NFATc1 to be the most highly induced transcription factor in osteoclast precursor cells.<sup>42)</sup> This induction is mediated by the autoamplification of NFATc1: NFATc1 binds to the *Nfatc1* promoter and induces itself.<sup>43)</sup> This strategy is often observed in hematologic cells that undergo irreversible differentiation.  $Nfatc1^{-/-}$ embryonic stem (ES) cells cannot differentiate into osteoclasts in vitro and overexpression of NFATc1 induces osteoclastogenesis. These results suggest that NFATc1 is the master regulator of osteoclastogenesis,<sup>42)</sup> but it has proven difficult to show that this transcription factor is indispensable for osteoclast differentiation in vivo due to the embryonic lethality of  $Nfatc1^{-/-}$  mice. Recently, we provided genetic evidence that *Nfatc1* is essential for osteoclast differentiation in vivo by generating chimeric mice, in which the NFATc1 gene is disrupted in the osteoclast lineage (by adoptive transfer of  $Nfatc1^{-/-}$  hematopoietic stem cells to osteoclastdeficient  $Fos^{-/-}$  mice and by  $Fos^{-/-}$  blastocyst complementation.<sup>43</sup>) The role of NFAT in osteoblast differentiation and bone formation has also been unveiled recently.<sup>44)</sup> The NFAT family of transcription factors was originally identified in immune cells, but these results indicate that NFAT family members also play a crucial role in the regulation of both limbs of the bone remodeling process, i.e., bone resorption and formation.

### Immunoreceptors in osteoclastogenesis

The close relationship between the bone and immune system extends beyond the cytokines and transcription factors they share. Activation and nuclear localization of NFAT are dependent on its dephosphorylation by the phosphatase calcineurin, which is activated by calcium  $(Ca^{2+})$  signaling.  $Ca^{2+}$  oscillation is observed during osteoclastogenesis, and the calcineurin inhibitors cyclosporin A and FK506 strongly inhibit osteoclastogenesis.<sup>42</sup> However, it is not clear how calcium signaling is activated during osteoclastogenesis. DNAX-activating protein 12 (DAP12) is an adaptor molecule that associates with immunoglobulin-like receptors and harbors an immunoreceptor tyrosine-based activation motif (ITAM), an important signaling motif found in various receptor subunits in T, B, natural killer and myeloid cells. The osteopetrotic phenotype in DAP12-deficient mice made evident the importance of ITAM, which is known to be crucial for the activation of  $Ca^{2+}$  signaling in immune cells.<sup>45)</sup> Mice doubly deficient in DAP12 and the Fc receptor common  $\gamma$  subunit (FcR $\gamma$ ) have been shown to exhibit severe osteopetrosis owing to the lack of osteoclasts, indicating that immunoglobulinlike receptors (that associate with DAP12 or  $FcR\gamma$ ) provide the third essential signal required for osteoclastogenesis in addition to the RANK and M-CSF receptor.<sup>46</sup> Immunoreceptor signaling alone cannot induce osteoclastogenesis, suggesting that these receptors provide a costimulatory signal for RANKL.<sup>47)</sup> For the next step, it is important to identify immunoglobulin-like receptors and their ligand in bone cells.  $FcR\gamma$ -associating receptors include osteoclast-associated receptor (OSCAR) and paired immunoglobulin-like receptor (PIR)-A, and DAP12- associating receptors include triggering receptor expressed on myeloid cells (TREM)-2 and signal-regulatory protein (SIRP)- $\beta$ 1, although the ligands for these immunoreceptors are yet to be identified. Recent studies showed that calcium signaling activates the transcription factor cAMP response element-binding protein (CREB) through calcium/calmodulin kinase, which contributes to both osteoclast differentiation and function (Fig. 2).48)

## Toward novel strategies for anti-osteoclast therapy

Rheumatologists are now aware of the great impact that anti-TNF therapy has made on the management of RA, but other cytokines will soon be targeted by similar strategies. In addition, new findings in osteoimmunology are being applied to the development of new methods for the control of excessive osteoclastogenesis. The efficacy of an anti-RANKL antibody for postmenopausal osteoporosis in clinical trials has been reported.<sup>49)</sup> New NF- $\kappa$ B inhibitors are also under developement. Based on recent findings, NFATc1 is another important research target for future therapy against excessive osteoclastogenesis. Indeed, antirheumatic drugs inhibit osteoclastogenesis by suppressing the induction of NFATc1 in osteoclast precursor cells.<sup>50), 51)</sup> FK506 and cyclosporine A are used for the treatment of RA, but it is reported that NFATs play a critical role in bone formation<sup>44</sup>): caution will be needed therefore for long-term administration, and the development of an osteoclast-specific drug delivery system would be of great clinical utility.

### Conclusion

The emerging field of osteoimmunology originates from studies on bone destruction in RA. Increasing evidence suggests that the skeletal and immune systems are connected in complex ways, and it would be difficult to understand either system adequately without the insights afforded by studying their interaction in an osteoimmunological context. Osteoimmunology will also provide a molecular basis for the development of novel therapeutic approaches to bone and immune diseases, a number of which have been very difficult to treat.

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### Profile

Hiroshi Takayanagi was born in 1965 and was initially trained as an orthopaedic surgeon and rheumatologist in the Faculty of Medicine, University of Tokyo. He began his research career in 1996 with studies on the mechanism of bone destruction in arthritis in the Department of Orthopaedic Surgery (Prof. Kozo Nakamura) and the Department of Immunology (Prof. Tadatsugu Taniguchi), Graduate School of Medicine, University of Tokyo. He discovered that bone destruction in arthritis is attributable to the enhanced expression of osteoclast differentiation factor, RANKL. He was awarded Ph.D. in 2001 by University of Tokyo and became Associate at the Department of Immunology, University of Tokyo. He further studied the regulation of bone metabolism by the immune system by focusing on the control of osteoclast differentiation and revealed the role of immunomodulatory



factors including interferons, NFATc1 and immunoglobulin-like receptors in the skeletal system. In 2003, he was promoted to Professor at Tokyo Medical and Dental University and continued to explore the interdisciplinary field, osteoimmunology. He was awarded the Amersham Biosciences and Science Prize for Young Scientists in 2002, Japan Academy Medal in 2005 and the Japan Society for the Promotion of Science Prize in 2005. He has been the Associate Member, Science Council of Japan since 2006, the Vice President of the Asia Pacific League of Associations for Rheumatology (APLAR) since 2004 and the organizer of the International Conference on Osteoimmunology since 2005.