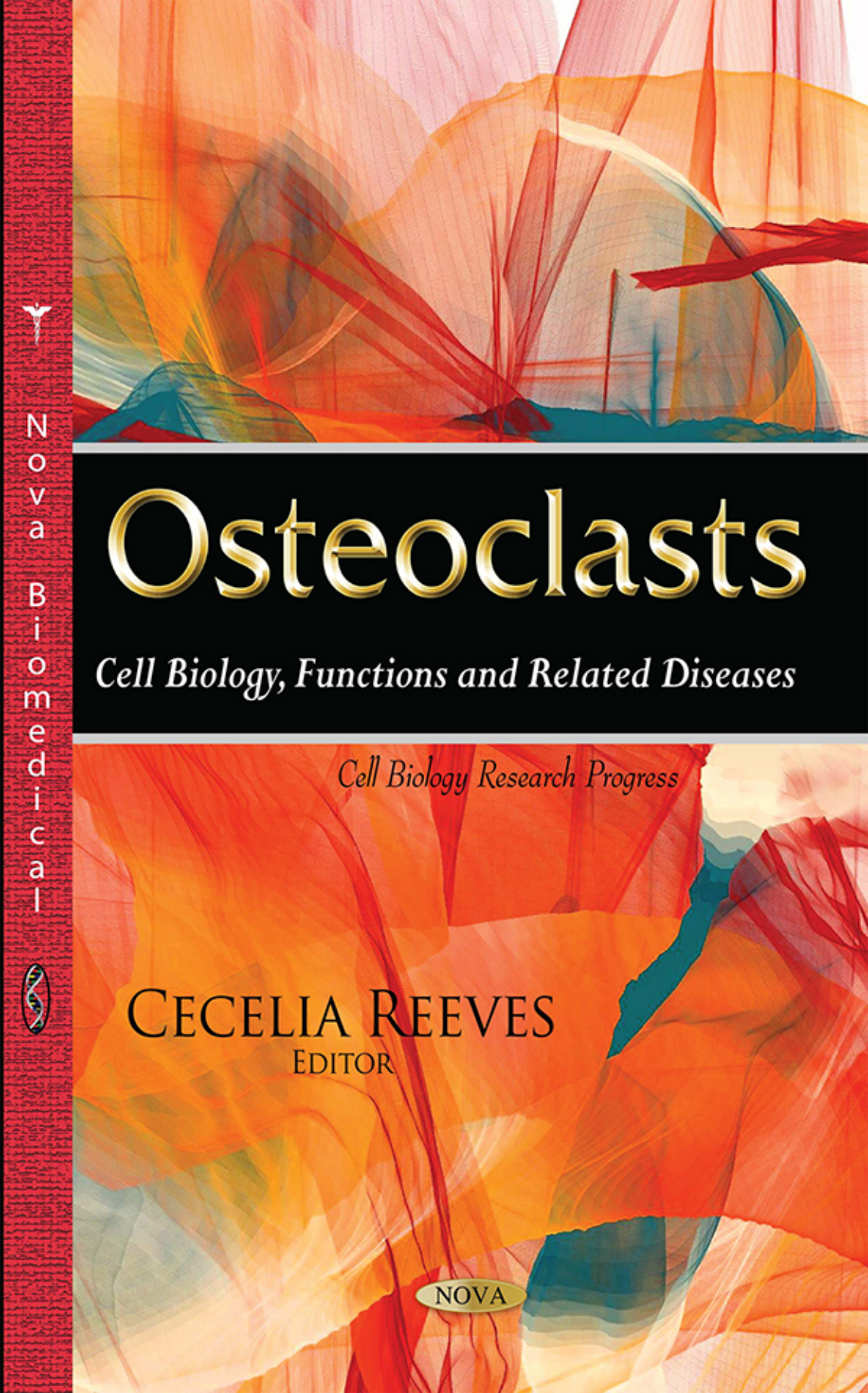
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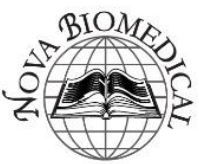

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CONTENTS

PREFACE

Bone mass is maintained by two specialized types of cells: bone-forming osteoblasts and bone-resorbing osteoclasts. Osteoclasts are tartrate-resistant acid phosphatase-positive, multinucleated giant cells that originate from the monocyte/macrophage lineage of hematopoietic stem cells. Chapter one of this book describes the role miRNAs have in osteoclast differentiation and function. Chapter two investigates how the inhibition of complement activity influenced joint destruction in relation to osteoclast differentiation. Chapter three is a discussion on the effect of sepsis and bacterial infection on immune system activation, bone remodeling and osteoclast activation. The final chapter of the book provides a current view of possible roles of DC-STAMP in signaling.

Chapter 1 – Bone is an organ that is remodeled continuously throughout life. Bone mass is maintained by two specialized types of cells: bone-forming osteoblasts and bone-resorbing osteoclasts. Osteoclasts are tartrate-resistant acid phosphatase-positive, multinucleated giant cells that originate from the monocyte/macrophage lineage of hematopoietic stem cells. Recently, the authors and other researchers demonstrated that microRNAs (miRNAs) are involved in osteoclast differentiation and function. miRNAs are single-stranded, non-coding, small (approximately 20–22 nucleotides) RNAs that are ubiquitous in plants and animals and act in a sequence-specific manner to regulate gene expression at the post-transcriptional level through cleavage or translational repression of their target mRNAs. They regulate cellular differentiation, proliferation, apoptosis, and cancer development. This chapter describes the roles of miRNAs in osteoclast differentiation and function.

Chapter 2 – Rheumatoid arthritis (RA) is a chronic joint inflammation resulting in cartilage and bone destruction. Complement activation is a part of pathological processes involved in many autoimmune disorders including RA.

Complement components and their activation products have been detected in the synovial fluid as well as in the synovial membrane of the affected joints. C5 and C3 components and their receptors are expressed in human mesenchymal stem cells, osteoblasts, and osteoclasts. The inhibition of complement activity provides a reliable approach for the control of inflammation and amelioration of joint damage. The progression of RA is dependent on the balance between erosive processes and homeostatic or remodeling events. The aim of this study was to investigate how the inhibition of complement activity influenced joint destruction in relation to osteoclast differentiation. The experiments were carried out in zymosan-induced arthritis (ZIA) in mice depleted of complement by injection of cobra venom factor (CVF). *In vitro* CVF triggered osteoclast differentiation of bone marrow (BM) and acted synergistically with macrophage colony-stimulating factor (M-CSF), along with an inhibition of osteoblast differentiation of calvarial cells. The absence of functional complement lowered the expression of receptor activator of nuclear factor kappa-B ligand (RANKL) in the synovium and joints. Bone erosion is based on the differentiation of mononuclear precursor cells to mature osteoclasts within the synovial membrane. The lack of complement activity at the initiation of ZIA resulted in a reduction of the number of mature osteoclasts in the synovium and bone which limited bone resorption. The role of TNF-related apoptosis-induced ligand (TRAIL) and its death receptor DR5 for the progression of ZIA was not clearly defined and needed further experiments. Their results proved the important role of complement system in the osteoclast generation in a model of RA and they might be helpful for the development of new therapeutic approaches.

Chapter 3 – It is well known that diseases affect the general condition of the hosts. The hosts are usually much weaker in strength and vitality after bacterial infection. It has long been appreciated that bacterial diseases produce detrimental effects on the metabolism and remodeling of the bones. Gram-negative bacteria produced lipopolysaccharide is a major mediator of bone loss. LPS enhances differentiation and activation of the osteoclast cells. Intracellular signaling induced by LPS includes NF-κB activation, p65 translocation and IκB-α degradation in mature osteoclast and osteoclast precursor cells. LPS also augments TNFα enhanced osteolysis. There are many Gram-negative bacterial toxins involved in the inhibition of osteoblast differentiation and bone loss. Cell wall virulence factors and surface associated proteins of Gram-positive bacteria are also involved in osteoclast differentiation. Virulence factor lipoteichoic acid inhibits bone remodeling function by osteroclasts. *Staphylococcus aureus* infected osteoblasts show

heightened TRAIL receptor expression and the apoptosis. Infection-induced general or local activation of B and T lymphocytes are also related with osteoclast activation. General activation of the T lymphocytes is associated with septic loosening of the bone implants. OPG ligand expression on $CD4^+$ T cells, increased local $CD8^+$ T cells, and increased RANKL expressing T cells has been documented upon bacterial infection-related bone loss. All these infection-related activation of immune functions are intertwined with poor bone remodeling and bone loss.

Chapter 4 – Bone is a dynamic tissue that continuously undergoes remodeling with diverse functions including locomotion, hemopoiesis, and protection of internal organs. Osteoimmunology has recently become an emerging field of musculoskeletal research, which connects the bone and immune systems. Results from osteoimmunology studies provide a new concept that bone is not only an essential component of the musculoskeletal system, but it also participates in immune regulation. Many important factors involved in osteoimmunology regulation also participate in bone homeostasis. Bone homeostasis is achieved by a coordinated action between bone-synthesizing osteoblasts and bone-degrading osteoclasts. An imbalanced action between osteoblasts and osteoclasts often results in pathological bone diseases: osteoporosis is caused by an excessive osteoclast activity over that of osteoblasts, whereas osteopetrosis results from an increased osteoblast activity. This review focus on Dendritic Cell-Specific Transmembrane Protein (DC-STAMP), an important protein currently considered as the master regulator of osteoclastogenesis. DC-STAMP is required for cell-cell fusion during osteoclast differentiation. Intriguingly, the frequency of circulating DC-STAMP+ cells is elevated during the pathogenesis of psoriatic diseases. Current data collectively suggest that DC-STAMP also plays an imperative role in bone homeostasis by regulating the differentiation of both osteoclasts and osteoblasts. This article summarizes our current knowledge on DC-STAMP by focusing on its interacting proteins, its regulation on osteoclastogenesis-related genes, its possible involvement in immunoreceptor tyrosine-based inhibitory motif (ITIM)-mediated signaling cascade, and its potential of developing therapeutics for clinical applications.

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Chapter 1

ROLES OF MICRORNAS IN OSTEOCLAST DIFFERENTIATION AND FUNCTION

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ABSTRACT

Bone is an organ that is remodeled continuously throughout life. Bone mass is maintained by two specialized types of cells: bone-forming osteoblasts and bone-resorbing osteoclasts. Osteoclasts are tartrate-resistant acid phosphatase-positive, multinucleated giant cells that originate from the monocyte/macrophage lineage of hematopoietic stem cells. Recently, we and other researchers demonstrated that microRNAs (miRNAs) are involved in osteoclast differentiation and function. miRNAs are single-stranded, non-coding, small (approximately 20–22 nucleotides) RNAs that are ubiquitous in plants and animals and act in a sequence-specific manner to regulate gene expression at the post-transcriptional level through cleavage or translational repression of their target mRNAs. They regulate cellular differentiation, proliferation, apoptosis, and cancer development. This chapter describes the roles of miRNAs in osteoclast differentiation and function.

Keywords: Osteoclasts, microRNAs, exosomes, extracellular vesicles

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INTRODUCTION

The amount of bone in the body is determined by the balance between bone formation and bone resorption. Numerous diseases can be caused by disruption of this balance. For example, osteoporosis, rheumatoid arthritis, and alveolar bone loss in periodontitis are all caused by excessive bone resorption, while osteopetrosis is attributable to excessive bone formation. Osteoclasts, the cells that mediate bone resorption, are formed by the fusion of mononuclear preosteoclasts, which are hematopoietic stem cell-derived cells of the monocyte/macrophage lineage. Mature osteoclasts are large, tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells [1–4]. The molecular mechanism of osteoclastogenesis has been elucidated. Macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL), which are expressed in osteoblasts and stromal cells, are both essential for osteoclast differentiation. The binding of M-CSF to its receptor, c-Fms, induces the transcription factor c-Fos, whereas the binding of RANKL to its receptor, receptor activator of nuclear factor κB (RANK), leads to the recruitment of TNF-receptor-associated factor 6 (TRAF6), which activates nuclear factor κB (NF-κB) and c-Jun N-terminal kinase (JNK). JNK in turn activates the transcription factor c-Jun [3, 5, 6]. RANKL/RANK also induces c-Fos to form activator protein-1 (AP-1), a heterodimeric transcription factor, with c-Jun. AP-1 and NF-κB then induce nuclear factor of activated T cell cytoplasmic 1 (NFATc1), a master transcription factor that regulates osteoclast differentiation. Thus, M-CSF and RANKL signaling induce NFATc1 and osteoclast-specific genes, including *Trap*, *Cathepsin k*, and *Calcitonin receptor* [4, 5, 7, 8].

We and other researchers recently showed that, in addition to these mechanisms, microRNAs (miRNAs) are involved in osteoclast differentiation and function [9-13]. miRNAs are small, endogenous, noncoding RNAs approximately 20–22 nucleotides in length [9-14]. They act in a sequence-specific manner to regulate gene expression at the post-transcriptional level through cleavage or translational repression of their target mRNAs [9-14]. To date 1,915 and 2,588 miRNAs have been identified in mice and humans, respectively (miRBase database, http://www.mirbase.org/). Although the biological functions of most miRNAs are not yet fully understood, they participate in the regulation of many kinds of biological activities such as cellular differentiation, proliferation, apoptosis, and cancer development [9,

10]. This chapter focuses on miRNAs and describes their roles in osteoclast differentiation and function.

1. BIOGENESIS OF MICRORNA AND EXTRACELLULAR VESICLES

In the nucleus, miRNA is either transcribed from its own promoter in the intergenic region or is processed from the intronic region of a coding gene as a long primary transcript, pri-miRNA, which is characterized by hairpin structures [14, 15]. This pri-miRNA is processed into 70–100 nucleotide precursor miRNAs (pre-miRNA) by the RNase III enzymes Drosha and its co-factor DGCR8 [9, 14, 15]. The RNA is exported to the cytoplasm by a transport protein, Exportin-5 [14, 15]. In the cytoplasm, it is further processed by another RNase III enzyme, Dicer, with the help, in some instances, of transactivating response RNA-binding protein (TRBP) and protein kinase RNA activator (PACT) [14, 15]. Thus, pre-miRNA is cleaved into a mature miRNA duplex. The resulting single-stranded mature miRNAs are ultimately incorporated into an RNA-induced silencing complex (RISC) that contains argonaute (AGO) family proteins (Figure 1) [9-11, 14, 15]. The AGO proteins recruit miRNAs specific for target mRNAs. The selectivity of miRNA action is conferred by nucleotides 2–7 at their 5' end, termed the "seed region," which pairs to its complementary site in the 3'UTR of the targeted mRNA [16]. Thus, the RISC inhibits the translation of the target mRNAs and/or degrades the target mRNAs [9-11, 14-16].

Recently, miRNAs were reported to be present in exosomes, a type of extracellular vesicle (EV), and to function in other cells [10, 17-19]. EVs are lipid bilayered vesicles that exist outside cells in three main types: apoptotic bodies, microvesicles, and exosomes. Apoptotic bodies are 800–5000 nm in diameter and are released by apoptotic cells. Microvesicles are 50–1000 nm in diameter and are formed by budding directly from the plasma membrane. Exosomes are 40–100 nm in diameter and are derived from multivesicular bodies (MVBs) [20, 21]. Following endocytosis into early endosomes, the cargo is packaged into intraluminal vesicles (ILVs) within MVBs. Then these MVBs may fuse with lysosomes resulting in degradation of the cargo, or alternatively, may fuse with the plasma membrane, resulting in the release of ILVs as exosomes [17, 22].

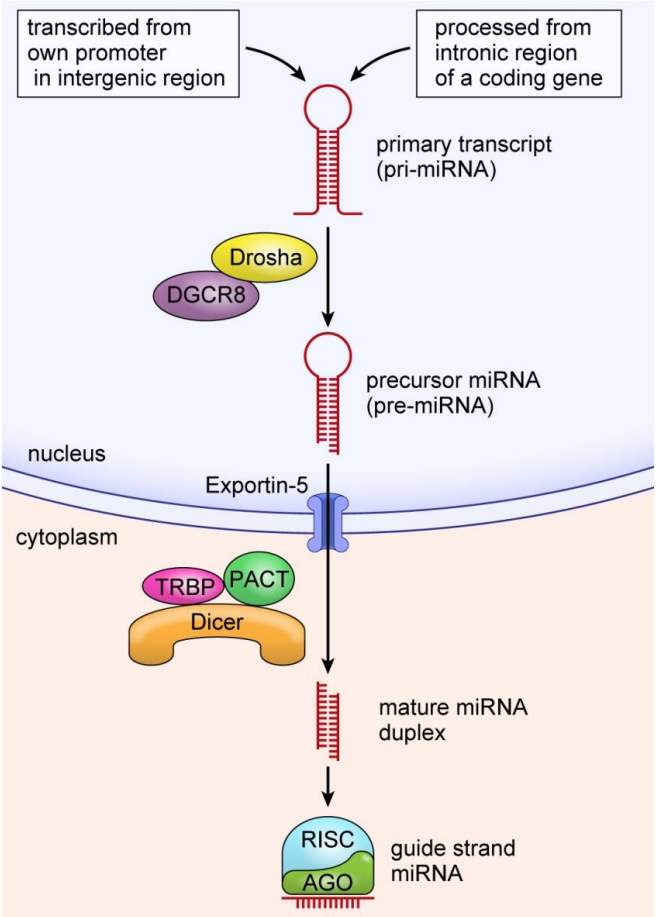

Figure 1. Biogenesis of microRNA.
In the nucleus, microRNA (miRNA) is either transcribed from its own promoter in the intergenic region or is processed from the intronic region of a coding gene as a long primary transcript, pri-miRNA, which is characterized by hairpin structures. This pri-miRNA is processed into a 70–100 nucleotide precursor miRNA (pre-miRNA) by the RNase III enzymes Drosha and its co-factor DGCR8. The RNA is exported to the cytoplasm by a transport protein Exportin-5. In the cytoplasm, pre-miRNA is processed further by the RNase III enzyme Dicer with the help, in some instances, of transactivating response RNA-binding protein (TRBP) and protein kinase RNA activator (PACT). Thus, pre-miRNA is cleaved into a mature miRNA duplex. The resulting single-stranded mature miRNAs are ultimately incorporated into an RNA-induced silencing complex (RISC) that contains argonaute (AGO) family proteins. The AGO proteins recruit miRNAs specific for target mRNAs and the RISC inhibits the translation of the target mRNAs and/or degrades the target mRNAs.

Exosomes contain many types of miRNAs, mRNAs, and proteins. Exosomes are considered to be important in cell-to-cell communication via the transfer of miRNAs, mRNAs, proteins, and bioactive lipids to target cells. The secretion of exosomes containing miRNAs depends on cell type, biological condition, and the types of miRNAs the cells contain [10, 17-22]. Because consensus is still developing regarding the origin and nomenclature of exosomes and microvesicles [21, 23], this chapter does not use the term "exosomes" but rather uses the term "EVs."

2. OSTEOCLASTS AND MICRORNAS

Table 1 lists miRNAs that are important for osteoclast differentiation and function.

This section discusses selected important miRNAs and EVs.

miR-21

miR-21 has attracted the attention of researchers in various fields and has become one of the most studied miRNAs [24]. It is highly conserved in mammals and has its own promoter, which has several conserved binding sites for transcription factors for osteoclast differentiation such as AP-1 and PU.1 [13]. miR-21 is highly expressed not only in cells during osteoclastogenesis, but also in the EVs of osteoclasts [9, 10, 13, 25]. RANKL-induced c-Fos upregulates miR-21 gene expression, which downregulates the expression of programmed cell death 4 (PDCD4), a negative regulator of osteoclastogenesis [13]. miR-21 is an NF-κB transactivational gene and the combination of TNF-α and RANKL treatment increases miR-21 expression compared to RANKL treatment alone during osteoclast differentiation [9].

Estrogen is the major hormonal regulator of bone metabolism not only in women but also in men. Sugatani et al. [25] reported that miR-21 plays a crucial role in estrogen-controlled osteoclast survival. Osteoclast precursors and osteoclasts are direct targets of estrogen during RANKL-induced osteoclastogenesis, and the proapoptotic effect of estrogen is controlled by a post-transcriptional increase in Fas ligand protein levels by downregulated miR-21 biogenesis [25].

Table 1. Important microRNAs for osteoclast differentiation and function

miRNA	Target(s)	Reference(s)
let-7e	*ITGA4, THBS1*	[9, 10, 39]
miR-7b	*DC-stamp*	[54]
miR-16	Not reported	[53]
miR-20a	*Atg16l1*	[55]
miR-21	Pdcd4, FasL	[13, 25]
miR-26a	*Ctgf*	[56]
miR-29a	Not reported	[31]
miR-29b	C-FOS, MMP-2	[30]
miR-29a/b/c	*Calcr, Cd93, Cdc42, Gpr85, Nfia, Srgap2*	[27]
miR-31	RhoA	[34]
miR-33a	Not reported	[9, 10, 53]
miR-34a	*Tgif2*	[35]
miR-99b	*IGF1R*	[39]
miR-124	*NFATc1*	[36, 37]
miR-125a	*TRAF6, TNFIP3*	[39, 40]
miR-132	*PTGS2*	[39]
miR-133a	*Mitf, Mmp14*	[53]
miR-141	*Mitf, Calcr*	[53]
miR-146a	*TRAF6*	[42]
miR-148a	*MAFB*	[57]
miR-150	Opg	[58]
miR-155	*Mitf, Socs1*,Pu.1	[12, 59]
miR-183	*Ho-1*	[60]
miR-190	Calcr	[53]
miR-212	*PTGS2, IL15*	[39]
miR-214	*Pten*	[61]
miR-218	p38Mapk signaling	[62]
miR-219	*Mitf, Traf6*	[53]
miR-223	NFI-A	[48, 49, 50]
miR-378	Not reported	[9, 10, 53]
miR-422a	Putative targets (*CBL, CD226, IGF1, PAG1, TOB2*)	[63]
miR-503	*RANK*	[64]
miR-9718	*PIAS3*	[65]

miR-29

The miR-29 family includes miR-29a, miR-29b, and miR-29c, which have been identified as tumor suppressors that are downregulated in association with various cancers [26, 27]. In bone tissues, miR-29 plays important roles in osteoblast and osteoclast differentiation. Kapinas et al. [28] showed that miR-29a and miR-29c are upregulated during osteoblast differentiation, implicating these miRNAs in the downregulation of osteonectin and probably in the regulation of other proteins critical for osteoblast function. Li et al. [26] have shown that miR-29b promotes osteogenesis by directly downregulating inhibitors of osteoblast differentiation. We and the other group [29] have shown that miR-29b is induced by NF-κB and upregulated in cells treated with TNF-α/RANKL relative to the levels in RANKL-treated cells during osteoclastogenesis. Rossi et al. [30] reported that miR-29b expression decreases progressively during human osteoclast differentiation. Forced expression of miR-29b suppresses c-Fos and matrix metalloproteinase 2 (MMP-2) expression and impairs osteoclastogenesis. Wang et al. [31] have shown that osteoporosis caused by glucocorticoids is associated with reduced miR-29a expression and that a gain of miR-29a function reduces glucocorticoid-induced osteoclastogenesis *in vitro*. In contrast, Franceshetti et al. [27] reported that miR-29 family members are positive regulators of osteoclast formation. Expression of all miR-29 family members increases during osteoclast differentiation. Knockdown of miR-29 causes impaired osteoclastic commitment and migration of pre-osteoclasts without affecting cell viability, actin ring formation, or apoptosis in mature osteoclasts. In addition, Franceshetti et al. [27] demonstrated that miR-29 negatively regulates cell division control protein 42 (Cdc42), SLIT-ROBO Rho GTPase-activating protein 2 (Srgap2), G protein-coupled receptor 85 (Gpr85), nuclear factor I/A (NFI-A), CD93, and calcitonin receptor (Calcr). Despite conflicting reports, miR-29 is considered to play a critical role in osteoclast differentiation and function.

miR-31

miR-31 is the only member of a broadly conserved miRNA “seed family” that is present in vertebrates and *Drosophila*. It is encoded by a single locus and is expressed in a wide variety of tissues and cells [32]. The activated osteoclast has a ring-shaped osteoclast-specific podosome belt termed the

"actin ring." The actin ring forms the sealing zone where the osteoclast adheres tightly to the bone surface [33]. Mizoguchi et al. [34] reported that inhibition of miR-31 severely impairs actin ring formation in osteoclasts and demonstrated that Rho A, a molecular switch that transduces extracellular signals to actin and the microtubule cytoskeleton, might be a target of miR-31 in osteoclasts.

miR-34a

Although miR-34 (miR-34a/b/c) is generally considered to be a critical mediator of p53 function and a potential tumor suppressor, it has recently been shown to be involved in bone metabolism. Krzezinski et al. [35] reported that miR-34a blocks osteoporosis and bone metastasis by inhibiting osteoclastogenesis. The expression level of miR-34a decreases during osteoclastogenesis and knockdown of miR-34a promotes osteoclast differentiation, while ectopic miR-34a inhibits this differentiation. In contrast, osteoblast differentiation is reduced in miR-34a knockout mice, but is increased in osteoblastic miR-34a conditional transgenic mice. Krzezinski et al. [35] identified transforming growth factor-b-induced factor 2 (Tgif2) as an essential direct target that is pro-osteogenic.

miR-124

miR-124 is a highly abundant miRNA particularly abundant in the brain, which contributes to the differentiation of neural progenitors into mature neurons [36]. An *in vitro* study revealed that, in bone tissues, the expression level of miR-124 decreases in a time-dependent manner during murine osteoclastogenesis [37]. A synthetic inhibitor of miR-124 enhances osteoclast differentiation and the expression of NFATc1, a master transcription factor of osteoclastogenesis. Ectopic miR-124 expression inhibits osteoclast differentiation and NFATc1 expression without affecting the expression of NF-κB p65 subunit or c-Fos [37]. Nakamachi et al. [36] demonstrated that NFATc1 is a direct target of miR-124 in human osteoclasts and that miR-124 inhibits the progression of adjuvant-induced arthritis in rats by reducing osteoclast formation.

miR-125a

An miRNA cluster containing miR-99b, let-7e, and miR-125a is expressed preferentially in hematopoietic stem cells [38]. miR-125a protects hematopoietic stem cells from apoptosis by directly downregulating the proapoptotic protein Bak1, and promotes extensive expansion of the hematopoietic stem cell pool [38]. We have observed that treating RAW264.7 cells with TNF-α/RANKL and RANKL triggers time-dependent upregulation of miR-125a expression during murine osteoclast differentiation [9]. De la Rica et al. [39] found that two miRNA clusters, miR-212/132 and miR-99b/let-7e/125a, exhibit rapid upregulation during human osteoclast differentiation. These miRNAs are activated directly by NF-κB, and their inhibition impairs osteoclastogenesis; however, Guo et al. [40] found that miR-125a expression is dramatically downregulated during human osteoclastogenesis caused by M-CSF/RANKL treatment. Overexpression of miR-125a inhibits osteoclastogenesis, while its inhibition has the opposite effect. Guo et al. [40] reported that TRAF6 is a direct target of miR-125a and that NFATc1, a downstream target of TRAF6, binds to the miR-125a promoter to modulate the expression of miR-125a. In summary, miR-125a plays a critical role in osteoclast differentiation, although there are conflicting reports as to the mechanism of action.

miR-146a

miR-146a was initially found in a human monocytic cell line, THP-1 [41]. It is an NF-κB-dependent gene that plays an important role in innate immunity by regulating cytokine production [29, 42]. For example, lipopolysaccharide, a contributing factor to some infectious diseases, is the most potent stimulator of miR-146a [29]. Mature miR-146a is highly expressed in human osteoclasts (unpublished observations). We have reported that the expression of miR-146a increases during TNF-α-regulated osteoclast differentiation [9]; however, miR-146a overexpression inhibits osteoclast formation [43]. TRAF6, which is an adaptor protein of RANK, is a target gene of miR-146a [42]. Without TNF-α, the time-dependent expression decreases in RAW264.7 cells during osteoclastogenesis [9]. Considering these reports, miR-146a expression induced by TNF-α-regulated-osteoclast differentiation may serve as a negative feedback regulator of osteoclastogenesis.

miR-155

miR-155 is an inflammation-associated miRNA that regulates inflammation and immune cell function at multiple levels [23]. We have reported that the expression of miR-155 in murine bone marrow macrophages (BMMs) is upregulated by TNF-α/RANKL/M-CSF treatment, but is modestly downregulated by RANKL/M-CSF treatment during osteoclastogenesis [9]. The upregulation of miR-155 expression in murine macrophages treated with TNF-α has been reported [44], and Mann et al. [12] found that miR-155 expression decreases during osteoclastogenesis. Our findings in BMMs are compatible with these reports; however, in our previous study, miR-155 expression was not significantly different between untreated and RANKL-treated RAW264.7 cells during osteoclastogenesis [9]. Mizoguchi et al. [45] reported that miR-155 levels in BMMs from wild-type mice are not significantly changed by RANKL treatment. Fewer osteoclasts were generated *in vitro* from BMMs of miR-155-deficient mice than from those of wild-type mice [46]. In contrast, Mann et al. [12] reported that overexpression of miR-155 blocks osteoclast differentiation by repressing MITF and PU.1, which are transcription factors that are crucial for osteoclast differentiation. Taken together, these reports suggest that miR-155 plays an important role in osteoclastogenesis and that its downregulation may not be necessary for osteoclastogenesis.

miR-223

miR-223 is specifically expressed in mouse $CD11b^+$ myeloid cell lineages and in human monocytes, granulocytes, and platelets [9]. It is a central modulator of myeloid differentiation [47]. Osteoclasts are hematopoietic stem cell-derived cells of the monocyte/macrophage lineage, and miR-223 plays a key role in osteoclastogenesis. We and other researchers [9, 11] have observed that miR-223 expression decreases during osteoclast differentiation. miR-223 regulates NFI-A and the expression of M-CSF receptor, which is critical for osteoclast differentiation and function [48]. Overexpression of miR-223 blocks osteoclast differentiation, whereas its inhibition has the opposite effect [11]. miR-223 is highly expressed in the synovium of rheumatoid arthritis patients [49, 50]. TNF-α is a crucial cytokine in rheumatoid arthritis. Given that miR-223 expression significantly decreases during TNF-α-regulated osteoclast

differentiation [9], controlling miR-223 expression may be important for rheumatoid arthritis therapy.

miR-378

miR-378 is generally considered to be an onco-miRNA that enhances tumor growth [51]; however, it is also involved in bone metabolism. miR-378 promotes osteoblast differentiation and bone formation [52]. We have reported that miR-378 expression increases during osteoclast differentiation in both RAW264.7 cells and BMMs, exhibiting a 21-fold increase in RANKL/M-CSF-treated BMMs [9]. It is highly expressed not only in cells during osteoclastogenesis but also in the EVs of osteoclasts [9, 10]. We originally hypothesized that osteoclastogenesis would be inhibited by miR-378 knockdown and promoted by miR-378 overexpression [9]. However, unexpectedly, in that study, both knockdown and overexpression of miR-378 inhibited osteoclast formation [9]. Ell et al. [53] also reported that miR-378 is significantly upregulated during osteoclastogenesis, but no significant difference was observed between ectopic expression and repression of miR-378. Thus, miR-378 is highly expressed during osteoclast differentiation, but its role in osteoclasts remains unclear.

Extracellular Vesicles

Although miRNAs have been known to play important roles in bone metabolism, whether osteoclasts secrete EVs containing miRNAs was unknown until recently.

We [10] investigated eight miRNAs in the EVs deemed important for osteoclastogenesis including let-7e, miR-21, miR-33, miR-155, miR-210, miR-223, miR-378, and miR-1224. Of these, the expression levels of miR-378, miR-21, and miR-210 were very high, while no significant expression of miR-33 or miR-1224 was detected. These results suggest that osteoclasts secrete EVs containing specific miRNAs, but do not contain the entire set of intracellular miRNAs. Another group [53] reported that miR-16 and miR-378 are present at higher levels in serum from mice with highly metastatic breast cancer cells and in serum from patients with breast cancer metastatic to bone than in healthy female donors. miRNAs in serum and plasma are divided into two populations: a vesicle-associated, membrane-bound form and a

ribonucleoprotein-associated, non-membrane-bound form. Given that most miR-16 in human serum is in the latter form [66], the increased levels of miR-16 in the serum of patients with bone metastasis may be of that same form. While these reports are important, the functions of miRNAs and EVs were not addressed in those studies. Deng et al. [67] reported that EVs from parathyroid hormone (PTH)-treated UAMS-32P cells from a stromal/osteoblastic cell line promoted osteoclast differentiation. The EVs containing RANK, RANKL receptor, and RANKL antibody treatment inhibited osteoclastogenesis. Thus, EVs from PTH-treated osteoblastic cells promote osteoclast differentiation via RANK/RANKL signaling.

CONCLUSION

Recent studies have demonstrated that miRNAs are novel, key regulators of bone formation, remodeling, and degeneration. The fact that exosomes and "matrix vesicles" are homologous structures is noteworthy. Since their discovery in 1967, numerous studies have reported that matrix vesicles are unique EVs that provide initial sites for mineral formation in endochondral bone. They serve as the nidus for apatite generation in mineralizing tissues [68]. Although whether matrix vesicles contain miRNAs is unknown, matrix vesicle miRNAs are likely to post-transcriptionally regulate the gene expression of chondrocytes, osteoblasts, osteocytes, and osteoclasts. Recently, pri-miRNAs were shown to contain short, open reading frame sequences that encode regulatory peptides in plants despite miRNAs being noncoding RNAs [69]. Thus, miRNAs provide the potential for understanding many important biological activities. We anticipate that miRNAs will open the next door in the field of bone biology research.

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Conflicts of Interest

The author declares no conflict of interest.

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Chapter 2

OSTEOCLAST FORMATION IS DELAYED IN THE ABSENCE OF FUNCTIONAL COMPLEMENT ACTIVITY IN A MODEL OF RHEUMATOID ARTHRITIS

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic joint inflammation resulting in cartilage and bone destruction. Complement activation is a part of pathological processes involved in many autoimmune disorders including RA. Complement components and their activation products have been detected in the synovial fluid as well as in the synovial membrane of the affected joints. C5 and C3 components and their receptors are expressed in human mesenchymal stem cells, osteoblasts, and osteoclasts. The inhibition of complement activity provides a reliable approach for the control of inflammation and amelioration of joint damage. The progression of RA is dependent on the balance between erosive processes and homeostatic or remodeling events. The aim of this study was to investigate how the inhibition of complement activity influenced joint

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destruction in relation to osteoclast differentiation. The experiments were carried out in zymosan-induced arthritis (ZIA) in mice depleted of complement by injection of cobra venom factor (CVF). *In vitro* CVF triggered osteoclast differentiation of bone marrow (BM) and acted synergistically with macrophage colony-stimulating factor (M-CSF), along with an inhibition of osteoblast differentiation of calvarial cells. The absence of functional complement lowered the expression of receptor activator of nuclear factor kappa-B ligand (RANKL) in the synovium and joints. Bone erosion is based on the differentiation of mononuclear precursor cells to mature osteoclasts within the synovial membrane. The lack of complement activity at the initiation of ZIA resulted in a reduction of the number of mature osteoclasts in the synovium and bone which limited bone resorption. The role of TNF-related apoptosis-induced ligand (TRAIL) and its death receptor DR5 for the progression of ZIA was not clearly defined and needed further experiments. Our results proved the important role of complement system in the osteoclast generation in a model of RA and they might be helpful for the development of new therapeutic approaches.

Keywords: Cobra venom factor, complement activity, osteoclasts, RANKL, TRAIL, zymosan-induced arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease chracterized with cell infiltration into the synovium, hyperplasia of the synovial lining, decreased cell death of synovial macrophages and fibroblasts and progressive cartilage and bone destruction [1, 2]. The development of RA is attended with the production of autoantibodies such as rheumatoid factor and anticitrullinated peptide antibody, directed against common autoantigens expressed outside the joints but leading to local bone erosion [3]. The accumulation of pro-inflammatory molecules incluing TNF-α, IL-1, IL-6, IL-7, and IL-17, activates osteoclast differentiation leading to impaired bone homeostasis [4-6]. Also, these cytokines caused the overexpression of receptor activator of nuclear factor kappaB ligand (RANKL) and reduced the levels of osteoprotegerin (OPG), serving as a decoy receptor of RANK. When the RANKL/OPG ratio is shifted towards RANKL it leads to enhanced osteoclastogenesis [7]. At the same time, a number of anti-inflammatory cytokines such as IL-10, IL-13, and Transforming growth factor beta (TGF-β) are found in high amounts aiming to limit joint destruction [8].

Osteoclasts are multinucleated cells formed in the bone marrow in the presence of M-CSF, and RANKL [9]. The latter stimulates the activation of mitogen-activated protein kinase/activator protein-1 (MAPK)/AP-1) and Ca^{2+}–nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) signaling pathways needed for efficient osteoclastogenesis. The increased expression of NFATc1 in osteoclast precursors provokes osteoclast formation in the absence of RANKL stimulation [10]. The immunoreceptor tyrosine-based activation motif (ITAM)-mediated signal acts as co-stimulatory signal in RANKL-induced osteoclas differentiation. RANKL activation of JNK phosphorylates the transcription factor c-Jun which binds c-Fos and forms activator protein-1 (AP-1) complex important for osteoclast differentiation [11, 12]. Osteoclast precursors pass from the bone marrow to the bloodstream where under the action of certain chemokines released at sites of resorption they return back into bones and differentiate into OCs [13]. They attach to bone surfaces by integrin vitronectin receptor αVβ3 and activates a canonical signaling complex consisting of c-Src, Syk, Dap12, Slp76, Vav 3, and Rac [14]. These large multinucleated cells expressed mature phenotypic markers, tartarate-resistant acid phosphatase (TRAP), calcitonin receptor, matrix metalloproteinase 9 (MMP9), and cathepsin K [15-17].

Activation of the complement system is a major event that underlies various inflammatory responses in a number of diseases including RA. This activation can be generated by each of three distinct pathways; the classical (CP), alternative (AP), and lectin (LP) pathways. While the LP and the CP are initiated by the recognition of target molecules, the activation of the AP can also be started when properdin recognizes targets and promotes initial C3b deposition [18]. The C3bBb dimer and C3bC3bBb multimers form the convertases of the AP that participate in the amplification of complement activity of the three pathways, whereas C4bC2a and C3bC4bC2a are convertases needed for CP and MBL pathways. All pathways of complement activation lead to cleavage of the C5 component into the anaphylatoxin C5a and, C5b that further forms the terminal complement complex (C5b-9). The complement activation products bind to their receptors. After the enzymatic activities of C3/C5 convertases, the resultant complement-activation products function as chemoattractants and activators of neutrophils and inflammatory macrophages facilitating cell migration, phagocytosis, as well as other cellular activities.

Complement activation plays an eminent destructive role in the inflamed joints of RA patients. Elevated levels of mRNA for C3, factor B, C3aR, and C5aR are detected in rheumatoid synovial tissues, pointing that these

components are locally produced [19, 20]. The differentiation of BM cells into osteoclasts is regulated by osteoblastic cells and might be stimulated by 1 alpha, 25$(OH)_2$ vitamin D_3 [21]. In response to the vitamin osteoblasts produce C3 component of the complement which directly participates in the osteoclast differentiation proven by blockade of this process through monoclonal anti-C3 antibodies [22]. The inhibitory effect of antibodies on osteoclast differentiation corresponded to the late proliferative phase and the early differentiation phase of osteoclast formation and similar inhibition was also expressed by anti-C3 receptor antibodies.

TRAIL has attracted much attention after its successful application as an anti-tumor agent without toxic side effects. Recent studies have revealed the important role for TRAIL in regulating immune responses to viruses, self-antigens and allergens. In humans TRAIL binds to two death-inducing receptors while only one is present in mice. The signaling induced by these receptors leads to apoptosis but might also result in activation of survival signals. One reason for the persistence of the joint inflammation might be the inhibited apoptosis of synovial lymphocytes, macrophages, and fibroblasts and also of osteoclasts. The results by Song et al. showed that the overexpression of TRAIL ameliorated collagen induced arthritis (CIA), while TRAIL deficiency and the administration of soluble TRAIL R2 exacerbated CIA [23].

The active form of Vitamin D_3, 1α,25-dihydroxyvitamin (1α,25$(OH)_2$) induces the production of factor B, factor D, and C5 by BM cells as well as C3aR and C5aR are also involved. C3 activation is needed for OC differentiation which was realized through altered IL-6 production [24]. Cobra venom factor (CVF) is structurally identical to C3b fragment of complement component C3. It forms a stable complex with CVFBb which acts as C3-C5 convertase thus leading to complement exhaustion in the presence of Factor B, Factor D and Mg^{2+}. Snake venom, which contains small basic peptides, phospholipase A_2 (PLA_2), and PLA_2 homologs, has a significant effect on RA. The experiments in animals depleted with CVF showed decreased susceptibility to CIA, thus demonstrating that functional complement has an important role in joint inflammation [25]. Also, decomplementation by CVF has been reported to suppress *Yersinia*-induced reactive arthritis [26].

Previously, in a model of RA induced by intraarticular (i.a.) injection of zymosan in properdin-deficient mice we have shown that the alternative pathway is definitely involved in the development and progression of zymosan-induced arthritis [27]. The induction of ZIA in the absence of complement activity provoked by pre-treatment with CVF, strongly inhibited the development of chronic synovitis, while complement activation during

ZIA exacerbated the synovitis through an increase of macrophage infiltration, C3aR and C5aR expression in the joints, and elevated levels of C5a and soluble RANKL(sRANKL) in the synovial fluid (SF) [28]. The aim of the present study was to estimate the role of functional complement system in the processes of osteoclastogenesis in a murine model of RA.

MATERIALS AND METHODS

Mice

Experiments were performed using BALB/c mice (7 to 8 weeks old; Charles River Laboratories, MA, USA). They were acclimated for 1 week and then randomly assigned to treatments. Animals were maintained on a 12:12 h light: dark cycle and received tap water and pelleted chow *ad libitum*. All experiments were carried out in accordance with the Bulgarian Food Safety Agency Guidelines №352 06.01.2012 and the protocols were approved by the Animal Care Committee at the Institute of Microbiology, Sofia, (Decree No. 14/19.07.2000).

Zmosan-Induced Arthritis

ZIA was induced by a single intraarticular (i.a.) injection of 180 µg zymosan A from *Saccharomyces cerevisiae* (Sigma-Aldrich, Germany) assigned as day 0. Control animals received an i.a. injection of an equal volume of sterile phosphate buffered saline (PBS).

Cobra Venom Factor Treatment and Complement Activity Assay

The first group (CVF) was injected intraperitoneally (i.p.) with 10 ng/g body weight of CVF factor (*Naja naja* CVF, cobra venom anti-complement protein, Sigma-Aldrich, St. Louis, MO) 72 and 48 h before zymosan. The second group of mice (CVF1) was injected i.p. with 10 ng/g body weight of CVF 7, 12 and 17 days after zymosan injection according to our previous experiments [28]. To evaluate the efficiency of the decomplementing effect of

CVF, 5-7 BALB/c mice in each experiment were injected with CVF at the same time points, as the experimental groups. Blood was collected by retro-orbital puncture and the AP complement activity in sera was determined by the method of Klerx et al. [29]. The pretreatment value was set to 100%. CVF treatment resulted in drastic suppression of serum complement activity which persisted for 5 days.

Osteoclast Differentiation

Bone marrow-derived macrophages were isolated from the long bones of 6-week-old mice. Using a 27G needle/1 ml syringe filled with α-minimal essential medium (Sigma-Aldrich) the bone marrow from both ends of the bone was expelled. The suspension was gently aspirated to disrupt cell aggregates and centrifuged at 1000 rpm for 5 min. Bone marrow precursors ($1x10^6$/ml, 96 well plate) were cultivated with 10 ng M-CSF (GenScript) for 1 day, followed by 50 ng M-CSF for 3 days and then with 50 ng M-CSF+50 ng sRANKL (Biolegend, UK) for 3 days. The cells were cultivated in the presence or absence of 5 ng/ml CVF and the specific tartarate-resistant acid phosphatase (TRAP) staining was performed. Fixation solution was added to the cells and then removed with PBS. TRAP staining solution (50 mM acetate buffer, 30 mM sodium tartrate, 0.1 mg/ml of Naphtol AS-MX phosphate, 0.1% w/v Triton X-100, and 0.3 mg/ml of Fast Red Violet LB stain) was added to the cells for 1 hr. The solution was then removed, and the cells were washed twice with PBS. Cell morphology was detected by microscopic observation. TRAP activity in the cell lysates was determined using TRAP solution (0.1 M sodium acetate, 1 mM ascorbic acid, 0.15 M KCl, 10 mM disodium tartarate, and 10 mM *p*-nytrophenil phosphate). The reaction was stopped with 0.2 N NaOH, and the absorbance was measured at 405 nm.

Determination of IFN-γ

The amount of IFN-γ in the supernatants of BM cells at day 7 of cultivation was quantified by ELISA kit (ab 46025, Abcam, Cambridge, UK), according to manufacturer's instructions. The detection limit was 5.0 pg/ml.

Calvarial Osteoblast Cultures

Mouse osteoblastic cell cultures were established from calvarial cells isolated from 4 day-old neonatal mice. After surgical isolation from skull and removal of sutures and adherent mesenchymal tissues, calvaria were subjected to four sequential 15-min enzyme digestions at 37°C in solution containing 0.1% trypsin-EDTA and 0.5% collagenase (Sigma-Aldrich). Cells released from the 2^{nd} to 4^{th} digestions were collected, centrifuged, re-suspended, and plated at a concentration of 1×10^5 cells/100 µl. The cells were then incubated at 37°C in 5% CO_2 for 24 hours. After that, the media were changed and the cells were incubated in α-MEM supplemented with 10% FCS (Sigma-Aldrich), antibiotics (streptomycin 100 U/ml, penicillin G 100 µg/ml, Sigma-Aldrich), L-ascorbic acid (50 µg/ml), supplemented with 5 mM β-glycerophosphate (Sigma-Aldrich) and 10^{-8} M dexamethasone (Sigma-Aldrich).

CVF at a concentration of 5 ng/ml presented for the whole period of cultivation. After 7 day cell culture, the fixed cells were incubated in 50 mM $MgCl_2$ and 0.1 M Tris–HCl (pH 7.4) for 30 min. Cells were then stained for alkaline phosphatase (ALP) with a mixture of 0.1 mg/ml naphthol AS-MX (Sigma-Aldrich) phosphate and 0.6 mg/ml fast blue RR salt (Sigma-Aldrich) in 0.2 M Tris–HCl (pH 8.5). Cells were washed with distilled water and ALP-positive cell nodules were quantified.

Histotopathological Examination

The dissected legs were fixed in 4% paraformaldehyde/PBS and decalcified in 14% EDTA for 2 weeks. The tissue samples were dehydrated and embedded in paraffin before sections of 5 to 7 µm were prepared and deparaffinised.

For histopathological evaluation, sections were stained by using a conventional hematoxylin and eosin (H&E) tissue stain. Bone erosion was scored on the following scale: 0 = none, 1 = mild, 2 = moderate and 3 = severe cartilage loss and bone erosion. For TRAP staining, sections were incubated for one hour in 1 mg/ml naphthol AS-TR phosphate (N-(4-Chloro-2-methylphenyl)-3-(phosphonooxy)naphthalene-2-carboxamide), 60 nmol/l NN-dimethylformamide, 100 nmol/l sodium tartrate, and 1 mg/ml Fast red TR salt solution (all from Sigma-Aldrich, St. Louis, USA). The number of OCs was

determined at high magnification (×100) and the results were expressed as the total number of osteoclasts per slide.

RANKL Expression in the Joints

The slides were permeabilized with 0.05% triton for 10 min and then for 10 min with 3% H_2O_2 to block endogenous peroxidase, followed by washing procedure with PBS and immediately blocked with 5% BSA at room temperature for 10 min.

The sections were then incubated for 30 min for 2 h at room temperature with biotinylated antibodies RANKL (10 μg/ml, Abcam UK). Isotype antibody (biotinylated rat anti-mouse IgG, Sigma-Aldrich) was used as a background staining control. Further, the cells were washed with PBS and HRP-streptavidin (1:100 diluted; Sigma-Aldrich, Germany) was added for 10 min. The slides were washed and incubated with DAB solution kit (3',3'diaminobenzididne kit, Abcam) for 10 minutes and counterstained with Gill's hematoxylin for 3 minutes.

Flowcytometry

BM cells were stained on ice with PE-conjugated anti-mouse CD253 mAb (TRAIL, clone: N2B2), or PE-conjugated anti-CD262 (DR5, TRAIL-R2. clone: MD5-1), or biotin anti-mouse CD254 (TRANCE, RANKL, clone: IK22/5) (all antibodies from Biolegend). After 20 min incubation in the dark, cells were rinsed twice with 2%FCS/PBS buffer and resuspended in PBS. After four times washing with PBS, the samples were analyzed by a flowcytometer (BDTMLSR II), using FCS ExpressTM Diva Software (Beckton and Dickinson).

Statistics

Statistical analyses were performed using InStat 3.0 and GraphicPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). The data were presented as the mean ± SEM. Group mean values were compared by two-way ANOVA. Differences were considered significant when $p < 0.05$.

RESULTS

Osteoclast Differentiation of BM Cells and Osteoblast Formation of Calvarial Cells

Cultivation of BM cells alone (non-stimulated) produced few osteoclasts (< 5 per well) which were defined as multinucleated (MNCs) and having positive staining for TRAP (Figure 1A, E).

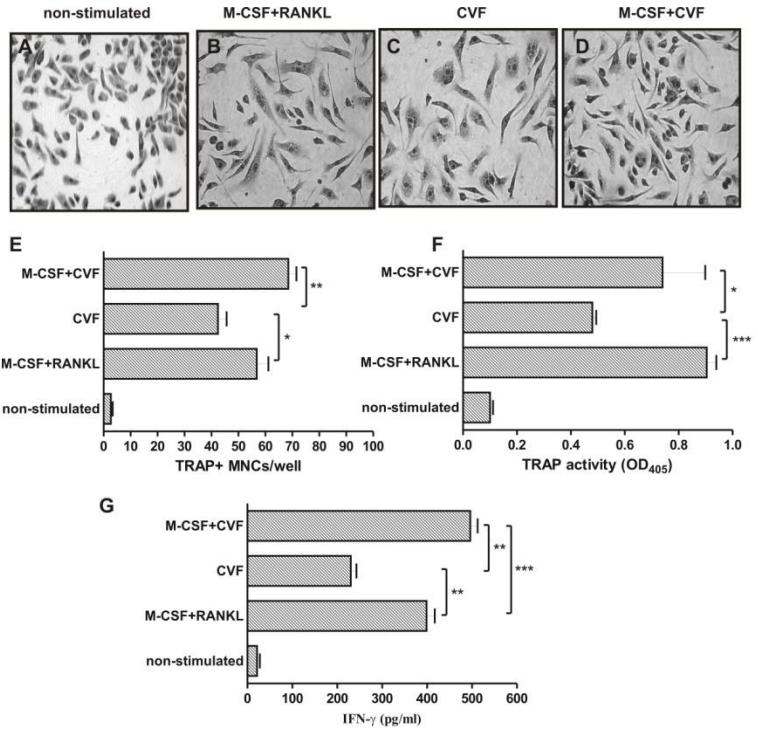

Figure 1. Osteoclast differentiation of bone marrow (BM) cells *in vitro*. Photomicrogrphs show tartarate-resistant acid phosphatase (TRAP) stained mature osteoclasts. (A) Non-stimulated cells. (B) BM cells were treated with 10 ng/ml M-CSF (1day) , 30 ng/ml M-CSF (3 days) and with 50 ng/ml M-CSF+50 ng/ml RANKL (3 days). (C) BM cells cultivated in the presence of 5 ng/ml CVF (7 days). (D) BM cells cultivated with 30 ng/ml M-CSF (1 day), 30 ng/ml M-CSF (3 days) and 50 ng/ml M-CSF+5 ng/ml CVF (3 days). (E) Figure data present the number of multinucleated cells (MNCs). (F) TRAP activity. (G) The concentration of IFN-γ in the supernatants at day 7 of cultivation. Data are means ± SEM from 3 determinations (n=6 per group). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, two-way ANOVA.

Treatment of the cells with M-CSF only lead to the appearance of a few number of TRAP+ MNCs (~ 10 per well; data not shown). In contrast, treatment of the cultures with 30 ng/ml M-CSF (1-3 day), followed by 50 ng M-CSF+50 ng/ml RANKL (4-7 day) caused an increase of the number of osteoclasts up to ~ 60 per/well (Figure 1B, E). When BM cells were cultivated with 5 ng/ml of CVF for 7 days the number of TRAP+ MNCs was ~ 40 per/well (Figure 1C, E). We observed that the addition of CVF instead of RANKL (10 ng/ml M-CSF for 1 day, 30 ng/ml M-CSF for 3 days, followed by 30 ng/ml CVF + 5 ng/ml CVF for 3 days) resulted in more pronounced effect compared to CVF-treatment alone (5 ng/ml for 7 days) and a relatively similar number compared to M-CSF + RANKL treatment (Figure 1D, E). The same tendency was established in concern to TRAP activity. Cells treated with M-CSF alone slightly increased TRAP activity while BM cells stimulated with M-CSF+RANKL or M-CSF+CVF showed significant TRAP activity increase. Intermediate TRAP activity was expressed by the cells treated with CVF alone (Figure 1F).

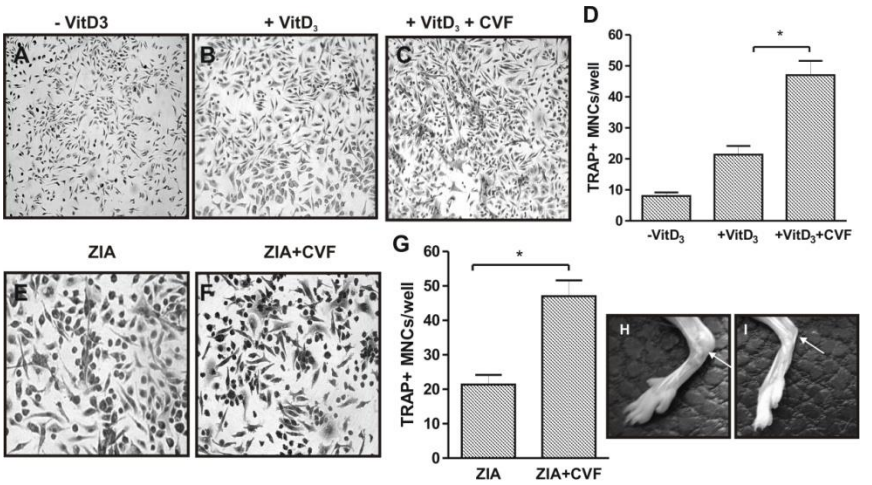

Figure 2. CVF enhanced $VitD_3$-induced osteoclast differentiation of BM cells. (A) Cells cultivated in the absence of $VitD_3$. (B) Cells cultivated in the presence of 10^{-8}M $VitD_3$ for 7 days. (C) Cells cultivated with 10^{-8}M $VitD_3$ and 5 ng/ml CVF for 7 days. (D) Figure data present the number of multinucleated cells (MNCs). CVF triggered osteoclast differentiation of BM isolated at day 7 of ZIA. Non-stimulated ZIA cells (E), ZIA cells cultivated in the presence of 5 ng/ml CVF for 7 days (F) and the number of multinucleated cells (G). Paw swelling at day 7 of ZIA (H) and paw swelling of the CVF-treated group (I). Data are means ± SEM from 3 determinations (n=5 per group). *$p < 0.05$, two-way ANOVA.

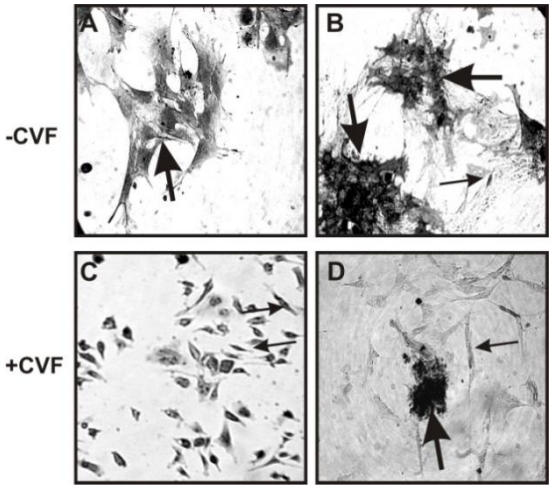

Figure 3. CVF inhibited the formation of osteoblast nodules. Calvarial cells were cultivated in dexamethasone-containing media in the absence of CVF (A, B) or in the presence of 5 ng/ml CVF (C, D). Thick arrows pointed osteoblast nodules and osteocytes and thin arrows point fibroblast-like cells. Representative microphotographs from 6 determinations.

The production of IFN-γ was assessed at the end of osteoclast differentiation (day 7) induced in BM cell cultures through M-CSF+RANKL as well as CVF alone or M-CSF+CVF. In the supernatants of cells stimulated with M-CSF+RANKL we observed a release of IFN-γ which was two fold higher than in the supernatants from cells grown in the presence of CVF (Figure 1G). The combination of M-CSF with CVF showed higher IFN-γ production compared to CVF and M-CSF + RANKL groups.

In another set of experiments we studied the influence of CVF on osteoclast formation induced *in vitro* with 1,25$(OH)_2$ Vitamin D_3. The results showed that when CVF (5 ng/ml) was present during the cultivation the number of TRAP positive MNCs was further increased (Figure 2A, B, C, D). Next, BM cells were obtained at day 7 of ZIA and *in vitro* cultivated in the presence of 5 ng/ml CVF for 7 days. This increased the number of MNCs (Figure 2E, F, G). Figure 2H, I shows that at day 7 of ZIA joint swelling of CVF-treated mice was visually reduced.

Calvarial cells were cultivated in media supplemented with dexamethasone in the presence or absence of CVF and after 7 days were stained with fast blue. Large nodules of osteoblasts surrounded with osteocytes were observed in the group without CVF (Figure 3A, B). In contrast, less

nodules was seen in CVF-treated cultures and more fibroblasts instead osteocytes were found (Figure 3C, D).

Complement Depletion Reduced RANKL Positive Cells in the Joint

RANK/RANKL/OPG axis is a key mechanism by which immune cells regulate or disrupt physiological bone turnover. We observed that at day 30 of established arthritis megacariocytes in BM stained positive for RANKL, both in untreated and CVF-treated groups (Figure 4A, B, C). Also, ZIA mice showed large areas stained positively in the synovium that were absent in non-arthritic and CVF injected mice (Figure 4D, E, F).

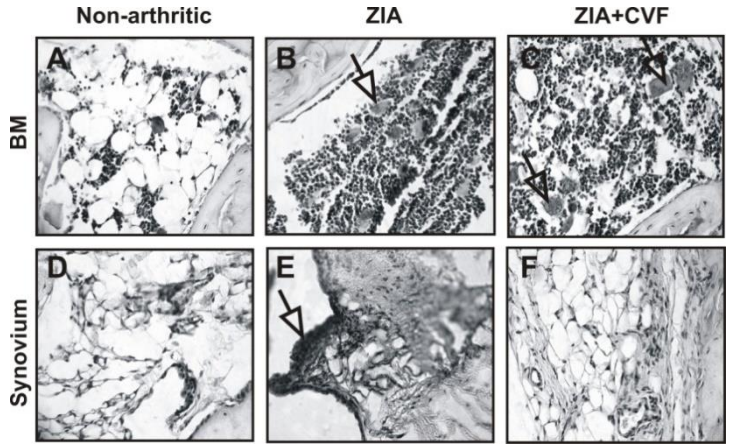

Figure 4. Immunohistochemical staining showed RANKL expression in bone and synovium at day 30 of ZIA. In BM of non-arthritic mice (A), mice with ZIA (B) and ZIA mice pretreated with CVF (C). Arrows indicate RANKL positive megakaryocytes (representative sections for 10 mice per group). RANKL positive cells in the synovium of non-arthritic mice (D), mice with ZIA (E) and ZIA mice pretreated with CVF (F). Arrow indicates RANKL positive area (representative sections for 10 mice per group).

Complement Depletion Reduced TRAP Positive Cells in the Joint

We determined the development of bone erosion at day 30 of ZIA in untreated mice and in arthritic mice pretreated with CVF or treated at days 7,

12 an 15 (CVF1 group). Previously, we have established that these two schemes provoke opposite effects [28]. TRAP staining of joint sections showed that the initiation of ZIA in the absence of functional complement activity limited bone erosion (Figure 5C, E). However, when CVF injection started at the peak of inflammation it exacerbated bone erosion in correlation with the intensity of TRAP staining (Figure 5D, E, F). A lot of TRAP positive cells were observed in the zone between cartilage and bone and deeply in the bone in ZIA group (Figure 5B, F) and in the group repeatedly treated with CVF (Figure 5 D, F). In the group pretreated with CVF osteoclasts were found only in the cartilage/bone zone (Figure 5C). Figure 5A represents healthy bone.

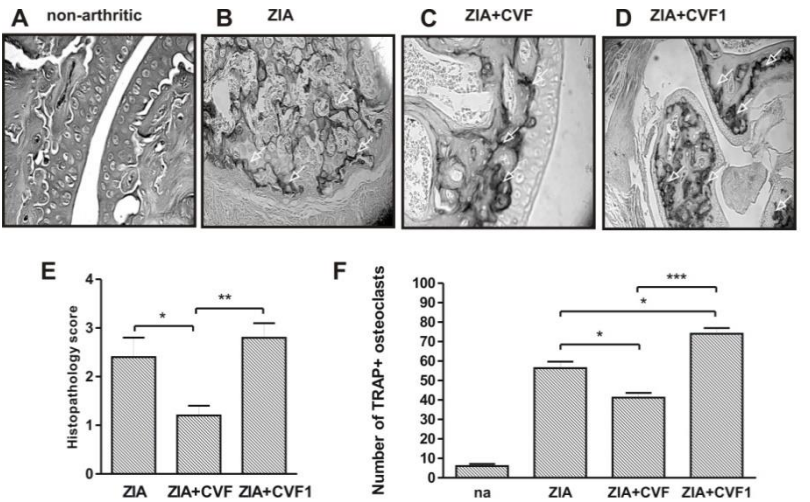

Figure 5. Representative TRAP (tartrate resistant acid phosphatase positive cells) stained joint sections at day 30 of ZIA. (A) Non-arthritic mice, (B) mice with ZIA, (C) a group pretreated with CVF (CVF) and (D) a group treated with CVF at days 7, 12 and 17 (CVF1). Arrows indicate TRAP positive cells. (E) Histopathological score of H&E stained joint sections. (F) Figure data representing the number of TRAP+ osteclasts. Data are means ± SEM from 3 determinations (n=5 per group). *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$, two-way ANOVA.

Influence of CVF-Treatment on RANKL, TRAIL and DR5 Expression by BM Cells

The assessment of RANKL, TRAIL and DR5 expression showed that the injection of CVF did not change the number of TRAIL and DR5 positive BM

cells while RANKL positive cells were increased in CVF-treated mice (Figure 6A, B, C, D). At day 7 of ZIA elevated number of RANKL expression was registered in arthritic mice prevented by CVF pretreatment (Figure 6E, H). The onset of arthritis was attended with an increase of TRAIL positive cells less exerted in CVF injected mice (Figure 6F, H).

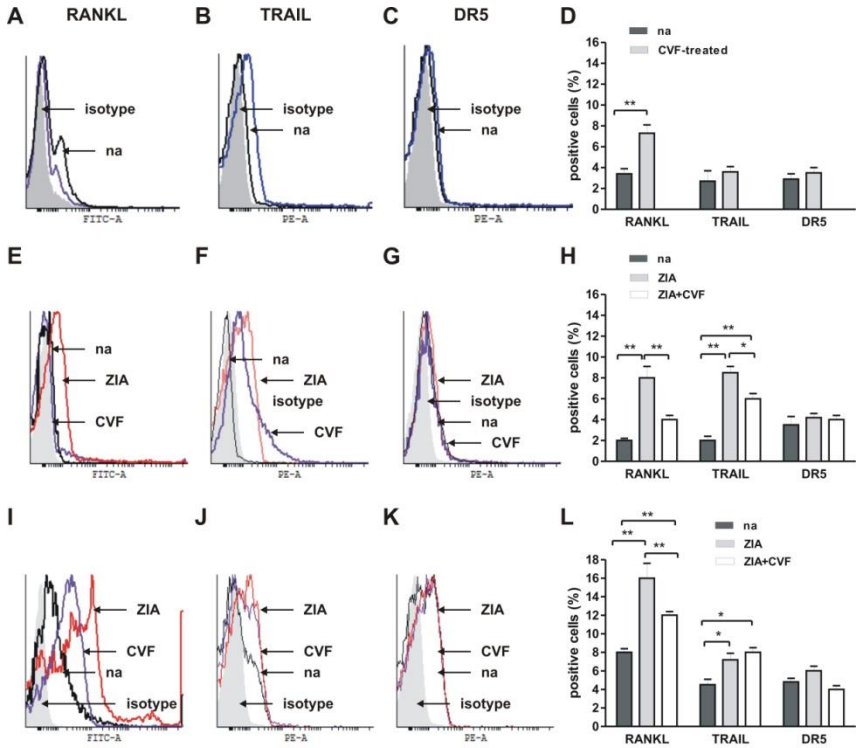

Figure 6. Flow cytometric analysis of RANKL, TRAIL and DR5 expression. BM cells were isolated from non-arthritic (na A, B, C) mice or 72 h after CVF injection (A, B, C) and graphical data (D). BM were isolated from na mice (E), from mice at day 7 of ZIA (F) and from mice at day 7 of ZIA pretreated with CVF (G) and figure data to these groups (H). BM were isolated from na mice (I), from mice at day 7 of ZIA (J) and from mice at day 7 of ZIA pretreated with CVF (K) and *in vitro* cultivated with 10 ng/ml M-CSF (1 day), 30 ng/ml M-CSF (3 days), followed by 50 ng/ml M-CSF+50 ng/ml RANKL (3 days), and figure data to these groups (L). Data are means ± SEM from 3 determinations (n=5 per group). *$p < 0.05$; **$p < 0.01$, two-way ANOVA.

The *in vitro* stimulation with M-CSF+RANKL induced RANKL expression in all groups, the highest effect being observed in cells isolated from mice with ZIA. This effect was diminished in CVF treated group (Figure

6I, L). There was no difference in the number of TRAIL positive cells between unstimulated and M-CSF+RANKL stimulated cells (Figure 6F, H and Figure 6J, L). Similar results were obtained for non-arthritic, ZIA and CVF-treated ZIA mice in regard to DR5 positive cells nevertheless stimulated or not stimulated (Figure 6G, H and 6K, L).

DISCUSSION

In the present study we used a murine model of RA to evaluate the effect of the depletion of complement system on the *in vitro* and *in vivo* osteoclast formation. CVF is a fragment of cobra C3 which unlike C3b is not cleaved by factor H and factor I. This leads to an unregulated activation of the AP, ending up in a total abolishment of complement activity. The essential role of the complement system for the tissue damage in RA is proved by the elevated levels of complement fragments and their pro-inflammatory receptors in the rheumatoid joints [30, 31]. There are relatively limited data on the role of complement activity in rheumatic disorders. Most of the synoviocytes in chronic joint inflammation express receptors for complement factor C3 and the complement decay-accelerating factor (DAF) [32, 33]. Under normal conditions bone is maintained by two groups of cells, bone-resorbing osteoclasts and bone-forming osteoblats. The impaired bone homeostasis in RA patients is a result of prevalent action of osteoclasts [34]. They are multinucleated giant cells derived from mononuclear cells of the monocyte/macrophage lineage in the presence of two stimulating factors named M-CSF and RANKL. *In vitro* M-CSF pretreatment of BM cells for up to four days is used to increase the number of osteoclast progenitors [35]. Based on this scheme we observed that CVF alone was able to replace the action of both M-CSF and RANKL, promoting osteoclast differentiation of BM cells. It might be suggested that CVF locally provoked the release of M-CSF and RANKL and thus facilitated osteoclastogenesis. The increased number of MNCs well correlated with the elevated TRAP activity. We also found that the combination of CVF and RANKL had more powerful effect (Figure 1) compared to each of them alone, which suggests their additive action. IFN-γ is one of the cytokines with dual effect on osteoclastogenesis and bone resorption. The overall effect probably depends on the balance between its direct anti-osteoclastogenic action and the indirect pro-resorptive activity [36]. IFN-γ causes degradation of the RANK adapter protein TNF receptor–associated factor 6 (TRAF6) that provokes a decrease of the

RANKL-induced activation of NF-κB and c-Jun N-terminal kinase [37]. IFN-γ also inhibits RANKL-stimulated cathepsin K and lowers cathepsin S and cathepsin L gene expression in preosteoclastic cells [38]. Another possibility for the influence of osteoclast differentiation is through apoptosis of osteoclast progenitors [39]. On the other hand, IFN-γ induces class II expression thus supporting antigen presentation and, causing T cell activation and release of RANKL and TNF-α [40, 41]. In the present experiments we established that M-CSF/RANKL stimulation increased IFN-γ production by BM cells. CVF itself raised IFN-γ production and showed additive effect with M-CSF. These results support the concept that the increased amounts of IFN-γ can favor osteoclast formation at least *in vitro*.

Vitamin D_3, is a hormone capable to enhance RANKL expression *in vitro*. Treating osteoblastic cells with Vitamin D_3 induces RANKL expression, which in turn provokes osteoclastogenic processes. At the same time, active vitamin D compounds such as calcitriol, alfacalcidol and eldecalcitol have been used as therapeutic drugs due to their ability to increase bone mineral density in patients with osteoporosis [42]. Thus, the effects of active vitamin D compounds on bone resorption *in vitro* and *in vivo* might be opposite. Vitamin D3 receptors are indentified in many cell types of the skeleton like chondrocytes, osteoblasts, osteocytes, and osteoclasts. Osteoclast precursors from vitamin D_3 deficient mice can be induced by vitamin to form osteoclasts in the presence of osteoblasts, indicating that osteoblast is the object of vitamin action with respect to osteoclast formation [43]. In the present study, we established that the addition of Vitamin D_3 during the cultivation moderately enhanced osteoclast formation in BM cell cultures but this effect may be amplified by co-culture with CVF. On the other hand, our results showed that CVF inhibited osteoblast and osteocyte formation in calvarial cell cultures induced in the presence of dexamethasone. The number of osteoblast nodules was significantly reduced by venom treatment in parallel with increased appearance of fibroblasts. It should be noted that at the peak of inflammation (day 7) in BM of ZIA mice mature osteoclasts were detected. *Ex vivo* stimulation of BM cells derived from arthritic mice pretreated with CVF increased the percentage of MNCs. This result might be explained with CVF-triggered release of RANKL, leading to osteoclast generation. We identified a number of osteoclasts in cultures with CVF, although no osteoclastogenic stimuli were added.

RANKL is an important molecule in bone metabolism and is produced by infiltrating active T cells and macrophages in the synovial tissues of patients with active RA [44, 45]. The development of ZIA was coincided with an

increase of RANKL positive megakaryocytes in BM but was not the case in the CVF pretreated group. Therefore, venom pretreatment lowered the number of RANKL positive cells in the synovium. Evidently, the lack of functional complement at the point of arthritis initiation was important for the limited osteoclast persistence in the synovium. Periodically repeated complement activation was associated with high osteoclast accumulation in the bone of ZIA mice. Our results are relevant to the situation in RA patients when joint inflammation might go along with another complement activating processes such as infection, leading to re-activation of RA. We looked for a correlation between complement activity and the presence of RANKL positive cells in BM as well as we aimed to find whether the expression of TRAIL and its receptor DR5 was altered. Three days after CVF treatment (that corresponded to the point of ZIA initiation) we observed an increase of the percentage of RANKL positive cells in the BM compared to non-treated controls. Approximately 8% of BM cells obtained at day 7 of ZIA expressed RANKL and this number was increased by 2 fold after *in vitro* stimulation with M-CSF+RANKL. The presence of functional complement activity when arthritis was induced appeared to be decisive for the late bone erosion. The percentage of RANKL positive BM cells from mice pretreated with CVF was lower and moreover, they were less sensitive to M-CSF+RANKL stimulation *in vitro*. These results clearly show that complement participates in the regulation of bone resorption through RANKL-dependant mechanisms.

Elevated TRAIL levels in the synovial fluid were detected in patients with RA but not in patients with OA or SpA [46]. Fibroblast-like synoviocytes (FLS) can be divided into TRAIL-sensitive and TRAIL-resistant. TRAIL-sensitive to apoptosis FLS express lower levels of TRAIL-DR5 and TRAIL-DR5 and thus decreasing the apoptosis of FLS. TRAIL and its receptor DR5 can play dual role in RA. The interactions between TRAIL and its receptor are important for the regulation of osteoclast apoptosis. *In vitro* data pointed that TRAIL receptors are up-regulated in the early stage of osteoclast differentiation and down-regulated at the late phase [47]. DR5 can cause osteoclast apoptosis, a process that might be canceled by the addition of anti-DR5 neutralizing antibodies. TRAIL-induced apoptosis activates intracellular caspase-B and BID pathways. BM cells obtained from CVF-injected mice at day 0 of ZIA induction did not cause any changes in the percentage of TRAIL and DR5 positive cells which was negligible alike in non-treated group. The peak of ZIA (day 7) was characterized with an appearance of TRAIL positive BM cells inhibited by CVF treatment. The *in vitro* stimulation with M-CSF+RANKL failed to increase the percentage of TRAIL positive cells as

well as DR5 expression of BM cells was not altered neither in non-treated nor in CVF-treated mice. In our previous studies we found that CVF-pretreatment in mice with ZIA reduced chronic synovitis [28]. Using this model of complement depletion associated with C3 and C5 inactivation, we extended our study on the complement-mediated mechanisms of osteoclastogenesis and the changes in bone resorption. In summary, CVF directly acted as triggering agent for *in vitro* generation of osteoclasts and reduction of pro-osteoblast differentiation. Complement elimination at the point of arthritic induction, delayed late bone resorption through inhibited RANKL expression and abolished osteoclast formation in the joints. The influence of functional complement on TRAIL/DR5 processes remains unclear. More investigations are needed to elucidate the role of complement in the apoptotic events in ZIA. The present model proved to be convenient for investigations of the bone remodeling complement-mediated mechanisms.

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COMPETING INTEREST STATEMENT

The authors declare no competing interests relevant to this work.

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Chapter 3

THE EFFECT OF SEPSIS AND BACTERIAL INFECTION ON IMMUNE SYSTEM ACTIVATION, BONE REMODELING AND OSTEOCLAST ACTIVATION

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ABSTRACT

It is well known that diseases affect the general condition of the hosts. The hosts are usually much weaker in strength and vitality after bacterial infection. It has long been appreciated that bacterial diseases produce detrimental effects on the metabolism and remodeling of the bones. Gram-negative bacteria produced lipopolysaccharide is a major mediator of bone loss. LPS enhances differentiation and activation of the osteoclast cells. Intracellular signaling induced by LPS includes NF-κB activation, p65 translocation and IκB-α degradation in mature osteoclast and osteoclast precursor cells. LPS also augments TNFα enhanced osteolysis.

There are many Gram-negative bacterial toxins involved in the inhibition of osteoblast differentiation and bone loss. Cell wall virulence

factors and surface associated proteins of Gram-positive bacteria are also involved in osteoclast differentiation. Virulence factor lipoteichoic acid inhibits bone remodeling function by osteroclasts. *Staphylococcus aureus* infected osteoblasts show heightened TRAIL receptor expression and the apoptosis. Infection-induced general or local activation of B and T lymphocytes are also related with osteoclast activation. General activation of the T lymphocytes is associated with septic loosening of the bone implants. OPG ligand expression on $CD4^+$ T cells, increased local $CD8^+$ T cells, and increased RANKL expressing T cells has been documented upon bacterial infection-related bone loss. All these infection-related activation of immune functions are intertwined with poor bone remodeling and bone loss.

INTRODUCTION

Bone is an organ far more active and ever changing than has been appreciated. Bone absorption and reshaping is a process named bone remodeling that is intricate and under constant control. The key players are RANK and its ligand, RANKL. RANK expressed on the surface of osteoclast precursor cells. The receptor-ligand binding promotes signaling cascade for osteoclast activation and differentiation. The action of RANKL on osteoclasts is opposed by the soluble receptor OPG as a decoy receptor. OPG ligand while present may alleviate the decoy effect of OPG. It is now evident that the bone and immune system are co-regulated by many shared cytokines and signaling molecules. Sepsis is the leading cause of death among critically ill patients. Focal bacterial infection such as periodontitis and osteomyelitis cause profound morbidity in the host. These bacterial infections shaped host immune system and bone remodeling process alike.

Bacteria and their products cause inflammatory bone loss in various infections, including periodontitis, chronic otitis media, infective joint disease and osteomyelitis. Infection of orthopedic implants, hospital acquired or idiopathic of the total knee or total hip replacement causes significant morbidity and loss of the implant. In case of chronic inflammation associated with gram-negative bacterial infection, the bacterial product endotoxin, lipopolysaccharide and mononuclear cell infiltration have been implicated as significant factors for aberrant bone remodeling, local osteoclatogenesis and bone loss (Nair 1996; Dumitrescu 2004; Taubman 2005; Doucet 2006; Walsh 2006). Lipopolysaccharide (LPS) is a large molecule consisting with a lipid and a polysaccharide joined by a covalent bond. It is found in the cell walls of

gram-negative bacteria and act as an endotoxin. LPS has been shown to induce osteoclast formation and bone loss in vitro and in vivo (Chiang 1999; Zou 2002; M¨ormann 2008). LPS induced NF-κ B activation, p65 translocation, Iκ Bα degradation in mature osteoclast and their precursors. NF-κ B activation is essential for LPS induced osteoclastogenesis and bone resorption (Miyazaki 2000; Itoh 2001; Yip 2004). LPS binds to the Toll-like receptor 4 (TLR4) on the surface of fibroblast and macrophages to induce the production of inflammatory cytokines (Kikuchi 2001; Itoh 2003). NF-κ B pathways are important for LPS-induced release of inflammatory mediators, including prostaglandin E2, TNFα, IL-1, IL-6, RANKL and macrophage-colony stimulating factors from cells adjacent to osteoclasts (Abu-Amer 1997; Ueda 1998; Bar-Shavit 2008). These mediators contribute to the development of osteoclast progenitor cells and osteoclast maturation and activation (Chiang 1999; Lam 2000). LPS also enhances the survival of osteoclast directly via TLR-4 signaling (Hou 2000; Takami 2002; Itoh 2003). In the skeletal bone remodeling unit, osteoblasts regulate osteoclast differentiation and activity through receptor activator of nuclear factor -κ B ligand (RANKL), osteoprotegerin (OPG) and cytokines, such as IL-6. RANKL induces osteoclast maturation by binding to its receptor, RANK, on osteoclast precursors. OPG alternatively named osteoclastogenesis inhibitory factor, functions as a soluble decoy receptor that inhibits osteoclast formation by competing with RANK for RANKL binding (Baud'huin 2013). Osteocytes commend the initiation of bone remodeling through RANKL expression. Osteocytes are embedded within the lacuna-cannliculum network of the trabecular bones. RANK is expressed on osteoclast progenitor cells and mature osteoclasts. Binding of RANK-RANKL starts osteoclast differentiation and maturation. Many of the osteotropic cytokines including IL-1, IL-6, IL-11 induced RANKL expression on osteoblasts (Arron 2000; Takayanagi 2007; Walsh 2014). RANK-RANKL interaction is the center of bone remodeling process. Variable bacterial product and subsequent astray biological effects sets off aberrant bone remodeling processes. More than one product from a single bacterium may induce osteolysis. For example the *Actinobacillus actinomycetemcomitans* infection produces surface associated protein, capsular polysaccharide, cpn60 and gapstatin that are related with osteolysis (Nair 1996). Almost all of the cells and cytokines involved in biological bone remodeling unit have been documented being affected by bacterial product. For example LPS produced by P*orphyromonas gingivalis* and *E. coli* may induce osteoclast formation in the absence of osteoblasts. This has been shown

to be dependent on OPG ligand and TNFα (Jiang 2002). The non-LPS component of endotoxin, i.e., lipid-A associated protein from a number of periodontopathic bacteria can stimulate bone resorption and induce synthesis of IL-6 (Reddi 1995; Reddi 1995).

It is quite reasonable to find other bacterial toxins that augment the osteolytic effect of LPS. The 146-kDa *Pasteurella multocida* toxin (PMT) activates heterodimeric G proteins of the $G_{\alpha q/11}$-family which activates RhoA. Activated RhoA transactivates the mitogen activated protein (MAP) kinase cascade via Rho kinase, Ras, MEK and ERK, resulting in inhibition of osteoblast differentiation and a destruction of nasal turbinate bones in severe case of atrophic rhinitis. *P. multocida* is the causative agent of atrophic rhinitis and growth retardation of young pigs and causes similar symptoms in domestic pigs, rabbits, wild pigs and cattle (Wilkie 2012; Siegert 2013). Muramyl dipeptide (MDP), the minimal structure unit for the immunological activity of bacterial peptidoglycans, is ubiquitously expressed in gram-positive and gram-negative bacteria alike. MDP enhances LPS-induced proinflammatory cytokines in monocytes (Yang 2001). MDP upregulates RANKL and TLR4 expression in osteoclasts treated with LPS or TNFα, also enhances LPS-induced RANKL as well as TLR4 expression in bone stroma cells (Ishida 2015). MDP also enhances MAP kinase signaling, including ERK, p38, and JNK in bone stroma cells. This is another example that microorganisms often exert multiple routes to augment their pathophysiological effects in the host.

Gram-positive bacteria express lipoteichoic acid (LTA) which is comparable with LPS in gram-negative bacteria. LTA is a major cell-wall virulence factor of gram-positive bacteria being composed of lipidated polymer with repeating units of glycerol phosphate or ribitol phosphate. LTA invokes a MyD88-dependent signaling pathway and stimulates via TLR2 but not TLR4 (Ellingsen 2002). LTA dose-dependently inhibits the differentiation of osteoclast precursors to mature osteoclasts resulting in inhibition of bone resorption function. But instead LTA heightens inflammatory potential in the osteoclast precursors. LTA inhibits phosphorylation of ERK and JNK in osteoclast precursors stimulated with macrophage colony stimulating factor (M-CSF) and RANKL via MyD88 dependent and –independent pathways (Yang 2009). *Staphylococcus aureus* is a common pathogen in osteomyelitis. The osteolytic pathology of S. aureus is also involved in the induction of apoptosis of osteoblasts in the affected sites. Infected osteoblasts express the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5. In the meantime, the decoy receptor OPG is inhibited. Thus the

apoptosis of infected osteoblasts via TRAIL and TRAIL receptors signaling is enhanced and thus results in bone loss (Tucker 2000; Alexander 2003).

B and T lymphocytes are also involved in the pathology of bone loss due to bacterial infection. A number of anaerobic bacteria have been shown involved in periodontitis and the loss of teeth. In the case of *Actinobacillus actinomycetemcomitans*-induced periodontal disease high osteoclast formation, increased number of $CD8^+$ cytotoxic T cells and TNFα expression in the affected connective tissue has been detected (Bezerra 2012). Osteoclast differentiation factor, RANKL, is also expressed in activated B and T cells in affected tissue (Kawai 2006). Antigen-specific B cells from *A. actinomycetemcomitans* infected periodontitis tissue express RANKL and induce significant levels of osteoclast differentiation (Han 2005). The T cells over infected sites show heightened expression of RANKL. The expression of RANKL is predominantly in Foxp3 negative and $Foxp3^{dim}$ cells, but not in $Foxp3^{high}$ cells (Ernst 2007). It was generally known that Treg cells inhibit osteoclast differentiation, bone resorption with a mechanism dependent on IL-6, IL-10, and IFNγ (Buchward 2012). Adoptive transfer using Foxp3-positive $CD8^+$ Treg cells could decrease bone loss in response to RANKL application (Buchward 2013).These observation indicates that bacterial infection shape the local T cell population and exert impact on local osteogenesis and bone remodeling. $CD4^+$ T cells at the site of *A. actinomycetemcomitans* infection produce osteoprotegerin ligand (OPG-L) and trigger osteoclast differentiation (Lacey 1998; Teng 2000). The OPG-L binding to OPG abolishes the decoy effect of OPG molecule in RANK-RANKL interaction and osteoclastogenesis. Systemic T cell activation is detected in patients with infectious implant. While patient that lost the implant due to aseptic reasons did not show systemic T cell activation. Activated T cells are shown to initiate crosstalk between RANKL and IFNγ (Takayanagi 2000).

There are multiple cytokines that regulate the differentiation and maturation of osteoclast. The osteoclast stimulatory cytokines include: IL-1β, IL-6, IL-8, IL-11, IL-15, IL-32 and TNFα. The inhibitory cytokines are: IL-4, IL-10, IL-13, IL-18, IL-33 and IFNs (Cappariello 2014). Lastly but not the least important is the host factor in shaping the clinical outcome of the infection. *Porphyronomonas gingivalis* is a gram-negative anaerobic bacterium being important pathogen in chronic periodontitis and tooth loss. Host factors are associated with the disease severity and progression. Increased IL-18 production of the host is associated with reduced local INFγ

levels. INFγ was involved in accelerated degradation of the RANK adaptor protein, tumor necrosis factor receptor-associated factor 6 (TRAF6). Reduced INFγ levels in *P. gingivalis* infected tissues result in strong signaling of RANKL and osteoclast differentiation and activation (Yoshinaka 2014).

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Chapter 4

DC-STAMP: REGULATOR OF CELL FUSION AND BONE HOMEOSTASIS

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ABSTRACT

Bone is a dynamic tissue that continuously undergoes remodeling with diverse functions including locomotion, hemopoiesis, and protection of internal organs. Osteoimmunology has recently become an emerging field of musculoskeletal research, which connects the bone and immune systems. Results from osteoimmunology studies provide a new concept that bone is not only an essential component of the musculoskeletal system, but it also participates in immune regulation. Many important factors involved in osteoimmunology regulation also participate in bone homeostasis. Bone homeostasis is achieved by a coordinated action between bone-synthesizing osteoblasts and bone-degrading osteoclasts. An imbalanced action between osteoblasts and osteoclasts often results in pathological bone diseases: osteoporosis is caused by an excessive osteoclast activity over that of osteoblasts, whereas osteopetrosis results from an increased osteoblast activity. This review focus on Dendritic Cell-Specific Transmembrane Protein (DC-STAMP), an important

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protein currently considered as the master regulator of osteoclastogenesis. DC-STAMP is required for cell-cell fusion during osteoclast differentiation. Intriguingly, the frequency of circulating DC-STAMP+ cells is elevated during the pathogenesis of psoriatic diseases. Current data collectively suggest that DC-STAMP also plays an imperative role in bone homeostasis by regulating the differentiation of both osteoclasts and osteoblasts. This article summarizes our current knowledge on DC-STAMP by focusing on its interacting proteins, its regulation on osteoclastogenesis-related genes, its possible involvement in immunoreceptor tyrosine-based inhibitory motif (ITIM)-mediated signaling cascade, and its potential of developing therapeutics for clinical applications.

Keywords: DC-STAMP, osteoclasts, osteoblast, bone fracture, healing, RANK ligand, cell fusion

I. INTRODUCTION

Bone is a dynamic tissue that continuously undergoes remodeling through a concerted action between bone-resorbing osteoclasts (OC), bone-forming osteoblasts (OB), and osteocytes, the long-lived osteoblast-derived cells that reside within the bone matrix to monitor and orchestrate OC::OB mediated bone homeostasis [1-3]. Imbalanced bone homeostasis results in pathological inflammatory bone diseases. For example, osteoporosis results from excessive bone erosion activity of OC, whereas osteopetrosis arises from increased bone synthetic OB activity. Therefore, modulating the relative balance between OB and OC remains an effective therapeutic strategy for treating bone diseases such as osteoporosis and rheumatoid arthritis (RA).

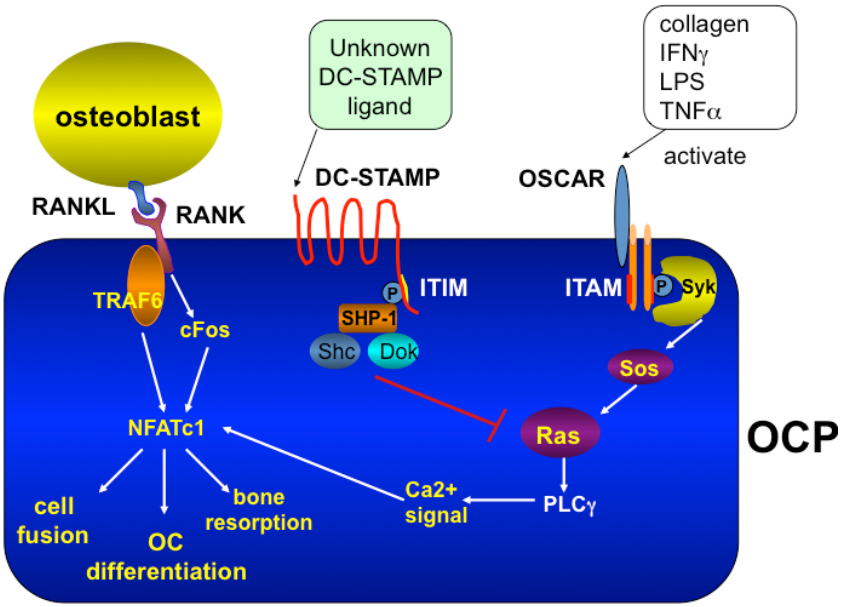
OC are multinucleated giant cells that are derived from myeloid precursors, and are the only cell type known to form ruffled membranes and degrade bone [3]. In response to macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-kB ligand (RANKL) stimulation, single nucleated osteoclast precursors (OCPs) differentiate into mature multinucleated OC with bone erosion activity through several rounds of cell-cell fusion. In addition to being “bone eaters”, intriguingly, OC can regulate OB in a mutual fashion and other cells through bone remodeling to maintain skeletal integrity. Thus, OC are the key mediators of bone homeostasis which determine the onset and outcome of the bone pathogenesis [4, 5].

The differentiation of OC, a process termed osteoclastogenesis, is a complex but orderly event that involves aggregation of cell surface proteins required for cell-cell fusion, initiation of signaling cascades after cytokine stimulation, and activation of gene transcription programs that direct the transition from a single nucleated monocyte to a functional bone-degrading multinucleated polykaryon [3]. Among all proteins and mediators essential for OC formation, Dendritic cell-specific transmembrane protein (DC-STAMP) is currently considered the master regulator of osteoclastogenesis [6-8]. DC-STAMP is a 470-amino acid protein that is encoded by a single long open reading frame, has a signal sequence, 7 putative transmembrane regions, 3 potential N-linked glycosylation sites, a protein kinase C phosphorylation site, and a 72-residue cytoplasmic tail containing multiple serines, 2 of which may be the targets of phosphorylation. Knocking-down of DC-STAMP completely abrogates cell-cell fusion during OCgenesis, resulting in a mouse strain that produces only single nucleated OC with a mild osteopetrosis phenotype [9]. We previously identified an immunoreceptor tyrosine-based inhibitory motif (ITIM) on the cytoplasmic tail of DC-STAMP, suggesting its role in signaling (Figure 1). The role of ITIM on DC-STAMP function remains currently unknown. Based on the recent studies of ours and others, in this review, we discussed the function of DC-STAMP beyond cell-cell fusion. First, we update current research progress on DC-STAMP and its potential on clinical application. Second, we review several important cellular events of osteoclastogenesis which is likely to be regulated by DC-STAMP.

II. Human Bone Diseases Caused by Osteoclast Dysregulation

Human and mouse studies have revealed that several bone diseases are linked to the dysregulation of OC function and differentiation. These diseases are recently well described and reviewed by Crockett et al. [10]. With this thorough review, in this chapter we will elaborate on only two diseases, pycnodysostosis and Paget's disease. Patients with pycnodysostosis, a genetic disease that causes short stature and brittle dense bones (osteosclerosis), have dysfunctional osteoclasts due to mutations in the cathepsin K gene [11]. Paget's disease of the bone (PDB) is characterized by a disorder in the bone metabolism, which is usually associated with impaired osteoclast activity [12]. In the early onset of Paget's disease, heterozygous insertion mutations are

found in the RANK signal peptide, suggesting a close link between RANK signaling, osteoclasts and human Paget's diseases.



Adapted from Chiu et al., 2012, JBMR, 27(1), 79-92.

Figure 1. A model proposing a putative role of DC-STAMP in RANKL-ITAM-ITIM signaling network. Dual signals, one from the activation of RANK receptor and one co-stimulatory signal from the immunoreceptor tyrosine-based activation motif-containing (ITAM-containing) receptors/adaptor, are required for osteoclastogenesis (76). Up to date, it is still poorly understood how co-stimulatory signals are integrated with RANK signaling. The presence of an ITIM on the cytoplasmic tail of DC-STAMP (21) and a common counteracting action between ITIM- and ITAM- bearing receptors suggests a possible involvement of DC-STAMP in the RANK-ITAM-ITIM signaling network. In this model, DC-STAMP was considered to regulate osteoclastogenesis through its ITIM and the Ca2+/NFATc1 axis.

Intriguingly, DC-STAMP was identified as a susceptible protein whose mutation is associated with Paget's disease by genome-wide screening [13-15]. Two variants, one close to and one within the DC-STAMP coding region showed a correlation with Paget's diseases. A bigger cohort of patients with Paget's disease needs to be analyzed to confirm this initial observation.

Human bone diseases are usually diagnosed by plain radiographs. Musculoskeletal ultrasound is recently becoming more popular based on its ability to visualize soft tissues, assess blood flow with Power Doppler and

high resolution images that reveal early bone erosion in inflammatory arthritis. Serum and cellular biomarkers are recently considered as surrogates of bone resorptive diseases at the early stages of pathogenesis. Identification of disease-specific biomarkers is the current trend for biomedical research, which if successful, may provide valid and reliable markers for the prevention and diagnosis of bone disease. In this regard, human peripheral blood monocytes (PBMs) may serve as an important source for biomarker identification, since circulating osteoclast precursors are attracted to bone to carry out homeostatic remodeling or in the case of inflammatory arthritis, direct pathologic bone resorption. Cytokines present in the blood have profound effects on osteoclast differentiation, activation, and apoptosis. Taken together, human PBMs are appropriate tools for bone- and inflammation-related study [16].

Along this line, osteoclast activity and subtypes are considered as appropriate biomarkers, which reflect the physiology and pathology of bone diseases [17]. Because the cell population of PBMs in human blood is greatly affected by several factors such as the time of blood collection, medication, patient BMI and exercise, precautions need to be taken into consideration when testing potential biomarkers in human peripheral blood.

III. Potential Clinical Applications of DC-STAMP

A. Biomarkers for Psoriatic Diseases and Bone Fractures

1. Ps to PsA Transition and Medication Response

Psoriatic arthritis (PsA) is an inflammatory joint disease that affects over 600,000 Americans [18]. Bone damage develops in half these patients within the first 2 years of disease, often leaving them with impaired function and diminished quality of life [19]. The advent of anti-Tumor Necrosis Factor therapies (TNFi) has greatly reduced bone damage in PsA patients, however, only 50-60% of patients respond to these agents. To improve these outcomes, we must address 2 major gaps: a limited understanding of key events that underlie pathologic bone destruction and the absence of biomarkers to predict TNFi response and identify early TNFi responders to facilitate optimization of therapy.

To fill these gaps, our research group has been focusing on testing the potential of DC-STAMP as the biomarkers of psoriatic diseases [20, 21]. Psoriatic disease is an attractive disease paradigm for identification of arthritis

biomarkers in psoriasis patients, since the transition from skin psoriasis (Ps) to inflammatory arthritis (PsA) is 10 years on average.

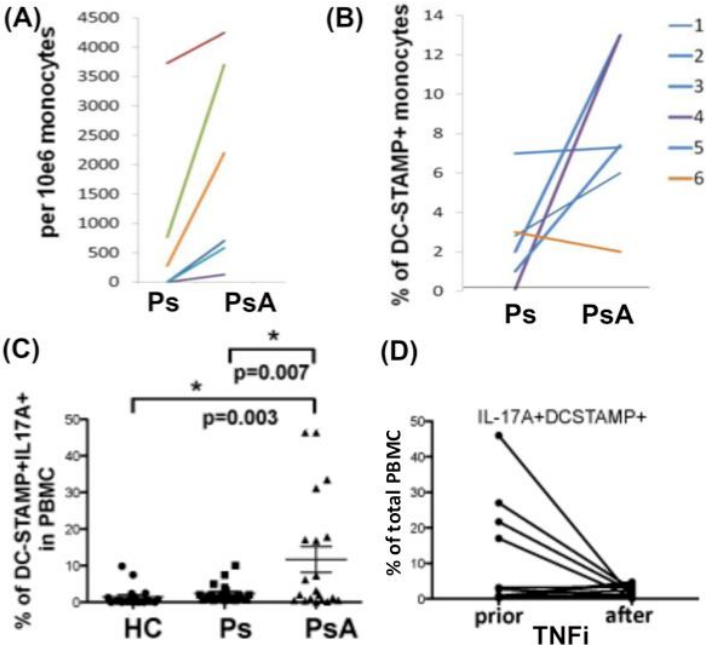


Figure 2. Two pilot studies suggest the potential of circulating DC-STAMP+ cells in human blood as psoriatic disease biomarker. (A)-(B) The frequency of DC-STAMP+ cells was elevated when psoriasis (Ps) patients progress into psoriatic arthritis (PsA) with musculoskeletal symptoms, suggesting the potential of DC-STAMP as a biomarker to predict disease status. (A) osteoclast counts and (B) frequency of DC-STAMP+ cells on the stage of Ps and PsA for 6 Ps to PsA patients. (C)-(D) Evaluate the potential of DC-STAMP+IL17A+ cells to serve as a TNFi response biomarker. The frequency of circulating DC-STAMP+IL17A+ cells were examined in HC, Ps and PsA cohorts (n=20/cohort, HC, healthy control).

Our preliminary data showed that DC-STAMP is not only a good biomarker whose expression is elevated after Ps to PsA transition (Figure 2B) with a positive correlation with elevated circulating OCP (Figure 2A), but also a valid response biomarker, which declines rapidly in responders to anti-TNFα medication (Figure 2C and 2D). The results derived from this small cohort of PsA patients were presented at the Annual Rheumatology meeting at year 2012 [20]. We are currently recruiting and analyzing a bigger cohort of PsA patients to establish a set of data, which can determine the potential of DC-STAMP as the biomarker for TNFi responses and psoriatic disease

conversion (Ps to PsA transition) with the power of statistical significance [22].

2. Fracture Healing

Bone fracture is one of the top clinical challenges worldwide, which substantially impacts the cost of healthcare and the life quality of patients. On average, more than 20 billion dollars are spent to treat 7.9 million bone fracture patients in the United States every year [23-25]. Delayed healing and non-union fractures, occurring in 10-15% of total bone fractures, are the major causes of complications in treating bone fractures. The life quality of patients with delayed and non-union healings is reduced due to a prolonged period of pain, disability, and repetitive operative interventions [26]. They have a higher comorbidity, are more susceptible to additional fractures, and medical expenses are twice as high as those without non-union bone repair [24]. Absence of efficient medications and reliable diagnostic tools are two major clinical challenges. In addition, a limited number of available animal models has hindered the understanding of the molecular mechanism underlying non-union fractures and delayed healing [27, 28]. Based on our preliminary data and experience of analyzing human blood for the past 8 years, our result on the cohort of bone fracture patients is extremely limited (unpublished data, manuscript in preparation) when combined with our results obtained from the murine bone fracture (unpublished data, manuscript in preparation), we considered that the expression level of DC-STAMP and the frequency of circulating DC-STAMP+ cells in peripheral blood have a great potential to be the biomarker that can monitor the status of bone healing and determine the types of bone healing (delayed, non-union, or normal healing).

B. Targeted Drug Delivery to Promote Bone Healing

Our preliminary data showed that DC-STAMP+ cells are recruited to the bone fracture sites (unpublished data, manuscript in preparation) and concentrated on OB proximity (submitted manuscript). An elevated DC-STAMP+ cell frequency in human blood was detected at a certain stage of bone healing (unpublished data, manuscript in preparation). Using video capturing, the trafficking of DC-STAMP+ cells to fracture sites occurs in an organized order (OB first followed by OC, unpublished data). Collectively, these data suggest that DC-STAMP+ cells are attracted to the bone fracture sites either through cytokines, chemokines, or unidentified DC-STAMP

ligands. Our data suggested that DC-STAMP, together with MMP-2 and MMP-9 [29, 30], can be a useful tool for monitoring, in parallel with other bone turnover markers, in order to evaluate the bone remodeling and tissue healing processes. Based on the characteristics that DC-STAMP+ cells are homing to bone fracture sites, a step forward of these observations is to molecular engineer a chimeric DC-STAMP protein by which DC-STAMP is able to bring small peptides/proteins such as bone morphogenetic protein 2 (BMP-2) or MMP-9 [31-35] with bone healing effects to bone fracture sites.

IV. Current Updates on DC-STAMP Research

A. OC-STAMP vs. DC-STAMP

In addition to DC-STAMP, OC-STAMP is another critical RANKL-induced, multipass transmembrane protein that promotes the formation and differentiation of osteoclasts [36, 37]. In addition to their structure similarity, DC-STAMP and OC-STAMP shared many common features. They both promote cell-cell fusion between osteoclast precursors when overexpressed; the presence of antibody or siRNA will block early events in osteoclastogenesis such as fusion, but leave late stage osteoclast differentiation unaffected; they are both induced by RANKL. There are so many similarities between DC-STAMP and OC-STAMP, however, they show differences in many ways as summarized in Table 1. Of note, although both DC-STAMP and OC-STAMP are essential for osteoclastogenesis, they are distinct proteins and are not interchangeable. DC-STAMP deficiency cannot be complemented by overexpression of OC-STAMP, and vice versa.

Table 1. Comparison between OC-STAMP vs. DC-STAMP

	OC-STAMP	DC-STAMP
TM	6-pass	7-pass
Motif	no ITIM	ITIM
bone phenotype	No	Increased BV/TV Trabecular bone volume
NFATc1	no effect	No effect on mRNA Has Effect on protein

DC-STAMP deficiency cannot be complemented by overexpression of OC-STAMP, and vice versa.

How DC-STAMP and OC-STAMP regulate osteoclastogenesis and whether they interact with each other remains to be further defined. Based on current data, 3 possibilities were proposed by Yang et al., for the relationship between DC-STAMP and OC-STAMP: (1) They are ligands of each other; (2) they have their own distinct ligands. Once engaged, they provide positive reciprocal signals to each other to allow the fusion to proceed; (3) they form a dimer as a receptor complex on the cell surface. Experiments such as immunoprecipitation and reciprocal cultures between DC-STAMP-/- and OC-STAMP-/- cells will help to determine these possibilities.

It will also be interesting to examine whether OC-STAMP glycosylation will affect DC-STAMP surface expression or function, by taking advantage of the available mutated glycosylation-deficient (N162D) OC-STAMP [36]. In addition, based on the structural similarity between DC-STAMP and OC-STAMP and current topology analysis by the TMHMM algorithm [36], it will be interesting to investigate whether DC-STAMP is a 7-pass transmembrane protein as originally considered [9]. According to a recent review and two studies on OC-STAMP [8, 36, 37], the topology of DC-STAMP (whether it's a 5, 6, or 7-pass transmembrane protein) remains an open question which needs further investigation to determine its similarity to OC-STAMP, a 6-pass transmembrane protein. To date, these two STAMPs are the only cell-type specific proteins essential for cell-cell fusion of generating osteoclasts and giant cells, making their role in osteoclastogenesis of particular interest and importance. Elucidating the involvements of DC-STAMP and OC-STAMP on the split and re-seal of the lipid bilayers of membranes during cell-cell fusion, and their possible interplay in the signaling

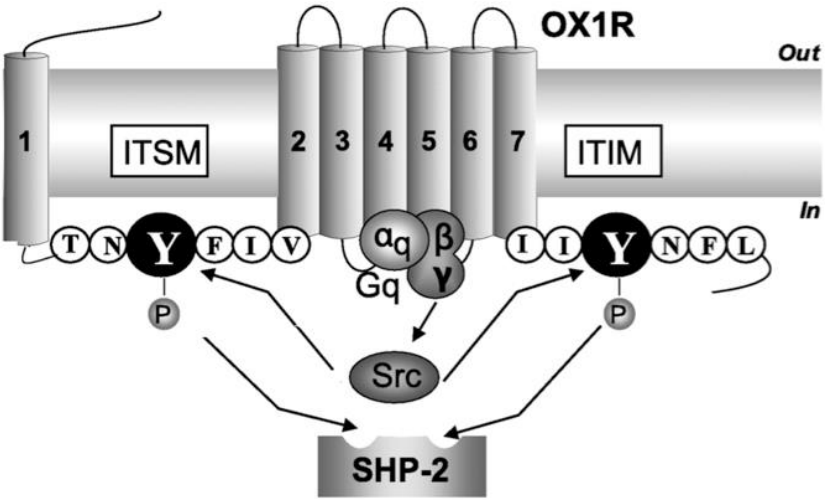
B. Is DC-STAMP a GPCR-Like Protein That Is Involved in the Apoptosis Regulation?

According to a recent good review on DC-STAMP [8], sequence alignment reveals a low similarity between DCSTAMP and G-protein-coupled receptor (GPCR), suggesting that DC-STAMP might belong to a new protein family. GPCRs are transmembrane receptor proteins that transfer signals across the cell membrane. The importance of GPCRs in chronic inflammatory bone diseases is recently highlighted by their involvement in many pathophysiological processes including pathways relevant to rheumatoid arthritis (RA), osteoarthritis (OA) and psoriatic arthritis (PsA) [38]. Based on

its 7-pass transmembrane feature, DC-STAMP was suggested to be a G protein-coupled receptor (GPCR)-like protein with similar function [39]. Our identification of an immunoreceptor tyrosine-based inhibitory motif (ITIM) on the cytoplasmic tail of DC-STAMP raised a possibility for its role in cell signaling [21], further supporting the role of DC-STAMP as a GPCR. Once activated by a yet to be identified soluble and/or membrane bound DC-STAMP-ligand, DC-STAMP subsequently induces a serial gene expression of fusogenic molecules [40], similar to a cascade employed by other GPCRs [41]. Intriguingly, although ITIM is not a common feature shared by the family of G proteins, this motif is included in a few G proteins, if not all, including orexin receptor OX1R [42] and bradykinin receptor B2 [41-46].

Given that ITIM is rarely present in GPCRs, the real biological functions of this group of GPCRs remains largely unknown. The studies of OX_1R could provide us some clues and shed light on the investigation of DC-STAMP function, if DC-STAMP belongs to this unique subset of proteins. El Firar et al., showed that the ITIM of OX_1R is phosphorylated on the tyrosine residue upon receptor activation, which subsequently allows the recruitment and activation of the tyrosine phosphatase SHP-2, leading to apoptosis. More intriguingly, another motif, immunoreceptor tyrosine-based switch motif (ITSM), is also present in OX1R and is mandatory for OX1R-mediated apoptosis [43]. The authors hypothesized that apoptosis is initiated and activated after activation of the tyrosine phosphatase SHP-2 via dual signals from ITIM and ITSM (Figure 3, modified and adopted from El Firar et al., [43]).

The apoptosis function of OX1R raises a possibility that the 7-pass transmembrane ITIM-bearing DC-STAMP is involved in the apoptosis of osteoclast precursors. Apoptosis of osteoclast is one important aspect of osteoclast research, since an overall enhanced OC survival is usually associated with an increased bone resorption. Updated research progress on osteoclast apoptosis has been recently well reviewed by Boyce [3]. Thus, we will not elaborate more. Briefly, apoptosis of osteoclasts, including osteoblast-induced osteoclast apoptosis [47], can be induced by cytokines and chemicals (such as estrogen, bisphosphonates) through various signaling pathways (such as Fas-ligand-induced, TNFα- induced). Genes that control the apoptosis of osteoclasts are potential new drug targets to treat osteoclast-mediated inflammatory bone diseases.



Adapted from El Firar et al., 2009, FASEB, 23(12), 4069-80.

Figure 3. Is DC-STAMP, like OX1R, an ITIM-expressing G protein-coupled receptor (GPCR)? The 7-pass transmembrane nature is a common feature of a GPCR. However, it is unusual to detect a tyrosine-based inhibitory motif ITIM on the cytoplasmic tail in the majority of GPCRs. To determine the likelihood of the 7-pass transmembrane, ITIM-containing DC-STAMP protein to be a GPCR, after a broad search, we identified OX1R, a GPCR which contain two unique motifs, one ITSM in its N'-terminus and one ITIM in it's cytoplasmic tail, as shown in this Figure. OX1R is the receptor of orexin and regulates apoptosis via Src phosphorylation and SHP-2-binding [43, 45].

Being a unique ITIM-bearing 7-pass transmembrane protein, whether DC-STAMP, like OX_1R, is also involved in the regulation of osteoclast apoptosis, remains an open question.

C. Bone Marrow Is the Major Reservoir of DC-STAMP+ OCPs

Osteoclast precursors are generated and reside within the bone marrow [48]. Osteoclasts were found at subchondral sites of erosion in the rheumatoid joint [49]. The local inflammation process is shown to be related to osteoclast differentiation on the marrow side of subchondral bone. Intriguingly, bone marrow Th17 TNFα+ cells have recently been shown to induce osteoclast differentiation and link bone destruction to inflammatory bowel diseases (IBD) [50, 51].

Taken together, these data suggest a functional role of bone marrow in local damage via cells residing and egressing from them [52]. Cells, usually at their naïve status, migrate out from bone marrow, circulate into peripheral blood, and are recruited to sites in bone where they will develop into mature osteoclasts after activation by local inflammatory cytokines and chemokines [3].

DC-STAMP was previously shown to be the biomarker of osteoclast precursors [21, 53]. DC-STAMP+ cells are present in both human and mouse bone marrow [54, 55] (unpublished data, manuscript in preparation). They egress rapidly from bone marrow and aggregate surrounding the bone fracture sites.

The signal which initiates the migration of OCP from bone marrow to blood remains largely unknown. Intriguingly, two pieces of information, one from human and one from mouse, suggest that the property of OCPs within the bone marrow could be altered by diseases, and thus could be a surrogate that reflects overall health conditions of individuals. For example, a lower frequency of osteoclasts were reported in the bone marrow of diabetic mice, which is accompanied by a decreased DC-STAMP expression [56]. In addition, our analyses on the OCP frequency of human bone marrow and blood suggest that the expression level of DC-STAMP and the frequency of DC-STAMP+ cells in bone marrow have the potential to serve as valid biomarkers of bone diseases. When comparing the DC-STAMP+ OCP frequency between BM and blood, >95% of PsA patients have more circulating DC-STAMP+ OCPs in the blood than bone marrow; conversely, healthy individuals have more DC-STAMP+ OCPs in their bone marrow than the blood (n=10, unpublished results). Sub-setting DC-STAMP+ cells in the blood and bone marrow by specific cell surface markers will be necessary and of interest to identify the elevated DC-STAMP+ cell populations in the circulation of patients.

A recent study demonstrated that heterogeneity determines the fusion efficiency between fusion partners [57], supporting the in vivo observations where mono-nucleated precursors migrating from the bone marrow fuse with more mature osteoclasts sitting on the bone surface. It will be of interest to investigate the reversed observation between healthy controls and the PsA patients.

D. DC-STAMP Expression on a Subset of T Cells

Despite DC-STAMP was initially identified from a dendritic cell-specific library [9] and thus was considered as a molecule that was preferentially expressed by dendritic cells [58], a unique DC-STAMP+ T cell subset was unexpectedly identified during our analysis of a large cohort of patients with psoriatic diseases (unpublished data and Chiu et al., [59]). In contrast to healthy controls whose majority of DC-STAMP+ cells were CD14+ monocytes (Figure 4A, upper right in the quadrant) [21], we detected a distinctive DC-STAMP+ cell population, which was CD14-negative (bottom right in the quadrant of Figure 4A) in 10 out of 26 PsA patients. Intriguingly, gated DC-STAMP+CD14+ cells did not express either CD3 or CD4 (Figure 4B), suggesting they were CD14+ monocytes.

On the contrast, the majority of gated DC-STAMP+CD14- single positive cells express both CD3 and CD4 (Figure 4C), suggesting they are CD3+CD4+ T cells.

This preliminary observation, together with our recent results showing a deficiency of T cell development in the thymus of DC-STAMP knock-out (KO) mice, suggest that a certain subset of DC-STAMP+CD3+CD4+ T cells was selectively proliferated in pathogenic disease conditions. In addition to flow data as shown in Figure 4, we also performed real-time PCR to confirm the co-expression of DC-STAMP, CD3ε, and TCR (unpublished data). The fact that DC-STAMP+ T cell subset was only present in the cohort of patients with psoriatic diseases but absent in the healthy control encouraged us to hypothesize that this T cell subset was a new T cell population preferentially selected under inflammatory pathogenic bone conditions. More studies will be necessary to investigate this hypothesis and determine the nature of this unique T cell subset.

E. Proteins that Interact with DC-STAMP and Regulate DC-STAMP Expression

Given that DC-STAMP ligand is still unknown, identification of DC-STAMP-interacting protein will help to elucidate the function of DC-STAMP in cell-cell fusion and downstream signaling. To date, CCN2, OS9 and Pin-1 are three proteins that were shown to have physical interaction with DC-STAMP [7, 60, 61].

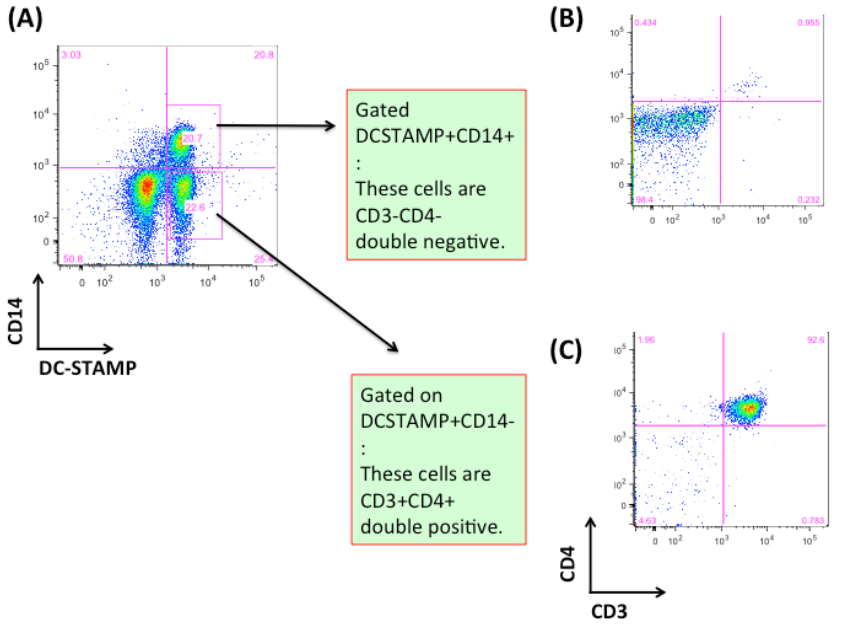


Figure 4. A unique DC-STAMP+CD3+ T cell subset is present only in the peripheral blood of psoriatic arthritis patients whereas it is absent in healthy controls. The expression pattern of DC-STAMP and CD14 was analyzed in 26 psoriatic arthritis (PsA) patients and 12 healthy controls. Their PBMC were isolated and subject to antibody staining for flow cytometry analysis. Ten out of 26 PsA patients showed a similar distribution of DC-STAMP, CD14 pattern shown in (A). This distribution pattern was never detected in healthy controls. DC-STAMP+ cells were gated into 2 populations, DC-STAMP+CD14+ double-positive and DC-STAMP+ single positive, based on their CD14 expression. The expression of CD3 and CD4 were analyzed on these two gated populations were shown in (B) and (C). The co-expression of CD3 and CD4 on DC-STAMP single positive cells but not on DC-STAMP+CD14+ double positive cells suggests that DC-STAMP+CD14- (DC-STAMP single positive cells) cells are CD3+CD4+ T cells.

E.1. DC-STAMP-Interacting Proteins

1.1. CCN2, one of the connective tissue growth factors, promotes endochondral ossification and significantly enhances the tartrate-resistant acid phosphatase (TRAP)+ multinucleated osteoclast formation in the presence of RANKL. The interaction between CCN2 and DC-STAMP was supported by real-time PCR, coimmunoprecipitation analysis, solid-phase binding assays, and a successful complementation of osteoclastogenesis deficiency of CCN2 -/- cells by overexpression of DC-STAMP.

1.2. OS9 was identified by screening a yeast 2-hybrid DNA library using the C terminus of DCSTAMP as bait. DC-STAMP and OS9 colocalized in endoplasmic reticulum (ER). TLR2- or TLR4-induced maturation of DCs led to translocation of DCSTAMP from the ER to the Golgi in a manner dependent on interaction with OS9; however, OS9 localization was unaffected. Based on these data, OS9 is considered to be involved in the modulation of ER-to-Golgi transport of DCSTAMP in response to TLR triggering.

1.3. Pin1 binds, isomerizes DC-STAMP, and affects the expression levels and localization of DC-STAMP at the plasma membrane.

E.2. Molecules That Affect the Expression of DC-STAMP

In addition to OS9, CCN2 and Pin1, the activity of DC-STAMP is modulated, either enhanced or suppressed, by a few modulators including proteins and miRNA involved in osteoclastogenesis (Table 2).

2.1. Strawberry Notch Homologue 2 (Sbno2) [62]

Sbno2 regulates osteoclast fusion by enhancing the expression of DC-STAMP [62].

Sbno2-null mice were osteopetrotic, with increases in trabecular bone volume and number and impaired osteoblastogenesis. Fusion of osteoclasts was also impaired in Sbno2-null mice, as was the formation of multinucleated osteoclasts in response to RANKL.

Stimulation of Sbno2 +/+, but not Sbno2-/- MCSF-derived macrophages with RANKL increased expression of DC-STAMP. Sbno2 bound to Tal1 and attenuated its inhibition of DC-STAMP expression, leading to activation of the DC-STAMP promoter by Mitf.

2.2. Pin1 [7]

Pin1 binds, isomerizes DC-STAMP, and affects the expression levels and localization of DC-STAMP at the plasma membrane. Pin1-/- osteoclasts are larger than wild-type osteoclasts and have higher nuclei numbers, indicating greater extent of fusion. RT-PCR analysis showed that DC-STAMP signal is significantly increased in Pin1(-/-) osteoclasts. Immunohistochemistry revealed that DC-STAMP expression is significantly increased in the tibias of Pin1 KO mice. Collectively, these results indicate that Pin1 regulates osteoclast fusion via suppressing DC-STAMP, supporting the concept that DC-STAMP is involved in the regulation of osteoclast volume.

Table 2. Summary of mediators that can regulate the expression of DC-STAMP at transcriptional or translational levels

enhance	Protein	Effect	Reference
	Sbno-2	Sbno-2 regulates osteoclast fusion by enhancing the expression of DC-STAMP.	Maruyama et al., [62]
	CCN2	CCN2 promotes osteoclastogenesis via induction of and interaction with DC-STAMP	Nishida et al., [60]
**	PU.1	Pu.1 activates the transcription of DC-STAMP	Courtial et al., [6]
**	MITF	Pu.1 activates the transcription of DC-STAMP	Courtial et al., [6]
suppress			
	Pin1	Pin1 regulates osteoclast fusion through suppression of the master regulator of cell fusion DC-STAMP.	Islam et al., [7]
	MiR-7b	MiR-7b directly targets DC-STAMP causing suppression of NFATc1 and c-Fos signaling during osteoclast fusion and differentiation	Dou et al., [63]
**	Tal1	Tal1 regulates osteoclast differentiation through suppression of the master regulator of cell fusion DC-STAMP.	Courtial et al., [6]

** Tal1, PU.1, and MITF are all transcription factors.
Tal1 counteracts the activating function of the transcription factors PU.1 and MITF.

2.3. Tal-1 [6]

Tal1 is a transcription factor that is expressed in osteoclasts. Deletion of Tal1 in osteoclast progenitors resulted in altered expression on more than 1200 genes. DC-STAMP is a direct target gene of Tal1. Tal1 represses DC-STAMP expression by counteracting the activating function of the transcription factors PU.1 and MITF.

2.4. MiR-7b [63]

A panel of microRNAs (miRNA) have been identified to play crucial roles in bone metabolism and osteoclast differentiation. Among them, one miRNA miR-7b was shown to be directly related to OCPs fusion and specifically target

DC-STAMP to inhibit osteoclastogenesis. Overexpression of miR-7b in RAW264.7 cells attenuated the number of TRAP-positive cells number and the formation of multinucleated cells, whereas the inhibition of miR-7b enhanced osteoclastogenesis.

The identification of ITIM on the cytoplasmic tail of DC-STAMP suggests its involvement in the ITAM-ITIM network [21]. This notion was supported by the changes of several key regulatory fusogenic genes including NFATc1, c-Fos, Akt, Irf8, Mapk1, and Traf6 after miR-7b targeting to DC-STAMP [63]. Thus, identification of more miRNAs, which block osteoclastogenesis via DC-STAMP, might serve as a potential therapeutic approach to treat osteoclast-related bone disorders. To date, in addition to miR-7b, many new miRNA that specifically target DC-STAMP have been identified (http://www.informatics.jax.org/interaction/explorer?markerIDs=MGI:1923016). It remains to be identified whether these miRNAs affect DC-STAMP-mediated osteoclastogenesis to a different extent at distinct regulation levels.

2.5. RNAi [64]

Using the lentiviral transgenic system for RNAi delivery, 80% gene transfer efficiency can be achieved at a multiplicity of infection of 15. The expression of DC-STAMP at both mRNA and protein levels can be significantly and specifically inhibited by RNAi [64]. Of note, DC-STAMP inhibition by RNAi consequently suppressed fusion and bone resorption of human osteoclasts, suggesting that inhibition of DC-STAMP by RNAi is an efficient and effective method of regulating osteoclast functions. The data from Zeng et al., [64] demonstrated that the lentivirus-mediated RNAi was capable of efficiently suppressing DC-STAMP expression in primary human osteoclasts and inhibiting osteoclastogenesis, providing direct evidence that DC-STAMP plays a pivotal role in the differentiation of human osteoclasts.

V. BIOLOGICAL PATHWAYS LIKELY TO BE MEDIATED BY DC-STAMP

Based on our preliminary observations on DC-STAMP KO mice and results from the comparison of WT or tail-deleted (TD)-overexpressing DC-STAMP, DC-STAMP is suggested to regulate multiple biological pathways during bone remodeling, homeostasis, and osteoclast differentiation in a direct

or indirect manner. In addition to the absence of mature differentiated multinucleated osteoclasts, DC-STAMP KO mice exhibited an increased rate in teeth malocclusion (unpublished data, manuscript in preparation) and impaired osteoblast differentiation (Figure 5) (unpublished data, manuscript in preparation). In accordance with previous publications demonstrating that the activation of DC-STAMP is tightly regulated by the binding of various transcription factors on the DC-STAMP promoter [6, 65], we also observed that the expression of DC-STAMP is regulated in a time-dependent manner (Figure 6).

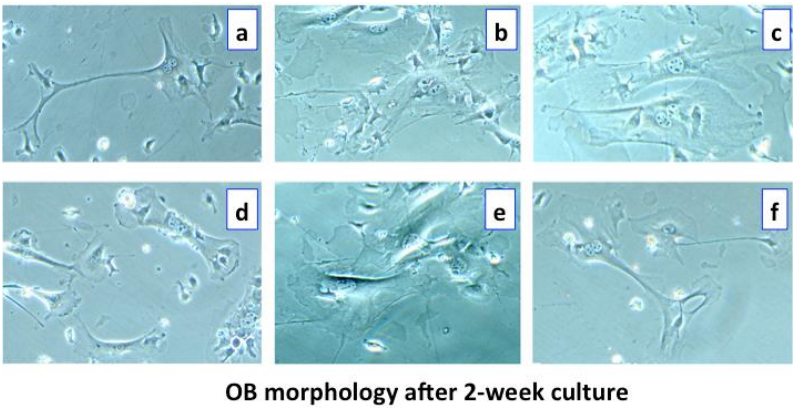


Figure 5. The morphology of mesenchymal stromal cells from WT and DC-STAMP KO mice is similar. Enriched CD45-Sca1+ mesenchymal stromal progenitor cells (BMSC) were cultured in osteoblast-promoting media for 14 days. (a)-(c): WT; (d)-(f): DC-STAMP KO.

Under OC-promoting culture condition (RANKL + M-CSF), the expression of DC-STAMP is not detectable until day 7 when eGFP was used as the surrogate of the DC-STAMP gene expression.

Intriguingly, our recent preliminary data suggested that the ITIM on the cytoplasmic tail of DC-STAMP is a function motif essential for osteoclastogenesis. Deletion of ITIM on DC-STAMP results in deficiencies in the generation of multi-nucleated osteoclasts (Figure 7), disturbance of DC-STAMP cell surface distribution and delayed osteoclast mobility (unpublished data, manuscript in preparation). Collectively, the data from DC-STAMP KO mice and studies from the overexpression of WT or TD DC-STAMP on DC-STAMP-/- cells suggested that DC-STAMP is likely to be involved in the regulation of multiple biological events during osteoclast differentiation as summarized below.

A. Cytoskeleton Rearrangement

An active rearrangement of cytoskeleton is constantly occurring during each cell-to-cell fusion, which involves actin-dependent cytoskeleton signaling network [66]. In order to form multi-nucleated osteoclasts, single nucleated osteoclast precursors need to go through many rounds of cell-cell fusions. As cell-cell fusion proceeds, the volume of cells keeps expanding and zipper-like actin superstructures appear transiently [67].

However, the molecular mechanism underlying the control of osteoclast cell size remains unclear. By using various inhibitors that specifically block the actin cytoskeleton signaling network [68], it has been shown that the size of osteoclasts is regulated by the actin-mediated cytoskeleton signaling network. Briefly, osteoclast polarization is accompanied by extensive reorganization of the actin cytoskeleton.

Several sensor proteins including Src, Rho and Rac1 [69-71] are involved in actin-mediated cytoskeleton reorganization signaling. Current data demonstrated that actin mediated positive regulators of podosome formation favor the generation of large osteoclasts [68]. Given that tailed-deleted DC-STAMP cannot complement OC-forming deficiency of DC-STAMP-/- cells and only generates cells with 3 or less nuclei without expanded cell volume (Figure 7) whereas WT DC-STAMP can fully complement OC-forming deficiency of DC-STAMP-/- cells associated with a larger volume of osteoclasts (Figure 7), DC-STAMP is likely to control the size of osteoclasts in a direct or indirect manner.

In addition to actin, integrin is the other critical protein essential for the attachment of osteoclasts to bone matrix to initiate bone resorption and degradation. Once osteoclasts are trafficking to skeletal tissue to initiate bone resorption, a privileged microenvironment space is established between the osteoclast and the bone surface, which is mediated by signals emanating from integrin and transits to its active high-affinity conformation by growth factor-initiated intracellular events targeting the matrix receptor's cytoplasmic domain. Integrin-mediated OC attachment to matrix and activation is well reviewed by Boyce [3]. Briefly, an active cytoskeleton remodeling occurs during two stages of osteoclast differentiation: cell-cell fusion and bone matrix attachment through a complex cascade, which connects the extracellular

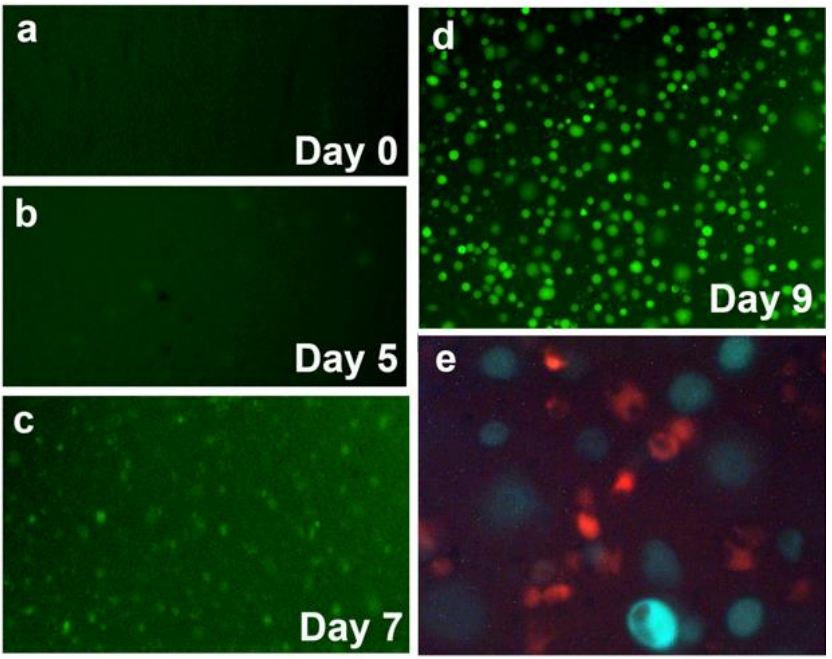


Figure 6. The promoter of DC-STAMP is activated in a timely and RANKL-dependent manner. Knockout of DC-STAMP was achieved by introducing the eGFP protein in the exon 2 of the DC-STAMP gene (9). Mature multinucleated osteoclasts could usually be generated after 4-5 day culture in the presence of RANKL and MCSF. Because eGFP was integrated into the DC-STAMP locus and theoretically those signals could be detected by microscope, Yagi. et al., needed to use the anti-EGFP antibody to detect the eGFP expression. To determine whether the tagged knock-in eGFP gene could be used to monitor the activation of the DC-STAMP gene, we cultured DC-STAMP -/- cells isolated from the DC-STAMP KO mice and examined the fluorescence signal elicited by eGFP without antibody staining at different time points. Minor eGFP signals started to be detected as early as day 5 (b), increasingly enhanced on day 7 (c) and reached the maximal on day 9 (d). Elicitation of eGFP signal from DC-STAMP -/- cells make these cells a good tool to examine the fusion partner between cells with different levels of DC-STAMP on their cell surface: red m-Cherry-infected cells were mixed with DC-STAMP -/- cells on day 5 to test fusion efficiency between these two cell types (e).

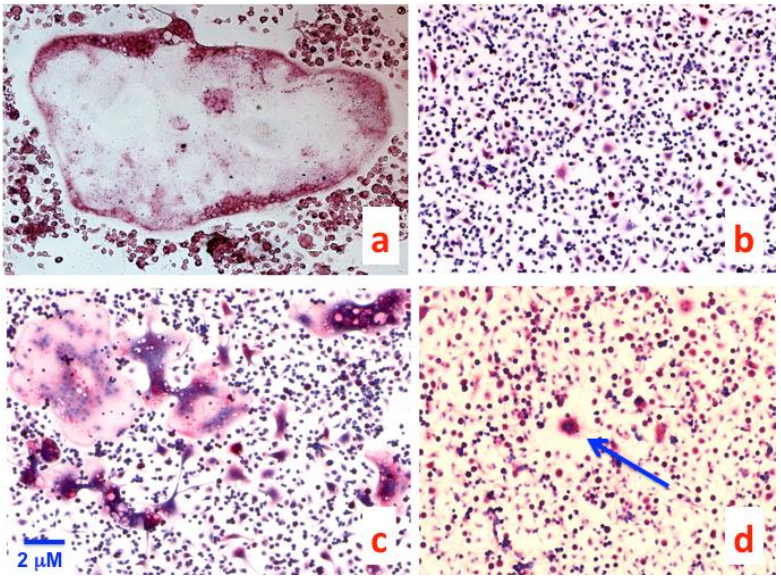


Figure 7. DC-STAMP is likely to be involved in the regulation of osteoclast volume. A huge multi-nucleated DC-STAMP+/+ WT osteoclast, which contain more than 30 nuclei; (b) mono-nucleated DC-STAMP-/- osteoclasts isolated from DC-STAMP KO mice, which fail to fuse due to the deficiency of DC-STAMP; (c) the OC-forming deficiency of DC-STAMP -/- cells can be complemented fully by overexpression of WT DC-STAMP; (d) ITIM-deleted (TD) DC-STAMP cannot complement OC forming deficiency. The maximal nuclear # in TD-overexpressing cells is 3, suggesting cell-cell fusion only occurs twice and is terminated.

integrin-mediated activation signal to the intracellular actin-mediated cytoskeleton rearrangement. c-Src links a RANK/αβ3 integrin complex to the osteoclast cytoskeleton [71]. In addition to c-Src, two proteins in the FAK kinase family, FAK and Pyk2, are also known to be involved in the regulation of cytoskeleton. The role of DC-STAMP in cytoskeleton remodeling of osteoclasts is suggested by 4 lines of evidence as follows. First, deletion of ITIM on DC-STAMP results in a failure of cytoskeleton remodeling and expanding of cell volume; second, there is a tyrosine residue in the ITIM motif of the DC-STAMP cytoplasmic tail, which is likely to be the target of SHIP-1 [21] or FAK-kinase family [72, 73]; third, similar to FAK kinases (FAK and Pyk2), DC-STAMP also regulates both OB and OC differentiation, suggesting that DC-STAMP and FAK kinases are in the same biological pathway to mediate cytoskeleton remodeling; fourth, like integrin, DC-STAMP is also a transmembrane protein. Given that c-Src links a RANK/αβ3 integrin complex

to the osteoclast cytoskeleton [71], one possibility remains to be investigated whether DC-STAMP is also one component of the RANK/αβ3 integrin complex. Of note, integrin was shown to regulate spleen tyrosine kinase (Syk) through an ITAM-independent pathway [74]. Thus, DC-STAMP and integrin are likely to regulate cytoskeleton remodeling, especially at each cell-cell fusion, through a complex signaling interplay between integrin-Syk-mediated signaling and ITAM- and ITIM-bearing receptors during osteoclastogenesis.

B. Fusion Frequency and Osteoclast Volume

In contrast to WT DC-STAMP infection to DC-STAMP -/- cells, which fully complement the OC-forming deficiency (Figure 7), deletion of ITIM on DC-STAMP caused a premature termination of cell-cell fusion, resulting in multinucleated cells with no more than 3 nuclei (Figure 7d, arrow). A recent study suggested that DC-STAMP is involved in the regulation of cell-cell fusion and control of osteoclast volume via Pin1. The interaction between Pin1 and DC-STAMP is summarized as the following: (1) Pin1-/- osteoclasts are larger than wild-type osteoclasts and have higher nuclei numbers, indicating a greater extent of fusion; (2) RT-PCR analysis showed that the DC-STAMP signal is significantly increased in Pin1(-/-) osteoclasts; (3) Immunohistochemistry revealed that DC-STAMP expression is significantly increased in the tibias of Pin1 KO mice; (4) Pin1 binds, isomerizes DC-STAMP, and affects the expression levels and localization of DC-STAMP at the plasma membrane. Taken together, as shown in Table 2, Pin1 is considered to regulate osteoclast fusion through suppression of the master regulator of cell fusion DC-STAMP.

C. Selection of Fusion Partner

Expression of DC-STAMP protein on the cell membrane is required for cell-cell fusion [40]. Intriguingly, we found that RANKL induces various expression of DC-STAMP on osteoclast precursors of which the DC-STAMP(lo) precursors are the master fusogens [39]. This finding suggests that the heterogeneous expression of DC-STAMP determines the fusion potentials of osteoclast precursors. This concept is supported by several recent findings from others. First, Soe et al. showed that cell fusion occurs preferentially between fusion partners that have a higher level of heterogeneity. These

heterogeneity includes the # of nuclei, the level of maturity, and the mobility [57]. Intriguingly, fusions between a mobile and an immobile partner were most frequent (62%), while fusion between two mobile (26%) or two immobile partners (12%) was less frequent. In addition, a more mature osteoclast prefers to fuse with a less mature pre-osteoclast. Interestingly, osteoclasts most often gain nuclei by the addition of one nucleus at a time, and a moving cell is usually the nucleus donor to an immobile cell [57]. Secondly, it has been recently shown that the relative cell surface localization of C-STAMP, CD47 and syncytin-1 determines the occurrence of cell-cell fusion and the site of fusion [75]. Taken together, these findings suggest that the expression level as well as the cellular localization of DC-STAMP determine the selection of the fusion partner and the frequency of cell-cell fusion.

D. RANKL-ITAM-ITIM Signaling

Costimulatory signals, one from RANK and one from immunoreceptor tyrosine-based activation motif-containing (ITAM-containing) receptors/ adaptors, are required for the activation of osteoclastogenesis in osteoclast precursors [76-78]. However, it is not well understood how ITAM costimulatory signals are integrated into RANK signaling. As an essential protein of osteoclastogenesis, identification of ITIM on the cytoplasmic tail of DC-STAMP suggests its role in signaling [21]. Based on a common counter action between the ITAM- and ITIM-bearing receptors in immune regulation [79, 80], we proposed that DC-STAMP is likely to participate in the network of RANK-ITAM costimulation. The collective interplay between RANK, ITAM- & ITIM- signals determines the consequent outcome of osteoclastogenesis activation in osteoclast precursors [21]. Our model and hypothesis were supported by a recent study, which demonstrated that targeting of DC-STAMP by MiR-7b caused a suppression of NFATc1 and c-Fos signaling during osteoclast fusion and differentiation [63]. Suppression of DC-STAMP results in altered downstream signals and changed fusogenic genes and key regulating genes including NFATc1, c-Fos, Akt, Irf8, Mapk1, and TRAF6 [65, 81-83].

E. OB::OC Coupling

Bone homeostasis, an equilibrium that is disturbed in many bone diseases, is achieved with a balance between bone-forming osteoblasts and bone-resorbing osteoclasts. Although DC-STAMP is recently considered as an essential protein required for osteoclast differentiation, our recent studies suggested that DC-STAMP also mediates the differentiation of osteoblasts either directly or indirectly. Impaired function and numbers of osteoblasts were detected in DC-STAMP knockout (KO) mice (unpublished data, manuscript submitted). This observation, together with the presence of DC-STAMP+ cells at fracture sites (unpublished data, manuscript in preparation) and delayed bone healing in DC-STAMP KO mice (unpublished data, manuscript submitted) suggested a role of DC-STAMP in the coupling of osteoclasts (OC) and osteoblasts (OB). The communication between OC and OB has been well reviewed by Charles and Aliprantis [5], which is mutual and bi-directional. Although OB::OC coupling was known a long time ago, the molecular mechanisms underlying the OB::OC communication is still largely unknown and many new concepts, including clastokines, have been proposed based on emerging novel findings.

Briefly, OC communicate with OB through secreting growth factors (TGFb and IGF1) and clastokines, or physical interaction through the engagement of ephrins and Eph receptors [5]. Conversely, OB can induce the apoptosis of OC through the FAS ligand/FAS pathway, a previously unrecognized mechanism that has an important role in the maintenance of bone mass in both physiological conditions and osteoporosis induced by ovariectomy [47].

In addition to mutual regulations between OB and OC, the OB::OC coupling can be affected by commonly shared factors which are involved in the regulation of both OB and OC, including IL-17A, NFATc1 and RunX2. IL-17A induces cathepsin K and MMP-9 expression in osteoclasts via celecoxib-blocked prostaglandin E2 in osteoblasts [84]. NFATc1 is a transcription factor that regulates many biological pathways including OB and OC differentiation [85-89]. In addition, Pin-1 is the other factor that regulates not only DC-STAMP for OC development but also Runx2 for OB differentiation [7].

It will be of interest to investigate whether RANKL-induced DC-STAMP(Hi) and DC-STAMP(low) OC employ distinct communication tools, either growth factors, clastokines, or ephrins/Eph receptors, to communicate with OB in murine and human cells. Intriguingly, OC were found surrounding

the cancer cells [90]. This finding is coincidently consistent with our recent finding showing that DC-STAMP+TRAP+ cells are recruited to the OB proximity at the bone fracture site (unpublished data, manuscript in preparation). The co-localization of OB and OC is never found in healthy bone without fractures. Collectively, our preliminary results suggest that DC-STAMP+ cells respond to unidentified factors by which they communicate with OB and are attracted to the OB proximity. Additional work will be required to further determine the molecular mechanism underlying this regulation.

F. Fracture Healing

Laser capture microdissection (LCM) showed that the expression of DC-STAMP was decreased in osteoclasts from diabetic mice whose bone healing is delayed [56].

This observation, together with our recent finding showing the presence of DC-STAMP+ cells at fresh bone fracture sites (unpublished data, manuscript in preparation), DC-STAMP KO mice have a delayed bone healing compared to wild-type mice (unpublished data, manuscript in preparation), and an elevated circulating DC-STAMP+ cells in human bone fractured patients during the bone healing stage (unpublished data, manuscript in preparation), suggest the participation of DC-STAMP in bone repair.

G. Tooth Development: Eruption and Remodeling

Teeth are part of the skeletal system where an active bone remodeling constantly occurs. Osteoclasts are involved in tooth development especially during the stage of tooth eruption [91]. Together with other cell subsets and cytokine profiles present at local tooth areas, osteoclasts play an important role in the pathogenesis of periodontal diseases [91-94]. The involvement of DC-STAMP in tooth development is suggested by our recent studies on DC-STAMP KO mice: An increased rate of tooth malocclusion was found in DC-STAMP KO mice, a phenotype which is closely linked to the homozygous DC-STAMP-/- loci (unpublished data, manuscript in preparation). The malocclusion phenotype of DC-STAMP KO was discovered unexpectedly due to a frequent diet-uptake problem reported from our animal facility. More studies will be necessary to investigate the role of DC-STAMP in teeth

development in the context of local cytokine profile including RANKL [91] receptors, and physiological inhibitors and growth factors during periodontal disease progression and tooth eruption.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

It has been 15 years since DC-STAMP was initially identified as a novel transmembrane protein preferentially expressed by dendritic cells [58]. DC-STAMP was considered to be involved in cell-cell fusion step during osteoclast differentiation, since knocking down of DC-STAMP completely abrogated the differentiation of osteoclasts and foreign body giant cells [9, 40]. Based on its critical role in osteoclast differentiation, DC-STAMP is currently well accepted as the master regulator in osteoclastogenesis [6-8]. Despite many significant advances in our knowledge of DC-STAMP over the past 15 years, the molecular mechanism underlying the DC-STAMP-mediated regulation remains largely unknown due to the absence of DC-STAMP ligand, one major hurdle in DC-STAMP research. If DC-STAMP is involved in signaling as suggested by the presence of ITIM on its cytoplasmic tail [21], dissecting its downstream signaling cascade after ligand engagement and activation is necessary to elucidate the DC-STAMP-mediated osteoclastogenesis. Before the ligand of DC-STAMP is identified, we are currently situated in a muddle of DC-STAMP research, mimicking the time before RANKL was identified as an essential ligand of RANK and differentiation factor for osteoclastogenesis in 1997 [95, 96]. Thus, identification of DC-STAMP ligand is an urgent priority, which may lead to a breakthrough in our current knowledge on OC differentiation and activation with similar impacts on OC research as RANK ligand (RANKL) was initially identified [95, 96].

We recently tried to address whether ITIM on the cytoplasmic tail of DC-STAMP is a functional motif by employing molecular engineering approaches. Our results showed that deletion of ITIM on DC-STAMP resulted in multiple alternations in osteoclast differentiation, including cell-cell fusion, bone erosion activity, cell surface distribution and rolling mobility of osteoclasts (unpublished data, manuscript in preparation). Since DC-STAMP null mutation impairs the differentiation of both osteoclast and osteoblast, we hypothesize that DC-STAMP regulates their differentiation through a common factor such as NFATc1. The fact that DC-STAMP+ cells were recruited into OB proximity close to the bone fracture site (unpublished data, manuscript in preparation) prompts us to propose that osteoclasts and osteoblasts

communicate with each other through clastokines or unidentified DC-STAMP ligand. Additional experiments are necessary to further test how deletion of ITIM on DC-STAMP causes multiple biological defects of osteoclast differentiation.

Based on its role as a master regulator in osteoclastogenesis, DC-STAMP holds great potential as the specific target for OC-based therapeutics. Bone diseases caused by excessive osteoclast activity such as osteoporosis and erosive arthritis can be moderated or prevented by medications that block DC-STAMP activity. To date, miRNA [63, 97-99] and RNA interference [64] have been shown to effectively block the biological activity and gene expression of DC-STAMP. In addition, since ITIM was present in the cytoplasmic tail of DC-STAMP, employing ITIM-bearing peptides to saturate and block DC-STAMP-interacting proteins remain an option to control DC-STAMP function. As a similar approach, synthetic peptides containing ITIM-like domains have been shown to block expression of inflammatory mediators and migration/invasion of cancer cells through activation of SHP-1 and PI3K [100, 101].

Following are the potential applications of DC-STAMP as a diagnostic tool and therapeutic agent: (1) biomarkers of psoriatic diseases [22]: we have previously shown that PsA patients have an elevated frequency of circulating DC-STAMP+ cells, which is associated with a higher surface expression of DC-STAMP [21]. Along this line, together with a recent finding showing a specific mutation on the cytoplasmic tail of DC-STAMP found in patients with Padget's diseases [13, 14], it will be of interest to examine whether the same mutation occurs in those Ps/PsA patients who have elevated circulating DC-STAMP+ cell frequency; (2) Role of DC-STAMP in tooth development: A higher tooth malocculation rate in DC-STAMP KO mice (unpublished data, manuscript in preparation) suggests a role of DC-STAMP in tooth development. It will be intriguing to investigate the interplay between DC-STAMP and other growth factors and cytokines previously known to regulate human periodontal diseases [91-94]; (3) Therapeutic approach for delayed bone healing: replenish circulating DC-STAMP+ cells by enriched BM-residing DC-STAMP+ cells; (4) Specific targeted medicine delivery to bone fracture to promote bone healing: because DC-STAMP+ cells are recruited to bone fracture sites in a specific manner (unpublished data, manuscript in preparation), it is likely that a certain motif in the DC-STAMP protein responds to signals, either cellular or structure, delivered and released at bone fracture and healing sites. Once this targeting sequence within the DC-STAMP

is identified, it can be subcloned and fused with bone-healing-promoting proteins such MMP-9 to facilitate bone repair.

Taken together, the data indicate that DC-STAMP is not only a master regulator of cell fusion, but that it may have other central functions that regulate bone homeostasis. Recent animal models combined with the ability to specifically target specific genes show that the DC-STAMP may act as an arthritis biomarker, is a potential marker of bone repair response, and may also play a pivotal role in Paget's disease. Additional studies and novel approaches will be required to elucidate the structure of DC-STAMP and its putative ligand. These new discoveries will open new avenues of investigation, which will hopefully accelerate the development of DC-STAMP as a biomarker and therapeutic agent in metabolic and inflammatory bone disorders. As several pieces of research data on DC-STAMP have been increasingly and gradually accumulated for the past 15 years, we anticipate important but currently unknown aspects of DC-STAMP will be investigated in the next 15 years. Novel and new findings derived from DC-STAMP research will assemble all pieces of puzzles together to reveal the big picture of DC-STAMP function in osteoclast differentiation and clinical application.

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