Yongwon Choi *Editor*

Osteoimmunology

Interactions of the Immune and Skeletal Systems

Springer

Osteoimmunology

Yongwon Choi
Editor

Osteoimmunology

Interactions of the Immune and Skeletal Systems

Springer

Editor
Yongwon Choi
Department of Pathology and Laboratory Medicine
University of Pennsylvania School of Medicine
Philadelphia, PA, USA

ISBN 978-1-4614-5365-9 ISBN 978-1-4614-5366-6 (eBook)
DOI 10.1007/978-1-4614-5366-6
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2012952101

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Contents

Contributors

Ulrike Baschant Institute for Age Research–Fritz Lipmann Institute, Jena, Germany

Kirsten Braem Laboratory of Tissue Homeostasis and Disease, Skeletal Biology and Engineering Research Center, Department of Development and Regeneration, Leuven, Belgium

Sun Wook Cho Department of Periodontics and Oral Medicine, Medical School, University of Michigan, Ann Arbor, MI, USA

Jennifer Davis Division of Bone and Mineral Diseases, Departments of Medicine and Pathology, Washington University School of Medicine, St. Louis, MO, USA

Laurie H. Glimcher Department of Immunology and Infectious Diseases, Harvard School of Public Health, and Department of Medicine, Harvard Medical School, Boston, MA, USA

Matthew B. Greenblatt Department of Immunology and Infectious Diseases, Harvard School of Public Health, and Department of Medicine, Harvard Medical School, Boston, MA, USA

Masaru Ishii, MD, PhD Laboratory of Cellular Dynamics, WPI-Immunology Frontier Research Center, Osaka University, Yamada-oka, Suita, Osaka, Japan

Junichi Kikuta Laboratory of Cellular Dynamics, WPI-Immunology Frontier Research Center, Osaka University, Yamada-oka, Suita, Osaka, Japan

Hong-Hee Kim Department of Cell and Developmental Biology, BK21 and Dental Research Institute, Seoul National University, Seoul, Republic of Korea

Department of Cell and Developmental Biology, School of Dentistry, Seoul National University, Seoul, Republic of Korea

Hyung Joon Kim Department of Cell and Developmental Biology, BK21 and Dental Research Institute, Seoul National University, Chongno-Gu, Seoul 110-749, Republic of Korea

Atsuko Kubo Laboratory of Cellular Dynamics, WPI-Immunology Frontier Research Center, Osaka University, Yamada-oka, Suita, Osaka, Japan

Youngkyun Lee Department of Cell and Developmental Biology, BK21 and Dental Research Institute, Seoul National University, Seoul, Republic of Korea

Joseph Lorenzo, MD University of Connecticut Health Center, Farmington, CT, USA

Rik J. Lories Laboratory of Tissue Homeostasis and Disease, Skeletal Biology and, Engineering Research Center, Department of Development and Regeneration, Leuven, Belgium

Laurie McCauley Department of Periodontics and Oral Medicine, University of Michigan, Ann Arbor, MI, USA

Pathology Department, Medical School, University of Michigan, Ann Arbor, MI, USA

Katherine McCoy Division of Bone and Mineral Diseases, Departments of Medicine and Pathology, Washington University School of Medicine, St. Louis, MO, USA

Deborah Novack Division of Bone and Mineral Diseases, Departments of Medicine and Pathology, Washington University School of Medicine, St. Louis, MO, USA

Roberto Pacifici, MD Division of Endocrinology, Metabolism and Lipids, Department of Medicine, Emory University School of Medicine, Atlanta, GA, USA

Immunology and Molecular Pathogenesis Program, Emory University, Atlanta, GA, USA

Georg Schett, MD Department of Internal Medicine 3, University of Erlangen-Nuremberg, Erlangen, Germany

Alexander Rauch Institute for Age Research – Fritz Lipmann Institute, Jena, Germany

Jae-Hyuck Shim Department of Immunology and Infectious Diseases, Harvard School of Public Health, and Department of Medicine, Harvard Medical School, Boston, MA, USA

Hiroshi Takayanagi Department of Cell Signaling, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan

Global Center of Excellence (GCOE) Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo, Japan

Japan Science and Technology Agency (JST), ERATO, Takayanagi Osteonetwork Project, Tokyo, Japan

Sakae Tanaka Department of Orthopaedic Surgery, Faculty of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

Steven L. Teitelbaum Department of Pathology and Immunology, Washington University in St. Louis School of Medicine, St. Louis, MO, USA

Anna Teti Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, L'Aquila, Italy

Jan Tuckermann Institute for Age Research – Fritz Lipmann Institute, Jena, Germany

Chang Yang Division of Bone and Mineral Diseases, Departments of Medicine and Pathology, Washington University School of Medicine, St. Louis, MO, USA

Tetsuro Yasui Department of Orthopaedic Surgery, Faculty of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

Hisataka Yasuda Nagahama Institute for Biochemical Science, Oriental Yeast Co., Nagahama, Shiga, Japan

Weiguo Zou Department of Immunology and Infectious Diseases, Harvard School of Public Health, and Department of Medicine, Harvard Medical School, Boston, MA, USA

Wei Zou Department of Pathology and Immunology, Washington University in St. Louis School of Medicine, St. Louis, MO, USA

Osteoimmunology: An Introduction

Joseph Lorenzo

It has been almost 40 years since it was first observed that cells of the immune system could influence the functions of bone [1]. Since that time, significant strides have been made in our understanding of the interactions between hematopoietic, immune, and bone cells. This field is now known as "osteoimmunology," which is a term that was first coined by Arron and Choi in an commentary in Nature in 2000 [2]. In the 11 years since the field has grown markedly. PubMed now lists over 2,000 references that combine the terms immunology with osteoclasts or osteoblasts. The 3rd International Conference on Osteoimmunology was designed to further nurture the development of this field. It met at the Nomikos Conference Center Fira, Santorini, Greece, from June 20 to 25, 2010. Over 145 participants heard a rich program that was composed of 22 half hour talks and 11 short 10-min talks. In addition, there were 65 abstracts presented as posters. The subjects covered in these presentations spanned all the current topic areas of this field. Talks were divided into sessions. These included Niche and Soil, three session on the Basic Concepts of Osteoimmunology: Osteoblasts, Osteoclasts and Cross Talk, and a session on emerging areas entitled "Up and Coming."

However, the most important function of this meeting was to bring together researchers from the sometimes-disparate fields of immunology and bone biology so that they could interact, exchange ideas, and develop new collaborations. The Organizing Committee for this meeting is deeply indebted to its many sponsors in both the public and private sector who provided the resources that made the meeting an overwhelming success.

The goal was to speed our understanding of the mechanisms regulating the interactions of immune and bone cells. It has now become clear that both systems

J. Lorenzo, M.D. (✉)
University of Connecticut Health Center, Farmington, CT, USA
e-mail: Lorenzo@nso2.uchc.edu

Y. Choi (ed.), *Osteoimmunology: Interactions of the Immune and Skeletal Systems,*
DOI 10.1007/978-1-4614-5366-6_1,

interact and regulate each other in both health and disease. By better understanding these interactions it is hoped that novel insights will be developed, which may lead to new therapies to prevent or reverse human diseases that affect these organ systems.

References

1. Horton JE, Raisz LG, Simmons HA, Oppenheim JJ, Mergenhagen SE (1972) Bone resorbing activity in supernatant fluid from cultured human peripheral blood leukocytes. Science 177:793–795
2. Arron JR, Choi Y (2000) Bone versus immune system [news]. Nature 408:535–536

NF-κB and Inflammatory Bone Loss: "Alternative" Family Members Take Their Place at the Table

Deborah Novack, Chang Yang, Jennifer Davis, and Katherine McCoy

1 Regulation of Alternative NF-κB Signaling by RANKL

In unstimulated cells, alternative NF-κB is negatively regulated in two ways, by destabilization of NIK protein and retention of RelB in the cytoplasm (Fig. 1). NIK binds TRAF3, which recruits cIAP1/2 via TRAF2 [3, 4]. In this complex, the E3 ubiquitin ligase of cIAP1/2 targets NIK, leading to its proteosomal degradation. RelB is held inactive in the cytoplasm by p100, a bifunctional protein that serves as a precursor for the NF-κB subunit p52 and contains a C-terminal IκB domain. Upon RANKL stimulation, TRAF3 is recruited to its receptor RANK, and intact NIK is released. NIK phosphorylates IKKα, which then phosphorylates p100 causing its processing via the proteosome. The IκB domain of p100 is degraded and RelB/p52 dimers are released, allowing their accumulation in the nucleus. Downstream targets of this NIK/RelB pathway include c-fos and NFATc1, both transcription factors required for OC differentiation. NIK-deficient mice are unable to process p100 to p52, preventing nuclear translocation, and thus activation, of RelB [5].

D. Novack (✉) • C. Yang • J. Davis • K. McCoy
Division of Bone and Mineral Diseases, Departments of Medicine and Pathology,
Washington University School of Medicine,
St. Louis, MO 63110, USA
e-mail: novack@wustl.edu; cyang@dom.wustl.edu; jldavis@dom.wustl.edu;
beddowk@wusm.wustl.edu

Y. Choi (ed.), *Osteoimmunology: Interactions of the Immune and Skeletal Systems,*
DOI 10.1007/978-1-4614-5366-6_2,

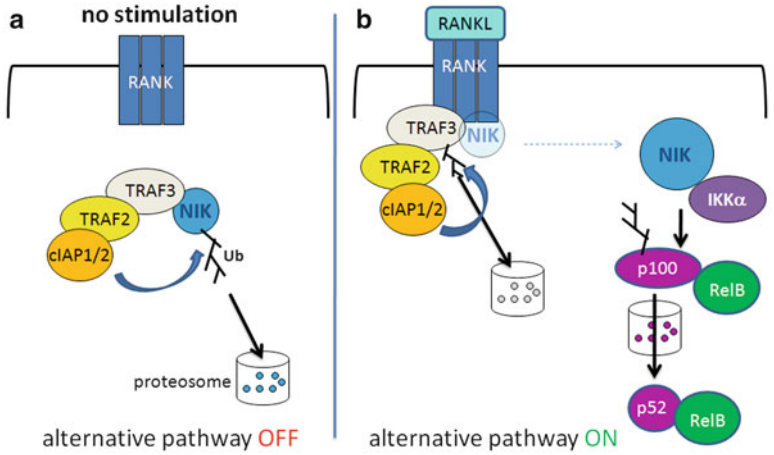

Fig. 1 Activation of alternative NF-κB pathway by RANKL. (**a**) In resting cells, NIK protein levels are low because cIAP1/2 ubiquitinates NIK within a TRAF3/TRAF2 complex, targeting it for proteosomal degradation. Under these conditions, the alternative pathway is off. (**b**) Upon RANKL binding, RANK recruits TRAF3, causing a rearrangement of the complex that changes the substrate of cIAP1/2 from NIK to TRAF3. TRAF3 is degraded by the proteosome, allowing NIK to accumulate. High NIK levels activate IKKα, causing processing of p100 to p52 by the proteosome. RelB/p52 dimers are released to travel to the nucleus, thus activating the downstream targets of the alternative pathway. In OC precursors in vitro, this pathway remains on for several days of RANKL stimulation

2 NIK-Deficient Mice Have Defects in Stimulated Osteoclastogenesis

Unmanipulated NIK−/− mice have only a small increase in bone mass, with a normal number of TRAP+ osteoclasts [2, 5]. Mice lacking RelB, the NF-κB subunit downstream of NIK, have a similar phenotype. Strikingly, however, NIK−/− precursors are unable to form OCs in vitro with RANKL stimulation or in coculture with osteoblasts [5], a property shared with RelB−/− precursors [2]. This led us to examine models of stimulated osteoclastogenesis and bone loss in vivo. Direct injection of RANKL above the calvarium induced a very weak osteoclastogenic response in NIK−/− mice, compared to WT littermates [5]. Using the serum transfer arthritis model, in which arthritogenic serum from K/BxN mice was injected into WT and NIK−/− mice on days 0, 2, and 7, we found that NIK-deficiency did not alter the inflammatory response, but did block osteolysis [6]. NIK−/− mice had few osteoclasts on periosteal surfaces of the hindpaw bones, showed significantly less bone erosion, and had no significant increase in serum TRAP5b levels. Thus, the alternative NF-κB pathway, mediated by NIK and RelB, is important for pathological bone loss, but not basal bone homeostasis.

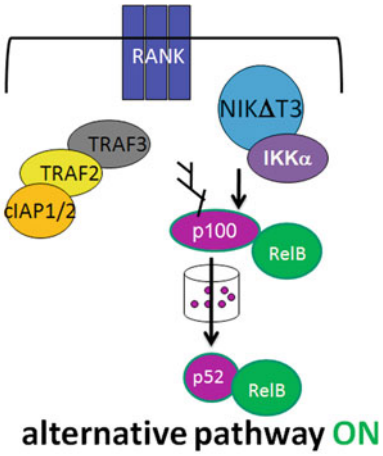

Fig. 2 Alternative NF-κB pathway is activated by NIKΔT3. NIK lacking its TRAF3 binding domain (NIKΔT3) is unable to bind the TRAF3/TRAF2/cIAP complex. Therefore, NIKΔT3 is stabilized in the cell, even without RANKL stimulation. In consequence, p100 is constitutively processed to p52, and RelB/p52 can be found in the nucleus of resting cells

3 Expression of Constitutively Active NIK in OCs Causes Osteoporosis

Mutation of the TRAF3 binding domain of NIK prevents its rapid degradation in resting cells [7], leading to constitutive activation of the alternative NF-κB pathway (Fig. 2). Recently, transgenic mice bearing cDNA for TRAF3-binding defective NIK (NIKΔT3) in the ROSA26 locus with an upstream lox-STOP-lox sequence were generated [8]. In order to express NIKΔT3 in the osteoclast lineage, we mated these mice to both cathepsin K Cre (catK-Cre) and lysozyme M Cre (lysM-Cre) mice. In NIKΔT3.lysM-Cre bone marrow macrophages, NIK is stabilized, processing of p100 to p52 is enhanced, and both p65 and RelB are present in the nucleus constitutively [9]. In the NIKΔT3.catK-Cre line, the NIKΔT3 is recombined with 2–3 days of RANKL stimulation in culture, with an associated increase in NIK protein levels and p100 processing. Using either Cre, expression of activated NIK drives osteoclastogenesis at lower doses of RANKL, leading to increased expression of markers of osteoclast differentiation. Furthermore, NIKΔT3+ osteoclasts have larger actin rings and have more resorptive activity than nontransgenic controls, in vitro. In vivo, transgenic expression of constitutively active NIK in the OC lineage causes osteoporosis. NIKΔT3.catK-Cre mice have low bone mass (BV/TV and BMD) by microCT. Serum levels of CTX and osteocalcin are increased; by histomorphometry, numbers of osteoclasts are increased along with bone formation rates. Thus, despite expression only in the osteoclast lineage, NIKΔT3 generates high-turnover osteoporosis. Furthermore, NIKΔT3.catK-Cre mice demonstrate significantly more osteolysis in response to serum transfer arthritis than littermate controls.

4 Summary

Although it has been known that NF-κB are an important signal for osteoclast differentiation since the late 1990s [10, 11], the specific role of each NF-κB pathway has been less defined. By studying both globally NIK-deficient mice and mice expressing constitutively active NIK in the OC lineage, as well as RelB-deficient mice, we have demonstrated that the alternative NF-κB pathway controls osteoclastogenesis and bone mass in vivo. RANKL-induced NIK activation sends a pro-differentiation precursors to OC precursors that are critical for the osteolytic response in inflammatory arthritis, without large changes in basal bone mass. Constitutive NIK activation causes both osteoporosis and enhanced inflammatory osteolysis.

References

1. Vaira S, Alhawagri M, Anwisye I, Kitaura H, Faccio R, Novack DV (2008) RelA/p65 promotes osteoclast differentiation by blocking a RANKL-induced apoptotic JNK pathway in mice. J Clin Invest 118:2088–2097
2. Vaira S, Johnson T, Hirbe AC et al (2008) RelB is the NF-κB subunit downstream of NIK responsible for osteoclast differentiation. Proc Natl Acad Sci USA 105:3897–3902
3. Vallabhapurapu S, Matsuzawa A, Zhang W et al (2008) Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-κB signaling. Nat Immunol 9:1364–1370
4. Zarnegar BJ, Wang Y, Mahoney DJ et al (2008) Noncanonical NF-κB activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK. Nat Immunol 9:1371–1378
5. Novack DV, Yin L, Hagen-Stapleton A et al (2003) The IκB function of NF-κB2 p100 controls stimulated osteoclastogenesis. J Exp Med 198:771–781
6. Aya K, Alhawagri M, Hagen-Stapleton A, Kitaura H, Kanagawa O, Novack DV (2005) NF-κB-inducing kinase controls lymphocyte and osteoclast activities in inflammatory arthritis. J Clin Invest 115:1848–1854
7. Liao G, Zhang M, Harhaj EW, Sun SC (2004) Regulation of the NF-kappaB-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation. J Biol Chem 279:26243–26250
8. Sasaki Y, Calado DP, Derudder E et al (2008) NIK overexpression amplifies, whereas ablation of its TRAF3-binding domain replaces BAFF:BAFF-R-mediated survival signals in B cells. Proc Natl Acad Sci USA 105:10883–10888
9. Yang C, McCoy K, Davis JL et al (2010) NIK stabilization in osteoclasts results in osteoporosis and enhanced inflammatory osteolysis. PLoS One 5:e15383
10. Iotsova V, Caamano J, Loy J, Yang Y, Lewin A, Bravo R (1997) Osteopetrosis in mice lacking NF-κB1 and NF-κB2. Nat Med 3:1285–1289
11. Franzoso G, Carlson L, Xing L et al (1997) Requirement for NF-κB in osteoclast and B-cell development. Genes Dev 11:3482–3496

Adaptive Immune Responses and Bone

Hiroshi Takayanagi

1 Introduction

The bone enables locomotive activity, the storage of calcium and the harboring of hematopoietic stem cells (HSCs) [1]. The multifunctional tissue is continuously renewed by a process, called bone remodeling. This is dependent on the dynamic balance between bone formation and resorption, which are mediated by osteoblasts and osteoclasts, respectively. A delicate regulation of this process is requisite for normal bone homeostasis, and an imbalance is often related to bone and joint diseases [2].

Accumulating evidence has indicated that the immune and skeletal systems share a number of regulatory molecules, including cytokines, receptors, signaling molecules, and transcription factors. Furthermore, immune cells are formed and HSCs are maintained in the bone marrow where they interact with bone cells. Therefore, the evidence that the physiology and pathology of one system might affect the other is compelling and the term osteoimmunology was coined to cover these overlapping scientific fields. The most typical example of the interaction between the skeletal and adaptive immune systems is seen in the abnormal and/or prolonged activation of the immune system in autoimmune diseases such as rheumatoid arthritis (RA), which is characterized by progressive multiple joint destruction. Since autoreactive T lymphocytes are considered to play a key role in the pathogenesis of RA, attention

H. Takayanagi (✉)
Department of Cell Signaling, Graduate School of Medical and Dental Sciences,
Tokyo Medical and Dental University,
Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8549, Japan

Global Center of Excellence (GCOE) Program, International Research Center for Molecular Science in Tooth and Bone Diseases,
Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8549, Japan

Japan Science and Technology Agency (JST), ERATO, Takayanagi Osteonetwork Project,
Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8549, Japan
e-mail: taka.csi@tmd.ac.jp

Y. Choi (ed.), *Osteoimmunology: Interactions of the Immune and Skeletal Systems,*
DOI 10.1007/978-1-4614-5366-6_3,

must be paid to the relationship between osteoclast-mediated bone destruction and aberrant adaptive immune responses in order to develop effective therapeutic strategies against RA. Here, we summarize recent progress in the understanding of the relationship between bone and the adaptive immune system in arthritis by focusing mainly on osteoclasts and osteoclastogenic helper T cells, Th17 cells.

2 RANK/RANKL in Osteoclastogenesis

Osteoclasts are large, multinucleated cells formed by the fusion of precursor cells of monocyte/macrophage lineage [2]. Mature osteoclasts degrade bone matrix proteins by secreting proteolytic enzymes, such as cathepsin K and matrix metalloproteinase, and decalcify the inorganic components of bone by releasing hydrochloric acid. In the late 1980s, an in vitro osteoclast formation system was established which utilizes a system of culturing bone marrow-derived cells of monocyte/macrophage lineage together with osteoclastogenesis-supporting cells such as osteoblasts [3, 4]. These supporting mesenchymal cells provide certain factors that are necessary for osteoclast differentiation [5]. Analysis of *op*/*op* mice with osteopetrosis revealed one of these essential factors to be macrophage colony-stimulating factor (M-CSF) [6]. M-CSF stimulation alone, however, does not induce the differentiation of osteoclasts. Forced expression of anti-apoptotic molecule Bcl-2 partially rescues the osteopetrotic phenotype of the *op*/*op* mice [7], suggesting that M-CSF is a survival factor for osteoclast precursor cells. Ultimately, Yasuda et al. and Lacey et al. did clone the long-sought ligand mediating the essential signal for osteoclast differentiation in 1998, which was called ODF and osteoprotegerin ligand, respectively [8, 9]. Interestingly, this cytokine, which belongs to the tumor necrosis factor (TNF) family, was shown to be identical to receptor activator of nuclear factor-κB ligand (RANKL) and TNF-related activation-induced cytokine (TRANCE), which had been cloned in the immune system [10, 11]. The cloning of ODF (RANKL, hereafter) enabled investigation of the differentiation process in a sophisticated culture system employing recombinant RANKL and M-CSF [12].

The receptor for RANKL is RANK, a type I transmembrane protein which possesses a high homology with CD40. RANK is expressed on osteoclast precursor cells and mature osteoclasts, and the binding of RANKL to RANK is inhibited by the decoy receptor osteoprotegerin (OPG) [13, 14]. In bone, RANKL is expressed by osteoclastogenesis-supporting cells including osteoblasts, in response to osteoclastogenic factors, such as 1,25-dihydroxyvitamin D_3, prostaglandin E_2, and parathyroid hormone, and is a crucial determinant of the level of bone resorption in vivo [5, 12]. Mice with a disruption of either *Rank* or *Rankl* exhibit severe osteopetrosis accompanied by a defect in tooth eruption resulting from a complete lack of osteoclasts [15–17]. In contrast, mice lacking *Opg* exhibit a severe form of osteoporosis caused by both an increased number and enhanced activity of osteoclasts [18, 19]. These genetic findings clearly demonstrate that RANK/RANKL signaling is essential

for osteoclastogenesis in vivo. Furthermore, mutations in RANK, RANKL, and OPG have been identified in human patients with bone disorders such as familial expansile osteolysis, autosomal recessive osteopetrosis, and juvenile Paget's disease of bone, respectively [20–23].

3 Signal Transduction Downstream of RANK

The ligation of RANK with RANKL results in trimerization of RANK and recruitment of adaptor molecules such as the TNF receptor-associated factor (TRAF) family of proteins, among which TRAF6 has been shown to be the major adaptor molecule [24, 25]. TRAF6 trimerizes upon RANK stimulation, and activates nuclear factor-κB (NF-κB) and mitogen-activated protein kinases, including Jun N-terminal kinase (JNK) and p38. RANK also activates the transcription-factor complex, activator protein 1 (AP-1), through the induction of its component c-Fos [26]. The induction mechanism of c-Fos is dependent on the activation of Ca^{2+}/calmodulin-dependent kinase IV (CaMKIV) and cyclic adenosine monophosphate responsive-element-binding protein (CREB) [27], as well as the activation of NF-κB [28]. Importantly, RANKL specifically and potently induces nuclear factor of activated T cells cytoplasmic 1 (NFATc1), the master regulator of osteoclast differentiation, and this induction is dependent on both the TRAF6 and c-Fos pathways [29]. The activation of NFAT is mediated by a specific phosphatase, calcineurin, which is activated by calcium–calmodulin signaling. The *NFATc1* promoter contains NFAT binding sites, and NFATc1 specifically autoregulates its own promoter during osteoclastogenesis, thus enabling the robust induction of NFATc [30]. The essential role of NFATc1 has been conclusively demonstrated by genetic experiments [30–32]. NFATc1 regulates a number of osteoclast-specific genes, such as cathepsin K, tartrate-resistant acid phosphatase (TRAP), calcitonin receptor, osteoclast-associated receptor (OSCAR), and β3 integrin, in cooperation with other transcription factors such as AP-1, PU.1, microphthalmia-associated transcription factor (MITF), and CREB (Fig. 1).

During osteoclastogenesis, activation of calcium signaling is dependent on costimulatory receptors for RANK, which are immunoglobulin-like receptors, such as OSCAR and triggering receptor expressed in myeloid cells-2 (TREM-2). These receptors associate with the adaptor molecules Fc receptor common γ subunit (FcRγ) and DNAX-activating protein 12 (DAP12), transducing signals by the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) within the adaptor proteins, which, in turn, recruits the spleen tyrosine kinase (Syk) [33, 34] (Fig. 1). It has been recently shown that Tec family tyrosine kinases (Tec and Btk) activated by RANK cooperate with Syk to induce efficient phosphorylation of phospholipase Cγ (PLCγ), which induces the release of calcium from the endoplasmic reticulum through the generation of inositol trisphosphate [35]. Although a series of genetically modified mice has clearly shown that ITAM-mediated signals are essential for osteoclastogenesis, the ligands for the costimulatory receptors remain to be identified [33–35].

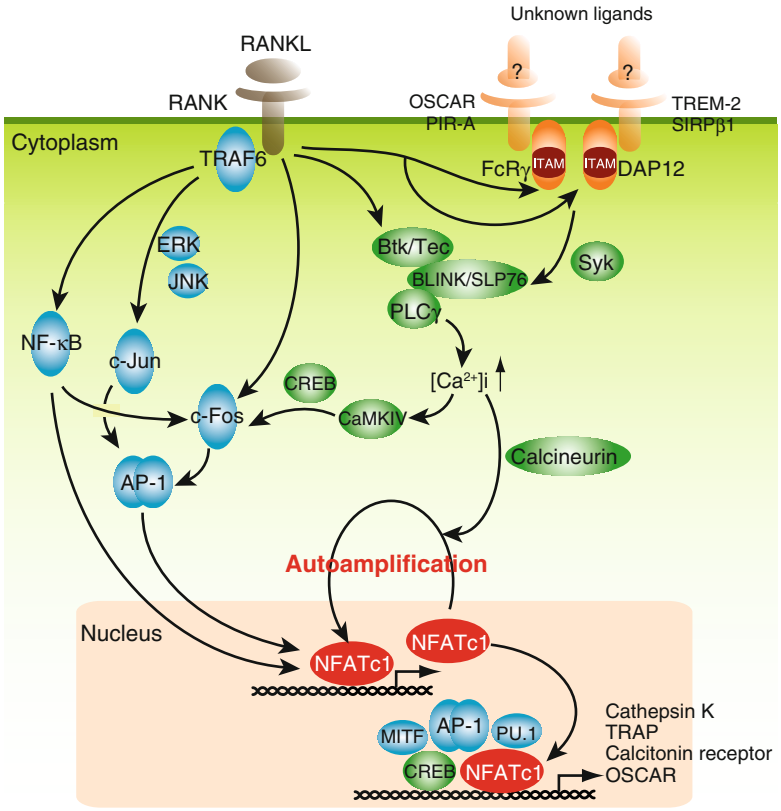

Fig. 1 Signal transduction during osteoclastogenesis. Receptor activator of nuclear factor-κB ligand (RANKL)-RANK binding results in the recruitment of tumor necrosis factor receptor-associated factor (TRAF)6, which activates nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs). RANKL also stimulates the induction of c-Fos through NF-κB and Ca^{2+}/calmodulin-dependent kinase IV (CaMKIV). NF-κB and c-Fos are important for the robust induction of nuclear factor of activated T cells cytoplasmic 1 (NFATc1). Several costimulatory receptors associate with the immunoreceptor tyrosine-based activation motif (ITAM)-harboring adaptors, Fc receptor common γ subunit (FcRγ), and DNAX-activating protein 12 (DAP12): osteoclast-associated receptor (OSCAR) and triggering receptor expressed in myeloid cells-2 (TREM2) associate with (FcRγ), and signal-regulatory protein β1 (SIRPβ1) and paired immunoglobulin-like receptor-A (PIR-A) associate with DAP12. RANK and ITAM signaling cooperate to phosphorylate phospholipase Cγ (PLCγ), and activate calcium signaling, which is critical for the activation and autoamplification of NFATc1. Tec family tyrosine kinases (Tec and Btk) activated by RANK are important for the formation of the osteoclastogenic signaling complex composed of Tec kinases, B-cell linker (BLNK)/SH2 domain-containing leukocyte protein of 76 kDa (SLP76) (activated by ITAM-spleen tyrosine kinase (Syk)) and PLCγ, all of which are essential for the efficient phosphorylation of PLCγ

4 The Essential Role of Osteoclasts in Bone Destruction in RA

The bone destruction observed in the joints of patients with RA presents a challenging clinical problem. In the early 1980s, researchers observed osteoclast-like cells at the bone destruction sites [36], but it was not until RANKL was cloned that the importance of osteoclasts became generally accepted. We previously demonstrated efficient osteoclast formation in synovial cell cultures obtained from patients with RA [37]. Moreover, the expression of RANKL was detected specifically in the synovium of patients with RA [38, 39]. Recent studies have provided further direct genetic evidence: RANKL-deficient mice, which lack osteoclasts, were protected from bone destruction in an arthritis model induced by serum transfer [40]. Bone erosion was not observed in osteopetrotic *Fos*$^{-/-}$ mice, even when they were crossed with TNF-α transgenic mice, which develop erosive arthritis spontaneously [41]. In both cases, a similar level of inflammation was observed, indicating that RANKL and osteoclasts are indispensable for the bone loss but not the inflammation. Consistent with this, anti-RANKL and anti-osteoclast therapies have been shown to be beneficial in the treatment of bone damage in animal models of arthritis [42, 43]. Inflammatory cytokines such as TNF-α, interleukin (IL)-1, and IL-6 have a potent capacity to induce RANKL expression on synovial fibroblasts/osteoblasts and to facilitate RANKL signaling, thus directly contributing to the bone destruction process. In particular, TNF-α is considered of special importance since anti-TNF therapy reduces bone erosion as well as inflammation [44].

5 Effect of T Cells on Osteoclastogenesis

As infiltration of T cells into the synovium is a pathological hallmark of RA, it is vital to address how T-cell immunity is linked to the enhanced expression of RANKL and eventual osteoclastic bone resorption. More specifically, as RANKL is known to be expressed in activated T cells, it is important to determine whether this source of RANKL can directly induce osteoclast differentiation. In 1999, Kong et al. showed that the RANKL expressed on activated T cells directly acts on osteoclast precursor cells and induces osteoclastogenesis in vitro [42]. Horwood et al. also reported that osteoclastogenesis could be induced in vitro by activated T cells [45]. However, it is important to note that T cells produce various cytokines, including interferon (IFN)-γ, IL-4, and IL10, which exert potent inhibitory effects on osteoclast differentiation [2]. In the former study, the T cells were fixed by formaldehyde and were thus unable to release any humoral factors [42]. In the latter study, the T cells and osteoclast precursor cells were derived from different species, suggesting that the effect of cytokines would in all likelihood be much lower than that on cells of the same species [45]. The question then arises as to how T-cell cytokines other than RANKL affect osteoclast differentiation.

Upon activation, naïve $CD4^+$ T cells differentiate into different lineages of helper T (Th) cells, depending on the cytokine milieu [46]. Th1 and Th2 cells are traditionally thought to be the major subsets generated upon antigenic stimulation. Th1 cells, which are induced by IL-12, produce mainly IFN-γ and are involved in cellular immunity; Th2 cells mainly produce IL-4, IL-5, and IL-10 and contribute to humoral immunity. RA was previously considered to be a disease in which the Th1–Th2 balance is skewed towards Th1. However, IFN-γ is not highly expressed in the joints of RA patients [47]. Notably, IFN-γ strongly inhibits osteoclastogenesis, even at minute concentrations, through ubiquitin-proteasome-mediated degradation of TRAF6 [48]. Moreover, the severity of collagen-induced arthritis was reported to be exaggerated in the absence of IFN-γ signaling [49, 50], suggesting that Th1 cells are not linked to bone damage in arthritis.

6 Th17 Cells Function as Osteoclastogenic Th Cells

It is worthwhile to define what is believed to be a very rare but pathologically important Th cell subset which is responsible for abnormal bone resorption as osteoclastogenic Th cells. Previous investigations in our laboratory together with other studies on synovial T cells in RA have clarified the characteristics of osteoclastogenic Th cells in autoimmune arthritis [51]. First, osteoclastogenic Th cells do not produce a large amount of IFN-γ. Second, they trigger both local inflammation and the production of inflammatory cytokines that induce RANKL expression on synovial fibroblasts. Third, osteoclastogenic Th cells express RANKL and might thereby directly participate in accelerated osteoclastogenesis. Because these Th cells have such osteoclastogenic characteristics, they can tip the balance in favor of osteoclastogenesis synergistically.

Th17 cells have recently been identified as a new effector Th cell subset characterized by the production of proinflammatory cytokines including IL-17, IL-17F, IL-21, and IL-22. Th17 cell differentiation is induced by the combination of IL-6 and transforming growth factor (TGF)-β. IL-23 is dispensable for the lineage commitment of Th17 cells, but is required for the growth, survival, and effector functions of Th17 cells [52, 53]. Importantly, this unique subset plays a critical role in host defense against certain extracellular pathogens and also contributes to the pathogenesis of various autoimmune diseases [53]. Recent data from our laboratory indicate that Th17 cells represent the long sought-after osteoclastogenic Th-cell subset, fulfilling all of the criteria mentioned above [54]. IL-17 induces RANKL on osteoclastogenesis-supporting mesenchymal cells, such as osteoblasts and synovial fibroblasts [55]. IL-17 also enhances local inflammation and increases the production of inflammatory cytokines, which further promote RANKL expression and activity. Therefore, the infiltration of Th17 cells into the inflammatory lesion is the link between the abnormal T-cell response and bone damage (Fig. 2).

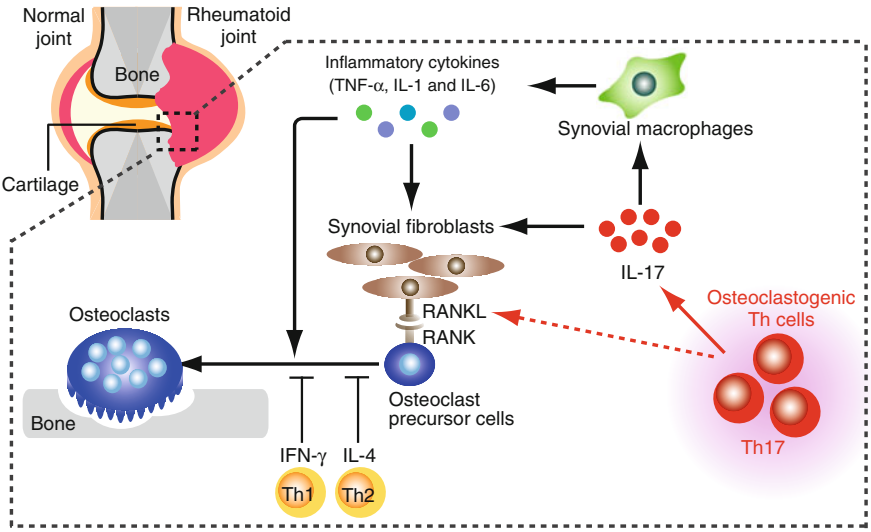

Fig. 2 Osteoclast regulation by T cells in rheumatoid arthritis. Interleukin (IL)-17-producing helper T (Th17) cells have stimulatory effects on osteoclastogenesis and play an important role in the pathogenesis of rheumatoid arthritis through IL-17, while Th1 and Th2 cells have inhibitory effects on osteoclastogenesis through interferon (IFN)-γ and IL-4, respectively. IL-17 not only induces receptor activator of nuclear factor-κB ligand (RANKL) on synovial fibroblasts of mesenchymal origin but also activates local inflammation, leading to the upregulation of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1, and IL-6. These cytokines activate osteoclastogenesis by either directly acting on osteoclast precursor cells or inducing RANKL on synovial fibroblasts. Th17 cells also express RANKL on their cellular membrane, which partly contributes to the enhanced osteoclastogenesis

7 Effects of Regulatory T Cells on Osteoclastogenesis

$CD4^+$ $CD25^+$ regulatory T (Treg) cells are a specialized T cell subset that engages in the maintenance of immunological self-tolerance and immune homeostasis, as evidenced by the development of severe autoimmune disease, allergy, and immunopathology in humans and mice with a mutation of forkhead box P3 (Foxp3), an master regulator for the Treg cell lineage [56]. Treg cells can be classified into two main populations, $FoxP3^+$ naturally occurring Treg cells generated in the thymus and $FoxP3^+$ Treg cells induced by antigen stimulation in a milieu rich in TGF-β in the periphery. Although the differences and similarities between these two populations are yet to be fully elucidated, both have been considered to be essential for immune homeostasis. Notably, Th17 cells and Treg cells are reciprocally regulated during differentiation, but exert the opposite effects on autoimmunity, and the balance between these populations is associated with inflammation and autoimmune diseases [53, 56]. In many studies, Treg cells were found in high number within joint fluid from RA patients [57–59]. However, Treg cells in joint fluid from RA patients failed to suppress effector T cell proliferation or cytokine production. This is because

inflammatory cytokines including IL-6 and TNF-α attenuate Treg function. Effector T cells in joint fluid were also reported to be resistant to suppression by Treg cells. In addition, serum markers of bone resorption such as CTx inversely correlated with the number of $CD4^+CD25^+$ Treg cells in peripheral blood of healthy control and RA patients [60]. Thus, it is of key interest whether Treg cells affect inflammatory-associated bone destruction. Several groups have reported the inhibitory effect of Treg cells on osteoclastogenesis and bone resorption, but no consensus regarding their inhibitory mechanisms has been established. Dr. Kim et al. reported the human $CD4^+CD25^+$ Treg cells isolated from peripheral blood mononuclear cells (PBMCs) suppress osteoclast differentiation in a cytokine-dependent manner, and proposed that TGF-β and IL-4 are required for the suppressive function of Treg cells [61]. Dr. Zaiss et al. demonstrated the inhibitory effect of $CD4^+CD25^+$ Treg cells purified from mouse spleen on osteoclast differentiation [62]. However, they showed that $CD4^+CD25^+$ Treg cells inhibit osteoclastogenesis partially via IL-4 and IL-10 production, but mainly through cell-to-cell contact via cytotoxic T lymphocyte antigen 4. It is notable that wild-type Treg cells failed to inhibit the differentiation of osteoclasts from $CD80/86^{-/-}$ monocytes [63]. A decrease in osteoclast number and bone resorption was observed after transfer of $CD4^+CD25^+$ Treg cells into Rag1-deficient mice, indicating that Treg cells could directly block osteoclastogenesis without engaging effector T cells [63]. Furthermore, Dr. Luo et al. have recently reported that human PBMC-derived $CD4^+CD25^+$ Treg cells suppress osteoclastogenesis and bone resorption in a TGF-β1 and IL-10 cytokine-dependent manner [64]. Since TGF-β, IL-10, and IL-4 are well-known cytokines to inhibit osteoclastogenesis, these cytokines produced by Treg cells may be, at least partially, involved in the suppressive function of Treg cells on osteoclastogenesis. In all studies by these three groups, Treg cells were activated before coculture experiments, but their culture conditions varied, which may cause the difference among their results. Dr. Zaiss et al. also reported increased bone mass and partial protection from bone loss after ovariectomy in Foxp3 transgenic mice [63]. $Foxp3^+$ Treg cells have been shown to protect against local and systemic bone destruction in the mouse model of TNF-α-induced arthritis [60]. Taken as a whole, it is likely that $Foxp3^+$ Treg cells exert inhibitory effects on inflammatory-associated bone destruction, but it is important to consider the possibility that the characteristics of Treg cells are affected by the specific microenvironment such as autoimmune inflammation, as described above. Additional studies would be needed to determine how Treg cells affect osteoclast-mediated bone destruction under inflammatory conditions.

8 Mechanisms Underlying Th17 Cell Differentiation

Th17 cell subset has emerged as attractive therapeutic targets for both inflammation and bone destruction. It is therefore important to understand the molecular mechanism underlying Th17 development in order to develop novel therapeutic strategies.

Helper T cell differentiation is initiated by the T cell receptor signal in combination with other cytokine receptor signals. These signals induce the activation of specific transcription factors to promote lineage-specific cytokine production [46]. For example, the T-box-containing protein expressed in T cells, which is activated by IL-12 and IFN-γ, is required for Th1 cell differentiation. Th2 cell differentiation requires the function of the GATA binding protein 3 which is induced by the IL-4-activated signal transducer and activator of transcription (Stat) 6.

Soon after the discovery of Th17 cells, Dr. Littman and his colleagues reported that retinoid-related orphan receptor (ROR)γt is selectively expressed in Th17 cells and is required for Th17 cell differentiation [65]. RORγt expression is induced by the combination of IL-6 and TGF-β through STAT3. Furthermore, RORγt deficiency was shown to lead to an impairment of Th17 cell differentiation both in vitro and in vivo. Subsequent studies by Dr. Dong and his colleagues showed that another ROR family member, RORα, is also highly induced during Th17 cell differentiation in a STAT3-dependent manner [66]. Although RORα deletion in mice had only a minimal effect on IL-17 production, the deficiency of both RORα and RORγt completely abolished IL-17 production and protected mice from EAE. Thus, RORγt and RORα have redundant functions, but RORγt seems to be the major player in Th17 cell differentiation. Although the mechanisms by which the ROR nuclear receptors drive Th17 development and production of Th17-related cytokines such as IL-17 have not yet been fully elucidated, they are considered to be essential factors for Th17 development.

9 Regulation of Th17 Development by IκBζ

We found that a nuclear IκB family member, IκBζ, was most highly expressed in Th17 cells among the helper T cell subsets [67]. IκBζ is a nuclear protein highly homologous to Bcl-3, which interacts with the NF-κB subunit via the ankyrin repeat domain [68]. Its expression is rapidly induced by TLR ligands or IL-1 stimulation in peritoneal macrophages. Yamamoto et al. demonstrated using IκBζ-deficient mice that IκBζ is essential for the LPS induction of a subset of secondary response genes, including IL-6 and the IL-12 p40 subunit in macrophages [69]. However, no attempt to determine the function of IκBζ in T cells was reported in their study.

IκBζ expression was shown to be upregulated by the combination of IL-6 and TGF-β. IκBζ induction was mediated by Stat3, but not by RORγt, in Th17 cells. Importantly, not only IκBζ-deficient mice but also Rag2-deficient mice transferred with IκBζ-deficient $CD4^+$ T cells were shown to be highly resistant to EAE. When naïve $CD4^+$ T cells were activated in vitro under Th1- and Th2-polarizing conditions, IκBζ-deficient naïve $CD4^+$ T cells normally produced IFN-γ and IL-4, respectively. On the other hand, when activated under Th17-polarizing conditions, IL-17 production in IκBζ-deficient T cells was markedly reduced compared to wild-type T cells. Since the expression of RORγt and RORα was shown to be

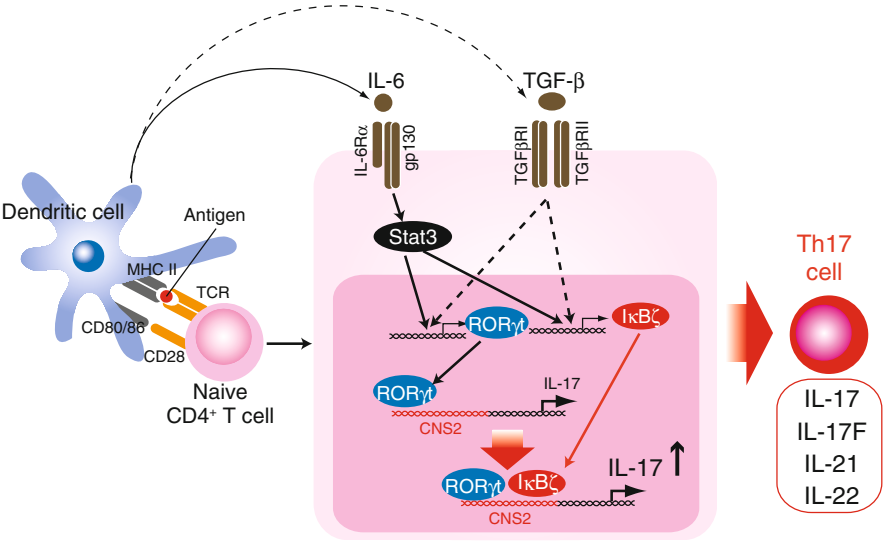

Fig. 3 IκBζ and ROR nuclear receptors are essential for Th17 development. Interleukin (IL)-6 and transforming growth factor (TGF)-β induce Th17 cell differentiation, in which the ROR nuclear receptors, RORγt and RORα, have an indispensable role. The expression of IκBζ is induced by the combination of IL-6 and TGF-β. IκBζ induction is mediated by signal transducer and activator of transcription (Stat) 3 but not RORγt. IκBζ and ROR nuclear receptor bind directly to the CNS2 region of the *Il17* promoter and cooperatively activate the *Il17* promoter. Notably, recruitment of IκBζ to the CNS2 region was dependent on RORγt, suggesting that the binding of both IκBζ and ROR nuclear receptors to the *Il17* promoter leads to an efficient recruitment of transcriptional coactivators having histone acetylase activity. *TCR* T cell receptor, *MHC II* major histocompatibility complex class II

normal in IκBζ-deficient T cells, it is unlikely that ROR nuclear receptors function downstream of IκBζ or vice versa.

Although ROR nuclear receptors have been proposed as essential regulators for Th17 development as described above, several groups have reported that the ectopic expression of RORγt or RORα leads to only modest IL-17 production in the absence of IL-6 and TGF-β [66, 70]. The ectopic expression of IκBζ in naïve CD4+ T cells did not induce IL-17 production in the absence of IL-6 and TGF-β. Interestingly, however, even in the absence of IL-6 and TGF-β, the ectopic expression of IκBζ, together with RORγt or RORα, potently induced IL-17 production. A reporter assay system showed that IκBζ moderately activated the promoter of the mouse *Il17* gene as well as RORγt and RORα. When the ROR nuclear receptor was expressed, IκBζ highly activated the *Il17* promoter. Previous studies showed that an evolutionarily conserved noncoding sequences (CNS) 2 region in the *Il17* locus is associated with histone H3 acetylation in a Th17 lineage-specific manner and that the ROR nuclear receptor is recruited to the CNS2 region during Th17 development [66, 71, 72]. In combination with RORγt and RORα, IκBζ potently induced the CNS2 enhancer activity. IκBζ was recruited to the CNS2 region in Th17 cells, and recruitment of

IκBζ to the CNS2 region was dependent on RORγt function (Fig. 3). Moreover, the expression of IL-17F, IL-21, and IL-23 receptor was decreased in IκBζ-deficient T cells. IκBζ also bound to the promoter or the enhancer region of these genes in Th17 cells. Collectively, these findings indicate that IκBζ is critical for the transcriptional program in Th17 cell lineage commitment [67].

10 Conclusion

Th17 cell subset is an auspicious target for future therapeutic investigation, and cytokines related to Th17 cell differentiation and function will be of great clinical importance. Antibodies against IL-17 or IL-23 would be expected to exert beneficial effects in autoimmune diseases, and antibodies targeting the IL-6 receptor might also inhibit Th17 development in RA, in addition to effecting a direct inhibition of local inflammation and osteoclastogenesis [73, 74]. Although further studies will be required to determine whether or how IκBζ synergizes with other transcriptional regulators of Th17 cells, our results raise the possibility that the targeting of IκBζ may prove effective in the treatment of autoimmune diseases. Th17 cells are also implicated in host defense against a number of microorganism, therefore great care will be required so as to effectively treat autoimmune diseases without compromising the host defense system.

Acknowledgments I am grateful to Dr. Kazuo Okamoto for the assistance in preparing the manuscript. This work was supported in part by Grants-in-Aid for GCOE Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), and ERATO, Takayanagi Osteonetwork Project from JST.

References

1. Seeman E, Delmas PD (2006) Bone quality – the material and structural basis of bone strength and fragility. N Engl J Med 354(21):2250–2261
2. Takayanagi H (2007) Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat Rev Immunol 7(4):292–304
3. Takahashi N, Akatsu T, Udagawa N, Sasaki T, Yamaguchi A, Moseley JM, Martin TJ, Suda T (1988) Osteoblastic cells are involved in osteoclast formation. Endocrinology 123(5):2600–2602
4. Takahashi N, Yamana H, Yoshiki S, Roodman GD, Mundy GR, Jones SJ, Boyde A, Suda T (1988) Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. Endocrinology 122(4):1373–1382
5. Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ (1999) Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 20(3):345–357
6. Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD (1990) The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 345(6274):442–444
7. Lagasse E, Weissman IL (1997) Enforced expression of Bcl-2 in monocytes rescues macrophages and partially reverses osteopetrosis in *op/op* mice. Cell 89(7):1021–1031

8. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A et al (1998) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 95(7):3597–3602
9. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S et al (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93(2):165–176
10. Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, DuBose RF, Cosman D, Galibert L (1997) A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 390(6656):175–179
11. Wong BR, Rho J, Arron J, Robinson E, Orlinick J, Chao M, Kalachikov S, Cayani E, Bartlett FS 3rd, Frankel WN et al (1997) TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. J Biol Chem 272(40):25190–25194
12. Theill LE, Boyle WJ, Penninger JM (2002) RANK-L and RANK: T cells, bone loss, and mammalian evolution. Annu Rev Immunol 20:795–823
13. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T et al (1997) Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 89(2):309–319
14. Tsuda E, Goto M, Mochizuki S, Yano K, Kobayashi F, Morinaga T, Higashio K (1997) Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. Biochem Biophys Res Commun 234(1):137–142
15. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A et al (1999) OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 397(6717):315–323
16. Dougall WC, Glaccum M, Charrier K, Rohrbach K, Brasel K, De Smedt T, Daro E, Smith J, Tometsko ME, Maliszewski CR et al (1999) RANK is essential for osteoclast and lymph node development. Genes Dev 13(18):2412–2424
17. Li J, Sarosi I, Yan XQ, Morony S, Capparelli C, Tan HL, McCabe S, Elliott R, Scully S, Van G et al (2000) RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. Proc Natl Acad Sci USA 97(4):1566–1571
18. Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL et al (1998) *osteoprotegerin*-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 12(9):1260–1268
19. Mizuno A, Amizuka N, Irie K, Murakami A, Fujise N, Kanno T, Sato Y, Nakagawa N, Yasuda H, Mochizuki S et al (1998) Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. Biochem Biophys Res Commun 247(3):610–615
20. Hughes AE, Ralston SH, Marken J, Bell C, MacPherson H, Wallace RG, van Hul W, Whyte MP, Nakatsuka K, Hovy L et al (2000) Mutations in *TNFRSF11A*, affecting the signal peptide of RANK, cause familial expansile osteolysis. Nat Genet 24(1):45–48
21. Whyte MP, Obrecht SE, Finnegan PM, Jones JL, Podgornik MN, McAlister WH, Mumm S (2002) Osteoprotegerin deficiency and juvenile Paget's disease. N Engl J Med 347(3):175–184
22. Sobacchi C, Frattini A, Guerrini MM, Abinun M, Pangrazio A, Susani L, Bredius R, Mancini G, Cant A, Bishop N et al (2007) Osteoclast-poor human osteopetrosis due to mutations in the gene encoding RANKL. Nat Genet 39(8):960–962
23. Guerrini MM, Sobacchi C, Cassani B, Abinun M, Kilic SS, Pangrazio A, Moratto D, Mazzolari E, Clayton-Smith J, Orchard P et al (2008) Human osteoclast-poor osteopetrosis with hypogammaglobulinemia due to *TNFRSF11A* (*RANK*) mutations. Am J Hum Genet 83(1):64–76
24. Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A, Morony S, Capparelli C, Van G, Kaufman S et al (1999) TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev 13(8):1015–1024
25. Naito A, Azuma S, Tanaka S, Miyazaki T, Takaki S, Takatsu K, Nakao K, Nakamura K, Katsuki M, Yamamoto T et al (1999) Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in *TRAF6*-deficient mice. Genes Cells 4(6):353–362

26. Wagner EF, Eferl R (2005) Fos/AP-1 proteins in bone and the immune system. Immunol Rev 208:126–140
27. Sato K, Suematsu A, Nakashima T, Takemoto-Kimura S, Aoki K, Morishita Y, Asahara H, Ohya K, Yamaguchi A, Takai T et al (2006) Regulation of osteoclast differentiation and function by the CaMK-CREB pathway. Nat Med 12(12):1410–1416
28. Yamashita T, Yao Z, Li F, Zhang Q, Badell IR, Schwarz EM, Takeshita S, Wagner EF, Noda M, Matsuo K et al (2007) NF-κB p50 and p52 regulate receptor activator of NF-κB ligand (RANKL) and tumor necrosis factor-induced osteoclast precursor differentiation by activating c-Fos and NFATc1. J Biol Chem 282(25):18245–18253
29. Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, Saiura A, Isobe M, Yokochi T, Inoue J et al (2002) Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cell 3(6):889–901
30. Asagiri M, Sato K, Usami T, Ochi S, Nishina H, Yoshida H, Morita I, Wagner EF, Mak TW, Serfling E et al (2005) Autoamplification of NFATc1 expression determines its essential role in bone homeostasis. J Exp Med 202(9):1261–1269
31. Winslow MM, Pan M, Starbuck M, Gallo EM, Deng L, Karsenty G, Crabtree GR (2006) Calcineurin/NFAT signaling in osteoblasts regulates bone mass. Dev Cell 10(6):771–782
32. Aliprantis AO, Ueki Y, Sulyanto R, Park A, Sigrist KS, Sharma SM, Ostrowski MC, Olsen BR, Glimcher LH (2008) NFATc1 in mice represses osteoprotegerin during osteoclastogenesis and dissociates systemic osteopenia from inflammation in cherubism. J Clin Invest 118(11):3775–3789
33. Koga T, Inui M, Inoue K, Kim S, Suematsu A, Kobayashi E, Iwata T, Ohnishi H, Matozaki T, Kodama T et al (2004) Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. Nature 428(6984):758–763
34. Mocsai A, Humphrey MB, Van Ziffle JA, Hu Y, Burghardt A, Spusta SC, Majumdar S, Lanier LL, Lowell CA, Nakamura MC (2004) The immunomodulatory adapter proteins DAP12 and Fc receptor γ-chain (FcRγ) regulate development of functional osteoclasts through the Syk tyrosine kinase. Proc Natl Acad Sci USA 101(16):6158–6163
35. Shinohara M, Koga T, Okamoto K, Sakaguchi S, Arai K, Yasuda H, Takai T, Kodama T, Morio T, Geha RS et al (2008) Tyrosine kinases Btk and Tec regulate osteoclast differentiation by linking RANK and ITAM signals. Cell 132(5):794–806
36. Bromley M, Woolley DE (1984) Chondroclasts and osteoclasts at subchondral sites of erosion in the rheumatoid joint. Arthritis Rheum 27(9):968–975
37. Takayanagi H, Oda H, Yamamoto S, Kawaguchi H, Tanaka S, Nishikawa T, Koshihara Y (1997) A new mechanism of bone destruction in rheumatoid arthritis: synovial fibroblasts induce osteoclastogenesis. Biochem Biophys Res Commun 240(2):279–286
38. Gravallese EM, Manning C, Tsay A, Naito A, Pan C, Amento E, Goldring SR (2000) Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. Arthritis Rheum 43(2):250–258
39. Takayanagi H, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, Koshihara Y, Oda H, Nakamura K, Tanaka S (2000) Involvement of receptor activator of nuclear factor κB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. Arthritis Rheum 43(2):259–269
40. Pettit AR, Ji H, von Stechow D, Muller R, Goldring SR, Choi Y, Benoist C, Gravallese EM (2001) TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. Am J Pathol 159(5):1689–1699
41. Redlich K, Hayer S, Ricci R, David JP, Tohidast-Akrad M, Kollias G, Steiner G, Smolen JS, Wagner EF, Schett G (2002) Osteoclasts are essential for TNF-α-mediated joint destruction. J Clin Invest 110(10):1419–1427
42. Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, Capparelli C, Li J, Elliott R, McCabe S et al (1999) Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature 402(6759):304–309
43. Takayanagi H, Juji T, Miyazaki T, Iizuka H, Takahashi T, Isshiki M, Okada M, Tanaka Y, Koshihara Y, Oda H et al (1999) Suppression of arthritic bone destruction by adenovirus-mediated *csk* gene transfer to synoviocytes and osteoclasts. J Clin Invest 104(2):137–146

44. Takayanagi H (2009) Osteoimmunology and the effects of the immune system on bone. Nat Rev Rheumatol 5(12):667–676
45. Horwood NJ, Kartsogiannis V, Quinn JM, Romas E, Martin TJ, Gillespie MT (1999) Activated T lymphocytes support osteoclast formation in vitro. Biochem Biophys Res Commun 265(1):144–150
46. Zhou L, Chong MM, Littman DR (2009) Plasticity of $CD4^+$ T cell lineage differentiation. Immunity 30(5):646–655
47. Firestein GS, Zvaifler NJ (1990) How important are T cells in chronic rheumatoid synovitis? Arthritis Rheum 33(6):768–773
48. Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, Takaoka A, Yokochi T, Oda H, Tanaka K et al (2000) T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-γ. Nature 408(6812):600–605
49. Manoury-Schwartz B, Chiocchia G, Bessis N, Abehsira-Amar O, Batteux F, Muller S, Huang S, Boissier MC, Fournier C (1997) High susceptibility to collagen-induced arthritis in mice lacking IFN-γ receptors. J Immunol 158(11):5501–5506
50. Vermeire K, Heremans H, Vandeputte M, Huang S, Billiau A, Matthys P (1997) Accelerated collagen-induced arthritis in IFN-γ receptor-deficient mice. J Immunol 158(11):5507–5513
51. Sato K, Takayanagi H (2006) Osteoclasts, rheumatoid arthritis, and osteoimmunology. Curr Opin Rheumatol 18(4):419–426
52. Kastelein RA, Hunter CA, Cua DJ (2007) Discovery and biology of IL-23 and IL-27: related but functionally distinct regulators of inflammation. Annu Rev Immunol 25:221–242
53. Korn T, Bettelli E, Oukka M, Kuchroo VK (2009) IL-17 and Th17 cells. Annu Rev Immunol 27:485–517
54. Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, Tanaka S, Kodama T, Akira S, Iwakura Y et al (2006) Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. J Exp Med 203(12):2673–2682
55. Kotake S, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, Saito S, Inoue K, Kamatani N, Gillespie MT et al (1999) IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. J Clin Invest 103(9):1345–1352
56. Sakaguchi S, Yamaguchi T, Nomura T, Ono M (2008) Regulatory T cells and immune tolerance. Cell 133(5):775–787
57. Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, Mauri C (2004) Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFα therapy. J Exp Med 200(3):277–285
58. Boissier MC, Assier E, Falgarone G, Bessis N (2008) Shifting the imbalance from Th1/Th2 to Th17/treg: the changing rheumatoid arthritis paradigm. Joint Bone Spine 75(4):373–375
59. Nistala K, Wedderburn LR (2009) Th17 and regulatory T cells: rebalancing pro- and anti-inflammatory forces in autoimmune arthritis. Rheumatology (Oxford) 48(6):602–606
60. Zaiss MM, Frey B, Hess A, Zwerina J, Luther J, Nimmerjahn F, Engelke K, Kollias G, Hunig T, Schett G et al (2010) Regulatory T cells protect from local and systemic bone destruction in arthritis. J Immunol 184(12):7238–7246
61. Kim YG, Lee CK, Nah SS, Mun SH, Yoo B, Moon HB (2007) Human CD4+CD25+ regulatory T cells inhibit the differentiation of osteoclasts from peripheral blood mononuclear cells. Biochem Biophys Res Commun 357(4):1046–1052
62. Zaiss MM, Axmann R, Zwerina J, Polzer K, Guckel E, Skapenko A, Schulze-Koops H, Horwood N, Cope A, Schett G (2007) Treg cells suppress osteoclast formation: a new link between the immune system and bone. Arthritis Rheum 56(12):4104–4112
63. Zaiss MM, Sarter K, Hess A, Engelke K, Bohm C, Nimmerjahn F, Voll R, Schett G, David JP (2010) Increased bone density and resistance to ovariectomy-induced bone loss in FoxP3-transgenic mice based on impaired osteoclast differentiation. Arthritis Rheum 62(8):2328–2338
64. Luo CY, Wang L, Sun C, Li DJ (2011) Estrogen enhances the functions of $CD4^+CD25^+Foxp3^+$ regulatory T cells that suppress osteoclast differentiation and bone resorption in vitro. Cell Mol Immunol 8(1):50–58

65. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR (2006) The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17$^+$ T helper cells. Cell 126(6):1121–1133
66. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, Ma L, Shah B, Panopoulos AD, Schluns KS et al (2008) T helper 17 lineage differentiation is programmed by orphan nuclear receptors RORα and RORγ. Immunity 28(1):29–39
67. Okamoto K, Iwai Y, Oh-hora M, Yamamoto M, Morio T, Aoki K, Ohya K, Jetten AM, Akira S, Muta T et al (2010) IκBζ regulates T_H17 development by cooperating with ROR nuclear receptors. Nature 464(7293):1381–1385
68. Muta T (2006) IκB-ζ: an inducible regulator of nuclear factor-κB. Vitam Horm 74:301–316
69. Yamamoto M, Yamazaki S, Uematsu S, Sato S, Hemmi H, Hoshino K, Kaisho T, Kuwata H, Takeuchi O, Takeshige K et al (2004) Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IκBζ. Nature 430(6996):218–222
70. Brustle A, Heink S, Huber M, Rosenplanter C, Stadelmann C, Yu P, Arpaia E, Mak TW, Kamradt T, Lohoff M (2007) The development of inflammatory T_H-17 cells requires interferon-regulatory factor 4. Nat Immunol 8(9):958–966
71. Zhang F, Meng G, Strober W (2008) Interactions among the transcription factors Runx1, RORγt and Foxp3 regulate the differentiation of interleukin 17-producing T cells. Nature Immunol 9(11):1297–1306
72. Akimzhanov AM, Yang XO, Dong C (2007) Chromatin remodeling of interleukin-17 (IL-17)-IL-17F cytokine gene locus during inflammatory helper T cell differentiation. J Biol Chem 282(9):5969–5972
73. Takatori H, Kanno Y, Chen Z, O'Shea JJ (2008) New complexities in helper T cell fate determination and the implications for autoimmune diseases. Mod Rheumatol 18(6):533–541
74. Mihara M, Ohsugi Y, Kishimoto T (2009) Evidence for the role of Th17 cell inhibition in the prevention of autoimmune diseases by anti-interluekin-6 receptor antibody. Biofactors 35(1):47–51

Intracellular Calcium Signaling for Osteoclast Differentiation

Hyung Joon Kim, Youngkyun Lee, and Hong-Hee Kim

1 Introduction

The concentration of calcium in the cytoplasm of the cell is tightly regulated to be about 100 nM in general [1]. In contrast, calcium concentrations in the extracellular fluid (~1 mM) and intracellular endoplasmic reticulum (ER; ~100 μM) are high. Various factors signal to the cell via regulation of the cytosolic calcium concentration by modulating the calcium trafficking through the plasma and ER membranes. The calcium signaling pathway ultimately results in changes in gene expression and cell metabolism and plays critical roles in determining diverse cellular responses such as proliferation, differentiation, apoptosis, and secretion [2]. Interestingly, the intracellular calcium response to an external stimulus displays different patterns depending on the signaling stimulus and the responding cell type. While a brief rise-and-fall pattern of spike is one mode of the calcium signal, repetition of transient calcium spikes forming an oscillatory pattern is also present in various systems [1, 3, 4]. Variation in both the frequency and the amplitude of the oscillatory calcium spikes may contribute to the modulation of versatile responses by calcium signals. In fact, the frequency of calcium spikes has been shown to be as various as from a few seconds to several hours. The difference in calcium frequency was even reported to be responsible for the activation of different transcription factors [5].

H.J. Kim • Y. Lee
Department of Cell and Developmental Biology, BK21 and Dental Research Institute, Seoul National University, Seoul 110-749, Republic of Korea

H.-H. Kim (✉)
Department of Cell and Developmental Biology, BK21 and Dental Research Institute, Seoul National University, Seoul 110-749, Republic of Korea

Department of Cell and Developmental Biology, School of Dentistry, Seoul National University, 28 Yeongon-Dong, Chongno-Gu, Seoul 110-749, Republic of Korea
e-mail: hhbkim@snu.ac.kr

Y. Choi (ed.), *Osteoimmunology: Interactions of the Immune and Skeletal Systems,*
DOI 10.1007/978-1-4614-5366-6_4,

Osteoclasts differentiate from monocyte/macrophage lineage of hematopoietic cells [6]. The commitment to osteoclast lineage is triggered by receptor activator of nuclear factor kappa B ligand (RANKL) that binds to its receptor RANK on the precursor cells [7, 8]. The RANK–RANKL interaction leads to recruitment of TNF receptor-associated factor (TRAF) members to RANK and subsequent activation of several intracellular signaling pathways [9]. The activated signaling molecules include mitogen-activated protein kinases (MAPKs) and Src family members, PI-3K, Akt, TAK, NIK, and IKK. [9, 10]. In addition, intracellular calcium signaling was found to be essential for osteoclast differentiation by RANKL [11]. These signaling pathways ultimately converge on the regulation of transcription factors that control the expression of genes required for osteoclastic phenotype manifestation and bone resorption function. The transcription factors targeted downstream RANK include c-Fos, CREB, NFATc1, as well as NFκB [9, 12].

2 Ca^{2+} Oscillation in Osteoclasts

Genomic approaches to identify genes differentially expressed by RANKL treatment in bone marrow-derived macrophages (BMMs; osteoclast precursor cells) led to the identification of NFATc1 as one of RANKL target genes [11]. Initial increase of the NFATc1 protein level in the cytoplasm is succeeded by accumulation of the transcription factor in the nucleus during RANKL-induced osteoclastogenesis [11]. As the nuclear translocation of NFAT proteins requires dephosphorylation by calcineurin which is activated by calcium/calmodulin [1, 13, 14], it was reasoned that calcium signaling could be involved in the RANKL induction of NFATc1. Subsequent time lapse tracing experiments of intracellular calcium revealed an oscillatory pattern of calcium spikes in RANKL-treated BMMs [11]. The calcium oscillation appeared to be specific to RANKL as the calcium response was not induced in BMMs treated with IL-1 or M-CSF. Intriguingly, there was about a 24 h time lag before appearance of calcium oscillation after RANKL treatment, suggesting that new gene transcription and protein synthesis might be required for the regulation of intracellular calcium levels in differentiating osteoclasts. Blocking the calcium response by using a calcium chelator-suppressed NFATc1 induction by RANKL and reduced the generation of osteoclasts from BMMs [11]. Therefore, the calcium oscillation seems to be critical for RANKL stimulation of osteoclastogenesis. However, Kuroda et al. reported that, in the presence of osteoblasts, BMMs could generate osteoclasts under conditions where calcium oscillation and calcineurin activation were absent, although the extent of osteoclastogenesis was weaker than when calcium oscillation was normal [15]. This observation led to a suggestion that both calcium oscillation-dependent and -independent signaling operate for the activation of NFATc1 in osteoclast differentiation depending on the environment that osteoclast precursors encounter.

3 Ca^{2+} Channels in OC Intracellular Organelles

Since studies linking phosphatidylinositol (PI) hydrolysis to calcium signaling [16–18], inositol 1,4,5-trisphosphate (IP_3) was found to release calcium from ER [19–21]. IP_3 binds to its receptor on ER membrane and activates the receptor channel to transport calcium to the cytoplasm. The IP_3 receptor is also sensitive to calcium [22, 23], which generates calcium-induced calcium release like the ryanodine receptor in muscle cells. The generation of IP_3 via hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) is stimulated by various external signals. When these signals bind to their receptors, the activation of phospholipase C (PLC) is triggered [2]. While PLCβ is activated by G-protein-coupled receptors, PLCγ is stimulated by tyrosine kinase-coupled receptors.

In osteoclast precursor cells, the activation of PLCγ was observed upon RANKL treatment [24]. The PLCγ activation was defective in osteoclast precursors from mice lacking immune cell adaptors Fc receptor common γ subunit (FcRγ) and DNAX-activating protein (DAP) 12 that harbor immunoreceptor tyrosine-based activation motif (ITAM) [24]. These ITAM-containing adaptors associate with immunoglobulin-like receptors osteoclast-associated receptor (OSCAR), paired immunoglobulin-like receptor (PIR)-A, triggering receptor expressed by myeloid cells (TREM)-2 and signal-regulatory protein (SIRP) β1 [24]. Consistently with the lack of PLCγ activation, calcium oscillation and NFATc1 induction were defective in the $DAP12^{-/-}$ $FcR\gamma^{-/-}$ precursor cells. Consequently, osteoclast differentiation was severely impaired and the $DAP12^{-/-}$ $FcR\gamma^{-/-}$ mice displayed osteopetrotic phenotype [24]. This observation led to the recognition that RANK signaling needs additional costimulatory signals for induction of calcium oscillation for efficient osteoclastogenesis.

The activation of PLCγ is achieved by phosphorylation. The ITAM motifs of immune adaptor molecules associate with Syk family kinases via SH2 domain binding [25]. Indeed, Syk knockout precursor cells showed defective osteoclastogenesis and bone resorption [26]. The defect could be rescued by exogenous expression of the SH2 domains of Syk [26]. Likewise, forced expression of intact ITAM domains could reconstitute osteoclast development from $DAP12^{-/-}$ $FcR\gamma^{-/-}$ cells [26]. The binding of a SH2 domain to an ITAM motif requires the phosphorylation of tyrosine residues. In B cells, Btk/Tec family tyrosine kinases form a complex with B cell receptor and phosphorylate the ITAM motifs in the BCR [27]. Shinohara et al. found by a genome-wide screening of nonreceptor tyrosine kinases differentially expressed during osteoclast differentiation that Btk and Tec kinases are highly expressed in osteoclasts [28]. $Btk^{-/-}$ $Tec^{-/-}$ mice displayed osteopetrosis and osteoclastogenesis was abrogated in $Btk^{-/-}$ $Tec^{-/-}$ cells. RANKL activated Btk and Tec. Btk associated with the adaptor protein BLNK, which was abrogated in $DAP12^{-/-}$ $FcR\gamma^{-/-}$ cells. Precursor cells deficient in BLNK and SLP-76 (a T-cell homologue of BLNK) also showed defective osteoclastogenesis. Furthermore, the activation of PLCγ, calcium oscillation, and NFATc1 by RANKL was suppressed in $Btk^{-/-}$ $Tec^{-/-}$ cells. Taken together, both the RANK activation of Btk/Tec and the ITAM-mediated activation of BLNK/SLP-76 were required for PLCγ activation and subsequent calcium response for osteoclast differentiation.

The generation of IP_3 by activated PLC leads to increase in intracellular calcium as IP_3 binds to its receptor IP_3R in the ER. IP_3R itself is a calcium channel and the activity is modulated by ATP, calcium, regulatory proteins as well as IP_3 [29–31]. There are three isoforms of IP_3Rs. Kuroda et al. found that BMMs from IP_3R2 knockout mice were incapable of osteoclastogenesis whereas BMMs from IP_3R1 and IP_3R3 were able to differentiate to osteoclasts as effectively as wild-type BMMs [15]. IP_3R2 knockout BMMs also could not elicit calcium oscillation and NFATc1 induction upon RANKL stimulation. Strikingly, IP_3R2 knockout BMMs could generate osteoclasts when cocultured with osteoblasts or stromal cells despite lack of calcium oscillation. NFATc1 activation was also achieved in the coculture of IP_3R2 knockout BMMs. These findings suggested that both calcium/calcineurin-dependent and -independent mechanisms of NFATc1 activation operate for osteoclastogenesis in the presence of supporting cells.

Oscillation in the cytosolic calcium level inevitably requires repetitive activity of both calcium channels/pumps that transport calcium from intracellular stores such as IP_3R and those that import calcium back into intracellular stores. The sarco/endoplasmic reticulum calcium ATPase (SERCA) pump that reuptakes calcium into ER is thus critical for cytosolic calcium oscillation [32]. Indeed, SERCA+/– mice displayed reduced frequency of calcium oscillation [33]. Yang et al. examined osteoclastogenesis and bone metabolism with SERCA2+/– mice [34]. SERCA2+/– mice were osteopetrotic and had ~1.5-fold higher bone mineral density. BMMs from SERCA2+/– mice did not elicit calcium oscillation during RANKL-induced osteoclastogenesis. The extent of osteoclast formation was lower in BMMs from SERCA2+/– mice than in wild-type BMMs. Consistently, the induction and activation of NFATc1 was reduced in SERCA2+/– BMMs.

4 Ca^{2+} Channels in OC Plasma membranes

Calcium influx and efflux through the plasma membrane also play a major role in the regulation of cytosolic calcium concentrations. In addition, extracellular calcium influx is often coupled with intracellular calcium release [2]. There was a report on a plasma membrane calcium channel involved in intracellular calcium signaling in osteoclasts [35]. In the report it was found that $TRPV4^{-/-}$ mice had increased bone mass after weaning. The bone mass increase in adult $TRPV4^{-/-}$ mice was associated with reduced bone resorption, but not with increased bone formation. Osteoclastogenesis from bone marrow cells of $TRPV4^{-/-}$ mice generated lower number of large osteoclasts compared with wild-type cells. While NFATc1 mRNA level was also lower in $TRPV4^{-/-}$ osteoclasts, TRPV4 activation in wild-type osteoclasts led to NFATc1 nuclear translocation. Intriguingly, the oscillatory calcium response was observed only in small and medium size osteoclasts, but not in large osteoclasts [35]. Instead, calcium influx occurred when TRPV4 was active in large, but not in small osteoclasts. Consistent with its function as a calcium influx channel for intracellular signaling, TRPV4 was located on the basolateral membrane of large

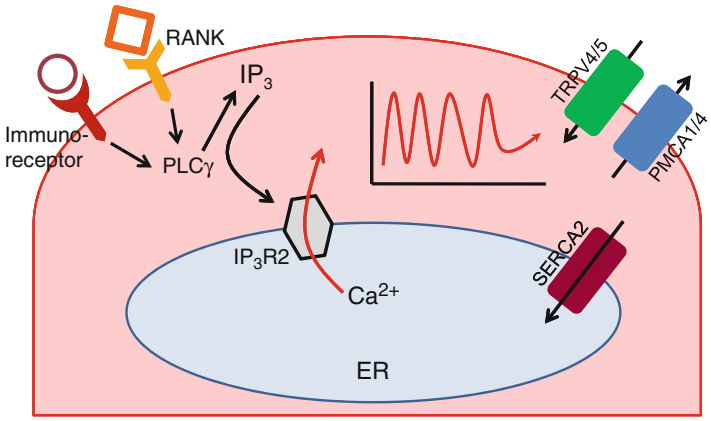

Fig. 1 Calcium regulators in osteoclasts. Intracellular calcium channel IP_3 receptor increases cytosolic calcium by exporting calcium from internal stores, while SERCA takes cytosolic calcium back to the stores. Plasma membrane proteins including TRPV and NCX also participate in regulation of intracellular calcium concentrations in osteoclasts

osteoclasts [35]. From these observations, it appears that the intracellular calcium signaling is mediated by calcium oscillations via IP_3R2 in early stage and by basolateral calcium influx via TRPV4 in late stage of osteoclastogenesis.

Other calcium channels implicated in calcium influx through the plasma membrane of osteoclasts include Na^+/Ca^{2+} exchangers (NCXs) [36], ryanodine receptor calcium channel [37], receptor-linked calcium channel [38], voltage-gated calcium channels [39], and TRPV5 [40]. Some of these channels were located to the apical side of osteoclast plasma membrane and proposed to be involved in calcium transcytosis from the bone resorbing surface to the basolateral side of cells [36, 40]. The molecules involved in calcium efflux through plasma membrane in osteoclasts have remained elusive. As the NCX is bidirectional transporter, it can mediate calcium efflux depending on the electrochemical gradients. We identified molecules mediating calcium efflux on the basolateral plasma membrane of osteoclasts. The expression of these molecules, plasma membrane calcium ATPase (PMCA) 1 and 4, increased during RANKL-induced osteoclastogenesis. Gene knockdown of PMCA increased intracellular calcium oscillation and osteoclast differentiation (data not shown), suggesting involvement of plasma membrane calcium efflux in the regulation of intracellular calcium response during osteoclast differentiation.

5 Conclusion

Intracellular cytosolic calcium plays a pivotal role in osteoclastogenesis by activating calcium-dependent phosphatase calcineurin that activates NFATc1, the transcription factor critically involved in the expression of osteoclast marker genes.

Intracellular calcium channels IP_3R2 and SERCA mediate calcium transport between ER and cytosol generating an oscillatory pattern of calcium concentration changes in differentiating osteoclasts. In addition, plasma membrane proteins TRPV and NCX channels and PMCA pumps are also involved in regulating calcium transport between cytosol and extracellular environment of osteoclasts (Fig. 1). The manifestation of bone phenotype of mice deficient in these calcium channels and pumps suggest that calcium regulators in osteoclasts may be useful targets for development of bone-destructive diseases. However, further studies are required to ensure selectivity of the identified calcium channels and pumps toward osteoclasts with a reasonable window compared to their contributions to cytosolic calcium regulation in other cell types.

Acknowledgements This work was supported by the National Research Foundation of Korea (NRF) grants to the 21C Frontier Functional Proteomics Center (FPR08B1-170) and Science Research Center (20110001028) funded by the Korean government (MEST).

References

1. Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 1:11–21
2. Berridge MJ, Bootman MD, Roderick HL (2003) Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol 4:517–529
3. Carafoli E, Santella L, Branca D, Brini M (2001) Generation, control, and processing of cellular calcium signals. Crit Rev Biochem Mol Biol 36:107–260
4. Kiselyov K, Shin DM, Muallem S (2003) Signalling specificity in GPCR-dependent Ca^{2+} signalling. Cell Signal 15:243–253
5. Dolmetsch RE, Xu K, Lewis RS (1998) Calcium oscillations increase the efficiency and specificity of gene expression. Nature 392:933–936
6. Boyle WJ, Simonet WS, Lacey DL (2003) Osteoclast differentiation and activation. Nature 423:337–342
7. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176
8. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T (1998) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 95:3597–3602
9. Lee ZH, Kim HH (2003) Signal transduction by receptor activator of nuclear factor kappa B in osteoclasts. Biochem Biophys Res Commun 305:211–214
10. Takayanagi H (2005) Mechanistic insight into osteoclast differentiation in osteoimmunology. J Mol Med 83:170–179
11. Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, Saiura A, Isobe M, Yokochi T, Inoue J, Wagner EF, Mak TW, Kodama T, Taniguchi T (2002) Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cell 3:889–901
12. Nakashima T, Takayanagi H (2008) The dynamic interplay between osteoclasts and the immune system. Arch Biochem Biophys 473:166–171

13. Rao A, Luo C, Hogan PG (1997) Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol 15:707–747
14. Crabtree GR, Olson EN (2002) NFAT signaling: choreographing the social lives of cells. Cell 109 Suppl: S67–S79
15. Kuroda Y, Hisatsune C, Nakamura T, Matsuo K, Mikoshiba K (2008) Osteoblasts induce Ca^{2+} oscillation-independent NFATc1 activation during osteoclastogenesis. Proc Natl Acad Sci USA 105:8643–8648
16. Fain JN, Berridge MJ (1979) Relationship between hormonal activation of phosphatidylinositol hydrolysis, fluid secretion and calcium flux in the blowfly salivary gland. Biochem J 178:45–58
17. Berridge MJ, Lipke H (1979) Changes in calcium transport across Calliphora salivary glands induced by 5-hydroxytryptamine and cyclic nucleotides. J Exp Biol 78:137–148
18. Berridge MJ, Fain JN (1979) Inhibition of phosphatidylinositol synthesis and the inactivation of calcium entry after prolonged exposure of the blowfly salivary gland to 5-hydroxytryptamine. Biochem J 178:56–68
19. Streb H, Irvine RF, Berridge MJ, Schulz I (1983) Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol 1,4,5-trisphosphate. Nature 306:67–69
20. Burgess GM, Godfrey PP, McKinney JS, Berridge MJ, Irvine RF, Putney JW (1984) The second messenger linking receptor activation to internal calcium release in liver. Nature 309:63–66
21. Berridge MJ, Heslop JP, Irvine RF, Brown KD (1991) Inositol trisphosphate formation and calcium mobilization in Swiss 3T3 cells in response to platelet-derived growth factor. Biochem J 222:195–201
22. Bezprozvanny I, Watras J, Ehrlich BE (1991) Bell-shaped calcium response curves of Ins(1,4,5)P3– and calcium gated channels from endoplasmic reticulum of cerebellum. Nature 351:751–754
23. Finch EA, Turner TJ, Goldin SM (1991) Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. Science 252:443–446
24. Koga T, Inui M, Inoue K, Kim S, Suematsu A, Kobayashi E, Iwata T, Ohnishi H, Matozaki T, Kodama T et al (2004) Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. Nature 428:758–763
25. Lanier LL, Corliss BC, Wu J, Leong C, Phillips JH (1998) Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. Nature 391:703–707
26. Mocsai A, Humphrey MB, Van Ziffle JA, Hu Y, Burghardt A, Spusta SC, Majumdar S, Lanier LL, Lowell CA, Nakamura MC (2004) The immunomodulatory adapter proteins DAP12 and Fc receptor γ-chain (FcRγ) regulate development of functional osteoclasts through the Syk tyrosine kinase. Proc Natl Acad Sci USA 101:6158–6163
27. Schmidt U, Boucheron N, Unger B, Ellmeier W (2004) The role of Tec family kinases in myeloid cells. Int Arch Allergy Immunol 134:65–78
28. Shinohara M, Koga T, Okamoto K, Sakaguchi S, Arai K, Yasuda H, Takai T, Kodama T, Morio T, Geha RS, Kitamura D, Kurosaki T, Ellmeier W, Takayanagi H (2008) Tyrosine kinases Btk and Tec regulate osteoclast differentiation by linking RANK and ITAM signals. Cell 132:794–806
29. Patterson RL, Boehning D, Snyder SH (2004) Inositol 1,4,5-trisphosphate receptors as signal integrators. Annu Rev Biochem 73:437–465
30. Higo T, Hattori M, Nakamura T, Natsume T, Michikawa T, Mikoshiba K (2005) Subtype-specific and ER lumenal environment-dependent regulation of inositol 1,4,5-trisphosphate receptor type 1 by ERp44. Cell 120:85–98
31. Ando H, Mizutani A, Kiefer H, Tsuzurugi D, Michikawa T, Mikoshiba K (2006) IRBIT suppresses IP3 receptor activity by competing with IP3 for the common binding site on the IP3 receptor. Mol Cell 22:795–806
32. Lee MG, Xu X, Zeng W, Diaz J, Kuo TH, Wuytack F, Racymaekers L, Muallem S (1997) Polarized expression of Ca^{2+} pumps in pancreatic and salivary gland cells. Role in initiation and propagation of $[Ca^{2+}]i$ waves. J Biol Chem 272:15771–15776

33. Zhao XS, Shin DM, Liu LH, Shull GE, Muallem S (2001) Plasticity and adaptation of Ca^{2+} signaling and Ca^{2+}-dependent exocytosis in SERCA2(+/2) mice. EMBO J 20:2680–2689
34. Yang YM, Kim MS, Son A, Hong JH, Kim KH, Seo JT, Lee SI, Shin DM (2009) Alteration of RANKL-induced osteoclastogenesis in primary cultured osteoclasts from SERCA2+/− mice. J Bone Miner Res 24:1763–1769
35. Masuyama R, Vriens J, Voets T, Karashima Y, Owsianik G, Vennekens R, Lieben L, Torrekens S, Moermans K, Vanden Bosch A, Bouillon R, Nilius B, Carmeliet G (2008) TRPV4-mediated calcium influx regulates terminal differentiation of osteoclasts. Cell Metab 8:257–265
36. Li JP, Kajiya H, Okamoto F, Nakao A, Iwamoto T, Okabe K (2007) Three Na^{+}/Ca^{2+} exchanger (NCX) variants are expressed in mouse osteoclasts and mediate calcium transport during bone resorption. Endocrinology 148:2116–2125
37. Moonga BS, Li S, Iqbal J, Davidson R, Shankar VS, Bevis PJ, Inzerillo A, Abe E, Huang CL, Zaidi M (2002) Ca^{2+} influx through the osteoclastic plasma membrane ryanodine receptor. Am J Physiol Renal Physiol 282:F921–F932
38. Bennett BD, Alvarez U, Hruska KA (2001) Receptor-operated osteoclast calcium sensing. Endocrinology 142:1968–1974
39. Miyauchi A, Hruska KA, Greenfield EM, Duncan R, Alvarez J, Barattolo R, Colucci S, Zambonin-Zallone A, Teitelbaum SL, Teti A (1990) Osteoclast cytosolic calcium, regulated by voltage-gated calcium channels and extracellular calcium, controls podosome assembly and bone resorption. J Cell Biol 111:2543–2552
40. van der Eerden BC, Hoenderop JG, de Vries TJ, Schoenmaker T, Buurman CJ, Uitterlinden AG, Pols HA, Bindels RJ, van Leeuwen JP (2005) The epithelial Ca^{2+} channel TRPV5 is essential for proper osteoclastic bone resorption. Proc Natl Acad Sci USA 102: 17507–17512

Lessons from Glucocorticoid Receptor Action in Bone: New Ways to Avoid Side Effects of Steroid Therapy

Alexander Rauch, Ulrike Baschant, and Jan Tuckermann

1 Introduction

Glucocorticoids (GCs) as anti-inflammatory agents proved successful for the first time in patients suffering from rheumatoid arthritis (RA) in 1948 [1] and this finding was awarded with the Nobel prize in 1950. RA is currently one of the most investigated diseases in the emerging research area "osteoimmunology" addressing the interactions between bone and hematopoietic tissues. RA is still treated with GCs in combination with disease modifying antirheumatic drugs (DMARDs) [2] to ameliorate the most pro-inflammatory boosts. Soon it became evident that steroid therapy is hampered by a multitude of side effects acting on metabolism, cardiovascular system, and tissue integrity. A general loss of bone at long-term GC treatment is considered to be one of the major complications. To improve steroid therapy and avoiding bone loss in RA, a detailed understanding of the cellular and molecular mechanisms is required. Work from our laboratory and others shed some light into the molecular mechanisms implicated in GC action in suppression of inflammation and GC-induced bone loss. We review here these recent advances and also define new criteria for selective acting GCs that avoid GC-induced bone loss, but may retain therapeutic potential.

1.1 The Glucocorticoid Receptor

GCs, such as the endogenous secreted hydrocortisol or corticosterol as well the synthetic GCs prednisolone, dexamethasone, and others bind to a member of the

A. Rauch • U. Baschant • J. Tuckermann (✉)
Institute for Age Research – Fritz Lipmann Institute,
Beutenbergstr. 11, Jena 07745, Germany
e-mail: jan@fli-leibniz.de

Y. Choi (ed.), *Osteoimmunology: Interactions of the Immune and Skeletal Systems,*
DOI 10.1007/978-1-4614-5366-6_5,

nuclear receptor family, the glucocorticoid receptor (GR). The GR is composed of an N-terminal transcriptional activation domain (AF-1) followed by a two Zn finger containing DNA binding domain (DBD), a short hinge region, the ligand binding domain (LBD), and a C-terminal transactivation domain (AF-2) [3]. The GR resides in the absence of ligands in the cytoplasm and is associated with a complex of chaperonic molecules composed of heat shock proteins and so called immunophillins. Hypothalamo–pituitary–adrenal axis triggered or pharmacologically applied GCs diffuse through the cell membrane. Within the cells 11-β-hydroxysteroid dehydrogenase type 1 (11-β-HSD1) converts the inactive GCs, such as cortisone and corticosterone to their active forms cortisol and corticosterol, respectively. Active GCs bind to the high affinity GR-heat shock protein complex [4]. Upon binding this complex disrupts and allows the released GR molecule to interact in the cytoplasm with signal transduction components, such as JNK, PI3K, 14-3-3 proteins or in T cells with the T cell receptor associated kinases lck and fyn [3]. The majority of the GR molecules translocate to the nucleus, facilitated by HSP90, the co-chaperone immunopholin FKBP52 [5], importin alpha/beta, and importin 7 [6]. Within the nucleus the GR executes two major activities: binding as a homodimer to palindromic response elements (GRE) of GC-induced genes and associating to sites of proinflammatory transcription factors. Homodimerization of the GR at the DNA is mediated by dimerization motifs within the 2nd Zn finger of the DBD [7], dimerization interfaces at the LBDs and presumably by LBD/DBD interaction as shown for other nuclear receptors [8]. The transactivation domains AF-1 and AF-2 of DNA bound GR serve as platforms for the recruitment of coactivators. Chromatin remodeling complexes of the SWI/SNF/Brg1 family are interacting with AF-1 in a ligand independent manner. In contrast AF-2 recruits proteins of the p160 family and subsequently coactivators such as CBP/p300 only in the presence of ligand. The kind of coactivators are recruited further depends on the conformational change of the GR influenced by the palindromic DNA binding sequence itself. Different GRE sequences lead to different transactivation capacities [9]. Recent global chromatin-immunoprecipitation sequencing combined with studies of fluorescence-tagged GR molecules at a high time resolution shows a complex dynamic behavior of GR DNA occupancy [10]. The binding of the GR seems to follow the oscillating GC release during the day, which is different from permanent nuclear residing GR by high affine synthetic GCs. Thus a comprehensive picture of GR DNA binding activity—so far only defined for a limited amount of cell lines—is just being unraveled. These experiments once performed in mesenchymal and hematopoietic cells will give fundamental insights into gene regulation of GC effects in osteoimmunological processes.

The second mode of GR activity, the interaction with already DNA bound transcription factors had attracted much attention over the last two decades as one of the major mechanisms of immune suppression by the GR [3]. In particular the GR represses proinflammatory molecules based on tethering to AP-1 [11], NF-κB [12], or IRF-3 [13]. These interactions occur in the presence of integrator proteins like thyroid hormone receptor interactor 6 (TRIP6) [14] or SRC1 and TIF2-associated binding protein (STAMP) [15]. The GR is supposed to prevent the recruitment of coactivators to NF-κB [16] or to inhibiting RNA-polymerase II phosphorylation on

the C-terminal domain by recruitment of phosphatases [17]. In addition the GR inhibits Toll-like receptor signaling via sequestration of the p160 protein GRIP1 from IRF3 and STAT1 sites, thereby interfering with their transactivating activity [13, 18, 19]. Due to the plethora of pro-inflammatory mediators, such as cytokines, enzymes and adhesion molecules that are under the control of the aforementioned transcription factors it became almost a dogma in the field that immune suppression of GCs solely depends on this tethering mode of nuclear GR action. To which extend this dogma holds and has to be modified will be discussed below.

1.2 Selective Glucocorticoid Receptor Modulators

Based on the two major nuclear mechanisms of GR action—binding as a homodimer and tethering as a monomer towards pro-inflammatory transcription factors—pharmaceutical companies started an intensive search for dissociating GR ligands that exclusively address the monomer function of the GR [20]. Such ligands should maintain anti-inflammatory efficacy, but avoid side effects which were attributed to the GR dimer. So far only for the regulation of enzymes involved in glucose metabolism the requirement of the GR dimer was clearly demonstrated, at the time these programs were launched. Thus, screening programs were mainly based on GRE-, AP-1-, or NF-κB-dependent reporter gene assays. Compounds were identified that exert a certain GR affinity and failed to induce GRE driven reporters, but maintained transrepression of AP-1- and/or NF-κB-dependent reporter genes. Whereas the first compounds did not maintain their dissociative properties in vivo [21], other substances, including AL-438 [22], "compound A" [23], LGD-5552 [24], ZK 216348 [25], and ZK 245286 [26] continued to be successful in some inflammatory assays in rodents, such as phorbol ester-induced skin irritation, air pouch-induced inflammation, contact allergy, and EAE [27, 28]. CpdA also proved therapeutically successful in an example of inflammatory bone disease, collagen-induced arthritis [29].

Concerning side effects the AL-438 [22], CpdA [23], and ZK 216348 [25] failed to induce glucose levels and to decrease glucose tolerance in rodent models in comparison to the full GR agonist dexamethasone. The investigated selective GR modulators SEGRMs spared effects on thymus weight, adrenal weight, and bone growth [20]. Little has been done on the effects of SERGMs on primary bone cells and bone integrity. Only for AL-438 and LGD-5552 a lack of bone formation inhibition was reported for rats [22, 24]. In tissue culture cells a reduced repression of the RANKL/OPG ratio in comparison to the classical agonists had been observed for AL-438 and ZK 216348 [30]. This might indeed lead to a decreased osteoclastogenesis triggered by osteoblastic cells, which remains to be investigated.

So far, however, the SEGRMs were only defined on a few selected reporter genes and therefore it is not entirely clear, how selective they are in other promoter contexts and whether they act in a tissue selective manner in vivo. To solve this issue, even for classical GR action, target tissues and molecular mechanisms have to be defined.

2 Cell Type Specific Molecular Mechanisms of Anti-inflammatory Actions of the GR

To define which molecular action of the GR, homodimerization or the monomeric activity contributes to anti-inflammation and to GC-induced bone loss as the major side effect we utilized an approach using conditional mutant mice of the GR.

For the identification of cell types critical for anti-inflammatory effects we utilized mice with a conditional GR allele with exon 3 flanked with loxP sites. These mice are suitable for recombination by transgenic cre expression to create a conditional GRnull allele in selected cell types [31, 32]. This strategy has the advantage to overcome the perinatal lethality in complete GR knockout mice [32, 33] to address GR function in adult animals. Using this approach we could first define for GC treatment of contact allergy in the skin using a murine model of contact hypersensitivity (CHS) that antigen presenting dendritic cells, keratinocytes and—interestingly—T cells were not critical for GC therapy. Myeloid cells however, when devoid of the GR in vivo in $GR^{LysMCre}$ mice rendered resistant to GC suppression of the inflammatory response in CHS [34]. Myeloid cells including neutrophils and macrophages turned out to be also critical for the actions of endogenous GCs in septic shock experiments [35]. In contrast in experimental encephalitis the GR in T cells was most critical for GC actions. Mice lacking the GR in T cells in GR^{LckCre} mice had a stronger disease progression due to impaired action of endogenous GCs and a diminished response to GC therapy. $GR^{LysMCre}$ mice were comparable to wild-type animals in their response to GCs [36]. Thus, these data demonstrate that the cell type most important to execute an anti-inflammatory effect of GCs depends on the type of inflammation investigated.

The type of inflammatory response does not only require the GR in different cell types but also dictates whether the monomer GR is sufficient for immune suppression. To discriminate between dimerization dependent and independent action of the GR in vivo, mice with a functional GR mutation abrogating the dimerization of the receptor were generated (GR^{dim}). A458T substitution in the 4th exon encoding the 2nd zinc finger disrupts the GR dimerization interface of the DBD. Interestingly, these mice are viable despite loosing the dimerization dependent DNA binding. Furthermore they preserve tethering of the monomeric GR in particular for the repression of AP-1 and NF-κB activity [37, 38]. As expected from the current dogma that anti-inflammatory effects of GCs rely on the monomer function of the GR, GR^{dim} mice were fully responsive to suppression of phorbol ester-induced inflammation and of AP-1-mediated Mmp13 expression in skin [38, 39]. Surprisingly, in contact hypersensitivity it was shown that GR^{dim} mice were not treatable with glucocorticoids revealing a potent anti-inflammatory action of the dimerized DNA bound GR in vivo [34]. Thus, the classical dogma that GR DNA binding is not involved in immunosuppression cannot be applied to all inflammatory conditions. This is further supported by the identification of GC-induced anti-inflammatory genes such as glucocorticoid-induced leucine zipper (GILZ) [40], annexin A1 [41], or mitogen-activated protein kinase phosphatase 1 (MKP1/DUSP-1) [42, 43].

Indeed, DUSP-1 knockout mice failed to respond to GCs in the zymosan-induced air pouche model [44].

We just recently uncovered the requirement for GR dimerization in a model of rheumatoid arthritis (Baschant and Tuckermann, unpublished). Thus, although so-called dissociating SEGRMs are presumably therapeutically efficative in RA models, we clearly show here by genetic in vivo evidence that GR dimerization is involved in anti-inflammatory activities. This can be possibly explained by recent findings that SEGRMs are capable to induce the GR dimer-dependent anti-inflammatory acting gene *Dusp1* in some inflammatory cell types (Egene Jeanny poster-abstract on BES meeting 2010; http://www.endocrine-abstracts.org/ea/0021/ea0021p361.htm). This strongly suggests that SEGRMs are not fully dissociative concerning DNA dimerization versus non-dimerization in anti-inflammatory aspects, but they rather may induce differential coactivator recruitment and fulfill thereby differential effects in comparison with classical GCs.

Besides the identification of target cells and addressing the mechanism for the beneficial aspects of GC therapy in greater detail, only recently the critical cell types in bone loss as one of the major side effects have been identified. We discuss in the following sections recent advances concerning the mechanisms of GC-induced osteoporosis (GIO) and the consequences for the demands on selective ligands that spare the bone.

3 The Role of the Glucocorticoids Receptor in Bone Homeostasis

3.1 Glucocorticoid-Induced Osteoporosis

Bone homeostasis depends on the balance of bone formation and bone resorption. High levels of glucocorticoids are known to negatively influence bone homeostasis since the early 1930s due to characterization of people with Cushing syndrome [45]. By the time of their clinical use the detrimental effects on bone turned out to be one of the most frequent side effects. Moreover approximately 25% of all clinical relevant osteoporosis in particular with high fracture risk are linked to high GC exposure [46]. People subjected to more than 7.5 mg prednisolone per day over 3 months showed an 50% increase in bone fracture risk. In these patients bone mineral density declines very fast with the onset of steroid therapy during the first 3–6 months [47]. The inhibitory effect on bone formation, osteoid thickness, mineral apposition, and mineralization by GCs is undisputed, whereas differences in bone resorption seems to be controversial [48]. Also to which extend systemic effects of GCs, e.g., on calcium metabolism may contribute to bone loss was until recently not shown due to the lack of respective mouse models. Below we discuss the effects of GCs on systemic physiology, osteoclasts and osteoblasts in detail and focus of recent advances by our laboratory and others.

3.2 Systemic Effects by Glucocorticoids

Glucocorticoid exposure opposes vitamin D actions on serum Ca^{2+} levels by a decrease of intestinal calcium absorption [49] and an increase in renal Ca^{2+} excretion [50]. Decreased calcium levels cause hyperparathyroidism [51]. However bone turnover in glucocorticoid-induced osteoporosis is low [48] in contrast to elevated turnover in hyperparathyroidism [52]. Systemic glucocorticoids may affect bone integrity by the reduction of gonadal hormones. First they blunt pituitary luteinizing hormone secretion [53]. Second they inhibit production of testosterone and estrogen in testes and ovary, respectively [54, 55]. However estrogen deficiency and glucocorticoid excess were described to be additive in rats [56] suggesting a minor role for regulation of gonadal function in glucocorticoid-induced osteoporosis.

3.3 Effects of Glucocorticoids on Osteoclasts

In contrast to systemic effects direct GC actions on bone cells seem to be crucial for GC-induced osteoporosis (GIO). GCs have been shown to act on osteoclasts directly and indirectly via other cells, such as the osteoblasts. Glucocorticoids are potent inducers of osteoclastogenesis-promoting RANKL and suppress the osteoclast-inhibitor OPG [57]. Suppression of OPG expression was described by interfering with JNK activity and transrepression of AP-1 bound to the OPG gene promoter [58]. The mechanism of upregulation of *Tnfsf11* mRNA, encoding for RANKL, is less understood. There is a potential GRE in the *Tnfsf11* promoter [58] implicating a direct transcriptional control, but also evidence for a contribution of enhanced mRNA stability [59]. Cytokines like IL-1α, β, TNF-α [60], and IL-6 [61] are potent promoters of osteoclastogenesis in bone inflammation, but are rather suppressed by GCs, such as IL-6 in osteoblasts [62, 63].

In the presence of RANKL in monocytic cultures GCs stimulate osteoclastogenesis at low concentration, but inhibit osteoclast formation at high concentration [64]. These data might explain the differential effects of GCs observed in vivo.

Indeed findings of GC effects on resorption are controversial. In one study prednisolone treatment increased resorption in Balb/c mice but not in human RANKL knock-in mice. Furthermore osteoclast numbers were not increased significantly in both mouse strains [65]. Also in other studies osteoclast numbers were unaltered in prednisolone treated mice, but resorption even decreased around 20–30% [62, 66].

Although osteoclast numbers seem not to be changed to a large fraction in rodent studies, GCs have been reported to increase osteoclast life span, e.g., by inhibiting caspase-3 activity [66]. Mice lacking glucocorticoid signaling in mature osteoclasts, but not progenitor cells displayed reduced osteoclast numbers upon steroid exposure in contrast to wild-type mice. This finding suggests that GCs enhance life span in mature osteoclasts, whereas osteoclastogenesis from osteoclast progenitors is inhibited [66]. The inhibition of osteoclastogenesis by pharmacological GC

concentrations was observed in cocultures of osteoblast and osteoclast precursors and was dependent on GR expression in both cell types [62]. This observation was in line with the observed decrease of resorption *in vivo* reported by Teitelbaum and colleagues and by our group [62, 67]. Indeed the reduced resorption could depend in part by the GR in osteoblasts, since we found a less severe reduction of resorptive activity in prednisolone treated mice lacking the GR in osteoblasts [62].

Kim et al. explained the cell-autonomouse effects of GCs on osteoclastogenesis by prevention of M-CSF-induced activation of Ras homolog gene family GTPases, in particular RhoA, Rac, and Vav3, which consequently disrupts actin ring formation. Reorganization of cytoskeleton is crucial for formation of so-called ruffled boarders and consequently resorptive activity [67]. Thus, the osteoclasts may lose their potential to degrade bone. Interestingly reduced osteoclast activity by prednisolone depends on the monomeric GR in osteoblasts and osteoclasts [62].

Finally there was a role of the GR in osteoclast suggested to influence bone formation [67]. Despite there is no doubt that osteoclast communicate to osteoblasts in bone remodeling this could not be supported by the analysis of mice with inactivated GC signaling in osteoclasts [66] and in our study using mice lacking the GR in myeloid cells and thus osteoprogenitors [62].

Although as we will discuss below the inhibition of bone formation is the major mechanism of GIO it should be noted that a full suppression of osteoclastogenesis by the monoclonal antibody Desonosumab inhibiting human RANKL in hRANKL knock-in mice ameliorates bone loss [65].

3.4 Effects of Glucocorticoids on Osteoblasts and Osteocytes

A hallmark of GC-induced bone loss is the inhibition of bone formation and thus a suppression of osteoblast function accompanied with a loss of osteoblast and osteocyte number [51]. Using conditional knockout mice ($GR^{Runx2Cre}$ mice) we recently demonstrated that the GR in osteoblasts is not only required to mediate suppression of bone formation, but is also instrumental for GC-induced bone loss [62].

The reduced osteoblast activity and numbers are attributed to inhibition of proliferation, induction of apoptosis, and suppression of differentiation.

3.4.1 Proliferation

Most evidence of GC effects on osteoblast proliferation derives from tissue culture experiments involving primary calvarial cells and immortalized and/or transformed cell lines. For MC3T3-E1 cells there was a postconfluent antiproliferative effect by GCs postulated as a prerequisite for reduced differentiation [68]. The inhibition of proliferation was explained by antagonizing the Wnt pathway, e.g., activating with GSK-3β kinase [69], suppressing PKB/Akt [70], inactivating TCF/LEF or inducing the wnt antagonist DKK-1 [71, 72]. Recently, it was shown that GC-induced

MAPK phosphatase 1/dual-specific phosphatase (DUSP1) is functionally involved in the reduction of mitogenic signaling and thus participates in anti-proliferative effects of GCs [73]. This is in line with our results that GR^{dim} osteoblasts with a GR impaired in dimerization are unable to induce DUSP-1 (Rauch and Tuckermann unpublished) and indeed failed to exhibit a reduction of osteoblast proliferation. Nevertheless GR^{dim} mice have impaired bone formation upon GC exposure, indicating that effects of GCs on proliferation are only to a minor part involved in GC-induced bone loss [62].

3.4.2 Apoptosis

Apoptosis of osteocytes and osteoblasts is a well-described feature in GC exposed rodents and humans [74].

Interestingly dexamethasone increases caspase-3 activity and induce consequently apoptosis in osteoblasts [75] which is opposite to the reduction of caspase-3 observed in osteoclasts [66].

Surprisingly the mechanical activation of osteocytes leads to prevention of apoptosis [76]. Mechanical forces signal via focal adhesion kinas (FAK), SRC and finally activation of ERK [77]. This outside-in survival signaling is compromised by the GC-mediated activation of the proapoptotic proline-rich tyrosine kinase 2 (PYK2) via phosphorylation at Tyr^{402} [78]. Activated PYK2 triggers reorganization of the cytoskeleton, cell detachment by disruption of integrin matrix engagement and finally apoptosis [79]. It is hypothesized that this occurs via mechanisms independent of GR-mediated gene regulation [78]. From our studies we could show that induction of apoptosis in primary osteoblastic cells dependents on GR expression but not binding to DNA in vitro [62]. It remains to be clarified whether direct interaction between the GR and PYK2 at the cell membrane occurs in order to induce apoptosis or whether nuclear effects such as inhibition of transcription are involved.

Nonetheless GCs could cause apoptosis also by elevation of the potent pro-apoptotic protein BAX, that was recently found to be upregulated by dexamethasone in a proteomic study using MC3T3-E1 cells [80].

Of note it has to be stressed that the observation of apoptosis of osteoblasts by dexamethasone affected only a minor fraction of all osteoblasts in vitro (approx. 10%) [78]. The minor role of apoptosis in bone loss is supported by a study from O'Brien and colleagues [81]. Here they used mice overexpressing 11β-HSD2 under the osteocalcin promoter, inactivating GC signaling in terminally differentiated osteoblasts and presumably osteocytes. These mice displayed a reduced apoptotic rate under prednisolone treatment [81]. Nonetheless there was still an overall bone loss observed, indicating that apoptosis is not sufficient to cause bone loss. This was corroborated by the fact that in other mouse strains subjected to GIO an increase of osteoblast/osteocyte apoptosis was hardly to be observed under prednisolone treatment at different time points [62].

3.4.3 Differentiation

Whereas induction of apoptosis take place to a minor degree, inhibition of differentiation in terms of alkaline phosphatase activity and mineralization occurs around 70–90% in primary osteoblasts at high concentration of GCs. This effect of GCs is biphasic. Low and physiological concentrations of GCs promote differentiation of pre-osteoblasts in numerous tissue culture systems [82, 83] and may depend on a specific time window of GC exposure [84]. These so-called anabolic effects of endogenous GCs can be observed in vivo for bone mass. Mice lacking the GR in osteoblasts [62] as well as osteoblast-specific overexpression of 11β-HSD2 thereby disrupting glucocorticoid signaling [85] display a reduced bone mineral density, albeit with no abnormalities in bone growth and architecture [62, 86]. Indeed osteoblasts lacking the GR display a reduced differentiation potential when grown in normal tissue culture medium with vitamin C and β-glycerolphosphate ad differentiation conditions [62]. As GR^{dim} mice, carrying a dimerization deficient GR, have no obvious bone phenotype and a unaltered osteoblast differentiation, anabolic GC actions on bone are independent of dimerized induced DNA binding [62].

Nonetheless it has been generally accepted that the treatment of osteoblastic cells with high-dose glucocorticoids leads to a suppression of osteoblast differentiation, which could be monitored by the reduced expression of Runx2 [87], the master osteoblast transcription factor [88], and of other marker genes of differentiation. Following reduced differentiation osteoblast function in terms of collagen production declines. Transcription of α1-(I)-procollagen was shown to be effected by glucocorticoid treatment [89]. Accordingly we could show that the suppression of *Col1a1* mRNA in vivo as well in vitro depends on the expression of the GR in osteoblasts [62] since mice lacking the GR in osteoblasts had no reduction of *Col1a1* expression upon glucocorticoid exposure. For this reduction the GR monomer was sufficient suggesting that tethering mechanisms of the GR with transcriptional activators of the Col1a1 gene are involved. It is tempting to speculate that the monomeric GR interferes with TGFβ-triggered smad signaling to reduce collagen I expression [90], but remains to be proven. Furthermore $GR^{Runx2Cre}$ mice but not GR^{dim} mice are resistant to glucocorticoid-induced suppression of bone formation. Consequently bone mass was not affected after 2 weeks of prednisolone treatment in $GR^{Runx2Cre}$ mice. From these findings together with the observation of a strong suppression of primary osteoblast differentiation around 70–90% in vitro we conclude that inhibition of differentiation is a major mechanism of GIO.

The underlying mechanisms of suppression of osteoblast differentiation by GCs are not completely understood. A number of evidence was reported from the Smith and Frenkel lab although they performed their experiments almost exclusively in the MC3T3-E1 cell line. Their data suggest that glucocorticoids suppress differentiation by interference with BMP/TGFβ signaling. In particular BMP-2 seems to be a promising target for interference with suppression of differentiation. *Bmp2* expression declines by GC treatment [91] and its exogenous administration rescues mineralization of glucocorticoid treated cells but not collagen deposition in MC3T3-E1 cells [92]. Expression profiling of glucocorticoid-treated MC3T3-E1 cells identified

early growth response 2 (EGR2/Krox20), a zinc finger transcription factor as a glucocorticoid suppressed target gene and a potential mediator of suppression [93]. EGR2/Krox20 is involved in chondrocyte–osteoblast interactions and its ablation severely attenuates bone formation [94]. Intriguingly Krox20 is a transcriptional activator of follistatin [95], an extracellular inhibitor of bone morphogenic proteins (BMPs) [96]. Due to GC prompted downregulation of EGR2 follistatin expression increases and thereby potentially inhibits BMP signaling. Thus, by interfering BMP signaling on several levels GCs can suppress osteoblast differentiation.

Other studies suggest that glucocorticoids might reduce osteoblast differentiation by regulation of insulin like growth factor-1 (IGF-1) action. IGF-1 increases osteoblast lineage expansion, collagen synthesis, and matrix apposition [97, 98]. GCs can directly suppress *Igf1* transcription by upregulation of CAAT/enhancer binding proteins, in particular C/EBPβ and C/EBPδ that are transcriptional inducers of the *Igf1* gene [99]. Furthermore IGF-1 activity can be influenced by the regulation of the IGF activating IGF binding protein 5 (IGFBP-5) that is diminished upon GC treatment of primary osteoblasts [100]. However, the decreased bone formation in IGFBP-5 overexpressing mice [101] questions whether this is a major mechanism.

The induction of C/EBPs as regulators of adipogenic differentiation by GCs is in conformity with the idea that inhibition of osteoblastogenesis leads to a shift towards adipogenesis of mesenchymal progenitor cells [102]. In line with this idea congenic mice with allelic suppression of skeletal and hepatic *Igf1* had low bone mass with fatty infiltration of the bone marrow but no signs of obesity [103]. Furthermore magnetic resonance imaging in humans revealed that the drop in bone mass of osteoporotic men correlates with increased bone marrow adiposity [104]. Importantly, we could recently show GR binding to DNA is instrumental for promotion of adipogenesis, in particular by transcriptional activation of KLF-15 [105]. In the light that adipogenesis by GCs requires GR dimerization [105], which is dispensable for suppression of osteoblast differentiation [62], our findings suggest that adipogenic differentiation by GCs can be uncoupled from suppression of osteoblast differentiation. This also argues against a transdifferentiation of committed osteoblasts towards adipocytes by GCs. Whether the increased bone marrow adiposity in osteoporotic bones originates from switching lineage of osteoblast arrested cells by mechanisms independent of suppression of differentiation or infiltration of mesenchymal progenitor cells is still elusive and requires lineage tracing studies.

Using osteoblasts from GR^{dim} mice that are still capable to undergo GC suppression of osteoblast differentiation we were able to dissect a GR monomer-dependent osteoblast gene expression program (Rauch, unpublished). From this analysis we identified genes encoding members of the IL-6 family like *Il6*, *Lif*, and *Il11* being suppressed in a GR dimer-independent manner. Interestingly, exogenous supplementation of Il11 in GC-treated cultures reversed GC suppression of differentiation. Collagen 1a1 expression, alkaline phosphatase activity and mineralization were at normal levels despite the presence of GCs [62]. We further demonstrated that c-Jun-dependent *Il11* transcriptional upregulation is targeted by the GR, whereas NF-κB interactions were dispensable [62]. IL-11 act in an autocrine manner via the IL11 receptor and the common receptor gp130 which leads to STAT3 phosphorylation, that is reduced in the presence of Dex [63].

Likewise IL-11 itself is a potent inducer of osteoblastogenesis [63] and prevents adipogenesis in culture [106]. The importance of IL-11 in osteoblast function is underscored by the analysis of mice expressing a human *IL11* transgene [106] and mice with activated STAT3 signaling by a *Gp130*$^{F759/F759}$ knock in [107] that both display increased bone formation. Complementary, targeted deletion of the Il-11 receptor results in decreased osteoblast numbers and bone formation in vivo [108] similar as in mice with osteoblast-specific ablation of *Stat3* [107].

Thus, interference with IL-11, an active player in bone formation, is one of the mechanisms how GC suppress bone formation.

4 Novel Criteria for Selective GR Modulators for Therapeutic Efficacy and Avoidance of Osteoporosis

Our approach to dissect the molecular mechanisms of GC action on bone in vivo by the analysis of conditional and function selective GR mutant mice can be summarized as followed (Fig. 1). In GC-induced bone loss the GR in osteoblasts reduces osteoblast activity and numbers mainly by suppression of differentiation. The decreased differentiation of osteoblasts engages the monomer GR without interfering with NF-κB, but interacting with AP-1 bound at promoters, e.g., the *Il11* gene. Suppression of IL-11 release leads to impaired Jak-Stat signaling via gp130/IL11receptors and reduced active phospho-STAT3, important for osteoblast differentiation.

The requirement of the GR monomer for this side effect of GC action might be on the first glance disappointing, since selective ligands had been designed that should maintain the monomeric function, but omit dimerized induced binding of DNA by the GR. Due to our results those compounds would still harm the bone. However, our finding that NF-κB is not involved in suppression of osteoblast differentiation would allow a new profile required for a dissociating GR ligand. A dissociating ligand that should preserve the bone should not induce GR dimerization, in order to avoid anti-proliferation of osteoblasts, and not induce GR interaction with AP-1 to spare suppression of osteoblast differentiation. However this compound should still be able to reduce NF-κB-controlled cytokine expression.

Our analysis of the activity of the GR ligand Compound A (CpdA) on bone cells demonstrates that these criteria can be met [63].

CpdA displays potent anti-inflammatory actions in collagen-induced arthritis [29]. CpdA is in addition capable to suppress pro-inflammatory cytokines, such as CXCL10 and IL-6, and does not influence RANKL/OPG ratio in osteoblastic cell lines and primary cells [63, 109]. Most importantly expression of *Il11* and subsequently osteoblast differentiation are unaffected by CpdA in contrast to classical GCs. Finally mice receiving compound A at the immunosuppressive dose of collagen-induced arthritis have strikingly higher serum osteocalcin levels compared to dexamethasone-treated animals [63]. Unpublished results from De Bosscher and colleagues indeed demonstrate that CpdA does not favor an AP-1/GR interaction in mesenchymal cells in contrast to inflammatory cells. Future work will explain this cell type-specific differential activity of this dissociating ligand.

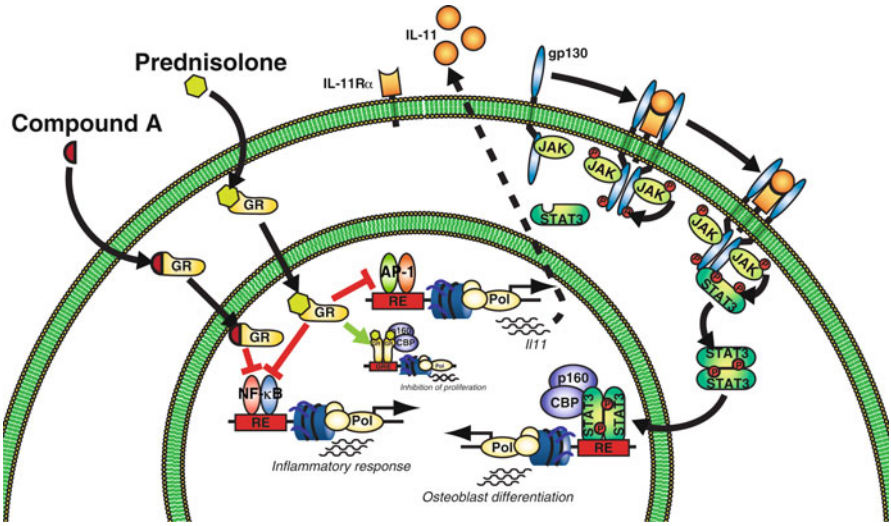

Fig. 1 Selective GR agonists avoiding GR dimerization and GR-AP-1 interaction and subsequent suppression of IL-11 signaling, preserve osteoblast differentiation and maintain anti-inflammatory capacities. Classical GCs such as prednisolone activate the GR in osteoblasts that translocates to the nucleus where it dimerize on DNA elements important for inhibition of proliferation, interferes with NF-κB activity leading to suppression of inflammatory cytokines and suppress AP-1-dependent IL-11 expression. IL-11 itself acts in an autocrine manner on osteoblasts through IL-11 receptor α and gp130 to mainly induce STAT3 activation. In line activated STAT3 and induction of its target genes is important for proper osteoblast differentiation. The dissociating GR ligand CpdA activates only partially the GR by omitting dimerization and GR-AP-1 interaction, but maintains suppression of NF-κB activity in osteoblasts. This allows autocrine IL11 signaling and osteoblast differentiation by keeping anti-inflammatory action

Although CpdA has a narrow therapeutic window we demonstrate here that such substances with optimized pharmacology could be of help in future to suppress inflammatory bone diseases and maintain bone integrity.

References

1. Hench PS, Kendall EC, Slocumb CH, Polley HF (1950) Effects of cortisone acetate and pituitary ACTH on rheumatoid arthritis, rheumatic fever and certain other conditions. Arch Intern Med 85:545–566
2. Hoes JN, Jacobs JW, Buttgereit F, Bijlsma JW (2010) Current view of glucocorticoid co-therapy with DMARDs in rheumatoid arthritis. Nat Rev Rheumatol 6(12):693–702
3. Baschant U, Tuckermann J (2010) The role of the glucocorticoid receptor in inflammation and immunity. J Steroid Biochem Mol Biol 120(2–3):69–75
4. Nemoto T, Ohara-Nemoto Y, Denis M, Gustafsson JA (1990) The transformed glucocorticoid receptor has a lower steroid-binding affinity than the nontransformed receptor. Biochemistry 29(7):1880–1886
5. Davies TH, Ning YM, Sanchez ER (2002) A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. J Biol Chem 277(7): 4597–4600

6. Freedman ND, Yamamoto KR (2004) Importin 7 and importin alpha/importin beta are nuclear import receptors for the glucocorticoid receptor. Mol Biol Cell 15(5):2276–2286
7. Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature 352(6335):497–505
8. Chandra V, Huang P, Hamuro Y, Raghuram S, Wang Y, Burris TP, Rastinejad F (2008) Structure of the intact PPAR-gamma-RXR-nuclear receptor complex on DNA. Nature 456(7220):350–356
9. Meijsing SH, Pufall MA, So AY, Bates DL, Chen L, Yamamoto KR (2009) DNA binding site sequence directs glucocorticoid receptor structure and activity. Science 324(5925):407–410
10. Stavreva DA, Wiench M, John S, Conway-Campbell BL, McKenna MA, Pooley JR, Johnson TA, Voss TC, Lightman SL, Hager GL (2009) Ultradian hormone stimulation induces glucocorticoid receptor-mediated pulses of gene transcription. Nat Cell Biol 11(9):1093–1102
11. Heck S, Kullmann M, Gast A, Ponta H, Rahmsdorf HJ, Herrlich P, Cato AC (1994) A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. EMBO J 13(17):4087–4095
12. Heck S, Bender K, Kullmann M, Gottlicher M, Herrlich P, Cato AC (1997) I kappaB alpha-independent downregulation of NF-kappaB activity by glucocorticoid receptor. EMBO J 16(15):4698–4707
13. Reily MM, Pantoja C, Hu X, Chinenov Y, Rogatsky I (2006) The GRIP1:IRF3 interaction as a target for glucocorticoid receptor-mediated immunosuppression. EMBO J 25(1):108–117
14. Kassel O, Schneider S, Heilbock C, Litfin M, Gottlicher M, Herrlich P (2004) A nuclear isoform of the focal adhesion LIM-domain protein Trip6 integrates activating and repressing signals at AP-1- and NF-kappaB-regulated promoters. Genes Dev 18(20):2518–2528
15. He Y, Simons SS Jr (2007) STAMP, a novel predicted factor assisting TIF2 actions in glucocorticoid receptor-mediated induction and repression. Mol Cell Biol 27(4):1467–1485
16. De Bosscher K, Vanden Berghe W, Haegeman G (2001) Glucocorticoid repression of AP-1 is not mediated by competition for nuclear coactivators. Mol Endocrinol 15(2):219–227
17. Nissen RM, Yamamoto KR (2000) The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. Genes Dev 14(18):2314–2329
18. Ogawa S, Lozach J, Benner C, Pascual G, Tangirala RK, Westin S, Hoffmann A, Subramaniam S, David M, Rosenfeld MG, Glass CK (2005) Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. Cell 122(5):707–721
19. Flammer JR, Dobrovolna J, Kennedy MA, Chinenov Y, Glass CK, Ivashkiv LB, Rogatsky I (2010) The type I interferon signaling pathway is a target for glucocorticoid inhibition. Mol Cell Biol 30(19):4564–4574
20. De Bosscher K, Beck IM, Haegeman G (2010) Classic glucocorticoids versus non-steroidal glucocorticoid receptor modulators: survival of the fittest regulator of the immune system? Brain Behav Immun 24(7):1035–1042
21. Belvisi MG, Wicks SL, Battram CH, Bottoms SE, Redford JE, Woodman P, Brown TJ, Webber SE, Foster ML (2001) Therapeutic benefit of a dissociated glucocorticoid and the relevance of in vitro separation of transrepression from transactivation activity. J Immunol 166(3):1975–1982
22. Coghlan MJ, Jacobson PB, Lane B, Nakane M, Lin CW, Elmore SW, Kym PR, Luly JR, Carter GW, Turner R, Tyree CM, Hu J, Elgort M, Rosen J, Miner JN (2003) A novel antiinflammatory maintains glucocorticoid efficacy with reduced side effects. Mol Endocrinol 17(5):860–869
23. De Bosscher K, Vanden Berghe W, Beck IM, Van Molle W, Hennuyer N, Hapgood J, Libert C, Staels B, Louw A, Haegeman G (2005) A fully dissociated compound of plant origin for inflammatory gene repression. Proc Natl Acad Sci USA 102(44):15827–15832
24. Lopez FJ, Ardecky RJ, Bebo B, Benbatoul K, De Grandpre L, Liu S, Leibowitz MD, Marschke K, Rosen J, Rungta D, Viveros HO, Yen WC, Zhi L, Negro-Vilar A, Miner JN (2008) LGD-5552, an antiinflammatory glucocorticoid receptor ligand with reduced side effects, in vivo. Endocrinology 149(5):2080–2089

25. Schacke H, Schottelius A, Docke WD, Strehlke P, Jaroch S, Schmees N, Rehwinkel H, Hennekes H, Asadullah K (2004) Dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist leads to separation of therapeutic effects from side effects. Proc Natl Acad Sci USA 101(1):227–232
26. Schacke H, Zollner TM, Docke WD, Rehwinkel H, Jaroch S, Skuballa W, Neuhaus R, May E, Zugel U, Asadullah K (2009) Characterization of ZK 245186, a novel, selective glucocorticoid receptor agonist for the topical treatment of inflammatory skin diseases. Br J Pharmacol 158(4):1088–1103
27. Wust S, Tischner D, John M, Tuckermann JP, Menzfeld C, Hanisch UK, van den Brandt J, Luhder F, Reichardt HM (2009) Therapeutic and adverse effects of a non-steroidal glucocorticoid receptor ligand in a mouse model of multiple sclerosis. PLoS One 4(12):e8202
28. van Loo G, Sze M, Bougarne N, Praet J, Mc Guire C, Ullrich A, Haegeman G, Prinz M, Beyaert R, De Bosscher K (2010) Antiinflammatory properties of a plant-derived nonsteroidal, dissociated glucocorticoid receptor modulator in experimental autoimmune encephalomyelitis. Mol Endocrinol 24(2):310–322
29. Dewint P, Gossye V, De Bosscher K, Vanden Berghe W, Van Beneden K, Deforce D, Van Calenbergh S, Muller-Ladner U, Vander Cruyssen B, Verbruggen G, Haegeman G, Elewaut D (2008) A plant-derived ligand favoring monomeric glucocorticoid receptor conformation with impaired transactivation potential attenuates collagen-induced arthritis. J Immunol 180(4):2608–2615
30. Humphrey EL, Williams JH, Davie MW, Marshall MJ (2006) Effects of dissociated glucocorticoids on OPG and RANKL in osteoblastic cells. Bone 38(5):652–661
31. Tronche F, Kellendonk C, Reichardt HM, Schutz G (1998) Genetic dissection of glucocorticoid receptor function in mice. Curr Opin Genet Dev 8(5):532–538
32. Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, Schutz G (1999) Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat Genet 23(1):99–103
33. Cole TJ, Blendy JA, Monaghan AP, Krieglstein K, Schmid W, Aguzzi A, Fantuzzi G, Hummler E, Unsicker K, Schütz G (1995) Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev 9(13):1608–1621
34. Tuckermann JP, Kleiman A, Moriggl R, Spanbroek R, Neumann A, Illing A, Clausen BE, Stride B, Forster I, Habenicht AJ, Reichardt HM, Tronche F, Schmid W, Schutz G (2007) Macrophages and neutrophils are the targets for immune suppression by glucocorticoids in contact allergy. J Clin Invest 117(5):1381–1390
35. Bhattacharyya S, Brown DE, Brewer JA, Vogt SK, Muglia LJ (2007) Macrophage glucocorticoid receptors regulate Toll-like receptor-4-mediated inflammatory responses by selective inhibition of p38 MAP kinase. Blood 109(10):4313–4319
36. Wust S, van den Brandt J, Tischner D, Kleiman A, Tuckermann JP, Gold R, Luhder F, Reichardt HM (2008) Peripheral T cells are the therapeutic targets of glucocorticoids in experimental autoimmune encephalomyelitis. J Immunol 180(12):8434–8443
37. Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, Gass P, Schmid W, Herrlich P, Angel P, Schutz G (1998) DNA binding of the glucocorticoid receptor is not essential for survival. Cell 93(4):531–541
38. Reichardt HM, Tuckermann JP, Göttlicher M, Vujic M, Weih F, Angel P, Herrlich P, Schütz G (2001) Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. EMBO J 20(24):7168–7173
39. Tuckermann JP, Reichardt HM, Arribas R, Richter KH, Schutz G, Angel P (1999) The DNA binding-independent function of the glucocorticoid receptor mediates repression of AP-1-dependent genes in skin. J Cell Biol 147(7):1365–1370
40. Ayroldi E, Migliorati G, Bruscoli S, Marchetti C, Zollo O, Cannarile L, D'Adamio F, Riccardi C (2001) Modulation of T-cell activation by the glucocorticoid-induced leucine zipper factor via inhibition of nuclear factor kappaB. Blood 98(3):743–753

41. Perretti M, D'Acquisto F (2009) Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. Nat Rev Immunol 9(1):62–70
42. Kassel O, Sancono A, Kratzschmar J, Kreft B, Stassen M, Cato AC (2001) Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. EMBO J 20(24):7108–7116
43. Lasa M, Abraham SM, Boucheron C, Saklatvala J, Clark AR (2002) Dexamethasone causes sustained expression of mitogen-activated protein kinase (MAPK) phosphatase 1 and phosphatase-mediated inhibition of MAPK p38. Mol Cell Biol 22(22):7802–7811
44. Abraham SM, Lawrence T, Kleiman A, Warden P, Medghalchi M, Tuckermann J, Saklatvala J, Clark AR (2006) Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1. J Exp Med 203(8):1883–1889
45. Cushing H (1932) Further concerning a parasympathetic center in the interbrain: VII. The effect of intraventricularly-injected histamine. Proc Natl Acad Sci USA 18(7):500–510
46. Eastell R, Reid DM, Compston J, Cooper C, Fogelman I, Francis RM, Hosking DJ, Purdie DW, Ralston SH, Reeve J, Russell RG, Stevenson JC, Torgerson DJ (1998) A UK Consensus Group on management of glucocorticoid-induced osteoporosis: an update. J Intern Med 244(4):271–292
47. Van Staa TP, Leufkens HG, Abenhaim L, Zhang B, Cooper C (2000) Use of oral corticosteroids and risk of fractures. J Bone Miner Res 15(6):993–1000
48. Dalle Carbonare L, Arlot ME, Chavassieux PM, Roux JP, Portero NR, Meunier PJ (2001) Comparison of trabecular bone microarchitecture and remodeling in glucocorticoid-induced and postmenopausal osteoporosis. J Bone Miner Res 16(1):97–103
49. Klein RG, Arnaud SB, Gallagher JC, Deluca HF, Riggs BL (1977) Intestinal calcium absorption in exogenous hypercortisonism. Role of 25-hydroxyvitamin D and corticosteroid dose. J Clin Invest 60(1):253–259
50. Nielsen HK, Thomsen K, Eriksen EF, Charles P, Storm T, Mosekilde L (1988) The effects of high-dose glucocorticoid administration on serum bone gamma carboxyglutamic acid-containing protein, serum alkaline phosphatase and vitamin D metabolites in normal subjects. Bone Miner 4(1):105–113
51. Mazziotti G, Angeli A, Bilezikian JP, Canalis E, Giustina A (2006) Glucocorticoid-induced osteoporosis: an update. Trends Endocrinol Metab 17(4):144–149
52. Parisien M, Silverberg SJ, Shane E, de la Cruz L, Lindsay R, Bilezikian JP, Dempster DW (1990) The histomorphometry of bone in primary hyperparathyroidism: preservation of cancellous bone structure. J Clin Endocrinol Metab 70(4):930–938
53. Sakakura M, Takebe K, Nakagawa S (1975) Inhibition of luteinizing hormone secretion induced by synthetic LRH by long-term treatment with glucocorticoids in human subjects. J Clin Endocrinol Metab 40(5):774–779
54. Hsueh AJ, Erickson GF (1978) Glucocorticoid inhibition of FSH-induced estrogen production in cultured rat granulosa cells. Steroids 32(5):639–648
55. Doerr P, Pirke KM (1976) Cortisol-induced suppression of plasma testosterone in normal adult males. J Clin Endocrinol Metab 43(3):622–629
56. Goulding A, Gold E (1988) Effects of chronic prednisolone treatment on bone resorption and bone composition in intact and ovariectomized rats and in ovariectomized rats receiving beta-estradiol. Endocrinology 122(2):482–487
57. Hofbauer LC, Gori F, Riggs BL, Lacey DL, Dunstan CR, Spelsberg TC, Khosla S (1999) Stimulation of osteoprotegerin ligand and inhibition of osteoprotegerin production by glucocorticoids in human osteoblastic lineage cells: potential paracrine mechanisms of glucocorticoid-induced osteoporosis. Endocrinology 140(10):4382–4389
58. Kondo T, Kitazawa R, Yamaguchi A, Kitazawa S (2008) Dexamethasone promotes osteoclastogenesis by inhibiting osteoprotegerin through multiple levels. J Cell Biochem 103(1):335–345
59. Hirano F, Kobayashi A, Maruyama N, Komura K, Okamoto K, Makino Y, Haneda M (2008) Parathyroid hormone and glucocorticoid cooperatively induce RANKL mRNA expression in

osteoblasts through different mechanisms. In: European congress of endocrinology, vol 16. Berlin, p. 72
60. Pfeilschifter J, Chenu C, Bird A, Mundy GR, Roodman GD (1989) Interleukin-1 and tumor necrosis factor stimulate the formation of human osteoclastlike cells in vitro. J Bone Miner Res 4(1):113–118
61. Jilka RL, Hangoc G, Girasole G, Passeri G, Williams DC, Abrams JS, Boyce B, Broxmeyer H, Manolagas SC (1992) Increased osteoclast development after estrogen loss: mediation by interleukin-6. Science 257(5066):88–91
62. Rauch A, Seitz S, Baschant U, Schilling AF, Illing A, Stride B, Kirilov M, Mandic V, Takacz A, Schmidt-Ullrich R, Ostermay S, Schinke T, Spanbroek R, Zaiss MM, Angel PE, Lerner UH, David JP, Reichardt HM, Amling M, Schutz G, Tuckermann JP (2010) Glucocorticoids suppress bone formation by attenuating osteoblast differentiation via the monomeric glucocorticoid receptor. Cell Metab 11(6):517–531
63. Rauch A, Gossye V, Bracke D, Gevaert E, Jacques P, Van Beneden K, Vandooren B, Rauner M, Hofbauer LC, Haegeman G, Elewaut D, Tuckermann JP, De Bosscher K (2011) An anti-inflammatory selective glucocorticoid receptor modulator preserves osteoblast differentiation. FASEB J 25(4):1323–1332
64. Takuma A, Kaneda T, Sato T, Ninomiya S, Kumegawa M, Hakeda Y (2003) Dexamethasone enhances osteoclast formation synergistically with transforming growth factor-beta by stimulating the priming of osteoclast progenitors for differentiation into osteoclasts. J Biol Chem 278(45):44667–44674
65. Hofbauer LC, Zeitz U, Schoppet M, Skalicky M, Schuler C, Stolina M, Kostenuik PJ, Erben RG (2009) Prevention of glucocorticoid-induced bone loss in mice by inhibition of RANKL. Arthritis Rheum 60(5):1427–1437
66. Jia D, O'Brien CA, Stewart SA, Manolagas SC, Weinstein RS (2006) Glucocorticoids act directly on osteoclasts to increase their life span and reduce bone density. Endocrinology 147(12):5592–5599
67. Kim HJ, Zhao H, Kitaura H, Bhattacharyya S, Brewer JA, Muglia LJ, Ross FP, Teitelbaum SL (2006) Glucocorticoids suppress bone formation via the osteoclast. J Clin Invest 116(8):2152–2160
68. Smith E, Redman RA, Logg CR, Coetzee GA, Kasahara N, Frenkel B (2000) Glucocorticoids inhibit developmental stage-specific osteoblast cell cycle. Dissociation of cyclin A-cyclin-dependent kinase 2 from E2F4-p130 complexes. J Biol Chem 275(26):19992–20001
69. Smith E, Coetzee GA, Frenkel B (2002) Glucocorticoids inhibit cell cycle progression in differentiating osteoblasts via glycogen synthase kinase-3beta. J Biol Chem 277(20):18191–18197
70. Smith E, Frenkel B (2005) Glucocorticoids inhibit the transcriptional activity of LEF/TCF in differentiating osteoblasts in a glycogen synthase kinase-3beta-dependent and -independent manner. J Biol Chem 280(3):2388–2394
71. Ohnaka K, Taniguchi H, Kawate H, Nawata H, Takayanagi R (2004) Glucocorticoid enhances the expression of dickkopf-1 in human osteoblasts: novel mechanism of glucocorticoid-induced osteoporosis. Biochem Biophys Res Commun 318(1):259–264
72. Butler JS, Queally JM, Devitt BM, Murray DW, Doran PP, O'Byrne JM (2010) Silencing Dkk1 expression rescues dexamethasone-induced suppression of primary human osteoblast differentiation. BMC Musculoskelet Disord 11:210
73. Horsch K, de Wet H, Schuurmans MM, Allie-Reid F, Cato AC, Cunningham J, Burrin JM, Hough FS, Hulley PA (2007) Mitogen-activated protein kinase phosphatase 1/dual specificity phosphatase 1 mediates glucocorticoid inhibition of osteoblast proliferation. Mol Endocrinol 21(12):2929–2940
74. Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC (1998) Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Invest 102(2):274–282
75. Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC, Bellido T (1999) Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. J Clin Invest 104(10):1363–1374

76. Plotkin LI, Mathov I, Aguirre JI, Parfitt AM, Manolagas SC, Bellido T (2005) Mechanical stimulation prevents osteocyte apoptosis: requirement of integrins, Src kinases, and ERKs. Am J Physiol Cell Physiol 289(3):C633–C643
77. Bellido T (2010) Antagonistic interplay between mechanical forces and glucocorticoids in bone: a tale of kinases. J Cell Biochem 111(1):1–6
78. Plotkin LI, Manolagas SC, Bellido T (2007) Glucocorticoids induce osteocyte apoptosis by blocking focal adhesion kinase-mediated survival: evidence for inside-out signaling leading to anoikis. J Biol Chem 282(33):24120–24130
79. Xiong W, Parsons JT (1997) Induction of apoptosis after expression of PYK2, a tyrosine kinase structurally related to focal adhesion kinase. J Cell Biol 139(2):529–539
80. Hong D, Chen HX, Yu HQ, Wang C, Deng HT, Lian QQ, Ge RS (2010) Quantitative proteomic analysis of dexamethasone-induced effects on osteoblast differentiation, proliferation, and apoptosis in MC3T3-E1 cells using SILAC. Osteoporos Int 22(7):2175–2186
81. O'Brien CA, Jia D, Plotkin LI, Bellido T, Powers CC, Stewart SA, Manolagas SC, Weinstein RS (2004) Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. Endocrinology 145(4):1835–1841
82. Cheng SL, Yang JW, Rifas L, Zhang SF, Avioli LV (1994) Differentiation of human bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone. Endocrinology 134(1):277–286
83. Bellows CG, Aubin JE, Heersche JN (1987) Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells in vitro. Endocrinology 121(6):1985–1992
84. Eijken M, Koedam M, van Driel M, Buurman CJ, Pols HA, van Leeuwen JP (2006) The essential role of glucocorticoids for proper human osteoblast differentiation and matrix mineralization. Mol Cell Endocrinol 248(1–2):87–93
85. Sher LB, Woitge HW, Adams DJ, Gronowicz GA, Krozowski Z, Harrison JR, Kream BE (2004) Transgenic expression of 11beta-hydroxysteroid dehydrogenase type 2 in osteoblasts reveals an anabolic role for endogenous glucocorticoids in bone. Endocrinology 145(2): 922–929
86. Sher LB, Harrison JR, Adams DJ, Kream BE (2006) Impaired cortical bone acquisition and osteoblast differentiation in mice with osteoblast-targeted disruption of glucocorticoid signaling. Calcif Tissue Int 79(2):118–125
87. Chang DJ, Ji C, Kim KK, Casinghino S, McCarthy TL, Centrella M (1998) Reduction in transforming growth factor beta receptor I expression and transcription factor CBFa1 on bone cells by glucocorticoid. J Biol Chem 273(9):4892–4896
88. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89(5):755–764
89. Delany AM, Gabbitas BY, Canalis E (1995) Cortisol downregulates osteoblast alpha 1 (I) procollagen mRNA by transcriptional and posttranscriptional mechanisms. J Cell Biochem 57(3):488–494
90. Centrella M, McCarthy TL, Canalis E (1991) Glucocorticoid regulation of transforming growth factor beta 1 activity and binding in osteoblast-enriched cultures from fetal rat bone. Mol Cell Biol 11(9):4490–4496
91. Luppen CA, Chandler RL, Noh T, Mortlock DP, Frenkel B (2008) BMP-2 vs. BMP-4 expression and activity in glucocorticoid-arrested MC3T3-E1 osteoblasts: Smad signaling, not alkaline phosphatase activity, predicts rescue of mineralization. Growth Factors 26(4):226–237
92. Luppen CA, Leclerc N, Noh T, Barski A, Khokhar A, Boskey AL, Smith E, Frenkel B (2003) Brief bone morphogenetic protein 2 treatment of glucocorticoid-inhibited MC3T3-E1 osteoblasts rescues commitment-associated cell cycle and mineralization without alteration of Runx2. J Biol Chem 278(45):44995–45003
93. Leclerc N, Luppen CA, Ho VV, Nagpal S, Hacia JG, Smith E, Frenkel B (2004) Gene expression profiling of glucocorticoid-inhibited osteoblasts. J Mol Endocrinol 33(1):175–193

94. Levi G, Topilko P, Schneider-Maunoury S, Lasagna M, Mantero S, Cancedda R, Charnay P (1996) Defective bone formation in Krox-20 mutant mice. Development 122(1):113–120
95. Seitanidou T, Schneider-Maunoury S, Desmarquet C, Wilkinson DG, Charnay P (1997) Krox-20 is a key regulator of rhombomere-specific gene expression in the developing hindbrain. Mech Dev 65(1–2):31–42
96. Fainsod A, Deissler K, Yelin R, Marom K, Epstein M, Pillemer G, Steinbeisser H, Blum M (1997) The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4. Mech Dev 63(1):39–50
97. Delany AM, Rydziel S, Canalis E (1996) Autocrine down-regulation of collagenase-3 in rat bone cell cultures by insulin-like growth factors. Endocrinology 137(11):4665–4670
98. Canalis E, Centrella M, Burch W, McCarthy TL (1989) Insulin-like growth factor I mediates selective anabolic effects of parathyroid hormone in bone cultures. J Clin Invest 83(1): 60–65
99. Delany AM, Durant D, Canalis E (2001) Glucocorticoid suppression of IGF I transcription in osteoblasts. Mol Endocrinol 15(10):1781–1789
100. Gabbitas B, Pash JM, Delany AM, Canalis E (1996) Cortisol inhibits the synthesis of insulin-like growth factor-binding protein-5 in bone cell cultures by transcriptional mechanisms. J Biol Chem 271(15):9033–9038
101. Devlin RD, Du Z, Buccilli V, Jorgetti V, Canalis E (2002) Transgenic mice overexpressing insulin-like growth factor binding protein-5 display transiently decreased osteoblastic function and osteopenia. Endocrinology 143(10):3955–3962
102. Rosen CJ, Bouxsein ML (2006) Mechanisms of disease: is osteoporosis the obesity of bone? Nat Clin Pract Rheumatol 2(1):35–43
103. Rosen CJ, Ackert-Bicknell C, Beamer WG, Nelson T, Adamo M, Cohen P, Bouxsein ML, Horowitz MC (2005) Allelic differences in a quantitative trait locus affecting insulin-like growth factor-I impact skeletal acquisition and body composition. Pediatr Nephrol 20(3):255–260
104. Wehrli FW, Hopkins JA, Hwang SN, Song HK, Snyder PJ, Haddad JG (2000) Cross-sectional study of osteopenia with quantitative MR imaging and bone densitometry. Radiology 217(2):527–538
105. Asada M, Rauch A, Shimizu H, Maruyama H, Miyaki S, Shibamori M, Kawasome H, Ishiyama H, Tuckermann J, Asahara H (2010) DNA binding-dependent glucocorticoid receptor activity promotes adipogenesis via Kruppel-like factor 15 gene expression. Lab Invest 91(2):203–215
106. Takeuchi Y, Watanabe S, Ishii G, Takeda S, Nakayama K, Fukumoto S, Kaneta Y, Inoue D, Matsumoto T, Harigaya K, Fujita T (2002) Interleukin-11 as a stimulatory factor for bone formation prevents bone loss with advancing age in mice. J Biol Chem 277(50): 49011–49018
107. Itoh S, Udagawa N, Takahashi N, Yoshitake F, Narita H, Ebisu S, Ishihara K (2006) A critical role for interleukin-6 family-mediated Stat3 activation in osteoblast differentiation and bone formation. Bone 39(3):505–512
108. Sims NA, Jenkins BJ, Nakamura A, Quinn JM, Li R, Gillespie MT, Ernst M, Robb L, Martin TJ (2005) Interleukin-11 receptor signaling is required for normal bone remodeling. J Bone Miner Res 20(7):1093–1102
109. Rauner M, Goettsch C, Stein N, Thiele S, Bornhaeuser M, De Bosscher K, Haegeman G, Tuckermann J, Hofbauer LC (2011) Dissociation of osteogenic and immunological effects by the selective glucocorticoid receptor agonist, compound a, in human bone marrow stromal cells. Endocrinology 152(1):103–112

A Tak1/p38 Signaling Axis Regulates Runx2 Activity and Osteoblast Functions

Matthew B. Greenblatt, Jae-Hyuck Shim, Weiguo Zou, and Laurie H. Glimcher

1 Introduction

Skeletal modeling during development and remodeling during adult life depends on coupling osteoblast differentiation to extracellular cues. The transcription factor Runx2 is the master regulator of osteoblast differentiation and function, and therefore its activity must be tied to these extracellular cues [1, 2]. However, relatively little is understood about what signal transduction pathways might accomplish this.

Prior genetic evidence indicates that the mitogen-activated protein kinases (MAPKs), in particular ERK MAPK, might serve this function by regulating Runx2 [3]. Some of the MAPKs were first identified as mediators of inflammatory responses, especially signaling by toll-like receptors [4–6]. We were interested in the possibility that a wide range of molecules identified as inflammatory mediators might also function to regulate osteoblast differentiation. In particular, we focused on TAK1 (MAP3K7), an MAP3K remarkable in that it is poised at the confluence of both numerous upstream pathways such as antigen receptors, TLR, IL1, and TNF signaling and numerous downstream pathways such as NF-kB, p38, and JNK MAPKs [7–11].

M.B. Greenblatt • J.-H. Shim • W. Zou • L.H. Glimcher (✉)
Department of Immunology and Infectious Diseases,
Harvard School of Public Health,
and Department of Medicine, Harvard Medical School,
Boston, MA 02115, USA
e-mail: lglimche@hsph.harvard.edu

Y. Choi (ed.), *Osteoimmunology: Interactions of the Immune and Skeletal Systems,*
DOI 10.1007/978-1-4614-5366-6_6,

2 TAK1 Deletion in Osteoblasts in In Vivo Results in Mice with Features of Cleidocranial Dysplasia

To evaluate the function of TAK1 in osteoblasts, mice with a conditional deletion of a *Tak1* floxed allele driven by the osterix promoter were bred (*Tak1*^{osx}). The resulting *Tak1*^{osx} strain displays profound osteopenia and stigmata associated with the human skeletal disorder cleidocranial dysplasia, calvarial hypomineralization, and clavicular hypoplasia [12]. Since human and mouse versions of cleidocranial dysplasia are both caused by haploinsufficiency of Runx2, TAK1 might be involved in the regulation of Runx2 [1, 13]. Examination of Runx2 levels revealed no detectable alterations in Runx2 transcript levels in the bones of *Tak1*^{osx} mice or in Runx2 protein levels in TAK1-deficient calvarial osteoblasts, suggesting that TAK1 is instead involved in the posttranslational regulation of Runx2 activity.

When examined in vitro, TAK1-deficient osteoblasts displayed defects in both early and late stages osteoblast differentiation. Defects in early differentiation were apparent though decreased alkaline phosphatase (ALP) and osterix (Osx) levels. Both ALP and Osx are known Runx2 target genes [14]. Additionally, later stage markers such as osteocalcin and collagen 1 were also reduced. These reductions could also be confirmed in vivo using in situ hybridization and real-time PCR. Runx2 activity was directly probed using the OG2 luciferase reporter and found to be reduced in TAK1-deficient osteoblasts. Enforced expression of Runx2 in WT osteoblasts, but not in TAK1 deficient osteoblasts, greatly potentiates osteoblast differentiation, demonstrating that TAK1 is required for Runx2 activity via a posttranslational mechanism.

In order to identify the pathway downstream of TAK1 that might regulate Runx2, we examined JNK, ERK, and p38 signaling in TAK1-deficient osteoblasts. Activation of the p38 pathway was deficient in response to BMP2/7 stimulation both in vivo via immunohistochemistry and in vitro in cultured osteoblasts. In contrast, activation of JNK ERK was preserved under the conditions examined. Given that TAK1 has been closely associated with activation of JNK in other contexts, this suggests that signaling molecules best understood as inflammatory mediators are likely to have very different the roles and "wiring" to upstream and downstream pathways when studied in osteoblasts [10, 15]. Thus, assumptions regarding the basic connectivity of MAPK pathways will have to be revisited in the context of bone biology.

3 The p38 MAPK Pathway Regulates Early Osteoblast Differentiation in In Vivo and In Vitro

The findings that TAK1 is a critical regulator of both osteoblast differentiation and p38 MAPK activation suggest that the p38 MAPK pathway in turn plays a critical role in osteoblasts. To evaluate this, mice lacking either of the two MAP2Ks connecting TAK1 and p38, MKK3 and MKK6, were examined. Both MKK3- and

MKK6-deficient mice were osteopenic at 3–4 weeks of age, and *Mkk3*$^{-/-}$*Mkk6*$^{+/-}$ mice displayed a more profound phenotype than mice lacking either MAP2K alone. Double knockout mice could not be examined due to embryonic lethality [16].

In contrast to the overlapping roles of MKK3 and MKK6 in the regulation of overall bone mass, only *Mkk3*$^{-/-}$ mice displayed a reduction in calvarial mineralization, with no effect seen in *Mkk6*$^{-/-}$ mice, and no additional contribution was seen in *Mkk3*$^{-/-}$*Mkk6*$^{+/-}$ mice. Whereas it is well appreciated that the functions of MAP3Ks vary from tissue to tissue and from stimulus to stimulus, MAP2Ks tend to behave in a more stereotyped fashion. These findings shed new light on tissue specificity at the MAP2K level, suggesting that the function of MAP2Ks is also highly context specific. This argues that a systematic and parallel analysis of MKK3 and MKK6 functions in systems where p38 is known to function will likely demonstrate a mixture of overlapping and unique roles that are tissue and stimulus specific.

p38 MAPK has 4 isoforms, labeled p38α, β, γ, and δ [17]. During in vitro studies, we found that the p38 inhibitor SB203580, which targets p38 α and β, is a potent inhibitor of early osteoblast differentiation as monitored by acquisition of alkaline phosphatase activity [18]. Thus, we focused on determining the role of p38α and β in vivo, though we cannot exclude an additional contribution from p38 γ and δ. The contribution of p38α to bone mineralization was assessed in vivo by injecting neonatal homozygous floxed allele mice with a concentrated cre recombinase-encoding lentivirus over the calvarium. One week later, mice were examined by microCT, and deletion of p38α was found to significantly retard progression of the mineralization fronts along the calvarial sutures. Thus, p38α is a physiologic regulator of skeletal mineralization.

p38β-deficient mice have been examined in detail and have not been appreciated to have a detectable phenotype [19]. In particular, T cell receptor, toll-like receptor, and IGF signaling have been examined and found to be normal. However, when the bones of 4-week-old p38β mice were scanned by μCT, they were found to be osteopenic. Interestingly, whereas p38α clearly contributes to calviarial mineralization, p38β knockout mice show only very subtle alterations in calvarial mineralization. Thus, p38β is not simply redundant with p38α and appears to play a unique role in bone metabolism in vivo.

Despite the fact that both p38α and p38β contribute to skeletal mineralization in vivo, the phenotype of p38α- and p38β-deficient osteoblasts in vitro are quite distinct. Whereas p38α-deficient osteoblasts display defects in both markers of early and late osteoblast differentiation, p38β-deficient osteoblasts only display defects in the later stages of osteoblast differentiation. In particular, only p38α-deficient osteoblasts display reduced ALP levels. This distinction was not simply due to redundancy or overlap in the roles of p38α and p38β, as knockdown of p38α in both WT and p38β$^{-/-}$ osteoblasts produced a similar decrease in ALP levels, with no additional contribution from the absence of p38β.

Examination of the kinetics of MKK3 and MKK6 expression explains the difference in function between p38α and p38β and also ties our observations regarding the MAP2Ks and MAPKs in the p38 pathway together. Previous biochemical studies have identified that whereas MKK6 can phosphorylate and activate both p38α and p38β, MKK3 can only activate p38α [18]. Given that no evidence existed for

a physiological role for p38β, no rationale could be offered for the evolution of this specificity in MKK3 action. Whereas we found that MKK3 is expressed at steady levels throughout early to late osteoblast differentiation, MKK6 is upregulated starting after 10 days in culture. This results in the activation of p38α throughout osteoblast differentiation, whereas p38β is activated only during later stages. Moreover, this reliance of p38β on MKK6 for activation is consistent with their similar phenotypes in vivo, in that both contribute to long bone but not calvarial mineralization.

Thus, every component of the p38 MAPK pathway examined plays a role in skeletal mineralization in vivo, with the observation of osteopenia in p38β-deficient mice being the first recorded function for p38β in vivo.

4 Activation of the TAK1/p38 MAPK Axis Results in Runx2 Phosphorylation

The phenotype of *Tak1*^{osx} mice implicates TAK1 in the regulation of Runx2 activity. In order to identify how this occurs, the ability of p38 isoforms to interact with Runx2 was examined. All four p38 isoforms were able to bind Runx2 in a co-expression IP. Additionally, a robust interaction between endogenous p38 and Runx2 could be detected using an antibody that recognizes all four p38 isoforms. In contrast, we could not detect any direct interaction between TAK1 and Runx2.

The p38-mediated phosphorylation sites in Runx2 were mapped by co-expressing constitutively active MKK6, p38α, and Myc-tagged Runx2 in an HEK293 cell system. Three inducible Runx2 phosphorylation sites were identified by phospho mass spectrometry, corresponding to serines 17, 261, and 298.

To test this observation and examine if TAK1 or the p38 pathway were physiological regulators of Runx2 phosphorylation in osteoblasts, overall Runx2 phosphorylation levels in osteoblasts deficient for either TAK1 or p38α was measured using radioactive orthophosphate labeling. Absence of TAK1 caused an approximately 50% reduction in overall Runx2 phosphorylation levels. Consistent with both p38α and p38β playing a role downstream of TAK1, absence of p38α caused a more modest ~25% reduction in overall Runx2 phosphorylation levels. The partial rather than complete reduction in Runx2 phosphorylation likely reflects the action of kinases outside the TAK1/p38 MAPK pathway and is consistent with the constitutive phosphorylation sites noted in the mass spectrometry study.

5 Phosphorylation of Runx2 by p38 Activates Runx2 by Promoting Association with the Cofactor CBP

To determine how alterations in p38 activation might impact Runx2, the functional relationship between p38, Runx2 activity, and osteoblast differentiation was explored. As previously published, treatment of osteoblasts with the p38 inhibitor SB203580 blocks their differentiation. Additionally, enforcing activation of the p38

pathway by expressing a constitutively active MKK6 mutant (Mkk6-glu) augmented osteoblast differentiation as measured by ALP acquisition and extracellular matrix mineralization. Moreover, expression of Mkk6-glu was able to rescue the defect in the differentiation of TAK1-deficient osteoblasts, providing functional evidence that the p38 MAPK pathway is a key downstream mediator of the TAK1 phenotype. Expression of Mkk6-glu also increased the activity of the Runx2-responsive OSE2 luciferase reporter, and treatment with the p38 inhibitor SB203580-reduced OSE2 activity. Thus, modulation of p38 activity levels produces a corresponding modulation of Runx2 transcriptional activity.

To link this back to the three phosphorylation sites identified above, a Runx2 mutant was constructed with these three sites mutated to alanine (Runx2-3SA). Mutation of all three sites blunted the induction of Runx2 activity achieved by coexpression of Mkk6-glu. Comparison of Runx2-3SA to Runx2 mutants with only 1 or 2 of the 3 sites mutated to alanine showed a gradual loss of activity corresponding to the total number of sites mutated, with no single site showing a dominant effect. This makes it unlikely that phosphorylation of Runx2 by p38 is highly processive. To tie this observation back to osteoblast differentiation, WT Runx2 was expressed in osteoblasts and found to potentiate differentiation. However, this activity was ablated in the Runx2-3SA mutant. Thus, the three Runx2 phosphorylation sites identified are crucial to the overall function of Runx2.

We postulated that these phosphorylation events might alter Runx2 activity by modulating cofactor association. Indeed, expression of Mkk6-glu alongside Myc-Runx2 promoted association with the cofactor CBP as determined by co-immunoprecipitation. This ability of Mkk6-glu to promote CBP association was ablated in the Runx2-3SA mutant. Endogenous levels of CBP/Runx2 association were examined in TAK1-deficient osteoblasts using co-immunoprecipitation and found to be reduced. Thus, activation of TAK1/p38 results in increased recruitment of CBP by Runx2 and increased osteoblast differentiation.

6 Conclusions and Summary

Based on the sum of these studies, we propose a model whereby TAK1 functions upstream of MKK3/6 and p38α/β to regulate Runx2 activity and osteoblast differentiation (Fig. 1). Given the level of context and signal specificity that characterize MAP3K function, it is highly likely that other MAP3Ks will also contribute to osteoblast differentiation, and under conditions different than those tested here, these may also function upstream of p38 MAPK. Given that the p38-induced Runx2 phosphorylation sites we identified partially overlap with the ERK phosphorylation sites, it is likely that p38 and ERK MAPK play overall similar roles in osteoblasts [20]. This is especially true given that both contribute specifically to the early stages of osteoblast differentiation in vitro. If so, it will be of particular interest to identify the MAP3K responsible for ERK activation in osteoblasts, as this will allow for an examination of if and how the p38 and ERK pathways are coordinately regulated to achieve roughly similar functional outcomes in osteoblast differentiation.

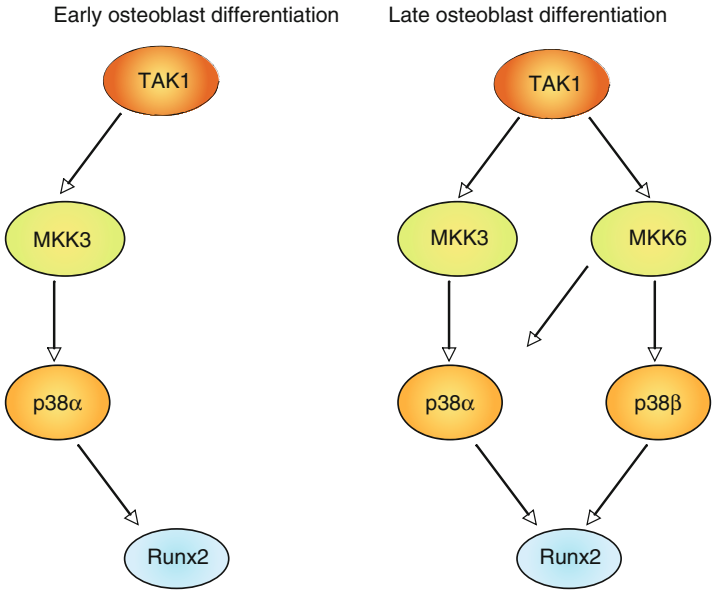

Fig. 1 A schematic depicting the Tak1/MAP2K/p38/Runx2 axis in early and later stages of osteoblast differentiation

It is of special note that p38β appears to function in the regulation of bone mass and osteoblast differentiation, despite having no appreciable contribution to other pathways such as TLR signaling where overall p38 activity is known to be crucial. Currently, it is unclear if this difference reflects factors intrinsic to p38β activity or extrinsic factors regulating p38β activity or substrate association. A better understanding of the biochemistry regulating both p38β and overall p38 activity will be essential to understand how our observations can be leveraged for the development of therapeutics to modulate osteoblast activity. One possibility is that p38 inhibitors might be useful for treatment of osteoblastic bone metastases or in the treatment of nerve entrapment caused by bony overgrowth. Conversely, p38 agonists might promote increased bone mass, though more needs to be understood regarding the systemic effects of increased p38 activity before implementing such an approach.

Lastly, it is of interest that the Runx2 phosphorylation sites identified are conserved and present in Runx1 and Runx3. This suggests the possibility that p38 might regulate the whole family of Runx transcription factors. Moreover, this role may extend beyond bone to other tissues. Runx transcription factors have been implicated in the pathogenesis of acute myelogenous leukemia, and perhaps p38 inhibitors may have clinical utility in that setting [21, 22]. Additionally, the function of Runx transcription factors have been linked to the induction of the Foxp3 transcription factor and the differentiation of regulatory T cells [23, 24]. Given that TAK1 is likely to play a similar role in activating p38 downstream of the regulatory T cell differentiation factor TGFβ, it is possible that the TAK1/p38/Runx pathway

we have described in osteoblasts might also contribute to the decision of a T cell to commit to the Th17 versus the T regulatory lineages. Thus, we are just beginning to understand how MAPKs contribute overall to regulation of Runx transcription factors, and there are many avenues to extend how the TAK1/p38/Runx axis contributes to both bone biology and the biology of other systems.

Acknowledgments We gratefully acknowledge the support of our collaborators in this study: Drs. Roland Baron, Jin Mo Park, Simon Arthur, Min Xie, Michael D. Schneider, Bo Zhai, Steven Gygi, Roger Davis, and Dorothy Hu, Sutada Lotinun, Yasuyo Sano, and Judy Reilly. This work was funded by NIH grant HD055601 (LHG).

LHG is a member of the board of directors of and holds equity in Bristol-Myers Squibb.

References

1. Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89:765–771
2. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89:747–754
3. Ge C, Xiao G, Jiang D, Franceschi RT (2007) Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development. J Cell Biol 176:709–718
4. Han J, Lee JD, Bibbs L, Ulevitch RJ (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 265:808–811
5. Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, Avruch J, Woodgett JR (1994) The stress-activated protein kinase subfamily of c-Jun kinases. Nature 369:156–160
6. Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M, Davis RJ (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell 76:1025–1037
7. Shim JH, Greenblatt MB, Xie M, Schneider MD, Zou W, Zhai B, Gygi S, Glimcher LH (2009) TAK1 is an essential regulator of BMP signalling in cartilage. EMBO J 28:2028–2041
8. Shim JH, Xiao C, Paschal AE, Bailey ST, Rao P, Hayden MS, Lee KY, Bussey C, Steckel M, Tanaka N, Yamada G, Akira S, Matsumoto K, Ghosh S (2005) TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. Genes Dev 19:2668–2681
9. Lee J, Mira-Arbibe L, Ulevitch RJ (2000) TAK1 regulates multiple protein kinase cascades activated by bacterial lipopolysaccharide. J Leukoc Biol 68:909–915
10. Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 412:346–351
11. Sato S, Sanjo H, Takeda K, Ninomiya-Tsuji J, Yamamoto M, Kawai T, Matsumoto K, Takeuchi O, Akira S (2005) Essential function for the kinase TAK1 in innate and adaptive immune responses. Nat Immunol 6:1087–1095
12. Greenblatt MB, Shim JH, Zou W, Sitara D, Schweitzer M, Hu D, Lotinun S, Sano Y, Baron R, Park JM, Arthur S, Xie M, Schneider MD, Zhai B, Gygi S, Davis R, Glimcher LH (2010) The p38 MAPK pathway is essential for skeletogenesis and bone homeostasis in mice. J Clin Invest 120:2457–2473
13. Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S, Lindhout D, Cole WG, Henn W, Knoll JH, Owen MJ, Mertelsmann R, Zabel BU, Olsen BR (1997) Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. Cell 89:773–779

14. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108:17–29
15. Shirakabe K, Yamaguchi K, Shibuya H, Irie K, Matsuda S, Moriguchi T, Gotoh Y, Matsumoto K, Nishida E (1997) TAK1 mediates the ceramide signaling to stress-activated protein kinase/c-Jun N-terminal kinase. J Biol Chem 272:8141–8144
16. Brancho D, Tanaka N, Jaeschke A, Ventura JJ, Kelkar N, Tanaka Y, Kyuuma M, Takeshita T, Flavell RA, Davis RJ (2003) Mechanism of p38 MAP kinase activation in vivo. Genes Dev 17:1969–1978
17. Zarubin T, Han J (2005) Activation and signaling of the p38 MAP kinase pathway. Cell Res 15:11–18
18. Enslen H, Raingeaud J, Davis RJ (1998) Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. J Biol Chem 273:1741–1748
19. Beardmore VA, Hinton HJ, Eftychi C, Apostolaki M, Armaka M, Darragh J, McIlrath J, Carr JM, Armit LJ, Clacher C, Malone L, Kollias G, Arthur JS (2005) Generation and characterization of p38beta (MAPK11) gene-targeted mice. Mol Cell Biol 25:10454–10464
20. Ge C, Xiao G, Jiang D, Yang Q, Hatch NE, Roca H, Franceschi RT (2009) Identification and functional characterization of ERK/MAPK phosphorylation sites in the Runx2 transcription factor. J Biol Chem 284:32533–32543
21. Tang JL, Hou HA, Chen CY, Liu CY, Chou WC, Tseng MH, Huang CF, Lee FY, Liu MC, Yao M, Huang SY, Ko BS, Hsu SC, Wu SJ, Tsay W, Chen YC, Lin LI, Tien HF (2009) AML1/RUNX1 mutations in 470 adult patients with de novo acute myeloid leukemia: prognostic implication and interaction with other gene alterations. Blood 114:5352–5361
22. Watanabe-Okochi N, Kitaura J, Ono R, Harada H, Harada Y, Komeno Y, Nakajima H, Nosaka T, Inaba T, Kitamura T (2008) AML1 mutations induced MDS and MDS/AML in a mouse BMT model. Blood 111:4297–4308
23. Rudra D, Egawa T, Chong MM, Treuting P, Littman DR, Rudensky AY (2009) Runx-CBFbeta complexes control expression of the transcription factor Foxp3 in regulatory T cells. Nat Immunol 10:1170–1177
24. Kitoh A, Ono M, Naoe Y, Ohkura N, Yamaguchi T, Yaguchi H, Kitabayashi I, Tsukada T, Nomura T, Miyachi Y, Taniuchi I, Sakaguchi S (2009) Indispensable role of the Runx1-Cbfbeta transcription complex for in vivo-suppressive function of FoxP3+ regulatory T cells. Immunity 31:609–620

Parathyroid Hormone: A Dynamic Regulator of Bone, Immune, and Hematopoietic Cells

Sun Wook Cho and Laurie McCauley

1 PTH Background

The bone marrow microenvironment is a rich locale for a wide variety of cells, growth factors, hormones, and minerals. Parathyroid hormone (PTH) is an endocrine mediator that circulates from the parathyroid gland to the bone marrow where it interacts with its classic target receptor on osteoblasts. The actions of PTH in bone have focused mainly on its role in stimulating osteoclastogenesis via indirectly targeting cells of the osteoclast lineage as well as its role in stimulating bone formation via increasing osteoblast numbers. Less well characterized but a critical component of PTH actions is its role in modulating other cells of the hematopoietic lineage in the bone marrow microenvironment. Through historic and recent findings of PTH action in a variety of hematopoietic lineage cells, PTH is emerging as a prominent and active member of the "osteoimmunology team."

PTH binds to a seven transmembrane domain G-protein-linked receptor on the surface of osteoblasts and kidney cells. This receptor is designated the PTH/PTHrP receptor, also termed the PTH-1 receptor or PPR. The *PTH1R* gene is located on human chromosome 3 and murine chromosome 9. Other cells in the bone marrow microenvironment that have been reported to have receptors for PTH include

S.W. Cho
Department of Periodontics and Oral Medicine, Medical School, University of Michigan, Ann Arbor, MI 48109-1078, USA

L. McCauley (✉)
Department of Periodontics and Oral Medicine, Medical School, University of Michigan, Ann Arbor, MI 48109-1078, USA

Pathology Department, Medical School, University of Michigan, Ann Arbor, MI 48109-1078, USA
e-mail: mccauley@umich.edu

Y. Choi (ed.), *Osteoimmunology: Interactions of the Immune and Skeletal Systems,*
DOI 10.1007/978-1-4614-5366-6_7,

osteoclasts (although most sources argue against biologically active receptors) and lymphocytes [1–3]. PTH and PTHrP signal through the PPR to evoke second messengers including protein kinase A (PKA), protein kinase C (PKC) and to a lesser extent, the MAPK pathway [4–6]. These second messenger pathways lead to a wide variety of gene transcription events followed by the production of many different proteins identified especially in cells of the osteoblast lineage. Many of these proteins have noted roles as cytokines impacting cells of the hematopoietic lineage.

2 PTH Mediates Cytokines that Impact Hematopoietic Cells

Osteoblasts are the source of a wide variety of cytokines that mediate hematopoietic cell development and function and PTH drives the production of many of these hematotropic factors. The most extensively characterized of these is RANKL. PTH increases RANKL synthesis in osteoblasts as well as reducing osteoprotegerin (OPG) [7]. The PTH mediation of RANKL and OPG occur via the cAMP/PKA pathway [8] and leads to regulation of osteoclast differentiation. PTH also supports osteoclastogenesis by stimulating osteoblast production of GM-CSF, M-CSF, and interleukin-6 (IL-6) [9–11]. IL-6 has been extensively characterized as a highly PTH-upregulated cytokine in osteoblasts [6, 11–13]. Inhibition of osteoclastogenesis is also found via PTH-mediated increases in leukemia inhibitory factor (LIF) [14, 15]. Osteoblasts synthesize cyclooxygenase (COX)-2 and prostaglandin E2 (PGE2) in response to PTH which could have effects on a variety of cells in the bone marrow microenvironment [16, 17]. Furthermore, monocyte chemotactic protein (MCP-1) also known as CCL2 is a prominent osteoblast-derived cytokine produced in response to PTH and important in monocyte chemotaxis, osteoclastogenesis, and angiogenesis in bone [18]. Stem cell factor (SCF; kit ligand) was found more than 10 years ago to be upregulated in osteoblasts in response to PTH [19]. SCF/Kit ligand is the receptor for c-kit and has been suggested to be responsible, in the context of increased marrow mast cells, for the osteitis fibrosis associated with hyperparathyroidism. However, serum levels were not found to be elevated in human patients with primary hyperparathyroidism [20, 21] so the physiologic significance of this cytokine relative to PTH is unclear. Vascular cell adhesion molecule-1 (VCAM-1) is well known as an adhesion factor for lymphocytes, monocytes, eosinophils, and basophils to vascular endothelium [22]. VCAM-1 is also responsible for hematopoietic stem cell localization and trafficking. PTH and PTHrP upregulate VCAM-1 expression in a JunB-dependent manner in osteoblasts [23].

3 Effect of PTH on Osteoclasts

It has long been known that PTH stimulates bone resorption by mediating the differentiation of osteoclasts. Although there are reports of PTH receptors on osteoclasts, most evidence suggests against a direct effect of PTH to stimulate

osteoclastogenesis [24, 25]. Instead, PTH upregulates RANKL expression in osteoblasts and decreases OPG expression resulting in multiple levels of osteoclastogenesis promotion. That PTH is catabolic for bone has been widely reported and notably, a cardinal sign of the human condition of primary hyperparathyroidism is loss of cortical bone. More controversial is the dependence of PTH on osteoclasts for anabolic actions in bone.

One of the first studies to suggest that PTH relied on osteoclasts for anabolic actions in bone was a description of the lack of a bone forming impact of PTH in c-*fos* mutant mice [26]. The proto-oncogene c-Fos is essential for osteoclastogenesis and hence mice with deletion of c-*fos* are osteopetrotic. Their anabolic response to intermittent PTH 1–34 was completely abolished despite typical PTH-mediated gene expression in osteoblasts [27]. Human clinical data corroborated these findings when reports of blunted anabolic responses to PTH were found in patients coadministered bisphosphonates with teriparatide [28]. Animal studies have both supported and refuted the findings of anti-resorptives blunting PTH anabolic actions [29–31]. More recent work suggests a mechanism that involves PTH-mediated resorptive activity resulting in release of TGFβ from the bone matrix that in turn supports mesenchymal stem cell recruitment and bone formation [32]. This is an area worthy of further clarification as it will likely reveal fundamental processes of musculoskeletal homeostasis and repair.

4 Effect of PTH on Immune Cells

Interactions between PTH and immune cells were first suggested in the context of patients with chronic renal failure (reviewed in ref [33]) who have both secondary hyperparathyroidism, which results in continuous high blood levels of PTH, as well as impaired immunity. These associations suggested that PTH could impact lymphocytic cells and early data reported that immune cells (neutrophils, B and T cells) have receptors for PTH [3, 34, 35]. Several lines of evidence support this hypothesis. PTH stimulates rat thymic lymphocyte proliferation [36] and both PTH 1–34 and PTH 1–84 also significantly stimulate phytohemagglutinin-induced T cell proliferation [37]. This pro-proliferative effect of PTH on T cells is altered in the uremic state associated with hyperparathyroidism [38, 39] and is reversed by parathyroidectomy [40–42]. Regarding humoral immunity, high doses of PTH inhibit B cell proliferation as well as the production of immunoglobin both in vitro and in vivo [43, 44]. An in vivo rat model of chronic renal failure with secondary hyperparathyroidism also showed impaired antibody response to antigens [45]. Collectively, these early reports suggested that continuous exposure of greater than physiologic levels of PTH could negatively impact immune cells. Recently with the pharmacologic use of the amino terminal PTH 1–34 analogue (teriparatide) in the management of osteoporosis, the immunomodulatory function of PTH is once again under consideration.

4.1 B Cells and PTH

B lymphocytes are well-characterized antigen producing cells in the bone marrow recognized for their ability to produce antibodies. Recently, the regulatory function of B cells in bone resorption via the production of OPG was established [46]. Li et al. suggested that cells of the B lineage are more likely the dominant producers of OPG in the bone marrow microenvironment rather than osteoblast lineages. Consequently, B cell knockout mice have an osteoporotic bone phenotype with markedly enhanced osteoclastic bone resorption. Both mRNA expression and protein production for OPG was reduced in B cell knockout bone marrow and reconstitution of young B cell knockout mice with adoptive transfer completely rescued the phenotype of osteoporosis [46].

The interaction between B cells and PTH is mediated by lymphopoietic cytokines, especially SDF-1 and IL-7. These powerful cytokines are produced by cells of the osteoblast lineage, and their production is enhanced in response to PTH [47–49]. Zhu et al. demonstrated that differentiation of B cells from bone marrow precursor cells requires cell–cell communication with osteoblasts in vitro and this process is mediated by VCAM-1, SDF-1, and IL-7 signaling induced in response to PTH [47] (Fig. 1). The G-protein coupled PPR uses the heterotrimeric G protein subunit $G_s\alpha$-protein kinase A signaling as a major downstream mediator [5]. Interestingly, specific deletion of $G_s\alpha$ in early osteogenic progenitor cells using Cre-recombinase driven by osterix ($G_s\alpha^{OsxKO}$ mice) showed not only a reduction in trabecular bone but also a significant reduction in B-cell precursors in the bone marrow and a consequent reduction in circulating B-cells [50]. Within the bone marrow, impairment of B lymphopoiesis was limited to the pro-B to pre-B cell transition. Prepro-B cells which are associated with SDF-1^+ cells are not affected in this model and there is no reduction of SDF-1 expression in $G_s\alpha^{OsxKO}$ osteoblasts. In contrast, mRNA expression of IL-7 is significantly decreased and the reduction of pre-B cells and pro-B cells is similar to that of either IL-7 or IL-7 receptor KO mice [51, 52]. These data suggest that $G_s\alpha$ signaling within cells of the osteoblast lineage is required for normal bone marrow B lymphopoiesis and likely involves IL-7 production. Collectively, PTH indirectly affects the proliferation and differentiation of B cells by stimulating cytokine production in osteoblastic stromal cells. While B cells can affect osteoclast activity by secreting OPG, less is known regarding effects of B cells on osteoblastic cells.

4.2 T Cells and PTH

A molecular link between T lymphocytes and bone emerged prominently with the discovery of RANKL and its receptor RANK. These molecules were first identified in T cells and dendritic cells (DCs) and have been shown to play a pivotal role in DC-mediated naïve T cell proliferation and DC survival. Soon after, they were identified as key osteoclastogenic factors (reviewed in ref. [53]). Much earlier studies

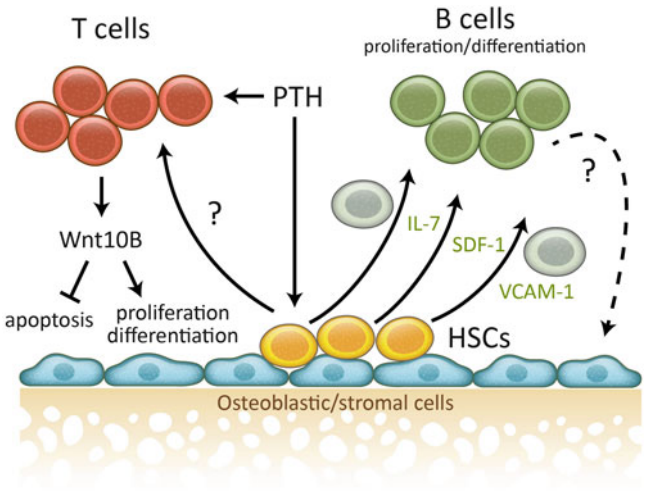

Fig. 1 The interaction between osteoblastic/stromal cells and lymphocytes mediated by PTH. In response to direct stimulation by PTH, bone marrow T lymphocytes secrete the Wnt ligand Wnt10b. After binding to its receptors on osteoblastic/stromal cells, Wnt10b activates canonical Wnt/β-catenin signaling which stimulates osteoblast proliferation/differentiation and inhibits apoptosis. PTH also stimulates osteoblastic/stromal cells, however, whether these PTH-stimulated osteoblastic/stromal cells can affect T lymphocytes is unclear. Better clarified, PTH-stimulated osteoblastic/stromal cells support bone marrow B lymphopoiesis. Differentiation of HSCs to B lymphocytes requires cell–cell contact with osteoblastic/stromal cells and this process is mediated via VCAM-1, SDF-1, and IL-7 signaling induced by PTH. Less is known about the effect of B cells on osteoblastic/stromal cells

focused on the functional role of T lymphocytes in bone physiology comparing athymic (nude) and euthymic mice [54]. These studies reported that athymic mice had altered skeletal phenotypes compared to euthymic mice despite little differences at the cellular level. Using a classic calvarial bone resorption assay, the calvaria of athymic mice was found to respond with similar levels of calcium release reflecting similar bone resorption in response to PTH as compared to euthymic mice. This study suggested that T cells could play a role in the skeleton, but lacked definitive identification of the mediators of the gross skeletal differences.

Furthermore, T lymphocytes were found to express functional PPR receptors which linked to an intracellular calcium response to PTH/PTHrP [55, 56] and resulted in the stimulation of osteoblastic differentiation [57–59]. More recently there has been improved understanding of the role of T cells in PTH-mediated skeletal homeostasis. Hory et al. first reported that transplantation of human parathyroid tissues into nude mice failed to stimulate bone resorption which suggested a possible role of T cells in PTH effects on bone [60]. Subsequently, Pettway et al. performed intermittent administration of PTH for up to 7 weeks in an ectopic "ossicle" implanted nude mice model [61]. This investigation disclosed that intermittent PTH increased the bone content of the implanted ossicles, a structure which contains normal bone marrow, but had little effect on vertebral bone growth in host nude

mice. This site differential response to intermittent PTH in nude mice could be due to species-specific phenomenon, T cell deficiency in the host, or a responsivity associated with the wound healing phenomenon induced with the ossicle model. Recently, Terauchi et al. investigated four different strains of T cell deficient mice (TCRb ko, RAG2 ko, class I and II MHC double KO mice, and nude mice) and revealed that mice lacking T cells, exhibit a blunted increase in bone formation and trabecular bone volume in response to intermittent PTH [62]. Furthermore, adoptive transfer of T cells into T cell deficient mice restored a normal response to intermittent PTH, and T cell produced Wnt10b was suggested as a key player in the mechanism of anabolic actions of intermittent PTH (Fig. 1).

To summarize the PTH effects on immune cells, PTH has both positive and negative effects not only on the skeleton but also on the immune system. Based on rodent studies, intermittent administration of PTH showed supportive effects on immunity, whereas data from hyperparathyroidism studies suggested that continuous increase of PTH at pathologic levels has opposite effects. To verify the possibility of clinical applications of PTH in altered immune conditions such as end stage renal failure or HIV infections, more epidemiologic and basic research is required.

5 PTH Support of the Hematopoietic Stem Cell Niche

The hematopoietic stem cell (HSC) niche is a specific microenvironment in which HSCs exist and contribute to stem cell fate. There is a long-standing interest in the effect of PTH on hematopoiesis (reviewed in ref. [63]). This story started with Eli Lilly's bovine parathyroid extract which was found to increase the 30-day survival of irradiated rats [64–66]. Subsequent studies revealed that it was the PTH activity in the Lilly parathyroid extract that improved the survival of irradiated rats and that PTH controls hematopoiesis in mice and rats [67–69]. In 1974, Gallien-Lartigue et al. demonstrated that administration of PTH stimulated proliferation of murine colony-forming unit spleen and bone marrow cells via cAMP, indicating that PTH could directly modulate mouse hematopoietic stem cells [70]. Recently, these intriguing findings have been reevaluated. Regarding the issue of the existence of PTH receptor on HSCs, Adams et al. clearly rebutted the old study performed by Gallien-Lartigue et al. demonstrating that purified $Lin^{-}Sca1^{+}c\text{-}kit^{+}$ HSCs showed undetectable mRNA for the PPR [71]. In 2003, Calvi et al. more clearly defined the story of the beneficial role of PTH in HSC niche and suggested Jagged1/Notch signaling as one of the possible mechanisms. Osteoblast-specific overexpression of the PPR increased the number of $Lin^{-}Sca1^{+}c\text{-}kit^{+}$ HSCs and osteoblastic cells in the bone marrow. The protein production of Jagged1 from osteoblastic cells was increased and the Notch1 intracellular domain in the HSC fraction was activated in vivo. Additionally, inactivation of Notch signaling by γ-secretase inhibitor blocked HSC expansion in vitro, which suggested that PPR-stimulated osteoblastic cells supported HSC expansion via Jagged1/Notch signaling. Furthermore, administration of

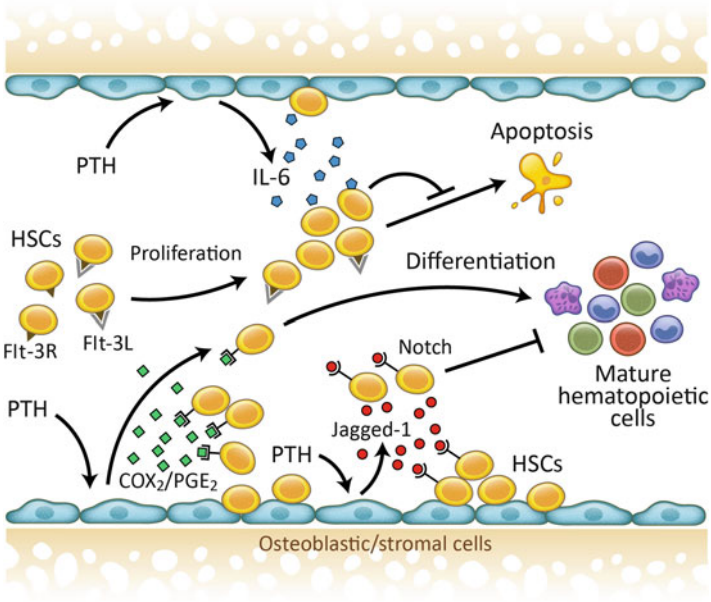

Fig. 2 Working model: How PTH controls the bone marrow microenvironment to support the HSC niche. PTH stimulates osteoblastic/stromal cells to increase Jagged1, cyclooxygenase (COX) 2/PGE2 and IL-6 levels in the HSC niche. Jagged1 with binding to the Notch receptor on HSCs increases HSC numbers and inhibits differentiation of HSC/progenitor cells. On the other hand, COX-2/PGE2 specifically increases short-term HSCs without altering long-term HSCs or inhibiting their lineage-specific differentiation. Flt-3 ligand (Flt-3L), a stem cell factor which is enriched in the HSC niche, increases hematopoietic progenitor cell proliferation. IL-6 produced in response to PTH action on stromal cells supports the Flt-3L-mediated HSC expansion by inhibiting apoptosis of responsive cells

PTH (1–34) to myeloablated mice showed an increase of bone marrow cellularity and a survival benefit at 28 days after bone marrow transplantation. This study concluded that the strengthening effect of PTH on the bone marrow microenvironment can result in improved engraftment [48]. The ability of PTH to augment the Jagged1 expression on osteoblasts in a cAMP/PKA-dependent manner was further confirmed. Five days of PTH administration to C57/Bl6 mice showed that Jagged1 protein was increased in specific populations of osteoblasts including those at the endosteum and spindle-shaped cells in the bone marrow cavity [72] (Fig. 2).

Recently, another potential mechanism for PTH actions on hematopoietic cells was elucidated using an ex vivo culture model of bone marrow cells [73]. It is well known that PTH induces IL-6 secretion in stromal and osteoblastic cells. In the ex vivo culture system, IL-6 supported hematopoietic progenitor cell expansion through inhibiting apoptosis of proliferating hematopoietic progenitor cells in response to a stem cell factor, Fms-like tyrosine kinase-3 ligand (Flt-3 L). Notably, this report

found no evidence of biologically active PPRs in the hematopoietic cell populations. Three weeks of PTH treatment to wild-type mice resulted in increased $Lin^{-}Sca1^{+}c\text{-}kit^{+}$ (LSK) hematopoietic cells, whereas in IL-6 deficient mice, PTH failed to increase LSK cells in the bone marrow. This study suggested that IL-6 plays a critical role in PTH-dependent hematopoietic cell expansion [73] (Fig. 2).

Another putative mediator of the interaction between HSCs and PTH is PGE2. The synthesis of PGE2 in bone is mainly regulated by inducible COX-2, and PTH is a potent inducer of COX-2 expression and PGE2 production in osteoblasts [74, 75]. Frisch et al. showed that in vivo treatment with PGE2 (twice daily IP injection for 16 days) resulted in an altered bone marrow microenvironment by decreasing numbers of bony trabeculae and increasing short-term HSCs/multipotent progenitor cells without impacting long-term HSC numbers, lineage distribution or programmed differentiation. They suggested that unlike Jagged1/Notch signaling which inhibits the differentiation of HSCs or progenitor cells [76, 77], PGE2 most likely expands short-term HSCs without altering long-term HSC or hematopoietic progenitor cells through a combination of direct and microenvironmental actions. Considering such an association, it is likely that PTH could impact the HSC niche via COX-2/PGE2 (Fig. 2), however, further investigation is warranted.

6 Evidence for Therapeutic Application of PTH

The therapeutic potential of PTH-mediated HSC stimulation was further investigated with mouse models that are relevant to clinical use [71]. Stimulation of the HSC niche with PTH during multiple rounds of chemotherapy showed that PTH can protect or even expand the resident HSC pool in the bone marrow during repetitive myelotoxic chemotherapy. Mice received cyclophosphamide every 2 weeks for four cycles. One day after chemotherapy, mice were treated with either saline, G-CSF, PTH alone, or the combination of PTH and G-CSF. PTH treatment led to an increase in the HSC pool in mice which did not receive G-CSF and a preservation of the HSC pool in G-CSF-treated mice. In an allogenic myeloablative murine HSC transplant model, PTH treatment after transplantation led to increased engraftment of the HSC compartment through increased expansion of the HSC pool. These studies pave the way to clinical trials aimed at increasing the effectiveness of HS cell therapies through targeting of the HSC niche.

To date, evidence of the supportive role of PTH in the HSC niche is limited to rodent systems and the mechanisms are still unclear. There is an increasing need for new therapeutic agents to treat the hematopoietic damage experienced in cancer patients and PTH, which is already on the market, could be a feasible tool; however, teriparatide has a “black box” warning against its use in patients with cancer or radiation therapy. It will be important to confirm whether PTH can stimulate human as well as rodent HSCs and the precise mechanisms by which this occurs.

7 Effect of PTH on Hematopoietic Lineage Cell Mobilization

Autologous stem cell transplantation is a key strategy to recover the hematopoietic system following myeloablation. Even with current improvements in cell mobilization techniques, low cell numbers of HSCs harvested from peripheral blood is a major limitation for successful reconstitution. Recently, several lines of evidence suggest that PTH could be a promising agent to facilitate stem cell mobilization [71, 78].

Adams et al. demonstrated that in addition to standard mobilization with G-CSF, PTH could increase the number of stem cells mobilized into the circulation [71]. Mice were treated with PTH or vehicle alone for 5 weeks followed by 5 days of G-CSF mobilization. There was no difference in the number of CFU-Cs mobilization in the vehicle or PTH-treated mobilized mice, demonstrating that PTH has no direct effect on progenitor cells. However, circulating HSCs showed a twofold increase in PTH mobilized mice assessed using two methods: (1) the number of $Lin^{-}Sca1^{+}c\text{-}Kit^{+}$ $Flt\text{-}2^{-}$ cells, and (2) the number of cells capable of competitively reconstituting irradiated hosts. These data suggest that PTH treatment increases the number of HSCs in the bone marrow that can be mobilized into the peripheral circulation, although it does not result in any alteration of mature cell counts or progenitor cell mobilization with a standard mobilization regimen [71]. Another study was designed to test the potency of PTH compared to G-CSF in the mobilization of stem cells and its regenerative capacity on bone marrow. Mice were treated with PTH, G-CSF, or saline for 6 days and LSK cells, as well as subpopulations ($CD31^{+}$, $c\text{-}kit^{+}$, $Sca\text{-}1^{+}$, $CXCR4^{+}$) of $CD45^{+}/CD34^{+}$ and $CD45^{+}/CD34L$ cells were analyzed. Treatment with PTH increased all characterized subpopulations of bone marrow-derived progenitor cells in peripheral blood similar to G-CSF. In contrast to G-CSF, PTH did not result in a depletion of $Lin^{-}/Sca\text{-}1^{+}/c\text{-}kit^{+}$ cells and $CD34^{+}$ stem cells in bone marrow. PTH treatment was associated with increased G-CSF serum levels and the mobilizing effect could be inhibited by blocking endogenous G-CSF possibly mediating release of progenitor cells from bone marrow [78].

Recently, several human studies have investigated the effect of PTH on the mobilization of bone marrow-derived progenitor cells (BMCs). A human prospective study of patients with primary hyperparathyroidism (PHPT) showed an increased number of circulating stem and progenitor cells in the peripheral blood [20]. Twenty-two patients with PHPT and 10 controls were recruited and circulating BMCs were analyzed with the following markers: (1) $CD45^{+}/CD34^{+}/CD31^{+}$, representing endothelial progenitor cells, (2) $CD45^{+}/CD34^{+}/c\text{-}kit^{+}$ representing hematopoietic stem cells, and (3) $CD45^{+}/CD34^{+}/CXCR4^{+}$, representing progenitor cells with the homing receptor CXCR4. Patients with PHPT showed an increase in circulating BMCs before surgery which returned to control levels postsurgery. There was a positive correlation of PTH levels with the number of BMCs in all subpopulations analyzed. Serum levels of G-CSF, EPO, and SCF, all known to mobilize BMCs, were reported as decreased or remained unchanged in PHPT patients. This study suggested that PTH may have a direct effect on stem cell mobilization in humans.

In contrast, patients with hypoparathyroidism treated with PTH showed no increase in circulating HSCs [79]. Nineteen controls and 19 hypoparathyroidism patients were recruited and administered PTH (1–84) for 12 months. Unlike osteocalcin-positive cells which were increased with PTH treatment, the hematopoietic cell populations which included total CD34+ cells and OCN−/CD34+ cells, did not change with PTH administration. The authors explained that such an increase may occur before the 1-month time point that was analyzed in this study. In fact, rodent studies which showed the ability of PTH to modulate stem cell mobilization were performed after 6 or 14 days of PTH injection [78]. Another human study was performed with a cohort of uremic patients [80]. The patients were divided into three groups (low—PTH less than 150 pg/ml, intermediate—between 150 and 300 pg/ml, and high—PTH levels greater than 300 pg/ml) and hematopoietic and endothelial progenitor cells were analyzed. The high-PTH groups showed higher levels of hematopoietic progenitor cells ($CD45^+/CD34^+/c\text{-}kit^+$) compared to intermediate or low-PTH groups, whereas endothelial progenitor cells ($CD45^+/CD34^+/CD31^+$) were highest in the intermediate group. All patients [13] in the high-PTH group who received 4 months of PTH lowering treatment (IV paracalcitriol, IV calcitriol, and P binder or cinacalcet) had increased endothelial progenitor cells after achieving intermediate PTH levels. Taken together, it seems clear that PTH mediates stem or progenitor cell mobilization in humans and the effects of PTH on specific subsets of progenitor cells are specific to the pathologic conditions.

Interestingly, using a small group of human patients who had previously failed to produce a sufficient number of $CD34^+$ HSC in their peripheral blood following mobilization, a Phase I trial of pretreatment with PTH for stem cell mobilization was performed. Patients who previously had failed one or two attempts at mobilization were treated with PTH for up to 14 days. As a result, 40–50% of patients could meet therapeutic mobilization criteria with PTH pretreatment followed by G-CSF treatment [81]. Overall, these studies suggest that an HSC mobilization strategy which includes PTH may be an effective means of circumventing low HSC yield issues.

8 Conclusions

Recently, there has been exciting progress relative to new findings of the impact of PTH on the bone marrow microenvironment; however, the precise interactions between the cells of hematopoietic and stromal lineages in the bone marrow microenvironment still remain unclear. Evidence described here substantiates actions of PTH as a key modulator of the bone marrow microenvironment. PTH regulates not only osteoclastogenesis but also modulates lymphocytes, monocytes, and HSCs by stimulating osteoblast production of various cytokines. T lymphocytes have their own PPR and are directly regulated by PTH to support the characteristic osteoblastic response of anabolic PTH actions. The recent literature regarding PTH and the immune system provides insight that PTH actions are complex, being both

direct and indirect and mediated via numerous cytokines produced in the bone marrow. The therapeutic potential of PTH-mediated HSC stimulation has been identified in various rodent models that mimic myeloablative conditions in humans. Human studies are beginning to embark on the use of PTH to mobilize HSCs into circulation. Although the data discussed here supports the thesis that PTH modulates the cells of hematopoietic linage, the physiologic relevance and therapeutic potential for humans deserves more investigation yet remains remarkably intriguing.

References

1. Rao LG, Murray TM, Heersche JN (1983) Immunohistochemical demonstration of parathyroid hormone binding to specific cell types in fixed rat bone tissue. Endocrinology 113:805–810
2. Murray TM, Rao LG, Divieti P, Bringhurst FR (2005) Parathyroid hormone secretion and action: evidence for discrete receptors for the carboxyl-terminal region and related biological actions of carboxyl- terminal ligands. Endocr Rev 26:78–113
3. Yamamoto I, Potts JT Jr, Segre GV (1983) Circulating bovine lymphocytes contain receptors for parathyroid hormone. J Clin Invest 71:404–407
4. Tamura T, Sakamoto H, Filburn CR (1989) Parathyroid hormone 1–34, but not 3–34 or 7–34, transiently translocates protein kinase C in cultured renal (OK) cells. Biochem Biophys Res Commun 159:1352–1358
5. Juppner H, Abou-Samra AB, Freeman M, Kong XF, Schipani E, Richards J, Kolakowski LF Jr, Hock J, Potts JT Jr, Kronenberg HM et al (1991) A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. Science 254:1024–1026
6. Chen C, Koh AJ, Datta NS, Zhang J, Keller ET, Xiao G, Franceschi RT, D'Silva NJ, McCauley LK (2004) Impact of the mitogen-activated protein kinase pathway on parathyroid hormone-related protein actions in osteoblasts. J Biol Chem 279:29121–29129
7. Lee SK, Lorenzo JA (1999) Parathyroid hormone stimulates TRANCE and inhibits osteoprotegerin messenger ribonucleic acid expression in murine bone marrow cultures: correlation with osteoclast-like cell formation. Endocrinology 140:3552–3561
8. Fu Q, Jilka RL, Manolagas SC, O'Brien CA (2002) Parathyroid hormone stimulates receptor activator of NFkappa B ligand and inhibits osteoprotegerin expression via protein kinase A activation of cAMP-response element-binding protein. J Biol Chem 277:48868–48875
9. Horowitz MC, Coleman DL, Flood PM, Kupper TS, Jilka RL (1989) Parathyroid hormone and lipopolysaccharide induce murine osteoblast-like cells to secrete a cytokine indistinguishable from granulocyte-macrophage colony-stimulating factor. J Clin Invest 83:149–157
10. Weir EC, Lowik CW, Paliwal I, Insogna KL (1996) Colony stimulating factor-1 plays a role in osteoclast formation and function in bone resorption induced by parathyroid hormone and parathyroid hormone-related protein. J Bone Miner Res 11:1474–1481
11. Feyen JH, Elford P, Di Padova FE, Trechsel U (1989) Interleukin-6 is produced by bone and modulated by parathyroid hormone. J Bone Miner Res 4:633–638
12. de la Mata J, Uy HL, Guise TA, Story B, Boyce BF, Mundy GR, Roodman GD (1995) Interleukin-6 enhances hypercalcemia and bone resorption mediated by parathyroid hormone-related protein in vivo. J Clin Invest 95:2846–2852
13. Onyia JE, Libermann TA, Bidwell J, Arnold D, Tu Y, McClelland P, Hock JM (1997) Parathyroid hormone (1–34)-mediated interleukin-6 induction. J Cell Biochem 67:265–274
14. Greenfield EM, Gornik SA, Horowitz MC, Donahue HJ, Shaw SM (1993) Regulation of cytokine expression in osteoblasts by parathyroid hormone: rapid stimulation of interleukin-6 and leukemia inhibitory factor mRNA. J Bone Miner Res 8:1163–1171
15. Lorenzo JA, Sousa SL, Leahy CL (1990) Leukemia inhibitory factor (LIF) inhibits basal bone resorption in fetal rat long bone cultures. Cytokine 2:266–271

16. Maciel FM, Sarrazin P, Morisset S, Lora M, Patry C, Dumais R, de Brum-Fernandes AJ (1997) Induction of cyclooxygenase-2 by parathyroid hormone in human osteoblasts in culture. J Rheumatol 24:2429–2435
17. Tetradis S, Pilbeam CC, Liu Y, Kream BE (1996) Parathyroid hormone induces prostaglandin G/H synthase-2 expression by a cyclic adenosine 3′,5′-monophosphate-mediated pathway in the murine osteoblastic cell line MC3T3-E1. Endocrinology 137:5435–5440
18. Li X, Qin L, Bergenstock M, Bevelock LM, Novack DV, Partridge NC (2007) Parathyroid hormone stimulates osteoblastic expression of MCP-1 to recruit and increase the fusion of pre/osteoclasts. J Biol Chem 282:33098–33106
19. Blair HC, Julian BA, Cao X, Jordan SE, Dong SS (1999) Parathyroid hormone-regulated production of stem cell factor in human osteoblasts and osteoblast-like cells. Biochem Biophys Res Commun 255:778–784
20. Brunner S, Theiss HD, Murr A, Negele T, Franz WM (2007) Primary hyperparathyroidism is associated with increased circulating bone marrow-derived progenitor cells. Am J Physiol Endocrinol Metab 293:E1670–E1675
21. Turner RT, Iwaniec UT, Marley K, Sibonga JD (2010) The role of mast cells in parathyroid bone disease. J Bone Miner Res 25:1637–1649
22. Mazo IB, von Andrian UH (1999) Adhesion and homing of blood-borne cells in bone marrow microvessels. J Leukoc Biol 66:25–32
23. Berry JE, Pettway GJ, Cordell KG, Jin T, Datta NS, McCauley LK (2008) JunB as a potential mediator of PTHrP actions: new gene targets Ephrin B1 and VCAM-1. Oral Dis 14:713–726
24. Itoh K, Udagawa N, Matsuzaki K, Takami M, Amano H, Shinki T, Ueno Y, Takahashi N, Suda T (2000) Importance of membrane- or matrix-associated forms of M-CSF and RANKL/ODF in osteoclastogenesis supported by SaOS-4/3 cells expressing recombinant PTH/PTHrP receptors. J Bone Miner Res 15:1766–1775
25. Udagawa N, Takahashi N, Jimi E, Matsuzaki K, Tsurukai T, Itoh K, Nakagawa N, Yasuda H, Goto M, Tsuda E et al (1999) Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor: receptor activator of NF-kappa B ligand. Bone 25:517–523
26. Demiralp B, Chen HL, Koh AJ, Keller ET, McCauley LK (2002) Anabolic actions of parathyroid hormone during bone growth are dependent on c-fos. Endocrinology 143:4038–4047
27. Koh AJ, Demiralp B, Neiva KG, Hooten J, Nohutcu RM, Shim H, Datta NS, Taichman RS, McCauley LK (2005) Cells of the osteoclast lineage as mediators of the anabolic actions of parathyroid hormone in bone. Endocrinology 146:4584–4596
28. Black DM, Delmas PD, Eastell R, Reid IR, Boonen S, Cauley JA, Cosman F, Lakatos P, Leung PC, Man Z et al (2007) Once-yearly zoledronic acid for treatment of postmenopausal osteoporosis. N Engl J Med 356:1809–1822
29. Ma YL, Bryant HU, Zeng Q, Schmidt A, Hoover J, Cole HW, Yao W, Jee WS, Sato M (2003) New bone formation with teriparatide [human parathyroid hormone-(1–34)] is not retarded by long-term pretreatment with alendronate, estrogen, or raloxifene in ovariectomized rats. Endocrinology 144:2008–2015
30. Pierroz DD, Bonnet N, Baldock PA, Ominsky MS, Stolina M, Kostenuik PJ, Ferrari SL (2010) Are osteoclasts needed for the bone anabolic response to parathyroid hormone? A study of intermittent parathyroid hormone with denosumab or alendronate in knock-in mice expressing humanized RANKL. J Biol Chem 285:28164–28173
31. Samadfam R, Xia Q, Goltzman D (2007) Co-treatment of PTH with osteoprotegerin or alendronate increases its anabolic effect on the skeleton of oophorectomized mice. J Bone Miner Res 22:55–63
32. Wu X, Pang L, Lei W, Lu W, Li J, Li Z, Frassica FJ, Chen X, Wan M, Cao X (2010) Inhibition of Sca-1-positive skeletal stem cell recruitment by alendronate blunts the anabolic effects of parathyroid hormone on bone remodeling. Cell Stem Cell 7:571–580
33. Shurtz-Swirski R, Shkolnik T, Shasha SM (1995) Parathyroid hormone and the cellular immune system. Nephron 70:21–24

34. Yamamoto I, Bringhurst FR, Potts JT Jr, Segre GV (1988) Properties of parathyroid hormone receptors on circulating bovine lymphocytes. J Bone Miner Res 3:289–295
35. Perry HM 3rd, Chappel JC, Bellorin-Font E, Tamao J, Martin KJ, Teitelbaum SL (1984) Parathyroid hormone receptors in circulating human mononuclear leukocytes. J Biol Chem 259:5531–5535
36. Atkinson MJ, Hesch RD, Cade C, Wadwah M, Perris AD (1987) Parathyroid hormone stimulation of mitosis in rat thymic lymphocytes is independent of cyclic AMP. J Bone Miner Res 2:303–309
37. Klinger M, Alexiewicz JM, Linker-Israeli M, Pitts TO, Gaciong Z, Fadda GZ, Massry SG (1990) Effect of parathyroid hormone on human T cell activation. Kidney Int 37:1543–1551
38. Kaneko T, Osono E, Hayama N, Lino Y, Terashi A (1997) T-cell activation modified by parathyroid hormone (PTH) in patients with end-stage renal disease. Clin Nephrol 48:353–358
39. Alexiewicz JM, Gaciong Z, Klinger M, Linker-Israeli M, Pitts TO, Massry SG (1990) Evidence of impaired T cell function in hemodialysis patients: potential role for secondary hyperparathyroidism. Am J Nephrol 10:495–501
40. Tzanno-Martins C, Futata E, Jorgetti V, Duarte AJ (2000) Restoration of impaired T-cell proliferation after parathyroidectomy in hemodialysis patients. Nephron 84:224–227
41. Shasha SM, Kristal B, Steinberg O, Shkolnik T (1989) Effect of parathyroidectomy on T cell functions in patients with primary hyperparathyroidism. Am J Nephrol 9:25–29
42. Kotzmann H, Koller M, Abela C, Clodi M, Riedl M, Graninger W, Niederle B, Luger A (1998) Effects of parathyroid hormone and serum calcium on the phenotype and function of mononuclear cells in patients with primary hyperparathyroidism. Eur J Clin Invest 28:353–358
43. Gaciong Z, Alexiewicz JM, Linker-Israeli M, Shulman IA, Pitts TO, Massry SG (1991) Inhibition of immunoglobulin production by parathyroid hormone. Implications in chronic renal failure. Kidney Int 40:96–106
44. Alexiewicz JM, Klinger M, Pitts TO, Gaciong Z, Linker-Israeli M, Massry SG (1990) Parathyroid hormone inhibits B cell proliferation: implications in chronic renal failure. J Am Soc Nephrol 1:236–244
45. Gaciong Z, Alexiewicz JM, Massry SG (1991) Impaired in vivo antibody production in CRF rats: role of secondary hyperparathyroidism. Kidney Int 40:862–867
46. Li Y, Toraldo G, Li A, Yang X, Zhang H, Qian WP, Weitzmann MN (2007) B cells and T cells are critical for the preservation of bone homeostasis and attainment of peak bone mass in vivo. Blood 109:3839–3848
47. Zhu J, Garrett R, Jung Y, Zhang Y, Kim N, Wang J, Joe GJ, Hexner E, Choi Y, Taichman RS et al (2007) Osteoblasts support B-lymphocyte commitment and differentiation from hematopoietic stem cells. Blood 109:3706–3712
48. Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR et al (2003) Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425:841–846
49. Jung Y, Wang J, Schneider A, Sun YX, Koh-Paige AJ, Osman NI, McCauley LK, Taichman RS (2006) Regulation of SDF-1 (CXCL12) production by osteoblasts; a possible mechanism for stem cell homing. Bone 38:497–508
50. Wu JY, Purton LE, Rodda SJ, Chen M, Weinstein LS, McMahon AP, Scadden DT, Kronenberg HM (2008) Osteoblastic regulation of B lymphopoiesis is mediated by Gs{alpha}-dependent signaling pathways. Proc Natl Acad Sci USA 105:16976–16981
51. Peschon JJ, Morrissey PJ, Grabstein KH, Ramsdell FJ, Maraskovsky E, Gliniak BC, Park LS, Ziegler SF, Williams DE, Ware CB et al (1994) Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. J Exp Med 180:1955–1960
52. von Freeden-Jeffry U, Vieira P, Lucian LA, McNeil T, Burdach SE, Murray R (1995) Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J Exp Med 181:1519–1526
53. Theill LE, Boyle WJ, Penninger JM (2002) RANK-L and RANK: T cells, bone loss, and mammalian evolution. Annu Rev Immunol 20:795–823

54. McCauley LK, Rosol TJ, Capen CC, Horton JE (1989) A comparison of bone turnover in athymic (nude) and euthymic mice: biochemical, histomorphometric, bone ash and in vitro studies. Bone 10:29–34
55. Stojceva-Taneva O, Fadda GZ, Smogorzewski M, Massry SG (1993) Parathyroid hormone increases cytosolic calcium of thymocytes. Nephron 64:592–599
56. McCauley LK, Rosol TJ, Merryman JI, Capen CC (1992) Parathyroid hormone-related protein binding to human T-cell lymphotropic virus type I-infected lymphocytes. Endocrinology 130:300–306
57. Rifas L (2006) T-cell cytokine induction of BMP-2 regulates human mesenchymal stromal cell differentiation and mineralization. J Cell Biochem 98:706–714
58. Rifas L, Arackal S, Weitzmann MN (2003) Inflammatory T cells rapidly induce differentiation of human bone marrow stromal cells into mature osteoblasts. J Cell Biochem 88:650–659
59. Rifas L, Arackal S (2003) T cells regulate the expression of matrix metalloproteinase in human osteoblasts via a dual mitogen-activated protein kinase mechanism. Arthritis Rheum 48:993–1001
60. Hory BG, Roussanne MC, Rostand S, Bourdeau A, Drueke TB, Gogusev J (2000) Absence of response to human parathyroid hormone in athymic mice grafted with human parathyroid adenoma, hyperplasia or parathyroid cells maintained in culture. J Endocrinol Invest 23:273–279
61. Pettway GJ, Schneider A, Koh AJ, Widjaja E, Morris MD, Meganck JA, Goldstein SA, McCauley LK (2005) Anabolic actions of PTH (1–34): use of a novel tissue engineering model to investigate temporal effects on bone. Bone 36:959–970
62. Terauchi M, Li JY, Bedi B, Baek KH, Tawfeek H, Galley S, Gilbert L, Nanes MS, Zayzafoon M, Guldberg R et al (2009) T lymphocytes amplify the anabolic activity of parathyroid hormone through Wnt10b signaling. Cell Metab 10:229–240
63. Whitfield JF (2005) Parathyroid hormone (PTH) and hematopoiesis: new support for some old observations. J Cell Biochem 96:278–284
64. Larizza P, Chirico G (1952) Experimental study on the effect of prolonged administration of parathyroid extracts on hemopoiesis. Haematologica 36:653–670
65. Rixon RH, Whitfield JF, Youdale T (1958) Increased survival of rats irradiated with x-rays and treated with parathyroid extract. Nature 182:1374
66. Rixon RH, Whitfield JF (1961) The radioprotective action of parathyroid extract. Int J Radiat Biol 3:361–367
67. Rixon RH, Whitfield JF (1972) Hypoplasia of the bone marrow in rats following removal of the parathyroid glands. J Cell Physiol 79:343–352
68. Swierenga SH, MacManus JP, Braceland BM, Youdale T (1976) REgulation of the primary immune response in vivo by parathyroid hormone. J Immunol 117:1608–1611
69. Hastings RH, Araiza F, Burton DW, Bedley M, Deftos LJ (2004) Parathyroid hormone-related protein regulates apoptosis in lung cancer cells through protein kinase A. Am J Physiol Cell Physiol 287:C1616–C1622
70. Gallien-Lartigue O, Carrez D (1974) In vitro induction of the S phase in multipotent stem cells of bone marrow by parathyroid hormone. C R Acad Sci Hebd Seances Acad Sci D 278:1765–1768
71. Adams GB, Martin RP, Alley IR, Chabner KT, Cohen KS, Calvi LM, Kronenberg HM, Scadden DT (2007) Therapeutic targeting of a stem cell niche. Nat Biotechnol 25:238–243
72. Weber JM, Forsythe SR, Christianson CA, Frisch BJ, Gigliotti BJ, Jordan CT, Milner LA, Guzman ML, Calvi LM (2006) Parathyroid hormone stimulates expression of the Notch ligand Jagged1 in osteoblastic cells. Bone 39:485–493
73. Pirih FQ, Michalski MN, Cho SW, Koh AJ, Berry JE, Ghaname E, Kamarajan P, Bonnelye E, Ross CW, Kapila YL et al (2010) Parathyroid hormone mediates hematopoietic cell expansion through interleukin-6. PLoS One 5:e13657
74. Tetradis S, Pilbeam CC, Liu Y, Herschman HR, Kream BE (1997) Parathyroid hormone increases prostaglandin G/H synthase-2 transcription by a cyclic adenosine 3′,5′-monophosphate-mediated pathway in murine osteoblastic MC3T3-E1 cells. Endocrinology 138:3594–3600

75. Kawaguchi H, Raisz LG, Voznesensky OS, Alander CB, Hakeda Y, Pilbeam CC (1994) Regulation of the two prostaglandin G/H synthases by parathyroid hormone, interleukin-1, cortisol, and prostaglandin E2 in cultured neonatal mouse calvariae. Endocrinology 135:1157–1164
76. Varnum-Finney B, Xu L, Brashem-Stein C, Nourigat C, Flowers D, Bakkour S, Pear WS, Bernstein ID (2000) Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. Nat Med 6:1278–1281
77. Stier S, Cheng T, Dombkowski D, Carlesso N, Scadden DT (2002) Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. Blood 99:2369–2378
78. Brunner S, Zaruba MM, Huber B, David R, Vallaster M, Assmann G, Mueller-Hoecker J, Franz WM (2008) Parathyroid hormone effectively induces mobilization of progenitor cells without depletion of bone marrow. Exp Hematol 36:1157–1166
79. Rubin MR, Manavalan JS, Dempster DW, Shah J, Cremers S, Kousteni S, Zhou H, McMahon DJ, Kode A, Sliney J et al (2010) Parathyroid hormone stimulates circulating osteogenic cells in hypoparathyroidism. J Clin Endocrinol Metab 96:176–186
80. Coppolino G, Bolignano D, De Paola L, Giulino C, Mannella A, Riccio M, Mascaro MA, Lombardi G, Fuiano G, Lombardi L et al (2011) Parathyroid hormone and mobilization of circulating bone marrow-derived cells in uremic patients. J Investig Med 59(5):823–828
81. Ballen KK, Shpall EJ, Avigan D, Yeap BY, Fisher DC, McDermott K, Dey BR, Attar E, McAfee S, Konopleva M et al (2007) Phase I trial of parathyroid hormone to facilitate stem cell mobilization. Biol Blood Marrow Transplant 13:838–843

Sphingosine-1-Phosphate and Chemokines in the Control of Osteoclast Precursors Migration Visualized by Intravital Multiphoton Microscopy

Junichi Kikuta, Atsuko Kubo, and Masaru Ishii

1 Introduction

Bone is a dynamic organ that is continuously turned over during growth, even in adults. During bone remodeling, homeostasis is regulated by the balance between bone formation by osteoblasts and bone resorption by osteoclasts [1, 2]. However, in pathological conditions such as osteoporosis, osteopetrosis, arthritic joint destruction, and bone metastasis, this equilibrium is disrupted. Since osteoclasts are excessively activated in osteolytic diseases, the inhibition of osteoclast function has been a major therapeutic strategy. Bisphosphonates, the most widely used group of anti-osteoporosis drugs, bind to hydroxyapatite, enter osteoclasts via endocytosis, and induce osteoclast apoptosis [3]. Recently, the inactivation of osteoclasts, as opposed to their elimination, has generated interest as an alternative treatment strategy [4, 5]. One promising regulation point is the recruitment of osteoclast precursors. In addition to several chemokines that are known regulators of migration, including CXCL12 [6], we have shown that sphingosine 1-phosphate (S1P), a lysophospholipid abundant in the plasma, plays an important role as both a chemoattractant and a chemorepellent [7, 8]. In this review, we summarize the bidirectional regulation of osteoclast precursor migration by S1P and describe intravital bone imaging in living animals.

J. Kikuta • A. Kubo • M. Ishii, M.D., Ph.D. (✉)
Laboratory of Cellular Dynamics,
WPI-Immunology Frontier Research Center,
Osaka University, 3-1 Yamada-oka, Suita,
Osaka 565-0871, Japan
e-mail: mishii@ifrec.osaka-u.ac.jp

Y. Choi (ed.), *Osteoimmunology: Interactions of the Immune and Skeletal Systems,*
DOI 10.1007/978-1-4614-5366-6_8,

2 Biological Function of S1P

S1P is a bioactive sphingolipid metabolite that regulates diverse biological functions including cell proliferation, motility, and survival [9–12]. Sphingolipids are essential plasma membrane constituents composed of a serine head group and one or two fatty acid tails. They are easily metabolized and converted to sphingosines, which are ATP-dependently phosphorylated by sphingosine kinases 1 and 2 (SPHK1 and SPHK2) in most cells, yielding S1P [13]. SPHKs, which are regulated by a variety of growth factors, hormones, and cytokines, control acute reactive generation of S1P and homeostasis in the circulation [13]. Immediately after its synthesis, free S1P is irreversibly degraded by intracellular S1P lyase or dephosphorylated by S1P phosphatases. As a result, the levels of S1P in most tissues, including bone marrow, are relatively low. In contrast, large amounts of S1P are continuously produced in the plasma, especially by erythrocytes, and the serum concentration of S1P is extremely high (several hundred nanomolar to low-micromolar range). Most S1P in the circulation is bound to high-density lipoprotein (HDL) and albumin, which serve as stable reservoirs and efficiently deliver S1P to epithelial cell-surface receptors. In addition, because S1P is an amphiphilic molecule that cannot easily cross membranes, an S1P gradient between the blood and tissues is maintained.

S1P signals via five 7-transmembrane receptors or G protein-coupled receptors (GPCRs), S1PR1 to S1PR5, previously referred to as endothelial differentiation gene (Edg) receptors [11, 12]. Because of the different distribution of these receptors and their different coupling to signal-transducing G proteins, S1P shows a broad range of bioactivities. S1PR1 is ubiquitously expressed and primarily coupled to PTX-sensitive Gi/o proteins, whereas S1PR2 and S1PR3, whose distributions are more limited, are coupled to G12/13 as well as Gq, Gs, and Gi. The expression of S1PR4 and S1PR5 is much lower than that of S1PR1, S1PR2, and S1PR3, and their functions remain to be elucidated. However, it has been reported that they are coupled to Gi/o and G12/13.

S1P receptors have key roles in the regulation of cellular motility. S1PR1 activates Rac through Gi and promotes cell migration and intercellular connection, whereas S1PR2 activates Rho signaling via G12/13, thereby counteracting the effects of S1PR1 and inhibiting Rac activity [13]. These differences account for the different biological functions of S1PR1 and S1PR2, which produce opposite effects on migration toward/against S1P gradients in vitro [14].

3 Control of Osteoclast Precursors Migration by S1P and Chemokines

Osteoclasts are derived from macrophage/monocyte-lineage cells that express both S1PR1 and S1PR2 [7]. As described above, S1PR1 and S1PR2 have opposite effects on the migration of osteoclast precursors. Osteoclast precursors are chemoattracted to S1P in vitro, a response that is blocked by PTX. In addition, treatment with S1P increases osteoclast precursor levels of the active form of Rac (GTP-Rac),

suggesting that Rac and Gi are involved in S1PR1 chemotactic signaling in osteoclast precursors. On the other hand, S1PR2 requires a higher concentration of S1P for activation and induces negative chemotactic responses, "chemorepulsion," to S1P gradients. S1PR2 activation causes cells to move from the bloodstream into bone marrow cavities [8]. As in leukocytes, the migration of osteoclast precursors is regulated by chemokines. Like the S1PRs, chemokine receptors are GPCRs and signal via Gi components. One of the best-known chemoattractants for osteoclast precursors is CXCL12 (also known as stromal derived factor-1), a CXCR4 ligand [6]. CXCL12 is constitutively expressed at high levels by osteoblastic stromal cells and vascular endothelial cells in bone, whereas CXCR4 is expressed on a wide variety of cells types, including circulating monocytes and osteoclast precursors. CXCL12 has chemotactic effects on osteoclast precursors, which express large amounts of CXCR4.

4 Intravital Multiphoton Imaging of Bone Tissues Revealing Migration of Osteoclast Precursors In Vivo

4.1 Development of New Imaging Method for Bone

To study the behavior of osteoclasts and their precursors in vivo, we developed a new intravital two-photon imaging system for use in the analysis of bone tissues [7, 8, 15]. Recent advances in microscope, laser, and fluorophore technology have made it possible to visualize living cells in intact organs and to analyze their mobility and interactions in a quantitative manner.

As calcium phosphate, the main structural component of the bone matrix, can easily scatter laser beams, it was difficult to access the deep interior of bone tissues, even using a near-infrared laser. We decided to use parietal bone in which the distance from the bone surface to the bone marrow cavity is 80–120 μm (within the appropriate range for two-photon microscopy). Using this new intravital two-photon imaging method, we showed that S1P controls the migratory behavior of osteoclast precursors, dynamically regulating bone mineral homeostasis, and we identified a critical control point in osteoclastogenesis. While monocytoid cells containing osteoclast precursors (CSF1R-EGFP-positive or CX_3CR1-EGFP-positive cells) were stationary at the steady state, osteoclast precursors were stimulated and moved into vessels when a potent S1PR1-specific agonist, SEW2871 [16], was injected intravenously.

4.2 An Application of This Method for Revealing In Vivo Behavior of Osteoclast Precursors

To clarify the physiological significance of S1P-directed chemotaxis of osteoclast precursors in bone homeostasis, we examined osteoclast/monocyte-specific S1PR1-deficient ($S1PR1^{-/-}$) mice. Global S1PR1 deficiency causes embryonic lethally at

e12.5–e14.5 due to defective blood vessel development [17]. The attachment of osteoclast precursors to bone surfaces was significantly enhanced in S1PR1$^{-/-}$ animals compared with controls. S1PR1$^{-/-}$ osteoclasts precursors on bone surfaces subsequently develop into mature osteoclasts and absorb bone tissues. S1P-mediated chemotaxis of osteoclast precursors would thus be expected to contribute to their redistribution from bone tissues to blood vessels.

We also performed intravital two-photon imaging of bone tissues to define the role of S1PR2 in vivo [8]. We showed that certain osteoclast precursors (CX_3CR1-EGFP-positive cells) moved into the bloodstream when a potent S1PR2 antagonist, JTE013 [18], was injected intravenously. The effect of JTE013 was less pronounced than that of the S1PR1 agonist SEW2871. Furthermore, to clarify the physiological significance of S1P-directed chemotaxis of osteoclast precursors in bone homeostasis, we examined S1PR2-deficient (S1PR2$^{-/-}$) mice. Although S1PR2-deficient mice suffer auditory impairment due to vessel defects in the inner ear, they survive and reproduce [19]. Although bone resorption of osteoclasts was significantly lower in S1PR2$^{-/-}$ animals than in controls, in vitro osteoclast formation was not significantly affected. In a high-S1P environment such as the bloodstream, S1PR1 is activated and rapidly internalized, allowing S1PR2 to predominate. Osteoclast precursors enter the bone marrow as a result of chemorepulsion mediated by S1PR2, and other chemokines attract them to bone surfaces. After they enter a low-S1P environment such as bone marrow, S1PR1 is transported back to the cell surface, and osteoclast precursors return from bone tissues to blood vessels as a result of chemotaxis to an S1P gradient.

4.3 Migration and Positioning of Osteoclast Precursors In Vivo

The number of osteoclast precursors on bone surfaces is determined by the balance between the trafficking of osteoclast precursors to and from the circulation. These data provide evidence that S1P controls the migratory behavior of osteoclast precursors, dynamically regulating bone mineral homeostasis, and identify a critical control point in osteoclastogenesis. Based on our findings, we propose that regulation of the migratory behavior of osteoclast precursors controls osteoclast differentiation. This control mechanism is summarized in Fig. 1. This critical control point in osteoclastogenesis may represent an attractive target for new treatments for osteoporosis. We previously showed that treatment with FTY720, which is metabolized by SPHK2 to a compound that acts as an agonist for four of the five S1P receptors (not S1PR2) [9, 20], relieved ovariectomy-induced osteoporosis in mice by reducing the number of mature osteoclasts attached to bone surfaces [7]. The mechanism of action of S1P is completely different from that of conventional treatments such as bisphosphonates, which suppress mature osteoclasts. We anticipate that the regulation of osteoclast precursor migration may be a useful clinical strategy in the near future.

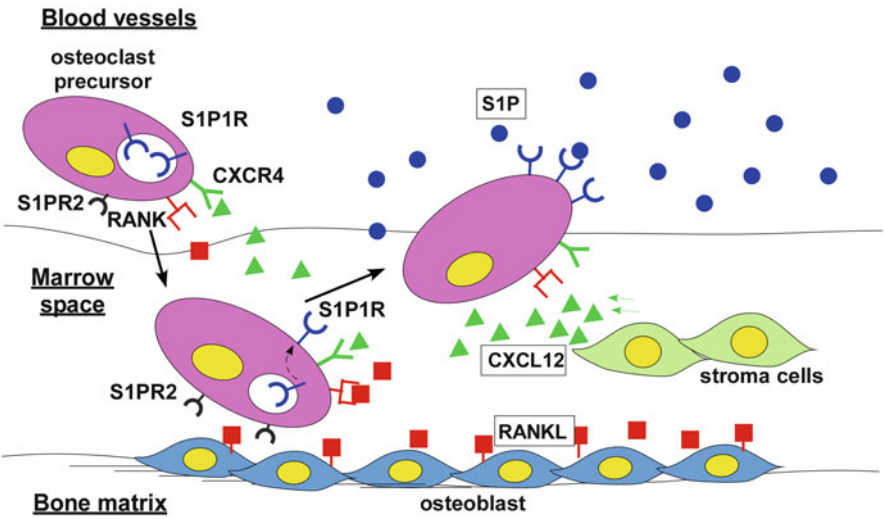

Fig. 1 A schematic model for migration and localization of osteoclast precursors by S1P and bone-enriched chemokines. The entry of osteoclast precursors from blood vessels, where S1P is at high concentration, is initiated by chemorepulsion through S1PR2. Once enter in bone marrow, osteoclast precursors migrate toward chemokines enriched in bone marrow cavity, such as CXCL12. On the other hand, their recirculation toward blood vessels is regulated by chemoattraction through S1PR1

5 Future Perspective on Intravital Multiphoton Microscopy

Intravital multiphoton imaging has revealed, and continues to reveal, dynamic features of physiological and pathological process. Its greatest strength is its ability to provide spatiotemporal information in living organisms, which cannot be achieved using other methods. However, current two-photon microscopy imaging techniques have several limitations. First, we cannot see everything in the visual fields in two-photon microscopy. Although fluorescence labeling and second-harmonic generation enable us to observe target cells and organs, the lack of a signal does never reflect an open field, as diverse structures and cellular components should be present. To avoid misinterpretation, we must interpret our observations with caution. Second, although two-photon microscopy has greater penetration depth than conventional confocal microscopy, its penetration depth is only 800–1,000 μm in soft tissues (e.g., brain cortex) and 200 μm in hard tissues (e.g., bone). Because of these resolution limitations, it may only be applied to small animals, such as mice and rats. Moreover, due to the wide scattering of light by the skin, it is necessary that target organs be exteriorized. It is possible that the necessary operative invasion and changes in oxygen concentration and humidity may influence cellular behavior. To resolve these problems, technical innovations in fluorochrome and optical systems, including improvements in light emission and amelioration of resolution problems [21], are needed.

Intravital microscopy has begun to be applied not only to observational studies but also to functional analysis and interventions. Recently, several new fluorescence tools have been developed. These include cell-cycle indicators [22] and light-sensing devices such as photoactivatable fluorescent proteins [23] and light-induced activators of G protein-coupled receptors [24].

6 Conclusion

As the recruitment of osteoclast precursors during osteoclastogenesis is dynamic and dependent on the microenvironment of the bone marrow cavity, temporospatial information is very important. Intravital imaging has made a huge contribution to improving our understanding of these processes. It enables us to visualize, temporospatially, complicated systems in living organisms. This new technique has revealed that S1P acts in concert with several chemoattractants to shepherd osteoclast precursors to appropriate sites. Controlling the recruitment and migration of osteoclast precursors represents a promising new therapeutic strategy for combating bone diseases. Although their limitations remain to be resolved, the range of applications for in vivo imaging techniques continues to expand.

References

1. Teitelbaum SL, Ross FP (2003) Genetic regulation of osteoclast development and function. Nat Rev Genet 4:638–649
2. Takayanagi H (2007) Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat Rev Immunol 7:292–304
3. Russell RGG, Xia Z, Dunford JE, Oppernann U, Kwaasi A, Hulley PA, Kavanagh KL, Triffitt JT, Lundy MW, Phipps RJ et al (2007) Bisphosphonates. An update on mechanisms of action and how these relate to clinical efiicacy. Ann N Y Acad Sci 1117:209–257
4. Yasuda Y, Kaleta J, Bromme D (2005) The role of cathepsins in osteoporosis and arthritis: rationale for the design of new therapeutics. Adv Drug Deliv Rev 57:973–993
5. Deal C (2009) Future therapeutic targets in osteoporosis. Curr Opin Rheumatol 4:380–385
6. Yu X, Huang Y, Collin-Osdoby P, Osdoby P (2003) Stromal cell-derived factor-1 (SDF-1) recruits osteoclast precursors by inducing chemotaxis, matrix metalloproteinase-9 (MMP-9) acrivity, and collagen transmigration. J Bone Miner Res 18:1404–1418
7. Ishii M, Egen JG, Klauschen F, Meier-Schellersheim M, Saeki Y, Vacher J, Proia RL, Germain RN (2009) Sphingosine-1-phosphate mobilizes osteoclast precursors and regulates bone homeostasis. Nature 458:524–528
8. Ishii M, Kikuta J, Shimazu Y, Meier-Schellersheim M, Germain RN (2010) Chemorepulsion by blood S1P regulates osteoclast precursor mobilization and obne remodeling in vivo. J Exp Med 207:2793–2798
9. Cyster JG (2005) Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu Rev Immunol 23:127–159
10. Rosen H, Goetzl EJ (2005) Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. Nat Rev Immunol 5:560–570

11. Rosen H, Sanna MG, Cahalan SM, Gonzalez-Cabrera PJ (2007) Tipping the gatekeeper: S1P regulation of endothelial barrier function. Trends Immunol 28:102–107
12. Rivera J, Proia RL, Olivera A (2008) The alliance of sphingosine-1-phosphate and its receptors in immunity. Nat Rev Immunol 8:753–763
13. Takuwa Y (2002) Subtype-specific differential regulation of Rho family G proteins and cell migration by the Edg family sphingosine-1-phosphate receptors. Biochem Biophys Acta 1682:112–120
14. Okamoto H, Takuwa N, Yokomizo T, Sugimoto N, Sakurada S, Shigematsu H, Takuwa Y (2000) Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. Mol Cell Biol 20:9247–9261
15. Klauschen F, Ishii M, Qi H, Bajénoff M, Egen JG, Germain RN, Meier-Schellersheim M (2009) Quantifying cellular interaction dynamics in 3D fluorescence microscopy data. Nat Protoc 4:1305–1311
16. Wei SH, Rosen H, Matheu MP, Sanna MG, Wang SK, Jo E, Wong CH, Parker I, Cahalan MD (2005) Sphingosine 1-phospate type 1 receptor agonism inhibits transendothelial migration of medullary T cells to lymphatic sinuses. Nat Immunol 12:1228–1235
17. Liu Y, Wada R, Yamashita T, Mi Y, Deng CX, Hobson JP, Rosenfeldt HM, Nava VE, Chae SS, Lee MJ et al (2000) Edg-1, the G-protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. J Clin Invest 106:951–961
18. Osada M, Yatomi Y, Ohmori T, Ikeda H, Ozaki Y (2002) Enhancement of sphingosine 1-phosphate-induced migration of vascular endothelial cells and smooth muscle cells by an EDG-5 antagonist. Biochem Biophys Res Commun 299:483–487
19. Kono M, Belyantseva IA, Skoura A, Frolenkov GI, Starost MF, Dreier JL, Lidngton D, Bolz SS, Friedman TB, Hla T, Proia RL (2007) Deafness and stria vascularis defects in S1P2 receptor-null mice. J Biol Chem 282:10690–10696
20. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, Allende ML, Proia RL, Cyster JG (2004) Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 6972:355–360
21. Ntziachristos V (2010) Going deeper than microscopy: the optical imaging frontier in biology. Nat Methods 7:603–614
22. Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, Osawa H, Kashiwagi S, Fukami K, Miyata T, Miyoshi H et al (2008) Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. Cell 132:487–498
23. Victora GD, Schwichkert TA, Fooksman DR, Kamphorst AO, Meyer-Hermann M, Dustin ML, Nussenzweig MC (2010) Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. Cell 143:592–605
24. Airan RD, Thompson KR, Fenno LE, Bernstein H, Deisseroth K (2009) Temporally precise in vivo control of intracellular signalling. Nature 458:1025–1029

T Cells Mediate the Effects of PTH in Bone

Roberto Pacifici

1 Introduction

Parathyroid hormone (PTH) causes bone loss when produced in a continuous fashion. However, PTH induces a potent bone anabolic effect when injected intermittently. The mechanism of action of PTH remains largely unknown. This article reviews the evidence in favor of the hypothesis that T cells play an unexpected critical role in the mechanism of action of PTH in bone.

2 Effects of PTH on Bone

Primary hyperparathyroidism is a common bone disease caused by continuous overproduction of PTH. This disorder causes cortical bone loss [1] and leads to a loss or gain of trabecular bone, depending on its severity, duration, and age of the patient [1–3]. Primary hyperparathyroidism is modeled by continuous PTH (cPTH) infusion, which, like hyperparathyroidism, stimulates bone resorption and causes cortical bone loss [4–6]. cPTH treatment may lead to modest gain or loss of cancellous

R. Pacifici, M.D. (✉)
Division of Endocrinology, Metabolism and Lipids, Department of Medicine, Emory University School of Medicine, 101 Woodruff Circle, Room 1309, Atlanta, GA 30322, USA

Immunology and Molecular Pathogenesis Program, Emory University, Atlanta, GA 30322, USA
e-mail: roberto.pacifici@emory.edu

Y. Choi (ed.), *Osteoimmunology: Interactions of the Immune and Skeletal Systems,*
DOI 10.1007/978-1-4614-5366-6_9,

bone depending on the age of the mouse and the dose and duration of the cPTH treatment [4–6]. By contrast, intermittent PTH (iPTH) treatment markedly increases bone volume and strength in the cortical and trabecular compartments.

Both cPTH and iPTH increase bone turnover [6–9]. The stimulation of bone formation induced by iPTH far exceeds bone resorption, leading to a net bone anabolic effect in the cortical and trabecular compartments [10]. By contrast, the stimulation of bone formation induced by cPTH is not sufficient to offset the increase in resorption, leading to a net cortical bone loss, which, in some conditions, is associated to trabecular bone loss [10]. PTH stimulates bone resorption by enhancing the production of RANKL, and M-CSF, and decreasing the production of OPG by stromal cells (SCs) and osteoblasts (OBs) [11, 12]. Recently, we have shown that another critical mechanism is the capacity of PTH to increase the production of TNF by T cells [5]. Enhanced bone resorption is accompanied by a stimulation of bone formation driven by an increase in the number of OBs [13–15] achieved through activation of quiescent lining cells [16], increased OB proliferation [17, 18] and differentiation [17, 19, 20], attenuation of OB apoptosis [21–24], and signaling in osteocytes [25]. However, the specific contribution of each of these effects of PTH remains controversial. The expansion of the osteoblastic pool induced by PTH is initiated by the release from the matrix undergoing resorption of TGFβ, IGF-1 and other growth factors that recruit SCs to remodeling areas [26–29]. Subsequent events are driven primarily by the activation of Wnt signaling in osteoblastic cells [30]. Activation of Wnt signaling induces OB proliferation [31] and differentiation [30, 32], prevents OB apoptosis [23, 24, 33], and augments OB production of OPG [34]. Wnt proteins initiate a canonical signaling cascade by binding to receptors of the Frizzled family together with the coreceptors LRP4-5-6, which results in the stabilization of cytosolic β-catenin. A nuclear complex of beta-catenin and the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors then interacts with DNA to regulate the transcription of Wnt target genes [35]. Wnt proteins also signal through noncanonical pathways which involve the Src/ERK and Pi3K/Akt cascades [23].

PTH is a canonical Wnt signaling agonist which increases β catenin levels in osteoblastic cells [36, 37], an effect which occurs through modulation of both the protein kinase A and protein kinase C pathways [36]. PTH, once bound to PPR, is also capable of forming a complex with LRP6 which results in LRP6 signaling and β catenin activation [38]. Thus, PTH activates Wnt signaling in osteoblastic cells through both Wnt ligands-dependent and Wnt ligands-independent mechanisms. Moreover, PTH down regulates the production of sclerostin, an osteocyte-derived Wnt antagonist which blocks Wnt signaling by binding to LRP5 and LRP6 [39, 40]. Recently, convincing evidence has emerged that PTH receptor signaling in osteocytes and the resulting direct regulation of sclerostin production play a particularly relevant role in the anabolic activity of PTH (O'Brien, 2008 #11387). PTH also regulates Dickkopf-1, a soluble LRP5 and LRP6 signaling inhibitor [37], and Sfrp-4, a factor which binds Wnt proteins thus antagonizing both canonical and noncanonical Wnt signaling [41]. Uncertainty remains with regard to the identity and the source

of Wnt ligands which activate Wnt signaling in response to PTH treatment are not completely understood.

3 Role of T Cells in the Anabolic Activity of Intermittent PTH Treatment

T cells express functional the PTH receptor PPR [42, 43] and respond to PTH. This prompted us to investigate whether T cells contribute to the anabolic response to iPTH. Studies were conducted in four strains of T cell-deficient mice (TCRβ−/−, RAG2−/−, class I and II MHC double KO mice, and nude mice). Analysis by DEXA and μCT revealed that in mice lacking T cells, iPTH induced a ~ 50 % smaller increase in bone density and bone volume as compared to T cell-replete controls [43]. Furthermore, adoptive transfer of T cells into T cell-deficient mice restored a normal response to iPTH. T cells were found to augment the capacity of iPTH to improve architecture in trabecular but not in cortical bone. Although the reason of this selectivity is unknown, a lack of access of T cells to cortical surfaces is not a likely explanation, as T cells reach endosteal and periosteal bone surface through blood vessels and recirculate in and out of the BM. In addition, direct measurements of bone strength by 4-point bending revealed that the capacity of iPTH to improve bone strength was abolished in T cell-deficient mice. Although the reason for this discrepancy is unknown, it is possible that T cells might be required to improve the material property of bone.

With regard of the mechanism by which T cells potentiate the bone anabolic activity of iPTH, studies have disclosed that in the absence of T cells iPTH is unable to increase the commitment of SCs to the osteoblastic lineage, induce OB proliferation and differentiation, and mitigate OB apoptosis. All of these actions of PTH were found to hinge on the capacity of T cells to activate Wnt signaling in osteoblastic cells [43]. Although it is well established that Wnt activation is a key mechanism by which iPTH expands the osteoblastic pool, little information is available on the nature and the source of the Wnt ligand required to activate Wnt signaling in OBs. We have found that PTH stimulates BM CD8+ T cells to produce large amounts of Wnt10b [43], a Wnt protein which activates Wnt signaling in SCs and OBs, thus increasing OB proliferation, differentiation, and life span Treatment with iPTH also caused a small increase in the production of Wnt10b by BM CD4+ cells which was associated with a slightly diminished anabolic response in class II MHC−/− mice, suggesting that production of Wnt10b by CD4+ cells contributes, in small part, to the anabolic activity of iPTH. The relevance of CD8+ cells was demonstrated by the inability of iPTH to promote bone anabolism in class I MHC−/− mice, a strain that lacks CD8+ cells [43]. Additional studies revealed that iPTH does not improve bone architecture in T cell-deficient mice reconstituted with CD4+ cells, while it does so in mice adoptively transferred with CD8+ cells [43]. The pivotal role of T cell-produced Wnt10b was revealed by the hampered effect of iPTH on bone volume in TCRβ−/− mice reconstituted with T cells from Wnt10b−/− mice. It is likely that iPTH directly targets CD8+ T cells and stimulates their

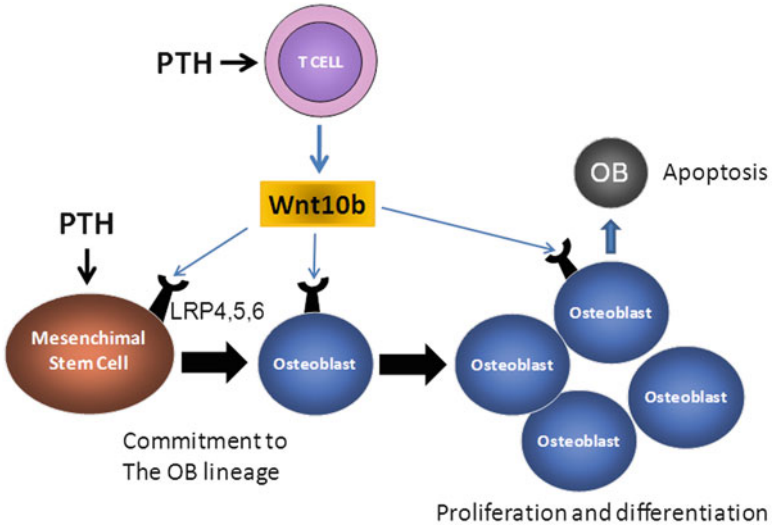

Fig. 1 Schematic representation of the role of T cells in the mechanism by which intermittent PTH treatment stimulates bone formation. PTH stimulates T cells to secrete Wnt10b, a Wnt ligand required to activate Wnt signaling in SCs and OBs. In the presence of T cell-produced Wnt10b, stimulation of osteoblastic cells by PTH result in the activation of the Wnt signaling pathway. This event leads to increased commitment of mesenchimal stem cells to the osteoblastic lineage, increased osteoblast proliferation and differentiation, and decreased osteoblast apoptosis

production of Wnt10b as in vitro PTH treatment potently stimulates Wnt10b production by T cells.

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

Together the data indicate that CD8+ T cells potentiate the anabolic activity of PTH by providing Wnt10b, which is a critical Wnt ligand required for activating Wnt signaling in osteoblastic cells Therefore in the absence of CD8+ cells, stimulation of osteoblastic cells by PTH is not sufficient to elicit maximal Wnt activation due to the lack of a critical Wnt ligand (Fig. 1). The residual bone anabolic activity of PTH observed in T cell-deficient mice is presumably due to ligand-independent activation of LRP6 [38], and suppressed production of sclerostin [39, 40, 44].

The anabolic activity of iPTH is not identical in all strains of T cell-deficient mice. In fact, while some strains had no increase in bone volume in response to PTH, other exhibited a blunted but not a completely absent response. Osteoblastic cells produce several bone anabolic Wnt ligands including Wnt10b, Wnt7a and Wnt3b [45, 46]. These factors are likely to contribute to the T cell-independent

anabolic activity of iPTH, and quantitative differences in their production may account, in part, to the strain-dependent variability in the response to iPTH observed herein. Furthermore, the magnitude of the anabolic response to iPTH in T cell null mice may be related to a strain- and age-dependent capacity of iPTH to inhibit the bone cells production of Wnt inhibitors such as sclerostin, [39, 40], Dickkopf-1 [37] and Sfrp-4 [41]. These factors have been shown to contribute to the anabolic activity of iPTH through T cell-independent mechanisms. Since B cells are regulated by PTH [47], the response of RAG2−/− mice to iPTH might also have been determined by the lack of B cells which is a feature of RAG2−/− mice.

The enhancement of bone formation induced by iPTH is accompanied by a stimulation of bone resorption which is driven by increased production of RANKL and decreased release of OPG in the bone microenvironment [48]. The direct effects of PTH on RANKL/OPG production are mitigated, in part, by the iPTH-induced activation of β catenin in OBs, as this transcriptional regulator stimulates their production of OPG [49] and represses that of RANKL [50]. The latter is one of the mechanisms that prevent bone resorption from offsetting the anabolic activity of iPTH.

Osteoblastic cells from WT mice treated with iPTH in vivo exhibited increased commitment to the osteoblastic lineage, proliferation, differentiation, and life span in vitro, as compared to the corresponding cells from T cell-deficient mice. Thus, T cells, like PTH, affect all aspects of OB life cycle. Remarkably, these differences were demonstrated in OBs purified from BM cultured for 7 days without the addition of PTH, suggesting that in vivo the hormone regulates early commitment steps of SCs and their osteoblastic progeny through T cell-produced Wnt10b, and that these steps are not reversed by the absence of PTH and T cells in vitro. This model is consistent with the capacity of Wnt signaling to guide cell fate determination [51]. A similar paradigm has been described in ovariectomized mice, a model where estrogen withdrawal in vivo leads to the formation of SCs which exhibit an increased osteoclastogenic activity which persists in vitro for 4 weeks [52].

4 T Cells and PTH-Induced Bone Loss

Studies designed to investigate the role of T cells in the bone catabolic activity of cPTH revealed that an infusion of cPTH that mimics hyperparathyroidism fails to induce OC formation, bone resorption, and cortical bone loss in mice lacking T cells [35]. A second study conducted in older mice, a model in which cPTH causes cortical and cancellous bone loss, revealed that T cell-deficient mice are also protected against trabecular bone loss [5].

An important finding of these studies was that cPTH equally stimulated bone formation in T cell-replete and T cell-deficient mice [35] [5]. It should be noted that while the stimulation of bone formation induced by cPTH was completely T cell independent, the lack of T cells blocked the increase in bone formation induced by iPTH. The reason for this critical difference remains to be determined.

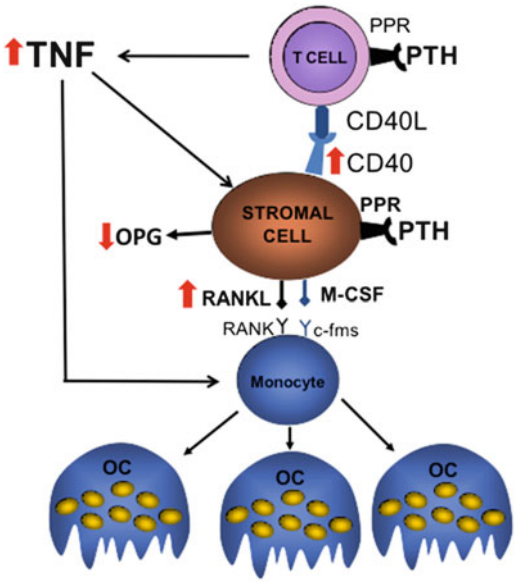

Fig. 2 Schematic representation of the role of T cells in the mechanism by which continuous PTH stimulates OC formation. Continuous PPR signaling in T cells induced by continuous PTH (cPTH) treatment stimulates the production of TNF. This cytokine increases CD40 expression by SCs. Binding to CD40 of T cell expressed CD40L increases SC sensitivity to PTH resulting in enhanced SC production of RANKL and diminished secretion of OPG in response to PTH. T cell-produced TNF further stimulates OC formation through its direct effects on maturing OC precursors. The *red arrows* represent the main modifications induced by activation of PPR signaling in T cells

These studies further revealed the existence of a cross-talk between T cells and SCs mediated by the CD40L/CD40 signaling system. T cells provide proliferative and survival cues to SCs and sensitize SCs to PTH through CD40L, a surface molecule of activated T cells that induces CD40 signaling in SCs. An important element of this regulatory loop is the capacity of PTH to upregulate the expression of CD40 in SC from T cell-replete mice but not from T cell-deficient mice [35]. Thus T cells contribute to the CD40L/CD40-mediated exchange of information between T cells and SCs in two ways: first, by providing CD40L and secondly, by upregulating the expression of CD40 on SCs. As a result, mice lacking T cells or T cell-expressed CD40L have lower number of SCs. Furthermore, these SCs produce lower amount of RANKL and have an even smaller suppression of OPG secretion in response to PTH. Therefore, SCs from T cell-deficient mice have a lower capacity to support OC formation in vivo and in vitro. The alteration in SC function is the ultimate reason why deletion of T cells or T cell-expressed CD40L blunts the bone catabolic activity of PTH [35].

Studies have also shown that cPTH increases the T cell production of TNF. This cytokine not only increases OC formation directly, but also TNF upregulates the

expression of CD40 in SCs, thus increasing their response to T cell expressed CD40L. Attesting to the relevance TNF, cPTH fails to induce bone loss and stimulate bone resorption in mice lacking T cell TNF production [5].

To determine whether PTH targets T cells directly, we have conditionally silenced the PTH receptor PPR in T cells. We found that removal of PPR signaling in T cells blunts the stimulation of bone resorption induced by cPTH without affecting bone formation. As a result, silencing of PPR signaling in T cells prevents the loss of cortical bone induced by cPTH. Strikingly, the disruption of PPR signaling in T cells converts the effects of cPTH in trabecular bone from catabolic to anabolic [5].

Collectively, our data reveal that the effects of cPTH on bone are the result of a mechanism that involves PPR activation and TNF production in T cells (Fig. 2). T cell-produced TNF stimulates bone resorption directly by potentiating the sensitivity of maturing OCs to RANKL. In addition, TNF enhances CD40L/CD40 signaling from T cells to SCs by upregulating CD40 expression, an effect resulting in the increased capacity of SCs to support OC formation. Thus, a complex cross-talk between T cells and the osteoclastogenic machinery of the BM is central for the bone catabolic activity of cPTH.

References

1. Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, Capparelli C, Li J, Elliott R, McCabe S et al (1999) Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature 402:304–309
2. Di Rosa F, Santoni A (2002) Bone marrow CD8 T cells are in a different activation state than those in lymphoid periphery. Eur J Immunol 32:1873–1880
3. Westermann J, Pabst R (1992) Distribution of lymphocyte subsets and natural killer cells in the human body. Clin Investig 70:539–544
4. Kotake S, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, Saito S, Inoue K, Kamatani N, Gillespie MT et al (1999) IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. J Clin Invest 103:1345–1352
5. Sipkins DA, Wei X, Wu JW, Runnels JM, Cote D, Means TK, Luster AD, Scadden DT, Lin CP (2005) In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature 435:969–973
6. Becker TC, Coley SM, Wherry EJ, Ahmed R (2005) Bone marrow is a preferred site for homeostatic proliferation of memory CD8 T cells. J Immunol 174:1269–1273
7. Wilson A, Trumpp A (2006) Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol 6:93–106
8. Tokoyoda K, Zehentmeier S, Chang HD, Radbruch A (2009) Organization and maintenance of immunological memory by stroma niches. Eur J Immunol 39:2095–2099
9. Uccelli A, Moretta L, Pistoia V (2008) Mesenchymal stem cells in health and disease. Nat Rev Immunol 8:726–736
10. Mak TW, Ferrick DA (1998) The gammadelta T-cell bridge: linking innate and acquired immunity. Nat Med 4:764–765
11. Nauta AJ, Fibbe WE (2007) Immunomodulatory properties of mesenchymal stromal cells. Blood 110:3499–3506
12. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y (2008) Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2:141–150

13. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M et al (2008) Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 371:1579–1586
14. Huang W, La Russa V, Alzoubi A, Schwarzenberger P (2006) Interleukin-17A: a T-cell-derived growth factor for murine and human mesenchymal stem cells. Stem Cells 24:1512–1518
15. Yamaza T, Miura Y, Bi Y, Liu Y, Akiyama K, Sonoyama W, Patel V, Gutkind S, Young M, Gronthos S et al (2008) Pharmacologic stem cell based intervention as a new approach to osteoporosis treatment in rodents. PLoS One 3:e2615
16. Grewal IS, Flavell RA (1998) CD40 and CD154 in cell-mediated immunity. Annu Rev Immunol 16:111–135
17. Quezada SA, Jarvinen LZ, Lind EF, Noelle RJ (2004) CD40/CD154 interactions at the interface of tolerance and immunity. Annu Rev Immunol 22:307–328
18. Andre P, Prasad KS, Denis CV, He M, Papalia JM, Hynes RO, Phillips DR, Wagner DD (2002) CD40L stabilizes arterial thrombi by a beta3 integrin–dependent mechanism. Nat Med 8:247–252
19. Prasad KS, Andre P, He M, Bao M, Manganello J, Phillips DR (2003) Soluble CD40 ligand induces beta3 integrin tyrosine phosphorylation and triggers platelet activation by outside-in signaling. Proc Natl Acad Sci USA 100:12367–12371
20. Zirlik A, Maier C, Gerdes N, MacFarlane L, Soosairajah J, Bavendiek U, Ahrens I, Ernst S, Bassler N, Missiou A et al (2007) CD40 ligand mediates inflammation independently of CD40 by interaction with Mac-1. Circulation 115:1571–1580
21. Leveille C, Bouillon M, Guo W, Bolduc J, Sharif-Askari E, El-Fakhry Y, Reyes-Moreno C, Lapointe R, Merhi Y, Wilkins JA et al (2007) CD40 ligand binds to alpha5beta1 integrin and triggers cell signaling. J Biol Chem 282:5143–5151
22. Grammer AC, Lipsky PE (2000) CD40-mediated regulation of immune responses by TRAF-dependent and TRAF-independent signaling mechanisms. Adv Immunol 76:61–178
23. Saeland S, Duvert V, Caux C, Pandrau D, Favre C, Valle A, Durand I, Charbord P, de Vries J, Banchereau J (1992) Distribution of surface-membrane molecules on bone marrow and cord blood CD34+ hematopoietic cells. Exp Hematol 20:24–33
24. Ahuja SS, Zhao S, Bellido T, Plotkin LI, Jimenez F, Bonewald LF (2003) CD40 ligand blocks apoptosis induced by tumor necrosis factor alpha, glucocorticoids, and etoposide in osteoblasts and the osteocyte-like cell line murine long bone osteocyte-Y4. Endocrinology 144:1761–1769
25. Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, Tanaka S, Kodama T, Akira S, Iwakura Y et al (2006) Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. J Exp Med 203:2673–2682
26. Grewal IS, Flavell RA (1996) The role of CD40 ligand in costimulation and T-cell activation. Immunol Rev 153:85–106
27. Solanilla A, Dechanet J, El Andaloussi A, Dupouy M, Godard F, Chabrol J, Charbord P, Reiffers J, Nurden AT, Weksler B et al (2000) CD40-ligand stimulates myelopoiesis by regulating flt3-ligand and thrombopoietin production in bone marrow stromal cells. Blood 95:3758–3764
28. Funakoshi S, Taub DD, Anver MR, Raziuddin A, Asai O, Reddy V, Rager H, Fanslow WC, Longo DL, Murphy WJ (1997) Immunologic and hematopoietic effects of CD40 stimulation after syngeneic bone marrow transplantation in mice. J Clin Invest 99:484–491
29. Teitelbaum SL (2000) Osteoclasts, integrins, and osteoporosis. J Bone Miner Metab 18: 344–349
30. Haas W, Pereira P, Tonegawa S (1993) Gamma/delta cells. Annu Rev Immunol 11:637–685
31. Yokota T, Oritani K, Mitsui H, Aoyama K, Ishikawa J, Sugahara H, Matsumura I, Tsai S, Tomiyama Y, Kanakura Y et al (1998) Growth-supporting activities of fibronectin on hematopoietic stem/progenitor cells in vitro and in vivo: structural requirement for fibronectin activities of CS1 and cell-binding domains. Blood 91:3263–3272

32. Cantor JM, Ginsberg MH, Rose DM (2008) Integrin-associated proteins as potential therapeutic targets. Immunol Rev 223:236–251
33. Li Y, Toraldo G, Li A, Yang X, Zhang H, Qian WP, Weitzmann MN (2007) B cells and T cells are critical for the preservation of bone homeostasis and attainment of peak bone mass in vivo. Blood 109:3839–3848
34. Lopez-Granados E, Temmerman ST, Wu L, Reynolds JC, Follmann D, Liu S, Nelson DL, Rauch F, Jain A (2007) Osteopenia in X-linked hyper-IgM syndrome reveals a regulatory role for CD40 ligand in osteoclastogenesis. Proc Natl Acad Sci USA 104:5056–5061
35. Gao Y, Wu X, Terauchi M, Li JY, Grassi F, Galley S, Yang X, Weitzmann MN, Pacifici R (2008) T cells potentiate PTH-induced cortical bone loss through CD40L signaling. Cell Metab 8:132–145
36. Tobimatsu T, Kaji H, Sowa H, Naito J, Canaff L, Hendy GN, Sugimoto T, Chihara K (2006) Parathyroid hormone increases beta-catenin levels through Smad3 in mouse osteoblastic cells. Endocrinology 147:2583–2590
37. Kulkarni NH, Halladay DL, Miles RR, Gilbert LM, Frolik CA, Galvin RJ, Martin TJ, Gillespie MT, Onyia JE (2005) Effects of parathyroid hormone on Wnt signaling pathway in bone. J Cell Biochem 95:1178–1190
38. Wan M, Yang C, Li J, Wu X, Yuan H, Ma H, He X, Nie S, Chang C, Cao X (2008) Parathyroid hormone signaling through low-density lipoprotein-related protein 6. Genes Dev 22:2968–2979
39. Bellido T, Ali AA, Gubrij I, Plotkin LI, Fu Q, O'Brien CA, Manolagas SC, Jilka RL (2005) Chronic elevation of parathyroid hormone in mice reduces expression of sclerostin by osteocytes: a novel mechanism for hormonal control of osteoblastogenesis. Endocrinology 146:4577–4583
40. Keller H, Kneissel M (2005) SOST is a target gene for PTH in bone. Bone 37:148–158
41. Qin L, Qiu P, Wang L, Li X, Swarthout JT, Soteropoulos P, Tolias P, Partridge NC (2003) Gene expression profiles and transcription factors involved in parathyroid hormone signaling in osteoblasts revealed by microarray and bioinformatics. J Biol Chem 278:19723–19731
42. Young N, Mikhalkevich N, Yan Y, Chen D, Zheng WP (2005) Differential regulation of osteoblast activity by Th cell subsets mediated by parathyroid hormone and IFN-gamma. J Immunol 175:8287–8295
43. Terauchi M, Li JY, Bedi B, Baek KH, Tawfeek H, Galley S, Gilbert L, Nanes MS, Zayzafoon M, Guldberg R et al (2009) T lymphocytes amplify the anabolic activity of parathyroid hormone through Wnt10b signaling. Cell Metab 10:229–240
44. O'Brien CA, Plotkin LI, Galli C, Goellner JJ, Gortazar AR, Allen MR, Robling AG, Bouxsein M, Schipani E, Turner CH et al (2008) Control of bone mass and remodeling by PTH receptor signaling in osteocytes. PLoS One 3:e2942
45. Rawadi G, Vayssiere B, Dunn F, Baron R, Roman-Roman S (2003) BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. J Bone Miner Res 18:1842–1853
46. Luo Q, Kang Q, Si W, Jiang W, Park JK, Peng Y, Li X, Luu HH, Luo J, Montag AG et al (2004) Connective tissue growth factor (CTGF) is regulated by Wnt and bone morphogenetic proteins signaling in osteoblast differentiation of mesenchymal stem cells. J Biol Chem 279:55958–55968
47. Alexiewicz JM, Klinger M, Pitts TO, Gaciong Z, Linker-Israeli M, Massry SG (1990) Parathyroid hormone inhibits B cell proliferation: implications in chronic renal failure. J Am Soc Nephrol 1:236–244
48. Ma YL, Cain RL, Halladay DL, Yang X, Zeng Q, Miles RR, Chandrasekhar S, Martin TJ, Onyia JE (2001) Catabolic effects of continuous human PTH (1–38) in vivo is associated with sustained stimulation of RANKL and inhibition of osteoprotegerin and gene-associated bone formation. Endocrinology 142:4047–4054
49. Glass DA 2nd, Bialek P, Ahn JD, Starbuck M, Patel MS, Clevers H, Taketo MM, Long F, McMahon AP, Lang RA et al (2005) Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. Dev Cell 8:751–764

50. Spencer GJ, Utting JC, Etheridge SL, Arnett TR, Genever PG (2006) Wnt signalling in osteoblasts regulates expression of the receptor activator of NFkappaB ligand and inhibits osteoclastogenesis in vitro. J Cell Sci 119:1283–1296
51. Moon RT, Bowerman B, Boutros M, Perrimon N (2002) The promise and perils of Wnt signaling through beta-catenin. Science 296:1644–1646
52. Kimble RB, Srivastava S, Ross FP, Matayoshi A, Pacifici R (1996) Estrogen deficiency increases the ability of stromal cells to support murine osteoclastogenesis via an interleukin-1and tumor necrosis factor-mediated stimulation of macrophage colony-stimulating factor production. J Biol Chem 271:28890–28897

The Relationship Between Inflammation, Destruction, and Remodeling in Chronic Joint Diseases

Kirsten Braem and Rik J. Lories

1 Introduction: Chronic Joint Diseases, a Major Health Problem

Chronic joint diseases are a major health problem as they are linked to pain, loss of function, and increasing disability. Osteoarthritis (OA) is the most prevalent disorder and is generally considered a degenerative disease associated with aging, tear-and-wear, trauma, and acquired factors such as obesity [1]. In contrast, rheumatoid arthritis (RA) and the different forms of spondyloarthritis (SpA) are chronic inflammatory diseases, affecting younger people, steered on by persistent activation of the immune system [2, 3]. These inflammatory joint diseases can be further defined as a group of chronic musculoskeletal disorders with common inflammatory pathways, characterized by joint organ and tissue damage, increased morbidity and mortality, and reduced quality of life. From a pathological perspective, not only changes in the adaptive and innate immune system but also molecular and cellular pathways that determine joint tissue homeostasis, repair and remodeling will determine the outcome of these diseases [4].

RA is the best-known form of chronic arthritis and typically affects the peripheral joints in a symmetric fashion. The small joints of hand and feet are most commonly involved. RA affects more females than males and is associated with specific HLA

K. Braem • R.J. Lories (✉)
Laboratory of Tissue Homeostasis and Disease, Skeletal Biology and Engineering Research Center, Department of Development and Regeneration , O&N1, Box 813, B3000 KU Leuven, Belgium
e-mail: Rik.Lories@uz.kuleuven.be

Y. Choi (ed.), *Osteoimmunology: Interactions of the Immune and Skeletal Systems,*
DOI 10.1007/978-1-4614-5366-6_10,

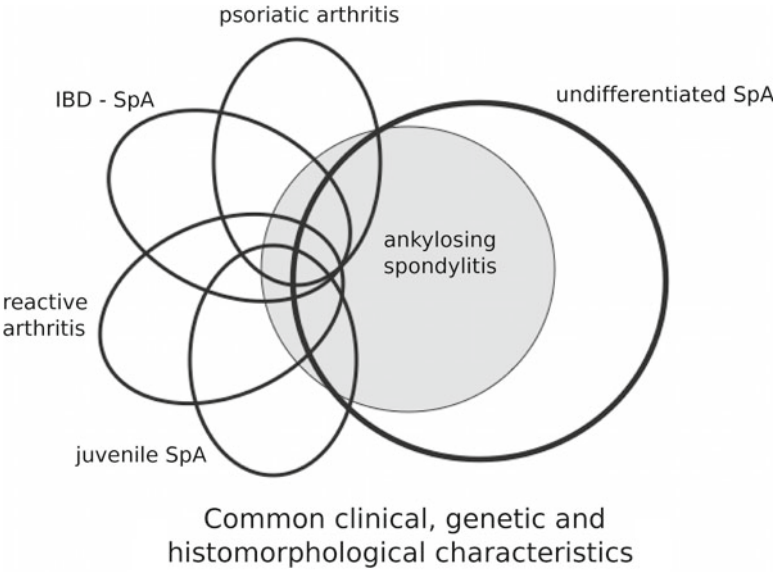

Fig. 1 The spondyloarthritis concept. Ankylosing spondylitis represents that paradigm disorder for this group of related diseases. Other diagnostic entities include psoriatic arthritis, reactive arthritis, arthritis associated with inflammatory bowel disease (IBD), a juvenile and an undifferentiated form. As clinical symptoms overlap, the first diagnosis may be made by the initial presentation but the disease phenotype may change over time resulting in either reclassification or fit in different entities

genes (HLA-DRB1) as well as with other polymorphisms in genes with a role in the immune system [5]. Autoantibodies against specific antigens play a role in pathogenesis, course and diagnosis of the disease. Among these, antibodies directed against citrullinated proteins and rheumatoid factor appear most important. RA affects between 0.3 and 1% of the population and typically starts between the ages of 30 and 60 years.

The SpA concept groups distinct diagnostic entities that share common clinical, genetic, and morphological characteristics [6]. Ankylosing spondylitis (AS) formerly known as Bechterew's disease, reactive arthritis, psoriatic arthritis, arthritis associated with inflammatory bowel disease, a juvenile and an undifferentiated form are all part of the SpA concept (Fig. 1). The axial skeleton, in particular the sacroiliac joints and the lower spine, are commonly involved. Peripheral arthritis, if present, mainly manifests as a nonsymmetrical oligoarticular disease. SpAs and in particular AS affect more males than females and are genetically strongly associated with the HLA-B27 antigen. Other genetic factors have recently been identified and include a number of genes linked to immunity or inflammation [7]. In contrast with RA, these diseases are not associated with autoantibody formation. Like RA, the disorders that make up the SpA concept affect between 0.3 and 1% of the population in the Western world.

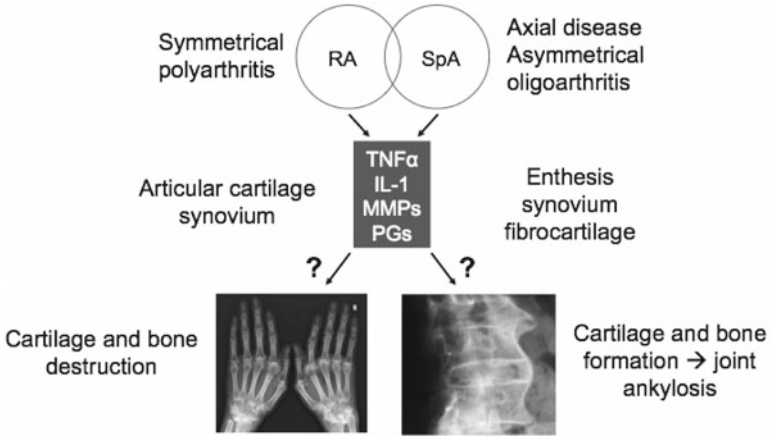

Fig. 2 Differences and similarities between different rheumatoid arthritis and spondyloarthritis. Despite the presence of similar symptoms at the individual joint level and the existence of comparable immune mechanisms, anatomic sites where the disease processes start as well as the long-term outcome may be very different. Rheumatoid arthritis is associated with the synovium and the articular cartilage, whereas increasing evidence supports a central role for the enthesis in spondyloarthritis. In rheumatoid arthritis structural damage is characterized by joint destruction, in spondyloarthritis by ankylosis

2 Similar Symptoms But a Strikingly Different Outcome of the Diseases

Clinical manifestations of RA and the different SpAs are much alike (Fig. 2). Affected joints show swelling, redness, pain, warmth, and loss of function. As mentioned above, the pattern of joints involved may be very different, in particular with the dominance of axial disease in SpA. Nevertheless at the tissue level, common effector mechanisms and inflammation-driving processes are easily recognized [8]. Proinflammatory cytokines such as interleukin-1 (IL1) and tumor necrosis factor-alpha (TNFa) are present, different types of immune cells invade the joint tissues, prostaglandins are activated and a number of tissue destructive enzymes are activated including matrix metalloproteinases (MMPs). However, the specific tissues within the joint that are the primary target of the disease process may be different between RA and SpA (Fig. 2) [8]. In RA, the synovium and the articular cartilage appear primarily involved. In SpA, strong evidence links the onset of disease to the enthesis, an anatomical zone in which tendons and ligaments insert into the underlying bone and thus a site in which biomechanical stresses are transferred from the soft tissues to the skeleton.

The most surprising feature when considering both groups of chronic arthritis is found in the outcome of the diseases (Fig. 2). RA is typically characterized by extensive cartilage and bone destruction, whereas in AS and related SpAs often new

cartilage and bone formation can be seen which is leading to the formation of syndesmophytes, osteophytes, or enthesophytes and which may result in progressive ankylosis of the sacroiliac joints and the spine.

3 Arthritis: Research Progress Translating Symptoms into Molecular Pathology

Over the last decades considerable progress has been made in our understanding of the basic mechanisms that underlie the signs, symptoms, and outcome in the different forms of chronic arthritis. Most progress has been made in understanding the inflammatory cascades [2]. This has, among others, resulted in the identification of key cytokines (TNFa and IL6), key cell populations (T cells, macrophages, B cells) in these diseases and some of these findings have been translated into new advanced therapeutic strategies that have an unprecedented impact on the management and wellbeing of patients. Current biological therapies thus include antibodies and soluble receptors directed against TNFa, antibodies against the IL6 receptors, T cell modulators such as CTLA4-Ig and antibodies depleting B cell precursors [9].

The rapidly emerging field of osteoimmunology research has also unveiled many of the molecular mechanisms that underlie progressive joint destruction. Osteoclasts have been identified as key cells in the destruction of bone and the formation of bone erosions and the molecular system supporting their differentiation, maturation, and activation has been discovered [10]. Recently antibodies against receptor of NFkB ligand (RANKL), one of the key factors, have also been introduced in clinical practice. These are currently used in the treatment of osteoporosis but are also studied in different joint diseases [11, 12]. Similarly, the research community has better understood mechanisms leading to activation and transformation of synovial fibroblasts, mainly in rheumatoid arthritis [13]. By producing tissue destructive enzymes such as MMPs and by expressing RANKL these cells play an essential role in the progression of joint destruction.

The molecular cascades underlying new cartilage and bone formation that is leading to ankylosis, have been less studied. However, in the last couple of years, we and other groups have started to understand some of the basic mechanisms that steer ankylosis and how these are linked to the inflammation characteristic of chronic arthritis [14]. These studies have been hindered by the limited availability of tissue samples from human patients as biopsies from spinal lesions can only rarely be obtained. Advanced imaging techniques in patients including the widespread availability of nuclear magnetic resonance have clearly demonstrated the inflammatory lesions in AS and related SpAs but new bone formation, which is a slower process, remains difficult to dynamically visualize and even consistently measure on convential X-ray images [15].

4 Successful Treatment of Signs and Symptoms in Arthritis May Not Always Be Sufficient to Obtain Full Control of the Diseases

The introduction of anti-TNF treatments in RA has not only transformed the management of the patients as signs and symptoms could be more effectively controlled but also changed the outcome of the disease. Anti-TNF therapy, in particular when combined with conventional immunomodulating drugs such as methotrexate, also appears to successfully stop the radiographic progression of disease [16]. Joint erosions and damage predict loss of function and disability and control of these processes therefore adds an additional level of benefits for patients and society. However, such a structural effect has only been recognized for joint destructive processes as seen in RA and some forms of psoriatic arthritis [17] but has not been demonstrated for joint remodeling leading to ankylosis in AS and other SpAs. Three different studies comparing treatment with anti-TNF to a historical cohort over a 2-year period could not demonstrate a benefit in terms of radiographic disease progression [18–20]. Nevertheless, it would be a mistake to downplay the effect that anti-TNF drugs have on both the short- and long-term outcome of patients with AS [21]. A recent study demonstrates that this outcome is determined by both inflammation and structural damage [22]. However, these observations also show that other mechanisms may play a role in the remodeling processes and that there is additional room for other or better interventions. Moreover, the differences between RA and SpA may also point towards specific mechanisms of disease and change the prevalent concepts of chronic arthritis.

Different hypotheses have been put forward to explain these differences between RA and SpA or between effects of anti-TNF on joint destruction and remodeling. Sieper et al. propose the existence of fundamental differences in the pattern and duration of inflammation between RA and AS [23]. In RA, inflammation is hypothesized to be a continuous and persisting process leading to progressive erosive disease. In AS and related SpAs, inflammation may be more fluctuating, leading to minor erosive damage and when it subsides, would leave a window of opportunity for repair processes to occur. This repair phenomenon, called osteoproliferation, however does not respect the original confinement of the damaged tissues and is exaggerated leading to syndesmophyte formation and ankylosis.

Although differences in inflammation between RA and AS are clear, some aspects of this hypothesis may be challenged. We and others demonstrated that inhibition of osteoclasts does not have an effect on joint remodeling and ankylosis in different mouse and rat models of arthritis thereby suggesting that erosion of bone is not necessary to trigger new bone formation [24, 25]. Also, in OA, osteophyte formation is often considered a stabilizing effort in a damaged joint. Moreover, the striking paradox in AS that new bone formation from the edges of the vertebra and trabecular bone loss are occurring at the same time at sites that are in close proximity suggests that the mechanisms underlying new bone formation are at least

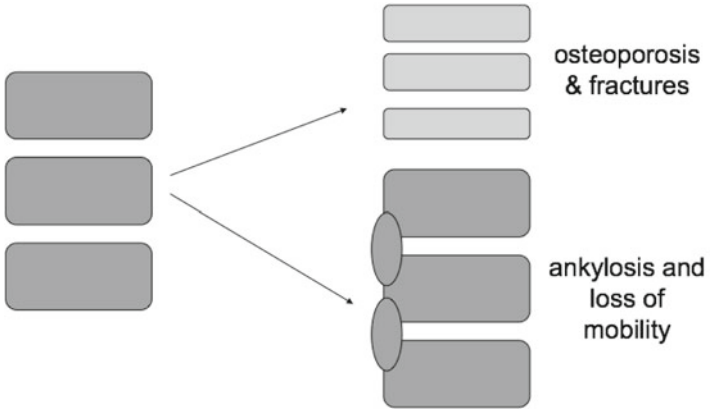

Fig. 3 The bone paradox in ankylosing spondylitis. Inflammation causes loss of trabecular bone leading to osteoporosis and enhanced fracture risk. In the same vertebrae, new bone formation may take place at the edges leading to ankylosis

partly independent from inflammation (Fig. 3). This view is further corroborated by ultrasound studies in AS patients, which show that sites of inflammation and bone erosion are distinct from the sites in which new bone formation occurs [26].

5 Molecular Aspects of Ankylosis

As mentioned above, molecular studies on human bone samples are not easily performed and therefore most experimental evidence has been obtained in animal models. This first lead to the observation that ankylosis in different models mainly occurs through a process of endochondral bone formation [27] that is well known from bone development [28]. Here, progenitor cells at the enthesis or periosteum appear to proliferate, condensate and start differentiating into chondrocyte-like cells. Subsequently the core of these cells further differentiates into hypertrophic chondrocytes. These cells produce not only collagen type X but also MMPs and Vascular Endothelial Growth Factor which leads to invasion by vessels, osteoclastic breakdown of the matrix and growth of osteoblasts and bone. Much alike the developmental cascades an outer core of direct bone formation appears present and the whole process is driven by a number of feedback mechanisms stimulating growth of the osteo- or enthesophyte.

Based on this striking resemblance between pathological bone formation leading to ankylosis and developmental cascades, different signaling pathways could play a role in ankylosis. We have extensively studied the role of bone morphogenetic proteins (BMPs) [29, 30]. BMPs were originally identified for their in vivo bone inductive properties but, as members of the transforming growth factor superfamily, have distinct effects on a variety of cell types. Some BMPs, including BMP2, play an important role in early chondrogenesis. We therefore targeted BMP signaling in a

specific mouse model. DBA/1 mice, an inbred strain that is considered immunologically normal, spontaneously develop arthritis in the hind paws upon grouped caging of aging males from different litters [27]. This unusual arthritis is characterized by entheseal cell proliferation, local endochondral bone formation, and progressive joint ankylosis. Different BMPs are expressed in these processes and overexpression of a broad BMP extracellular antagonist noggin inhibits both onset and progression of disease. BMP target cells were identified in the early processes in which progenitor cells are progressing towards chondrogenic differentiation [29].

The Wnt signaling pathway is another key player during skeletal development. Wnts are strongly associated with osteoblast differentiation but also have different effects depending on the specific family member, on early chondrogenic differentiation during endochondral bone formation [14]. In a series of experiments, Diarra et al. demonstrated that Wnt signaling may determine the phenotypical outcome of arthritis in mouse models [31]. Human TNF transgenic mice typically develop an erosive polyarthritis that shares many similarities with RA. However, when these mice are treated with an antibody directed against Dickkopf-1 (DKK1), a soluble Wnt co-receptor antagonist, bone destruction is inhibited and new bone formation by osteophytes becomes apparent. The inflammatory reaction however remains unchanged. Such observations are not only made in the peripheral joints but also in the sacroiliac joints [32]. Inhibition of DKK1 results in upregulation of osteoprotegerin, inhibiting osteoclast formation. At the same time, bone formation appears directly stimulated. Additional data suggest that functional levels of DKK1 in AS patients are low, although the absolute levels may be increased [33].

6 An Alternative View on the Relationship Between Inflammation and Ankylosis

The cohort observations in patients with AS were further corroborated by data obtained in the DBA/1 model [34]. Treatment with TNF antagonist etanercept had no effect on arthritis or ankylosis suggesting that inflammation and joint remodeling may be largely molecularly uncoupled events. As an alternative or complementary approach we have put forward the entheseal stress hypothesis (Fig. 4) in which we suggests that both inflammation and remodeling have a common trigger but then evolve separately thereby not excluding the potential crosstalk between the pathways [14]. As AS and related SpAs have been strongly associated with the enthesis, we put forward that biomechanical forces and local micro damage to the tissue may play a critical role in the onset of the diseases. In most normal individuals, local homeostatic and repair mechanisms, including minor acute inflammation will be sufficient to restore the tissue but under specific circumstances, for instance in genetically predisposed individuals, inflammation may become a chronic process and new tissue formation may become a pathological rather than a repair process. In the former process cytokines like TNF are essential and their targeting represents an effective therapeutic option. In the latter, BMPs and Wnts may be critical mediators and could be considered as new therapeutic targets.

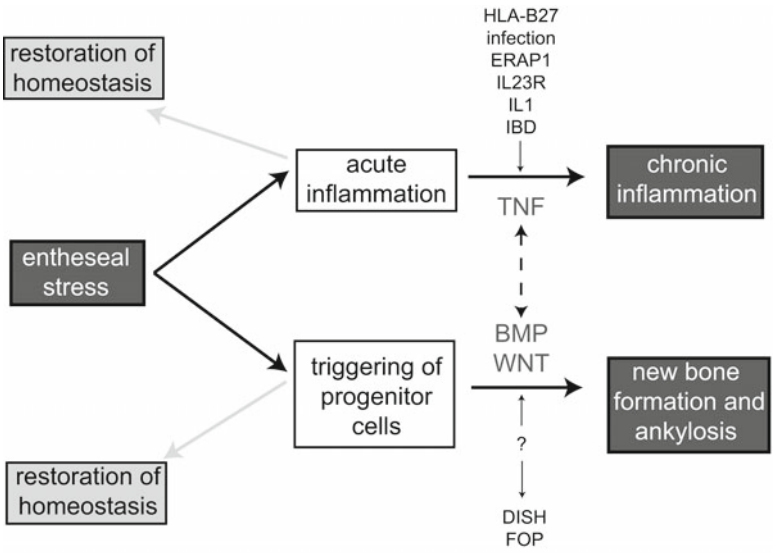

Fig. 4 The entheseal stress hypothesis. The primary event is considered as "entheseal stress." Biomechanical factors and microdamage are likely to play a part in this. Entheseal stress leads to triggering of an acute inflammatory reaction and of progenitor cells. In most instances, the acute events are unnoticed and homeostasis is restored. Under specific circumstances, the acute events can turn into a chronic situation in which both inflammation or ankylosis appears at the forefront. Different pathways regulate chronic inflammation and new tissue formation but these pathways are likely to influence each other. Genetic factors are likely to steer chronic inflammation and new tissue formation. For the latter aspects, clues may be found in other bone forming diseases (*IBD* inflammatory bowel disease, *IL23R* interleukin 23 receptor, *ERAP1* endoplasmic reticulum aminopeptidase 1). This figure is reproduced from Lories et al., Arthritis Research and Therapy 2009, 11(2):221 [14] with permission from the Publisher

The specific anatomic site in which both processes develop may be different. Inflammation develops in the synovium and the bone marrow (osteitis) underlying entheseal sites. The enthesis itself is largely resistant to cell invasion. We have therefore proposed the existence of a functional synovio-entheseal complex to understand the development of inflammation in AS, PsA, and other SpAs [35]. In contrast new bone formation develops from these mechanoprivileged sites [27].

In this context, mesenchymal or stromal cells in the enthesis, bone marrow, and synovium may have a key role in the onset and perpetuation of the inflammation. In a series of elegant experiments in the TNFdARE mice, a mouse model of arthritis and colitis caused by the disruption of a regulator element in the mouse TNFa gene resulting in enhanced endogenous expression, showed that the presence of a TNF receptor on stromal cells is sufficient for the model to develop even when the inflammatory cells cannot respond to the key cytokine [36].

The "chicken and egg" question whether stromal cells rather than inflammatory cells and by extension growth factors or proinflammatory cytokines, provide the

first signals for disease onset in AS remains open. Although the cohort data and animal studies with anti-TNF suggest some degree of uncoupling between inflammation and tissue remodeling, recent evidence obtained in studies on a rare genetic disorder shed additional light on this issue. Fibrodysplasia ossificans progressiva (FOP) is a rare disorder characterized by extensive new bone formation in muscles upon (mild) injury [37]. The disease is often lethal at a young age as an exoskeleton develops with aging. FOP has been associated with activating mutations in the Activin A type I receptor (ACVR1) gene, which is also a BMP receptor [38]. Yu et al. recently engineered a mouse model with overexpression of a constitutively active ACVR1 gene in the muscle [39]. However, to allow a controlled expression of the transgene, an additional removal of a genomic stop-cassette is necessary. Removal of the stop-cassette using an adenovirus overexpressing a cre recombinase enzyme leads to new bone formation in the adenovirus-injected muscle. However, chemical induction of the transgene tamoxifen treatment in contrast is not sufficient to trigger this cascade but requires a nonspecific injection of adenovirus in the target muscle. These experiments suggest that a full cascade only develops after initial microdamage or inflammation even in the presence of a constitutively active system leading to endochondral bone formation. These data are in line with the entheseal stress hypothesis and support its further investigation.

7 Conclusions

Current evidence from patient cohorts and from animal models suggests that inflammation and new bone formation are unique features of some forms of arthritis that also contribute to disability and thus represent a therapeutic challenge. Current strategies based on control of inflammation by targeting cytokines such as TNFa have no specific effect on these disease features despite their overwhelming effect on signs and symptoms. This suggest that inflammation and new bone formation in AS and related SpAs are linked but largely molecularly uncoupled processes.

Further research in AS and related disorders should consider osteoimmunology concepts in the context of microdamage and biomechanical factors contributing to acute and chronic inflammation and also to tissue remodeling. BMP and Wnt signaling pathways have been identified as targets but their modulation may represent specific pharmacological and safety challenges. In addition, further evidence may come from genetic disorders such as FOP and also from more common diseases such as diffuse idiopathic skeletal hyperostosis in which new tissue formation is seen without a clear association with inflammation [40].

Acknowledgments Kirsten Braem is the recipient of a PhD fellowship from the IWT (Agency for innovation by science and technology). Rik Lories was the recipient of a postdoctoral fellowship from the Flanders Research Foundation (FWO-Vlaanderen) and is currently a KU Leuven Research Professor. Their studies were supported by a GOA grant from KU Leuven, FWO grants and an grant from Abbott Immunology to the SPIRAL consortium.

References

1. Hunter DJ, Felson DT (2006) Osteoarthritis. BMJ 332:639–642
2. McInnes IB, Schett G (2007) Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol 7:429–442
3. Braun J, Sieper J (2007) Ankylosing spondylitis. Lancet 369:1379–1390
4. Luyten FP, Lories RJU, Verschueren P, de Vlam K, Westhovens R (2006) Contemporary concepts of inflammation, damage and repair in rheumatic diseases. Best Pract Res Clin Rheumatol 20:829–848
5. Klareskog L, Catrina AI, Paget S (2009) Rheumatoid arthritis. Lancet 373:659–672
6. Khan MA (2002) Update on spondyloarthropathies. Ann Intern Med 136:896–907
7. Thomas GP, Brown MA (2010) Genetics and genomics of ankylosing spondylitis. Immunol Rev 233:162–180
8. Lories RJ, Baeten DLP (2009) Differences in pathophysiology between rheumatoid arthritis and ankylosing spondylitis. Clin Exp Rheumatol 27:S10–S14
9. McInnes IB, O'Dell JR (2010) State-of-the-art: rheumatoid arthritis. Ann Rheum Dis 69:1898–1906
10. Schett G, Teitelbaum SL (2009) Osteoclasts and arthritis. J Bone Miner Res 24:1142–1146
11. Iqbal J, Sun L, Zaidi M (2010) Denosumab for the treatment of osteoporosis. Curr Osteoporos Rep 8:163–167
12. Lewiecki EM (2009) Denosumab for joints and bones. Curr Rheumatol Rep 11:196–201
13. Neumann E, Lefèvre S, Zimmermann B, Gay S, Müller-Ladner U (2010) Rheumatoid arthritis progression mediated by activated synovial fibroblasts. Trends Mol Med 16:458–468
14. Lories RJU, Luyten FP, de Vlam K (2009) Progress in spondylarthritis. Mechanisms of new bone formation in spondyloarthritis. Arthritis Res Ther 11:221
15. Maksymowych WP (2009) Progress in spondylarthritis. Spondyloarthritis: lessons from imaging. Arthritis Res Ther 11:222
16. van der Heijde D, Klareskog L, Landewé R, Bruyn GAW, Cantagrel A, Durez P, Herrero-Beaumont G, Molad Y, Codreanu C, Valentini G, Zahora R, Pedersen R, MacPeek D, Wajdula J, Fatenejad S (2007) Disease remission and sustained halting of radiographic progression with combination etanercept and methotrexate in patients with rheumatoid arthritis. Arthritis Rheum 56:3928–3939
17. van der Heijde D, Kavanaugh A, Gladman DD, Antoni C, Krueger GG, Guzzo C, Zhou B, Dooley LT, de Vlam K, Geusens P, Birbara C, Halter D, Beutler A (2007) Infliximab inhibits progression of radiographic damage in patients with active psoriatic arthritis through one year of treatment: results from the induction and maintenance psoriatic arthritis clinical trial 2. Arthritis Rheum 56:2698–2707
18. van der Heijde D, Landewé R, Einstein S, Ory P, Vosse D, Ni L, Lin S, Tsuji W, Davis JC Jr (2008) Radiographic progression of ankylosing spondylitis after up to two years of treatment with etanercept. Arthritis Rheum 58:1324–1331
19. van der Heijde D, Landewé R, Baraliakos X, Houben H, van Tubergen A, Williamson P, Xu W, Baker D, Goldstein N, Braun J, Ankylosing Spondylitis Study for the Evaluation of Recombinant Infliximab Therapy Study Group (2008) Radiographic findings following two years of infliximab therapy in patients with ankylosing spondylitis. Arthritis Rheum 58:3063–3070
20. van der Heijde D, Salonen D, Weissman BN, Landewé R, Maksymowych WP, Kupper H, Ballal S, Gibson E, Wong R, Canadian (M03-606) Study Group, ATLAS Study Group (2009) Assessment of radiographic progression in the spines of patients with ankylosing spondylitis treated with adalimumab for up to 2 years. Arthritis Res Ther 11:R127
21. Lories RJU, de Vlam K, Luyten FP (2010) Are current available therapies disease-modifying in spondyloarthritis? Best Pract Res Clin Rheumatol 24:625–635
22. Machado P, Landewé R, Braun J, Hermann KA, Baker D, van der Heijde D (2010) Both structural damage and inflammation of the spine contribute to impairment of spinal mobility in patients with ankylosing spondylitis. Ann Rheum Dis 69:1465–1470

23. Sieper J, Appel H, Braun J, Rudwaleit M (2008) Critical appraisal of assessment of structural damage in ankylosing spondylitis: implications for treatment outcomes. Arthritis Rheum 58:649–656
24. Lories RJU, Derese I, Luyten FP (2008) Inhibition of osteoclasts does not prevent joint ankylosis in a mouse model of spondyloarthritis. Rheumatology (Oxford) 47:605–608
25. Schett G, Stolina M, Dwyer D, Zack D, Uderhardt S, Krönke G, Kostenuik P, Feige U (2009) Tumor necrosis factor alpha and RANKL blockade cannot halt bony spur formation in experimental inflammatory arthritis. Arthritis Rheum 60:2644–2654
26. McGonagle D, Wakefield RJ, Tan AL, D'Agostino MA, Toumi H, Hayashi K, Emery P, Benjamin M (2008) Distinct topography of erosion and new bone formation in achilles tendon enthesitis: implications for understanding the link between inflammation and bone formation in spondylarthritis. Arthritis Rheum 58:2694–2699
27. Lories RJU, Matthys P, de Vlam K, Derese I, Luyten FP (2004) Ankylosing enthesitis, dactylitis, and onychoperiostitis in male DBA/1 mice: a model of psoriatic arthritis. Ann Rheum Dis 63:595–598
28. Kronenberg HM (2003) Developmental regulation of the growth plate. Nature 423:332–336
29. Lories RJU, Derese I, Luyten FP (2005) Modulation of bone morphogenetic protein signaling inhibits the onset and progression of ankylosing enthesitis. J Clin Invest 115:1571–1579
30. Lories RJU, Daans M, Derese I, Matthys P, Kasran A, Tylzanowski P, Ceuppens JL, Luyten FP (2006) Noggin haploinsufficiency differentially affects tissue responses in destructive and remodeling arthritis. Arthritis Rheum 54:1736–1746
31. Diarra D, Stolina M, Polzer K, Zwerina J, Ominsky MS, Dwyer D, Korb A, Smolen J, Hoffmann M, Scheinecker C, van der Heide D, Landewe R, Lacey D, Richards WG, Schett G (2007) Dickkopf-1 is a master regulator of joint remodeling. Nat Med 13:156–163
32. Uderhardt S, Diarra D, Katzenbeisser J, David J, Zwerina J, Richards W, Kronke G, Schett G (2010) Blockade of Dickkopf (DKK)-1 induces fusion of sacroiliac joints. Ann Rheum Dis 69:592–597
33. Daoussis D, Liossis SC, Solomou EE, Tsanaktsi A, Bounia K, Karampetsou M, Yiannopoulos G, Andonopoulos AP (2010) Evidence that Dkk-1 is dysfunctional in ankylosing spondylitis. Arthritis Rheum 62:150–158
34. Lories RJU, Derese I, de Bari C, Luyten FP (2007) Evidence for uncoupling of inflammation and joint remodeling in a mouse model of spondylarthritis. Arthritis Rheum 56:489–497
35. McGonagle D, Lories RJU, Tan AL, Benjamin M (2007) The concept of a "synovio-entheseal complex" and its implications for understanding joint inflammation and damage in psoriatic arthritis and beyond. Arthritis Rheum 56:2482–2491
36. Armaka M, Apostolaki M, Jacques P, Kontoyiannis DL, Elewaut D, Kollias G (2008) Mesenchymal cell targeting by TNF as a common pathogenic principle in chronic inflammatory joint and intestinal diseases. J Exp Med 205:331–337
37. Shore EM, Kaplan FS (2008) Insights from a rare genetic disorder of extra-skeletal bone formation, fibrodysplasia ossificans progressiva (FOP). Bone 43:427–433
38. Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Cho T, Choi IH, Connor JM, Delai P, Glaser DL, LeMerrer M, Morhart R, Rogers JG, Smith R, Triffitt JT, Urtizberea JA, Zasloff M, Brown MA, Kaplan FS (2006) A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat Genet 38:525–527
39. Yu PB, Deng DY, Lai CS, Hong CC, Cuny GD, Bouxsein ML, Hong DW, McManus PM, Katagiri T, Sachidanandan C, Kamiya N, Fukuda T, Mishina Y, Peterson RT, Bloch KD (2008) BMP type I receptor inhibition reduces heterotopic [corrected] ossification. Nat Med 14:1363–1369
40. Olivieri I, D'Angelo S, Palazzi C, Padula A, Mader R, Khan MA (2009) Diffuse idiopathic skeletal hyperostosis: differentiation from ankylosing spondylitis. Curr Rheumatol Rep 11: 321–328

Regulation of RANKL-Induced Osteoclastogenesis by TGF-β

Tetsuro Yasui, Hisataka Yasuda, and Sakae Tanaka

1 Introduction

Osteoclasts are multinucleated giant cells primarily responsible for bone resorption. Recent studies have revealed that receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) play central roles in osteoclast differentiation [1–3]. The binding of RANKL to its receptor RANK recruits TRAF6 to activate downstream signaling pathways, such as NF-κB, p38 mitogen-activated protein kinase (p38MAPK), and c-Jun N-terminal kinase (JNK), resulting in the differentiation, activation, and survival of osteoclasts[1, 4]. It was previously demonstrated that several cytokines either inhibit or promote RANKL and M-CSF-induced osteoclastogenesis. TGF-β is a cytokine with ubiquitous proliferation and differentiation activity in many types of cells, and is abundantly restored in bone [5, 6]. The binding of TGF-β to the type II receptor recruits and phosphorylates type I receptors, which in turn activate downstream signaling, including both Smad and non-Smad pathways [6, 7]. Although the role of TGF-β in osteoclast differentiation has been controversial, recent studies have revealed that TGF-β directly acts on M-CSF-dependent bone marrow macrophages (BMMs) to promote osteoclastogenesis [8–10], whereas it inhibits osteoclastogenesis by stimulating osteoprotegerin production in osteoblasts [11].

We here report that TGF-β signaling is essential for RANKL and M-CSF-induced osteoclastogenesis both in vitro and in vivo. In addition, TGF-β stimulates Cx3cr1 expression in BMMs, which may play important roles in osteoclastogenesis.

T. Yasui • S. Tanaka (✉)
Department of Orthopaedic Surgery, Faculty of Medicine, The University of Tokyo,
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
e-mail: tyasu-tky@umin.ac.jp; tanakas-ort@h.u-tokyo.ac.jp

H. Yasuda
Nagahama Institute for Biochemical Science, Oriental Yeast Co.,
50 Kano-cho, Nagahama, Shiga 526-0804, Japan
e-mail: hyasuda@oyc.co.jp

Y. Choi (ed.), *Osteoimmunology: Interactions of the Immune and Skeletal Systems,*
DOI 10.1007/978-1-4614-5366-6_11,

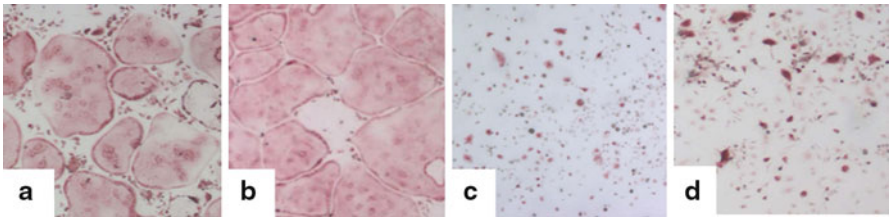

Fig. 1 Up- or downregulation of TGF-β signal enhanced or inhibited RANKL-induced osteoclastogenesis, respectively. BMMs were treated with 10 ng/ml M-CSF and 100 ng/ml RANKL for 3 days. TGF-β signal was upregulated by pretreatment with 2 ng/ml TGF-β, or downregulated either by treatment with SB431542 (3 μM) or by retroviral overexpression of dominant negative form of type II receptor of TGF-β (RxTβRIIDN). Cells were stained with TRAP. (**a**) Control, (**b**) +TGF-β (2 ng/ml), (**c**) +SB431542 (3 μM), (**d**) +RxTβRIIDN

2 TGF-β Is Indispensible for In Vitro Osteoclastogenesis

We first analyzed the effect of TGF-β on RANKL and M-CSF-induced osteoclastogenesis in vitro. When BMMs were treated with 10 ng/ml M-CSF and 100 ng/ml RANKL for 3 days, many tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts were formed. When the cultures were pretreated with TGF-β (2 ng/ml) before RANKL and M-CSF, osteoclastogenesis was increased and a number of large osteoclasts were formed (Fig. 1b). Conversely, when TGF-β signaling was blocked either by administration of a specific inhibitor SB431542 (3 μM) or by retroviral overexpression of dominant negative form of type II receptor of TGF-β (TβRIIDN), RANKL and M-CSF-induced osteoclastogenesis was almost completely abolished (Fig. 1c, d). These results clearly demonstrated that TGF-β is not only a stimulatory cytokine for osteoclastogenesis, but also it is indispensible for RANKL and M-CSF-induced osteoclast differentiation.

3 TGF-β Is Indispensable for RANKL-Induced Bone Resorption In Vivo

We then assessed the effect of TGF-β inhibition on RANKL-induced osteoclastogenesis in vivo. Five weeks old ddY mice were subcutaneously injected with 40 nmol GST-RANKL (kindly provided by Oriental Yeast Co.) into calvaria once a day for 3 days [12]. They were sacrificed 3 days after the last injection, and the calvaria were collected for histological analysis. As shown in Fig. 2, RANKL-induced osteoclast formation and bone resorption was strongly suppressed when SB431542 was concurrently administered with GST-RANKL. These data suggest that TGF-β is indispensable for RANKL-induced osteoclastogenesis both in vitro and in vivo.

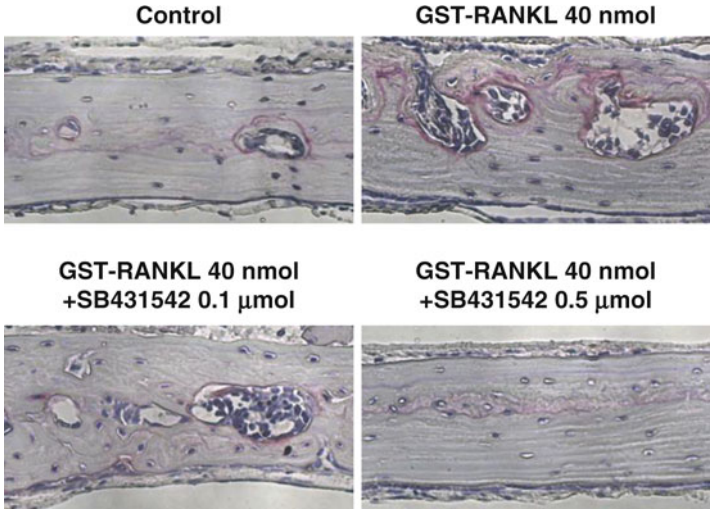

Fig. 2 Five weeks old ddY mice received subcutaneous injection into calvaria of 40 nmol GST-RANKL or PBS (*control*) together with the indicated amount of SB431542 once a day for 3 days. Three days after the last injection, calvaria bones were collected and fixed with 4% paraformaldehyde. After decalcification and embedment in paraffin, coronal sections were cut from the specimen. TRAP-stained sections near the sagittal suture were shown. Bar = 200 μm

4 Cx3cr1 Is a Downstream Molecule of TGF-β in BMMs and Plays a Critical Role in Osteoclastogenesis

We then underwent cDNA microarray analysis of BMMs in which TGF-β signaling was up- or downregulated by TGF-β stimulation or by retroviral overexpression of dominant negative form of TGF-β receptor type II (RxTβRIIDN) to identify genes regulated by TGF-β in BMMs. We picked up genes whose expression was upregulated more than twofold by TGF-β stimulation and downregulated to less than 0.5-fold by TβRIIDN introduction. Through confirmation by real-time RT-PCR, we extracted four candidate genes (Cx3cr1, Egr1, Enc1, and CD24a) as targets of TGF-β (Fig. 3). Of these four genes, overexpression of Cx3cr1 partially rescued the inhibitory effect of RxTβRIIDN on osteoclastogenesis (Fig. 4). Conversely, its silencing by short hairpin RNA (shCx3cr1) reduced the stimulatory effect of TGF-β on osteoclastogenesis (Fig. 5). Cx3cr1 is a receptor of Cx3cl1/fractalkine, a potent chemotactic factor reported to be involved in the synovial inflammation of rheumatoid arthritis. Recent study has suggested that Cx3cl/fractalkine induced migration of bone marrow cells containing osteoclast precursors, and anti-Cx3cl1 antibody significantly suppressed mature osteoclast differentiation [13], suggesting a critical role of the CX3CL1–CX3CR1 axis in osteoclast development.

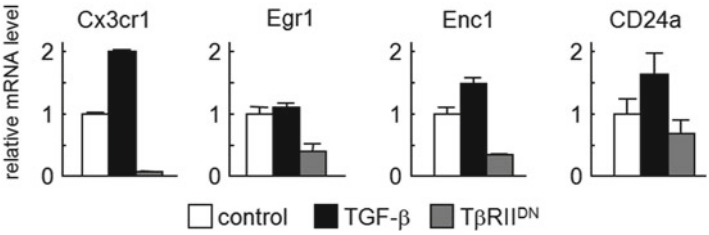

Fig. 3 In a comprehensive analysis using DNA array, several genes were picked up whose expression was upregulated more than twofold by TGF-β stimulation and downregulated less than 0.5-fold by retroviral TβRIIDN introduction. Real-time RT-PCR demonstrated that the expression of Cx3Cr1, Egr1, Enc1, and CD24a was increased in response to TGF-β (2 ng/ml) treatment, and suppressed by TβRIIDN expression

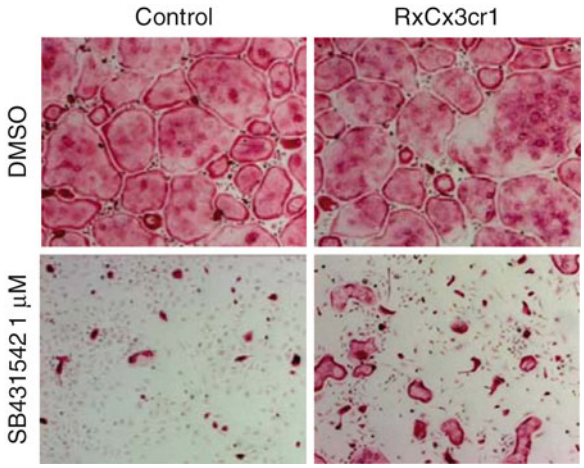

Fig. 4 Retroviral overexpression of Cx3cr1 partially recovered the inhibitory effect of SB431542 on RANKL and M-CSF-induced osteoclastogenesis. After Cx3cr1 was retrovirally introduced, BMMs were treated with 10 ng/ml M-CSF, 100 ng/ml RANKL in the presence or absence of 1 μM SB431542 for 3 days. Cells were stained with TRAP

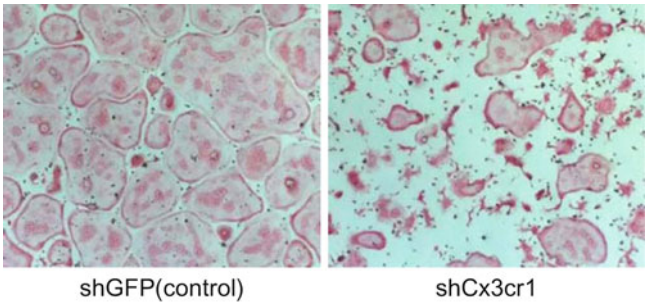

Fig. 5 Gene silencing of Cx3cr1 by retrovirus-mediated overexpression of short hairpin RNA (shCx3cr1) suppressed the stimulatory effect of TGF-β on RANKL and M-CSF-induced osteoclastogenesis. After shRNA of Cx3cr1 was retrovirally introduced, BMMs were treated with 10 ng/ml M-CSF, 100 ng/ml RANKL, and 2 ng/ml TGF-β for 3 days. Cells were stained with TRAP

5 Conclusion

In this study, we demonstrated that TGF-β signal is indispensable for RANKL and M-CSF-induced osteoclastogenesis both in vitro and in vivo, and Cx3cr1 may act as an important downstream effector of TGF-β in BMMs. Further studies are required to elucidate the precise mechanism of action of Cx3cr1 in physiological and/or pathological bone resorption.

Acknowledgments This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to S.T.

References

1. Tanaka S, Nakamura K, Takahasi N, Suda T (2005) Role of RANKL in physiological and pathological bone resorption and therapeutics targeting the RANKL-RANK signaling system. Immunol Rev 208:30–49
2. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T (1998) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 95:3597–3602
3. Tanaka S, Takahashi N, Udagawa N, Tamura T, Akatsu T, Stanley ER, Kurokawa T, Suda T (1993) Macrophage colony-stimulating factor is indispensable for both proliferation and differentiation of osteoclast progenitors. J Clin Invest 91:257–263
4. Takayanagi H (2009) Osteoimmunology and the effects of the immune system on bone. Nat Rev Rheumatol 5:667–676
5. Centrella M, Horowitz MC, Wozney JM, McCarthy TL (1994) Transforming growth factor-beta gene family members and bone. Endocr Rev 15:27–39
6. Derynck R, Zhang YE (2003) Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425:577–584
7. Massague J, Wotton D (2000) Transcriptional control by the TGF-beta/Smad signaling system. EMBO J 19:1745–1754
8. Fox SW, Fuller K, Bayley KE, Lean JM, Chambers TJ (2000) TGF-beta 1 and IFN-gamma direct macrophage activation by TNF-alpha to osteoclastic or cytocidal phenotype. J Immunol 165:4957–4963
9. Kaneda T, Nojima T, Nakagawa M, Ogasawara A, Kaneko H, Sato T, Mano H, Kumegawa M, Hakeda Y (2000) Endogenous production of TGF-beta is essential for osteoclastogenesis induced by a combination of receptor activator of NF-kappa B ligand and macrophage-colony-stimulating factor. J Immunol 165:4254–4263
10. Kim N, Kadono Y, Takami M, Lee J, Lee SH, Okada F, Kim JH, Kobayashi T, Odgren PR, Nakano H, Yeh WC, Lee SK, Lorenzo JA, Choi Y (2005) Osteoclast differentiation independent of the TRANCE-RANK-TRAF6 axis. J Exp Med 202:589–595
11. Takai H, Kanematsu M, Yano K, Tsuda E, Higashio K, Ikeda K, Watanabe K, Yamada Y (1998) Transforming growth factor-beta stimulates the production of osteoprotegerin/osteoclastogenesis inhibitory factor by bone marrow stromal cells. J Biol Chem 273:27091–27096
12. Tomimori Y, Mori K, Koide M, Nakamichi Y, Ninomiya T, Udagawa N, Yasuda H (2009) Evaluation of pharmaceuticals with a novel 50-hour animal model of bone loss. J Bone Miner Res 24:1194–1205
13. Koizumi K, Saitoh Y, Minami T, Takeno N, Tsuneyama K, Miyahara T, Nakayama T, Sakurai H, Takano Y, Nishimura M, Imai T, Yoshie O, Saiki I (2009) Role of CX3CL1/fractalkine in osteoclast differentiation and bone resorption. J Immunol 183:7825–7831

Effects of the Interleukin-1 Cytokine Family on Bone

Georg Schett

1 Introduction

Cytokines are major regulators of bone homeostasis and influence the function of both osteoblasts and osteoclasts. For instance, receptor activator of NF-kB ligand (RANKL) is a member of the tumor necrosis factor (TNF) family and an essential mediator of osteoclastogenesis. Apart from RANKL, proinflammatory cytokines modulate osteoclast differentiation, amongst them TNF-α is a potent stimulator of osteoclastogenesis. Other proinflammatory cytokines like IL-17 and IL-11 are also stimulators of bone resorption by inducing osteoclast differentiation, whereas others such as interferon-(IFN)-γ and IL-12 suppress osteoclastogenesis and balance enhanced bone resorption. In this chapter, we focus on the IL-1 of cytokine family and summarize their role on bone homeostasis. Members of the IL-1 cytokine family are involved in multiple cellular functions including the innate and adaptive immune system. They are key mediators of inflammation and govern the complex processes of cell trafficking, cytokine and matrix enzyme release, fever responses, and metabolic changes during inflammatory disease.

2 Interleukin-1

The pro-inflammatory cytokine IL-1 is induced by TNF-α and was first described as factor acting on T and B cells driving immune responses [1]. Since then, it became evident that IL-1 is one of the key players during acute inflammation and produced by multiple cell lineages including macrophages, lymphocytes as well as mesenchymal cells.

G. Schett (✉)
Department of Internal Medicine 3, University of Erlangen-Nuremberg,
Krankenhausstrasse 12, 91054 Erlangen, Germany
e-mail: georg.schett@uk-erlangen.de

Y. Choi (ed.), *Osteoimmunology: Interactions of the Immune and Skeletal Systems*,
DOI 10.1007/978-1-4614-5366-6_12,

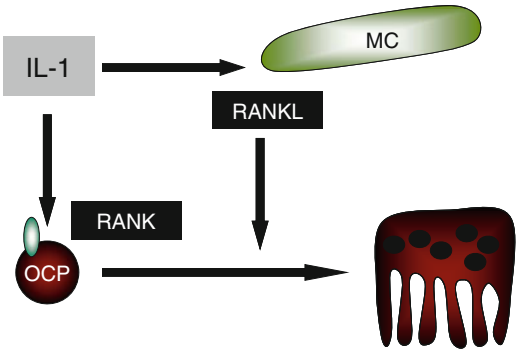

Fig. 1 Interleukin-1 induces osteoclastogenesis. *MC* mesenchymal cell, *OCP* osteoclast precursor, *RANKL* receptor activator of nuclear factor kappa B ligand, *RANK* receptor activator of nuclear factor kappa B

There are two polypeptides, IL-1α and IL-1β. Both polypeptides bind to the same cell surface receptors and exert similar biological functions. IL-1α is predominantly expressed in the cytoplasm and already active as a pro-form (pro-IL-1α), whereas IL-1β is functionally inactive as a pro-form and only exerts its action after cleavage by the interkeukin-1 converting enzyme. IL-1 has multiple biological functions: it contributes the activation of several leukocyte lineages, endothelial cells, and synovial fibroblasts. In addition, IL-1 is a highly potent inducer of matrix enzyme production by chondrocytes. In addition, IL-1 is essentially involved in the fever response and also plays an important role in glucose metabolism by promoting the death of beta cells in the pancreas and impairing glucose tolerance and promoting diabetes.

IL-1 was shown to exert potent effects on bone tissue by enhancing bone resorption activity in vitro and in vivo [2]. IL-1 has been shown to prolong the lifespan of osteoclasts [3] and thereby contributing to osteoclast survival and formation. Together with TNF-α, IL-1 appears to play a crucial role in recruiting osteoclasts together with inflammatory cells to inflammation sites and thereby induces local activation of joint destruction. Elegant studies by Wei et al. have shown that TNF induces expression of IL-1 and IL-1R in mesenchymal cells, which both support their osteoclastogenic effects on mononuclear cells [4]. Thus, IL-1 induces RANKL expression in mesenchymal cells and additionally acts directly on osteoclasts by enhancing expression of RANK [4–6] (Fig. 1). Blockade of IL-1 by its soluble receptor antagonist (IL-1ra) or by using mice deficient for the type I IL-1R receptor strongly reduced the potential of TNF to induce osteoclast formation, suggesting that IL-1 represents a major link between TNF and osteoclast formation in vitro and in vivo.

The central role of IL-1 in inflammatory osteoclastogenesis is also supported by the destructive nature of arthritis models, which depend on IL-1, such as collagen-induced arthritis or serum transfer-induced arthritis [7]. Thus, for instance, deficiency of the type I IL-1R does not only achieve excellent protection from inflammatory signs of arthritis in the serum transfer model but also protection from local bone destruction. Even in arthritis, where inflammatory signs of disease are fully TNF-dependent (TNFtg mice) and which do not require IL-1, this cytokine is pivotal for local bone erosion and systemic bone loss [5, 6]. These

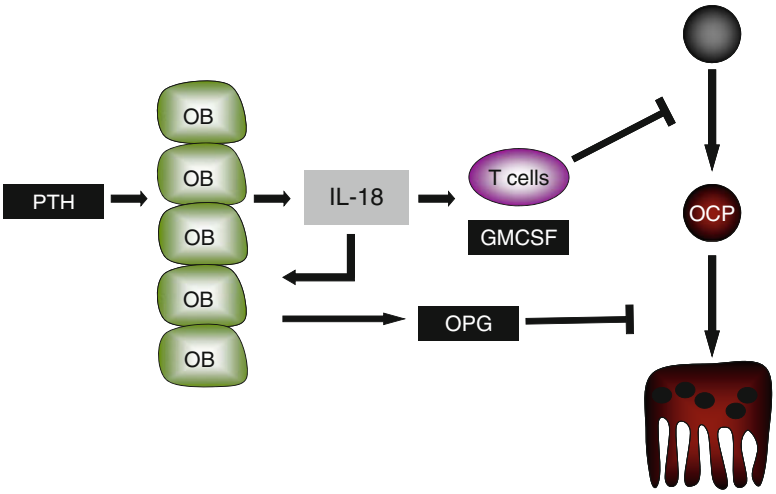

Fig. 2 Interleukin-18 blocks osteoclastogenesis. *OB* osteoblast, *OCP* osteoclast precursor, *PTH* parathyroid hormone, *OPG* osteoprotegerin, *GMCSF* granulocyte/macrophage colony stimulating factor

previous data as well as the observation that over-expression of IL-1 causes osteopenia [8] suggested that IL-1 plays a central role in TNF-mediated systemic inflammatory bone loss as well.

3 Interleukin-18

IL-18 has been described as IFNg inducing factor in the mid-1990s [9]. IL-18 thereby acts in synergy with Il-12 to stimulate the release of IL-18 from TH1 cells. Moreover, IL-18 is involved in the regulation of energy intake and insulin sensitivity. IL-18, like IL-1 is widely expressed in the hematopoietic lineage and also found in mesenchymal cells. It is secreted from the cells and acts in an autocrine and paracrine fashion by engaging its receptor, which shares structural homologies to the IL-1 receptor. The b-subunit thereby forms high affinity complexes with IL-18/IL-18R alpha chain dimmers. IL-18 is expressed in mesenchymal cells such as osteoblasts and its expression is induced by parathyroid hormone [10] (Fig. 2). In fact, the anabolic effect of parathyroid hormone is at least in part mediated by the induction of IL-18 in the osteoblast lineage. Furthermore, binding of IL-18 to osteoblasts stimulates the expression of osteoprotegerin [11]. It is known as a potent suppressor of osteoclastogenesis [12]. Anti-osteoclastogenic activity of IL-18 is particularly strong during the early phase of the differentiation process. The fact that IL-18 induces several potent cytokine inhibitors of

osteoclastogenesis such as IFNg and GM-CSF in T cells and explains its strong anti-osteoclastogenic activity, which is reflected by impaired bone resorption when IL-18 is administered. Thereby the induction of GMCSF appears to be essential in mediating the effects of IL-18 on the osteoclast and T cells have been identified as the primary source of GMCSF production elicited by IL-18 [13]. Thus IL-18 exerts profoundly different effects to the skeleton as compared to IL-1 by fostering osteoblast-mediated bone formation and inhibiting osteoclast-mediated bone erosion.

4 Interleukin-33

Interleukin (IL)-33 is a recently described member of the IL-1 family [14, 15], constitutively expressed in various tissues, particularly in endothelial cells and epithelial cells exposed to the environment, such as skin, gastrointestinal tract, and the lungs. Similar to IL-1β, IL-33 may act as both a cytokine and a nuclear factor. As a cytokine, IL-33 signals through its interaction with a heterodimeric receptor consisting of membrane-bound ST2L (member of the IL-1 receptor family) and IL-1R accessory protein (IL-1RAcP), leading to NF-kB and MAPkinase activation. ST2L is expressed on monocytes, macrophages, neutrophils, T cells, particularly TH2 (but not TH1 cells), and mast cells. IL-33 is involved in the polarization of IL-5-producing T cells, migration of TH2 cells, activation of basophils, mast cells, eosinophils, and alternatively activated macrophages, contributing to allergic response and asthma. IL-33 also promotes chemo-attraction of neutrophils to inflammatory sites and attenuates poly-microbial sepsis and mediates mast cell-dependent arthritis.

IL-33 is expressed in the bone tissue and acts as a bone protective cytokine by effectively blocking osteoclastogenesis and local bone erosions [16]. Exogenously administered IL-33 also blocks TNF-α-mediated local and systemic bone loss in vivo. Conversely, deletion of the IL-33 receptor ST2 in bone marrow cells enhances bone loss [17]. The mechanism by which IL-33 exerts its inhibitory effect on osteoclast differentiation is not likely by the regulation of OPG or RANKL synthesis. Instead IL-33 directly acts on human and mouse bone marrow $CD11b^+$ cells by inhibiting their development toward mature osteoclasts. IL-33 appears to shift the osteoclast precursor differentiation towards alternatively activated macrophages. IL-33-activated alternatively activated macrophages produce elevated levels of IL-4 and GM-CSF, which are known inhibitors of osteoclast differentiation [17] (Fig. 3). Interestingly, IL-33 is not able to affect osteoclast development when added to committed immature osteoclasts, suggesting that IL-33 acts on the very early step of cell commitment. This phenomenon may also explain the lack of an inhibitory effect of IL-33 on osteoclast development from human peripheral blood $CD14^+$ cells [18, 19].

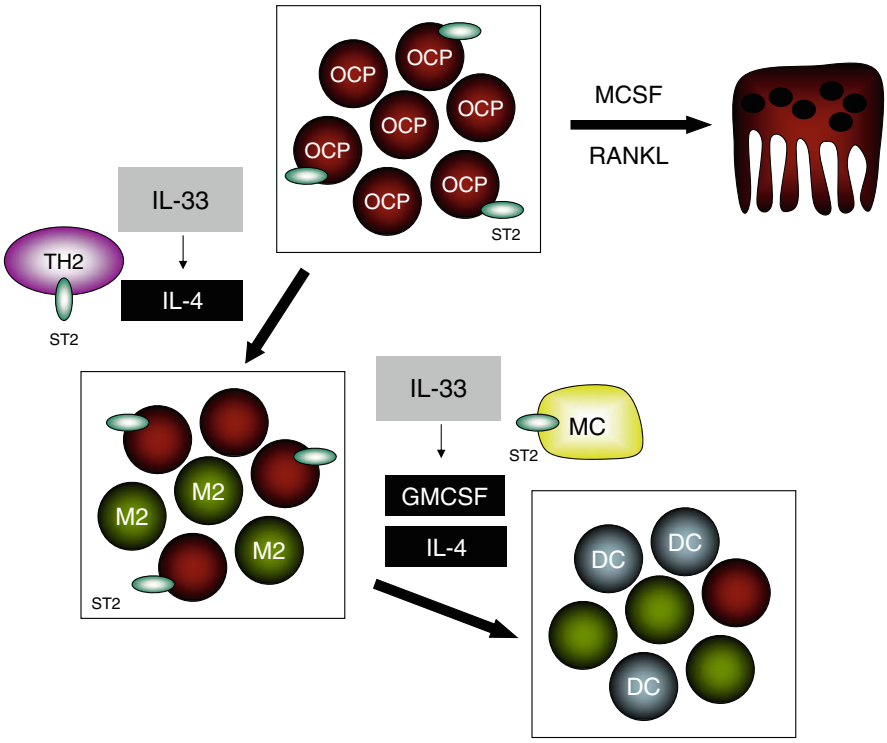

Fig. 3 Interleukin-33 blocks osteoclastogenesis. *OCP* osteoclast precursor, *RANKL* receptor activator of nuclear factor kappa B ligand, *MC* mast cell, *DC* dendritic cell, *GMCSF* granulocyte/macrophage colony stimulating factor, *IL-4* interleukin-4

5 Summary

IL-1 family members constitute a group of cytokines at the interphase between inflammation and the skeletal system. All three IL-1 family members have potent effects on bone and are particularly involved in the regulation of bone resorption by modulating osteoclast differentiation. Whereas IL-1 is a potent inducer of osteoclastogenesis and bone loss, IL-18 and IL-33 are strongly inhibitors of bone resorption. The pattern of IL-1 family member expression within inflammatory tissue is therefore of key importance for bone damage elicited by inflammation. Clinical relevance of this concept is strongly supported by the bone phenotype of gout, which is a highly IL-1-driven inflammatory process based on uric acid crystal deposition and inflammasome activation, which is accompanied by vast bone resorption induced by IL-1-driven osteoclastogenesis.

References

1. Gowen M et al (1983) An interleukin 1 like factor stimulates bone resorption in vitro. Nature 306(5941):378–380
2. Boyce BF et al (1989) Effects of interleukin-1 on bone turnover in normal mice. Endocrinology 125(3):1142–1150
3. Jimi E et al (1999) Interleukin 1 induces multinucleation and bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells. Exp Cell Res 247(1):84–93
4. Wei S, Kitaura H, Zhou P, Ross FP, Teitelbaum SL (2005) IL-1 mediates TNF-induced osteoclastogenesis. J Clin Invest 115:282–290
5. Zwerina J et al (2007) TNF-induced structural joint damage is mediated by IL-1. Proc Natl Acad Sci USA 104(28):11742–11747
6. Polzer K, Joosten L, Gasser J et al (2010) Interleukin-1 is essential for systemic inflammatory bone loss. Ann Rheum Dis 69(1):284–290
7. Ji H, Pettit A, Ohmura K, Ortiz-Lopez A, Duchatelle V, Degott C, Gravallese E, Mathis D, Benoist C (2002) Critical roles for interleukin 1 and tumor necrosis factor alpha in antibody-induced arthritis. J Exp Med 196(1):77–85
8. Aoki Y, Ichimura S, Kikuchi T, Yoshihara Y, Kogawa M, Tada N, Nemoto K et al (2005) Overexpression of the human Interleukin 1a gene causes osteopenia in mice. J Rheumatol 32:320–324
9. Okamura H et al (1995) Cloning of a new cyokine that induces IFN-g production by T cells. Nature 378:88–91
10. Ragatt LJ et al (2008) Interleukin-18 is regulated by parathyroid hormone and is required for its bone anabolic actions. J Biol Chem 283:6790–6798
11. Makiishi-Shimobayashi C, Tsujimura T, Iwasaki T, Yamada N, Sugihara A, Okamura H, Hayashi S, Terada N (2001) Interleukin-18 up-regulates osteoprotegerin expression in stromal/osteoblastic cells. Biochem Biophys Res Commun 281(2):361–366
12. Udagawa N et al (1997) Interleukin 18 (interferon-g-inducing factors) is produced by osteoblastst and acts via granulocyte/macrophage colony stimulating factor and not via interferon-g to inhibit osteoclast formation. J Exp Med 185:1005–1012
13. Horwood NJ et al (1998) Interleukin 18 inhibits osteoclast formation via T cell production of granulocyte macrophage colony-stimulating factor. J Clin Invest 101(3):595–603
14. Schmitz J et al (2005) IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity 23:479–490
15. Liew FY, Pitman NI, McInnes IB (2010) Disease-associated functions of IL-33: the new kid in the IL-1 family. Nat Rev Immunol 10:103–110
16. Schulze J, Bickert T, Beil FT et al (2011) Interleukin-33 is expressed in differentiated osteoblasts and blocks osteoclast formation from bone marrow precursor cells. J Bone Miner Res 26:704–717
17. Zaiss MM, Kurowska-Stolarska M, Böhm C et al (2011) Interleukin (IL)-33 shifts the balance from osteoclast to alternatively- activated macrophage differentiation and protects from TNFa-mediated bone loss. J Immunol 186(11):6097–6105
18. Mun SH et al (2010) Interleukin-33 stimulates formation of functional osteoclasts from human CD14(+) monocytes. Cell Mol Life Sci 67(22):3883–3892
19. Saidi S et al (2011) IL-33 is expressed in human osteoblasts, but has no direct effect on bone remodeling. Cytokine 53:347–354

DAP12 Regulates the Osteoclast Cytoskeleton

Wei Zou and Steven L. Teitelbaum

The primary ITAM-containing signaling adapters, in osteoclast lineage cells, are DAP12 and the FcεRIγ chain (FcRγ). Each associates with specific immunoreceptors. DAP12 associates with the receptors TREM2 and SIRPβ1 [1], while FcRγ recognizes OSCAR [2, 3]. Both molecules also contain an ITAM motif, within their cytoplasmic domains, whose phosphorylated tyrosines provide a high-affinity binding site for Syk family kinases.

DAP12 deficiency is purported to arrest osteoclast formation induced by RANKL and M-CSF, a defect rescued by co-culture with osteoblasts [4, 5]. While absence of DAP12 yields mild osteopetrosis, added deletion of FcRγ markedly enhances severity [4, 5]. These observations prompt the current posture that FcRγ, in association with its co-receptor, OSCAR, compensates for absence of DAP12 in the osteoclastogenic process. On the other hand, controversy exists as to whether DAP12 and/or FcRγ exert their resorptive effects by promoting osteoclastogenesis or by enabling the osteoclast to structure its cytoskeleton [4–7]. We document that Dap12 deficiency predominantly dysregulates the osteoclast cytoskeleton prohibiting the cell from transmigrating through an osteoblast layer and attaching to substrate. In consequence, removal of osteoblasts in co-culture with osteoclast precursors also eliminates resorptive polykaryons yielding the misconception that osteoclastogenesis is arrested [8].

W. Zou • S.L. Teitelbaum (✉)
Department of Pathology and Immunology,
Washington University in St. Louis School of Medicine,
St. Louis, MO 63110, USA
e-mail: teitelbs@wustl.edu

Y. Choi (ed.), *Osteoimmunology: Interactions of the Immune and Skeletal Systems,*
DOI 10.1007/978-1-4614-5366-6_13,

1 Dap12-Deficient Bone Marrow Macrophages in Co-culture Yield Few Osteoclasts Following Removal of Osteoblasts

To determine if osteoblasts compensate for absence of Dap12, we co-cultured bone marrow macrophages (BMMs) lacking the ITAM protein with WT calvarial osteoblasts. After 7 days, osteoblasts were removed with collagenase. Sheets of characteristic WT osteoclasts form, which are virtually absent in cells lacking DAP12, with or without FcRγ.

We next retrovirally expressed one or the other ITAM proteins in Dap12−/−/FcRγ−/− (DKO) BMMs and placed them in co-culture. DAP12, but not FcRγ transduction into DKO cells yielded a population of spread osteoclasts indistinguishable from WT. Thus DAP12, but not FcRγ, mediates osteoclast formation even in the presence of osteoblasts.

To identify the ITAM adaptor components mediating osteoclast formation and function, we retrovirally transduced DKO macrophages with chimeras containing various combinations of DAP12 and FcRγ extracellular, transmembrane, and cytoplasmic domains. The transduced osteoclast precursors were cultured with WT osteoblasts. DAP12's transmembrane domain is required to induce DKO macrophages to differentiate into normal appearing osteoclasts following removal of osteoblasts. While necessary, the DAP12 transmembrane region is not sufficient to rescue DKO osteoclasts as either the DAP12 extracellular or intracellular domain is also required. Consistent with these findings, substitution of the charged amino acid in the DAP12 transmembrane domain, which disrupts receptor association (R231A), and a nonfunctional ITAM mutation (2YF), fail to rescue DAP12−/− osteoclasts following osteoblast removal.

These data differ from the conclusions of Koga et al. [4] and Moscai et al. [5] who claim that the impaired osteoclastogenesis of DAP12-deficient macrophages is normalized by osteoblasts. A possible explanation for this discrepancy would be failure of DAP12−/− osteoclasts to transmigrate through the osteoblasts and adhere to plastic [9]. In this circumstance, collagenase treatment would remove not only osteoblasts but associated osteoclasts as well. In fact, DAP12−/− co-cultures not treated with collagenase contain osteoclasts in numbers approximating WT. On the other hand, whereas WT osteoclasts generated by osteoblasts form actin rings, these structures are absent in those lacking DAP12−/− which also fail to spread indicating cytoskeletal dysfunction (Fig. 1). To directly examine transmigration, DAP12−/− and WT pre-fusion osteoclasts were lifted and placed on osteoblast-like ST-2 cells. Whereas WT osteoclasts transit completely through the osteoblast layer and spread on plastic, DAP12−/− cells are incapable of doing so.

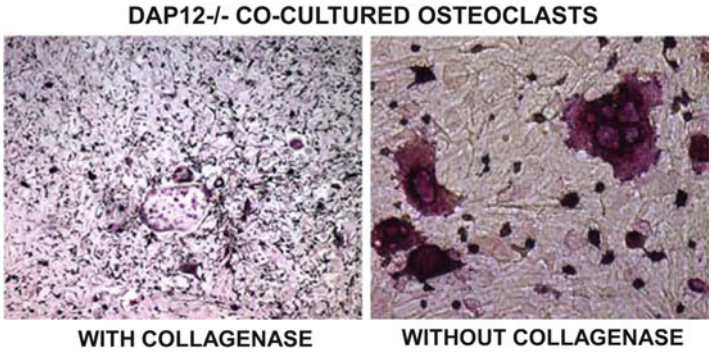

Fig. 1 Dap12–/– BMMs were cultured with WT osteoblasts. Seven days later some cultures were treated with collagenase to remove osteoblasts. All cells were TRAP stained to identify osteoclasts which are rare in collagenase-exposed wells. Osteoclasts are abundant in non-collagenase-treated co-cultures but their "crenated" appearance is indicative of cytoskeletal dysfunction

2 OSCAR-FLAG Activation Induces Fusion of Dap12–/– Osteoclasts

Evidence suggests that the OSCAR ligand(s) is likely expressed by osteoblasts, thus providing a presumptive mechanism for the generation of DAP12–/– osteoclasts in co-culture [4, 5, 10]. The abnormal appearance of DAP12–/– osteoclasts indicates, however, that the OSCAR/FcRγ axis, putatively activated by osteoblasts, is insufficient to rescue their cytoskeleton.

To explore the role of OSCAR in organizing the cytoskeleton of Dap12–/– osteoclasts, we designed a construct, with a FLAG-tag at the OSCAR carboxyl terminus which localizes to the plasma membrane and co-immunoprecipitates with FcRγ. FcRγ is phosphorylated in construct-transduced, but not vector bearing, DAP12–/– osteoclasts by anti-FLAG mAb. Furthermore, the stimulating antibody promotes osteoclast formation in DAP12–/– cells but not in the absence of its co-receptor, FcRγ. While activated OSCAR-FLAG substantially increases the size of DAP12–/– osteoclasts, façade of their cytoskeleton remains disorganized. In contrast to WT OSCAR, a transmembrane mutant, R231A, structurally predicted to inhibit FcRγ recognition [10], bound to FLAG, only partially increases poorly spread DAP12–/– osteoclasts. As in cells lacking FcRγ, absence of Syk, an OSCAR effector in other cells, abolishes OSCAR-mediated osteoclast organization.

3 OSCAR-FLAG Activation Suppresses Osteoclast Apoptosis But Not Differentiation

OSCAR-FLAG activation increases DAP12−/− osteoclast size but not cytoskeletal organization. Alternatively, markers of osteoclast differentiation are not increased in these cells, nor are RANKL-and M-CSF-stimulated osteoclastogenic signals. Hence, OSCAR does not impact precursor differentiation into osteoclasts and the same holds true regarding BMM proliferation. OSCAR-FLAG activation, however, inhibits apoptosis of osteoclasts deprived of either M-CSF or RANKL.

To determine if OSCAR mediates the capacity of FcRγ to partially compensate for DAP12 deficiency we generated osteoclasts, retrovirally transduced with OSCAR-FLAG, on anti-FLAG mAb- or IgG-coated coverslips. While WT cells form classical podosome belts, DAP12−/− transductants on anti-FLAG mAb contain a few small circular actin structures at their periphery. Vector-bearing cells exhibit no cytoskeletal organization on bone, but atypical "non-expanded actin rings" appear in OSCAR-FLAG-activated osteoclasts. Although smaller than those stimulated by anti-FLAG mAb, these abnormal actin structures are also induced by IgG. In keeping with this observation, anti-FLAG mAb also rescues the failed bone resorptive capacity of DAP12−/− osteoclasts. However, IgG exposure partially does so suggesting an OSCAR-activating ligand may reside in bone matrix [6, 11, 12].

Matrix-derived signals, mediated via the αvβ3 integrin and M-CSF, are particularly important in organizing the osteoclast cytoskeleton [12]. This signaling complex includes c-Src, Syk, DAP12, Vav3, the SLP adaptor proteins, and the small GTPase, Rac [6, 11–14]. Absence of any of these complex-residing proteins yields OCs which fail to spread. Indicating that the partial rescue of DAP12−/− osteoclast function and cytoskeletal organization, extant in OSCAR-FLAG-overexpressing mutant cells, is mediated by the αvβ3 integrin/M-CSF-activated complex, and phosphorylation of the guanine nucleotide exchange factor, Vav3, is phosphorylated by FLAG mAb [13].

This study was prompted by discrepant observations regarding the means by which DAP12 mediates bone degradation. Koga et al. [4] and Moscai et al. [5], who established the severe osteopetrosis of mice with combined deletion of the two ITAM proteins, conclude that DAP12 deficiency arrests osteoclastogenesis induced by RANKL and M-CSF. We find, however, DAP12-deficient osteoclasts do form when exposed to the cytokines but fail to resorb bone because of cytoskeletal dysfunction [6, 7]. We presently confirm our conclusion in living osteoclasts. Normal expression of osteoclast differentiation markers in DAP12-deficient M-CSF/RANKL cultures [5] challenges the concept of failed osteoclastogenesis.

Given the capacity of DAP12-deficient macrophages to differentiate into substrate-adherent osteoclasts in the presence of RANKL and M-CSF, we were surprised that few such cells were present when generated with osteoblasts if the latter cells were removed with collagenase. We therefore suspected that the

cytoskeletal abnormalities of DAP12–/– osteoclasts compromise their capacity to transmigrate through the osteoblast layer and attach to substrate, and such proves to be the case.

Indirect evidence suggests that the ligand(s) for the FcRγ co-receptor, OSCAR, is expressed by osteoblasts [10]. This speculation provided the rationale for assuming that the putative rescue of DAP12–/– osteoclastogenesis by the bone-forming cells reflects FcRγ activation. Because we find DAP12 deficiency does not impair recruitment of osteoclasts, but their function, we asked if OSCAR rescues the cell's compromised cytoskeleton. In contrast to their absence on FLAG mAb-coated glass, actin ring-like structures, albeit abnormal, form in OSCAR-FLAG-expressing DAP12–/– osteoclasts on similarly treated bone. It is therefore possible that, similar to its regulation of αvβ3 integrin conformation, bone matrix contains an OSCAR-binding molecule(s), which when faced with an abundance of the receptor is sufficient to partially organize the osteoclast cytoskeleton and promote resorption [11]. Thus, two possible scenarios present themselves. First, endogenous FcRγ, interacting with physiological amounts of OSCAR, is incapable of affecting the osteoclast cytoskeleton. Second, osteoblasts contain insufficient OSCAR ligand to activate FcRγ. In consequence, other components of the marrow environment, such as members of the immune system, deserve investigation as sources of OSCAR ligand [15]. Hence, the prevalent effect of DAP12 deficiency not arrested osteoclast recruitment, but cytoskeletal disorganization. Furthermore, failure of osteoblasts to normalize DAP12–/– osteoclasts indicates that functionally relevant quantities of OSCAR ligand do not reside in bone-forming cells.

References

1. Colonna M, Turnbull I, Klesney-Tait J (2007) The enigmatic function of TREM-2 in osteoclastogenesis. Adv Exp Med Biol 602:97–105
2. van Beek EM, de Vries TJ, Mulder L et al (2009) Inhibitory regulation of osteoclast bone resorption by signal regulatory protein alpha. FASEB J 23:4081–4090
3. Ishikawa S, Arase N, Suenaga T et al (2004) Involvement of FcRgamma in signal transduction of osteoclast-associated receptor (OSCAR). Int Immunol 16:1019–1025
4. Koga T, Inui M, Inoue K et al (2004) Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. Nature 428:758–763
5. Mocsai A, Humphrey MB, Van Ziffle JA et al (2004) The immunomodulatory adapter proteins DAP12 and Fc receptor gamma-chain (FcRgamma) regulate development of functional osteoclasts through the Syk tyrosine kinase. Proc Natl Acad Sci USA 101:6158–6163
6. Zou W, Reeve JL, Liu Y, Teitelbaum SL, Ross FP (2008) DAP12 couples c-Fms activation to the osteoclast cytoskeleton by recruitment of Syk. Mol Cell 31:422–431
7. Faccio R, Zou W, Colaianni G, Teitelbaum SL, Ross FP (2003) High dose M-CSF partially rescues the Dap12–/– osteoclast phenotype. J Cell Biochem 90:871–883
8. Zou W, Zhu T, Craft CS, Broekelmann TJ, Mecham RP, Teitelbaum SL (2010) Cytoskeletal dysfunction dominates in DAP12-deficient osteoclasts. J Cell Sci 123:2955–2963
9. Saltel F, Chabadel A, Zhao Y et al (2006) Transmigration: a new property of mature multinucleated osteoclasts. J Bone Miner Res 21:1913–1923

10. Kim N, Takami M, Rho J, Josien R, Choi Y (2002) A novel member of the leukocyte receptor complex regulates osteoclast differentiation. J Exp Med 195:201–209
11. Zou W, Kitaura H, Reeve J et al (2007) Syk, c-Src, the avb3 integrin, and ITAM immunoreceptors, in concert, regulate osteoclastic bone resorption. J Cell Biol 176:877–888
12. Ross FP, Teitelbaum SL (2005) avb3 and macrophage colony-stimulating factor: partners in osteoclast biology. Immunol Rev 208:88–105
13. Faccio R, Teitelbaum SL, Fujikawa K et al (2005) Vav3 regulates osteoclast function and bone mass. Nat Med 11:284–290
14. Reeve JL, Zou W, Liu Y, Maltzman JS, Ross FP, Teitelbaum SL (2009) SLP-76 couples Syk to the osteoclast cytoskeleton. J Immunol 183:1804–1812
15. Baron R (2004) Arming the osteoclast. Nat Med 10:458–460

Osteoclast Determinants and Implications for Therapy

Anna Teti

Abbreviations

CAII	Carbonic anhydrase II
CLC7	Chloride channel type 7
CtpK	Cathepsin K gene
DC-STAMP	Dendritic cell-specific transmembrane protein
ERK	Extracellular signal-regulated kinase
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MFR	Macrophage fusion receptor
MITF	Microphthalmia-associated transcription factor
MMPs	Metalloproteinases
NEMO	NF-κβ essential modulator
NF-kB	Nuclear factor-kB
OSTM1	Osteopetrosis associated transmembrane protein 1
PLEKHM1	Pleckstrin homology domain containing, family M (with RUN domain) member 1
PRELP	Proline/arginine-rich end leucine-rich repeat protein
RANK	Receptor activator of NF-kappaB
RANKL	Receptor activator of NF-kappaB ligand
RGD	Amino acid sequence Arg-Gly-Asp
S1P	Sphingosine-1-phosphate

A. Teti (✉)
Department of Biotechnological and Applied Clinical Sciences,
University of L'Aquila, Via Vetoio – Coppio 2
67100, L'Aquila, Italy
e-mail: teti@univaq.it

Y. Choi (ed.), *Osteoimmunology: Interactions of the Immune and Skeletal Systems,*
DOI 10.1007/978-1-4614-5366-6_14, © Springer Science+Business Media New York 2013

SERM	Selective estrogen receptor modulator
SIRP	Signal-regulatory-protein
TCIRG1	T-cell immune regulator 1
TFGβ	Transforming growth factor β
TNFSF11	Tumor necrosis factor ligand superfamily, member 11
TNFRSF11A	Tumor necrosis factor receptor superfamily, member 11A
TRAcP	Tartrate-resistant acid phosphatase

1 Introduction

Osteoclasts are exceptionally interesting cells as they belong to the only lineage known so far to be able to resorb bone [1]. They share various features with macrophages, including the ability to fuse into syncytial entities to became multinucleated, but resorb the bone by an unique mechanism not shared with any other cell type [2]. Isolation and in vitro differentiation of osteoclast precursors have provided over the last decades powerful tools to investigate the mechanisms of osteoclast differentiation, bone resorption, and interplay with the extracellular matrix and other cell types [3]. The field is still rapidly expanding providing new knowledge which is expected to lead promptly to new interventions to treat osteoclast-dependent diseases. Here I will describe the principal features of osteoclast biology, and will highlight new determinants that are likely to represent the basis for innovative therapies.

2 Osteoclast Biology and Bone Resorption

Osteoclasts are localized on resorbing bone surfaces (Fig. 1), both at the trabecular and cortical level. They appear polarized, tightly attached to the bone matrix, with nuclei and organelles located in the vascular side of the cell, and an apical membrane facing the bone surface, extensively infolded to form the ruffled border [4] (Figs. 1 and 2). This area is surrounded by a sealing zone whose cytoplasm is devoid of organelles, but is enriched in microfilaments which contribute to the organization of podosomes, the adhesion structures involved in the attachment of the cell to the bone surface. They recognize RGD-proteins by specific integrins, especially the αVβ3 receptor [5, 6].

Polarization is essential for osteoclast function [4, 7] and involves not only organelle distribution but also the polarized trafficking of intracellular acidic vesicles [8] which accumulate above the ruffled border and are then secreted with a threefold purpose: (1) to increase the extension of the ruffled border membrane, (2) to send the ruffled border membrane the ion transporters required for hydroxyapatite dissolution, and (3) to secrete the enzymes involved in the degradation of the bone organic matrix [8].

Bone resorption takes place extracellularly, in the area underneath the osteoclast, tightly and dynamically sealed by the sealing membrane, called resorbing lacuna

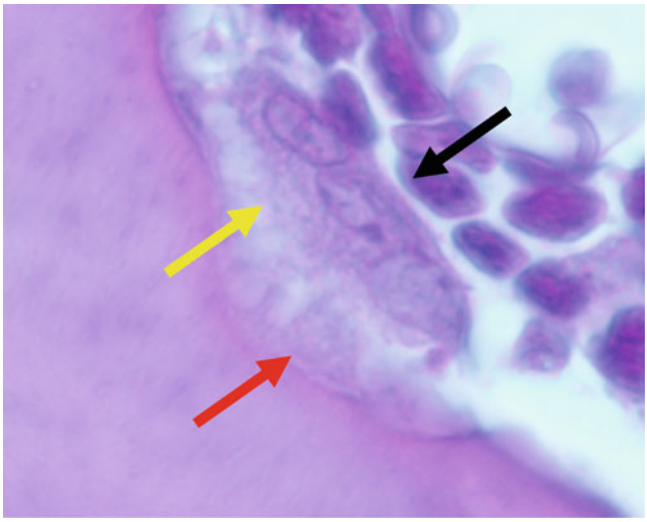

Fig. 1 A resorbing osteoclast (*black arrow*) is a polarized, multinucleated cell which attaches to the bone matrix delimitating the resorbing lacuna (*red arrow*) toward which it extends the ruffled border (*yellow arrow*). Nuclei are located in the area opposing the bone, and the cytoplasm appears finely granular. Original magnification ×100

(Figs. 1 and 2), in which acidification and acidic hydrolases, especially the cistein proteinase cathepsin K, dissolve all components of the bone matrix [1, 9, 10]. Several mechanisms are important for bone resorption and some of them are altered in the typical osteoclast disease, osteopetrosis [11], especially in those forms in which osteoclast are generated but are unable to resorb the mineralized matrix. Indeed, mutations of various genes involved in the generation of an acidic microenvironment as well as mutations of the cathepsin K gene are known to impair osteoclast function in humans [11] (Table 1).

Osteoclasts originate from the granulocyte-macrophage colony-forming unit through a series of steps leading to hematopoietic precursor commitment, differentiation, multinucleation, and activation [12, 13]. Many genes are involved in this process, the earliest being the PU.1 transcription factor which, along with M-CSF and the MITF, c-fos and c-jun transcription factors, induces committed precursor to differentiate into a pre-osteoclast sensitive to the osteoclastogenic cytokine RANKL [13, 14]. Expression of RANK is indeed the essential mechanism by which precursors are enabled to maturate into multinucleated osteoclasts ([15], Table 1) through the activity of fusion proteins, including DC-STAMP, MFR, and the d2 subunit of the V-H^+ATPase [16]. The final step of osteoclast activation requires cytoskeletal remodeling and integrin-mediated signals which induce osteoclast polarization and adhesion to bone, and trigger vesicular trafficking enabling the organization of the ruffled border, the transport of protons, and the secretion of the proteolytic enzymes into the resorbing lacuna (Fig. 2) [1, 7–10].

Until recently, osteoclasts were believe to depend on osteoblasts for their function with no return signals from osteoclasts to osteoblasts, except those released from

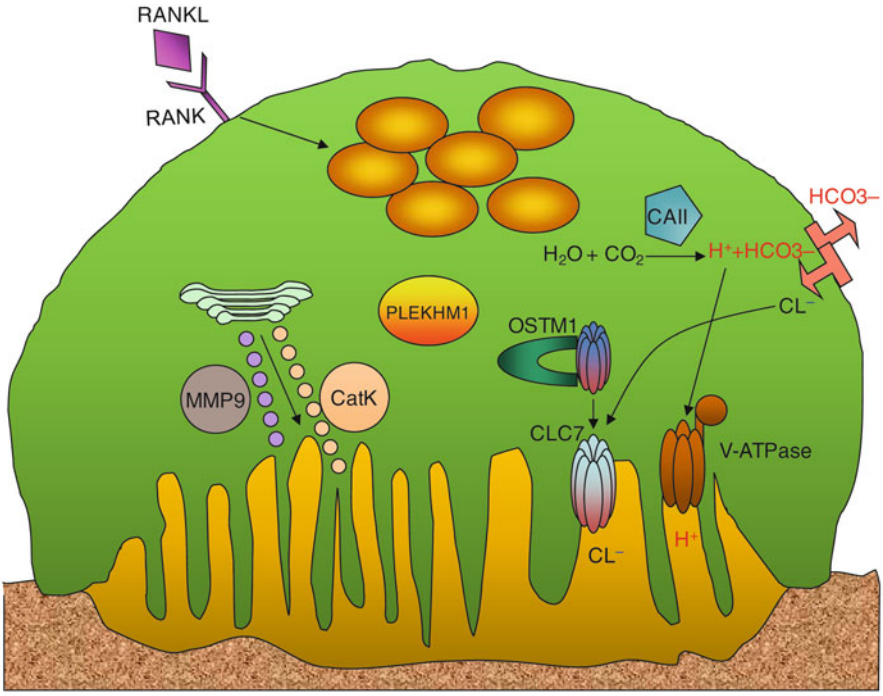

Fig. 2 The osteoclast and the molecular mechanisms involved in bone resorption

Table 1 Genes involved in diseases due to osteoclast impairment

Gene	Protein	Disease
TCIRG1	a3 subunit of the V-ATPase	Osteoclast-rich autosomal recessive osteopetrosis
CLC7	Cl^-/H^+ type 7 antiporter	Osteoclast-rich autosomal recessive osteopetrosis with neurodegeneration
CLC7	Cl^-/H^+ type 7 antiporter	Autosomal dominant osteopetrosis
OSTM1	Ostm1 protein involved in Cl^-/H^+ type 7 antiporter stabilization	Osteoclast-rich autosomal recessive osteopetrosis with neurodegeneration
CAII	Carbonic anhydrase type II	Osteoclast-rich autosomal recessive intermediate osteopetrosis with tubular acidosis and cerebral calcifications
PLEKHM1	Plekhm1 protein involved in vesicle acidification	Osteoclast-rich autosomal recessive intermediate osteopetrosis
TNFSF11	RANKL	Osteoclast-poor autosomal recessive osteopetrosis
TNFRSF11A	RANK	Osteoclast-poor autosomal recessive osteopetrosis
NEMO	Nemo protein involved in NF-κB signal	X-linked osteoclast-poor osteopetrosis
CTK	Cathepsin K	Pycnodysostosis or Toulouse-Lautrec disease
SNX10 [17]	Sorting nexin family	Osteoclast-rich autosomal recessive osteopetrosis

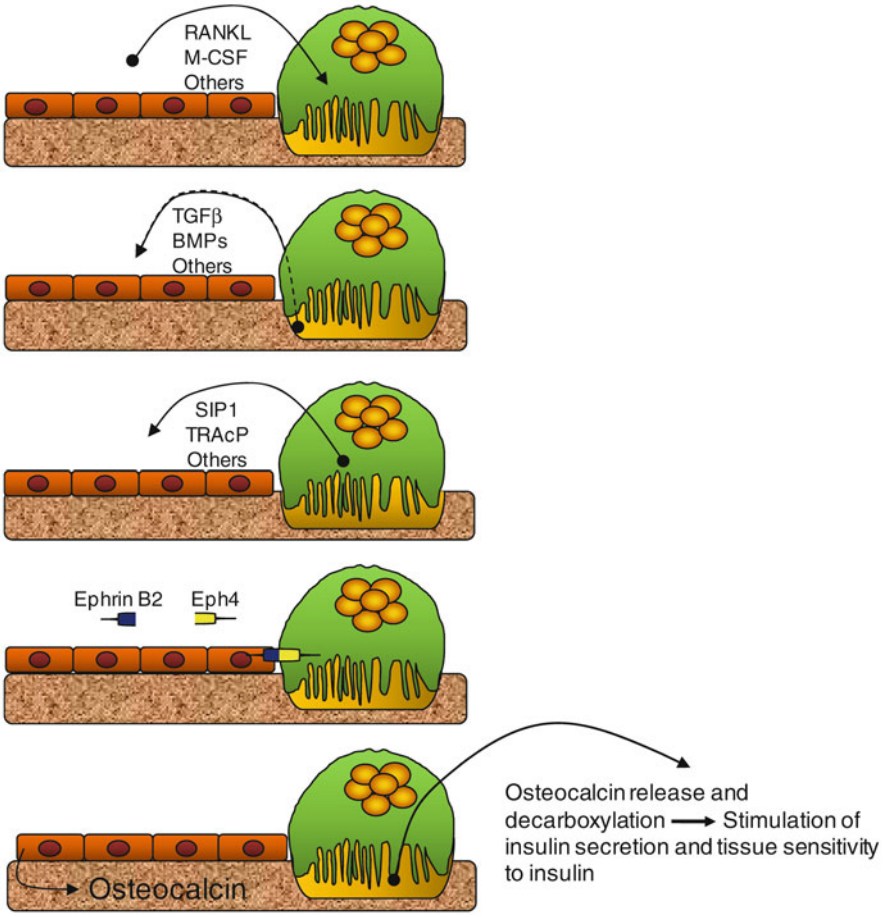

Fig. 3 Two-ways osteoblast–osteoclast cross-talk

bone matrix during resorption (i.e., TGFβ, BMPs, IFGs, FGFs, PDGF, etc.) [18]. This vision is now changed and it is generally accepted that osteoclasts may contribute to correct remodeling through cellular mechanisms which so far are known to involve the S1P and the TRAcP proteins released by the cell [11, 19]. These signals are able to recruit osteoblasts and increase their activity [20, 21]. In addition, the ephrinB2, expressed by osteoblasts, and its receptor eph4, expressed by osteoclasts, represent a two-ways signal that inhibits osteoclasts while enhancing osteoblast activity [22]. Furthermore, osteoblasts have indirect systemic effects on energy metabolism in response to insulin, enhancing osteoclast bone resorption which, in turn, releases and decarboxylates the bone matrix protein osteocalcin. Entering the circulation, decarboxylated osteocalcin acts as a systemic stimulator of islet β cell insulin secretion and peripheral tissue sensitivity to insulin [23] (Fig. 3).

Osteoblast–osteoclast cross-talk is essential for correct bone remodeling, which consists in the sequential and balanced activities of the two cell types at the same site, to ensure removal and replacement of the same amount of bone. Imbalance of resorption/formation cycles induces bone diseases, with increased bone mass if bone resorption is low (Table 1), and reduced bone mass if resorption is higher than formation [2]. In both cases, the skeleton becomes fragile and prone to fracture.

Antiresorptive treatments are still the gold standard therapy to combat osteoporosis, along with anabolic intervention [24]. However, existing antiresorptive drugs used chronically have revealed a number of pitfalls that are required to be resolved.

Bisphosphonates have been considered first line antiresorptive treatment for years [25], until their chronic use unveiled adverse effects [14]. They reduce bone turnover and improve trabecular microarchitecture [26]. However, they have high affinity for the mineralized bone matrix and persist in the bone matrix for years, leading to failure to repair microdamages, thus increasing bone fragility [26, 27].

Strontium ranelate has a peculiar mechanism of action, stimulating bone formation and, at the same time, inhibiting bone resorption [28]. Its anti-catabolic pathway is due to decrease of RANKL/OPG ratio by osteoblasts through which osteoclastogenesis is impaired [29]. It also has a direct effect on osteoclast function, reducing bone resorption with a mechanism that disrupts the actin ring and induces osteoclast apoptosis [29].

Hormone replacement therapy is used as a first line therapy for postmenopausal osteoporosis [30]. It provides an important osteoclast apoptotic agent, and also prevents osteoclast formation through the supporting cells [31, 32]. However, the prolonged use of the hormone is associated with an increased risk of mammary and endometrial cancer [33] and, for this reason, selective estrogen receptor modulators, SERMs, have been developed as they have an estrogenic activity in some organs, including the bone, while sparing the breast and the endometrium from undesirable effects as those exerted by the hormone replacement therapy [34]. However, SERMs exacerbate typical menopausal symptoms, including hot flushes, breast pain, vaginal bleeding, and thromboembolic events [35].

Denosumab is a fully human monoclonal antibody which blocks the principal osteoclastogenic cytokine RANKL [36]. It is being investigated in advanced clinical trials for post-menopausal osteoporosis, rheumatoid arthritis, and skeletal metastases [37]. However, a few aspects need still accurate assessments as the RANKL/RANK axis is involved in the development of the immune system, therefore safety issues about infection and cancers are currently being considered [37].

Since all these drugs are still not fully satisfactory, new antiresorptive agents are underway. The papain-like cysteine protease cathepsin K is considered targetable as antiresorptive drug due to fact that it is the most important enzyme with respect to bone resorption for its intense collagenolytic activity [38]. Odanacatib is a potent, reversible non-peptidic cathepsin K inhibitor that neutralizes the catalytic activity of the enzyme [39]. The inhibitor is currently in phase I and II clinical trials to determine the dose and evaluate safety and efficacy [39, 40]. The inhibitor showed no alteration of bone formation markers, and did not exhibit differences in adverse effects compared to placebo, proving efficacious in dose-dependently increasing bone mineral density in all sites evaluated [41].

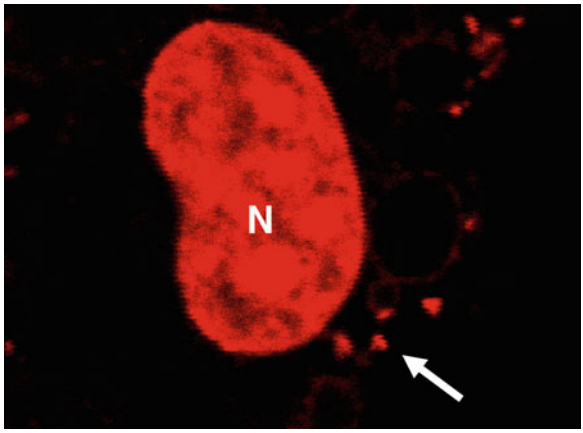

Fig. 4 Internalization of the N-terminal domain of PRELP in a pre-fusion osteoclast precursor. Vital incubation for 20 min with biotin-tagged N-terminal PRELP revealed by fluorescence microscopy. *N* nucleus, *arrow* intracellular vesicles. Original magnification ×100

A promising antiresorptive molecule that we tested in preclinical studies, is the proline/arginine and leucine rich repeat protein PRELP, especially its N-terminal domain [42], which was isolated for its capacity to bind glycosaminoglycans [43]. This is a protein that anchors the cells to collagen and is expressed in basement membranes, cartilage, and developing bone [44]. Its glycosaminoglycan-binding domain recognizes chondroitin sulfates and annexin 2 at the osteoclast precursor cell surface, reducing adhesion to substrate and forming a tri-molecular complex through which it is actively internalized by the cell (Figs. 4 and 5). Internalization is osteoclast-specific as it has not been noted in bone marrow macrophages, HEK239 and MDA-MB231 cell lines, while it is observed in osteoclast-like RAW264.7 cells. The internalized peptide is found in vesicles that translocate to the nucleus (Fig. 4), where it binds the p65NF-κB transcription factor subunit [42]. This interaction reduces p65NF-κB binding to the DNA and inhibits its transcriptional activity. These events prevent progression of osteoclast precursors toward a mature osteoclast phenotype [42], thus abolishing bone resorption. In fact, the transcription of downstream osteoclast-specific genes, including cathepsin K, calcitonin receptor, MMP-9, TRAcP, DC-STAMP, CD44, and RANK, is inhibited in the presence of the PRELP peptide. In contrast, the peptide has no effects on cell survival and phosphorylation of immediate MAPK signaling proteins, ERK, p38, and JNK. The peptide is completely inactive on the osteoblast lineage and its anti-osteoclastic effect is very potent in vivo [42]. In fact, in ovariectomized mice, the PRELP peptide given i.p. at a dose of 10 mg/kg reduces bone loss by preserving bone volume and trabecular number and preventing the increase of the osteoclast numbers and of the surface of trabecular bone covered by osteoclasts (Fig. 6). Again, no effect on osteoblast numbers and mineral apposition rate was observed in vivo upon treatment with the peptide [42].

In conclusion, osteoclast research is nowadays very active and it is identifying new targetable mechanisms which may lead to antiresorptive therapy more effective and with less adverse events. It is expected that in the near future insights into new

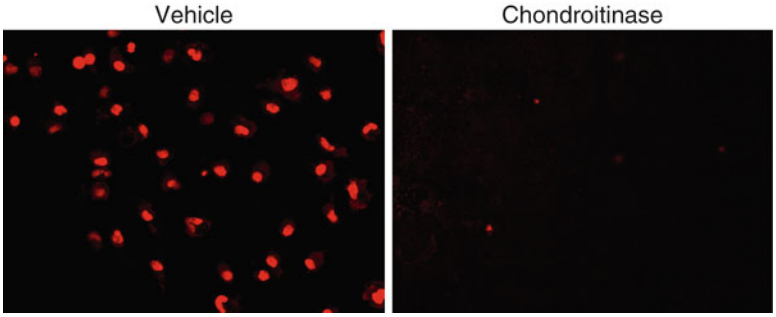

Fig. 5 Inhibition of vital biotin-tagged N-terminal PRELP internalization (red fluorescence) in prefusion osteoclasts treated for 30 min with 0.45 U/L chondroitinase ABC. Original magnification ×20

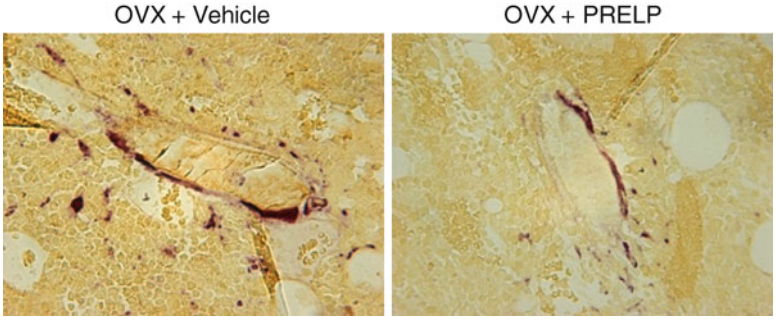

Fig. 6 Anti-osteoclastic effect of the N-terminal domain of PRELP in ovariectomized (OVX) mice treated with 10 mg/kg of the peptide, administered i.p. 5 days a week for 5 weeks. TRAcP staining (*purple*) of osteoclasts in histological sections of proximal tibia secondary spongiosa. Original magnification ×20

osteoclast determinants will be available, which may then translate into innovative treatments to relieve patients from suffering from osteoporosis.

Acknowledgments The original work was supported by grants from the EC (OSTEOGENE, contract No. LSHM-CT-2003-502941), the Italian Association for Cancer Research and the Swiss Bridge Foundation.

References

1. Teitelbaum SL (2007) Osteoclasts: what do they do and how do they do it? Am J Pathol 170:427–435
2. Raggatt LJ, Partridge NC (2010) Cellular and molecular mechanisms of bone remodeling. J Biol Chem 285:25103–25108
3. Nemeth K, Schoppet M, Al-Fakhri N, Helas S, Jessberger R, Hofbauer LC, Goettsch C (2011) The role of osteoclast-associated receptor in osteoimmunology. J Immunol 186:13–18

4. Stenbeck G (2002) Formation and function of the ruffled border in osteoclasts. Semin Cell Dev Biol 13:285–292
5. Marchisio PC, Cirillo D, Naldini L, Primavera MV, Teti A, Zambonin-Zallone A (1984) Cell-substratum interaction of cultured avian osteoclasts is mediated by specific adhesion structures. J Cell Biol 99:1696–1705
6. Nakamura I, Gailit J, Sasaki T (1996) Osteoclast integrin alphaVbeta3 is present in the clear zone and contributes to cellular polarization. Cell Tissue Res 286:507–515
7. Takahashi N, Ejiri S, Yanagisawa S, Ozawa H (2007) Regulation of osteoclast polarization. Odontology 95:1–9
8. Coxon FP, Taylor A (2000) Vesicular trafficking in osteoclasts. Semin Cell Dev Biol 19:424–433
9. Teitelbaum SL (2000) Bone resorption by osteoclasts. Science 289:1504–1508
10. Väänänen K (2005) Mechanism of osteoclast mediated bone resorption – rationale for the design of new therapeutics. Adv Drug Deliv Rev 57:959–971
11. Del Fattore A, Cappariello A, Teti A (2008) Genetics, pathogenesis and complications of osteopetrosis. Bone 42:19–29
12. Suda T, Takahashi N, Martin TJ (1992) Modulation of osteoclast differentiation. Endocr Rev 13:66–68
13. Zhao Q, Shao J, Chen W, Li YP (2007) Osteoclast differentiation and gene regulation. Front Biosci 12:2519–2529
14. Sakiyama H, Masuda R, Inoue N, Yamamoto K, Kuriiwa K, Nakagawa K, Yoshida K (2001) Establishment and characterization of macrophage-like cell lines expressing osteoclast-specific markers. J Bone Miner Metab 19:220–227
15. Villa A, Guerrini MM, Cassani B, Pangrazio A, Sobacchi C (2009) Infantile malignant, autosomal recessive osteopetrosis: the rich and the poor. Calcif Tissue Int 84:1–12
16. Vignery A (2009) Macrophage fusion: molecular mechanisms. Methods Mol Biol 475: 149–161
17. Aker M, Rouvinski A, Hashavia S, Ta-Shma A, Shaag A, Zenvirt S, Israel S, Weintraub M, Taraboulos A, Bar-Shavit Z, Elpeleg O (2012) An SNX10 mutation causes malignant osteopetrosis of infancy. J Med Genet 49:221–226
18. Matsuo K (2009) Cross-talk among bone cells. Curr Opin Nephrol Hypertens 18:292–297
19. Karsdal MA, Martin TJ, Bollerslev J, Christiansen C, Henriksen K (2007) Are nonresorbing osteoclasts sources of bone anabolic activity? J Bone Miner Res 22:487–494
20. Ryu J, Kim HJ, Chang EJ, Huang H, Banno Y, Kim HH (2006) Sphingosine 1-phosphate as a regulator of osteoclast differentiation and osteoclast-osteoblast coupling. EMBO J 25:5840–5851
21. Del Fattore A, Fornari R, Van Wesenbeeck L, de Freitas F, Timmermans JP, Peruzzi B, Cappariello A, Rucci N, Spera G, Helfrich MH, Van Hul W, Migliaccio S, Teti A (2008) A new heterozygous mutation (R714C) of the osteopetrosis gene, pleckstrin homolog domain containing family M (with run domain) member 1 (PLEKHM1), impairs vesicular acidification and increases TRACP secretion in osteoclasts. J Bone Miner Res 23:380–391
22. Zhao C, Irie N, Takada Y, Shimoda K, Miyamoto T, Nishiwaki T, Suda T, Matsuo K (2006) Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis. Cell Metab 4:111–121
23. Ferron M, Wei J, Yoshizawa T, Del Fattore A, DePinho RA, Teti A, Ducy P, Karsenty G (2010) Insulin signaling in osteoblasts integrates bone remodeling and energy metabolism. Cell 142:296–308
24. Reginster JY (2011) Antifracture efficacy of currently available therapies for postmenopausal osteoporosis. Drugs 71:65–78
25. Papapoulos SE (2011) Use of bisphosphonates in the management of postmenopausal osteoporosis. Ann N Y Acad Sci 1218:15–32
26. Diel IJ, Bergner R, Grotz KA (2007) Adverse effects of bisphosphonates: current issues. J Support Oncol 5:475–482
27. Abrahamsen B (2010) Adverse effects of bisphosphonates. Calcif Tissue Int 86:421–435
28. Marie PJ (2006) Strontium ranelate: a physiological approach for optimizing bone formation and resorption. Bone 38:S10–S14

29. Caudrillier A, Hurtel-Lemaire AS, Wattel A, Cournarie F, Godin C, Petit L, Petit JP, Terwilliger E, Kamel S, Brown EM, Mentaverri R, Brazier M (2010) Strontium ranelate decreases receptor activator of nuclear factor-KappaB ligand-induced osteoclastic differentiation in vitro: involvement of the calcium-sensing receptor. Mol Pharmacol 78:569–576
30. Doren M, Samsioe G (2000) Prevention of postmenopausal osteoporosis with oestrogen replacement therapy and associated compounds: update on clinical trials since 1995. Hum Reprod Update 6:419–426
31. Cenci S, Weitzmann MN, Roggia C, Namba N, Novack D, Woodring J, Pacifici R (2000) Estrogen deficiency induces bone loss by enhancing T-cell production of TNF-alpha. J Clin Invest 106:1229–1237
32. Manolagas SC, Kousteni S, Jilka RL (2002) Sex steroids and bone. Recent Prog Horm Res 57:385–409
33. Persson I, Weiderpass E, Bergkvist L, Bergstrom R, Schairer C (1999) Risks of breast and endometrial cancer after estrogen and estrogen-progestin replacement. Cancer Causes Control 10:253–260
34. Riggs BL, Hartmann LC (2003) Selective estrogen-receptor modulators - mechanisms of action and application to clinical practice. N Engl J Med 348:618–629
35. Archer DF (2010) Tissue-selective estrogen complexes: a promising option for the comprehensive management of menopausal symptoms. Drugs Aging 27:533–544
36. Charopoulos I, Orme S, Giannoudis PV (2011) The role and efficacy of denosumab in the treatment of osteoporosis: an update. Expert Opin Drug Saf 10(2):205–217
37. Moen MD, Keam SJ (2011) Denosumab: a review of its use in the treatment of postmenopausal osteoporosis. Drugs Aging 28:63–82
38. Gowen M (1997) Inhibition of cathepsin K – a novel approach to antiresorptive therapy. Expert Opin Investig Drugs 6:1199–1202
39. Bone HG, McClung MR, Roux C, Recker RR, Eisman JA, Verbruggen N, Hustad CM, DaSilva C, Santora AC, Ince BA (2010) Odanacatib, a cathepsin-K inhibitor for osteoporosis: a two-year study in postmenopausal women with low bone density. J Bone Miner Res 25:937–947
40. Lewiecki EM (2009) Odanacatib, a cathepsin K inhibitor for the treatment of osteoporosis and other skeletal disorders associated with excessive bone remodeling. Drugs 12:799–809
41. Eisman JA, Bone HG, Hosking DJ, McClung MR, Reid IR, Rizzoli R, Resch H, Verbruggen N, Hustad CM, Dasilva C, Petrovic R, Santora AC, Ince BA, Lombardi A (2011) Odanacatib in the treatment of postmenopausal women with low bone mineral density: three-year continued therapy and resolution of effect. J Bone Miner Res 26:242–251
42. Rucci N, Rufo A, Alamanou M, Capulli M, Del Fattore A, Ahrman E, Capece D, Iansante V, Zazzeroni F, Alesse E, Heinegard D, Teti A (2009) The glycosaminoglycan-binding domain of PRELP acts as a cell type-specific NF-kappaB inhibitor that impairs osteoclastogenesis. J Cell Biol 187:669–683
43. Bengtsson E, Aspberg A, Heinegard D, Sommarin Y, Spillmann D (2000) The amino-terminal part of PRELP binds to heparin and heparan sulfate. J Biol Chem 275:40695–40702
44. Bengtsson E, Morgelin M, Sasaki T, Timpl R, Heinegard D, Aspberg A (2002) The leucine-rich repeat protein PRELP binds perlecan and collagens and may function as a basement membrane anchor. J Biol Chem 277:15061–15068

Index

Y. Choi (ed.), *Osteoimmunology: Interactions of the Immune and Skeletal Systems,*
DOI 10.1007/978-1-4614-5366-6, © Springer Science+Business Media New York 2013