



Development of Novel Anti-DC-STAMP Monoclonal Antibody Targeting Osteolytic Periodontal Lesion

Citation

Wisitrasameewong, Wichaya. 2016. Development of Novel Anti-DC-STAMP Monoclonal Antibody Targeting Osteolytic Periodontal Lesion. Doctoral dissertation, Harvard School of Dental Medicine.

Permanent link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:33797369>

Terms of Use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA>

Share Your Story

The Harvard community has made this article openly available.
Please share how this access benefits you. [Submit a story](#).

[Accessibility](#)

**Development of Novel Anti-DC-STAMP Monoclonal Antibody
Targeting Osteolytic Periodontal Lesion**

A Thesis Presented by **Wichaya Wisitrasameewong, DDS, MS**

The Faculty of Medicine
in partial fulfillment of the requirements
for the degree of
Doctor of Medical Sciences

Research Mentor: **Toshihisa Kawai, DDS, PhD**

The Forsyth Institute

Harvard School of Dental Medicine

Boston, Massachusetts

April 2016

To my dad and mom

To my brother and sister

To Nat

For their love, support, caring, and understanding

ACKNOWLEDGEMENT

I am extremely indebted to my advisor, Dr. Toshihisa Kawai. I would like to express my sincere gratitude and appreciation to Dr. Kawai, for his guidance, encouragement, supervision, suggestion, kindness and being my inspiration. I wish to thank my advisory and thesis committee members; Dr. Martin A. Taubman, Dr. Yefu Li, Dr. Xiaozhe Han, Dr. Hajime Sasaki and Dr. Tatiana Besschetnova for their kindness in being my committee members and suggestions on my project. Sincere appreciation is expressed to all my friends in Dr. Kawai's lab for their kind advice and technical assistance in conducting the experiments.

My sincere appreciation is also extended to my faculties and coresidents at the Division of Periodontology, Department of Oral Medicine, Infection and Immunity, Harvard School of Dental Medicine. Finally, I would like to acknowledge King Ananda Mahidol Scholarship for the support throughout my study at Harvard School of Dental Medicine.

ABSTRACT

Background: Dendritic cell-specific transmembrane protein (DC-STAMP) plays a key role in the induction of osteoclast (OC) cell fusion. This conclusion is supported by evidence showing that osteoclast cell fusion is diminished, while systemic bone mineral density is significantly elevated, in DC-STAMP-KO mice, when compared to their wild-type mice. Clinically available anti-bone loss drugs, such as denosumab (anti-RANKL mAb) and bisphosphonates, cause severe side effects of MRONJ or medication related osteonecrosis of the jaw, presumably by targeting the complete suppression of osteoclastogenesis, which interferes with normal osteoclast-osteoblast coupling, or by interrupting antibacterial immune responses. Therefore, this study aimed to examine the effects of locally administered anti-DC-STAMP-mAb on bone and immunological responses in the context of periodontitis using a mouse model.

Methods: A silk ligature was placed around the second maxillary molar of C57BL/6J mice (male, 6- to 7-week-old, n=6-7/group) to induce alveolar bone resorption. Immediately after ligature placement, local injection of anti-DC-STAMP-mAb or control mAb (10 μ g/site) on the palatal tissue was performed. Alveolar bone loss, localization of OC, and IgG antibody response to the mouse periodontal opportunistic pathogen *Pasteurella pneumotropica* (*Pp*) were measured at Day-7 and Day-14 using μ CT, TRAP staining and ELISA. T-cells isolated from cervical lymph nodes were stimulated *in vitro* with mouse dendritic cells and *Pp*-antigen in the presence or absence of anti-DC-STAMP-mAb or control-mAb, followed by measurement of T-cell proliferation by 3H-thymidine incorporation assay.

Results: Immunofluorescence staining demonstrated higher expression of DC-STAMP on osteoclast-like cells on the alveolar bone surface in the ligature-induced periodontitis lesion

compared to the healthy non-ligated site. However, local injection of anti-DC-STAMP-mAb significantly suppressed the ligature-triggered alveolar bone resorption compared to control mAb at Day-7 (1.18 vs. 1.68 mm, $p < 0.05$) and Day-14 (1.58 vs. 2.28 mm, $p < 0.01$). According to histological analyses, anti-DC-STAMP-mAb decreased the number and size of multinucleated TRAP⁺ cells in the alveolar bone in comparison to those found in control. Neither *in vivo* anti-*Pp* IgG antibody nor *in vitro* anti-*Pp* T-cell response was affected by anti-DC-STAMP-mAb. These results suggested the robust efficacy of anti-DC-STAMP-mAb in suppressing alveolar bone loss by downregulating osteoclast cell fusion, but without affecting antibacterial immune responses.

Conclusions: Our study demonstrated, for the first time, that anti-DC-STAMP-mAb suppressed ligature-induced periodontal bone loss without affecting adaptive immune response to bacteria or total number of osteoclast precursors. Anti-DC-STAMP-mAb-mediated suppression of periodontal bone loss could be attributed to the inhibition of cell-cell fusion between osteoclast precursors. In sum, this anti-DC-STAMP-mAb could be developed as a lead candidate targeting osteoclast-mediated periodontal bone loss, resulting in normal homeostatic bone remodeling through rescue of OB-OC coupling, while, at the same time, avoiding the side effects associated with the currently applied therapies, as noted above.

CONTENTS	Page
Abstract	v
Contents.....	vii
List of Figures.....	ix
List of Abbreviations.....	x
 Chapters	
I Introduction.....	1
1.1 Background of the present study.....	1
1.2 Aims.....	3
1.3 Hypothesis.....	3
1.4 Field of research.....	3
1.5 Limitation of research.....	3
1.6 Application and expectation of research.....	4
1.7 Keywords.....	5
II Literature review.....	6
2.1 Periodontal disease.....	6
2.2 Osteoclastogenesis.....	8
2.3 Osteoclast fusion events.....	9
2.4 DC-STAMP	10
2.5 DC-STAMP and periodontal disease	12
2.6 Monoclonal antibody	14
III Materials and Methods.....	17
3.1 Cells.....	17
3.2 Animals.....	17
3.3 Osteoclastogenesis induction	17
3.4 Tartrate-resistant acid phosphatase (TRAP) staining	18
3.5 Pit formation assay.....	18
3.6 Flow cytometry analysis.....	18
3.7 Induction of periodontitis lesion in mice using silk ligature	19

3.8 Local delivery of anti-DC-STAMP-mAb	19
3.9 Measurement of alveolar bone resorption	20
3.10 Histological sample preparation	20
3.11 Immunofluorescence staining	21
3.12 Analysis of cytokine production in gingival tissue	21
3.13 Dendritic cell proliferation	22
3.14 T-cell isolation	22
3.15 Mixed lymphocyte reaction (MLR)	22
3.16 IgG response against bacteria	23
3.17 Statistical analysis	23
IV Results	24
4.1 Characterization of Anti-DC-STAMP mAb	24
4.2 Localization of DC-STAMP in osteoclast cell culture <i>in vitro</i>	27
4.3 Effect of anti-DC-STAMP-mAb on osteoclast formation <i>in vitro</i>	30
4.4 Effect of anti-DC-STAMP-mAb on resorptive activity of osteoclasts <i>in vitro</i>	35
4.5 Localization of DC-STAMP in ligature-induced periodontal lesion <i>in vivo</i>	38
4.6 Effect of anti-DC-STAMP-mAb on alveolar bone loss in ligature-induced periodontitis model	39
4.7 Evaluation of osteoclast formation <i>in vivo</i>	43
4.8 Effect of anti-DC-STAMP-mAb local administration on cytokine production	44
4.9 Effect of anti-DC-STAMP-mAb local administration on IgG-antibody response to bacterial challenge	49
4.10 The effect of anti-DC-STAMP-mAb local administration on T-cell response ...	51
V Discussion and conclusion	54
References.....	63

LIST OF FIGURES

Figures	Page
1 The possible mechanism by which anti-DC-STAMP-mAb inhibits preosteoclast fusion.....	16
2 Determination of anti-DC-STAMP-mAb isotype	25
3 Anti-DC-STAMP-mAb recognizes the 53 kDa and 106kDa DC-STAMP	25
4 Comparison of anti-DC-STAMP-mAb binding protein	26
5 Flow cytometry analysis of DC-STAMP expression on osteoclast precursor cells	28
6 localization of DC-STAMP in osteoclasts <i>in vitro</i>	29
7 Effect of anti-DC-STAMP-mAb on osteoclastogenesis in RAW264.7 cells	31
8 Effect of anti-DC-STAMP-mAb on osteoclastogenesis in mouse bone marrow-derived osteoclast cells	32
9 Effects of anti-DC-STAMP-mAb in mouse bone marrow-derived osteoclastogenesis in C57BL/6 wild-type compared to FcR-KO mice	33
10 TRAP staining of mouse bone marrow-derived osteoclast cell culture	34
11 Pit formation assay of mouse bone marrow-derived osteoclast cell culture	35
12 Effect of anti-DC-STAMP-mAb on resorptive activity in mouse bone marrow-derived osteoclast cells	36
13 Distribution of DC-STAMP detected in periodontal lesion by immunofluorescence staining	38
14 Alveolar bone resorption of maxilla under microscopy	40
15 Measurement of alveolar bone resorption	41
16 Effect of anti-DC-STAMP-mAb on alveolar bone loss in ligature-induced periodontitis model.....	42
17 Effect of anti-DC-STAMP-mAb on an <i>in vivo</i> ligature-caused osteoclast induction.....	43
18-21 Effect of anti-DC-STAMP-mAb on IL-1 β , IL-6, TNF- α and sRANKL responses in ligature-induced periodontitis model	45
22 Effect of local administration anti-DC-STAMP-mAb on antibacterial immune response	50
23 3H-thymidine incorporation assays of T-cell isolated from cervical lymph node of ligature-induced periodontitis mice	52

LIST OF ABBREVIATIONS

Atpv0d2	ATPase V(0) domain subunit d2
c.p.m.	Counts per minute
DC	Dendritic cell
DC-STAMP	Dendritic cell-specific transmembrane protein
DC-STAMP KO	Dendritic cell-specific transmembrane protein knock-out
DC-STAMP Tg	Dendritic cell-specific transmembrane protein transgenic
FBS	Fetal bovine serum
FcR	Fc receptor
FcR KO	Fc receptor knock-out
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association study
IgG	Immunoglobulin G
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
mAb	Monoclonal antibody
M-CSF	Macrophage colony-stimulating factor
MFR	Macrophage fusion receptor
MRONJ	Medication related osteonecrosis of the jaw
OC	Osteoclast
OC-STAMP	Osteoclast stimulatory transmembrane protein
ONJ	Osteonecrosis of the jaw
OPG	Osteoprotegerin
<i>Pp</i>	<i>Pasteurella pneumotropica</i>
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
TNF- α	Tumor necrosis factor- α
TRAP	Tartrate-resistant acid phosphatase

CHAPTER I

Introduction

1.1 Background of the present study

Periodontal disease is a chronic inflammatory disease that affects the supporting tissue around teeth. It causes the destruction of periodontal tissues, particularly alveolar bone resorption, which is the hallmark of periodontitis, resulting in multiple tooth loss. In physiological condition, a balance is struck between the activity of osteoblasts and osteoclasts, i.e., bone formation and bone resorption, respectively, to maintain normal homeostatic bone remodeling. However, in the pathological condition, this homeostasis can be disrupted by various molecular mechanisms triggered by the immune system. This demonstrates the close relationship between lymphocytes and bone cells, which is explored in the field of osteoimmunology (1). Imbalance between osteoblast and osteoclast activities in systemic bone can result in abnormal bone formation, such as osteopetrosis and osteoporosis (2, 3). However, in the context of periodontal disease, host immune and inflammatory response against bacterial components is thought to cause the aberrant activation of osteoclasts as a result of RANKL production from activated lymphocytes (4). Osteoclasts are exclusive bone-resorbing cells that gain their unique morphology based on fusion of their precursors (preosteoclasts) originating from hematopoietic progenitors in bone marrow (5). Multinucleated cell types are limited in mammals, including sperm and oocytes during fertilization, trophoblasts during placenta

formation, myoblasts in the formation of skeletal muscle and macrophages during giant cell and osteoclast formation (6, 7). Unlike other multinucleated cells which undergo fusion during developmental growth, osteoclast fusion occurs continuously (7). Therefore, a strategy that targets this event could result in a therapeutic approach for osteoclast-specific pathogenic bone loss with minimal side effects. Importantly, it is this fusion step in osteoclastogenesis that significantly increases the osteoclast activity of these bone-resorbing cells (8-10). DC-STAMP plays a critical role in the fusion of osteoclast precursor cells to become multinucleated osteoclasts, as determined through both *in vitro* and *in vivo* studies that used DC-STAMP-deficient mice (10, 11). DC-STAMP is a seven-transmembrane protein originally identified in dendritic cells, IL-4-induced macrophages, and osteoclasts (12, 13), whereas the molecular signal transductions upon DC-STAMP activation, as well as its ligands, remain elusive. Since bisphosphonate, one of the most potent drugs used for bone lytic diseases, has presented the emerging side effect of bisphosphonate-induced osteonecrosis of jaw (14), alternative drugs for bone lytic diseases, including periodontitis, are sought for development. Undoubtedly, the inhibition of DC-STAMP could be a potential therapeutic target for bone lytic diseases caused by osteoclastogenesis. In contrast to chemical compound drugs, monoclonal antibody drugs have sparked increasing interest by their specificity and minimal side effects. Thus, development of a DC-STAMP monoclonal antibody might generate an alternative approach to the treatment of various bone lytic diseases, but with fewer complications and side effects. Thus, this study aims to examine the effect of DC-STAMP monoclonal antibody on the alveolar bone resorption in a mouse model of periodontitis.

1.2 Aims

The specific aims are as follows:

Specific Aim 1 : To study the effect of DC-STAMP monoclonal antibody in osteoclast formation and function *in vitro*.

Specific Aim 2 : To investigate the effect of DC-STAMP monoclonal antibody on the alveolar bone resorption in ligature-induced periodontitis *in vivo* using mice model.

1.3 Hypothesis

Anti-DC-STAMP mAb will inhibit alveolar bone resorption in periodontitis.

1.4 Field of research

Effect of anti-DC-STAMP-mAb on osteoclastogenesis and its resorptive activity in mouse bone marrow-derived osteoclast cell culture and mouse model of ligature-induced periodontitis.

1.5 Limitation of research

Anti-DC-STAMP-mAb generated in this study is murine in origin, and its efficacy and side effects were examined in a mouse model. Its application in human will require further investigation.

1.6 Application and expectation of research

Periodontal disease is one of the most common chronic inflammatory diseases in humans. It affects the periodontium, which is the tooth attachment apparatus. Bacterial

plaque and its components trigger host inflammatory responses which subsequently lead to periodontal destruction, such as bone loss and, eventually, tooth loss. The main rationale for periodontal therapy is to restore periodontal health by promoting the regeneration of the destroyed periodontal structures. However, the destroyed tissue is clinically difficult to reconstruct. Various regenerative periodontal treatment options are available, including, for example, bone grafts, guided tissue regeneration and use of growth factors. However, the outcome of current procedures is limited and unpredictable. Therefore, inhibition of alveolar bone resorption is an alternative treatment that potentially overcomes the limitations of current periodontal treatment.

With our advanced scientific knowledge and techniques, the ability to humanize mouse antibody makes it possible to use monoclonal antibodies as a therapeutic approach in various diseases. Therefore, DC-STAMP monoclonal antibody could be developed as a lead candidate targeting osteoclast-mediated periodontal bone loss, but potentially other diseases characterized by systemic bone loss, such as osteoporosis, resulting in normal homeostatic bone remodeling through rescue of OB-OC coupling, while, at the same time, avoiding the side effects associated with the currently applied therapies, as noted above. In doing so, such mAb might eventually lead to a better understanding of bone homeostasis. However, further studies are needed in order to investigate the underlying mechanism of cell-cell fusion, since the signal transduction pathways regulating this event remain largely unclear.

1.7 Keywords

DC-STAMP, Osteoclast, Periodontitis, Mouse model, Cell fusion, Bone resorption

CHAPTER II

Literature review

2.1 Periodontal disease

Periodontal disease is a chronic inflammatory disease that affects the supporting tissue around the teeth. It causes the destruction of periodontal tissues which, in severe cases, leads to tooth loss. Among more than 700 species in the oral cavity, *Aggregatebacter actinomycetemcomitans* (*A. actinomycetemcomitans*), *Porphyromonas gingivalis* (*P. gingivalis*) and *Tannerella forsythia* (*T. forsythia*) are considered key periodontal pathogens (15). These microbes possess a variety of potent virulence factors that can destroy periodontal tissues (16). However, bacteria alone cannot trigger the onset of disease. Indeed, many individuals harboring these pathogens are able to limit the progression of periodontal disease, suggesting a complex multifactorial etiology associated with an imbalance between host immune response and microbial virulence factors (17). In a susceptible host, microbes and their virulence factors trigger the release of proinflammatory mediators in the periodontal tissue, leading to periodontal tissue destruction (15). Therefore, the interaction between microbial virulence factors and host immune response plays key roles in the pathogenesis and progression of periodontal disease (18).

Periodontal disease is recognized as a global health problem, albeit generally perceived as a non-life-threatening disease. However, epidemiologic studies have shown

a significant association between periodontal and other systemic diseases, such as cardiovascular disease, diabetes mellitus and preterm low birth weight (19-21). In addition, alveolar bone resorption, as a hallmark of periodontitis, often starts early in the disease process, and its progression is a cause of considerable patient morbidity. The interactions between microbes, microbial virulence factors and host immune response play key roles in the pathogenesis and progression of periodontal disease (18). Cells growing in biofilm adopt different phenotypic properties in which genes are differentially regulated (22), triggering host inflammatory responses which, subsequently, lead to periodontal bone loss or, in severe cases, tooth loss. The most recent theory supports that so called keystone bacteria can cause the dysbiosis or biofilm community which leads to the pathogenic immune responses that trigger periodontal bone loss. However, currently, the bacterial molecules that are responsible for causing dysregulation of host immune response that are engaged in progression of periodontal disease remains unclear (18, 23). In healthy condition, bone resorption by osteoclasts is followed by an equivalent amount of new bone formation by osteoblasts by the phenomenon of OC-OB coupling signals. Therefore, normal bone homeostasis is a reflection of the balance of functions between osteoclasts and osteoblasts (24). On the other hand, under pathological conditions, in particular, the inflammatory state, bone homeostasis is disrupted and favors the axis of bone resorption rather than bone formation. As a result, this imbalance between osteoblast and osteoclast activities eventually leads to net bone loss and disease, such as osteoporosis and, especially, periodontitis (2, 23, 25).

2.2 Osteoclastogenesis

Osteoclasts are unique bone-resorbing cells derived from the cells of monocyte-macrophage lineage. Osteoclastogenesis is a complicated process and includes many stages, for example, differentiation and multinucleation (26). Multinucleate, or multinucleated, cells are eukaryotic cells that have more than one nucleus per cell, i.e., multiple nuclei share one common cytoplasm. Osteoclast differentiation and function are known to be regulated by a molecular triad: the receptor activator of nuclear factor- κ B (RANK) and its ligand RANKL, as well as osteoprotegerin (OPG), as a natural antagonist of RANKL (27, 28). After binding of macrophage colony-stimulating factor, or M-CSF, a secreted cytokine which influences hematopoietic stem cells to differentiate into macrophages, these macrophages are driven to express RANK on their surface to become osteoclast precursor cells. Next, the binding of RANKL to RANK expressed on osteoclast precursor cells is required for osteoclast differentiation from their precursor to mononucleated osteoclasts. Meanwhile, OPG acts as a soluble decoy receptor to prevent RANKL from binding to RANK, thus inhibiting the formation of osteoclasts. Consequently, osteoclast differentiation is controlled by complex interactions among OPG, RANKL and RANK (28). However, for osteoclasts to perform their function whenever some pathological cause has interrupted the normal osteoblast-osteoclast balance noted above, multinucleation, as an essential cell-cell fusion step, takes place (9, 10). The cell-cell fusion mechanism in mammalian cells is cell-type specific. The fusion of somatic cells mostly occurs during the developmental stage from embryo to the mature adult, including skeletal muscle cells and trophoblasts. Macrophage fusion is, however, different in that it occurs throughout life (7).

As described above, the monocyte phagocytic system was recognized in early 1980 as a precursor of osteoclasts (29). Thus, the signaling cascade that eventually results in multinucleated osteoclast formation has been known for some time (30), but the molecular mechanism that mediates cell-cell fusion is largely unclear. DC-STAMP has only recently been reported as critically and specifically involved in macrophage-macrophage cell fusion (7, 10). However, it is the binding of RANK by RANKL that subsequently triggers the downstream signaling cascade which, in turn, induces the transcription of various osteoclast-specific genes, including DC-STAMP (9). Therefore, it is hypothesized that DC-STAMP expression is also regulated by transcription factors downstream of RANKL-RANK signaling. This hypothesis is supported by the study of Yagi et al. (2007) which demonstrates that c-Fos, AP-1 transcription factor, and NFATc1 cooperatively regulate DC-STAMP expression during osteoclastogenesis (31). Moreover, in the presence of RANKL, osteoclast cell-cell fusion significantly up-regulated in the DC-STAMP transgenic (DC-STAMP-Tg) cells that over express DC-STAMP. In contrast, when RANKL is absent, cell-cell fusion is not induced in DC-STAMP-Tg cells (31). These results suggest that RANKL stimulation is required for DC-STAMP-mediated cell-cell fusion event in osteoclasts.

2.3 Osteoclast Fusion Events

Unlike other multinucleated cells which undergo fusion during developmental growth, osteoclast fusion occurs continuously (7). Therefore, a strategy that targets the multinucleation event is thought to be specific to osteoclast bone loss and thus have minimal side effects. It should be noted that the multinucleation step is one that increases

the osteoclast activity of these bone resorbing cells (8-10). Even though the molecular mechanism of osteoclast fusion is largely unclear, studies point out various candidate molecules that mediate osteoclast fusion, such as CD44, macrophage fusion receptor (MFR) (7, 29, 30), dendritic cell-specific transmembrane protein (DC-STAMP), the d2 isoform of vacuolar ATPase V0 domain subunit d2 (Atpv0d2) and osteoclast stimulatory transmembrane protein (OC-STAMP), all of which appear to be specific and critical for osteoclast fusion (31, 32).

2.4 Dendritic cell-specific transmembrane protein; DC-STAMP

DC-STAMP was originally identified as a seven-transmembrane protein expressed in dendritic cells (12), then in IL-4-induced macrophages (13) and, most recently, in osteoclasts (9). The critical role of DC-STAMP in osteoclastogenesis was first demonstrated in an *in vitro* study in 2004 when it was found to be involved in the generation and function of osteoclasts (9). In the following year, the role of DC-STAMP as a fusogen was further clarified *in vivo* as the result of experimentation which showed decreased multinucleation in osteoclasts from DC-STAMP-deficient mice, while the expression of transcription factors required for osteoclast differentiation, including NFATc1, and a terminal differentiation marker of osteoclasts, cathepsin K, were unchanged. Kim et al. (31) concluded that “the NFATc1/Atp6v0d2 and DC-STAMP signaling axis plays a key role in the osteoclast multinucleation process, which is essential for efficient bone resorption.” This suggested that DC-STAMP is regulated by the pivotal osteoclastogenesis transcription factor NFATc1. RT-PCR analysis on the time course of DC-STAMP expression in mouse bone marrow-derived osteoclasts reached its

peak on the third day after RANKL stimulation (9, 31) and was detected until the sixth day (9).

The important role of DC-STAMP in the regulation of bone homeostasis was further studied in overexpressing DC-STAMP transgenic mice (DC-STAMP-Tg). Measurement of bone mineral density and bone morphometric analysis both show that DC-STAMP-deficient mice have increased bone mass, while DC-STAMP-Tg mice show decreased bone mass, compared to wild-type mice (10, 11). These results indicate that DC-STAMP negatively regulates bone volume by inducing preosteoclast cell fusion which, in turn, promotes the resorbing activity of osteoclasts, thereby disrupting bone homeostasis and causing such diseases as osteopetrosis, which was observed in the DC-STAMP-deficient mice.

Bone mass increase observed in DC-STAMP-deficient mice is the result of reduced osteoclast bone resorbing activity by the loss of DC-STAMP. More importantly, the loss of DC-STAMP not only inhibits osteoclast bone resorption, but also appears to stimulate osteoblast activity to generate bone *in vivo*, thereby increasing bone volume (11). Although the detailed mechanism underlying the activation of osteoblasts in the DC-STAMP-KO mice is not fully elucidated, such results indicate that a therapeutic approach targeting DC-STAMP could increase bone regeneration, while halting the bone resorption, which is an advantage over the currently available anti-osteoclastogenesis drugs which cannot induce osteoblast proliferation.

Previous studies reporting on DC-STAMP mostly focused on analyses of DC-STAMP gene expression, but not endogenous DC-STAMP protein levels (9, 33), as no appropriate agent was available that could bind DC-STAMP protein. Recently, Mensah

et al. (2010) generated an anti-DC-STAMP monoclonal antibody (1A2) from mice to investigate the surface expression of DC-STAMP on osteoclast precursor cells (34). Compared to rabbit polyclonal anti-DC-STAMP antibody (KR104), polyclonal anti-DC-STAMP antibody and 1A2 recognized different extracellular domains of DC-STAMP. Moreover, 1A2 shows stronger detection of surface DC-STAMP which is expressed as a dimer on the plasma membrane of murine (RAW 264.7) and human osteoclast precursor cells (human PBMC) (34). Similar to the study of KR104 in a murine cell line (9), 1A2 could block osteoclast formation in human monocytes *in vitro*. In addition, the inhibitory effect of 1A2 was shown to be dose-dependent (34). The level of DC-STAMP expression is, therefore, associated with the frequency of multinucleated osteoclast formation. Moreover, blockade of DC-STAMP with an anti-DC-STAMP antibody potentially inhibited multinucleated osteoclast formation. These findings underscore the essential role of DC-STAMP in osteoclastogenesis.

DC-STAMP has been suggested as a potential biomarker of osteoclast precursors in inflammatory arthritis, a susceptible biomarker of psoriasis (Ps), as well as severity marker of Psoriatic Arthritis (PsA) (35). Studies suggested DC-STAMP as a strong candidate gene involved in Paget's disease of bone, which is a common skeletal disorder characterized by focal areas of increased and disorganized bone turnover, primarily targeting the axial skeleton (36). In a genome-wide association study (GWAS) investigating the genetic risk for Paget's disease of bone, a significant association with a polymorphism from the chromosomal region around the TM7SF4 gene, encoding DC-STAMP, was found. However, genome-wide significance in the GWAS was not reached (37). Later, Teramachi and coworkers (2014) found a significantly elevated expression

of DC-STAMP, but only modestly elevated levels of ATP6v0d2 and ADAM8, in osteoclasts derived from a Paget's disease mouse model (38).

Until now, how DC-STAMP mediates osteoclast cell fusion has remained largely unclear, and its ligand is still unknown. As reported in the study of Yagi et al. (2005), supernatant from DC-STAMP^{+/+} could not induce osteoclast fusion of DC-STAMP^{-/-} cells, but multinucleated osteoclasts were found in the mixed culture of DC-STAMP^{+/+} and DC-STAMP^{-/-}. Since these results imply that direct cell contact is needed, the putative ligand of osteoclast fusion might be expressed on the cell surface. In addition, two subtypes of osteoclasts have been hypothesized, depending on the expression of DC-STAMP and its role in the fusion process, including DC-STAMP^{+/+} osteoclast precursors as master-fusing cells and DC-STAMP^{-/-} as fusion partner cells (10). However, in his review, Vignery has raised the possibility that DC-STAMP ligand might be a soluble protein released by osteoclasts in a constitutive or regulated manner. That ligand might be released by the master-fusing cells, the fusion partner cells, or both (39).

2.5 DC-STAMP and periodontal disease

In the cross-sectional clinical study, the mRNA expression level of DC-STAMP was detected in gingival tissues from periodontitis patients, but not in tissues from patients without periodontal disease. Moreover, the detection frequency of DC-STAMP expression increased with increasing severity of periodontitis (40). However, no further studies have investigated the role of DC-STAMP in periodontal disease. Therefore, although osteoclasts have been extensively studied as key contributors to alveolar bone

resorption, not much is known about the role of DC-STAMP in the evolution of the bony lesions that characterize periodontal disease.

2.6 Monoclonal Antibody

Monoclonal antibody therapy is the use of monoclonal antibodies (mAbs) to specifically bind target cells or proteins, which then block the interactions between receptors and their ligands, resulting in agonist or antagonist signaling in target cells. The mAbs derived from mice possess many limitations, including immunogenicity of these foreign proteins in patients, inefficient effector functions and half-lives that are typically less than 20 hours. However, these limitations are overcome by molecular engineering technologies which can modify the structure of murine mAbs to minimize immunogenicity, yet maximize specific therapeutic actions (41). These efforts have resulted in other subtypes of mAbs, including chimeric, humanized and fully human mAbs (42). Over the decades, mAbs have been accepted as an alternative approach of treating various diseases, in particular, cancer, inflammatory diseases and hematological disorders (43). These mAbs, or IgG-based molecules, have advantages over small-molecule drugs in many respects, including tissue-specific targeting, serum half-life and effector functions via complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity or drug conjugates (44).

Denosumab is a fully human monoclonal antibody neutralizing RANKL and, consequently, interfering with the differentiation, function and survival of osteoclast cells mediated by the RANKL-RANK signaling pathway (45). By blocking RANKL-RANK from binding on preosteoclasts, osteoclast maturation, function and survival are all

inhibited, leading to reduction of bone resorption. Denosumab (anti-RANKL neutralizing mAb) has been used as an alternative to bisphosphonates based on the disease type. In comparison to Denosumab that can neutralize the RANKL via circulation, bisphosphonates bind strongly to bone mineral which are absorbed by mature osteoclasts resulting in their apoptosis. These differences in mechanism of action result in a decreased number of osteoclasts (46). Therefore, Denosumab possesses the overlapping side effect profile attributed to the use of bisphosphonates, namely, osteonecrosis of the jaw (ONJ) (47).

An anti-DC-STAMP-mAb has recently been developed. However, no previous studies have investigated the effect of DC-STAMP inhibition on periodontal disease. Therefore, this study hypothesizes that an anti-DC-STAMP-mAb can inhibit osteoclast activity, resulting in the inhibition of alveolar bone resorption that occurs in periodontitis. Consequently, this anti-DC-STAMP-mAb could be the lead candidate for a novel therapeutic approach for treating periodontitis. Figure 1 shows the possible molecular mechanism by which anti-DC-STAMP-mAb inhibits preosteoclast fusion.

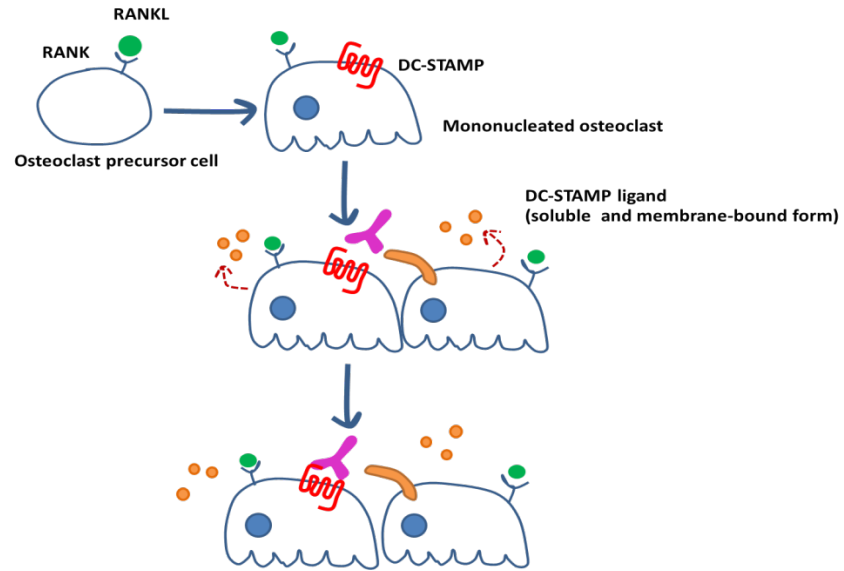


Figure 1. Schematic showing the possible molecular mechanism by which anti-DC-STAMP-mAb inhibits preosteoclast fusion.

CHAPTER III

Methodology

Cells

RAW 264.7 macrophage cell line was used for the osteoclastogenesis assay. For primary cells, mononuclear cells were derived from bone marrow of C57BL/6 mice. T-cells were isolated from cervical lymph nodes of C57BL/6 mice.

Animal

C57BL/6 mice from the Forsyth Institute were used throughout the study. The protocol to obtain mouse bone marrow cells and induce periodontitis lesion was approved by the Forsyth Institutional Animal Care and Use Committee (IACUC).

Osteoclastogenesis induction

Mononuclear bone marrow cells were isolated from femur and tibia of 8- to 12-week-old male wild-type mice. Cells were seeded into 96-well plates at 1.0×10^5 cells/well in 200 μ l of complete α -MEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 0.1% gentamycin, 1% MEM Nonessential Amino Acid (NEAA) and 30 ng/ml M-CSF (R&D Systems, Minneapolis, MN, USA) and incubated at 37°C. After 3 days, 100 μ l of the medium was replaced with fresh medium with 30 ng/ml M-CSF and 50 ng/ml murine soluble RANKL (sRANKL) (R&D Systems, Minneapolis, MN, USA). Cultures were applied with

different doses of mouse anti-DC-STAMP-mAb (10, 20 and 50 $\mu\text{g/ml}$) or mouse anti-*Pg10* (anti-*P. gingivalis* mAb, mouse IgG2a), as a control. After 3 days, 100 μl of the medium was replaced again with the medium as previously described. On differentiation day 10, TRAP staining or pit formation assay was performed.

Evaluation of osteoclastogenesis *in vitro*: Tartrate-resistant acid phosphatase (TRAP) staining

TRAP staining was performed with the leukocyte acid phosphatase kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. TRAP-positive multinuclear cells, which contained three nuclei or more, were considered as osteoclasts and were counted microscopically.

Evaluation of osteoclastogenesis *in vitro*: Pit formation assay

A pit formation assay was carried out as previously described (48). In brief, cells (3×10^5 cells/well) were plated on a Corning[®] Osteo Assay Surface 96-well plate (Corning Incorporated, Life Sciences, MA, USA) and cultured in α -MEM with 10% FBS, 30 ng/ml M-CSF and 50 ng/ml RANKL as described in osteoclastogenesis induction. On differentiation day 10, the plates were washed with 10% sodium hypochlorite solution to remove the cells. Resorbed areas on the slides were microscopically observed (48).

Flow cytometry analysis

Flow cytometry was used to quantitatively assess the change in DC-STAMP expression in response to sRANKL stimulation. RANK and CD11b were used as preosteoclast markers. The

cells were detached and scraped from culture wells. The cells were incubated in Mouse BD Fc Block™ on ice for 30 minutes prior to incubation with conjugated antibodies.

Induction of periodontitis lesion in mice using silk ligature

Silk ligature (5-0, Ethicon®, Guaynabo, Puerto Rico) was placed around the upper left second molar of C57BL/6j mice (male 6-7-week-old), whereas the upper right second molar without ligature was used as a control. The mice were fed with regular diet.

Local delivery of anti-DC-STAMP-mAb

After ligature placement, mice were randomly divided into two groups to receive either anti-DC-STAMP-mAb or control mAb. Anti-DC-STAMP-mAb or control mAb was locally injected at the palatal tissue close to the interdental papilla area. Ten µg of mAb were injected to ligature- and non-ligature-placed sites. The timeline of the experiment is shown in Figure 4. The injection of mAb was performed on Day 0.



Measurement of alveolar bone resorption

Mice were sacrificed on Day 3, 7 and 14. The maxilla bone was defleshed, and periodontal bone resorption was measured as previously described (49). Briefly, the distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) on the buccal side of each root was measured under a microscope with a reticule eyepiece at a magnification of $\times 25$. Recordings were made for the long axis of the root surface for all molar teeth. A total of six recordings were evaluated, including the distance measured in the first, second and third molars. Alveolar bone loss was calculated and presented as total bone loss, as determined by the following equation: [(total CEJ-to-ABC distance of ligatured site) – (total CEJ-to-ABC distance of non-ligatured site)].

Histological sample preparation

After sacrifice, the animal's whole maxilla was dissected and fixed in 5% formaldehyde-saline solution overnight at 4°C. The tissue was then decalcified in 10% Ethylene-diamine-tetraacetic acid (EDTA) containing 0.1 M Tris (pH 6.9) for 14 days at 4°C. The samples were then embedded in paraffin or frozen OCT compound (Tissue-Tek[®], Sakura Finetek, USA) for TRAP and immunofluorescence staining.

Evaluation of osteoclastogenesis *in vivo* by TRAP staining

Serial paraffin sections (7 μm) were cut. The formation and localization of osteoclasts were evaluated by TRAP staining as previously described (50). Briefly, the slides were incubated for 10 min in TRAP staining solution at 37°C in the dark. The slides were counterstained with

methyl green. Multinucleated dark brown cells with three or more nuclei lying along the alveolar bone surface were considered osteoclasts.

Immunofluorescence staining

Serial frozen sections (7 μm) were cut by cryostat sectioning and mounted on a glass slide. Frozen maxilla sections were fixed in ice-cold acetone at -20°C for 10 min and blocked with 5% bovine serum albumin (BSA) for 1 hour at room temperature in a humid chamber.

FITC- and Hoeschst 33342 (Thermo Fisher Scientific)-conjugated anti-mouse DC-STAMP monoclonal antibodies were used to stain murine DC-STAMP and nuclei, respectively. An inverted Zeiss LSM 780 confocal microscope with GAsP 34 channel detector was used to observe histological sections. Images were obtained with a Plan Apochromat 40x 1.40 NA oil immersion objective. Each scan was performed using identical laser power, gain, and offset values. Z-stack images were acquired at fixed intervals with a Z-depth of 4.0 μm . Bi-dimensional reconstruction was performed using the Zeiss LSM image browser and then saved as TIFF files. Final images were prepared for publication by using Adobe Photoshop CS, version 6.0.

Analysis of cytokine production in gingival tissue surrounded periodontal lesion

As previously described (51), gingival tissues were homogenized with Dounce glass homogenizer in phosphate-buffered saline supplemented with 0.05% Tween 20, Phenylmethanesulfonyl fluoride (1 mmol/L; Sigma, St. Louis, MP), and protease inhibitor cocktail (Sigma). After centrifugation, the supernatant was collected for ELISA analysis. For ELISA, TNF- α , IL-1 β and sRANKL levels in homogenized tissue were determined using

commercially available ELISA kits (DuoSet ELISA, R&D Systems, MN, USA), while the IL-6 ELISA kit was purchased from Peprtech (Rocky Hill, NJ).

Dendritic cell proliferation

Mononuclear bone marrow cells were isolated from femur and tibia of 8- to 12-week-old male wild-type mice. Cells were seeded into a T-25 flask at 1.0×10^6 cells/ml in 5 ml of complete RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 0.1% gentamycin, 1% MEM Nonessential Amino Acid (NEAA), 0.1% 2-Mercaptoethanol and 20 ng/ml GM-CSF (R&D Systems, Minneapolis, MN, USA) and incubated at 37 °C. Every two days, the medium was replaced with fresh medium with 20 ng/ml GM-CSF. At day 6 to 8, dislodged aggregates were collected and transferred to a new T-25 flask in fresh complete RPMI medium. Nonadherent cells were collected for the mixed lymphocyte reaction assay with T-cells. Dendritic cells were then incubated with Mitomycin C (Abcam, USA) 25µg/ml for 1 hour to inhibit cell growth.

T-cell isolation

T-cells from ligature-induced periodontal disease were isolated from cervical lymph node. T-cells were enriched by glass-wool column purification (52).

Mixed lymphocyte reaction (MLR) and T-cell proliferation assays

T-cells (2.5×10^5 cells/well) were cultured with 5×10^4 matured dendritic cells in RPMI medium supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin in a 96-well plate at 37 °C. T-cells were cultured with dendritic cells in the presence or absence of *Pp*

bacteria (10^7 /well) as an antigen. The anti-DC-STAMP-mAb or control mAb ($50\mu\text{g/ml}$) was also added to the T-cell cultures. ^3H -Thymidine ($0.5\mu\text{Ci/well}$) was applied for the last 24 hours of a total of 3-day culture. The proliferation and activation of T-cells were measured by radioactivity (c.p.m.) using a β -scintillation spectrometer (Beckham Coulter, Fullerton, CA, USA). The culture supernatant was collected for sRANKL detection by ELISA. Antigen-specific proliferation of T-cells was calculated and shown as a count per minute (c.p.m.) value and stimulation index (SI).

IgG response against bacteria

To detect antibacterial IgG levels in serum, the formalin-fixed bacteria/bacterial sonicate of *Pasteurella pneumotropica* (*Pp*) was used as antigen in a modified ELISA assay as previously described (53).

Statistical analysis

Student's t-test was used for comparison of two different outcomes of experiments performed. A *p*-value of less than 0.05 was considered statistically significant.

CHAPTER IV

Research results

Characterization of Anti-DC-STAMP mAb

The isotype of anti-DC-STAMP-mAb was determined using mouse IsoStrip™ Isotyping Kit (Roche, USA). Anti-DC-STAMP-mAb was shown to be an IgG2a subtype (Figure 2). Next, the ability of anti-DCSTAMP-mAb to recognize DC-STAMP protein was examined. Therefore, Western blot assay was performed on total protein extracted from RAW264.7 cell lysate after 7 days of sRANKL stimulation. The result demonstrated that anti-DC-STAMP-mAb could recognize approximately 53 kDa of DC-STAMP in monomer form and approximately 106 kDa of DC-STAMP in dimer form (Figure 3), similar to the results reported by the study using rabbit anti-DC-STAMP polyclonal Ab and another mouse anti-DC-STAMP-mAb (commercial Anti-DC-STAMP-mAb; clone 1A2, Millipore, USA) (9, 34). These results indicated that anti-DC-STAMP-mAb could recognize DC-STAMP expressed on osteoclasts.

While clone 1A2 recognizes DC-STAMP at the fourth extracellular domain (loop 3), the newly developed anti-DC-STAMP-mAb bound to the epitope present in the same loop 3. The difference in DC-STAMP binding sites between these two antibodies was confirmed by ELISA (Figure 4).



Figure 2. Determination of anti-DC-STAMP mAb (generated by Dr. Toshihisa Kawai) isotype. IsoStrip™ Mouse Monoclonal Antibody Isotyping Kit (Roche, USA) was used. The band appearing on the strip revealed that anti-DC-STAMP-mAb is an IgG2a isotype mAb.

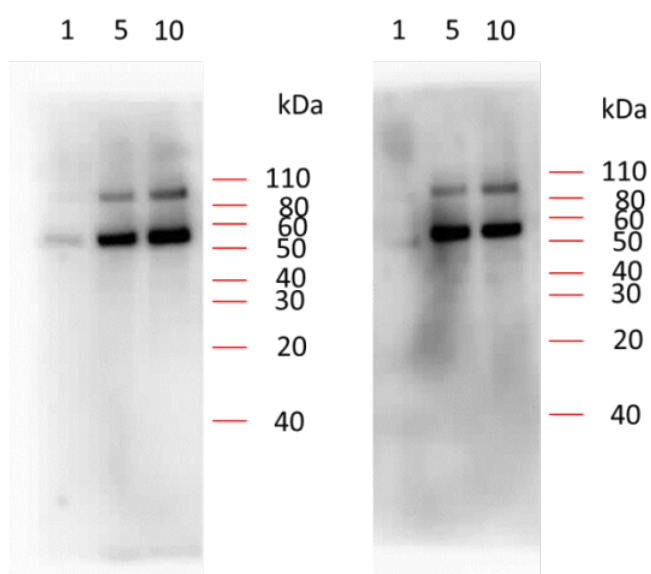


Figure 3. Anti-DC-STAMP-mAb (generated by Dr. Toshihisa Kawai) recognizes 53 kDa and 106kDa DC-STAMP in monomeric and dimeric forms, respectively. Total protein extracted from RAW264.7 cell lysate (1, 5 and 10 μ g, respectively). Western blotting analysis for DC-STAMP against specific anti-DC-STAMP-mAb (left and right panel represented the antibody dilution of 50 times and 200 times, respectively) demonstrated the strong detection of DC-STAMP protein, even with 200 times dilution. (The upper band represented dimeric form of DC-STAMP 106kDa, while the lower band represented monomeric form of DC-STAMP 53kDa.)

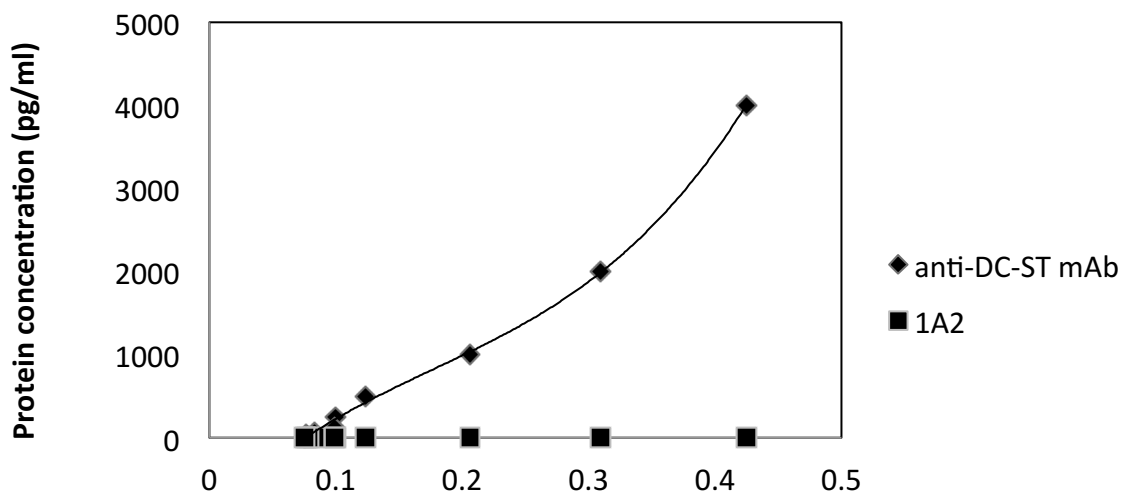


Figure 4. Comparison of developed anti-DC-STAMP-mAb (generated by Dr. Toshihisa Kawai) and commercial anti-DC-STAMP (clone 1A2, Millipore, USA) 1A2 for the detection DC-STAMP peptide. ELISA plates were coated with DC-STAMP protein fragment recognized by anti-DC-STAMP-mAb. ELISA result showed that anti-DC-STAMP mAb recognized a different DC-STAMP peptide compared to 1A2.

Localization of DC-STAMP in osteoclast cell culture *in vitro*

Next, we examined the expression of DC-STAMP on mouse bone marrow-derived mononuclear cells by anti-DC-STAMP-mAb. The cell surface expression of DC-STAMP in cell culture with or without sRANKL stimulation was analyzed by flow cytometry. In the absence of sRANKL stimulation, DC-STAMP was expressed in 18.5 % of the total cell number. DC-STAMP expression level significantly increased after sRANKL treatment for 3 days (Figure 5).

To visualize the presence of DC-STAMP on osteoclast cells, immunofluorescence staining of DC-STAMP and F-actin was performed in mouse bone marrow-derived osteoclast cell culture at day 5 and 7 of osteoclastogenesis (Figure 6). Results from confocal microscopy revealed the heterogeneity of DC-STAMP localization among cells. In addition, it was suggested that DC-STAMP was preferentially found in smaller osteoclasts, both in terms of size and number of nuclei.

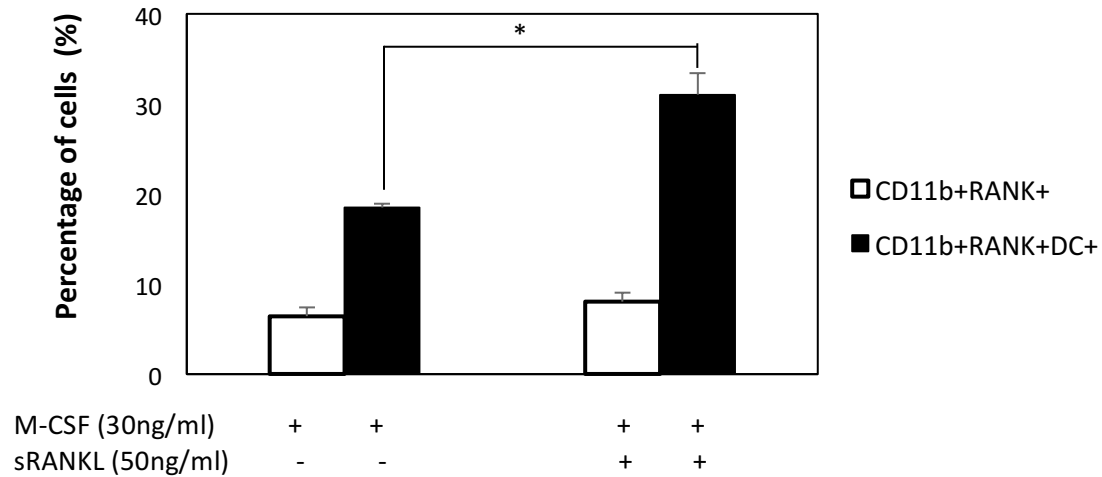


Figure 5. Flow cytometry analysis of DC-STAMP expression on osteoclast precursor cells. Osteoclasts derived from mouse bone marrow cells were incubated with or without sRANKL for 3 days, and the cell-surface expression levels of DC-STAMP were analyzed by flow cytometry. After 3 days of incubation with sRANKL treatment, the percentage of CD11b+RANK+DC-STAMP+ had significantly increased from 18.5% in untreated cells to 31.0% in sRANKL-treated cells ($p < 0.05$) (mean \pm SD; $n = 3$).

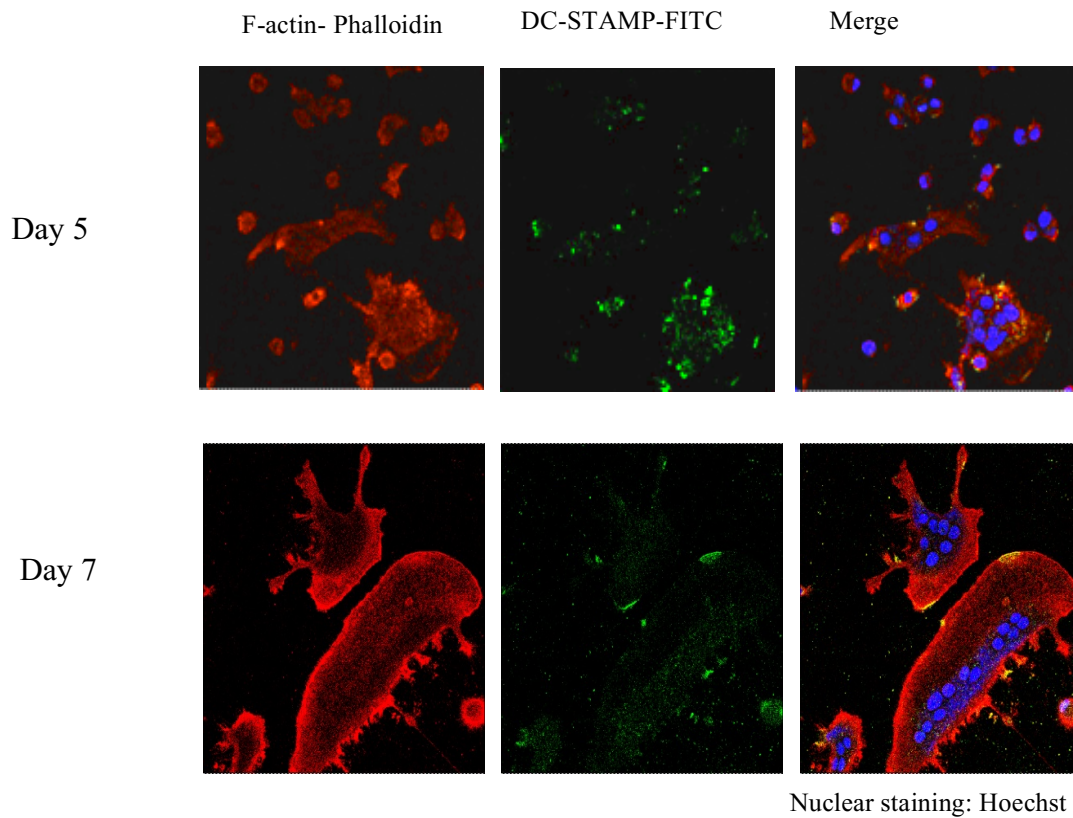


Figure 6. The localization of DC-STAMP in osteoclasts *in vitro*. Immunofluorescence staining for Hoechst-stained nuclei (blue), F-actin–phalloidin (red) and DC-STAMP-FITC (green) in osteoclast cell culture at day 5 and 7 of osteoclastogenesis was evaluated by confocal microscopy. The expression of DC-STAMP was observed in osteoclast cells. Results revealed heterogeneity in DC-STAMP localization among cells. In addition, it was suggested that DC-STAMP was preferentially found in smaller osteoclasts, both in terms of size and number of nuclei.

The effect of anti-DC-STAMP mAb on osteoclast formation *in vitro*

We next examined the effect of anti-DC-STAMP-mAb on osteoclastogenesis in RAW264.7 cells by TRAP staining. TRAP-positive multinuclear cells, containing three nuclei or more, were considered as osteoclasts and were counted microscopically. In RAW264.7 cell culture with M-CSF, no detectable TRAP staining was observed without RANKL stimulation. RANKL efficiently induced osteoclastogenesis, as shown by the TRAP-positive multinucleated cells. When anti-DC-STAMP-mAb (10, 20, 50 and 100 $\mu\text{g/ml}$) was added together with RANKL, the number of TRAP-positive multinucleated cells was diminished. In contrast, anti-Pg10 or control mAb had no effect on osteoclastogenesis. The most effective dose was 20 and 50 $\mu\text{g/ml}$ (Figure 7).

We also examined the inhibitory effect of anti-DC-STAMP-mAb on mouse bone marrow-derived cell culture. Results showed no detectable TRAP staining without RANKL, while osteoclast formation was detected when RANKL treatment was applied. Twenty and 50 $\mu\text{g/ml}$ of Anti-DC-STAMP-mAb significantly reduced the number of TRAP-positive multinucleated cells. The most effective dose was 50 $\mu\text{g/ml}$. Interestingly, when 100 $\mu\text{g/ml}$ of anti-DC-STAMP-mAb was added, osteoclastogenesis was not diminished. In addition, the number of TRAP-positive multinucleated cells was significantly increased with the addition of 100 $\mu\text{g/ml}$ of anti-Pg10-mAb (Figure 8).

We speculated that the Fc portion of mAb might contribute to the increase in osteoclastogenesis. We, therefore, examined osteoclastogenesis in FcR-KO mouse bone marrow-derived cells. In comparison to wild-type cells, the number of TRAP-positive multinucleated cells in the FcR-KO group was diminished in a dose-dependent manner (Figure 9). This

supported the hypothesis that the Fc portion of mAb might possibly bind to Fc receptor on osteoclasts and thus induce osteoclastogenesis.

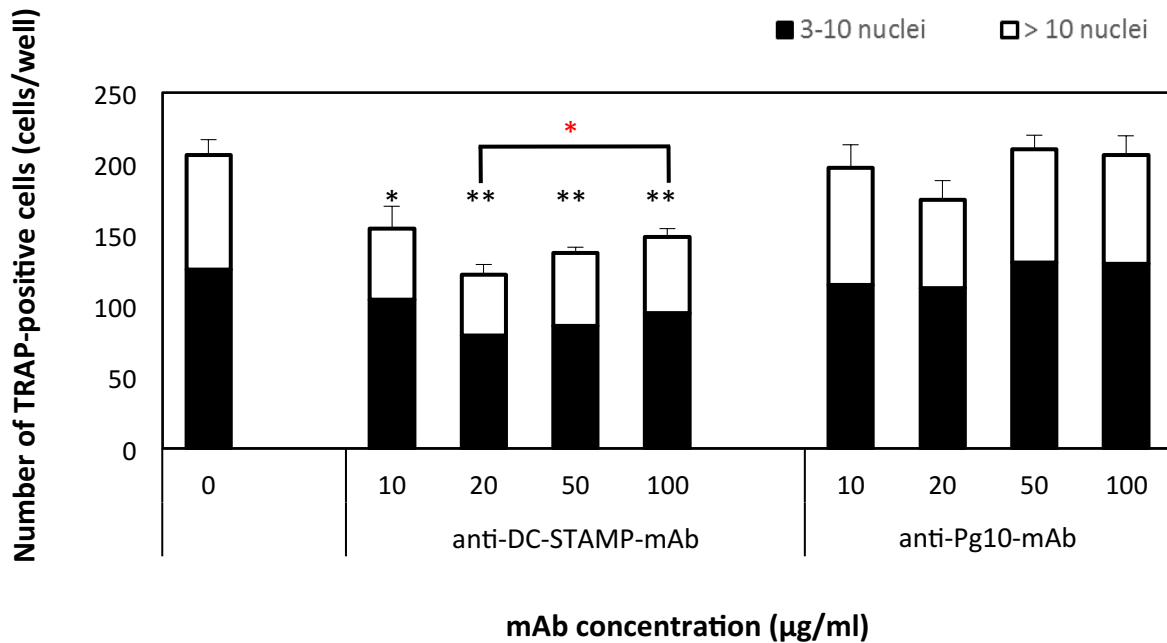


Figure 7. Effect of anti-DC-STAMP-mAb on osteoclastogenesis in RAW264.7 cells. Cells were incubated with M-CSF (30ng/ml) and sRANKL (50ng/ml) with or without mAb for 7 days. TRAP-positive multinucleated cells, containing three nuclei or more, were considered as osteoclasts and were counted microscopically. Anti-DC-STAMP-mAb at doses of 10, 20, 50 and 100 µg/ml significantly reduced osteoclast cell-cell fusion *in vitro*. Anti-Pg10-mAb had no effect. (n=8, * $p < 0.05$, ** $p < 0.01$, compared to negative control).

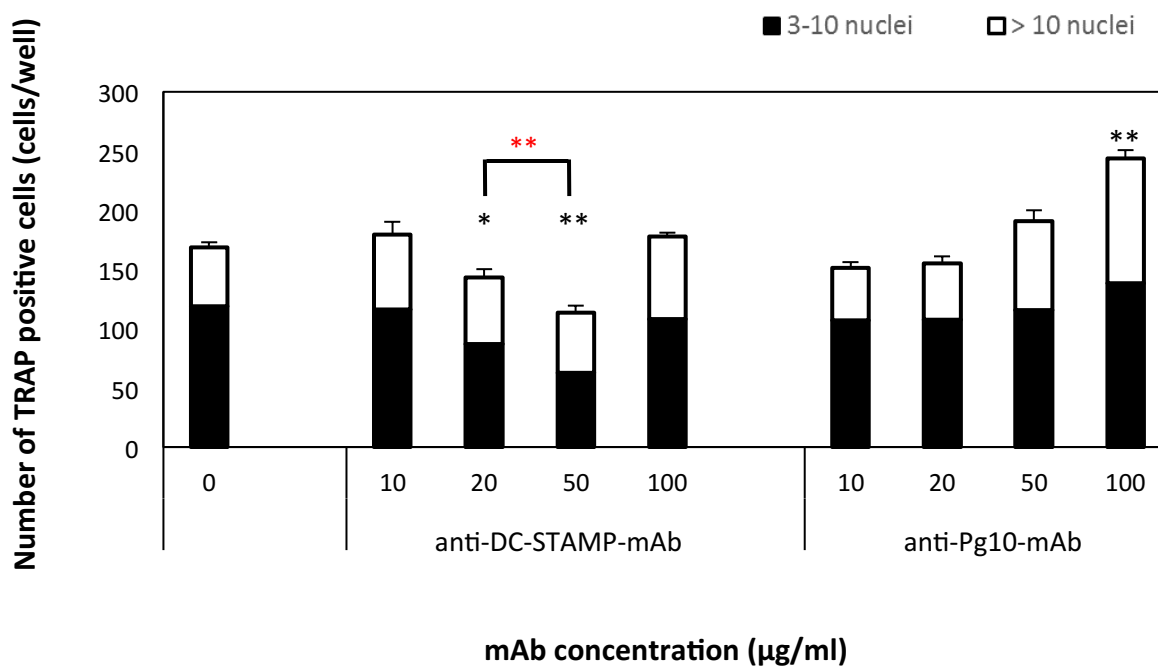


Figure 8. Effect of anti-DC-STAMP mAb on osteoclastogenesis in mouse bone marrow-derived osteoclast cells. Cells were incubated with M-CSF (30ng/ml) and sRANKL (50ng/ml) with or without mAb for 7 days. TRAP-positive multinucleated cells, containing three nuclei or more, were considered as osteoclasts and were counted microscopically. Anti-DC-STAMP-mAb at doses of 20 and 50 µg/ml significantly reduced osteoclast cell-cell fusion *in vitro*. Anti-Pg10-mAb had no effect on osteoclastogenesis at doses 10, 20 and 50µg/ml. Anti-Pg10-mAb significantly upregulated osteoclastogenesis at a dosage of 100µg/ml (n=8, * $p < 0.05$, ** $p < 0.01$, compared to negative control).

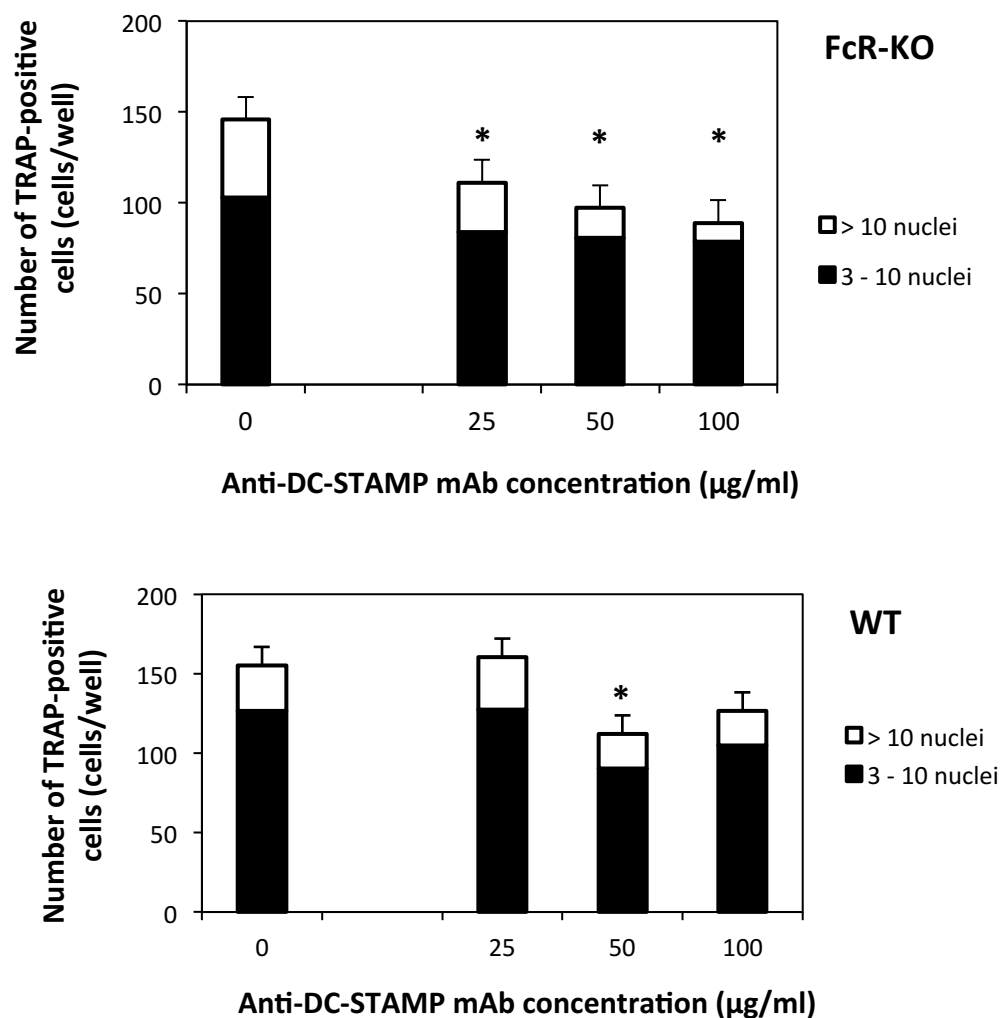


Figure 9. The effects of anti-DC-STAMP-mAb on osteoclastogenesis in C57BL/6 wild-type compared to bone marrow-derived osteoclastogenesis in FcR-KO mice. Cells were incubated with M-CSF (30ng/ml) and sRANKL (50ng/ml) with or without mAb for 7 days. TRAP-positive multinucleated cells, containing three nuclei or more, were considered as osteoclasts and were counted microscopically (n=6, * $p < 0.05$, compared to negative control).

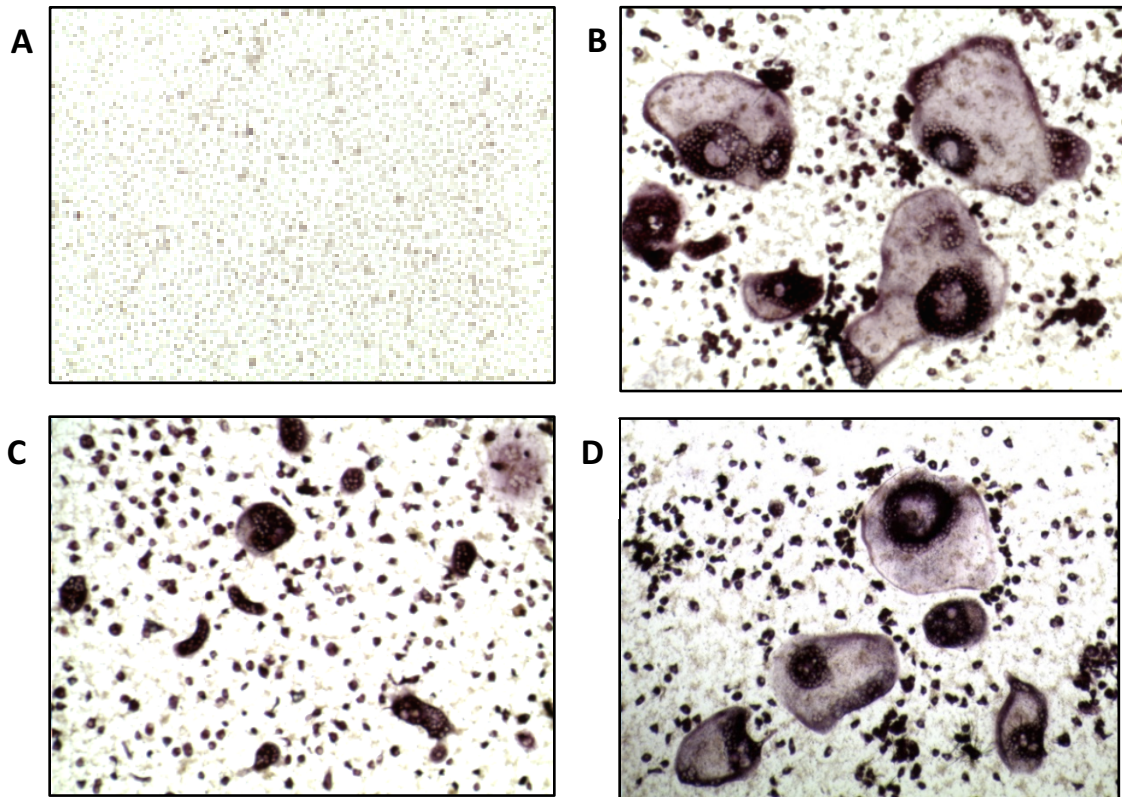


Figure 10. TRAP staining of mouse bone marrow-derived osteoclast cell culture under microscopy. TRAP staining was performed on day 7 after incubation with M-CSF (30ng/ml) alone (A) or M-CSF (30ng/ml) and sRANKL (50ng/ml) (B) and in the presence of anti-DC-STAMP-mAb (50µg/ml) (C) or anti-Pg10 mAb (50µg/ml) (D). Anti-DC-STAMP-mAb significantly reduced osteoclast cell-cell fusion *in vitro* (magnification 40x).

Effect of anti-DC-STAMP mAb on resorptive activity of osteoclasts in vitro

In a previous experiment, we found that 50 μ g/ml of anti-DC-STAMP-mAb effectively suppressed TRAP-positive multinucleated cell formation. To determine if this suppression affected resorptive activity, mouse bone marrow-derived mononuclear cells were cultured on Corning® Osteo Assay multiple-well plates. When compared to undifferentiated mononuclear cells, many resorption pits were observed with RANKL stimulation. Therefore, Anti-DC-STAMP-mAb had significantly reduced the resorption area, as shown in Figure 11 and 12.

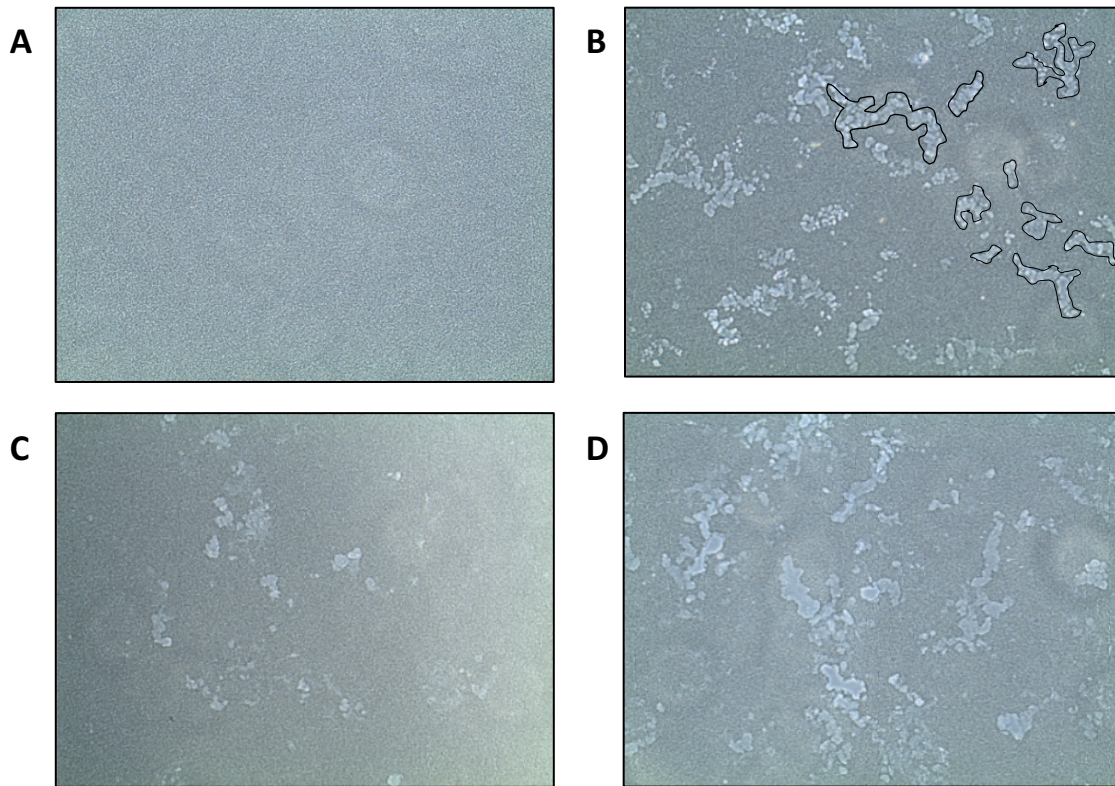


Figure 11. Pit formation assay of mouse bone marrow-derived osteoclast cell culture under microscopy. Cells were cultured on Corning® Osteo Assay multiple-well plates (Corning, USA). Pit formation assay was performed on day 7 after incubation with M-CSF (30ng/ml) alone (A) or M-CSF (30ng/ml) and sRANKL (50ng/ml) (B) and in the presence of anti-DC-STAMP-mAb (50µg/ml) (C) or anti-Pg10 mAb (50µg/ml) (D). Anti-DC-STAMP-mAb significantly reduced resorption pit *in vitro* (magnification 40x). The example of resorption pit was circled with black line.

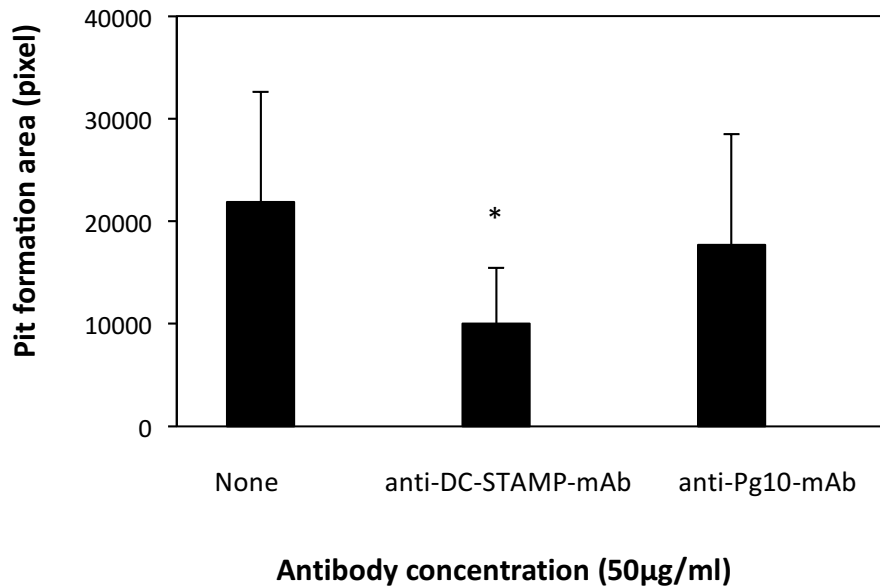


Figure 12. Effect of anti-DC-STAMP-mAb on resorptive activity in mouse bone marrow-derived osteoclast cells. Cells were cultured on Corning® Osteo Assay multiple-well plates (Corning, USA). Pit formation assay was performed on day 7 after incubation with M-CSF (30ng/ml) and sRANKL (50ng/ml) in the presence of anti-DC-STAMP-mAb (50µg/ml) or anti-Pg10 mAb (50µg/ml). Anti-DC-STAMP-mAb had significantly reduced resorption pit *in vitro* (n=8, * $p < 0.05$, compared to negative control).

Localization of DC-STAMP in ligature-induced periodontal lesion *in vivo*

Frozen sections were prepared from the maxilla mice with ligature-induced periodontitis sacrificed at day 7. Localization of DC-STAMP in periodontal lesion was determined by immunofluorescence using confocal microscopy (Figure 13). DC-STAMP expression was detected. Immunofluorescence staining demonstrated the higher expression of DC-STAMP on osteoclast-like cells on the alveolar bone surface in the ligature-induced periodontitis lesion compared to the healthy non-ligatured site.

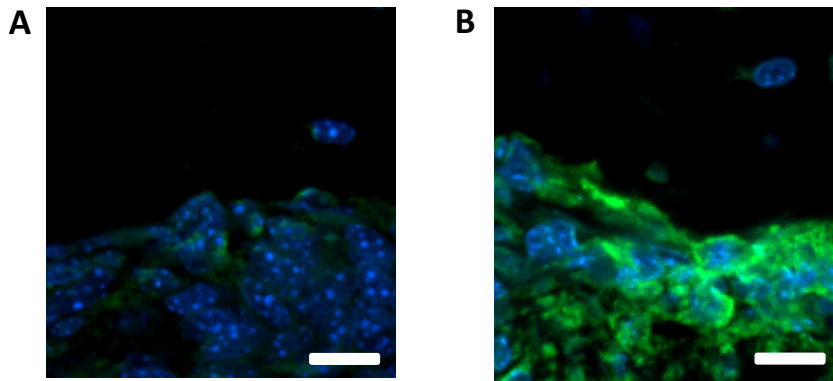


Figure 13. Distribution of DC-STAMP as detected in periodontal lesion by immunofluorescence staining. Immunofluorescence double-staining of frozen maxilla sections with monoclonal antibody against DC-STAMP (FITC; green) and nuclei (Hochest 33342; blue) were shown. DC-STAMP expression was higher in the ligature-induced periodontitis lesion (day 7) (B) in comparison to healthy site (without ligature) (A). The bars indicate 20 μm .

Effect of anti-DC-STAMP mAb on alveolar bone loss in ligature-induced periodontitis model

We first evaluated the effect of ligature placement on the amount of alveolar bone resorption in C57BL/6 mice at day 3, 7 and 14. Silk ligature promoted plaque accumulation and, as a result, induced alveolar bone loss (Figure 14). Bone loss was significant by day 7. Additional alveolar bone destruction could be observed from day 7 to 14, but not as significant (Figure 15). As previously mentioned, anti-DC-STAMP-mAb has a half-life of approximately 13 – 14 days. In this study, the anti-DC-STAMP-mAb was locally administered as a single dose at 10 μ g per site at the time of ligation. Mice were sacrificed at day 3, 7 and 14. Alveolar bone loss was observed in mice that received either anti-DC-STAMP-mAb or anti-Pg10-mAb. Anti-DC-STAMP-mAb, however, demonstrated a proportionately smaller amount of ligature-triggered alveolar bone resorption when compared to control mAb at Day 7 (1.18 vs. 1.68 mm, $p < 0.05$) and Day 14 (1.58 vs. 2.28 mm, $p < 0.01$) (Figure 16, 17). This result showed that anti-DC-STAMP-mAb effectively reduced periodontal bone resorption in ligature-induced periodontitis in mice.

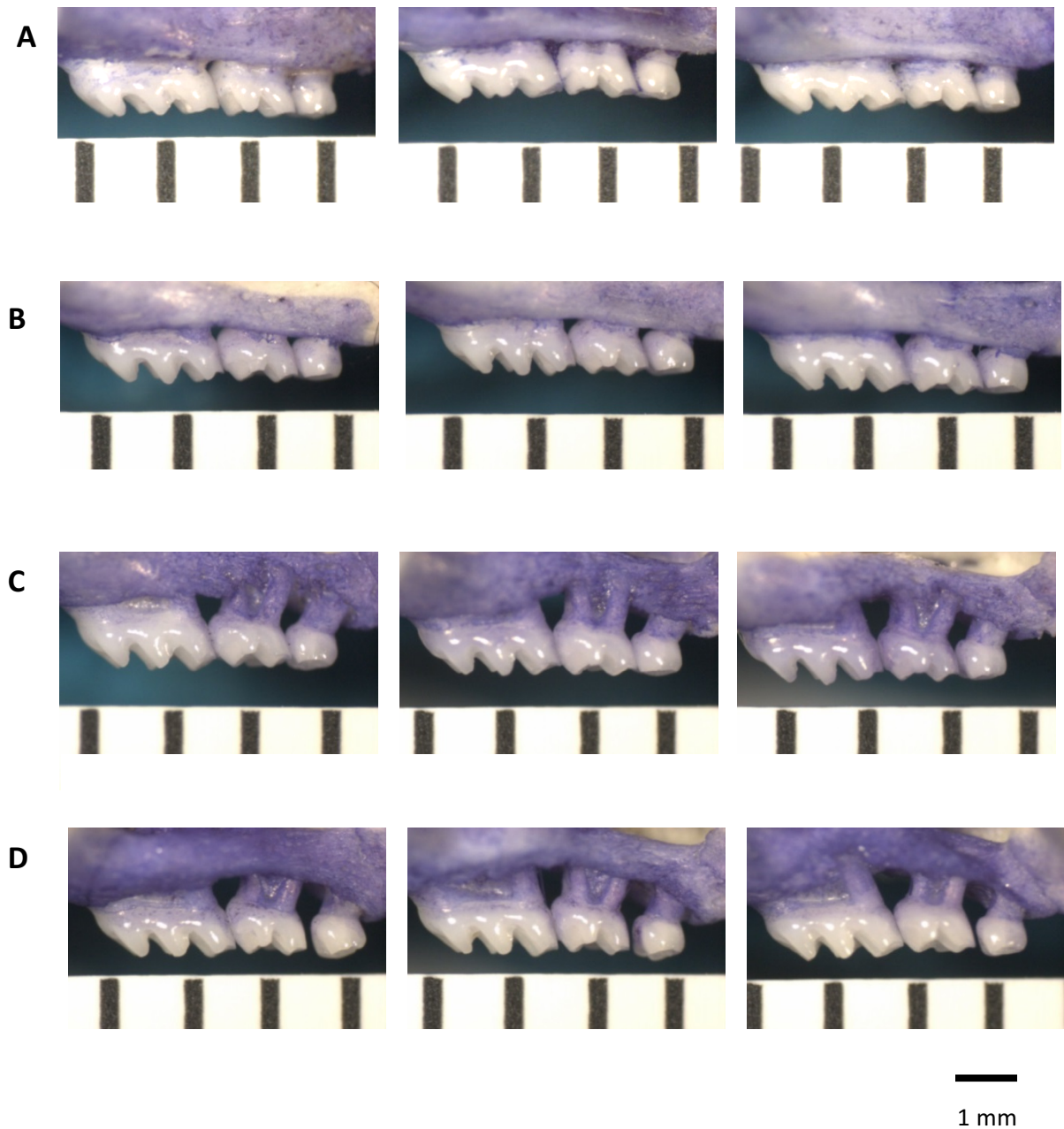


Figure 14. Alveolar bone resorption of maxilla under microscopy. The images presented ligature-induced bone resorption at maxillary buccal sites of mice without ligature (A). In B – D, 5-0 silk ligature (Ethicon, USA) was placed around maxillary second molars for 3, 7 and 14 days, respectively.

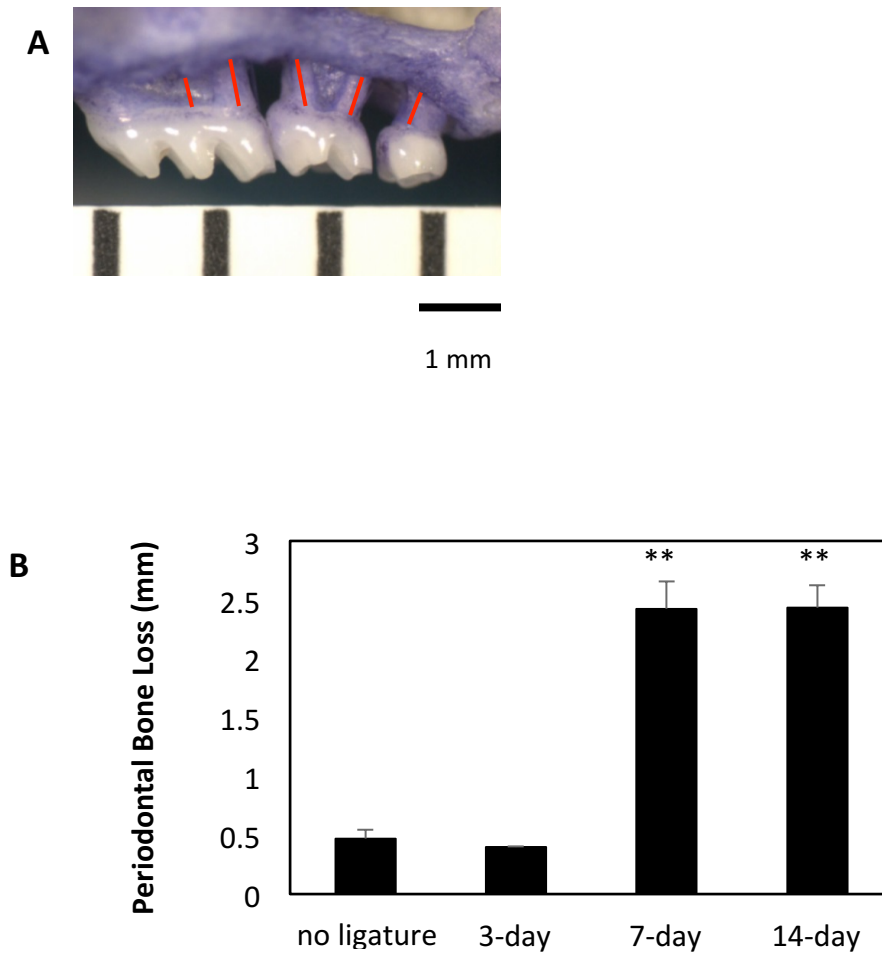


Figure 15. Measurement of alveolar bone resorption. Cervico-enamel junction (CEJ) to alveolar bone crest (ABC) distance was measured at the buccal side as indicated by the red line (A). The amount of bone resorption was calculated and presented in histogram (B) (Mean \pm SD, $n=3$, * $p<0.05$, ** $p<0.01$ compared to negative control).

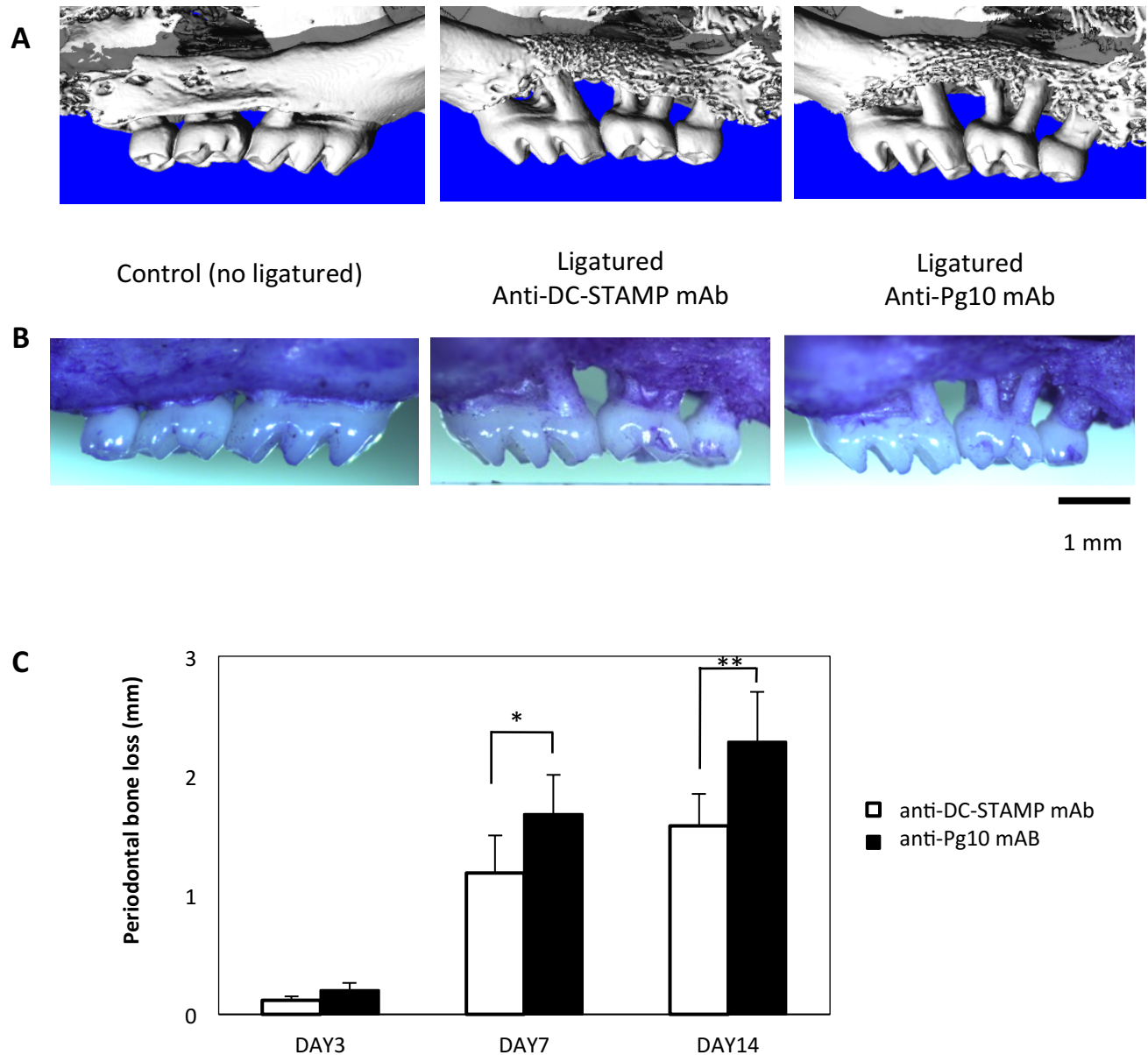


Figure 16. Effect of anti-DC-STAMP-mAb on alveolar bone loss in ligature-induced periodontitis model. Images of ligature-induced bone resorption at maxillary buccal sites of mice are shown (Day 7; A: microCT, B: dissection microscope). Anti-DC-STAMP-mAb significantly suppressed ligature-triggered alveolar bone resorption compared to control mAb at Day 7 and Day 14 presented in histogram (C) (1.18 vs. 1.68 mm, $p < 0.05$ and 1.58 vs. 2.28 mm, $p < 0.01$, respectively) (Mean + SD, $n = 6 - 7$ mice /group, * $p < 0.05$, ** $p < 0.01$).

Evaluation of osteoclast formation *in vivo*

Paraffin sections were prepared from maxilla of mice with ligature-induced periodontitis sacrificed at day 7. Localization of osteoclasts was examined in periodontal lesion by TRAP staining (Figure 17). According to the histological analyses, anti-DC-STAMP-mAb decreased the number and size of multinucleated TRAP⁺ cells in alveolar bone compared to control.

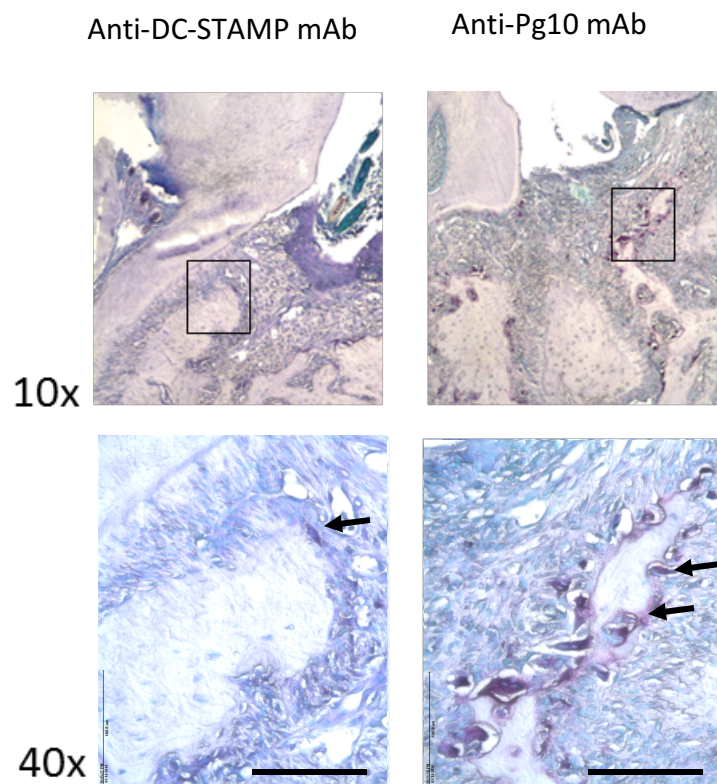


Figure 17. Effect of anti-DC-STAMP-mAb on *in vivo* ligature-caused osteoclast induction. At 7 days, paraffin sections from decalcified maxilla were submitted to TRAP staining. TRAP-positive multinucleated cells were indicated by arrowhead (Bar = 100 μ m). Anti-DC-STAMP-mAb decreased the local differentiation of TRAP-positive multinucleated cells.

Effect of locally administered anti-DC-STAMP-mAb on cytokine production

It is well known that TNF- α , IL-1 β , IL-6 and sRANKL are pro-inflammatory mediators involved in the pathogenesis of periodontitis (54). Therefore, cytokine levels were examined in the gingival tissue around periodontal lesion (Figure 18-21). Without ligature, clinically healthy periodontal tissue was assumed, and TNF- α , IL-1 β , IL-6 and sRANKL levels were readily detected. Induction of periodontitis with silk ligature increased TNF- α , IL-1 β , IL-6 and sRANKL production regardless of the administration with either anti-DC-STAMP-mAb or anti-Pg10-mAb. Indeed, there were no statistically significant difference in the production of those pro-inflammatory cytokines in the ligatured gingiva tissue between the anti-DC-STAMP-mAb and anti-Pg10-mAb treatments ($p > 0.05$), suggesting that DC-STAMP expressed in the inflamed periodontal tissue may not be engaged in the production of proinflammatory cytokines.

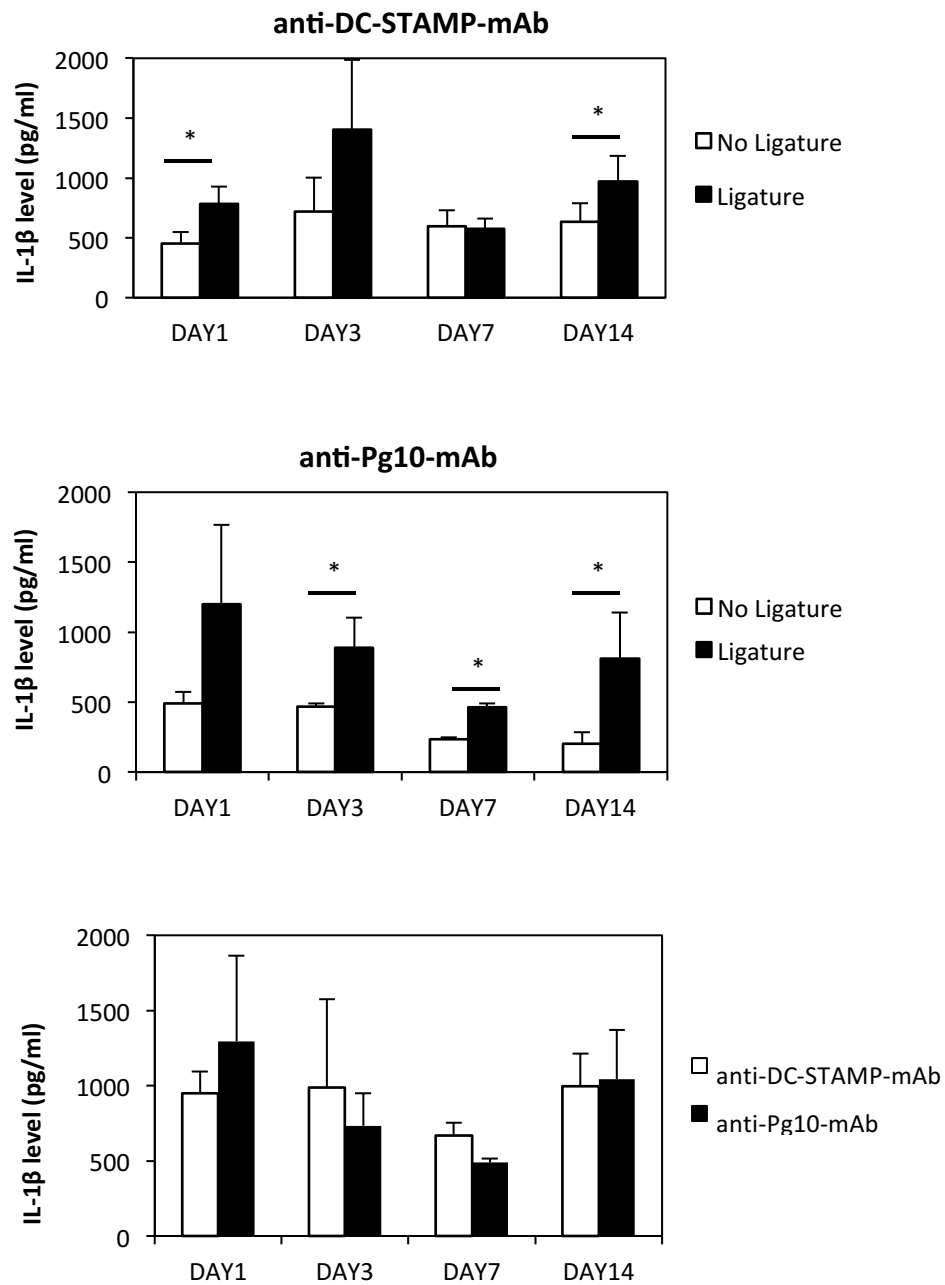


Figure 18. A-C. Effect of anti-DC-STAMP-mAb on IL-1 β response in ligature-induced periodontitis model. Proinflammatory cytokine IL-1 β in homogenized gingival tissue was measured by ELISA, and no statistical difference was found between groups receiving anti-DC-STAMP-mAb and control mAb (Mean \pm SD, n=4/group, * p <0.05).

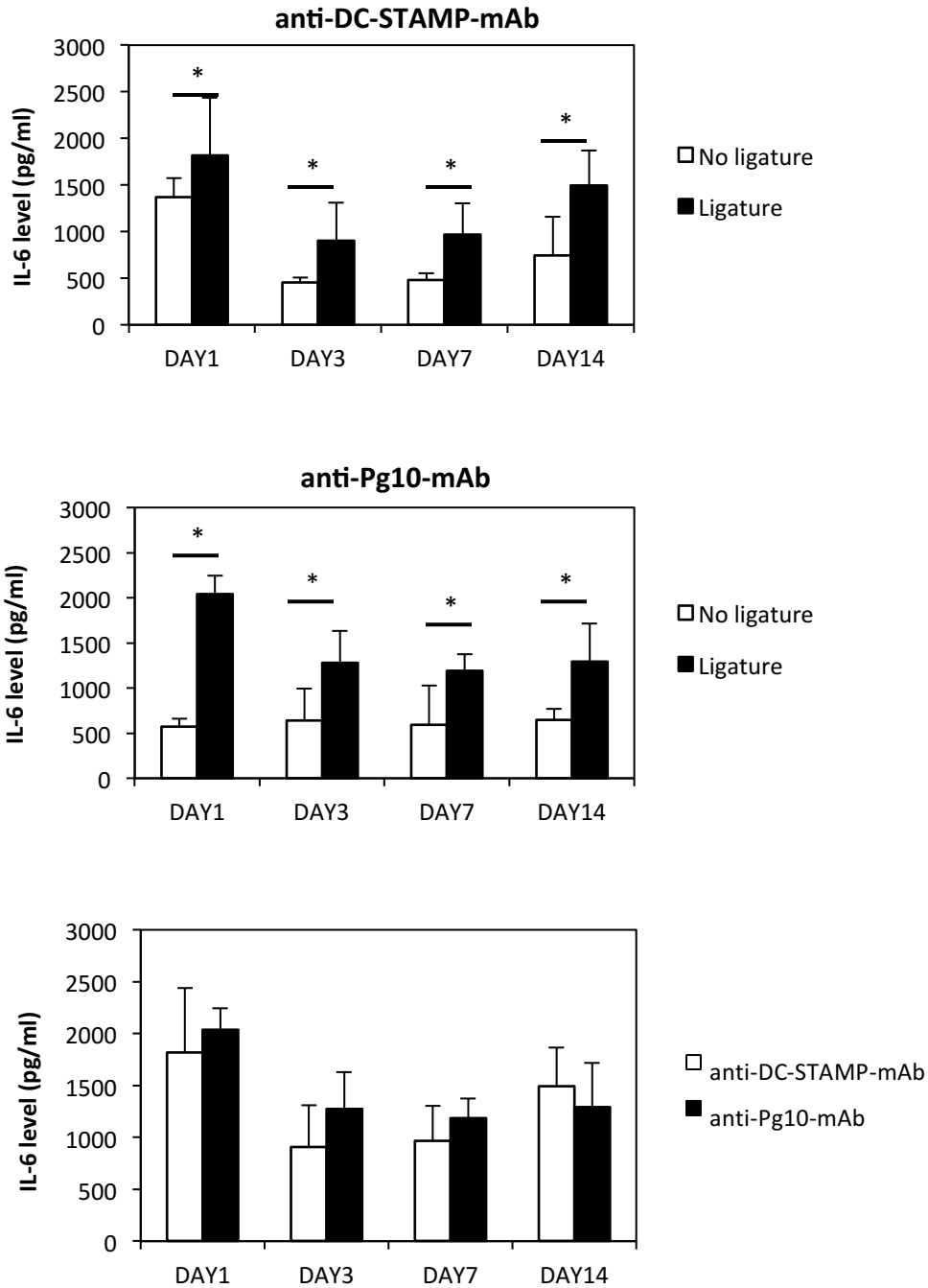


Figure 19. A-C. Effect of anti-DC-STAMP-mAb on IL-6 response in ligature-induced periodontitis model. Proinflammatory cytokine IL-6 in homogenized gingival tissue was measured by ELISA, and no statistical difference was found between groups receiving anti-DC-STAMP-mAb and control mAb (Mean \pm SD, n=4/group, * $p < 0.05$).

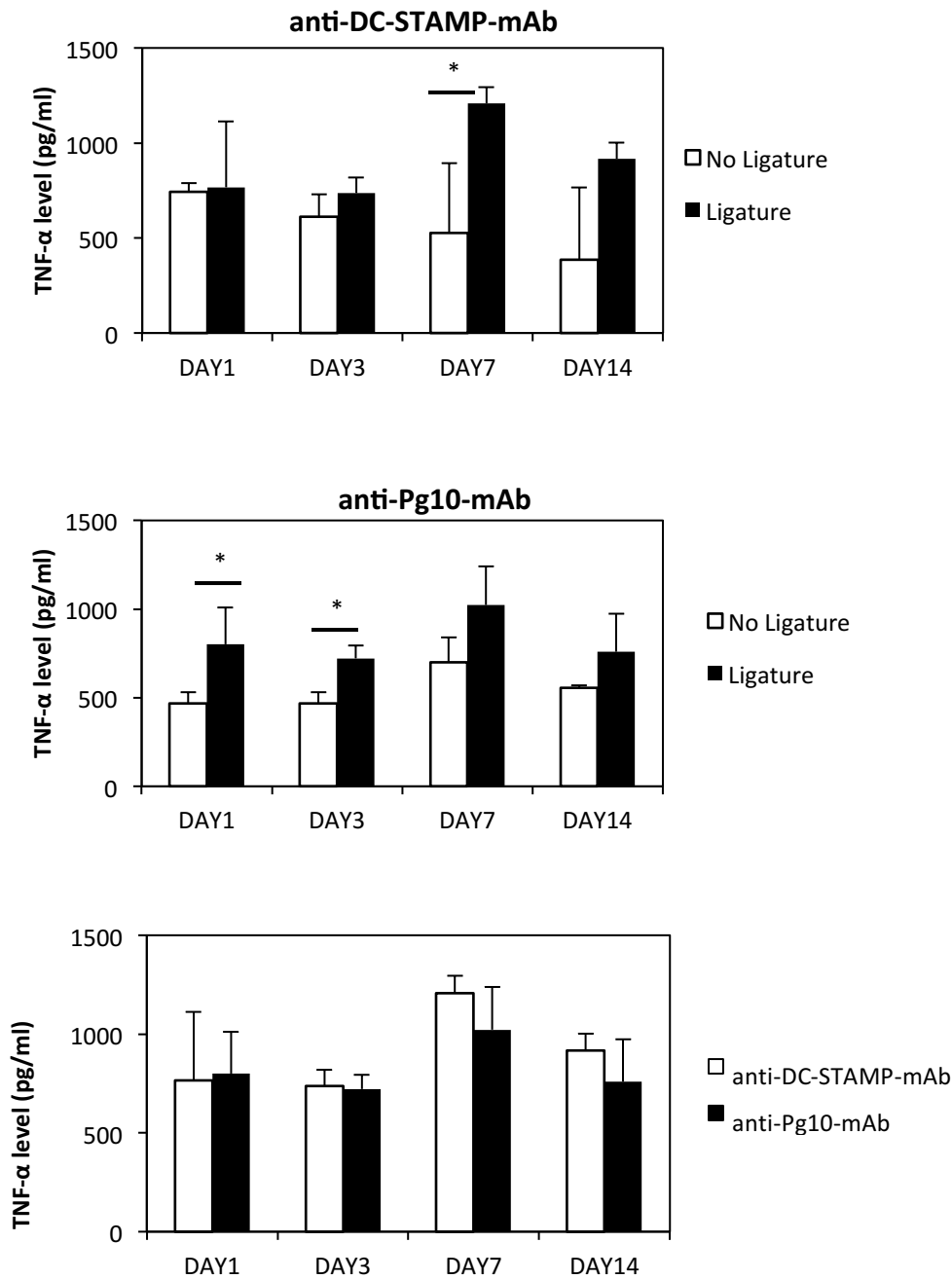


Figure 20. A-C. Effect of anti-DC-STAMP-mAb on TNF- α response in ligature-induced periodontitis model. Proinflammatory cytokine TNF- α in homogenized gingival tissue was measured by ELISA, and no statistical difference was found between groups receiving anti-DC-STAMP-mAb and control mAb (Mean \pm SD, n=4/group, * p <0.05).

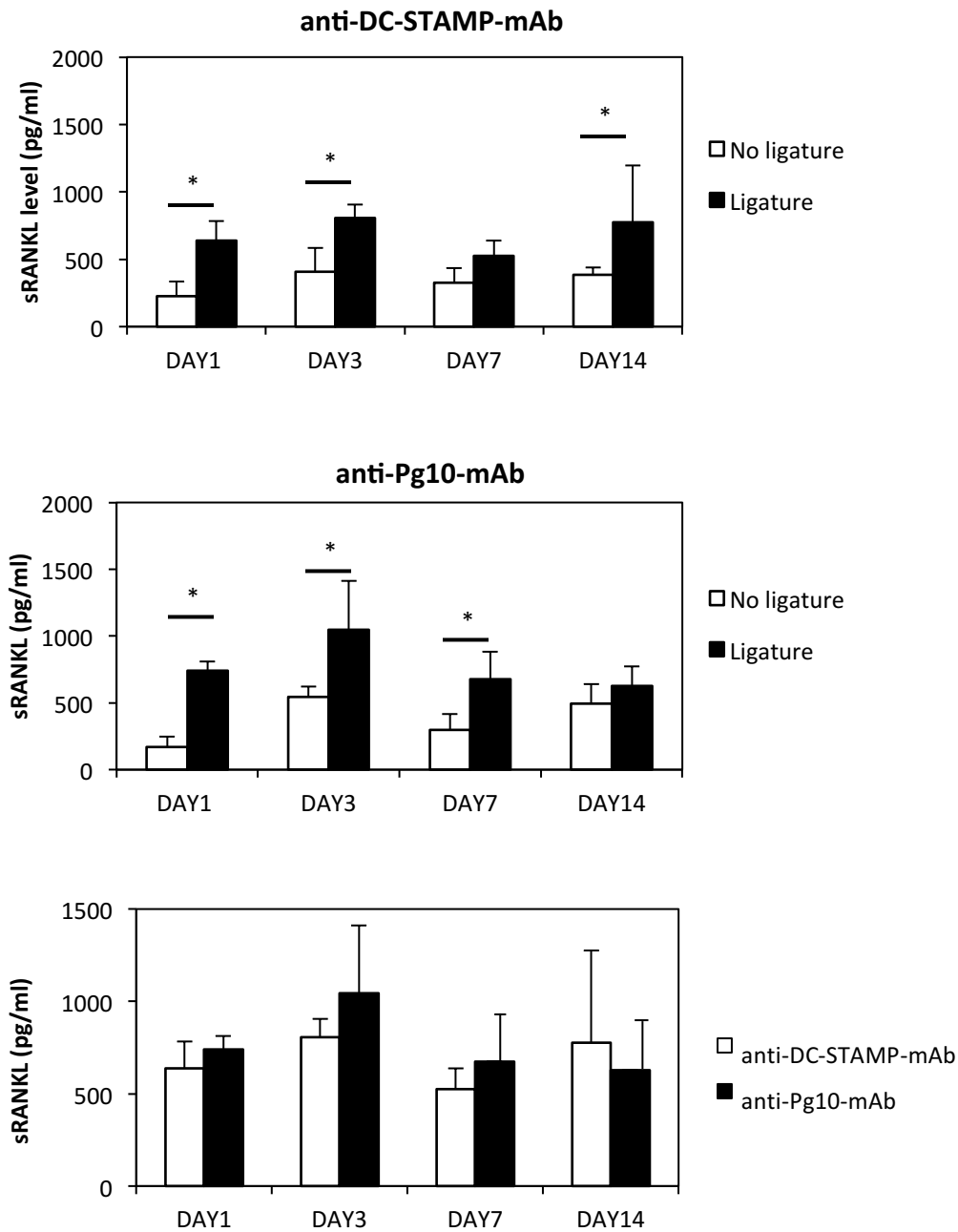


Figure 21. A-C. Effect of anti-DC-STAMP-mAb on sRANKL responses in ligature-induced periodontitis model. Proinflammatory cytokine sRANKL in homogenized gingival tissue was measured by ELISA, and no statistical difference was found between groups receiving anti-DC-STAMP-mAb and control mAb (Mean \pm SD, n=4/group, * $p < 0.05$).

Effect of locally administered anti-DC-STAMP-mAb on IgG-antibody response to bacterial challenge

We examined whether the local administration of anti-DC-STAMP- mAb would affect IgG antibody response against bacteria. We collected sera from mice that received anti-DC-STAMP or anti-*Pg*10 mAb locally injected at the gingival tissue after 7 and 14 days. We checked IgG antibody response against the mouse periodontal opportunistic pathogen *Pasteurella pneumotropica* (*Pp*) using ELISA. Results showed that anti-*Pp* IgG antibody was affected by anti-DC-STAMP-mAb (Figure 22).

These results suggested the robust efficacy of anti-DC-STAMP-mAb in suppressing alveolar bone loss by downregulating osteoclast cell fusion, but without affecting antibacterial immune responses.

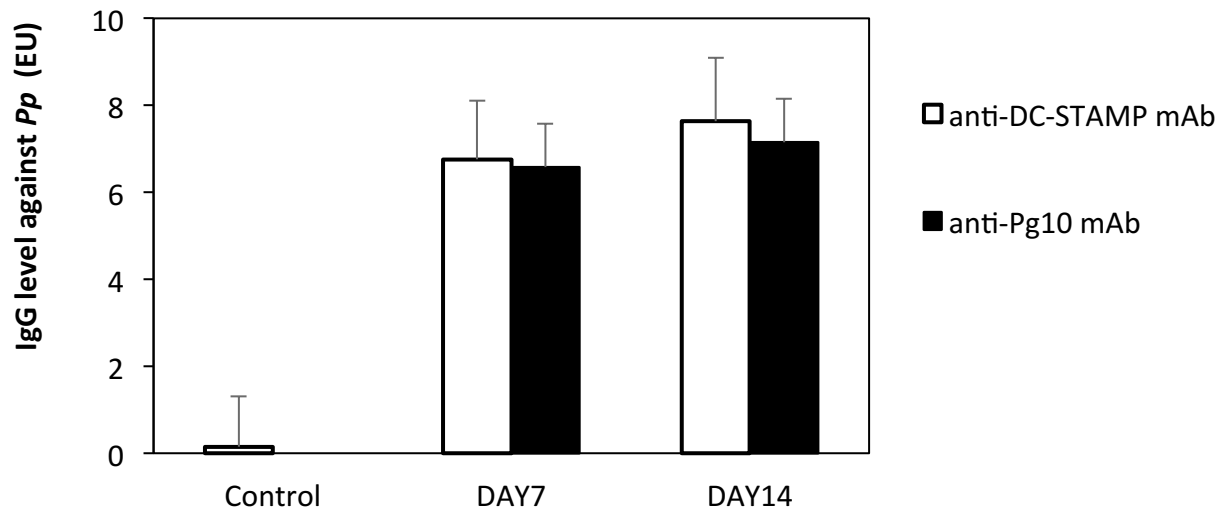


Figure 22. Effect of locally administered anti-DC-STAMP-mAb on antibacterial immune response. Serum IgG antibody response to *Pasteurella pneumotropica* (*Pp*) was analyzed by ELISA. No significant difference in IgG antibody response against *Pp* antigen was found when compared to control mAb (Mean \pm SD, n=5/group, * $p < 0.05$).

Effect of locally administered anti-DC-STAMP-mAb on T-cell response

Previous studies revealed the expression of DC-STAMP on dendritic cells (57, 58). Dendritic cells process and present antigen to T-cells. Therefore, we investigated the effect of locally administered anti-DC-STAMP-mAb on dendritic cells (DCs) in conjunction with T-cell response *in vitro*. In order to check the ability of DCs to induce T-cell proliferation, tritiated [3H] thymidine incorporation assays was used. T-cells were isolated from cervical lymph node of mice that received ligature placement around the second maxillary molars on both left and right sides for 2 days. T-cell proliferation was evaluated by 3H-thymidine incorporation assays. T-cell proliferation was increased upon *Pp* antigen challenge. Neither addition of anti-DC-STAMP nor control anti-*Pg*10-mAb affected T-cell proliferation. Anti-MHCII antibody significantly suppressed T-cell proliferation. As expected, T-cell response was not affected by local administration of anti-DC-STAMP-mAb (Figure 23-24).

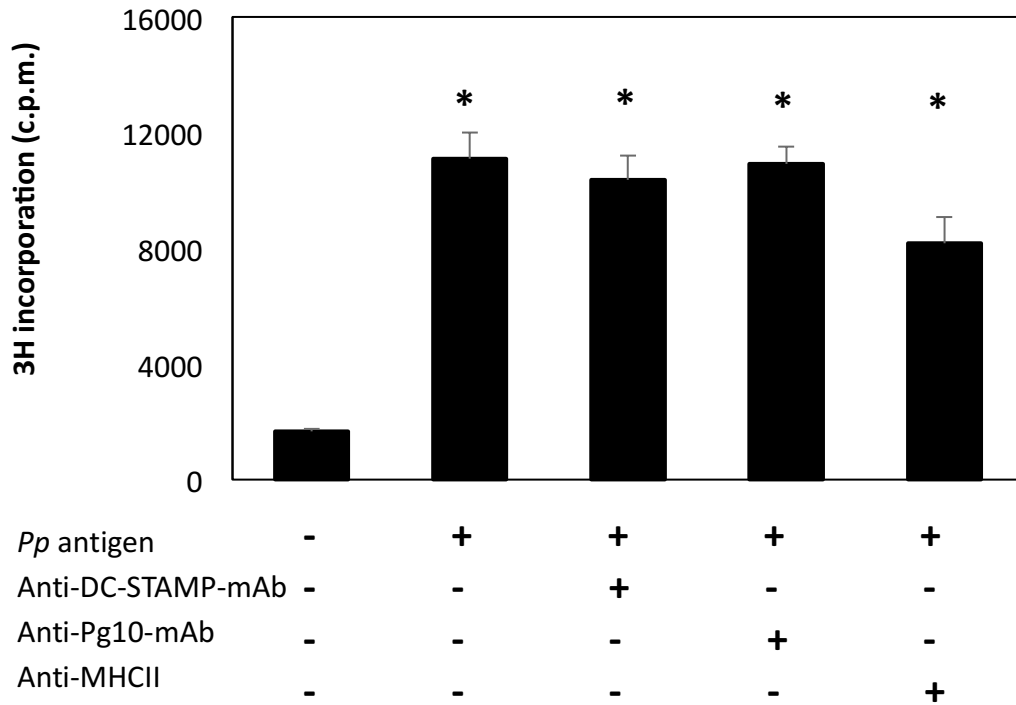


Figure 23. ^3H -thymidine incorporation assays of T-cells isolated from cervical lymph node of ligature-induced periodontitis mice. T-cell proliferation was upregulated when challenged with *Pp* antigen. The addition of anti-DC-STAMP-mAb or anti-*Pg*10 mAb did not alter T-cell proliferation in response to *Pp* antigen (Mean \pm SD, n=8/group, * $p < 0.01$, compared to control).

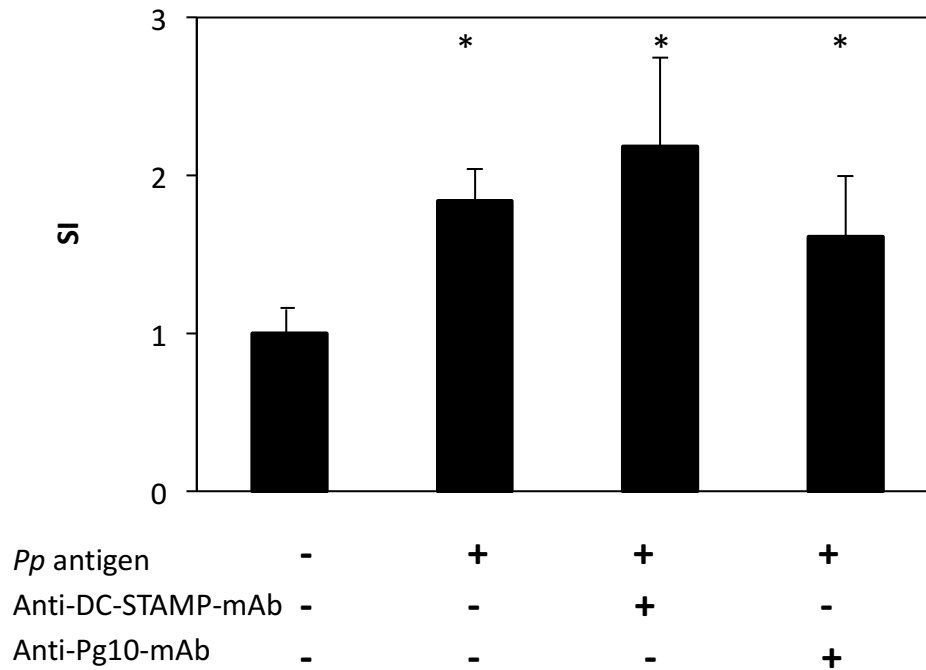


Figure 24. Stimulation indices (SI) of T-cells derived from cervical lymph node of mice that received ligature placement in response to bacterial antigen (*Pp*). Anti-DC-STAMP-mAb or anti-Pg10-mAb. T-cell proliferation was upregulated when challenged with *Pp* antigen. The addition of anti-DC-STAMP-mAb or anti-*Pg10* mAb, nor was T-cell proliferation altered in response to *Pp* antigen (Mean \pm SD, n=5/group, * $p < 0.05$, compared to control).

CHAPTER V

Discussion and Conclusion

In this study, we demonstrated that anti-DC-STAMP-mAb could inhibit ligature-induced periodontal bone resorption in a mouse model. Ligature-induced periodontitis has been used in various animal models, including rats, dogs, rabbits and mice. Compared to other animal models, several genetic strains of mice can be bred. Breeding these mice is inexpensive, and disease onset is rapid. Alveolar bone resorption in rat occurs predictably after 7 days of ligature placement (55, 56), but 5 to 7 days in mice (57, 58). In this study, we observed significant periodontal bone loss at day 7, gradually continuing until day 14. By the oral gavage method, on the other hand, significant bone loss typically takes longer than 4 weeks after the last inoculation (58-60). Ligature significantly facilitated the accumulation, as well as amount, of anaerobic bacteria. The importance of bacteria in the etiology of bone loss has been confirmed by antibiotic treatment, which significantly inhibited bone loss (57). In addition, ligatures do not induce inflammation or significant bone loss in germ-free rat (56, 61). The limitation of this model is the possibility of mechanical trauma during the placement of ligature. Regardless of this possibility, accumulated bacteria in the ligature do constitute a major factor in the induction of host immune response which, in turn, leads to the bone loss in this model.

In addition to RANKL-mediated osteoclastogenesis through RANK, co-stimulatory signaling has been reported (Kajiya et al. 2010). Fc γ RII and Fc γ RIII can bind polymeric IgG or IgG-immune complex and induce the phosphorylation of immunoreceptor tyrosine-based

activation motif (ITAM). Phospholipase C γ and calcium signaling are then upregulated providing the co-stimulatory signaling for RANKL-mediated osteoclastogenesis. This activation is induced by the mechanical force provided by multiple tightly connected IgG antibodies known as the capping effect (4). Therefore, it was speculated that the increase in osteoclast number in high concentrations of anti-DC-STAMP-mAb resulted from the formation of IgG-immune complex. Osteoclastogenesis was induced from bone marrow-derived cells from Fc γ R-KO mice in comparison to WT mice. As expected, anti-DC-STAMP-mAb at the same concentration as that used for suppression of osteoclastogenesis induced in WT-derived osteoclast showed the comparable inhibitory effect on Fc γ R-KO-derived osteoclasts (not shown).

Dendritic cells represent a large family of antigen presenting cells that circulate in the blood stream and also locate in tissue. Dendritic cells capture and process antigens to stimulate T-cell responses. Dendritic cells are critical in initiating an adaptive immune response. In human tissue with chronic periodontitis, immature dendritic cells, termed Langerhans cells, predominantly infiltrate the gingival epithelium. Mature dendritic cells, on the other hand, specifically infiltrate the CD4 (+) lymphoid-rich lamina propia (62). DC-STAMP was first shown to be expressed on dendritic cells (12). In their studies, DC-STAMP was constitutively expressed from day 3 to day 8 and detected on both immature and mature dendritic cells. The expression of DC-STAMP is first detectable at day 3 and reached its peak at day 7, then down-regulated as dendritic cell completed their maturation. The expression of DC-STAMP is restricted to monocyte-derived dendritic cell as well as myeloid CD11c⁺ blood dendritic cell, but not Langerhans cells or lymphoid CD11c⁻. In contrast, DC-STAMP expression was not found on monocyte, PBMC and B cells (63).

It is interesting that both osteoclasts and dendritic cells express the DC-STAMP molecule. However, multinucleation is only found in osteoclasts. One possible explanation is the difference in the localization of DC-STAMP between osteoclasts and dendritic cells. While DC-STAMP is expressed on the surface of osteoclasts (9), it is expressed on the endoplasmic reticulum of immature dendritic cells before translocation towards the Golgi compartment upon maturation (63, 64). However, the role of DC-STAMP in dendritic cells was not well understood. Role of DC-STAMP in dendritic cell was evaluated in DC-STAMP^{-/-} mice. Aged DC-STAMP^{-/-} mice, older than 12 months, developed autoimmune symptoms including multisystemic inflammations in the kidney, lung and salivary gland. These aged DC-STAMP^{-/-} mice compared to wild-type mice, showed increased splenic size and cellular infiltrates in the spleen, and T-cell infiltration into various organs and tissues. In addition, anti-anti-dsDNA antibodies was significantly increased with the IgG depositions in kidney glomeruli (65). In the *in vitro* experiment, DC-STAMP^{-/-} dendritic cells showed normal differentiation and proliferation. However, antigen presentation activity of DC-STAMP^{-/-} dendritic cells was significantly up-regulated through increased phagocytotic activity compared with wild-type dendritic cells. These results suggested that DC-STAMP is engaged in down-regulating the induction of autoimmune responses (65).

The same group of researchers further investigated the role of DC-STAMP in the antigen-presenting function to T cells using DC-STAMP-specific shRNAs to knock-down DC-STAMP in murine bone marrow-derived dendritic cells (66). There were no difference in term of morphology and the upregulation of costimulatory molecules upon LPS stimulation. Nevertheless, the secretions of cytokines; TNF- α , IL-6, IL-12, IL-10., MIP-1 α , were decreased whereas those secretions of IL-1 β and IL-1 α were increased. In addition, it was demonstrated

that DC-STAMP-/dendritic cells induced significantly less IFN- γ production from Th1 T cell and were less efficient in stimulating T cell proliferation compared to wild-type dendritic cells (66).

Our study demonstrated that anti-DC-STAMP-mAb could reduce alveolar bone resorption in periodontitis without affecting adaptive immune response. According to the *in vitro* assays, the inhibition of cell-cell fusion between preOCs by anti-DC-STAMP-mAb could account for its role in the suppression of periodontal bone loss. Interestingly, anti-DC-STAMP-mAb did not alter IgG antibody response against oral bacteria. Since antibodies play a major role in antibacterial adaptive immune response, this behavior could be considered beneficial to the host. Neither did anti-DC-STAMP-mAb cause dysregulated T cell- responses to bacteria (*Pp*) were also unaffected by anti-DC-STAMP-mAb *in vitro*. Furthermore, production of RANKL which is produced by activated T cells and B cells in the ligatured gingival tissue were not affected by the administration of anti-DC-STAMP-mAb. Based on these results, we speculated that the anti-bone loss effect of anti-DC-STAMP-mAb might be specific to the abrogation of osteoclast function, without affecting adaptive immune response. In this study, since anti-DC-STAMP-mAb was administered locally, but not systemically, the function of DCs to present antigen to T cells in lymph nodes was unaffected. Therefore, the possible effect of anti-DC-STAMP-mAb on dendritic cells (DCs) could not be ruled out. It would be intriguing to follow up the question of adaptive immune response against oral bacteria to determine any effects resulting from systemic administration of anti-DC-STAMP-mAb.

Fusion of preosteoclasts plays a vital role in the maturation of osteoclasts since this event increases the osteoclast activity of these bone-resorbing cells (8-10). Molecules involved in the fusion of preosteoclasts can be divided into two types, RANK-independent and RANK-dependent (67). Even though the molecular mechanism of osteoclast fusion is largely unclear,

studies point out various candidate molecules that mediate osteoclast fusion, such as CD44, macrophage fusion receptor (MFR) (7, 29, 30), dendritic cell-specific transmembrane protein (DC-STAMP), the d2 isoform of vacuolar ATPase Vo domain (Atpv0d2) and osteoclast stimulatory transmembrane protein (OC-STAMP), all of which appear to be specific and critical for osteoclast fusion (32, 48). This explains why the compensatory pathway is expected from other fusion molecules in the event of DC-STAMP inhibition.

OC-STAMP (osteoclast stimulatory transmembrane protein) is a novel protein introduced by Yang et al. in 2008 (32). OC-STAMP has been purposed to play an important role in pre-osteoclast fusion, the same as DC-STAMP, for several reasons. OC-STAMP protein has high similarity to DC-STAMP in consensus sequence. Both of them are significantly induced osteoclast precursors stimulation with RANKL. Anti-OC-STAMP and anti-DC-STAMP antibody can inhibit multinucleated osteoclast formation, while the overexpression of OC-STAMP and DC-STAMP promote multinucleated osteoclast formation in mouse BMC and RAW 264.7 cells (32).

Miyamoto et al. in 2012 (68) further showed that cell-cell fusion in osteoclasts is induced by both OC-STAMP and DC-STAMP, both of which are induced by RANKL-NFATc1 axis. The mixed cultures of OC-STAMP-deficient cells with DC-STAMP-deficient cells did not induce preosteoclast cell-cell fusion. OC-STAMP was required for osteoclast fusion independent of DC-STAMP since DC-STAMP was also equally expressed in OC-STAMP-deficient osteoclasts. OC-STAMP expression appears normal in DC-STAMP-deficient osteoclasts suggests that DC-STAMP and OC-STAMP likely do not regulate each other. In this study, they also generated OC-STAMP-deficient mice to analyze the role of OC-STAMP in vivo. The multinucleated osteoclast formation was completely abrogated in OC-STAMP deficient mice.

However, dexamethasone analysis of OC-STAMP-deficient mice demonstrated that some mice showed increased bone mass compared with wild-type mice, while the detected increase was not statistically significant (68). On the other hand, DC-STAMP-deficient mice have significantly increased bone mass, whereas DC-STAMP-Tg mice show decreased bone mass, compared to wild-type mice (10, 11). This suggests that OC-STAMP functions differently compared to DC-STAMP although both molecules are involved in osteoclast cell fusion. The presence of OC-STAMP cannot replace DC-STAMP. It must be examined in future studies how OC-STAMP and DC-STAMP regulate osteoclast fusion process. The putative ligand of DC-STAMP remains still unknown. In the study of Yagi et al. (10), supernatant from DC-STAMP^{+/+} could not induce osteoclast fusion of DC-STAMP^{-/-} cell. In the mixed culture of DC-STAMP^{+/+} and DC-STAMP^{-/-}, the multinucleated osteoclasts were found, suggesting that the direct cell contact is needed. The putative ligand of OC might be expressed on the OC cell surface. There are 2 subtypes of OC; master-fusing cells (DC-STAMP^{+/+}) and follower cells (DC-STAMP^{-/-}). These results showed that DC-STAMP ligand should be a membrane-bound molecule. In the review by Vignery (39), he stated that there is a possibility that DC-STAMP ligand might be a soluble protein that is released by the master cells, the follower cells, or both. He also proposed the possible DC-STAMP ligand which is MCP-1 or CCL2. The reasons are MCP-1 is involved in the osteoclast fusion and also foreign body giant cell formation. Both DC-STAMP and chemokine receptors are seven-transmembrane-spanning-proteins suggested the similarity in their structure. CCN2/CTGF (connective tissue growth factor; CTGF or CCN2) has been suggested as a putative ligand for DC-STAMP (69). It was demonstrated that CCN2/CTGF upregulated the expression of the dc-stamp gene and CCN2/CTGF directly bind to DC-STAMP molecule in RAW264.7 cells. The experiments in primary cells from fetal liver cells of Ccn2

null mice revealed that the formation of TRAP⁺ multinucleated cells were impaired in *Ccn2*^{-/-} OCs. When rCCN2 or dc-stamp was overexpressed into *Ccn2*^{-/-} OC, the impaired osteoclastogenesis was rescued. Since bisphosphonate, one of the most potent drugs used for bone lytic diseases, has presented the emerging side effect of bisphosphonate-induced osteonecrosis of jaw, alternative drugs for bone lytic diseases, including periodontitis, have been sought for development. In addition, unlike other multinucleated cells, such as trophoblasts, myoblasts and macrophages, which undergo fusion during developmental growth, osteoclast cell-cell fusion occurs continuously throughout the life span (6, 7). Therefore, a strategy that targets osteoclast multinucleation in the adult patients could lead to a therapeutic approach for osteoclast-specific pathogenic bone loss with minimal side effects.

Most studies have reported on DC-STAMP and osteoclastogenesis based on experiments performed with murine cells. Murine cells have advantages over human cells since they can be easily obtained due to their short life cycle, and it is possible to establish transgenic or knockout models. For human study, on the other hand, it is challenging to perform *in vivo* experiments without clearance of safety issue caused by the intervention. DC-STAMP is highly conserved. Eleveld et al 2005 characterized DC-STAMP and found that its sequence and genomic organization were highly conserved between murine and human. mDC-STAMP was found to be 95% homologous to its human and 99% to its rat counterpart (63). Nevertheless, the distinctive biological property of human-derived osteoclasts should not be ignored. Some studies have suggested differences between human and murine osteoclasts (70, 71) based on their responsiveness to RANKL or IL-17 which is different from that of mouse osteoclasts. However, Zeng et al. (72) suggested that DC-STAMP gene expression also regulates fusion and bone resorption of human osteoclasts in a manner similar to that of murine osteoclasts. By suppressing

DC-STAMP expression in human osteoclasts using RNAi, the fusion and osteoclast activity of human osteoclasts were suppressed, suggesting that DC-STAMP is still engaged in the cell fusion of human osteoclasts.

The expression of DC-STAMP gene in periodontal disease was first examined by Belibasakis et al. (40). In the cross-sectional clinical study, the expression level of DC-STAMP was elevated in gingival tissues from periodontitis patients compared to patients without periodontal disease. Although detection frequency of DC-STAMP was limited, the detection frequency and the expression levels of DC-STAMP tended to increase with the increased severity of periodontitis. Importantly, DC-STAMP was not detected at all in healthy subjects. Until the present work, no further studies have investigated the role of DC-STAMP in periodontal disease. In our study, DC-STAMP was detected in the decalcified tissue of the periodontal disease group under immunofluorescence microscopy, but not in the control group. These results provided sufficient evidence implicating the involvement of DC-STAMP in periodontal disease. Further studies, however, would be needed to confirm DC-STAMP localization, as well as identify its cellular source in periodontal tissue.

In contrast to chemical compound drugs, mAb drugs are increasingly of interest by the expected advantages of more specificity and fewer side effects (42). Over the decades, mAbs have been accepted as an alternative approach of treating various diseases, in particular, cancer, inflammatory diseases and hematological disorders (43). These mAbs, or IgG-based molecules, have advantages over small-molecule drugs in many respects, including tissue-specific targeting, serum half-life and effector functions via complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity or drug conjugates (44). An anti-DC-STAMP-mAb has recently been developed. However, no previous studies have investigated the effect of DC-

STAMP inhibition on the progression of periodontal disease. Thus, the results in this study may lay the groundwork for the development of a novel therapeutic target against bone loss in periodontal disease, as well as other diseases characterized by systemic bone loss, such as osteoporosis.

In conclusion, our study demonstrated, for the first time, that anti-DC-STAMP-mAb could suppress ligature-induced periodontal bone loss without affecting adaptive immune response to bacteria or total number of osteoclast precursors. Apparently, the suppressive effect of anti-DC-STAMP-mAb on periodontal disease can be attributed to its inhibition of cell-cell fusion between osteoclast precursors. Therefore, Anti-DC-STAMP-mAb could be developed as a potential lead candidate for a therapeutic approach that targets osteoclast-mediated periodontal bone loss with minimal, or no, side effects.

References

1. Takayanagi H. 2007. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nature reviews. Immunology* 7: 292-304
2. Harris WH, Heaney RP. 1969. Skeletal renewal and metabolic bone disease. *The New England journal of medicine* 280: 303-11 concl
3. Parfitt AM. 1982. The coupling of bone formation to bone resorption: a critical analysis of the concept and of its relevance to the pathogenesis of osteoporosis. *Metabolic bone disease & related research* 4: 1-6
4. Kajiya M, Giro G, Taubman MA, Han X, Mayer MP, Kawai T. 2010. Role of periodontal pathogenic bacteria in RANKL-mediated bone destruction in periodontal disease. *Journal of oral microbiology* 2
5. Bar-Shavit Z. 2007. The osteoclast: a multinucleated, hematopoietic-origin, bone-resorbing osteoimmune cell. *Journal of cellular biochemistry* 102: 1130-9
6. Horsley V, Pavlath GK. 2004. Forming a multinucleated cell: molecules that regulate myoblast fusion. *Cells, tissues, organs* 176: 67-78
7. Chen EH, Grote E, Mohler W, Vignery A. 2007. Cell-cell fusion. *FEBS letters* 581: 2181-93
8. Boyle WJ, Simonet WS, Lacey DL. 2003. Osteoclast differentiation and activation. *Nature* 423: 337-42
9. Kukita T, Wada N, Kukita A, Kakimoto T, Sandra F, Toh K, Nagata K, Iijima T, Horiuchi M, Matsusaki H, Hieshima K, Yoshie O, Nomiyama H. 2004. RANKL-induced DC-STAMP is essential for osteoclastogenesis. *The Journal of experimental medicine* 200: 941-6
10. Yagi M, Miyamoto T, Sawatani Y, Iwamoto K, Hosogane N, Fujita N, Morita K, Ninomiya K, Suzuki T, Miyamoto K, Oike Y, Takeya M, Toyama Y, Suda T. 2005. DC-STAMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells. *The Journal of experimental medicine* 202: 345-51
11. Iwasaki R, Ninomiya K, Miyamoto K, Suzuki T, Sato Y, Kawana H, Nakagawa T, Suda T, Miyamoto T. 2008. Cell fusion in osteoclasts plays a critical role in controlling bone

- mass and osteoblastic activity. *Biochemical and biophysical research communications* 377: 899-904
12. Hartgers FC, Vissers JL, Looman MW, van Zoelen C, Huffine C, Figdor CG, Adema GJ. 2000. DC-STAMP, a novel multimembrane-spanning molecule preferentially expressed by dendritic cells. *European journal of immunology* 30: 3585-90
 13. Staeger H, Brauchlin A, Schoedon G, Schaffner A. 2001. Two novel genes FIND and LIND differentially expressed in deactivated and Listeria-infected human macrophages. *Immunogenetics* 53: 105-13
 14. Asagiri M, Takayanagi H. 2007. The molecular understanding of osteoclast differentiation. *Bone* 40: 251-64
 15. 1996. Consensus report. Periodontal diseases: pathogenesis and microbial factors. *Annals of periodontology / the American Academy of Periodontology* 1: 926-32
 16. Socransky SS, Haffajee AD. 1991. Microbial mechanisms in the pathogenesis of destructive periodontal diseases: a critical assessment. *Journal of periodontal research* 26: 195-212
 17. Kinane DF, Riggio MP, Walker KF, MacKenzie D, Shearer B. 2005. Bacteraemia following periodontal procedures. *Journal of clinical periodontology* 32: 708-13
 18. Mahanonda R, Pichyangkul S. 2007. Toll-like receptors and their role in periodontal health and disease. *Periodontology 2000* 43: 41-55
 19. Beck JD, Offenbacher S. 2005. Systemic effects of periodontitis: epidemiology of periodontal disease and cardiovascular disease. *Journal of periodontology* 76: 2089-100
 20. Grossi SG, Genco RJ. 1998. Periodontal disease and diabetes mellitus: a two-way relationship. *Annals of periodontology / the American Academy of Periodontology* 3: 51-61
 21. Offenbacher S, Boggess KA, Murtha AP, Jared HL, Lieff S, McKaig RG, Mauriello SM, Moss KL, Beck JD. 2006. Progressive periodontal disease and risk of very preterm delivery. *Obstetrics and gynecology* 107: 29-36
 22. An D, Parsek MR. 2007. The promise and peril of transcriptional profiling in biofilm communities. *Current opinion in microbiology* 10: 292-6
 23. Taubman MA, Valverde P, Han X, Kawai T. 2005. Immune response: the key to bone resorption in periodontal disease. *Journal of periodontology* 76: 2033-41

24. Howard GA, Bottemiller BL, Turner RT, Rader JI, Baylink DJ. 1981. Parathyroid hormone stimulates bone formation and resorption in organ culture: evidence for a coupling mechanism. *Proceedings of the National Academy of Sciences of the United States of America* 78: 3204-8
25. Graves DT, Oates T, Garlet GP. 2011. Review of osteoimmunology and the host response in endodontic and periodontal lesions. *Journal of oral microbiology* 3
26. Yavropoulou MP, Yovos JG. 2008. Osteoclastogenesis--current knowledge and future perspectives. *Journal of musculoskeletal & neuronal interactions* 8: 204-16
27. Roodman GD. 1996. Advances in bone biology: the osteoclast. *Endocrine reviews* 17: 308-32
28. Theoleyre S, Wittrant Y, Tat SK, Fortun Y, Redini F, Heymann D. 2004. The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. *Cytokine & growth factor reviews* 15: 457-75
29. Saginario C, Sterling H, Beckers C, Kobayashi R, Solimena M, Ullu E, Vignery A. 1998. MFR, a putative receptor mediating the fusion of macrophages. *Molecular and cellular biology* 18: 6213-23
30. de Vries TJ, Schoenmaker T, Beertsen W, van der Neut R, Everts V. 2005. Effect of CD44 deficiency on in vitro and in vivo osteoclast formation. *Journal of cellular biochemistry* 94: 954-66
31. Kim K, Lee SH, Ha Kim J, Choi Y, Kim N. 2008. NFATc1 induces osteoclast fusion via up-regulation of Atp6v0d2 and the dendritic cell-specific transmembrane protein (DC-STAMP). *Molecular endocrinology* 22: 176-85
32. Yang M, Birnbaum MJ, MacKay CA, Mason-Savas A, Thompson B, Odgren PR. 2008. Osteoclast stimulatory transmembrane protein (OC-STAMP), a novel protein induced by RANKL that promotes osteoclast differentiation. *Journal of cellular physiology* 215: 497-505
33. Yagi M, Ninomiya K, Fujita N, Suzuki T, Iwasaki R, Morita K, Hosogane N, Matsuo K, Toyama Y, Suda T, Miyamoto T. 2007. Induction of DC-STAMP by alternative activation and downstream signaling mechanisms. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 22: 992-1001

34. Mensah KA, Ritchlin CT, Schwarz EM. 2010. RANKL induces heterogeneous DC-STAMP(lo) and DC-STAMP(hi) osteoclast precursors of which the DC-STAMP(lo) precursors are the master fusogens. *Journal of cellular physiology* 223: 76-83
35. Chiu YH, Ritchlin CT. 2012. Biomarkers to Diagnose Early Arthritis in Patients With Psoriasis. *Psoriasis forum / National Psoriasis Foundation* 18: 2-10
36. Albagha OM. 2015. Genetics of Paget's disease of bone. *BoneKEy reports* 4: 756
37. Chung PY, Beyens G, Riches PL, Van Wesenbeeck L, de Freitas F, Jennes K, Daroszewska A, Fransen E, Boonen S, Geusens P, Vanhoenacker F, Verbruggen L, Van Offel J, Goemaere S, Zmierzak HG, Westhovens R, Karperien M, Papapoulos S, Ralston SH, Devogelaer JP, Van Hul W. 2010. Genetic variation in the TNFRSF11A gene encoding RANK is associated with susceptibility to Paget's disease of bone. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 25: 2592-605
38. Teramachi J, Zhou H, Subler MA, Kitagawa Y, Galson DL, Dempster DW, Windle JJ, Kurihara N, Roodman GD. 2014. Increased IL-6 expression in osteoclasts is necessary but not sufficient for the development of Paget's disease of bone. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 29: 1456-65
39. Vignery A. 2005. Macrophage fusion: the making of osteoclasts and giant cells. *The Journal of experimental medicine* 202: 337-40
40. Belibasakis GN, Emingil G, Saygan B, Turkoglu O, Atilla G, Bostanci N. 2011. Gene expression of transcription factor NFATc1 in periodontal diseases. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica* 119: 167-72
41. Carter P. 2001. Improving the efficacy of antibody-based cancer therapies. *Nature reviews. Cancer* 1: 118-29
42. Hansel TT, Kropshofer H, Singer T, Mitchell JA, George AJ. 2010. The safety and side effects of monoclonal antibodies. *Nature reviews. Drug discovery* 9: 325-38
43. Keizer RJ, Huitema AD, Schellens JH, Beijnen JH. 2010. Clinical pharmacokinetics of therapeutic monoclonal antibodies. *Clinical pharmacokinetics* 49: 493-507
44. Jones EA, Waldmann TA. 1972. The mechanism of intestinal uptake and transcellular transport of IgG in the neonatal rat. *The Journal of clinical investigation* 51: 2916-27

45. McClung MR, Lewiecki EM, Cohen SB, Bolognese MA, Woodson GC, Moffett AH, Peacock M, Miller PD, Lederman SN, Chesnut CH, Lain D, Kivitz AJ, Holloway DL, Zhang C, Peterson MC, Bekker PJ. 2006. Denosumab in postmenopausal women with low bone mineral density. *The New England journal of medicine* 354: 821-31
46. Baron R, Ferrari S, Russell RG. 2011. Denosumab and bisphosphonates: different mechanisms of action and effects. *Bone* 48: 677-92
47. Henry DH, Costa L, Goldwasser F, Hirsh V, Hungria V, Prausova J, Scagliotti GV, Sleeboom H, Spencer A, Vadhan-Raj S, von Moos R, Willenbacher W, Woll PJ, Wang J, Jiang Q, Jun S, Dansey R, Yeh H. 2011. Randomized, double-blind study of denosumab versus zoledronic acid in the treatment of bone metastases in patients with advanced cancer (excluding breast and prostate cancer) or multiple myeloma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 29: 1125-32
48. Kim MH, Park M, Baek SH, Kim HJ, Kim SH. 2011. Molecules and signaling pathways involved in the expression of OC-STAMP during osteoclastogenesis. *Amino acids* 40: 1447-59
49. Kawai T, Eisen-Lev R, Seki M, Eastcott JW, Wilson ME, Taubman MA. 2000. Requirement of B7 costimulation for Th1-mediated inflammatory bone resorption in experimental periodontal disease. *Journal of immunology* 164: 2102-9
50. Lin X, Han X, Kawai T, Taubman MA. 2011. Antibody to receptor activator of NF-kappaB ligand ameliorates T cell-mediated periodontal bone resorption. *Infection and immunity* 79: 911-7
51. Kawai T, Matsuyama T, Hosokawa Y, Makihira S, Seki M, Karimbux NY, Goncalves RB, Valverde P, Dibart S, Li YP, Miranda LA, Ernst CW, Izumi Y, Taubman MA. 2006. B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease. *The American journal of pathology* 169: 987-98
52. Kawai T, Seki M, Hiromatsu K, Eastcott JW, Watts GF, Sugai M, Smith DJ, Porcelli SA, Taubman MA. 1999. Selective diapedesis of Th1 cells induced by endothelial cell RANTES. *Journal of immunology* 163: 3269-78
53. Ebersole JL, Frey DE, Taubman MA, Smith DJ. 1980. An ELISA for measuring serum antibodies to *Actinobacillus actinomycetemcomitans*. *Journal of periodontal research* 15: 621-32

54. Page RC. 1991. The role of inflammatory mediators in the pathogenesis of periodontal disease. *Journal of periodontal research* 26: 230-42
55. Kuhr A, Popa-Wagner A, Schmoll H, Schwahn C, Kocher T. 2004. Observations on experimental marginal periodontitis in rats. *Journal of periodontal research* 39: 101-6
56. Rovin S, Costich ER, Gordon HA. 1966. The influence of bacteria and irritation in the initiation of periodontal disease in germfree and conventional rats. *Journal of periodontal research* 1: 193-204
57. Abe T, Hajishengallis G. 2013. Optimization of the ligature-induced periodontitis model in mice. *Journal of immunological methods* 394: 49-54
58. de Molon RS, de Avila ED, Boas Nogueira AV, Chaves de Souza JA, Avila-Campos MJ, de Andrade CR, Cirelli JA. 2014. Evaluation of the host response in various models of induced periodontal disease in mice. *Journal of periodontology* 85: 465-77
59. Baker PJ, Dixon M, Roopenian DC. 2000. Genetic control of susceptibility to Porphyromonas gingivalis-induced alveolar bone loss in mice. *Infection and immunity* 68: 5864-8
60. Wilensky A, Polak D, Awawdi S, Halabi A, Shapira L, Houry-Haddad Y. 2009. Strain-dependent activation of the mouse immune response is correlated with Porphyromonas gingivalis-induced experimental periodontitis. *Journal of clinical periodontology* 36: 915-21
61. Graves DT, Fine D, Teng YT, Van Dyke TE, Hajishengallis G. 2008. The use of rodent models to investigate host-bacteria interactions related to periodontal diseases. *Journal of clinical periodontology* 35: 89-105
62. Jotwani R, Palucka AK, Al-Quotub M, Nouri-Shirazi M, Kim J, Bell D, Banchereau J, Cutler CW. 2001. Mature dendritic cells infiltrate the T cell-rich region of oral mucosa in chronic periodontitis: in situ, in vivo, and in vitro studies. *Journal of immunology* 167: 4693-700
63. Eleveld-Trancikova D, Triantis V, Moulin V, Looman MW, Wijers M, Fransen JA, Lemckert AA, Havenga MJ, Figdor CG, Janssen RA, Adema GJ. 2005. The dendritic cell-derived protein DC-STAMP is highly conserved and localizes to the endoplasmic reticulum. *Journal of leukocyte biology* 77: 337-43

64. Jansen BJ, Eleveld-Trancikova D, Sanecka A, van Hout-Kuijjer M, Hendriks IA, Looman MG, Leusen JH, Adema GJ. 2009. OS9 interacts with DC-STAMP and modulates its intracellular localization in response to TLR ligation. *Molecular immunology* 46: 505-15
65. Sawatani Y, Miyamoto T, Nagai S, Maruya M, Imai J, Miyamoto K, Fujita N, Ninomiya K, Suzuki T, Iwasaki R, Toyama Y, Shinohara M, Koyasu S, Suda T. 2008. The role of DC-STAMP in maintenance of immune tolerance through regulation of dendritic cell function. *International immunology* 20: 1259-68
66. Sanecka A, Ansems M, Prosser AC, Danielski K, Warner K, den Brok MH, Jansen BJ, Eleveld-Trancikova D, Adema GJ. 2011. DC-STAMP knock-down deregulates cytokine production and T-cell stimulatory capacity of LPS-matured dendritic cells. *BMC immunology* 12: 57
67. Xing L, Xiu Y, Boyce BF. 2012. Osteoclast fusion and regulation by RANKL-dependent and independent factors. *World journal of orthopedics* 3: 212-22
68. Miyamoto H, Suzuki T, Miyauchi Y, Iwasaki R, Kobayashi T, Sato Y, Miyamoto K, Hoshi H, Hashimoto K, Yoshida S, Hao W, Mori T, Kanagawa H, Katsuyama E, Fujie A, Morioka H, Matsumoto M, Chiba K, Takeya M, Toyama Y, Miyamoto T. 2012. Osteoclast stimulatory transmembrane protein and dendritic cell-specific transmembrane protein cooperatively modulate cell-cell fusion to form osteoclasts and foreign body giant cells. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 27: 1289-97
69. Nishida T, Emura K, Kubota S, Lyons KM, Takigawa M. 2011. CCN family 2/connective tissue growth factor (CCN2/CTGF) promotes osteoclastogenesis via induction of and interaction with dendritic cell-specific transmembrane protein (DC-STAMP). *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 26: 351-63
70. Kotake S, Yago T, Kawamoto M, Nanke Y. 2012. Role of osteoclasts and interleukin-17 in the pathogenesis of rheumatoid arthritis: crucial 'human osteoclastology'. *Journal of bone and mineral metabolism* 30: 125-35
71. Kanamaru F, Iwai H, Ikeda T, Nakajima A, Ishikawa I, Azuma M. 2004. Expression of membrane-bound and soluble receptor activator of NF-kappaB ligand (RANKL) in human T cells. *Immunology letters* 94: 239-46

72. Zeng Z, Zhang C, Chen J. 2013. Lentivirus-mediated RNA interference of DC-STAMP expression inhibits the fusion and resorptive activity of human osteoclasts. *Journal of bone and mineral metabolism* 31: 409-16