Resolvin E1 Regulation of Osteoclast Precursors and Osteoclasts

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DEDICATION

To my dear parents, **Abdulrahim Marghalani** and **Salha Bukhari**, for loving me unconditionally and teaching me that I can overcome anything with hard work in order to achieve my goals.

To my angel and the love of my life, **Alaa Bukhari**, for believing in me, standing beside me, and taking good care of our family, and to my cherished sons, **Yasser** and **Anas**.

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Abstract

Inflammatory bone diseases are pathological conditions characterized by the loss of balance between bone resorption and bone formation. These pathologies typically occur when target tissues are infiltrated by macrophages, neutrophils, and T cells leading to chronic inflammatory response, activation of osteoclasts, distorted bone turnover and gradual bone loss. Complete resolution of inflammation, defined as the complete removal of inflammatory cells with return to homeostasis, is considered the ideal and optimum outcome of acute inflammation. Endogenous specialized proresolving mediators (SPMs) are a very critical class of inflammation resolution mediators and have a significant impact on wound healing. These local mediators are highly efficient in treating a number of inflammatory diseases in which inflammation plays a significant role in animal models. Previous studies have demonstrated that resolvins (Rv) directly interact with bone cells to exert their activity. RvE1 treatment of isolated osteoclasts reduces cell fusion in the late stages of osteoclast differentiation.

The purpose of this work was to establish RvE1 preventive regulation of osteoclasts in a ligature-induced alveolar bone loss disease model in mice and to investigate RvE1 regulation of bone marrow-derived osteoclast precursor and osteoclast differentiation through the BLT1 receptor. Moreover, we examined RvE1 regulation of osteoclast precursor and osteoclast proliferation, survival and apoptosis and the molecular signaling pathways involved.

Our investigations showed that RvE1 significantly reduced bone loss area ($P<0.05$) and volume ($P<0.01$) at the maxillary second molar furcation and number of mono- ($P<0.01$) and multi-nucleated ($P<0.01$) TRAP-stained osteoclasts along the entire length of the tooth in ligature-induced periodontal disease model in mice. Also, RvE1 significantly down-regulated gene

expression of osteoclast markers ($P<0.05$) and hindered their differentiation ($P<0.01$) when induced at early preosteoclast ($P<0.01$) and late phases ($P<0.01$) in a dose-dependent manner. Moreover, BLT1 was revealed to be expressed on the cell surface of mice alveolar bone osteoclasts and bone marrow-derived osteoclast precursors and osteoclasts. Also, U75302-osteoclast cultures and U75302/RvE1-osteoclast cultures did not show any decrease in number of TRAP-stained multinucleated osteoclasts compared to control ones.

Proliferation and survival experiments showed that RvE1 significantly reduced osteoclast precursor and osteoclast proliferation ($P<0.01$) and viability ($P<0.01$) in a dose-dependent manner through BLT1 receptor. Also, RvE1 significantly increased osteoclast precursor and osteoclast apoptosis ($P<0.01$) through BLT1 receptor and significantly induced higher cleaved caspase 3 levels ($P<0.01$).

Finally, RvE1 demonstrated to induce significantly lower levels of phosphorylation of Akt ($P<0.01$) and to some extent ERK ($P<0.01$) when compared to control groups. Also, results obtained through using LTB_4-, U75302-, wortmannin- and PD98059-induced osteoclast precursor and osteoclast cultures validated the Akt pathway for the RvE1-BLT1-Rac1 signaling and uncovered possible cross-talks between Akt and ERK as well as possible ERK inducing receptors and intracellular signaling molecules for RvE1 other than the BLT1-Rac1 pathway.

To conclude, RvE1 decreases bone loss and number of osteoclasts in vivo in a ligature-induced periodontal disease model in mice, prevents bone marrow-derived osteoclast precursor and osteoclast in vitro differentiation and reduces their proliferation and survival through attenuating the activation of the Akt signaling pathway.

Key Words: Periodontal Disease, Osteoclastogenesis, Resolution, Resolvin E1, BLT1 Receptor.

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ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
ANOVA	analysis of variance
AP-1	activator protein 1
ATP6v0d2	v ATPase vO subunit d2
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CCL	CC chemokine ligand
CCR	CC-chemokine receptor
CD	clusters of differentiation
c-fms	colony stimulating factor 1 receptor
ChemR23	chemerin receptor 23
CK1	casein kinase 1
COX	cyclooxygenases
CT	calcitonin
DAPI	4',6-diamidino-2-phenylindole
DC-STAMP	dendritic cell specific transmembrane protein
DHA	docosahexaenoic acid
MEM	Eagle's minimal essential medium
EDTA	ethylenediaminetetraacetic acid
EFA	essential fatty acid
ERK	extracellular signal-regulated kinase

FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPCR	G-protein coupled receptors
Grb-2	growth factor receptor bound protein 2
HEPE	hydroxy eicosapentaenoic acid
HpEPE	hydroperoxy eicosapentaenoic acid
HPRT1	hypoxanthine guanine phosphoribosyl transferase 1
IFN	interferon
IKK1/2	inhibitor of NF- KB kinase 1/2
IL	interleukin
IP_3	inositol-1,4,5-trisphophate
JNK	c-Jun N-terminal kinase
LO	lipoxygenases
LT	leukotriene
LX	lipoxin
MAPK	mitogen-activated protein kinase
MaR	maresin
M-CSF	macrophage-colony stimulating factor
MFR	macrophage fusion receptor
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFAT	nuclear factor of activated T cells
NFATd	nuclear factor of activated T cells, cytoplasmic 1
NF-KB	nuclear factor-KB

OPG	osteoprotegerin
PCR	polymerase chain reaction
PD	protectin
PE	phycoerythrin
PG	prostaglandin
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PLCy	phospholipase Cy
PTH	parathyroid hormone
PUFA	poly unsaturated fatty acid
RANK	receptor activator of NF-KB
RANKL	receptor activator of NF-KB ligand
qRT-PCR	Quantitative real-time polymerase chain reaction Rv resolvin
RvE1	resolvin E1
SDS	sodium dodecyl sulfate
SPM	specific pro-resolving lipid mediator
TBE	tris buffered EDTA
TdT	deoxynucleotidyl transferase
TGF-B	transforming growth factor-P
Th	T helper
TNF-a	tumor-necrosis factor- a
TRAF	TNF receptor-associated factor
TRAP	tartrate-resistant acid phosphatase

TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
VEGF	vascular endothelial growth factor

CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Abnormal bone turnover is the hallmark of inflammatory bone diseases, which can occur systemically or locally (Clowes et al., 2005). Periodontitis, Rheumatoid arthritis and osteoporosis, are examples of conditions where the balance of bone resorption and bone formation is disrupted. These inflammatory bone diseases typically occur when target tissues are infiltrated by macrophages, neutrophils, and T cells leading to a chronic inflammatory response. This is followed by the activation of osteoclasts leading to excess bone resorption and gradual bone loss (Dar et al., 2018, Geusens and Lems, 2011, Nakashima and Takayanagi, 2009). Increased osteoclast activity represents a standard feature for many of these diseases. Osteoclasts are giant multinucleated bone resorbing cells of hematopoietic lineage. They share the same myelomonocytic progenitors with the macrophages and dendritic cells. Osteoclast transformation from progenitor cells into active bone resorbing cells requires specific factors that include macrophage colony-stimulating factor (M-CSF). Receptor Activator of Nuclear factor-kB Ligand (RANKL) and Osteoprotegerin (OPG) are secreted by osteoblasts as regulators of bone turnover. RANKL binds to RANK on osteoclasts to stimulate bone resorption; OPG is a scavenger receptor for RANKL and is secreted to inhibit the activity of RANKL. In inflammation, RANKL and OPG are secreted by inflammatory and stromal cells as well (need references). The early stages of osteoclast precursor differentiation and early cell fate determination are regulated by M-CSF, while RANKL and OPG act as physiological regulators of bone resorption at later stages of osteoclast differentiation (Boyle et al., 2003).

RANKL is a tumor-necrosis-factor-family molecule that is essential for osteoclast differentiation from the hematopoietic lineage. RANKL-deficient (RANKL-/-) mice exhibit a complete lack of osteoclasts with severe osteopetrosis. Also, these mice show defects in early stages of differentiation of T- and B-lymphocytes and complete lack of lymph nodes (Kong et al., 1999b). OPG is a protein that acts as a decoy receptor for RANKL. OPG activation in bone tissues leads to inhibition of osteoclast activation, differentiation, and survival leading to bone preservation (Clowes et al., 2005).

Cross-talk between immune cells and bone cells is a characteristic feature of inflammatory bone diseases driving their progression and leading to altered bone remodeling and loss of homeostasis. Osteoporosis, an inflammatory bone disease, can manifest in many forms in which the postmenopausal estrogen deficiency-induced type is the most common. Estrogen is an anti-inflammatory hormone that stimulates the production of OPG. Due to estrogen deficiency post-menopause, OPG levels decrease and osteoclast activation and bone resorption levels increase (Clowes et al., 2005). Rheumatoid arthritis is another inflammatory bone disease where synovial membrane accumulated T cells produce soluble and cell membrane-associated RANKL initiating the cascade of bone resorption events (Kong et al., 1999a).

Periodontal disease is a local bone disease of the jaw that is induced by commensal bacteria that form a biofilm on the teeth. The chronic nature of its inflammatory response leads to local soft and hard tissue destruction. T cells play a significant role in increased RANKL expression leading to osteoclast activation and bone resorption. Matrix metalloproteinase-8 is one of the collagenases released by neutrophils during periodontitis leading to further destruction of periodontal tissues (Taubman et al., 2005, Kong et al., 2000).

Proinflammatory molecules such as Prostaglandins (PG), Tumor necrosis factor alpha (TNFα), Interleukin-1 beta (IL- 1β), Interleukin-6 (IL-6), Interleukin-11 (IL-11), and Interleukin-17 (IL-17) are all key players in osteoclast regulation as they act indirectly moderating RANKL and OPG levels. They induce expression of RANKL through osteoblasts and bone marrow cells with or without a decrease in OPG. Conversely, anti-inflammatory mediators such as interferon-γ, Interleukin-4 (IL-4) and Interleukin-10 (IL-10) decrease RANKL levels and increase OPG levels leading to inhibition of bone resorption (Taubman et al., 2005).

Resolution of inflammation is considered the optimum result of inflammation. It used to be regarded as a passive process, but identification of novel pro-resolution mediators has established that resolution is an active process (Kong et al., 2000, Bannenberg et al., 2005, Levy et al., 2001). The first lipid mediators identified as pro-resolution mediators were Lipoxins (LXs). Previous studies showed their pro-resolving capabilities and their direct protective actions on bone cells has been suggested (Serhan et al., 2000, Serhan, 2007, Maderna and Godson, 2009).

Resolvins (Rvs) are autacoids derived from omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and have several subfamilies based on the long chain polyunsaturated fatty acids (PUFA) from which they are formed. Of these subfamilies, resolvin E (RvE1) has been shown to be effective in treating oral inflammation in rabbit periodontitis that results in spontaneous regeneration of associated bone loss (Hasturk et al., 2007, Hasturk et al., 2006, Herrera et al., 2008, Serhan et al., 2000, Serhan et al., 2002, Zhu et al., 2013).

RvE1 is an EPA-derived lipid mediator with pro-resolution properties. It is a subclass of the E family series of resolvins. It is produced by hypoxia-activated human endothelial cells that use Aspirin-acetylated cyclooxygenase-2 (ASA- acetylated COX-2) and p450-like reactions to convert eicosapentaenoic acid (EPA) to 18R-hydroxy-EPA, which in turn is transformed into a

5(6)-epoxide-18R-hydroxy by activated leukocytes. RvE1 was first identified *in vivo* from peritoneal exudates during the resolution phase of inflammation (Levy et al., 2001).

In vivo periodontal disease studies with RvE1 were performed in rabbits using silk ligatures with *P. gingivalis* (10^9 CFU) application. After six weeks, *P. gingivalis* application was stopped. RvE1 treatment (4 μg/tooth) was delivered for six weeks after and then animals were sacrificed. RvE1 application reversed periodontal inflammation and regenerated lost bone (Hasturk et al., 2007). Murine bone marrow primary osteoclast cultures performed in separate experiments indicated clearly that RvE1 indeed did act on osteoclasts and osteoblasts in a direct fashion. RvE1 was shown to inhibit osteoclast differentiation using *in vitro* resorption pit formation in a dose-dependent manner (Hasturk et al., 2007, Hasturk et al., 2006, Herrera et al., 2008, Serhan et al., 2000, Serhan et al., 2002, Zhu et al., 2013).

Further studies revealed that RvE1 treatment of isolated osteoclasts reduced cell fusion in the late stages of osteoclast differentiation leading to reduction in osteoclast formation by 32.8%. RvE1 specifically targeted Dendrocyte Expressed Seven Transmembrane Protein (DC-STAMP), which is an osteoclast fusion protein, down-regulating its expression by 65.4%. Moreover, RvE1 inhibited nuclear factor of activated T cells 1 (NFATc1), which is an essential expression factor for osteoclast differentiation, binding to the DC-STAMP promoter (Zhu et al., 2013).

NFATc1 is an essential master transcription factor for osteoclast terminal differentiation. Their induction is stimulated in the early phase of osteoclastogenesis through RANKL activated P13K/Akt, ERK/c-Fos, NF-κB, JNK/c-Jun and signaling pathways (Teitelbaum and Ross, 2003). PI3K/Akt and MAPKs pathways, activated through RANKL- recruited TRAF6, are essential for osteoclast differentiation and function. MEK1/2, MKK7, and MKK6 activate MAPK pathways, ERK, JNK, and p38 to induce activation of their downstream targets that include c-Fos and AP-1

transcription factors. The PI3K/Akt pathway regulates osteoclast proliferation, differentiation, survival and anti-apoptotic pathways. Moreover, PI3K/Akt and MAPK signaling pathways are involved in M-CSF-induced activation of bone marrow-derived macrophages through c-Fms receptors leading to increased osteoclast precursor proliferation and survival (David et al., 2002, Bonney et al., 2011, Li et al., 2002, Wong et al., 1999a, Teitelbaum and Ross, 2003).

NF-κB is a critically important transcription factor for osteoclast differentiation as shown in p50/p52 double-knockout mice, where osteoclastogenesis was prevented. RANKL stimulation activates through phosphorylation of IκB kinase (IKK), which is an inhibitory κB protein that forms a complex with NF-κB in the inactive state. This results in freeing NF-κB from the NF-κB/IκB complex to be translocated from the cytoplasm to the nucleus and binds to its target genes (Hayden and Ghosh, 2004). When transcriptionally active through RANKL-stimulated MAPK pathway, phosphorylated c-Jun (Ser63) and c-Fos (Ser32) dimerize to form activator protein-1 (AP-1) transcription factor required for osteoclastogenesis. Both c-Jun and c-Fos are essential for differentiation of osteoclasts, as shown in *c-Fos* knockout mice that exhibit a complete absence of osteoclasts; c-Jun deletion leads to arrest of osteoclast formation (Hayden and Ghosh, 2004).

Previous studies revealed that RvE1 interactions are mediated through two receptors; the resolvin E1 receptor (ERV1), also known as chemokine-like receptor 1 (ChemR23) and a leukotriene B4 receptor (BLT1). ChemR23 is expressed on monocytes, macrophages, dendritic cells, neutrophils and CD4+ T lymphocytes and osteoclasts, while BLT1 is expressed on polymorphonuclear leukocytes (PMN) and osteoclasts. (Arita et al., 2005, Yokomizo, 2011, Herrera et al., 2008). RvE1 down-regulates DC-STAMP and NFATc1 in osteoclasts by interacting with BLT1. (Zhu et al., 2013). However, there is limited data regarding RvE1 early cellular and

molecular regulatory interactions and the pathways involved in signal transduction through the BLT1 receptor prior to NFATc1 inhibition.

1.2. Literature Review

1.2.1. Inflammation

1.2.1.1. Inflammatory Response

Inflammation is defined as a response to a local triggering stimulus or a condition or an organ failure leading to a localized or generalized process of guiding the necessary white blood cells and plasma to a initiate the inflammatory reaction (Majno and Joris, 2004, Medzhitov, 2008). The acute episode of inflammation has been investigated heavily in the past to fully comprehend the cellular and molecular mechanisms involved. When an infection or tissue injury occurs, inflammation is instigated through the organized delivery of blood plasma and leukocytes to the site of infection or injury (Medzhitov, 2008). Receptors such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization-domain protein (NOD)-like receptors (NLRs) act as the first step in acute inflammation through recognition of microbial invaders (bacteria) and alerting the local macrophages and mast cells (Barton, 2008). As a result, inflammatory mediators that includes chemokines, cytokines, and lipid eicosanoids are released and the inflammatory cascade is in effect (Barton, 2008, Medzhitov, 2008).

However, localized and systemic chronic inflammations that are caused by chronic infections, autoimmune diseases and cardiovascular diseases are less understood compared to acute inflammation. The inflammatory response in these conditions is either triggered by an ongoing, long standing infection or substantial tissue injury or associated with a tissue

malfunction upsetting the host defense or tissue repair and causing an imbalance in homeostasis (Medzhitov, 2008).

Inflammation, when controlled, is considered a physiologic protective mechanism of the body against harmful stimuli, but when dysregulated, it can lead to pathological harmful results. This can be best described in the case of infection stimuli where there is a clear distinction between the physiologic and pathologic inflammatory response of the body. Other conditions, such as systemic inflammation, have a less clear difference of what constitutes a physiologic equal for the pathological inflammatory condition. Many researchers believe that the classic understanding of inflammation needs to be revised to accommodate for the other inflammatory responses stimulated by chronic and systemic conditions (Barton, 2008, Majno and Joris, 2004, Medzhitov, 2008).

After the release of chemokines, cytokines, lipid eicosanoids and other inflammation mediators, neutrophils and plasma proteins penetrate blood vessel walls to enter the local extravascular tissues where the mediators are released. This penetration action is allowed due to the activation of the endothelium of the blood vessels walls. Neutrophil integrins and chemokine receptors bind to selectins on endothelial cells to selectively pass through endothelium (McGill et al., 1998, Pober and Cotran, 1990, Pober and Sessa, 2007). When they reach the site of inflammation, they become active once in contact with cytokines or pathogens and release their highly potent toxic secretions. If the pathogens persist and the neutrophils fail to eliminate them, macrophages and T cell start to infiltrate the inflammation site leading to a transition of the inflammatory state from acute to chronic accompanied by granulomas and tertiary lymphoid tissue formation (Drayton et al., 2006, Nathan, 2002, Nathan, 2006, Nathan, 1987). Following inflammation and successful elimination of pathogens, inflammation

resolution starts to take effect. Resolution is characterized by the switch of pro-inflammatory lipid mediators into proresolution mediators that drive neutrophil apoptosis and attract monocytes with an anti-inflammatory phenotype leading to the initiation of tissue repair and remodeling (Serhan, 2007, Serhan and Chiang, 2008, Serhan and Chiang, 2013, Serhan et al., 2008a, Serhan et al., 2000, Serhan et al., 2008b, Freire and Van Dyke, 2013, Serhan, 2010, Serhan et al., 2007, Serhan and Savill, 2005) (Fig. 1). Resulting proresolving macrophages clear apoptotic neutrophils, bacteria and debris from the lesion through efferocytosis.

1.2.1.2. Inflammatory Signals

The inflammation cascade is regulated by a complex network of inducers, sensors, mediators and effectors that work together to initiate and regulate the inflammatory response. Each one of these regulatory signaling molecules has specific set of functions that complement the other signaling molecules in the inflammatory pathway. Inflammation inducers are the molecules responsible for initiating the inflammatory response and activating the inflammation sensors. When the sensors become activated, they provoke the release of inflammation mediators, which in turn regulate the process of inflammation through controlling inflammation effectors. The effectors are the tissues and organs that undergoes changes in their functional status as a part of the inflammatory response (Majno and Joris, 2004, Medzhitov, 2008, Rock and Kono, 2008, Medzhitov, 2010) (Fig. 2).

1.2.1.2.1. Inflammatory Inducers and Sensors

Inflammation inducers are categorized into exogenous or endogenous. **Exogenous** inducers are further sub-categorized into microbial and non-microbial inducers (Fig. 3). Pathogen-associated molecular patterns (PAMPs) and virulence factors are the two classes of the **microbial inducers**. PAMPs and virulence factors have their own specific receptors on host

cells that recognizes their presence and initiate the inflammatory response. **PAMPs** are specific conserved essential molecular structures in the invading microorganisms that serve as the target of recognition for a developed set of sensors in the host referred to as pattern recognition receptors (PRRs) (Medzhitov and Janeway, 1997, Janeway, 1989). PRRs such as CD14, DEC205 and collectins alert the immune system to recognize microbial inducers directly while other PRR sensors participate in alerting the immune system indirectly through other receptors such as nucleotide-binding oligomerization domain-like receptors (NLRs) and Toll-like receptors (TLRs) that recognize the PRR-PAMP recognition by-products (Fearon and Locksley, 1996).

Virulence factors are a group of exogenous microbial inducers that recognized by the host immune system indirectly through their adverse effects. A specialized set of sensors in the host tissues are responsible for identifying these adverse effects and initiating the inflammatory cascade. Cryopyrin (also known as NACHT) and leucine-rich-repeat- and pyrin-domain-containing protein (NALP3) inflammasome is an inflammation sensor that recognizes the efflux of K+ ions from pore formation of Gram-positive *Staphylococcus aureus* and *Listeria monocytogenes* bacteria exotoxins (Agostini et al., 2004, Dowds et al., 2004, Mariathasan et al., 2006). Also, a host-derived sensor in basophils acts as an inflammatory sensor that detects proteolytic activity when cleaved by the exogenous pathogen (Sokol et al., 2008, Wedemeyer et al., 2000).

The other class of exogenous inducers of inflammation is the **non-microbial inducers** which can be allergens, foreign bodies and toxic chemicals such as silica and asbestos particles. In many cases, the process to recognize these inducers occurs through detection of their adverse

effects on the host tissues using sensors that are mostly unknown such as the inflammatory sensors in basophils and macrophages (Dostert et al., 2008a, Dostert et al., 2008b).

Endogenous inducers of inflammation are signals produced through the desequestration of molecules or formation of crystal nucleation in stressed, damaged or malfunctioning tissues that disrupts the integrity cellular membranes, basement membranes and vascular endothelium surfaces (Medzhitov, 2008) (Fig. 3).

In **acute inflammation**, cellular membrane disruption results in the release of many cellular components, including ATP, high-mobility group box 1 protein (HMGB1) and numerous classes of the calcium-binding protein family (S100) during cell necrosis (Bianchi, 2007, Rock and Kono, 2008). ATP acts as an endogenous inducer of inflammation by alerting the nervous system of tissue injury by means of nociceptors and through activation of the NALP3 inflammasome by means of macrophage surface purinoceptors (Dowds et al., 2004, Julius and Basbaum, 2001, Mariathasan et al., 2006). HMGB1 and S100A12 initiate the inflammatory response by binding to the advanced glycation end-product-specific receptor (RAGE), followed by signaling through the TLRs (Hofmann et al., 1999, Park et al., 2006). Basement membrane disruption results in epithelial–mesenchymal interactions, which initiate an acute inflammatory response through unknown sensors. Airway epithelium is an example using basement membrane disruption as an inducer of acute inflammation. A growth factor known as heregulin is expressed at the apical part of the polarized airway epithelium to be separated from its sensors ERBB2, ERBB3 and ERRB4 that are expressed at the basolateral side. Any basement membrane desequestration that leads to epithelial–mesenchymal contact will provide access for the heregulin to its sensors to initiate an acute inflammatory response (Vermeer et al., 2003). Vascular endothelium disruption provides a portal of entrance for the

Hageman factor (factor XII) among other plasma proteins to the extracellular matrix where they become activated upon contact with collagen and many other molecules. When activated, they act as sensors for tissue damage and initiate an acute inflammatory response through proteolytic cascades (Majno and Joris, 2004, Pober and Cotran, 1990, Pober and Sessa, 2007).

In **chronic inflammation**, formation of crystal nucleation in stressed, damaged or malfunctioning tissues elicit macrophages to treat them as foreign bodies, which leads to the activation of the NALP3 inflammasome and caspase-1 substrates. Gout and pseudogout are chronic inflammatory conditions where crystal nucleations, comprising monosodium urate and calcium pyrophosphate dihydrate, are formed in the joints and periarticular tissues and act as chronic inflammation inducers (Dostert et al., 2008b, Martinon et al., 2006, Rock and Kono, 2008). Another example of a chronic inflammation endogenous inducer is the glycosaminoglycan hyaluronate. Upon tissue injury, hyaluronate breakdown from an inert high-molecular weight polymer into a low-molecular weight fragments activates TLR4 and initiates the inflammatory response (Jiang et al., 2005a, Jiang et al., 2007).

1.2.1.2.2. Inflammatory Mediators and Effectors

Inflammatory mediators are soluble molecules that are derived from plasma proteins or secreted by specialized cells to alter the functional status of the effector tissues and organs. Based on biochemical properties, inflammatory mediators are classified into seven categories: cytokines, chemokines, lipid mediators, proteolytic enzymes, vasoactive amines, vasoactive peptides, and complement fragments (Majno and Joris, 2004, Medzhitov, 2008).

Cytokines are inflammatory mediators produced mainly by macrophages and mast cells and participate in the activation of the endothelium and leukocytes and act as after induction inflammatory signals in the acute inflammation response. Tumor-necrosis factor-α (TNF-α),

Interleukin-1β (IL-1β) and IL-6 are the first secreted of inflammatory cytokines and are responsible for regulation of the inflammatory response, cell migration, and are involved in tissue destruction and bone resorption by stimulating matrix metalloproteinases (MMPs) and Receptor activator of nuclear factor kappa-B ligand (RANKL) (Freire and Van Dyke, 2013, Majno and Joris, 2004, Medzhitov, 2008, Nathan, 2002).

Chemokines are classified as inflammatory mediators because of their fundamental roles in regulating leukocyte extravasation and chemotaxis during inflammatory responses (Houshmand and Zlotnik, 2003, Zlotnik and Yoshie, 2012). C-X-C motif chemokine ligand 8 (CXCL8, also known as IL-8) and C-C motif chemokine ligand 2 (CCL2, also known as monocyte chemoattractant protein-1(MCP-1) are part of the chemokine superfamily responsible for attracting neutrophils and monocytes to the inflammatory site (Rollins, 1997, Yoshie et al., 2001, Zlotnik and Yoshie, 2000, Zlotnik et al., 2006). Although a subgroup of class A G protein-coupled receptors (GPCRs) has been identified as receptors for the chemokine superfamily, the chemokine ligand-receptor relationships is not exclusive; a single receptor recognizes many ligands and a single ligand binds to multiple receptors (Vassilatis et al., 2003).

Lipid mediators are derived from the phospholipid segment of the cellular membrane and are categorized into two classes: eicosanoids and platelet-activating factors. Arachidonic acid is the precursor of the eicosanoid family, whereas lysophosphatidic acid is the precursor of the platelet-activating factor family (Serhan et al., 2007, Serhan, 2007). Cyclooxygenases metabolize arachidonic acid to generate prostaglandins and thromboxanes, while lipoxygenases generate leukotrienes, lipoxins, resolvins and protectins. Prostaglandins and thromboxanes are pro-inflammatory mediators that trigger vasodilation, hyperalgesia and fever. Conversely, Lipoxins, resolvins and protectins are anti-inflammatory and pro-resolution inflammation

mediators (they will be reviewed more in details later) (Serhan, 2007, Serhan et al., 2007, Serhan and Savill, 2005, Serhan et al., 2008b, Serhan et al., 2008a, Serhan et al., 2000, Serhan and Chiang, 2013, Serhan and Chiang, 2008). Acetylation of lysophosphatidic acid results in products that are responsible for vasodilation, vasoconstriction, recruitment of leukocytes, vascular permeability and platelet activation during the inflammatory response (Medzhitov, 2008, Nathan, 2002).

Proteolytic enzymes act as inflammatory mediators by degrading extracellular matrix and basement-membrane proteins, remodeling effector tissues and contributing to leukocyte migration and host defense. Cathepsins and matrix metalloproteinases are some of protases involved largely in the inflammatory response as extracellular matrix and basement-membrane protein remodeling mediators (Medzhitov and Janeway, 1997, Nathan, 2002, Parks et al., 2004).

Vasoactive amines are released when mast cells and platelets degranulate and have diverse conflicting effects, depending on the inflammatory conditions at the time of their release. They could produce vasoconstriction effects, vasodilation effects and increased vascular permeability. Histamine and serotonin are vasoactive amines with instantaneous effects that might eventually cause vascular and respiratory collapse in uncontrolled conditions (Medzhitov, 2008, Nathan, 2002, Rock and Kono, 2008, Majno and Joris, 2004, Branco et al., 2018).

Vasoactive peptides are released as inflammatory mediators in an active form like substance P released from sensory neurons, or transformed from an inactive form into an active one by proteases like kinins, fibrinopeptide A and B. These mediators can trigger vasodilation, increased vascular permeability and hyperalgesia (Majno and Joris, 2004, Medzhitov, 2008, Nathan, 2002, Filippatos et al., 2001).

C3a, C4a and C5a are **complement fragments**, which are known as anaphylatoxins; they are produced by complement activation. They boost the process of chemotaxis of granulocytes and monocytes and induce mast-cell degranulation (Majno and Joris, 2004, Medzhitov, 2008, Laursen et al., 2012).

Inflammatory effectors are the cells, tissues and organs affected by inflammatory mediators leading to a shift in their functionality. The reaction of ubiquitous cells and tissues to TNF-α is an example of the effect of inflammatory mediators on effectors. Also, vasodilation, vasoconstriction and leukocyte chemotaxis and migration, and effects on neuroendocrine functions and metabolic activity are among some of the actions of inflammatory mediators on effectors (Majno and Joris, 2004, McGill et al., 1998, Nathan, 2002).

Figure 1. Lipid Mediator regulation of acute inflammation outcomes.

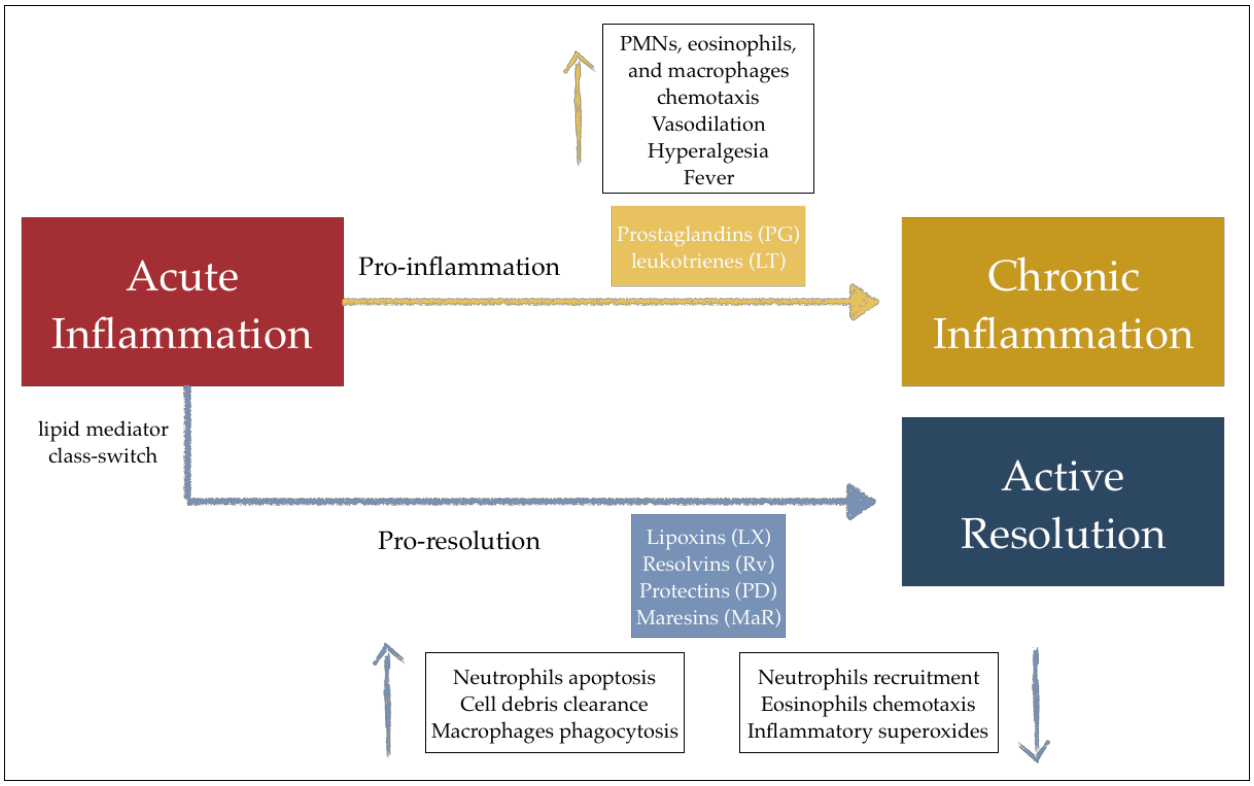


Figure 1. Lipid Mediator regulation of acute inflammation outcomes. The outcomes of acute inflammation are dictated by numerous mediators related to the type and duration of the injury or condition. Pro-inflammatory lipid mediators are involved in driving the acute inflammation cascade toward the chronic state. However, a class-switch in these lipid mediators plays a pivotal role in directing the cascade toward active resolution. Cellular outcomes of this event include increase in neutrophils apoptosis and non-phlogistic phagocytosis of cell debris and apoptotic cells by macrophages, reduction in neutrophil and basophil recruitment and dilution of inflammatory superoxide.

Figure 2. Inflammation cascade components.

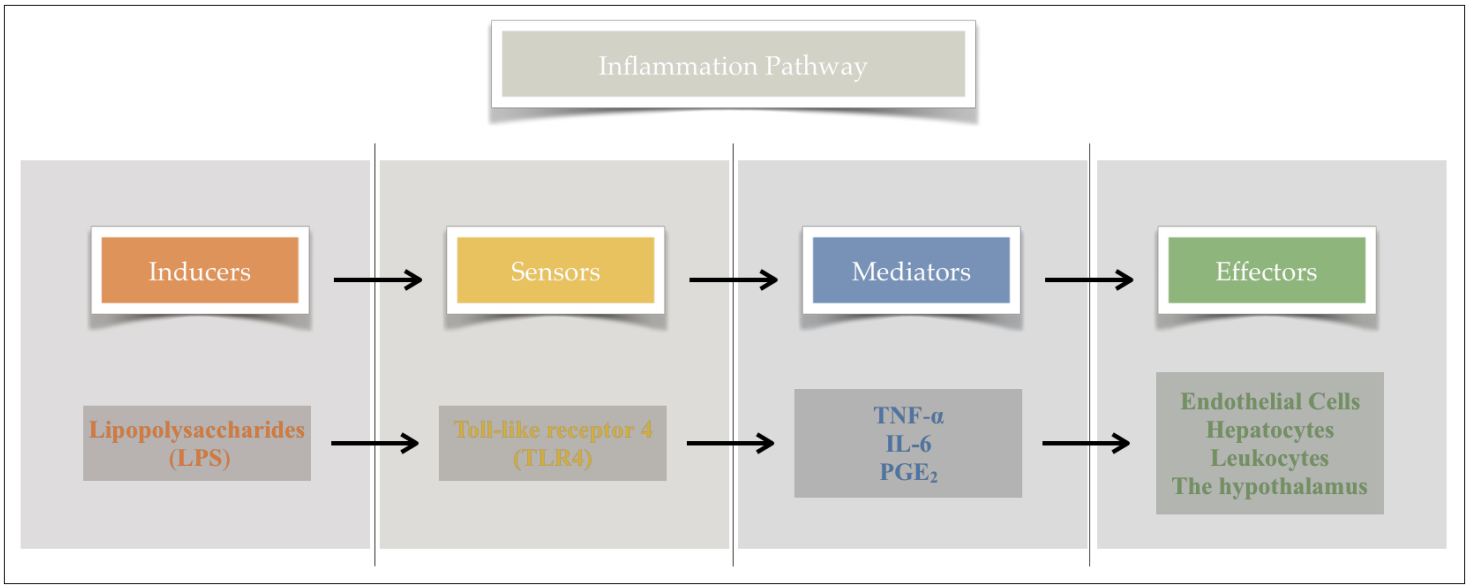


Figure 2. Inflammation cascade components. The inflammation cascade is regulated by a complex network of inducers, sensors, mediators and effectors that work together to initiate and regulate the inflammatory response. Each one of these regulatory signaling molecules has specific set of functions that complement the other signaling molecules in the inflammatory pathway. Inflammation inducers are the molecules responsible for initiating the inflammatory response and activating the inflammation sensors. When the sensors become activated, they provoke the release of inflammatory mediators, which in turn regulate the process of inflammation by controlling inflammation effector conditions. The effectors are the tissues and organs that undergo changes in their functional status as a part of the inflammatory response.

1.2.2. Osteoclastogenesis

Osteoclastogenesis is a multi-phase process that starts with securing immature osteoclast progenitor proliferation and survival followed by committing these progenitors to differentiate into mature osteoclasts and ending with executing bone resorption. **Osteoclasts** are the exclusive cells responsible for bone resorption through demineralization of inorganic constituents, crystalline hydroxyapatite, followed by proteolytic degradation of organic matrix, mainly type I collagen (Teitelbaum, 2000). Osteoclasts are multinucleated giant cells containing up to 20 nuclei derived from the myeloid tissues, monocyte/macrophage linage specifically, of bone marrow. They are the result of fusion of multiple mononuclear pre-osteoclast progenitors. They are characterized by their sealed ruffled border facing the bone matrix to build resorption lacunae (Howship's lacunae). (Boissy et al., 2002, Boyle et al., 2003, Dar et al., 2018, Wada et al., 2006).

Osteoclasts demineralize inorganic constituents via vacuolar H+-adenosine triphosphatase (H+-ATPase) that deliver protons to the resorption compartments leading to secretion of hydrochloric acid into the Howship's lacunae. To counter the flux of positive charge, osteoclasts release Cl- into the resorption compartment using Cl- charge-coupled to the H+- ATPase channels. Additionally, osteoclasts release various enzymes to degrade bone organic matrix such as tartrate resistant acid phosphatase (TRAP) and lysosomal proteolytic cathepsin K (Feng and Teitelbaum, 2013).

1.2.2.1. Osteoclastogenesis and Bone Remodeling

Osteoclastogenesis is part of the continuous remodeling process of bone. This unique feature of bone tissues requires reciprocal interactions between osteoclasts and the other two types of bone cells : osteoblasts and osteocytes (Arron and Choi, 2000, Dar et al., 2018). **Osteoblasts**

are metabolically highly active bone cells derived from bone marrow mesenchymal stem cells (MSCs) and responsible for producing the collagenous and non-collagenous bone matrix proteins. Wingless/int1 proteins (Wnt), bone morphogenic proteins (BMPs) and PGE2 with the aid of insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) and Parathyroid hormone (PTH) provide the necessary induction of MSCs to differentiate into osteoblasts through the expression of the transcription factors, Runx2 and Osterix and Catenin beta-1 (β-catenin). These Wnt, BMPs and PGE2 molecules communicate with each other and with the RANKL-OPG system, which is a key regulator of osteoclasts differentiation and function (Arboleya and Castaneda, 2013, Arron and Choi, 2000, Komori, 2006, Weitzmann and Ofotokun, 2016).

Osteocytes are bone cells entrapped inside the bone extracellular matrix and are derived from osteoblasts. They produce a specific molecule called sclerostin, encoded by the SOST gene, which inhibits Wnt signaling and bone formation. Moreover, osteocytes regulate osteoclastogenesis by responding to microdamage followed by cell apoptosis and activation of osteoclastic bone resorption (Arboleya and Castaneda, 2013, Arron and Choi, 2000, Schaffler and Kennedy, 2012, Weitzmann and Ofotokun, 2016).

1.2.2.2. Osteoclastogenesis and the Immune System

The interaction between the immune system and osteoclastogenesis is constructed by the functional network between bone and immune cells creating one integrated functional unit called the osteoimmune system. Cross-talks between the skeletal and immune systems regulate many vital body processes including inflammation and osteoclastogenesis (Arboleya and Castaneda, 2013, Dar et al., 2018).

The role of **B cells** in inflammation and osteoclastogenesis is very critical as demonstrated by active B cell production of osteoclastogenic RANKL and other pro-inflammatory cytokines

shown in inflammatory arthritis and periodontitis (Kawai et al., 2006, Schett, 2009). However, **T cells** have been shown to produce mixed positive and negative regulatory actions on osteoclastogenesis, simultaneously. Several subsets of T cells, namely CD4 T helper (Th)-cell subsets Th1 and Th2, release the anti-osteoclastogenic interferon (IFN)-y and IL-4. Conversely, Th17 subsets produce and release osteoclastogenic RANKL and IL-17 (Horwood et al., 1999, Kong et al., 1999a).

Human investigations and *in vitro* studies established that activated **neutrophils** express osteoclastogenic RANKL in acute inflammation and **macrophages** release osteoclastogenic inflammatory cytokines IL-1, TNF-a and IL-6 (Chakravarti et al., 2009). Lastly, in many diseases such as RA and periodontitis, **dendritic** cells exhibited predisposed interactive communications with T-cells to regulate osteoclasts indirectly (Cutler and Teng, 2007, Page and Miossec, 2005).

1.2.2.3. Osteoclastogenesis and Inflammation

The interaction between inflammation and osteoclastogenesis is an intricate reciprocal communication that exists as part of the functional network between the bone and the immune system. Many inflammatory mediators produced by immune cells and bone cells play pivotal roles in the progression of many osteoclastogenic bone pathologies leading to disturbed bone remodeling and homeostasis (Tables 1). Therefore, understanding inflammatory mediators' different roles in osteoclastogenesis and the disease mechanism in these various bone pathologies, such as post-menopausal osteoporosis, rheumatoid arthritis, osteoarthritis, and periodontitis, can help better understand the relationship between inflammation and osteoclastogenesis (Dar et al., 2018, Geusens and Lems, 2011, Nakashima and Takayanagi, 2009).

1.2.2.3.1. Inflammatory regulators of Osteoclastogenesis

Several inflammatory mediators participate in osteoclastogenesis as agonists or antagonists by regulating M-CSF, RANKL and OPG or through direct action on osteoclasts. There are numerous osteoclastogenic mediators that synergize with RANKL to activate osteoclastogenesis such as IL-1, IL-6, IL-8, IL-11, IL-15, IL-17, IL-32, LTs, PGs and TNFa. Conversely, anti-osteoclastogenic mediators antagonize RANKL-RANK signaling or osteoclastogenic mediators to inhibit osteoclastogenesis such as IFN-y, IFN-a, IFN-B, IL-4, IL-10, IL-13, IL-18, IL-33 (Table 1) (Arboleya and Castaneda, 2013, Dar et al., 2018, Feng and Teitelbaum, 2013, Schett and David, 2010).

1.2.2.3.2. Inflammatory Bone Diseases

Post-menopausal ovarian estrogen production deficiency leads to **osteoporosis** via increased osteoclastogenesis, diminished estrogens anti-inflammatory and bone protective effects, decreased release of Osteoprotegerin (OPG) and sclerostin and increased releases of macrophage colony-stimulating factor (M-CSF) and RANKL (Clowes et al., 2005, Di Gregorio et al., 2001, Kim et al., 2012, Srivastava et al., 2001, Srivastava et al., 1998, Szulc et al., 2001, Weitzmann and Pacifici, 2006).

The role that inflammatory mediators and immune cells play in postmenopausal osteoporosis have been confirmed to be vital through previous *in vivo* studies. Ovariectomy of T cell deficient mice and mice exposed to a T cell simulation blockage agent (Abatacept) showed no ovariectomy induced cortical and trabecular bone loss. T cells (Th17) induce osteoclastogenesis through the decreased release of IFN-γ and increased release of IL-17, RANKL, TNF-α (Cenci et al., 2000, Faienza et al., 2013, Gao et al., 2004, Grassi et al., 2007, Li et al., 2011, Moreland et al.,

2006, Polanczyk et al., 2004a, Polanczyk et al., 2004b, Roggia et al., 2001, Sato et al., 2006, van Amelsfort et al., 2004, Yamaza et al., 2008).

Rheumatoid arthritis (RA) is a disease of the synovial membrane, cartilage and bone where a sustained infiltration of activated T cells into the synovium causes a chronic inflammatory response (Edwards et al., 2004, Titanji et al., 2014). T cells differentiation and activation in affected synovial membranes and fluids requires the presence of IL-7 as shown in previous studies, where the inhibition of IL-7 causes reduction in T cell differentiation and activation leading to inhibition of RA. T cells diminishing effect in RA leads to reduced secretion of RANKL and TNF and other inflammatory cytokines important for driving bone loss (Fry and Mackall, 2001, Schett and David, 2010).

Osteoarthritis (OA) is a bone disease that causes disruption in the structure of the synovium and gradual damage of the articular cartilage, ligament and subchondral bones (Bhattaram and Chandrasekharan, 2017, Li et al., 2017). T cell infiltration into the synovial membrane is increased in OA affected patients, especially Th1, Th9 and Th17 cells (Hussein et al., 2008, Qi et al., 2016, Zhang et al., 2012). When activated, T cells secrete and induce the production of numerous pro-inflammatory cytokines (TNF-α, IL-1β, IL-9, IL-18, and IL-17) into the synovial membrane that drive the disease progression (Goldring et al., 1986).

Periodontitis is a progressive inflammatory disease of the periodontium initiated by an exogenous microbial inducer that leads to the destruction of the soft and hard tissues supporting the teeth. Bacteria initiate the inflammatory cascade leading to a flood of neutrophils followed by T cells, macrophages and other inflammatory cells infiltrating the periodontal tissues. Many inflammatory mediators are released (TNF-α, IL-1β, IL-6, IL-8, and Prostaglandin E2 (PGE2) that lead to the propagation of the inflammatory process (Kinane and Lappin, 2001, Kinane and

Lappin, 2002, Kornman et al., 1997, Van Dyke et al., 1993). Elevated levels of PGE2 and Leukotriene B4 (LTB4), produced mainly by activated leukocytes, lead to the discharge of granule-associated enzymes and osteoclast-mediated bone resorption. Periodontal disease in numerous cases presents as a chronic inflammatory condition and lipid mediators play a pivotal role in its chronicity (Offenbacher, 1996, Pouliot et al., 2000, Samuelsson et al., 1987, Varani and Ward, 1994).

1.2.2.4. Osteoclastogenesis Signal transduction

Bone resorption is a complex intricate process that involves several steps and requires the presence of many osteoclastogenesis signaling molecules (Feng and Teitelbaum, 2013, Park et al., 2017). Previous work by Utagawa et al in 1990 showed that the presence of marrow-derived stromal cell line is required for *in vitro* differentiation of macrophages into osteoclasts. At that time, it was not clear why the presence of this stromal cell line is essential for osteoclast maturation, but Utagawa et al. suggested that it may be related to the secretion of a homodimeric glycoprotein growth factor called **macrophage colony-stimulating factor CSF-1 (M-CSF)** (Teitelbaum, 2000, Udagawa et al., 1990). Later, it became clear that vital role of M-CSF in osteoclastogenesis can be accomplished *in vitro* with pure populations of macrophages exposed only to M-CSF with another essential growth factor called receptor for activation of nuclear factor kappa B ligand (RANKL), which is also known as OPGL and TRANCE (Lacey et al., 1998). M-CSF's primary role in macrophage maturation into osteoclasts is inducing receptor for activation of nuclear factor kappa B (RANK) expression, participating as a competence factor for differentiation and providing necessary signals for cell survival and proliferation (Arai et al., 1999).

M-CSF interacts with a transmembrane receptor called colony-stimulating factor-1 receptor (CSF-1R, also known as c-Fms), which acts as its sole receptor (Sherr et al., 1992, Stanley et al., 1997). Mice lacking the gene coding for c-Fms, *csf1r,* display a severe lack of osteoclasts leading to severe osteopetrosis and a striking decrease in tissue macrophages (Dai et al., 2002). The interaction of c-Fms with its ligand M-CSF results in c-Fms dimerization and auto-phosphorylation at certain tyrosine residues through the activation of the receptor tyrosine kinase (Schlessinger, 2000). Several studies explored a number of possible tyrosine residues that are subjected to phosphorylation following c-Fms dimerization and the most agreed upon ones are seven residues located at the cytoplasmic tail of c-Fms: Y559, Y697, Y706, Y721, Y807, Y921, and Y974 (Ross, 2006). More than 150 proteins involved in cell proliferation, survival, differentiation, and cytoskeletal reorganization, were found to bind to c-Fms following its activation indicating the complexity of the M-CSF/c-Fms signaling pathway (Schlessinger, 2000, Yeung et al., 1998). The central transducers of M-CSF/c-Fms signaling are the Phosphoinositide 3-kinase (PI3K)/Protein kinase B (PKB, also known as Akt) pathway and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) p42/44 pathway. There are limited data suggesting that the phospholipase C gamma (PLCγ) pathway may have a possible role in transducing M-CSF/c-Fms signaling (Nakamura et al., 2003, Newton, 2001).

As discussed earlier, M-CSF binding to c-Fms leads to phosphorylation of Y697 among other tyrosine residues in the cytoplasmic tail of c-Fms. This induces the recruitment of the docking protein complex Growth factor receptor-bound protein 2 (Grb2)/ Son of Sevenless (SOS) to bind to this particular phosphotyrosine residue through their Src Homology 2 (SH2) domain (Ross, 2006). SOS becomes activated following GRB2-SOS complex docking, which stimulates GDP removal from Ras stimulating the rest of the Ras/Raf/MEK/ p42/44 ERK pathway, but not JNK or

p38 (Cobrinik, 2005, Helgason et al., 1998, Takeshita et al., 2002). Moreover, following M-CSF/c-Fms contact, the p85 subunit of the PI3K complex binds to the phosphorylated c-Fms Y721 residue, activating Akt signaling. Akt promotes cell proliferation through inhibiting transcription factors GSK3β and FOXO responsible for cyclin D1, an essential cell cycle protein, degradation and inhibition (Cobrinik, 2005, Luo et al., 2003).

T lymphocytes, when activated, produce numerous inflammatory mediators and growth factors that can directly trigger osteoclastogenesis (Kong et al., 1999a). The most important molecule expressed by T lymphocytes in the context of osteoclastogenesis is **RANKL**, which is a tumor-necrosis-factor-family type II transmembrane protein. RANKL plays a key role in promoting osteoclast cell survival, cytoskeletal reorganization, bone resorption and cytokine production (Wong et al., 1999b). As shown by Kong et al., RANKL-deficient (*RANKL−/−*) mice exhibit a complete lack of osteoclasts with severe osteopetrosis. Also, these mice show defects in early stages of differentiation of T- and B-lymphocytes and complete lack of lymph nodes (Kong et al., 1999b). In humans, RANKL and RANK encoding gene mutations cause high bone density and osteopetrosis due to marked reduction in the number of osteoclasts (Guerrini et al., 2008, Sobacchi et al., 2007).

Another key molecule that affect the balance of osteoclastogenesis is Osteoprotegerin (OPG), which is a protein that acts as a decoy receptor for RANKL. OPG activation in bone tissues leads to inhibition of osteoclast activation, differentiation, and survival leading to bone preservation (Clowes et al., 2005). The fine regulated balance between the expression of RANKL and OPG controls the extent of osteoclastogenesis (Hofbauer et al., 1999a). As mentioned earlier, M-CSF induces osteoclast precursor proliferation and survival (Hofbauer et al., 1999b). Osteoclast precursor differentiation into osteoclasts requires the presence of RANKL to stimulate them to

commit to be osteoclasts. RANKL and its receptor, receptor for activation of nuclear factor kappa B (RANK), are members of the tumor necrosis factor (TNF) family and their TNF receptor superclass, confirming the pivotal role inflammatory mediators play in osteoclastogenesis (Teitelbaum, 2000).

The binding of RANKL to its receptor RANK stimulates its trimerization and activation. RANK transduces its signals intracellularly by recruiting adaptor protein TNFR-associated factors (TRAF) rather than eliciting an enzymatic response due to its lack of intrinsic enzymatic activity (Cheng et al., 2003, Wong et al., 1998, Ye et al., 2002). RANK binds TRAF6 at its three binding sites in its C-terminal cytoplasmic tail stimulating the rest of downstream signaling pathway (Wong et al., 1998, Ye et al., 2002). Previous reports showed that the other TNFR-associated factors such as TRAF2, TRAF3, and TRAF5 are able to bind to RANK and stimulate the rest of the signaling pathway but TRAF6 is the key adaptor protein as demonstrated in TRAF6-deficient mouse studies (Lomaga et al., 1999, Naito et al., 1999). Beside RANK, several immune receptors such as triggering receptor expressed in myeloid cells-2 (TREM-2) and osteoclast-associated receptor (OSCAR) are involved in transduction of NFATc1 signaling, solidifying the osteoimmunology nature of inflammatory bone diseases (Cella et al., 2003, Humphrey et al., 2006, Kim et al., 2002).

Following the activation of TRAF6, NF-κB signaling is stimulated by phosphorylation of transforming growth factor beta (TGFβ)-activated kinase 1 (TAK1)-dependent by TRAF6. Also, adaptor proteins TAK1-binding protein 1 (TAB1) and TAB2 bind with TRAF6 and TAK1 to form active complexes (Mizukami et al., 2002). When activated, TAK1 phosphorylates the IκB kinase (IKK) complex, which requires NF-κB essential modifier (NEMO)/IκB kinase gamma (IKKγ) ubiquitination to activate the NF-κB signaling pathway (Ninomiya-Tsuji et al., 1999). Moreover,

atypical protein kinase C (aPKC) phosphorylation by p62 protein and TRAF6 with IKKγ ubiquitination could also activate the IKK complex and stimulate the rest of the downstream NF-κB signaling (Duran et al., 2004, Ghosh and Karin, 2002, Mizukami et al., 2002). NF-κB signaling is very critical for osteoclastogenesis as shown by Franzoso et al. and Iotsova et al. in NF-κB $p50^{-/-}$/ $p52^{-/-}$ double-knockout mice exhibiting a severe osteopetrosis phenotype due to a significantly diminished number of osteoclasts (Franzoso et al., 1997, Iotsova et al., 1997).

Another critical downstream signaling pathway stimulated by RANKL/RANK/TRAF6 activation is the stimulation of AP-1 transcription factor (Wong et al., 1999a). The AP-1 transcription factor includes Fos (c-Fos, FosB, FosB2, Fra-1, and Fra-2), Jun (c-Jun, JunB, and JunD), and ATF (ATFa, ATF2, ATF4, and B-ATF) family members (Grigoriadis et al., 1994, Wagner, 2002). This signaling pathway is stimulated by active TRAF6 interaction with c-Src tyrosine kinase leading to its activation. c-Src is a member of a family of nonreceptor tyrosine kinases known by a common structure of SH2 and SH3 protein interaction domains and an inactivation tyrosine (Y527) that inactivates the kinase when phosphorylated (Funakoshi-Tago et al., 2003, Wong et al., 1999a, Xing et al., 2001). Activated c-Src tyrosine kinase stimulates AP-1 transcription factor through induction of c-Fos by adaptor proteins (Grigoriadis et al., 1994, Wagner, 2002). AP-1 transcription factor is a key factor for osteoclastogenesis as shown by Ikeda et al., and Wang et al. c-Fos knockout mice and transgenic overexpressed dominant negative c-Jun mice exhibit severe osteopetrosis caused by marked reduction in number of osteoclasts (Franzoso et al., 1997, Iotsova et al., 1997).

A third equally significant downstream signaling pathway activated through the RANKL/RANK/TRAF6 activation is the MAPKs signaling pathway. As mentioned earlier in NF-κB signaling pathway, activated TRAF6 stimulates the phosphorylation TAK1. When TAK1 is

activated, it activates the MKK6/ p38 signaling of MAPK pathway with the activated C kinase 1 (RACK1) acting as a linking scaffold protein. Furthermore, phosphorylation of Grb2-associated binder-2 (Gab2) leads to the activation of c-Jun N-terminal kinase (JNK) and p42/44 ERK of the MAPK signaling pathway and the PI3K/Akt signaling pathway (David et al., 2002, Bonney et al., 2011, Li et al., 2002, Wada et al., 2005, Wong et al., 1999a). These MAPK signaling pathways are essential for the initial RANKL differentiation phase of osteoclast precursors turning into osteoclasts and in the functional resorptive phase as well. Mice with deletion of the ERK1 gene (*Erk1*$^{-/-}$) showed decreased number of osteoclast progenitors and osteoclasts, impaired osteoclast migratory ability and resorptive activity, and increased bone mineral density (David et al., 2002, Bonney et al., 2011, Li et al., 2002).

Also, the PI3K/Akt signaling pathway is very critical for RANKL-induced cell survival by inhibiting numerous cell-apoptosis-inducing pathways. *In vitro c-Src*$^{-/-}$ osteoclasts and LY294002, a potent inhibitor of PI3K, exposed osteoclasts exhibited reduced RANKL-induced survival, but not differentiation, indicating that c-Src and PI3K regulated Akt activation is key in osteoclast survival (David et al., 2002, Bonney et al., 2011, Li et al., 2002, Wong et al., 1999a).

In the late stage of RANKL-induced osteoclast differentiation, RANK signaling induces GSK-3β, an inhibitor of NFATc1 phosphorylation and subsequent deactivation. This leads to NFATc1 dephosphorylation by calcineurin and translocation into the nucleus (Jang et al., 2011). Activated NFATc1 induces the expression of several genes encoding proteins involved in osteoclastogenesis. Proteins involved in cell-cell fusion such as dendritic cell-specific transmembrane protein (DC-STAMP) and in cell adhesion such as alpha v beta 3 vitronectin receptor are regulated by NFATc1 together with AP-1 and c-Fos (Kim et al., 2008, Yagi et al., 2007).

Table 1. Inflammatory regulators of Osteoclastogenesis.

Inflammatory Mediator	Source Cells	Osteoclastogenic Effects
TNF-α	Macrophages, Dendritic cells, Th17	Increased (Boyce and Xing, 2008)
TGF-β	Macrophages, Dendritic cells, Osteoblasts and other cells	Increased (Adamopoulos and Bowman, 2008)
IL-1	Macrophages, Dendritic cells	Increased (Adamopoulos et al., 2010)
IL-3	T cells	Decreased (Srivastava et al., 2011)
IL-4	Th2	Decreased (Mangashetti et al., 2005)
IL-6	Macrophages, Dendritic cells	Increased (Yun et al., 1998)
IL-7	Bone Marrow Stromal Cells	Increased (Giuliani et al., 2005)
IL-10	Treg	Decreased (Wing et al., 2011)
IL-17	T cells	Increased (Arboleya and Castaneda, 2013)
IL-18	Macrophages	Decreased (Sims et al., 2004)
IL-23	Macrophages, Dendritic cells	Increased (Sato et al., 2006)
IL-27	Macrophages, Dendritic cells	Decreased (Woodward, 2010)
IFN-γ	Th1, Natural killer lymphocyte	Decreased (Yun et al., 1998)
Prostaglandins	Monocytes and macrophages	Increased (Li et al., 2000)
Leukotrienes	Monocytes and macrophages	Increased (Jiang et al., 2005b)
Resolvins	Neutrophils, monocytes and macrophages	Decreased (Herrera et al., 2008)

Figure 3. Mechanism of Bone Resorption.

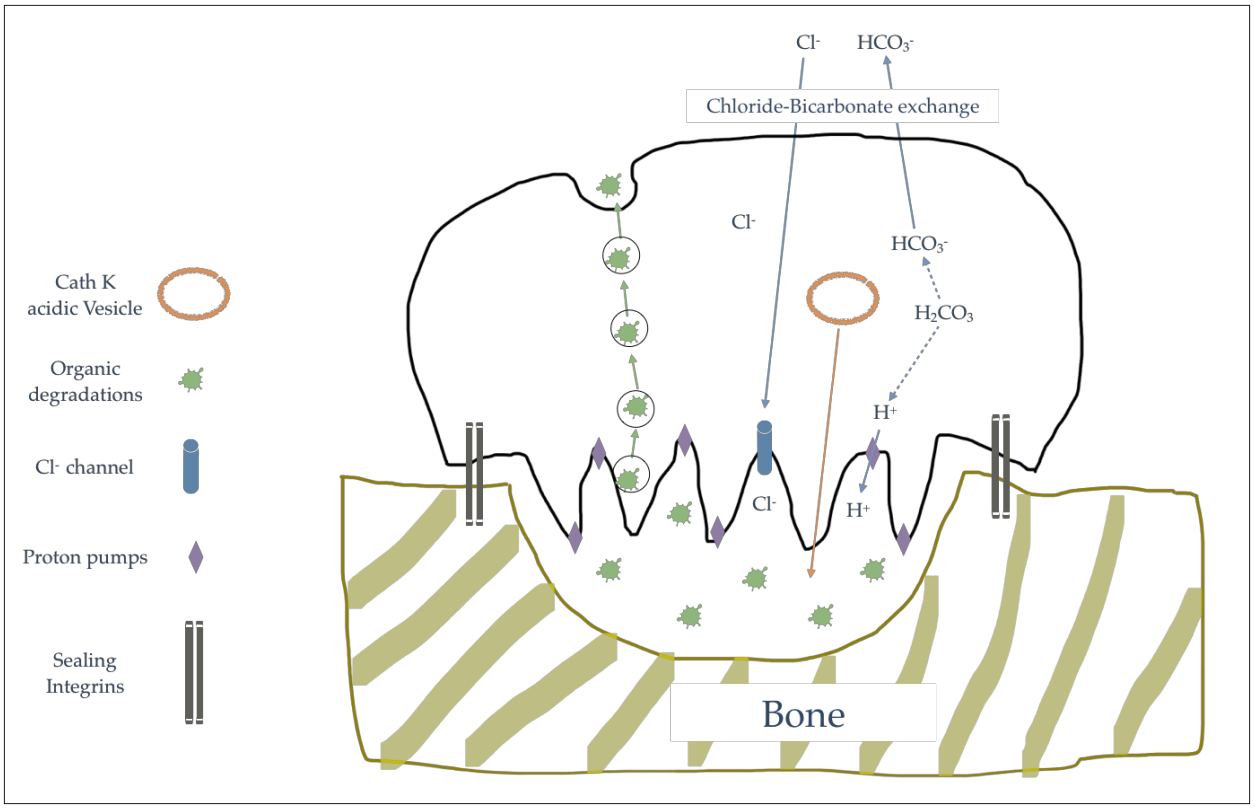


Figure 3. Mechanism of Bone Resorption. Osteoclastogenesis is a multi-phase process that starts with securing immature osteoclast progenitor proliferation and survival followed by committing these progenitors to differentiate into mature osteoclasts and ending with executing bone resorption. Osteoclasts are the exclusive cells responsible for bone resorption through demineralization of inorganic constituents, crystalline hydroxyapatite, followed by proteolytic degradation of organic matrix, mainly type I collagen. They are characterized by their sealed ruffled border facing the bone matrix to build resorption lacunae (Howship's lacunae). Osteoclasts demineralize inorganic constituents via vacuolar H+-adenosine triphosphatase (H+-ATPase) that delivers protons to the resorption compartment leading to secretion of hydrochloric acid into the Howship's lacunae. To counter the flux of positive charge, osteoclasts release Cl- into the resorption compartment using Cl- charge-coupled to the H+- ATPase channels. Additionally, osteoclasts release various enzymes to degrade bone organic matrix such as tartrate resistant acid phosphatase (TRAP) and lysosomal proteolytic cathepsin K.

Figure 4. Signaling pathways of c-Fms and RANK in the proliferation, survival and differentiation of osteoclast precursors and osteoclasts during osteoclastogenesis.

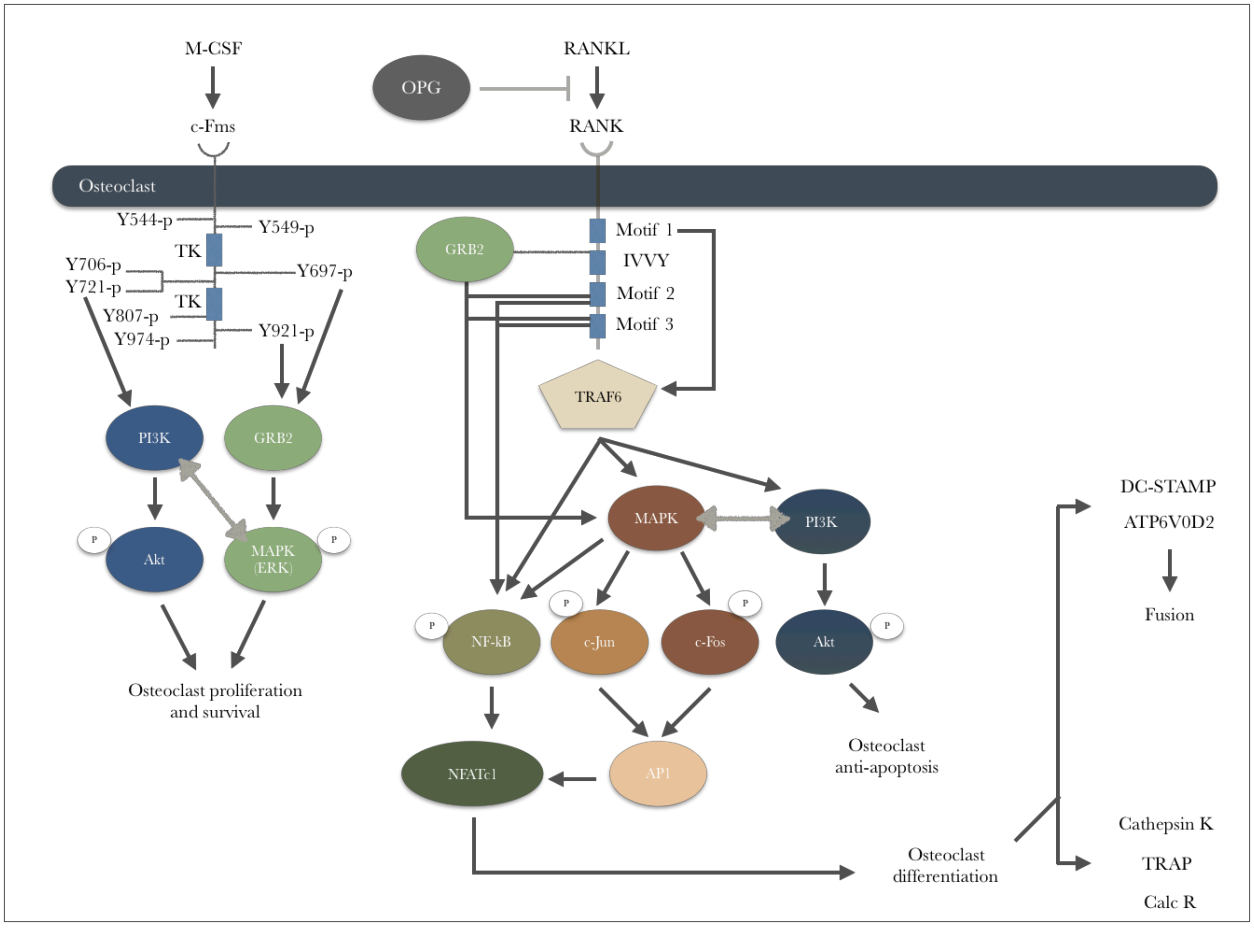


Figure 4. Signaling pathways of c-Fms and RANK in the proliferation, survival and differentiation of osteoclast precursors and osteoclasts during osteoclastogenesis. Bone resorption is a complex intricate process that involves several steps and requires the presence of many osteoclastogenesis signaling molecules. M-CSF interacts with a transmembrane receptor called colony-stimulating factor-1 receptor (CSF-1R, also known as c-Fms), which acts as its sole receptor. The interaction of c-Fms with its ligand M-CSF results in c-Fms dimerization and auto-phosphorylation at certain tyrosine residues through the activation of the receptor tyrosine kinase. Several studies explored possible tyrosine residues that are subjected to phosphorylation following c-Fms dimerization and the most agreed upon are seven residues located at the cytoplasmic tail of c-Fms: Y559, Y697, Y706, Y721, Y807, Y921, and Y974. The central transducers of the M-CSF/c-Fms signaling are Phosphoinositide 3-kinase (PI3K)/Protein kinase B (PKB, also known as Akt) pathway and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (ERK) p42/44 pathway responsible for cell proliferation, survival, differentiation, and cytoskeletal reorganization.

1.2.3. Inflammation Resolution

The first reported case of inflammation resolution was in the inflammatory disease, lobar streptococcal pneumonia. Most of the lesion in the lung was resolved without evident scaring and tissue destruction (Deepe and Eagleton, 1980, Jay et al., 1975). At that time, resolution was thought of as the passive outcome of dilution of the pro-inflammatory mediators (Majno and Joris, 2004). Today, we know that inflammation resolution is an active, programmed, protective biochemically-mediated response that is considered by pathologists to be an extension of the acute inflammatory response. The discovery of the highly regulated events of neutrophil apoptosis and subsequent removal by surrounding phagocytic tissue macrophages and the identification of the structurally unique pro-resolving mediators responsible for resolution called specialized pro-resolving mediators (SPMs) led to the recognition of resolution as an active response (Savill et al., 1989a, Savill et al., 1989b, Serhan, 2007, Serhan, 2010, Serhan, 2011). The cellular events of inflammation resolution are orchestrated by anti-inflammatory mediators and pro-resolving SPMs. Collectively, they activate the process of non-phlogistic recruitment of macrophages to facilitate removal of cell debris and apoptotic neutrophils, normalize endothelial vascular permeability and terminate neutrophil influx into the affected site (Serhan, 2010).

1.2.3.1. Inflammation Resolution Cellular Events

The events of acute inflammation resolution at a cellular level involve several cell types that permit the repair of injured tissues after eliminating invading organisms. Neutrophils play a major role in the initiation of resolution through the release of tertiary granules containing

annexin A1 and lactoferrin that diminishes neutrophil chemotaxis, stimulates their apoptosis and signals classical inflammatory scavenger macrophages (Bournazou et al., 2009, Elliott et al., 2009, Haslett, 1999, Lauber et al., 2003, Perretti and D'Acquisto, 2009, Peter et al., 2008, Scannell et al., 2007). Also, the interaction between neutrophils and epithelial cells and platelets incites the release of the specialized pro-resolving lipid mediators, which in turn attracts non-classical monocytes that develop into M2-like macrophages involved in promotion of wound healing and tissue repair (Auffray et al., 2007, Nahrendorf et al., 2007, Serhan, 1994, Serhan et al., 2008a, Ziegler-Heitbrock, 2007). These M2-like macrophages execute the non-phlogistic phagocytosis of apoptotic neutrophils and cell debris, while releasing anti-inflammatory mediators (interleukin-10 (IL-10) and transforming growth factor-β (TGFβ-(3)) and angiogenesis growth factors such as vascular endothelial growth factor (VEGF) (Fadok et al., 1998, Serhan et al., 2008a, Voll et al., 1997).

1.2.3.2. Inflammation Resolution Mediators

Inflammation resolution is mediated by various endogenous regulatory molecules that control the overall process of resolution. These mediators are released systemically and locally with diverse actions and include anti-inflammatory and pro-resolution mediators. Several locally-acting **cytokines** participate in the resolution response through stimulating immune cells to cease the release of pro-inflammatory mediators. For an example, TGF-β is a pro-inflammatory cytokine that in later stages of inflammation stimulates macrophages non-phlogistic phagocytosis of apoptotic neutrophils and cell debris (Ashcroft, 1999). Also, IL-10, which is released by monocytes, is another cytokine with anti-inflammatory actions that causes reduction in the

production of the very critical pro-inflammatory cytokines TNF-α and IL-1β (Murray, 2006, O'Shea and Murray, 2008).

Chemokines are pro-inflammatory mediators that are involved actively in the resolution response by creating the functionally-shifted anti-inflammatory chemokine-derived peptides. These molecules act as anti-inflammatory mediators to diminish neutrophil chemotaxis and infiltration and reduce any further release of pro-inflammatory mediators by M1-like macrophages (Cash et al., 2010, Cash et al., 2008, McQuibban et al., 2000, McQuibban et al., 2002). Other systemically released biochemical anti-inflammatory mediators such as **adrenalin, noradrenalin and glucocorticoids** regulate the inflammatory response via suppression of the production of pro-inflammatory mediators and up-regulation of the anti-inflammatory and pro-resolving mediators (Baschant and Tuckermann, 2010, Besedovsky and del Rey, 1996, Karin, 1998, Rhen and Cidlowski, 2005, Sigola and Zinyama, 2000, Spector et al., 1965, Spector and Willoughby, 1960, Zinyama et al., 2001).

The novel **SPMs** are the master activators and promoters of inflammation resolution. As discussed earlier, classic lipid mediators such as prostaglandins and leukotrienes are potent pro-inflammatory mediators released by monocytes and macrophages. They are synthesized by cyclooxygenases (COX) or lipoxygenases (LO) from membrane-derived arachidonic (Funk, 2001). Nevertheless, these pro-inflammatory mediators provoke a process called lipid mediator class-switch, where they are gradually replaced by anti-inflammatory and pro-resolving lipid mediator mediators. As an example, PGE_2 and PGD_2 progressively stimulate the production of the pro-resolving mediator lipoxin (Levy et al., 2001, Serhan et al., 2008a).

SPMs are structurally-unique autacoids with a short half-life (Serhan et al., 2000, Serhan et al., 2002). They are derived from several biochemical origins through different transcellular

biosynthesis pathways within exudates (Serhan et al., 2000, Serhan et al., 2002). Lipoxins (LX) are derived from arachidonic acid (AA), E-series resolvins (Rv) are derived from eicosapentaenoic acid (EPA) and D-series resolvins, protectins (PD) and maresins (MaR) are derived from docosahexaenoic acid (DHA) (Serhan and Chiang, 2008, Serhan et al., 2009).

The first member of the SPM family to be identifies was the **Lipoxins** (Serhan, 1994, Serhan et al., 2000). They hinder neutrophil recruitment and eosinophil chemotaxis, stimulate monocytes to reduce production of inflammatory superoxide anions and elicit macrophagic phagocytosis of apoptotic neutrophils and cell debris. Lipoxins are produced through leukocyte interactions with platelets leading to lipid mediator class shift such as leukotriene A4 (LTA_4) switch into to LXA_4 and LXB4 mediated by leukocyte 5-LO (Godson et al., 2000, Maddox et al., 1998, Maddox and Serhan, 1996, Serhan, 2005, Serhan, 2007, Soyombo et al., 1994).

Protectins are pro-resolution mediators with organ-protective abilities that are synthesized by glial cells. They diminish cells' ability to produce cytokines and reduce recruitment and chemotaxis of neutrophils (Hong et al., 2008, Serhan et al., 2006). Protectin D1 (PD1), for example, was found to promote tissue healing in Alzheimer's disease and corneal thermal injury among several other diseases by their ability to stimulate apoptosis of T cells (Ariel et al., 2005, Duffield et al., 2006, Gronert et al., 2005, Lukiw et al., 2005, Marcheselli et al., 2003).

The newest member of the SPM family is the **Maresins**, synthesized and produced by macrophages (Serhan et al., 2009). *In vivo* studies showed that maresin 1 (MaR1) stimulates macrophage phagocytosis of apoptotic neutrophils and zymosan, while reducing neutrophil chemotaxis and recruitment (Serhan et al., 2009). **Resolvins** are potent SPMs that act as antagonists of acute inflammation and agonists of the resolution phase of inflammation. They were

first identified in acute inflammation exudates extracted from mouse air-pouch models during the resolution response (Serhan, 2007).

1.2.3.3. RvE1

1.2.3.3.1. Biosynthesis and Metabolism

The endogenous RvE1 (5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E- eicosapentaenoic acid) is produced in humans as part of the resolution response to inflammation via vascular endothelial cells and leukocyte interactions. RvE1 biosynthesis is initiated by phospholipase mediated release of EPA from cell membranes (Kasuga et al., 2008). After EPA is released, it undergoes oxygenation by endothelial cells acetylated COX-2 to form 18R-hydroperoxy eicosapentaenoic acid (18R-HpEPE) followed by peroxidase reduction to generate 18R-HEPE. Another mechanism to transform EPA into 18R-HEPE exists through aspirin-independent Microbial cytochrome P_{450} monooxygenase. Upon vascular endothelial cell interactions with leukocytes, the later produce 5-LO to catalyze the lipoxygenation of 18R-HEPE to form hydroperoxide 5S-hydroperoxy-18R-hydroxy-EPE, which is converted later into epoxide 5S(6)-epoxy-18hydroxy-EPE. Then, the enzyme hydrolase generates RvE1 through an enzymatic hydrolysis process. RvE1 itself can undergo further metabolism to generate several metabolites based on tissue and cell type specificity and possible new roles. These metabolites include: 18-oxo-RvE1, 10,11-dihydro-RvE1, 19-hydroxy-RvE1 and 20-hydroxy-RvE1. It is worth mentioning that some of these metabolites are in fact biologically inactive forms of RvE1 such as the 10,11-dihydro-RvE1 (Arita et al., 2006, Hong et al., 2008).

1.2.3.3.2. Resolution Actions

Previous studies have demonstrated that RvE1 is key in reduction of the total number of neutrophils as well as in initiating and activating resolution earlier when compared to spontaneous resolution response through numerous mechanisms (Bannenberg et al., 2005). RvE1 stimulates macrophage non-phlogistic phagocytosis of apoptotic neutrophils and cell debris and subsequent clearance from tissues to the spleen and lymph nodes (Schwab et al., 2007). Also, RvE1 reduces the maximum number of neutrophils by inhibiting their infiltration, transmigration and chemotaxis. This is driven mainly by RvE1 attenuating the production of many local cytokines and chemokines such as CCR5, CCL3 and CCL5 expressed by apoptotic leukocytes (Ariel et al., 2006, Bannenberg et al., 2005, Haworth et al., 2008). Moreover, RvE1 blocks platelet aggregation by diminishing P-selectin mobilization and actin polymerization and facilitates mucosal epithelial surface cell clearance of neutrophils through increasing their CD55 expression (Campbell et al., 2007, Dona et al., 2008, Fredman et al., 2010). In addition to RvE1's anti-inflammatory and pro-resolution actions, previous studies demonstrated its effectiveness in inhibiting osteoclast differentiation and fusion *in vitro* using resorption pit formation in a dose-dependent manner (Herrera et al., 2008).

RvE1's pro-resolving actions induce inflammation resolution and stimulate tissue homeostasis *in vivo* in many inflammatory diseases and conditions such as periodontitis (Hasturk et al., 2007, Hasturk et al., 2006), colitis (Connor et al., 2007, Hasturk et al., 2006, Ishida et al., 2010), peritonitis (Bannenberg et al., 2005), cardiac ischemia/reperfusion injury (Keyes et al., 2010), allograft rejections (Levy et al., 2011), asthma (Aoki et al., 2008, Aoki et al., 2010, Haworth et al., 2008), obesity (Gonzalez-Periz et al., 2009), inflammatory pain (Xu et al., 2010),

retinopathy, dry eye and herpes simplex virus-induced ocular diseases (Connor et al., 2007, Li et al., 2010, Rajasagi et al., 2011).

1.2.3.3.3. Signal Transduction

Previous studies demonstrated that RvE1 interacts with two G protein-coupled receptors (GPCRs) to mediate its pro-resolving actions. The first receptor is Chemerin Receptor 23 (ChemR23). It was first identified and confirmed as a receptor for RvE1 during a functional screening of the interaction between lipoxin A4 receptor (ALX) and closely related GPCRs. It is expressed plentifully in macrophages, dendritic cells, monocytes and to a lesser extent in platelets and osteocytes. RvE1 promotes pro-resolving actions through the ChemR23 receptor by inhibiting the activation of NF-KB (Arita et al., 2005).

The second RvE1 receptor is the cell surface expressed Leukotriene B4 receptor 1 (BLT-1), which was originally defined as a high-affinity receptor for leukotriene B4 (LTB_4). BLT1 is expressed on the surface of several inflammatory and immune cells such as neutrophils, osteoclasts, granulocytes, eosinophils, differentiated Th1, Th2, Th17 cells and dendritic cells (Yokomizo, 2011). Although osteoclasts express ChemR23 and BLT-1 at a transcriptional level, binding specificity of RvE1 to BLT-1 is higher than to ChemR23 (Herrera et al., 2008)

LTB4 is a potent inflammatory mediator that stimulates PMN, eosinophil, and macrophage chemotaxis and promotes the release of numerous pro-inflammatory molecules by neutrophils (Savill et al., 1989b, Serhan, 2011). It can stimulate RANKL-independent osteoclastogenesis by increasing osteoclast number and resorptive activity (Garcia et al., 1996, Jiang et al., 2005b). BLT1 knock-out models exhibit reduced bone resorption generating osteopetrosis (Hikiji et al., 2009).

In leukocytes, RvE1 inhibits calcium mobilization stimulated by LTB4-BLT1 interaction and reduces the BLT1 intracellular signal activation of NF-kB. However, there is limited data

regarding RvE1 intracellular signaling events mediated via BLT1 in osteoclasts. Previous studies revealed that RvE1 treatment of isolated osteoclasts reduced cell fusion in the late stages of osteoclast differentiation leading to reduction in osteoclast formation by 32.8%. RvE1 specifically targeted Dendrocyte Expressed Seven Transmembrane Protein (DC-STAMP), which is an osteoclast fusion protein, down-regulating its expression by 65.4%. Moreover, RvE1 inhibited nuclear factor of activated T cells 1 (NFATc1), which is an essential expression factor for osteoclast differentiation, binding to the DC-STAMP promoter (Zhu et al., 2013).

LTB_4 intracellular signaling events mediated via BLT1 can shed some light on the possible signaling pathways involved in RvE1-BLT1 interactions. Previous studies established that BLT1 standard intracellular transduction is via two classes of G proteins, namely Gi- and G16-. These G proteins hinder cAMP formation and increase intracellular calcium influx (Yokomizo, 2011). Nevertheless, BLT1 transduces intracellular signals in osteoclasts rather through PTX-sensitive Gi protein and Rac1. The cell proliferation, survival and anti-apoptotic Akt pathway is activated either synergistically by Rac1 and PI3K or through a positive feedback loop between Rac1 and PI3K to control Akt activation (Hikiji et al., 2009). As shown by Fukuda et al., PI3K inhibitors prevented Rac1 survival and anti-apoptotic signaling while Mek, of the ERK pathway, inhibitors did not, indicating a possible BLT1/Rac1/PI3K/Akt pathway for RvE1-BLT1 interaction (Fukuda et al., 2005). Another possible pathway for transducing signals of RvE1-BLT1 interaction is through the activation of phospholipase C (PLC_B) and intracellular calcium flux mediated by calcium release–activated channel (CRAC). Subsequently, this leads to stimulation of the essential transcription factor nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) (Dixit et al., 2014).

Table 2. RvE1 in the different in vivo animal models.

In vivo Model	Actions
Periodontitis (Rabbit model)	Diminishes leukocytes chemotaxis and transmigration, stimulates hard and soft tissue regeneration and hinders osteoclastogenesis (Hasturk et al., 2007, Hasturk et al., 2006).
Colitis (Mouse model)	Promotes anti-inflammatory response via inhibiting the expression of pro-inflammatory genes (Connor et al., 2007, Hasturk et al., 2007, Hasturk et al., 2006).
Peritonitis (Mouse model)	Inhibits neutrophil infiltration, regulates chemokine/cytokine production and Induces phagocytes clearance, prevents neutrophil infiltration and shifts chemokine and cytokine production to produce anti-inflammatory response (Bannenberg et al., 2005).
Cardiac ischemic /reperfusion injury (Rat model)	Decreases size of the infarction (Keyes et al., 2010).
Allograft rejection (Mouse model)	Inhibits acute rejection of allografts (Levy et al., 2011).
Asthma (Mouse model)	Promotes anti-inflammatory response through increased IFN-y and LXA4 release and decreased IL-23 and IL-6 productions in airway of lungs (Aoki et al., 2008, Aoki et al., 2010, Haworth et al., 2008).

Obesity (Mouse model)	Provide protection from liver steatosis through adipokines regulation (Gonzalez-Periz et al., 2009).
Inflammatory pain (Mouse model)	Diminishes hypersensitivity to heat and mechanical stimuli and reduces spontaneous pain (Xu et al., 2010).
Retinopathy (Mouse model)	Provide protection from neovascularization (Connor et al., 2007).
Dry eye (Mouse model)	Stimulates tear production and provides protection for the corneal epithelial integrity (Li et al., 2010).
Herpes simplex virus-induced ocular diseases (Mouse model)	Induces anti-inflammatory mediators such as IL-10 and decreases angiogenesis and stromal keratitis (Rajasagi et al., 2011).

Figure 5. Pro-inflammatory and pro-resolving lipid mediators.

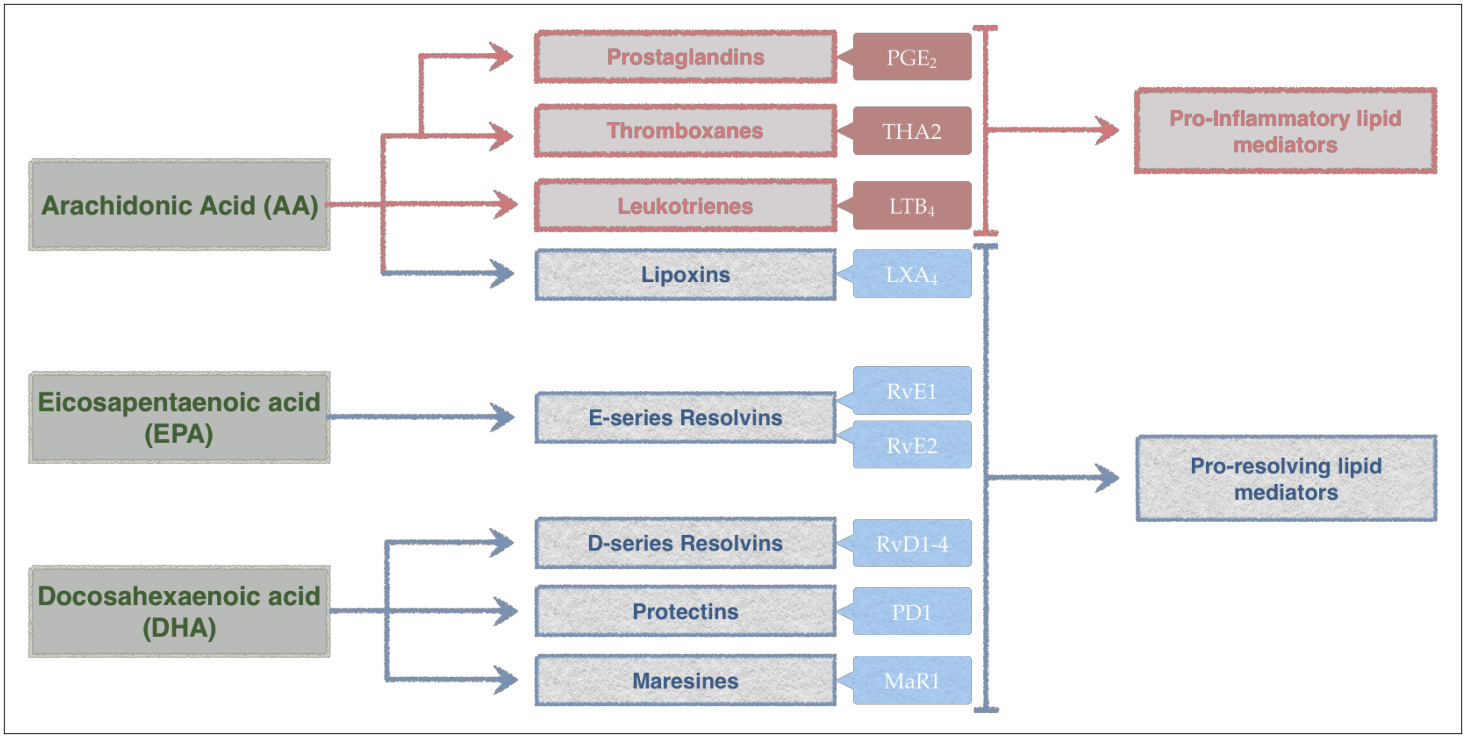


Figure 5. Pro-inflammatory and pro-resolving lipid mediators. Prostaglandins and leukotrienes are classic lipid mediators acting as potent pro-inflammatory mediators released by monocytes and macrophages. They are synthesized by cyclooxygenases (COX) or lipoxygenases (LO) from membrane-derived arachidonic acid. Nevertheless, these pro-inflammatory mediators provoke a process called lipid mediator class-switch, where they are gradually replaced by anti-inflammatory and pro-resolving lipid mediator mediators. Pro-resolving lipid mediators are structurally-unique autacoids with a short half-life. They are derived from several biochemical origins through different transcellular biosynthesis pathways within exudates. Lipoxins (LX) are derived from arachidonic acid (AA), E-series resolvins (Rv) are derived from eicosapentaenoic acid (EPA) and D-series resolvins, protectins (PD) and maresins (MaR) are derived from Docosahexaenoic acid (DHA).

Figure 6. Resolvin E1 biosynthesis pathways.

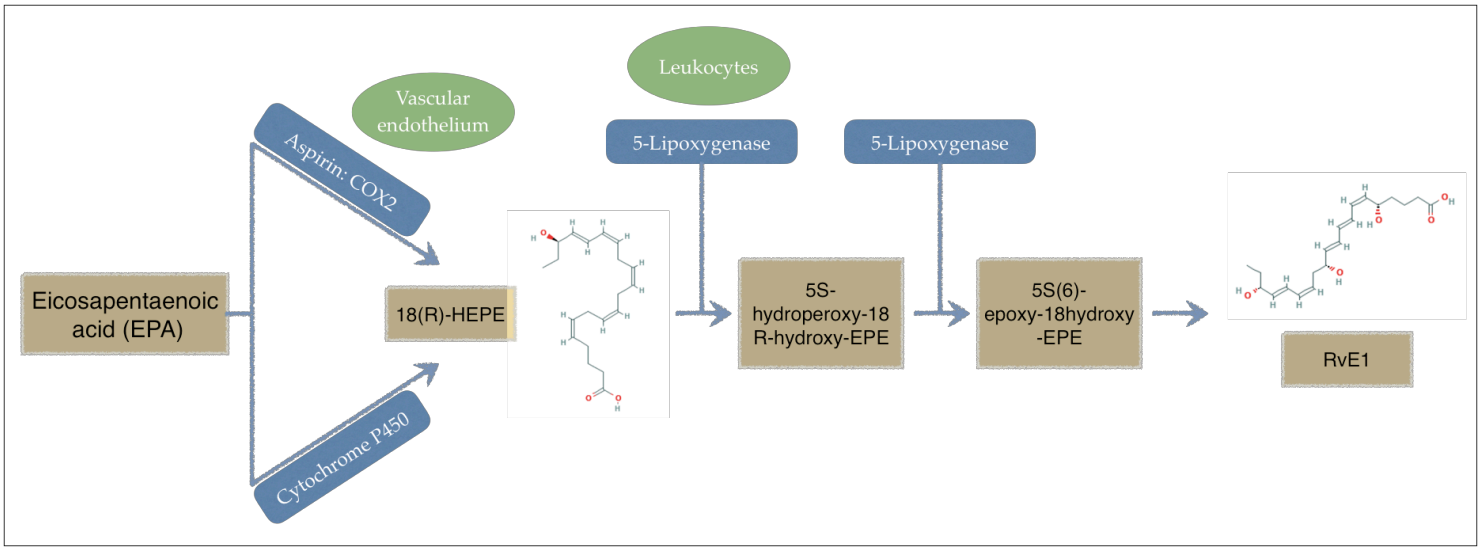


Figure 6. Resolvin E1 biosynthesis pathways. The endogenous RvE1 (5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E- eicosapentaenoic acid) is produced in humans as part of the resolution response to inflammation via vascular endothelial cells and leukocyte interactions. RvE1 biosynthesis is initiated by phospholipase enzymes mediated release of EPA from cell membranes. After EPA is released, it undergoes oxygenation by endothelial cells acetylated COX-2 to form 18R-hydroperoxy eicosapentaenoic acid (18R-HpEPE) followed by peroxidase reduction to generate 18R-HEPE. Another mechanism to transform EPA into 18R-HEPE exists through aspirin-independent Microbial cytochrome P_{450} monooxygenase. Upon vascular endothelial cells interactions with leukocyte, the later produce 5-LO to catalyze the lipoxygenation of 18R-HEPE to form hydroperoxide 5S-hydroperoxy-18R-hydroxy-EPE, which is converted later into epoxide 5S(6)-epoxy-18hydroxy-EPE. Then, the enzyme hydrolase generates RvE1 through an enzymatic hydrolysis process.

Figure 7. Resolvin E1 receptors mediated cellular events.

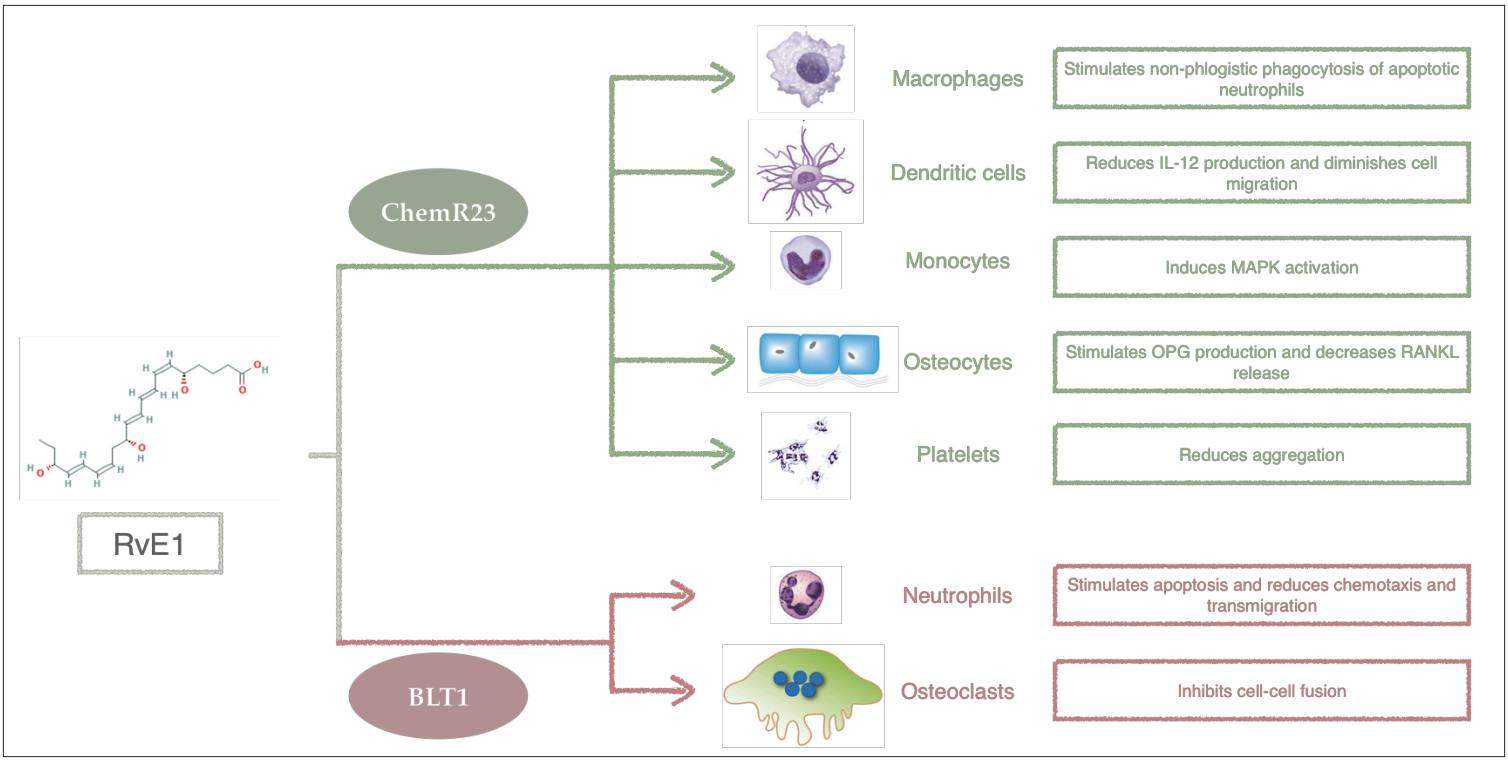


Figure 7. Resolvin E1 receptor mediated cellular events. RvE1 interacts with two G protein-coupled receptors (GPCRs) to mediate its pro-resolving actions. The first receptor is Chemerin Receptor 23 (ChemR23), which is expressed plentifully in macrophages, dendritic cells, monocytes and to a lesser extent in platelets and osteocytes. The second RvE1 receptor is the cell surface expressed Leukotriene B4 receptor 1 (BLT-1), which is originally defined as a high-affinity receptor for leukotriene B4 (LTB_4). BLT1 is expressed on the surface of several inflammatory and immune cells such as neutrophils, osteoclasts and differentiated Th1, Th2, Th17 cells. RvE1, via ChemR23, stimulate macrophages non-phlogistic phagocytosis of apoptotic neutrophils and cell debris, blocks platelet aggregation through diminishing P-selectin mobilization and actin polymerization, diminishes dendritic cells migration and IL-12 release, induce monocytes activation and stimulates osteocyte to produce OPG. Also, RvE1, via BLT1, reduces the maximum number of neutrophils through inhibiting their infiltration, transmigration and chemotaxis and inhibits osteoclasts differentiation and cell-cell fusion.

Figure 8. Osteoclastogenesis and involved signaling pathways and surface markers.

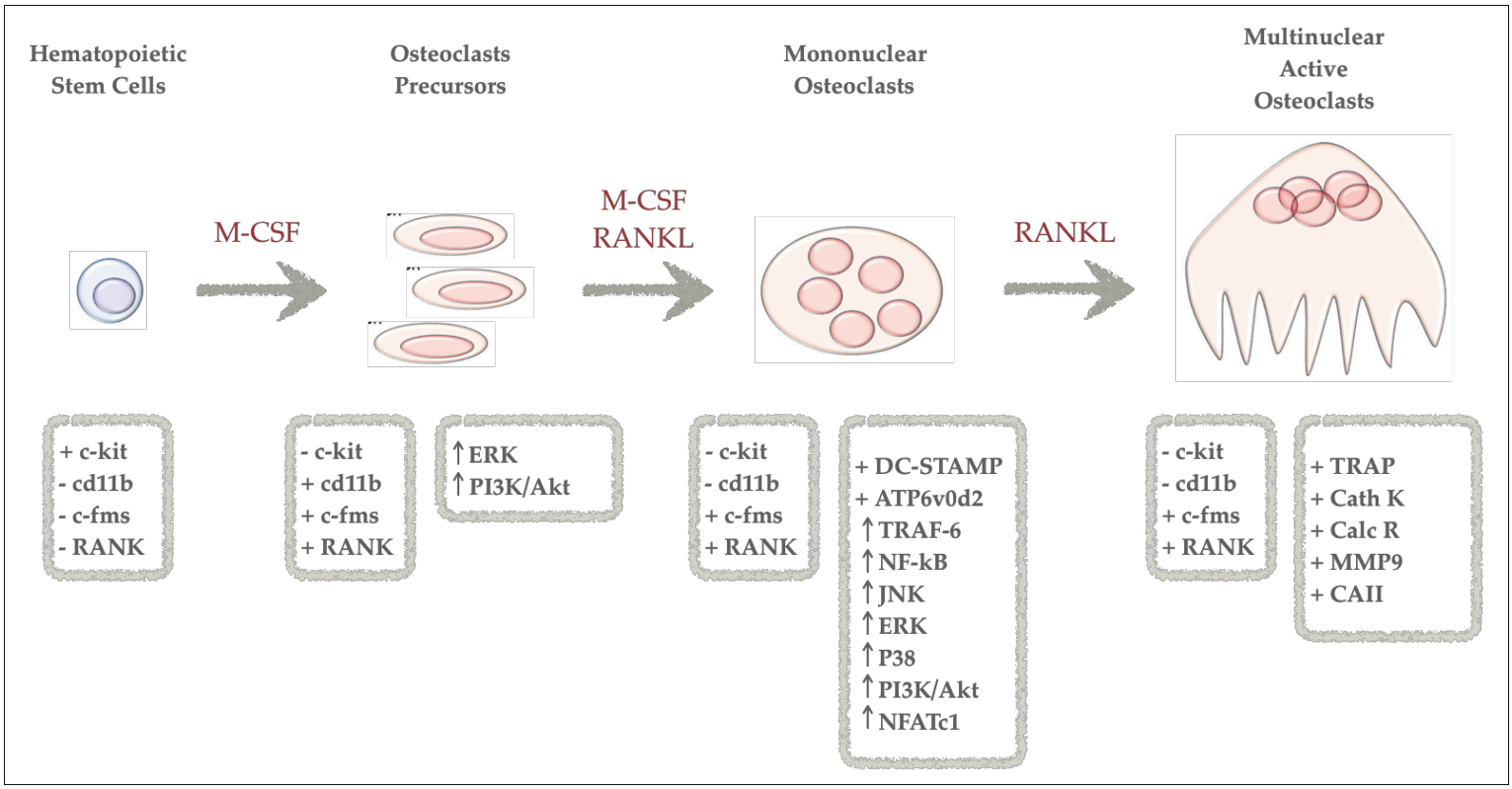


Figure 8. Osteoclastogenesis and involved signaling pathways and surface markers. Osteoclasts originate from the hematopoietic stem cells lineage. When induced by M-CSF, these stem cells differentiate into osteoclast precursors. However, RANKL induction is required for precursors to form committed mononuclear osteoclasts, which then undergo cell-cell fusion to form multinuclear osteoclasts followed by activated osteoclasts under the appropriate conditions. Several different cell surface markers are used to identify the cells in different stages. Also, cell signaling pathways are involved in the process of osteoclastogenesis from hematopoietic stem cells lineage into active osteoclasts.

1.3. Hypotheses and Specific Aims

In this study, our goal was to develop a verified periodontal disease model in mice using ligature to induce alveolar bone loss and to further investigate the underlying cellular and molecular mechanisms responsible for RvE1 regulatory actions on bone marrow-derived osteoclast precursor and osteoclast differentiation, survival, proliferation and apoptosis through addressing the following specific aims:

Specific Aim 1: Establish RvE1 preventive regulation of bone loss in a ligature-induced periodontal disease in mice. **Hypothesis 1:** RvE1 prevents bone loss in ligature-induced periodontal disease in mice:

- Does RvE1 reduce bone loss in ligature-induced periodontal disease in mice?
- Does RvE1 inversely regulate osteoclasts in ligature-induced periodontal disease in mice?

Specific Aim 2: Determine RvE1 regulation of bone marrow-derived osteoclast precursor and osteoclast differentiation through BLT1 receptors. **Hypothesis 2:** RvE1 prevents bone marrow-derived osteoclast precursor and osteoclast differentiation through BLT1 receptors.

- Does RvE1 impact the expression of differentiation markers in bone marrow-derived osteoclast precursors and osteoclasts?
- Does RvE1 hinder the differentiation of bone marrow-derived osteoclast precursors and osteoclasts?
- Are RvE1 regulatory actions on bone marrow-derived osteoclast precursor and osteoclast differentiation dose-dependent?
- Do bone marrow-derived osteoclast precursors and osteoclasts express BLT1 receptors?

- Does RvE1 hinder the differentiation of bone marrow-derived osteoclast precursors and osteoclasts through BLT1 receptors?

Specific Aim 3: Determine RvE1 regulation of bone marrow-derived osteoclast precursor and osteoclast proliferation, viability and apoptosis. **Hypothesis 3:** RvE1 reduces bone marrow-derived osteoclast precursor and osteoclast proliferation and viability and increases their apoptosis.

- Does RvE1 reduce bone marrow-derived osteoclast precursor and osteoclast proliferation in a dose-dependent manner?
- Does RvE1 reduce bone marrow-derived osteoclast precursor and osteoclast viability in a dose-dependent manner?
- Does RvE1 increase bone marrow-derived osteoclast precursor and osteoclast apoptosis?

Specific Aim 4: Determine the molecular signaling pathways involved in RvE1 regulation of bone marrow-derived osteoclast precursor and osteoclast differentiation, proliferation, viability and apoptosis. **Hypothesis 4:** RvE1 interferes with the activation/deactivation of the signaling pathways involved in bone marrow-derived osteoclast precursor and osteoclast differentiation, proliferation, viability and apoptosis through regulating the levels of phosphorylated signaling molecules.

- What are the signaling pathways involved in RvE1 regulation of bone marrow-derived osteoclast precursors and osteoclasts?

1.4. Significance

Periodontitis is a disease of inflammatory nature with bacterial etiology. The events of periodontitis start with biofilm accumulation on the teeth followed by a cascade of events that eventually will lead to host-mediated destruction of tooth supporting tissues, including junctional epithelium, connective tissue attachment and alveolar bone. Also, regeneration of hard and soft tissues lost to periodontal disease in uncontrolled inflammatory conditions is unpredictable and extremely restricted (Reynolds et al., 2003, Sculean et al., 2008).

Previous *in vivo* and *in vitro* studies showed that RvE1 halted alveolar bone loss in periodontal disease and inhibited osteoclast differentiation. Therefore, investigating the pro-resolution regulatory actions of RvE1 is key in the pursue to provide an environment that are protective for alveolar bone from destruction, and permissive for regeneration.

1.5. Innovation

There is very limited information concerning RvE1 regulatory actions on osteoclast precursor and osteoclast differentiation, proliferation, and survival. This proposal aims to implement innovative experiments that will target RvE1 regulation of these events using fundamental mechanistic studies. These studies will increase our knowledge to better understand the direct role of RvE1 as a resolution agonist in bone preservation and reversing osteoclast actions in bone diseases of inflammatory nature.

CHAPTER TWO
MATERIAL AND METHODS

2.1. Experimental ligature-induced periodontal disease model

Placement of ligatures on mice was performed under general anesthesia using 100-mg of ketamine per kg of body weight (Ketaset; Fort Dodge Animal Health, Fort Dodge, Iowa) and 5-mg of xylazine per kg of body weight (AnaSed; Ben Venue Laboratories, Bedford, OH) injections. Alveolar bone-loss was induced using a 7-0 silk suture that was placed into the gingival sulcus around the second molar of both maxillary quadrants with the knot placed toward the palatal side. Ligature placement was performed using a microscope to insure avoiding any gingival damage during procedure.

Twenty-four FVB wild-type (WT) mice were divided into three groups: baseline group that received no ligature placement nor RvE1 application, ligature group that received ligature placement only, and ligature+RvE1 that received ligature placement with RvE1 application (1 μg/tooth). The ligature placement and RvE1 treatment were delivered for one week and then animals were sacrificed. After sacrificing the animals, maxillae were split into right and left halves. For each mouse, the right half was fixed in 10% formalin for histological examination while the left half was defleshed and cleaned for morphometric analysis. The animals were euthanized using carbon monoxide (CO) that is delivered in a properly manufactured and equipped chamber according to the protocol approved by the IACUC. No adverse events were observed during experimental procedures throughout the study with regard to animal care.

2.2. Topical application of RvE1

Animals received topical treatment with 1 μM RvE1 or vehicle based on their group assignment daily for 7 days under inhalation anesthesia using isoflurane (4% induction and then 2% maintenance).

2.3. Bone destruction assessment using morphometric analysis

After the animals were sacrificed, the left half of maxilla was defleshed of muscles and soft tissues, cleaned and stained with methylene blue (1% in water) for good visual distinction between the bone and the tooth prior to bone loss morphometric evaluation. Then, the sectioned maxilla was mounted and photographed using an inverted microscope (Zeiss Axiovert 200, Zeiss, Thornwood, NY, USA) at ×10 magnification.

Four measurements were made to assess ligature-induced alveolar bone loss using ImageJ software and calculated in micrometers. The first measurement made was the total alveolar area bone loss bound by the alveolar bone crest level, the cementoenamel junction of teeth, the mesial surface of the first molar, and the distal surface of the second molar. The second one was the interproximal area bone loss between the first and second molars and bound by the alveolar bone crest level and the cementoenamel junction of teeth. The third one was the second molar furcation area bone loss bound by the alveolar bone crest level and cementoenamel junction of the second molar. The fourth one was the second molar furcation linear bone loss from the alveolar bone crest level to the furcation of the second molar. All measurements were taken on the buccal side.

2.4. Bone destruction assessment using micro-CT analysis

Evaluation of alveolar bone resorption were performed using a compact tomograph optical computed-tomography (μCT40, Scanco Medical AG, Basserdorf, Switzerland) at the Forsyth microCT core. The used parameters for the imaging were as follows: Tube voltage 70 kVp; current 140 mA; and integration time 300 ms. A μCT tubes were prepared with 5 samples in each for scanning. Each tube image included approximately 3500 micro-tomographic slices that were divided into about 350 slices for each sample with an increment of 17 μm, covering the entire width of the hemi-maxilla. The data were exported into DICOM format and the amount of bone loss were measured using Amira™ Software system (Thermo Scientific Pierce, Rockford, IL, USA).

Sample micro-tomographic slices were prepared following a standardized protocol and transformed into a 3-D image. Measurement were made through calculating first, second and third molar teeth volumes first. Then, total alveolar bone volume was calculated followed by calculating the total volume of the 3-D image of the sample outside the teeth and bone. The amount of remaining alveolar bone volume was divided by the total volume of the 3-D image to get the bone volume for each sample.

2.5. Histological analysis

Following the sacrifice, The right half of the maxilla were washed for 3 hours in running water, then immersed in 10% ethylenediaminetetraacetic acid (EDTA) for 3-4 weeks. The volume of the solution was at least 10 times the size of the specimen and was replaced every 2-3 days.

After decalcification, the tissues were washed aging for 3 hours in running water then and moved into formalin for at least 24 h before being embedded in paraffin. Sectioning of slices into thin sections (5 μm) were performed followed by staining procedures. Hematoxylin-eosin (HE) staining were used for bone destruction assessment and tartrate-resistant acid phosphatase (TRAP) staining were used to detect osteoclastic activity.

2.6. Osteoclast primary cell culture

Bone marrow-derived osteoclast precursor and osteoclast cells were harvested from 8-weeks-old mice. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Forsyth Institute University and performed in conformance to the standards of the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Bone marrow-derived cells were cultured in a medium that contains □α-MEM (Invitrogen, Carlsbad, CA, USA), 10% FBS (Atlanta Biologicals, Lawrenceville, GA, USA), 1% penicillin-streptomycin (Sigma, St. Louis, MO, USA). Osteoclast precursors were induced using pre-osteoclastogenesis differentiating medium (Pre-OC medium) that contains the previously mentioned medium plus 50 ng/ml M-CSF (Thermo Scientific). Osteoclast were induced using osteoclastogenesis differentiating medium (OC medium) that contains the previous medium plus 50 ng/ml M-CSF (Thermo Scientific) and 30 ng/ml RANKL (Thermo Scientific). Cells were plated in 96-well plates (2□x10^5 cells/well), 24-well plates (5x10^5 cells/well) or 6-well plates (10^6 cells/well). The following inhibitor were used in cell cultures based on their experimental design:

Table 3. List of inhibitors used.

Inhibitor
U-75302 #70705
Wortmannin #9951
PD98059 #9900

2.7. Tartrate Resistant Acid Phosphatase (TRAP) staining and Activity Assay

Osteoclastogenesis in histological sections of the in-vivo experiment and in cell cultures was evaluated by tartrate-resistant acid phosphatase (TRAP) staining following protocol described by Herrera et al (2008). TRAP+ multinuclear cells containing more than 3 nuclei were counted using 20X objectives in digital images of the cell culture that were taken using an inverted microscope (Zeiss Axiovert 200, Zeiss, Thornwood, NY, USA) with a video camera. TRAP activity assay was performed on 96-well plates (1□00x10^6 cells/well) culture cells and reading were performed using a microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA) at a wavelength of 570 nm.

2.8. RvE1 and control treatment

The process of RvE1 production was made through total organic synthesis and stored in 100% ethanol (17). The final concentration of RvE1 used was 10 nM based on our group prior study and a dose-dependent experiments (28). RvE1 and control treatment had identical culture medium except for the RvE1 itself, which was diluted immediately before use. Ethanol concentration in cell cultures never exceeded 1%.

2.9. Gene Expression analysis using qRT-PCR

Total cellular RNA was isolated from Bone marrow-derived osteoclast precursors and osteoclasts through TRIzol reagent (Life Technologies, Carlsbad, CA, USA) followed by testing the purity using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). After that, RNA was reverse transcribed using random hexamers, dNTP, buffer, nuclease-free water, ribonuclease (RNase) inhibitor and reverse transcriptase (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Carlsbad, CA, USA).

For the quantitative real-time PCR analysis, gene expression testing was performed using the Applied Biosystems StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and the detection assay used was the TaqMan™ Fast Advanced Master Mix (Thermo Scientific, Waltham, MA, USA). Data were analyzed using the $\Delta\Delta C_T$ method and the expression of each target gene was then calculated relative to the controls using GAPDH as an endogenous control. The following specific primers were used:

Table 4. List of specific primers used.

Name	Forward Sequence 5'-3'	Reverse Sequence 5'-3'
c-fms (Csf1r)	TGCGTCTACACAGTTCAGAG	ATGCTGTATATGTTCTTCGGT
cd11b (Itgam)	CAACAAGCACCTCTAGATGGT	GTGAGCCACACACAGAGCTTGCT
RANK (Tnfrsf11a)	CATGGCAGAGGCGGGAGTAC	GCCCGCTAGAGATGAACGTG

TRACP (Acp5)	CAGGAGACCTTTGAGGACGTG	GTGGAATTTTGAAGCGCAAAC
Cath K (Ctsk)	TGGCTCGGAATAAGAACAACG	GCACCAACGAGAGGAGAAATG
Calc R (Calcr)	TCATCATCCACCTGGTTGAG	GCTCGTCGGTAAACACAGC
GAPDH	GTCGGTGTGAACGGATTTGG	GACTCCACGACATACTCAGC

2.10. Cell viability analysis using MTT assay

Cell viability were assessed using MTT assay at days 5 and 7. Bone marrow-derived osteoclast precursor and osteoclast cells were plated in 96-well plates ($2x10^5$ cells/well) and incubated with MTT solution (0.5 mg/ml; Sigma-Aldrich; Merck KGaA) for 4 hours at 37°C. Using dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA), the formazan crystals formed were dissolved. bromide reduction was used to quantify cell viability. Absorbance reading were performed using a spectrophotometer microplate reader set at a wavelength of 560 nm (Spectramax 340PC 384 with a SoftMax Pro software 4.3LS, Molecular Devices).

2.11. Cell Proliferation analysis using BrdU incorporation assay

Cell proliferation were assessed using BrdU Assay Kit (GE Healthcare Life Sciences, Piscataway, NJ) at days 5 and 7. Bone marrow-derived osteoclast precursor and osteoclast cells were plated in 96-well plates ($2x10^5$ cells/well) and incubated with BrdU solution for 24 hours at 37°C. BrdU incorporation was used to quantify cell proliferation using the BrdU ELISA assay in the kit. Absorbance reading were performed using a spectrophotometer microplate reader set at dual

wavelength of 450/550 nm (Spectramax 340PC 384 with a SoftMax Pro software 4.3LS, Molecular Devices).

2.12. Cell apoptosis analysis using TUNEL assay

Cell apoptosis were assessed using TUNEL Assay Kit Click-iT™ TUNEL Alexa Fluor™ 488 Imaging Assay (Thermo Scientific) at days 5 and 7. Bone marrow-derived osteoclast precursor and osteoclast cells were plated in 24-well plates ($5x10^5$ cells/well). TUNEL staining was performed according to the manufacturer's instruction. TUNEL-positive cells were viewed at excitation 488 nm/emission 512 nm by fluorescence at a magnification of ×40. The ratio of TUNEL-positive nuclei was calculated as the number of TUNEL-labeled nuclei per the total number of cells.

2.13. Immunohistochemistry

Femur and tibia bones of mice were harvested from 8-10 weeks-old mice, immersed in 10% ethylenediaminetetracetic acid (EDTA) for 1-2 weeks. The volume of the solution was at least 10 times the size of the specimen and was replaced every day. After decalcification, the tissues were washed aging for 3 hours in running water then and moved into formalin for at least 24 h before being embedded in paraffin. Sectioning of slices into thin sections (5 μm) were performed followed by staining with anti-BLT1 (Thermo Scientific) and 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) for nuclei labeling.

2.14. Western blotting

Whole-cell proteins were extracted using CelLytic M solution (Sigma-Aldrich; Merck KGaA) with protease and phosphatase inhibitors. Protein concentration was determined using Bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit) and Absorbance was measured using a spectrophotometer microplate reader set at 562 nm (Spectramax 340PC384 with SoftMax 4.3LS, Molecular Devices).

Samples preparation then proceeded with mixing 10ug of lysate with 4x sample loading buffer with 2.5% volume β-ME and denatured by heating to 100°C for 8 min. Samples and a molecular weight standards then were loaded into the weals of a 12% polyacrylamide gels for electrophoresis in a running buffer (25 mM Trizma base, 192 mM glycine, 0.1% sodium dodecyl sulfate (SDS)) at 100 V for 1.5 hours. After that, Samples were transferred into a polyvinylidene fluoride (PVDF) membrane in a blotting buffer (25 mM Trizma base, 192 mM glycine, 20% 79 methanol) at 66 mA overnight at 4°C. Next morning, the membrane was incubated in blocking solution that contains 5% (w/v) BSA in 20mM Tris-HCI, 150mM NaCl, 0.1% Tween-20 + 5% BSA (TBS-T)) for 1-2 hours. The primary antibody of choice then used with 5% BSA in TBS-T to incubate the membrane overnight at 4°C. The following primary antibodies were used:

Table 5. List of primary antibodies used.

Primary Antibodies
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370
p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb #4695
Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060

Akt (pan) (C67E7) Rabbit mAb #4691

Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb #3033

NF-κB p65 (D14E12) XP® Rabbit mAb #8242

Phospho-IκBα (Ser32) (14D4) Rabbit mAb #2859

IκBα (44D4) Rabbit mAb #4812

Phospho-c-Jun (Ser63) II Antibody #9261

c-Jun (60A8) Rabbit mAb #9165

Phospho-c-Fos (Ser32) (D82C12) XP® Rabbit mAb #5348

c-Fos (9F6) Rabbit mAb #2250

BLT1 Receptor Polyclonal mAb #120114

Leukotriene B_4, Item # 20110, CAS # 71160-24-2

β-Actin (13E5) Rabbit mAb #4970

Anti-rabbit IgG, HRP-linked Antibody #7074

Next morning, the membrane was incubated with secondary antibody (goat anti-rabbit IgG-horseradish peroxidase (HRP)) in blocking solution (5% milk in TBS-T) for 1-2 hours at room temperature in a shaker. An HPR chemiluminescent substrate (Thermo Scientific) reaction was used to detect the HRP conjugate, followed by exposing the membrane to radiation to visualize protein bands. Quantification was carried out by measuring band density using Image J Software. β-actin was used as a loading control.

2.15. Statistical analyses

Experiments were performed in biological triplicates and repeated at least 3 times. Results are expressed as mean ± SEM. Statistical analysis was performed using smStata Software (StataCorp LLC, Texas, USA). Comparisons between groups were made using Student unpaired t test and analysis of variance (ANOVA) with Bonferroni correction and post hoc tests. Values of $P \leq 0.05$ were considered statically significant.

CHAPTER THREE
RESULTS

3.1. Specific Aim 1: Establish RvE1 preventive regulation of bone loss in ligature-induced alveolar bone loss in mice.

Rationale: The process of creating a successful periodontal disease model in animals requires constructing standardized experimental methods. Previous studies showed an experimental periodontal disease model induced using ligature combined with topical application of *P. gingivalis* in rabbits. These studies showed that destructive nature of periodontal disease resulting in periodontal tissues and alveolar bone loss. Moreover, the preventive regulation of inflammation by RvE1 led to diminished leukocyte infiltration and reduced alveolar bone loss. (Hasturk et al., 2007, Hasturk et al., 2006, Zhu et al., 2013). Establishing a successful experimental periodontal disease model in mice is very critical to further investigate the preventive regulation by RvE1 on alveolar bone loss and its direct actions on osteoclasts. Our **hypothesis** is that RvE1 prevents further bone loss in ligature-induced periodontitis in mice.

Experimental approach: twenty-four 8-weeks-old FVB wild-type (WT) mice were used in the ligature-induced experimental periodontal disease model. Animals were divided into three groups: baseline group that received no ligature placement nor RvE1 application, ligature group that received ligature placement only, and ligature+RvE1 that received ligature placement with RvE1 application (1 μg/tooth). Placement of ligature on mice was performed under general anesthesia using 100-mg of ketamine per kg of body weight (Ketaset; Fort Dodge Animal Health, Fort Dodge, Iowa) and 5-mg of xylazine per kg of body weight (AnaSed; Ben Venue Laboratories, Bedford,

OH) injections. Alveolar bone-loss was induced using a 7-0 silk suture that was placed into the gingival sulcus around the second molar of both maxillary quadrants with the knot placed toward the palatal side. Ligature placement was performed using a microscope to avoid any gingival damage during procedure (Fig. 10C).

The ligature placement and RvE1 treatment were delivered for one week and then animals were sacrificed. Animals received topical treatment with 1 μM RvE1 or vehicle based on their group assignment daily for 7 days under inhalation anesthesia using isoflurane (4% induction and then 2% maintenance). After sacrificing the animals, maxillae were split into right and left halves. For each mouse, the right half was fixed in 10% formalin for histological examination while the left half was defleshed and cleaned for morphometric analysis. The animals were euthanized using carbon monoxide (CO) that is delivered in a properly manufactured and equipped chamber according to the protocol approved by the IACUC. No adverse events were observed during experimental procedures throughout the study with regard to animal care (Fig. 9).

3.1.1. RvE1 reduced bone loss in ligature-induced periodontal disease in mice

Morphometric analyses showed that ligature placement for one week resulted in significant alveolar bone loss compared to no ligature placement. Also, RvE1 local application with ligature placement resulted in a significantly less alveolar bone loss compared ligature placement with vehicle. Measurements were made to assess ligature-induced alveolar bone loss using ImageJ software in two areas and calculated in micrometers. The first measurement made was the total alveolar area bone loss bound by the alveolar bone crest level, the cementoenamel junction of teeth, the mesial surface of the first molar, and the distal surface of the second molar (Fig 10A).

Ligature placement significantly increased total alveolar area bone loss by 103% ($P<0.01$) compared to no ligature placement and RvE1 local application significantly decreased total alveolar area bone loss by 29% ($P<0.05$) compared to ligature placement group (Fig. 10B). The second measurements made were to evaluate the second molar furcation area bone loss bound by the alveolar bone crest level and cementoenamel junction of the second molar (Fig. 10A). Ligature placement significantly increased second molar furcation area bone loss by 102% ($P<0.01$) compared to no ligature placement and RvE1 local application significantly decreased second molar furcation area bone loss by 68% ($P<0.05$) compared to ligature placement group (Fig. 10B).

Micro-CT analyses revealed that ligature placement for one week resulted in significant alveolar bone loss compared to no ligature placement. Also, RvE1 local application with ligature placement resulted in a significantly less alveolar bone loss compared ligature placement with vehicle. Micro-CT measurements were made to assess ligature-induced alveolar bone loss through calculating molar teeth volumes first. Then, total alveolar bone volume was calculated followed by calculating the total volume of the 3-D image of the sample outside the teeth and bone so it can be subtracted. The amount of remaining alveolar bone volume was divided by the remaining total volume of the 3-D image (Teeth and bone volume) to get the bone volume for each sample. Measurements were made using a compact tomograph optical computed-tomography (μCT40, Scanco Medical AG, Basserdorf, Switzerland) at the Forsyth microCT core (Fig. 11A). Ligature placement significantly decreased second molar alveolar bone volume by 24% ($P<0.01$) compared to no ligature placement and RvE1 local application significantly decreased second molar furcation area bone loss with more bone volume by 27% ($P<0.01$) compared to ligature placement group (Fig. 11B).

Histological analyses showed that ligature placement for one week resulted in significant alveolar bone loss compared to no ligature placement. Also, RvE1 local application with ligature placement

resulted in a significantly less alveolar bone loss compared to ligature placement with vehicle. Measurements were made to assess ligature-induced alveolar bone loss using an inverted microscope (Zeiss Axiovert 200, Zeiss, Thornwood, NY, USA) with a video camera in two areas and calculated in micrometers. Measurements were blindly through measuring the amount of remaining bone in the second molar area bound by the surface of tooth root and a line drawn between the tips of the two roots of the second molar (Fig. 12A). Ligature placement significantly decreased second molar furcation area by 89% ($P<0.01$) compared to no ligature placement and RvE1 local application significantly induced less second molar area bone loss with more bone area by 4-folds ($P<0.05$) compared to ligature placement group (Fig. 12B).

3.1.1. RvE1 reduced number of osteoclasts in ligature-induced periodontal disease model in Mice.

Histological analyses showed that ligature placement for one week resulted in significant increase in TRAP-stained multinucleated osteoclast cell count compared to no ligature placement. Also, RvE1 local application with ligature placement resulted in a significant decrease in TRAP-stained multinucleated osteoclast cell count compared to ligature placement with vehicle. Measurements of osteoclastogenesis were made by tartrate-resistant acid phosphatase (TRAP) staining in two areas following protocol described by Herrera et al (2008). TRAP+ multinuclear cells containing 3 nuclei or more were counted using 10X and 40X objectives in digital images of the histological sections that were taken using an inverted microscope (Zeiss Axiovert 200, Zeiss, Thornwood, NY, USA) with a video camera (Fig. 13A)

Measurements were made through counting the number of TRAP-stained multinucleated osteoclast in the second molar furcation area using 10X objectives field of view. Ligature placement significantly increased number of TRAP-stained multinucleated osteoclast by 6-folds ($P<0.01$) compared to no ligature placement and RvE1 local application significantly reduced number of TRAP-stained multinucleated osteoclast by 48% ($P<0.05$) compared to ligature placement group (Fig. 13B). A Second set of measurements was made to evaluate distribution and size of osteoclasts in the second molar furcation area. Osteoclasts distribution was evaluated by dividing the furcation area first into three parts bounded apically by a line drawn between the tips of the mesial and distal roots and cervically by a parallel line touching the highest point at the furcation. Then, the number of TRAP-stained multinucleated osteoclasts were counted at each area. Osteoclasts size was evaluated by classifying them first into three groups based on number of nuclei: (1&2) nuclei, (3-5) nuclei and (more than 5) nuclei groups. Then, the number of TRAP-stained osteoclasts were counted at based on the number of nuclei (Fig. 13A).

At the cervical third, ligature placement significantly increased number of TRAP-stained osteoclast (1&2) nuclei group by 113% ($P<0.01$) compared to no ligature placement and RvE1 local application significantly reduced number of TRAP-stained osteoclasts in the (1&2) nuclei group by 39% ($P<0.01$) compared to ligature placement group. Also, RvE1 local application significantly reduced number of TRAP-stained osteoclasts in the (3-5) nuclei group by 47% ($P<0.01$) compared to ligature placement group. However, ligature placement triggered no increase in number of TRAP-stained osteoclasts in the (3-5) nuclei group when compared to no ligature placement. Moreover, ligature placement significantly increased number of TRAP-stained osteoclasts in the (>5) nuclei group by 1.5-folds ($P<0.01$) compared to no ligature placement and

RvE1 local application significantly reduced number of TRAP-stained osteoclasts in the (>5) nuclei group by 38% (P<0.01) compared to ligature placement group (Fig. 13C).

At the middle third, ligature placement significantly increased number of TRAP-stained osteoclast (1&2) nuclei group by 3-folds (P<0.01) compared to no ligature placement and RvE1 local application significantly reduced number of TRAP-stained osteoclasts in the (1&2) nuclei group by 61% (P<0.01) compared to ligature placement group. Also, ligature placement significantly increased number of TRAP-stained osteoclasts in the (3-5) nuclei group by 102% (P<0.01) compared to no ligature placement and RvE1 local application significantly reduced number of TRAP-stained osteoclasts in the (3-5) nuclei group by 68% (P<0.01) compared to ligature placement group. Moreover, ligature placement significantly increased number of TRAP-stained osteoclasts in the (>5) nuclei group by 2.5-folds (P<0.01) compared to no ligature placement and RvE1 local application significantly reduced number of TRAP-stained osteoclasts in the (>5) nuclei group by 52% (P<0.01) compared to ligature placement group (Fig. 13C).

At the apical third, ligature placement significantly increased number of TRAP-stained osteoclast (1&2) nuclei group by 6-folds (P<0.01) compared to no ligature placement and RvE1 local application significantly reduced number of TRAP-stained osteoclasts in the (1&2) nuclei group by 62% (P<0.01) compared to ligature placement group. Also, ligature placement significantly increased number of TRAP-stained osteoclasts in the (3-5) nuclei group by 2.5-folds (P<0.01) compared to no ligature placement and RvE1 local application significantly reduced number of TRAP-stained osteoclasts in the (3-5) nuclei group by 29% (P<0.01) compared to ligature placement group. Moreover, ligature placement significantly increased number of TRAP-stained osteoclasts in the (>5) nuclei group by 10-folds (P<0.01) compared to no ligature placement and

RvE1 local application significantly reduced number of TRAP-stained osteoclasts in the (>5) nuclei group by 66% ($P<0.01$) compared to ligature placement group (Fig. 13C).

Figure 9. Experimental design of the *in vivo* ligature-induced periodontal disease model.

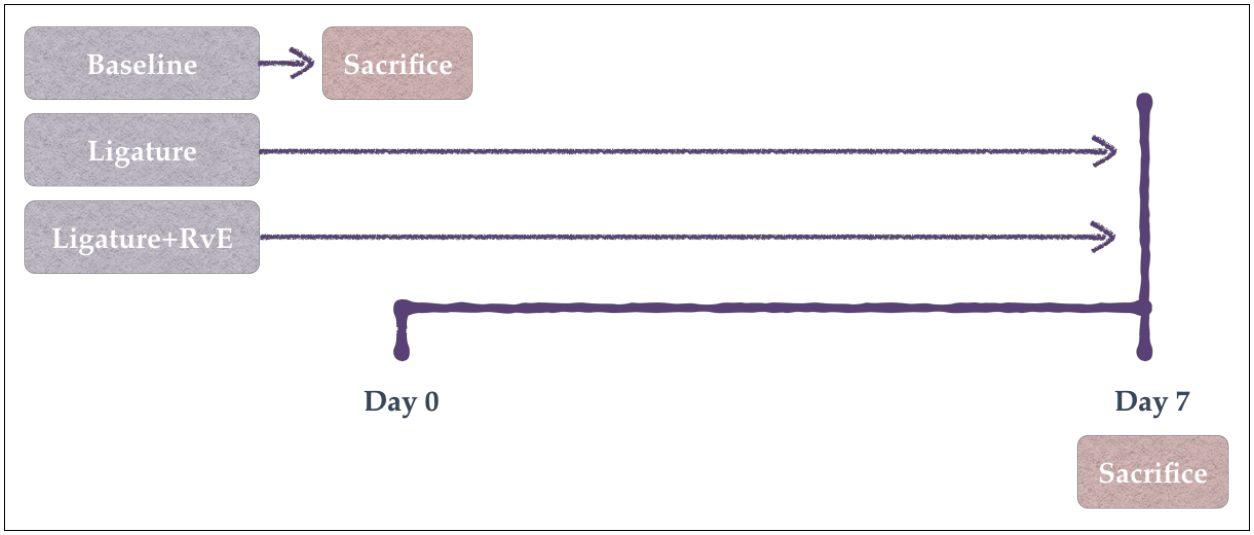


Figure 9. Experimental design of the *in vivo* ligature-induced periodontal disease model. Twenty-four 8-weeks-old FVB wild-type (WT) mice were used in the ligature-induced experimental periodontal disease model. Animals were divided into three groups: baseline group that received no ligature placement nor RvE1 application, ligature group that received ligature placement only, and ligature+RvE1 that received ligature placement with RvE1 application. Alveolar bone-loss was induced using a 7-0 silk suture that was placed into the gingival sulcus around the second molar of both maxillary quadrants with the knot placed toward the palatal side. The ligature placement and RvE1 treatment were delivered for one week and then animals were sacrificed. Animals received topical treatment with 1 μM RvE1 or vehicle based on their group assignment daily for 7 days under inhalation anesthesia. After sacrificing the animals, maxillae were split into right and left halves. For each mouse, the right half was fixed in 10% formalin for histological examination while the left half was defleshed and cleaned for morphometric analysis.

Figure 10. Morphometric analyses showed RvE1 local application significantly reduced alveolar bone loss compared ligature placement with vehicle.

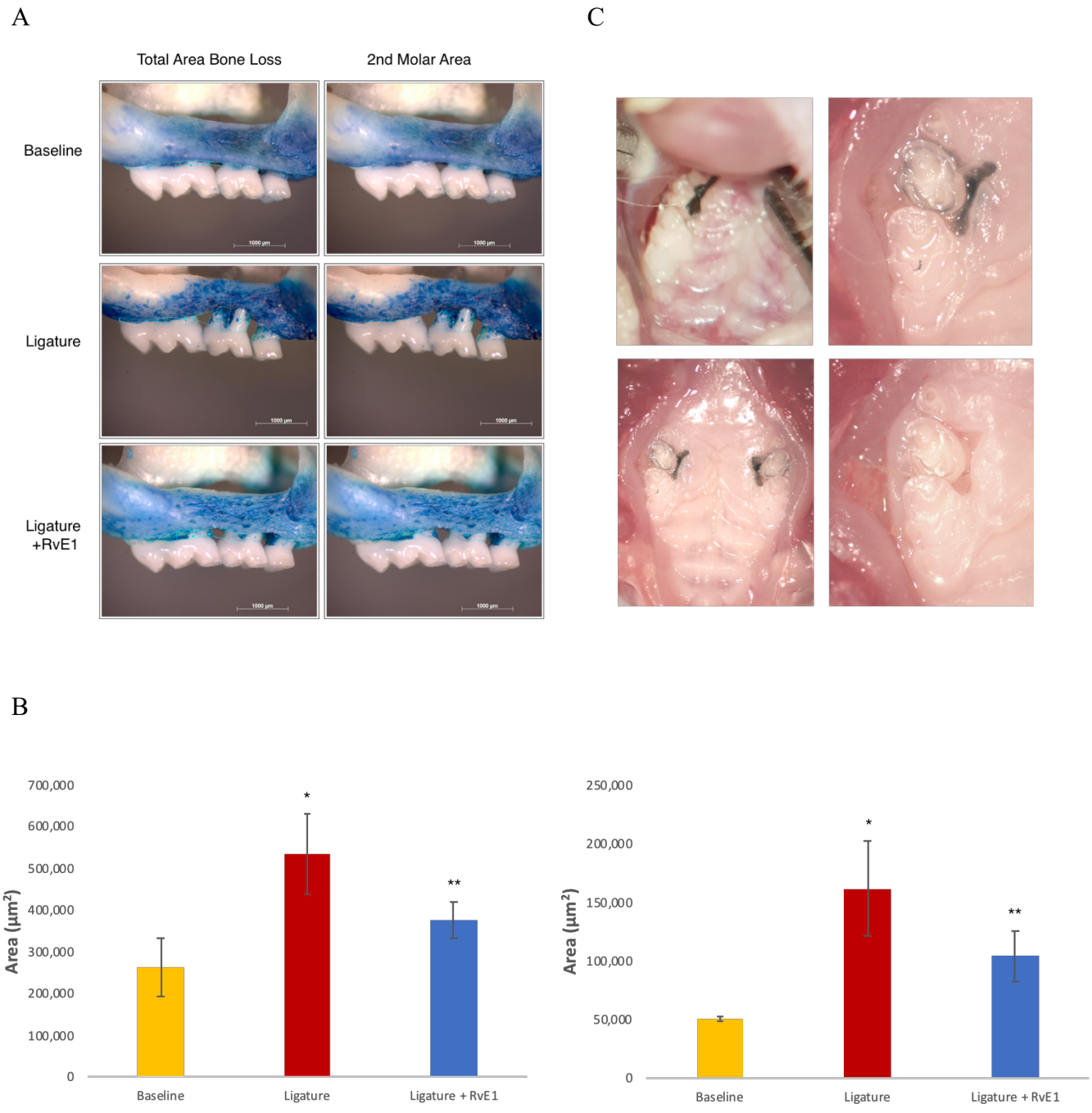


Figure 10. Morphometric analyses showed RvE1 local application significantly reduced alveolar bone loss compared ligature placement with vehicle. Measurements were made to assess ligature-induced alveolar bone loss using ImageJ software in two areas and calculated in micrometers. **(A, left side)** The first measurement made was the total alveolar area bone loss bound by the alveolar bone crest level, the cementoenamel junction of teeth, the mesial surface of the first molar, and the distal surface of the second molar. **(B, left side)** Ligature placement significantly increased total alveolar area bone loss by 103% ($P<0.01$) compared to no ligature placement and RvE1 local application significantly decreased total alveolar area bone loss by 29% ($P<0.05$) compared to ligature placement group. **(A, right side)** The second measurements made were to evaluate the second molar furcation area bone loss bound by the alveolar bone crest level and cementoenamel junction of the second molar. **(B, right side)** Ligature placement significantly increased second molar furcation area bone loss by 102% ($P<0.01$) compared to no ligature placement and RvE1 local application significantly decreased second molar furcation area bone loss by 68% ($P<0.05$) compared to ligature placement group. **(C)** Photos of the ligature (7-0 silk suture) that was placed into the gingival sulcus around the second molar of both maxillary quadrants with the knot placed toward the palatal side.

(Mean $\pm$ SD, n=24, ANOVA, *, ** $P<0.05$).

* statistically significant compared to the baseline group.

** statistically significant compared to the ligature group.

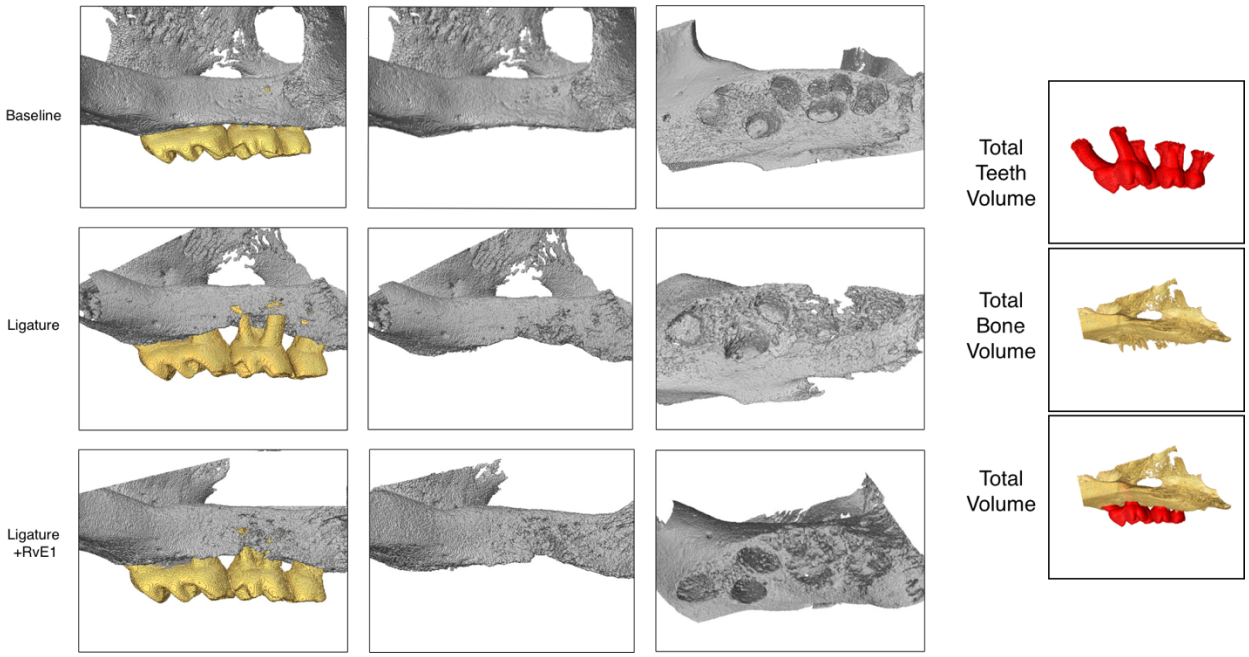
Baseline: WT mice with no ligature placement nor RvE1 treatment.

Ligature: WT mice with ligature placement for 7 days but without RvE1 treatment.

Ligature: WT mice with ligature placement and RvE1 treatment (1 μg/tooth) for 7 days.

Figure 11. Micro-CT analyses revealed RvE1 local application significantly reduced alveolar bone loss compared ligature placement with vehicle.

A



B

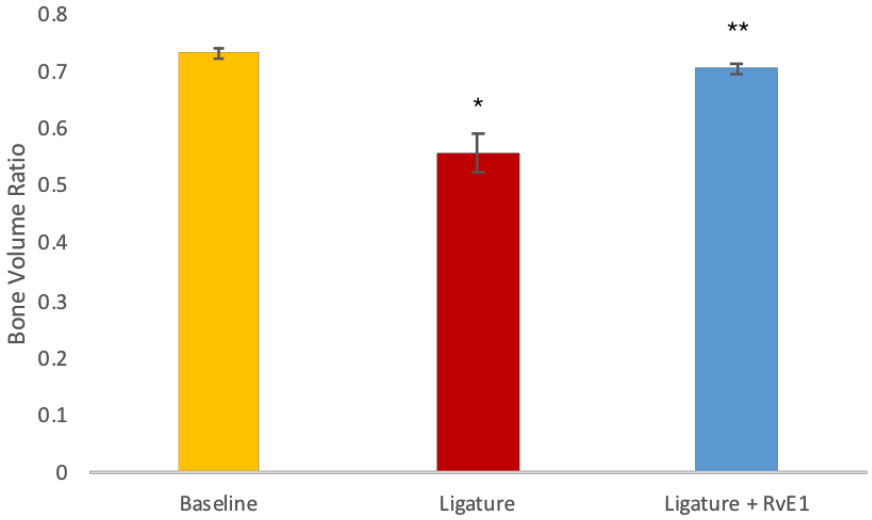


Figure 11. Micro-CT analyses revealed RvE1 local application significantly reduced alveolar bone loss compared ligature placement with vehicle. (A) Micro-CT measurements were made to assess ligature-induced alveolar bone loss through calculating molar teeth volumes first. Then, total alveolar bone volume was calculated followed by calculating the total volume of the 3-D image of the sample outside the teeth and bone so it can be subtracted. The amount of remaining alveolar bone volume was divided by the remaining total volume of the 3-D image (Teeth and bone volume) to get the bone volume for each sample. **(B)** Ligature placement significantly decreased second molar alveolar bone volume by 24% ($P<0.01$) compared to no ligature placement and RvE1 local application significantly decreased second molar furcation area bone loss with more bone volume by 27% ($P<0.01$) compared to ligature placement group.

(Mean $\pm$ SD, n=24, ANOVA, *, ** $P<0.05$).

* statistically significant compared to the baseline group.

** statistically significant compared to the ligature group.

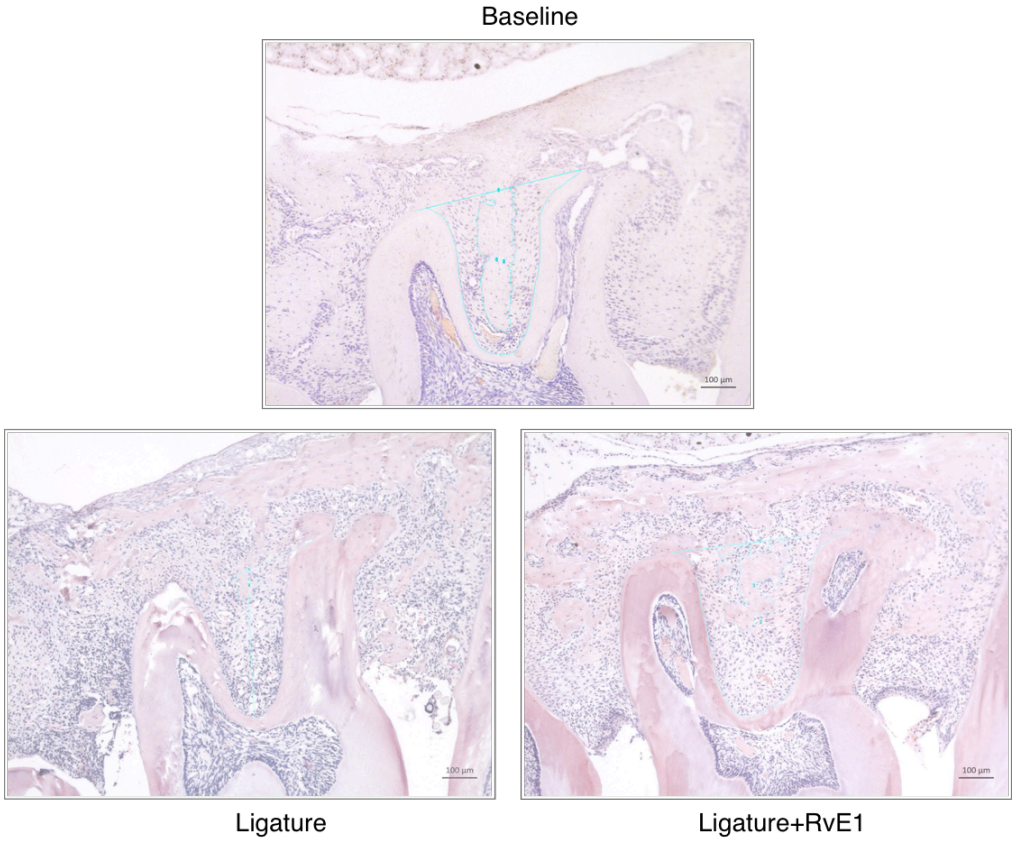
Baseline: WT mice with no ligature placement nor RvE1 treatment.

Ligature: WT mice with ligature placement for 7 days but without RvE1 treatment.

Ligature: WT mice with ligature placement and RvE1 treatment (1 μg/tooth) for 7 days.

Figure 12. Histological analyses showed RvE1 local application significantly reduced alveolar bone loss compared ligature placement with vehicle.

A



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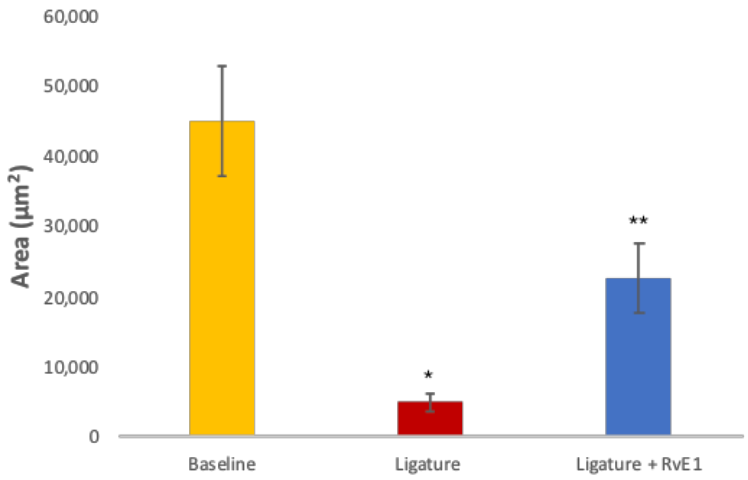


Figure 12. Histological analyses showed RvE1 local application significantly reduced alveolar bone loss compared ligature placement with vehicle. (A) Measurements were blindly performed through calculating the amount of remaining bone in the second molar area bound by the surface of tooth root and a line drawn between the tips of the two roots of the second molar. **(B)** Ligature placement significantly decreased second molar furcation area by 89% ($P<0.01$) compared to no ligature placement and RvE1 local application significantly induced less second molar area bone loss with more bone area by 4-folds ($P<0.05$) compared to ligature placement group.

(Mean $\pm$ SD, n=24, ANOVA, *, ** $P<0.05$).

* statistically significant compared to the baseline group.

** statistically significant compared to the ligature group.

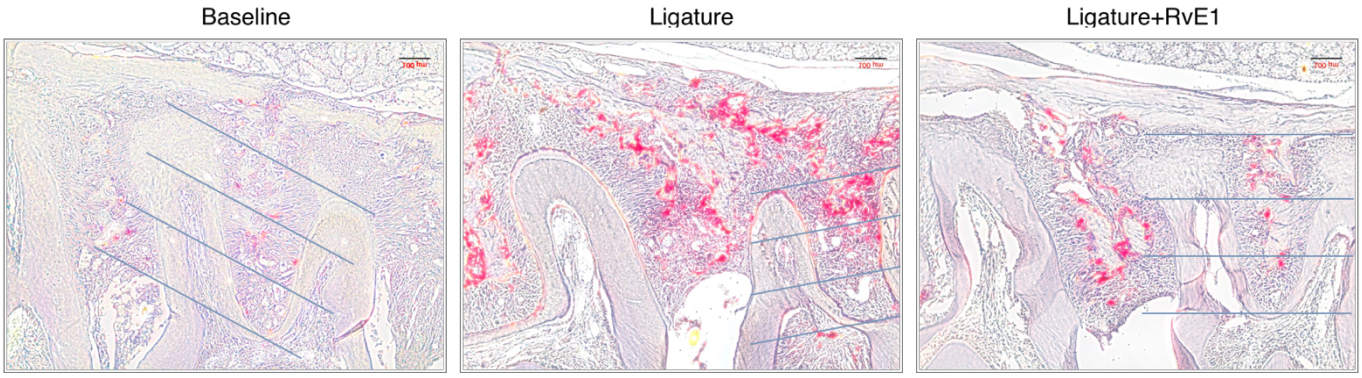
Baseline: WT mice with no ligature placement nor RvE1 treatment.

Ligature: WT mice with ligature placement for 7 days but without RvE1 treatment.

Ligature: WT mice with ligature placement and RvE1 treatment (1 μg/tooth) for 7 days.

Figure 13. Histological analyses showed RvE1 local application significantly decreased number of TRAP-stained multinucleated osteoclast cell count compared to ligature placement with vehicle.

A



B

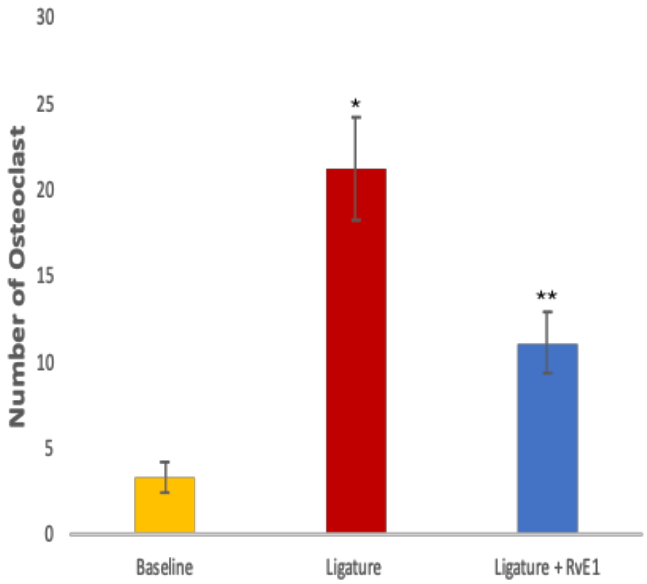


Figure 13. Histological analyses showed RvE1 local application significantly decreased number of TRAP-stained multinucleated osteoclast cell count compared to ligature placement with vehicle.

C

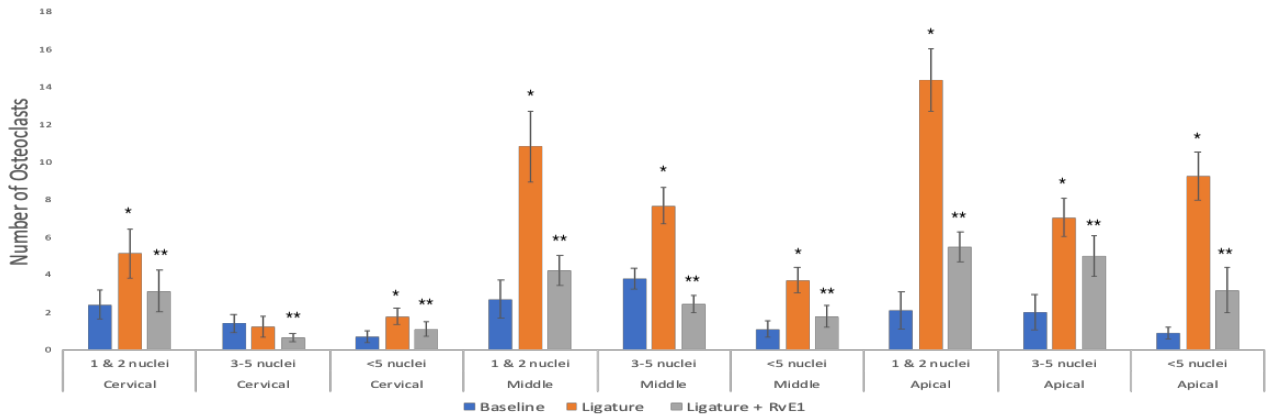


Figure 13. Histological analyses showed RvE1 local application significantly decreased number of TRAP-stained multinucleated osteoclast cell count compared to ligature placement with vehicle. (A) Measurements were made through counting the number of TRAP+ multinuclear cells containing 3 nuclei or more in the second molar furcation area using 10X objectives field of view. A Second set of measurements was made to evaluate distribution and size of osteoclasts in the second molar furcation area. Osteoclasts distribution was evaluated by dividing the furcation area first into three parts bounded apically by a line drawn between the tips of the mesial and distal roots and cervically by a parallel line touching the highest point at the furcation. Then, the number of TRAP-stained multinucleated osteoclasts were counted at each area. Osteoclasts size was evaluated by classifying them first into three groups based on number of nuclei: (1&2) nuclei, (3-5) nuclei and (more than 5) nuclei groups. Then, the number of TRAP-stained osteoclasts were counted at based on the number of nuclei. **(B)** Ligature placement significantly increased number of TRAP-stained multinucleated osteoclast by 6-folds ($P<0.01$) compared to no ligature placement and RvE1 local application significantly reduced number of TRAP-stained multinucleated osteoclast by 48% ($P<0.05$) compared to ligature placement group**. (C)** There is a significant increase in the number of TRAP-stained multinucleated osteoclast (3 and more) in the middle and apical thirds after ligature placement. Also, RvE1 significantly reduced number of TRAP-stained multinucleated osteoclast in all thirds.

(Mean $\pm$ SD, n=24, ANOVA, *, ** $P<0.05$).

* statistically significant compared to the baseline group.

** statistically significant compared to the ligature group.

Baseline: WT mice with no ligature placement nor RvE1 treatment.

Ligature: WT mice with ligature placement for 7 days but without RvE1 treatment.

Ligature: WT mice with ligature placement and RvE1 treatment (1 μg/tooth) for 7 days.

3.2. Specific Aim 2: Examine RvE1 inhibition of osteoclast precursor and osteoclast differentiation through the BLT1 receptor in bone marrow-derived cells.

Rationale: RvE1 bone protective actions during inflammation are complex, intricate and involve regulation of PMN, macrophages and several other cells. Recent studies from our group have shown that RvE1 has direct actions through the BLT1 receptor on osteoclasts leading to inhibition of osteoclast fusion and favoring bone preservation (Zhu et al., 2013). However, there are limited resources that have investigated RvE1 regulatory actions through the BLT1 receptor on osteoclast precursor differentiation, as well as information regarding other pro-resolving mediators' direct actions on osteoclast precursors and osteoclasts. We **hypothesize** that resolvin RvE1 has direct impact on osteoclast precursors and osteoclasts through preventing cell differentiation.

Experimental approach: Different osteoclast precursor and osteoclast cell cultures were generated from bone marrow of 8- to 10-week-old mice for the different aims. Based on previous studies, the concentrations of RvE1 used were 1, 10 and 100 nM (Zhu et al., 2013). The control groups were identical culture medium supplemented with the corresponding amount of ethanol (vehicle). The time of RvE1 induction plays a critical role in detecting its hypothesized actions on osteoclast precursors and osteoclasts. Bone Marrow Derived macrophage cultures were exposed to M-CSF (50 ngm/L1) in 6-well plates for two days until 75% confluency is reached then cells were passed to 24-well plates ($5x10^5$ cells/well) for qRT-PCR experiments, to 6-well plates (10^6 cells/well) for western blot, TRAP staining and activity assay.

Osteoclast precursor cultures were exposed to M-CSF (50 ngm/L1) at day 1 to induce osteoclast precursor differentiation and medium was replaced every day. RvE1 was delivered to the culture

at two different time points depending on the group; RvE1(1&2) group, where the time of delivery of RvE1 is at days 1 and 2 and RvE1(3&4) group, where the time of delivery of RvE1 is at days 3 and 4. RNA was isolated from test and control tissue cultures at day 5. RvE1 regulatory actions on osteoclast precursor differentiation were investigated by detecting the expression of RANK, cluster of differentiation 11b (CD11b) and c-fms (Fig. 14A).

Osteoclast cultures were exposed to M-CSF (50 ngm/L1) at day 1 to induce osteoclast precursor differentiation and medium was replaced every day. RANKL (30 ngm/L1) was introduced to cultures at day 3 to induce osteoclast differentiation and medium was replaced every day. RvE1 was delivered to the culture at three different time points depending on the group; RvE1(1&2) group, where the time of delivery of RvE1 is at days 1 and 2 to investigate the RvE1 regulatory actions on osteoclast differentiation when cultures exposed to RvE1 at osteoclast precursor phase. RvE1 (3&4) group was induced by RvE1 at days 3 and 4 and RvE1 (5&6) group was induced by RvE1 at days 5 and 6. RNA was isolated from test and control tissue cultures at day 7. RvE1 regulatory actions on osteoclast differentiation were investigated by detecting the expression of TRACP, cathepsin K and Calcitonin R. Also, TRAP staining and activity assay was performed for test and control tissue cultures at day 7 and whole-cell protein was extracted 15 minutes after RvE1 induction at day 7 for western blot analysis. (Fig 14B).

Measurements of osteoclast differentiation were made by tartrate-resistant acid phosphatase (TRAP) staining following protocol described by Herrera et al (2008). TRAP+ multinuclear cells containing 3 nuclei, and more were counted in osteoclast cultures (made as previously described) at day 7 for all three groups; RvE1 (1&2), RvE1 (3&4) and RvE1 (5&6) using three RvE1 different doses (1nM, 10nM, 100nM). Furthermore, TRAP activity assay measurements were performed in 96-well plates and read at day 7 for all three groups.

Right side hemimaxilla, Femur and tibia bones of WT mice were harvested from 8-week-old mice to investigate BLT1 receptor expression on the cell surface using anti-BLT1 staining (Thermo Scientific) and 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) for nuclei labeling.

U75302, a BLT1 inhibitor, was used in the osteoclast cultures as previously described with and without RvE1 induction two days before TRAP staining to investigate BLT1 as the cell surface receptor that interacts with RvE1 to negatively regulate osteoclast differentiation. Also, Western blot analysis was performed to validate BLT1 inhibition. U75302 and RvE1 were delivered into the cultures at day 7. U75302 delivered 1 hour prior to RvE1 followed by whole-cell protein extraction 15 minutes after using CelLytic M solution (Sigma-Aldrich; Merck KGaA) with protease inhibitors for Western blot analysis.

3.2.1. RvE1 down-regulated gene expression of osteoclast differentiation markers but not osteoclast precursors.

In osteoclast precursor cell cultures, mRNA expression of RANK, CD11b and c-fms genes using qRT-PCR were investigated. RvE1 exposure did not affect the expression of c-fms and RANK genes in RvE1 (1&2) and RvE1 (1&2) groups compared to control. However, RvE1 significantly increased the expression of CD11b gene in the RvE1 (1&2) group ($P<0.05$) and in the RvE1 (3&4) group ($P<0.05$) compared to control. (Fig. 15A, B & C).

In osteoclast cell cultures, mRNA expression of TRACP, cathepsin K and Calcitonin R genes using qR-PCR was investigated. RvE1 exposure did not affect the expression of the TRACP gene in the RvE1 (1&2) group, but significantly decreased gene expression in the RvE1 (3&4) group ($P<0.01$) and the RvE1 (5&6) group ($P<0.05$) compared to control. Also, RvE1 significantly decreased the

expression of the cathepsin K gene in the RvE1 (1&2) group ($P<0.05$), in the RvE1 (3&4) group ($P<0.01$) and in the RvE1 (5&6) group ($P<0.01$) compared to control. In addition, RvE1 did not affect the expression of Calcitonin R gene in the RvE1 (1&2) group, but significantly decreased gene expression in the RvE1 (3&4) group ($P<0.01$) and in the RvE1 (5&6) group ($P<0.01$) compared to control (Fig. 15D, E & F).

3.2.2. RvE1 hindered osteoclasts differentiation in a dose-dependent manner.

Counting the number of TRAP-stained multinucleated osteoclasts was performed using 20X objective field of view images. In the RvE1 (1&2) group, RvE1 significantly decreased the number of TRAP-stained multinucleated osteoclasts at 1 nM concentration by 44% ($P<0.01$), at 10nM concentration by 76% ($P<0.01$) and at 100nM concentration by 84% ($P<0.01$) compared to control. Also, RvE1 significantly decreased the number of TRAP-stained multinucleated osteoclasts at the 100nM concentration compared to 10nM concentration ($P<0.01$) and the 10nM concentration compared to 1nM concentration ($P<0.01$). In the RvE1 (3&4) group, RvE1 significantly decreased the number of TRAP-stained multinucleated osteoclasts at 1nM concentration by 60% ($P<0.01$), at 10nM concentration by 71% ($P<0.01$) and at 100nM concentration by 79% ($P<0.01$) compared to control. Also, RvE1 significantly decreased the number of TRAP-stained multinucleated osteoclasts at the 100nM concentration compared to 10nM and 1nM concentrations ($P<0.05$). In the RvE1 (5&6) group, RvE1 significantly decreased the number of TRAP-stained multinucleated osteoclasts at 1nM concentration by 67% ($P<0.01$), at 10nM concentration by 87% ($P<0.01$) and at 100nM concentration by 84% ($P<0.01$) compared to control. Also, RvE1 significantly decreased the number of TRAP-stained multinucleated osteoclasts at the 10nM concentration compared to 1nM concentration ($P<0.01$) (Fig. 16A, B & C).

In addition, the TRAP activity assay showed that RvE1 significantly decreased TRAP concentration levels in osteoclast cell cultures in the RvE1 (1&2) group by 57% ($P<0.01$), in the RvE1 (3&4) group by 68% ($P<0.01$), and in the RvE1 (5&6) group by 83% ($P<0.01$) compared to control (Fig. 16D).

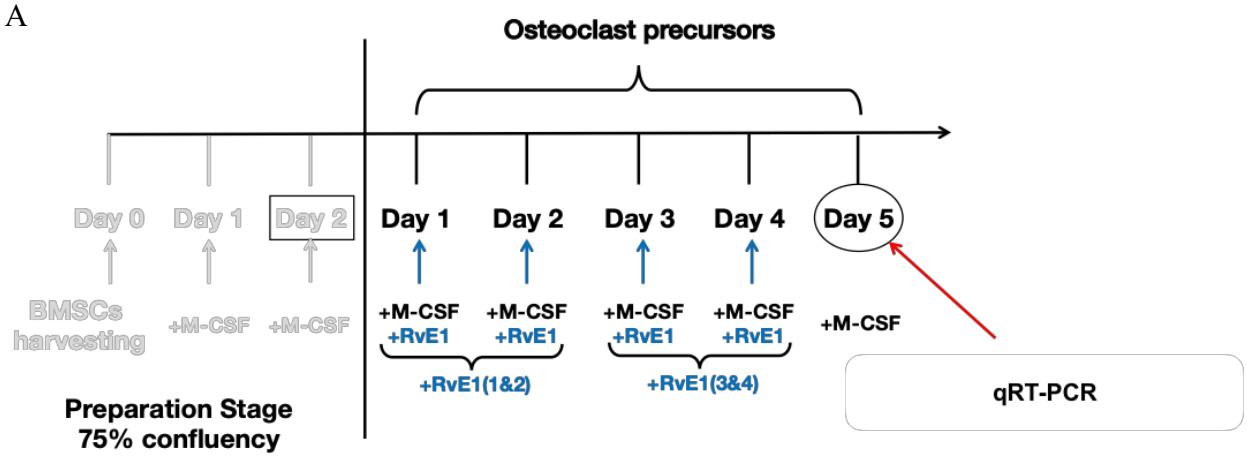
3.2.3. RvE1 reduced osteoclasts differentiation through BLT1 receptor.

Immunohistochemistry analyses performed on right side hemimaxilla, femur and tibia bones of mice using anti-BLT1 staining (Thermo Scientific) showed BLT1 receptor expression on the cell surface of mouse alveolar bone osteoclasts and bone marrow-derived osteoclast precursors and osteoclasts (Fig. 17A & B).

U75302-osteoclast cultures and U75302/RvE1-osteoclast cultures did not show any decrease in the number of TRAP-stained multinucleated osteoclasts compared to control-osteoclast cultures. Also, western blot analysis showed that U75302 reduced levels of BLT1 protein by 47% ($P<0.01$) compared to no U75302-osteoclast cultures (Fig. 17C & D).

Figure 14. Experimental design of qRT-PCR, Western blot, TRAP staining and activity assay.

A



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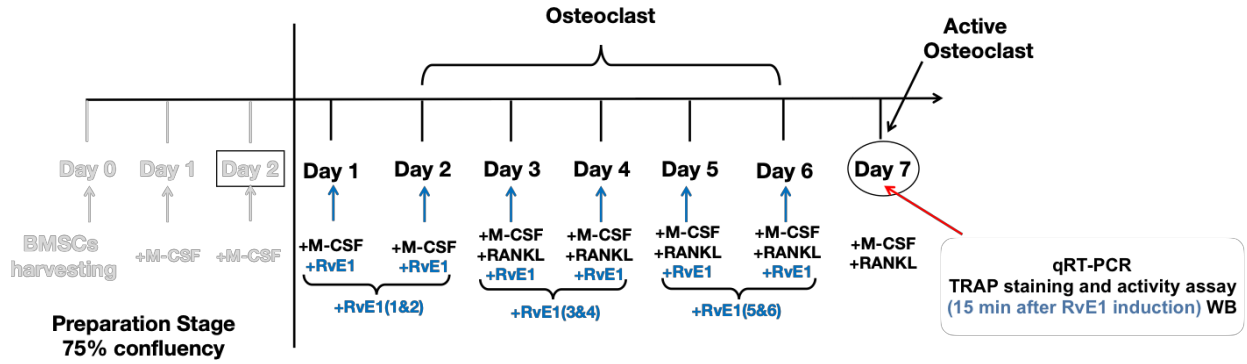
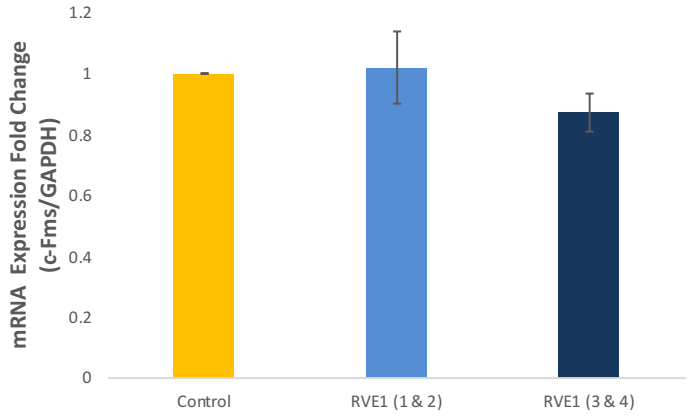


Figure 14. Experimental design of qRT-PCR, western blot, TRAP staining and activity assay. Different osteoclast precursor and osteoclast cell cultures were generated from bone marrow of 8- to 10-week-old mice for the different aims. Bone Marrow Derived macrophages cultures were exposed to M-CSF (50 ngm/L1) in 6-well plates for two days until 75% confluency is reached then cells were passed to 24-well plates ($5x10^5$ cells/well) for qRT-PCR experiments, to 6-well plates (10^6 cells/well) for western blot, TRAP staining and activity assay. **(A)** Osteoclast precursor cultures were exposed to M-CSF (50 ngm/L1) at day 1 to induce osteoclast precursor differentiation and were replaced every day. RvE1 were delivered to the culture at two different time sets depending on the group; RvE1(1&2) group, where the time of delivery of RvE1 is at days 1 and 2 and RvE1(3&4) group, where the time of delivery of RvE1 is at days 3 and 4. RNA was isolated from test and control tissue cultures at day 5. **(B)** Osteoclast cultures were exposed to M-CSF (50 ngm/L1) at day 1 to induce osteoclast precursor differentiation and were replaced every day. RANKL (30 ngm/L1) introduced to cultures at day 3 to induce osteoclast differentiation and were replaced every day. RvE1 were delivered to the culture at three different time sets depending on the group; RvE1(1&2) group, where the time of delivery of RvE1 is at days 1 and 2 to investigate the RvE1 regulatory actions on osteoclast differentiation when cultures exposed to RvE1 at osteoclast precursor phase. RvE1(3&4) group was induced by RvE1 at days 3 and 4 and RvE1(5&6) group was induced by RvE1 at days 5 and 6. RNA was isolated and TRAP staining and activity assay were performed for test and control tissue cultures at day 7. Also, whole-cell proteins were extracted 15 minutes after RvE1 induction at day 7 for western blot analysis.

Figure 15. RvE1 down-regulated gene expression of osteoclast differentiation markers but not osteoclast precursors.

A



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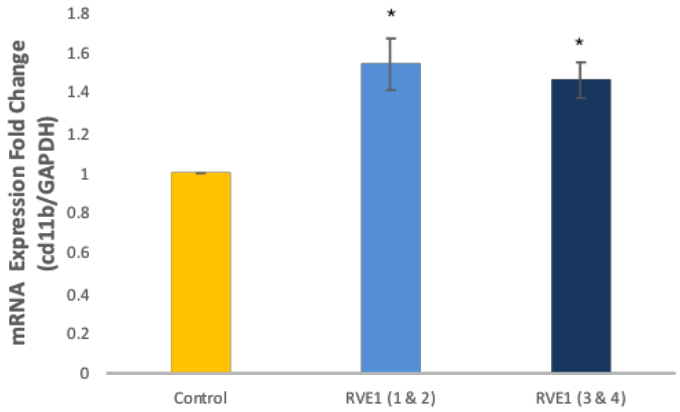

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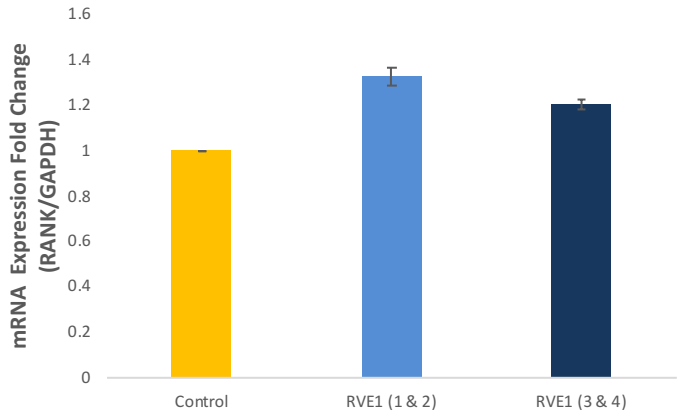
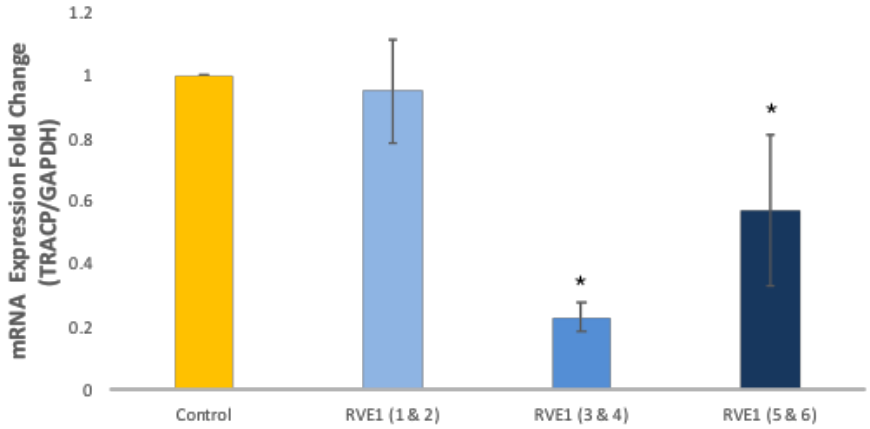


Figure 15. RvE1 down-regulated expression markers of osteoclast but not osteoclast precursors.

D



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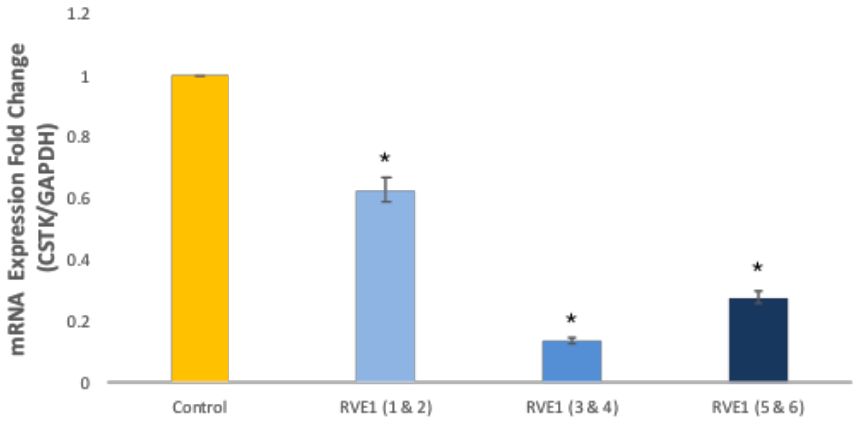

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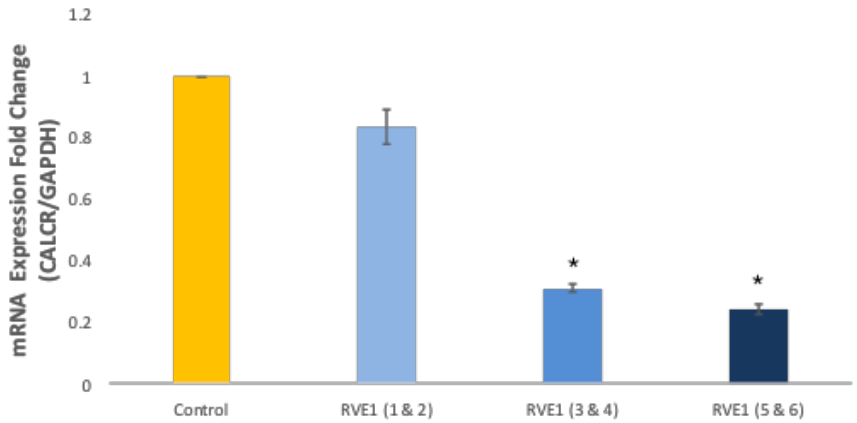


Figure 15. RvE1 down-regulated expression markers of osteoclast but not osteoclast precursors.

RvE1 exposure did not affect the expression of **(A)** c-fms and **(C)** RANK genes at RvE1(1&2) and RvE1(1&2) groups compared to control. However, RvE1 significantly increased the expression of **(B)** cd11b gene in RvE1(1&2) group ($P<0.05$) and in RvE1(3&4) group ($P<0.05$) compared to control. Also, RvE1 exposure did not affect the expression of **(D)** TRACP gene at RvE1(1&2) group, but significantly decreased gene expression at RvE1(3&4) group ($P<0.01$) and at RvE1(5&6) group ($P<0.05$) compared to control. Moreover, RvE1 significantly decreased the expression of **(E)** cathepsin K gene in RvE1(1&2) group ($P<0.05$), in RvE1(3&4) group ($P<0.01$) and in RvE1(5&6) group ($P<0.01$) compared to control. In addition, RvE1 did not affect the expression of **(F)** Calcitonin R gene in RvE1(1&2) group, but significantly decreased gene expression in RvE1(3&4) group ($P<0.01$) and in RvE1(5&6) group ($P<0.01$) compared to control.

(Mean $\pm$ SD, ANOVA * $P< 0.05$). All comparisons were made to the control group.

RvE1 (1 & 2): +M-CSF (50 ngm/L1) at days 1-4. +RvE1(10nM) at days 1, and 2 only.

RvE1 (3 & 4): +M-CSF (50 ngm/L1) at days 1-4. +RvE1(10nM) at days 3, and 4 only.

RvE1 (1 & 2): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(10nM) at days 1, and 2 only.

RvE1 (3 & 4): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(10nM) at days 3, and 4 only.

RvE1 (5 & 6): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(10nM) at days 5, and 6 only.

Figure 16. RvE1 hindered osteoclasts differentiation in a dose-dependent manner.

A

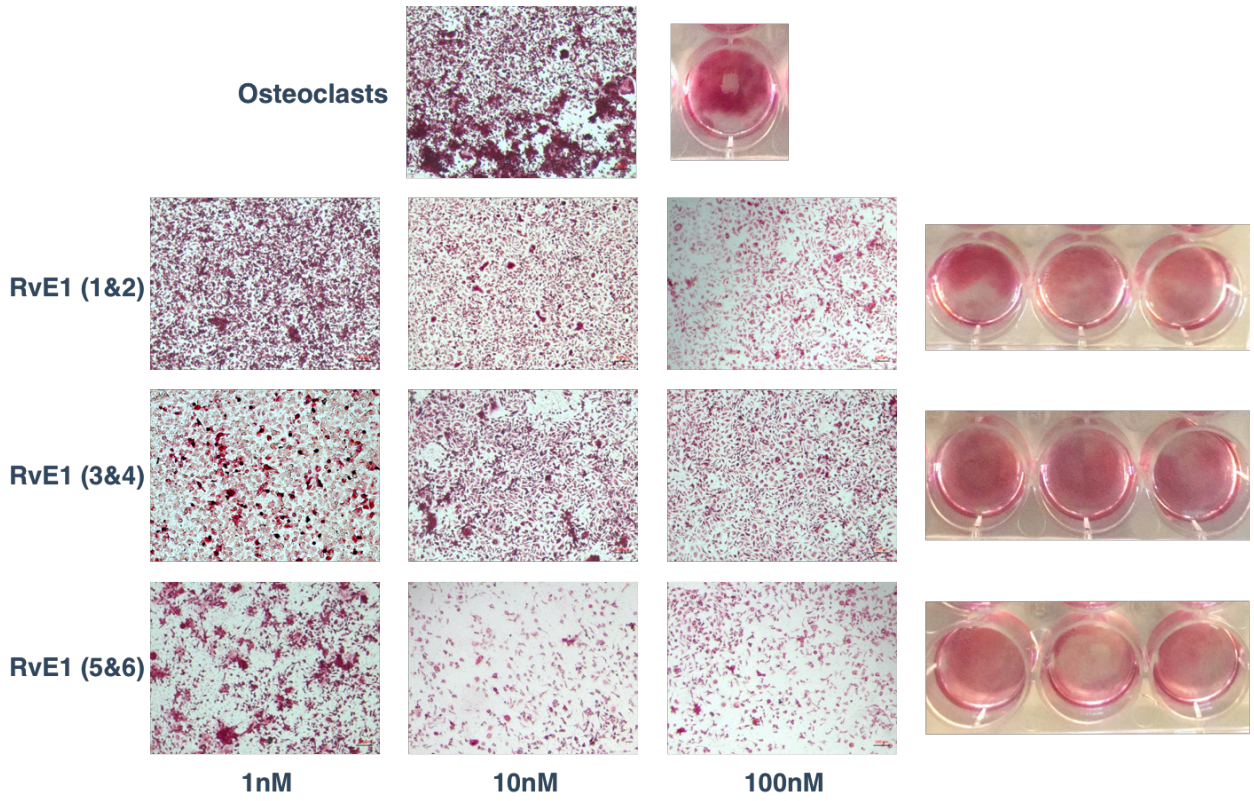
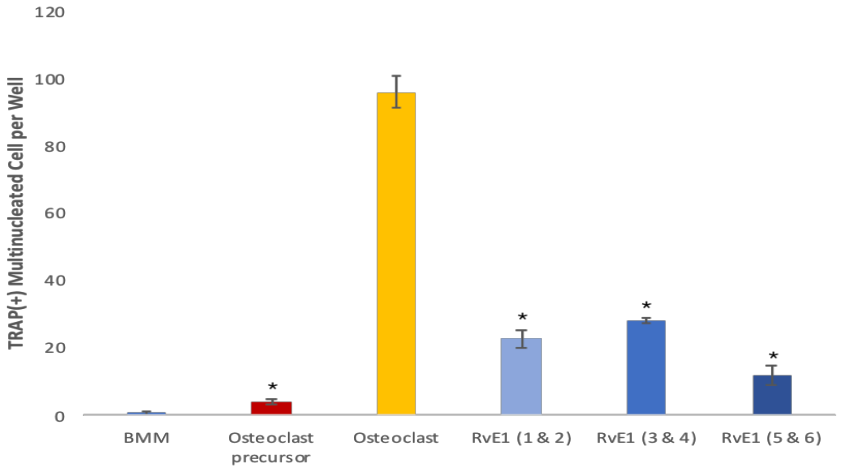


Figure 16. RvE1 hindered osteoclasts differentiation in a dose-dependent manner.

B



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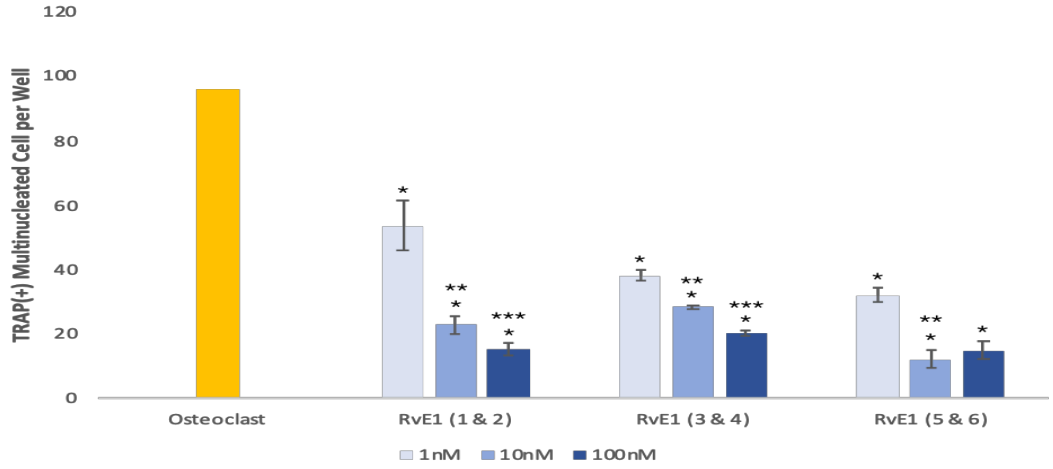

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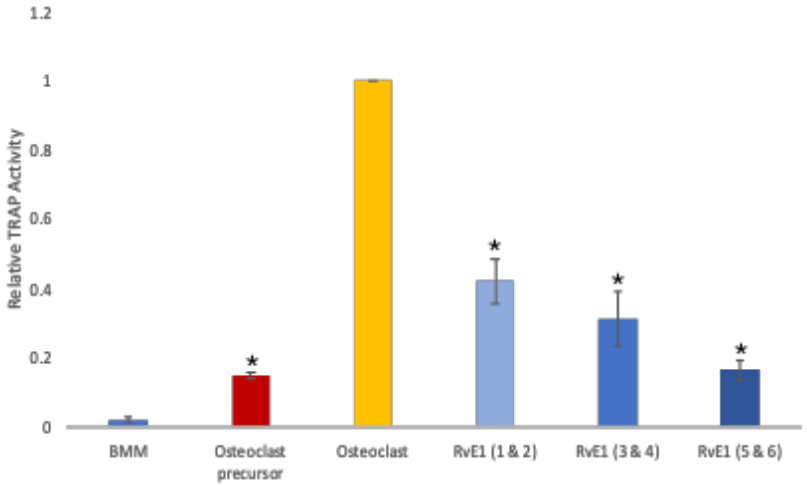


Figure 16. RvE1 hindered osteoclasts differentiation in a dose-dependent manner. (A) Counting the number of TRAP-stained multinucleated osteoclast were made using 20X objectives field of view images. **(B & C)** In RvE1(1&2) group, RvE1 significantly decreased number of TRAP-stained multinucleated osteoclast at 1nM concentration by 44% ($P<0.01$), at 10nM concentration by 76% ($P<0.01$) and at 100nM concentration by 84% ($P<0.01$) compared to control. In RvE1(3&4) group, RvE1 significantly decreased number of TRAP-stained multinucleated osteoclast at 1nM concentration by 60% ($P<0.01$), at 10nM concentration by 71% ($P<0.01$) and at 100nM concentration by 79% ($P<0.01$) compared to control. In RvE1(5&6) group, RvE1 significantly decreased number of TRAP-stained multinucleated osteoclast at 1nM concentration by 67% ($P<0.01$), at 10nM concentration by 87% ($P<0.01$) and at 100nM concentration by 84% ($P<0.01$) compared to control. **(C)** RvE1 significantly decreased number of TRAP-stained multinucleated osteoclasts in a dose dependent manner ($P<0.01$). **(D)** TRAP activity assay showed that RvE1 significantly decreased TRAP concentration levels in osteoclast cell cultures in RvE1(1&2) group by 57% ($P<0.01$), in RvE1(3&4) group by 68% ($P<0.01$), and in RvE1(5&6) group by 83% ($P<0.01$) compared to control.

(Mean $\pm$ SD, ANOVA *, **, *** $P< 0.05$). * comparisons were made to the osteoclasts. ** comparisons were made to 1nM RvE1. *** comparisons were made to 10nM RvE1

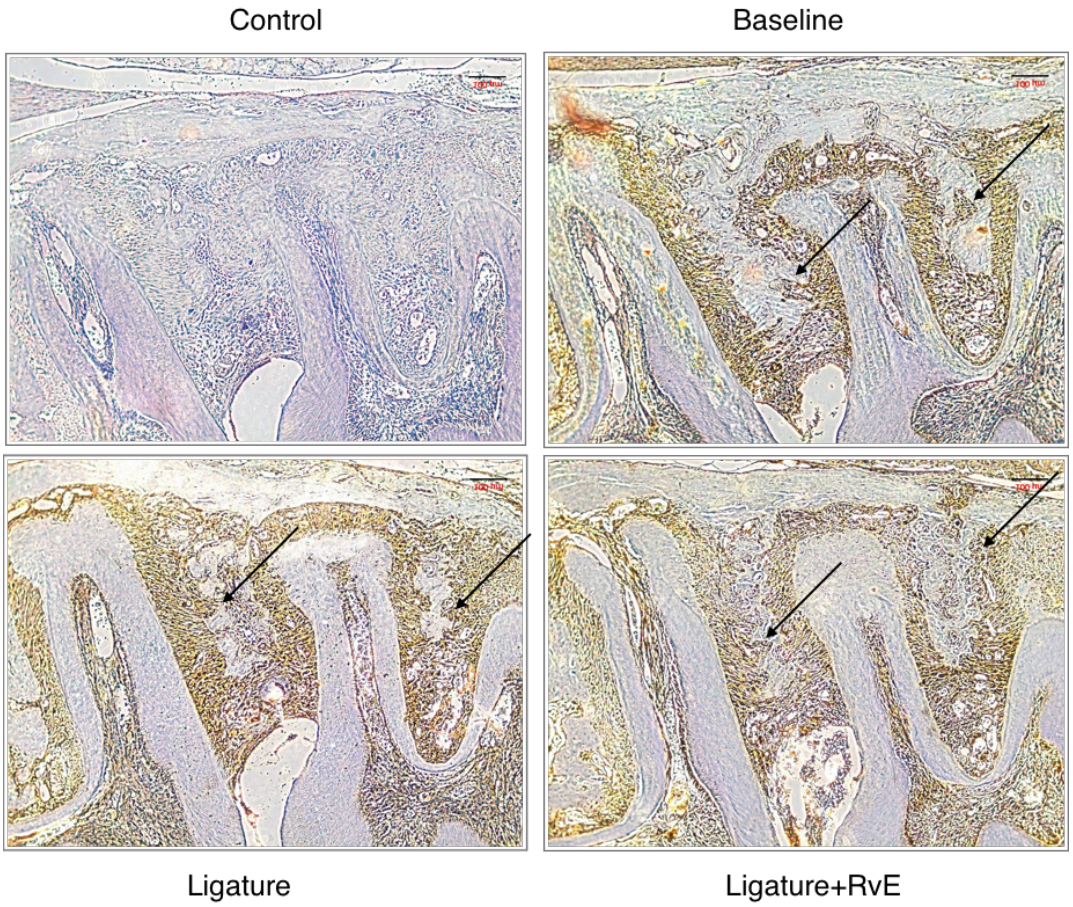
RvE1 (1 & 2): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(1nM, 10nM, 100nM) at days 1, and 2 only.

RvE1 (3 & 4): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(1nM, 10nM, 100nM) at days 3, and 4 only.

RvE1 (5 & 6): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(1nM, 10nM, 100nM) at days 5, and 6 only.

Figure 17. RvE1 reduced osteoclasts differentiation through BLT1 receptor.

A



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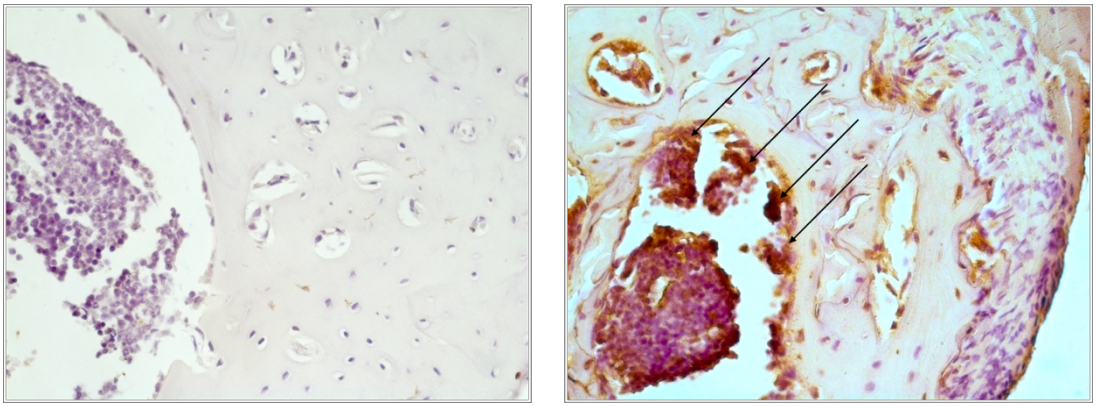
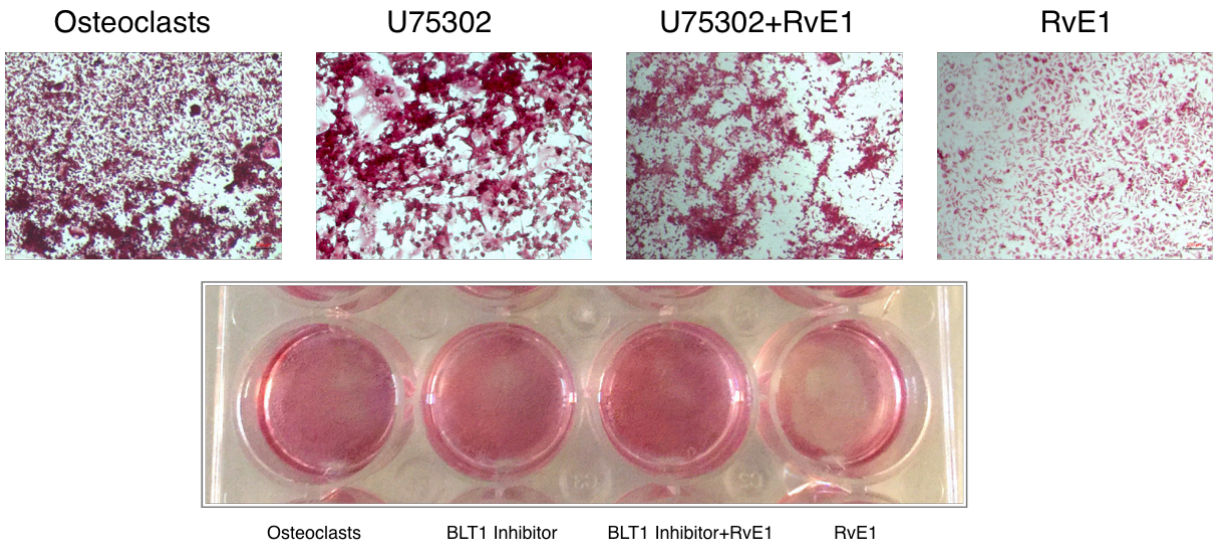


Figure 17. RvE1 reduced osteoclasts differentiation through BLT1 receptor.

C



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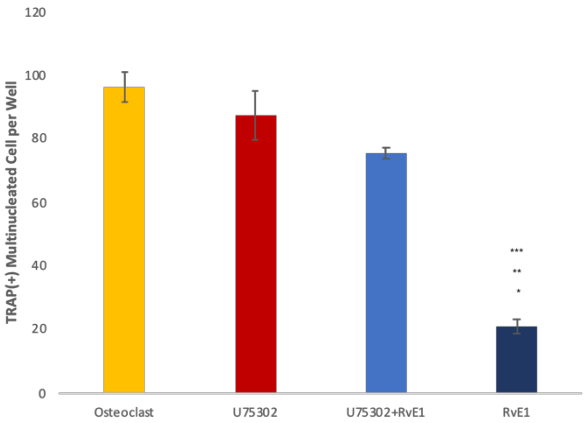

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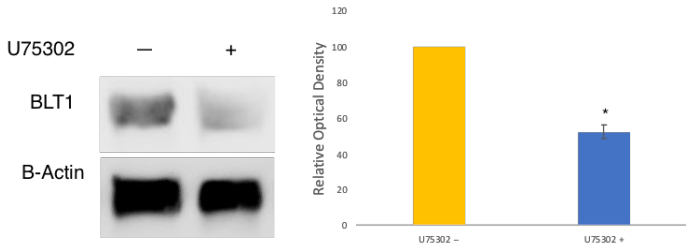


Figure 17. RvE1 reduced osteoclasts differentiation through BLT1 receptor. Immunohistochemistry analyses performed on **(A)** right side hemimaxilla, **(B)** femur and tibia bones of mice using anti-BLT1 staining (Thermo Scientific) showed BLT1 receptor expression on the cell surface of mice alveolar bone osteoclasts and bone marrow-derived osteoclast precursors and osteoclasts. **(C & D)** U75302-osteoclast cultures and U75302/RvE1-osteoclast cultures did not show any decrease in number of TRAP-stained multinucleated osteoclasts compared to control-osteoclast cultures. (**E)** Western blot analysis showed that U75302 reduced levels of BLT1 protein by 47% ($P<0.01$) compared to no U75302-osteoclast cultures.

(Mean $\pm$ SD, ANOVA *, **, *** $P< 0.05$). * comparisons were made to the osteoclasts. ** comparisons were made to U75302. *** comparisons were made to U75302+RvE1.

RvE1 (1 & 2): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(10nM) at days 1, and 2 only.

RvE1 (3 & 4): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(10nM) at days 3, and 4 only.

RvE1 (5 & 6): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(10nM) at days 5, and 6 only.

3.3. Specific Aim 3: Determine RvE1 deterrent regulation of bone marrow-derived osteoclast precursor and osteoclast proliferation and survival.

Rationale: Our group has revealed in previous studies that RvE1 reduces cell fusion in the late stages of osteoclast differentiation, which leads to reduced osteoclast formation through targeting of DC-STAMP and NFATc1 (Zhu et al., 2013). Also, as shown from the previous experiments, RvE1 exerted direct anti-catabolic actions on osteoclasts when delivered not only at late stages but at pre and early stages as well. We **hypothesize** that RvE1 has pleomorphic anti-catabolic actions on osteoclast precursors and osteoclasts in addition to preventing cell differentiation through reducing cell proliferation and survival.

Experimental approach: BrdU assay was performed to investigate the regulatory actions of RvE1 on osteoclast precursor and osteoclast proliferation rates. MTT and TUNEL assays were performed to investigate the regulatory actions of RvE1 on osteoclast precursor and osteoclast survival rates through assessing cell viability and apoptosis, respectively. BrdU and MTT assay were measured using a spectrophotometer microplate reader while for the TUNEL assay counting TUNEL-positive cells was viewed at excitation 488 nm/emission 512 nm by fluorescence. Different osteoclast precursor and osteoclast cultures were generated from bone marrow of 8-week-old mice for the different aims. Based on previous studies, the concentrations of RvE1 used were 1.0, 10.0 and 100.0 nM (Hasturk et al., 2007, Hasturk et al., 2006, Herrera et al., 2008). The control groups were identical culture medium supplemented with the corresponding amount of ethanol (vehicle). The time of RvE1 induction plays a critical role in detecting its hypothesized actions on osteoclast precursors and osteoclasts. Bone Marrow Derived macrophage cultures were exposed to M-CSF (50 ngm/L1) in 6-well plates for two days until 75% confluency is reached then cells are passed to

96-well plates ($2x10^5$ cells/well) for BrdU and MTT assays, and 24-well plates ($5x10^5$ cells/well) for the TUNEL assay.

Osteoclast precursor cultures were exposed to M-CSF (50 ngm/L1) at day 1 to induce osteoclast precursor differentiation and medium was replaced every day. RvE1 was delivered to the culture at two different time points depending on the group; RvE1 (1&2) group, where the time of delivery of RvE1 is at days 1 and 2 and RvE1 (3&4) group, where the time of delivery of RvE1 is at days 3 and 4. BrdU, MTT and TUNEL assays were performed at day 5 (Fig. 18A).

Osteoclast cultures were exposed to M-CSF (50 ngm/L1) at day 1 to induce osteoclast precursor differentiation and medium was replaced every day. RANKL (30 ngm/L1) was introduced to cultures at day 3 to induce osteoclast differentiation and medium was replaced every day. RvE1 was delivered to the culture at three different time points depending on the group; RvE1 (1&2) group, where the time of delivery of RvE1 is at days 1 and 2 to investigate the RvE1 regulatory actions on osteoclast differentiation when cultures exposed to RvE1 at osteoclast precursor phase. RvE1 (3&4) group was induced by RvE1 at days 3 and 4 and RvE1 (5&6) group was induced by RvE1 at days 5 and 6. BrdU, MTT and TUNEL assays were performed at day 7 (Fig. 18B).

U75302 was used in the osteoclast precursor and osteoclast cell cultures as previously described with and without RvE1 induction two days before TRAP staining to investigate BLT1 as the cell surface receptor that interacts with RvE1 to negatively regulate osteoclast proliferation and survival. Also, Western blot analysis was performed to validate RvE1 stimulation of osteoclast apoptosis through evaluating the presence of cleaved Caspase 3. U75302 and RvE1 were delivered into the cultures at day 7. U75302 delivered 1 hour prior to RvE1 followed by whole-cell protein extraction 15 minutes after using CelLytic M solution (Sigma-Aldrich; Merck KGaA) with protease inhibitors for Western blot analysis (Fig. 18B).

3.3.1. RvE1 reduced osteoclast precursor and osteoclast proliferation in a dose-dependent manner through BLT1 receptor.

In osteoclast precursor and osteoclast cell cultures, BrdU assays were performed to assess cell proliferation rate. BrdU incorporated osteoclast precursor and osteoclast cells is indicative of cell proliferation rate. In osteoclast precursor cell cultures RvE1 (1&2) group, RvE1 significantly decreased BrdU incorporation by osteoclast precursor cells at 1nM concentration by 27% ($P<0.01$), at 10nM concentration by 37% ($P<0.01$) and at 100nM concentration by 42% ($P<0.01$) compared to control. Also, RvE1 significantly decreased BrdU incorporation by osteoclast precursors at the 100nM concentration compared to 10nM concentration ($P<0.01$) and the 10nM concentration compared to 1nM concentration ($P<0.01$). In the RvE1 (3&4) group, RvE1 significantly decreased BrdU incorporation by osteoclast precursor cells at 1nM concentration by 25% ($P<0.01$), at 10nM concentration by 28% ($P<0.01$) and at 100nM concentration by 50% ($P<0.01$) compared to control. Also, RvE1 significantly decreased BrdU incorporation by osteoclast precursors at the 100nM concentration compared to 10nM concentration ($P<0.01$) (Fig. 19A).

In osteoclast cell cultures RvE1 (1&2) group, RvE1 significantly decreased BrdU incorporation by osteoclast cells at 1nM concentration by 18% ($P<0.01$) and at 10nM concentration by 34% ($P<0.01$) compared to control. However, RvE1 did not affect BrdU incorporation by osteoclast cells at 100nM concentration compared to control. Also, RvE1 significantly decreased BrdU incorporation by osteoclasts at the 10nM concentration compared to 1nM concentration ($P<0.01$). In the RvE1 (3&4) group, RvE1 did not BrdU incorporation by osteoclast cells at 1nM concentration compared to control. But, RvE1 significantly decreased BrdU incorporation by osteoclast cells at 10nM concentration by 34% ($P<0.01$) and at 100nM concentration by 25%

(P<0.01) compared to control. Also, RvE1 significantly decreased BrdU incorporation by osteoclasts at the 10nM concentration compared to 1nM concentration (P<0.01). In the RvE1 (5&6) group, RvE1 did not affect BrdU incorporation by osteoclast cells at 1nM concentration compared to control. But, RvE1 significantly decreased BrdU incorporation by osteoclast cells at 10nM concentration by 27% (P<0.01) and at 100nM concentration by 37% (P<0.01) compared to control. Also, RvE1 significantly decreased BrdU incorporation by osteoclasts at the 100nM concentration compared to 10nM concentration (P<0.01) and the 10nM concentration compared to 1nM concentration (P<0.01) (Fig. 19B). In addition, U75302-cell cultures and U75302/RvE1-cell cultures did not show any decrease in the proliferation rate of osteoclast precursors and osteoclasts compared to control-cell cultures (Fig. 19C & D).

3.3.2. RvE1 reduced osteoclast precursor and osteoclast viability in a dose-dependent manner through BLT1 receptor.

In osteoclast precursor and osteoclast cell cultures, MTT assays were performed to assess cell viability. The number of MTT bromide reducing osteoclast precursor and osteoclast cells is indicative of cell viability. In osteoclast precursor RvE1 (1&2) group, RvE1 did not affect number of MTT bromide reducing osteoclast precursor cells at 1nM concentration, but significantly decreased number of MTT bromide reducing osteoclast precursor cells at 10nM concentration by 24% (P<0.01) and at 100nM concentration by 37% (P<0.01) compared to control. Also, RvE1 significantly decreased the number of MTT bromide reducing osteoclast precursors at the 100nM concentration compared to 10nM concentration (P<0.01). In the RvE1 (3&4) group, RvE1 did not affect the number of MTT bromide reducing osteoclast precursor cells at 1nM concentration, but

significantly decreased number of MTT bromide reducing osteoclast precursor cells at 10nM concentration by 20% ($P<0.01$) and at 100nM concentration by 37% ($P<0.01$) compared to control. Also, RvE1 significantly decreased the number of MTT bromide reducing osteoclast precursors at the 100nM concentration compared to 10nM concentration ($P<0.01$). (Fig. 20A)

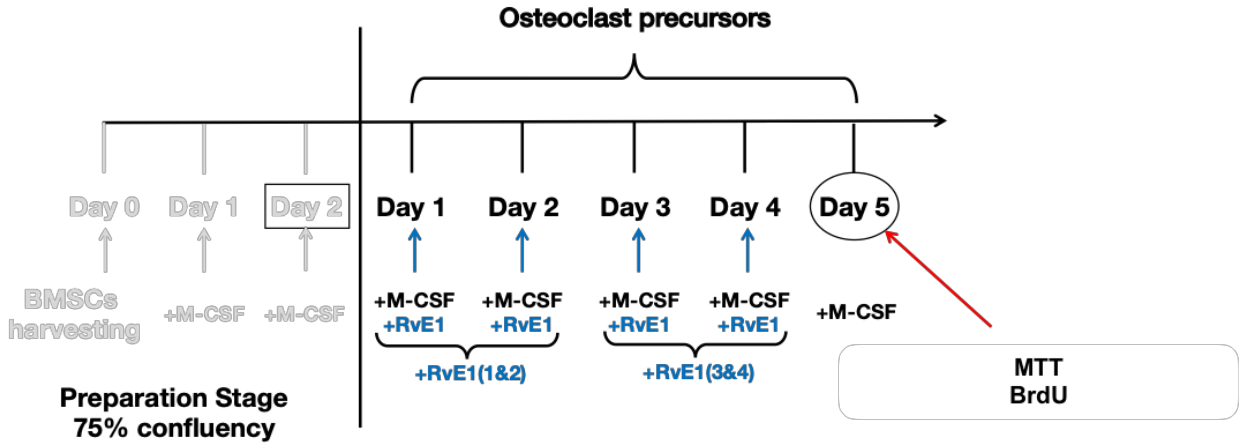
In osteoclast cell cultures RvE1 (1&2) group, RvE1 significantly decreased the number of MTT bromide reducing osteoclast cells at 1nM concentration by 45% ($P<0.01$), at 10nM concentration by 54% ($P<0.01$) and at 100nM concentration by 53% ($P<0.01$) compared to control. Also, RvE1 significantly the decreased number of MTT bromide reducing osteoclasts at the 10nM concentration compared to 1nM concentration ($P<0.01$). In the RvE1 (3&4) group, RvE1 significantly decreased the number of MTT bromide reducing osteoclast cells at 1nM concentration by 37% ($P<0.01$), at 10nM concentration by 34% ($P<0.01$) and at 100nM concentration by 49% ($P<0.01$) compared to control. Also, RvE1 significantly decreased the number of MTT bromide reducing osteoclasts at the 100nM concentration compared to 10nM concentration ($P<0.01$). In the RvE1 (5&6) group, RvE1 significantly decreased number of MTT bromide reducing osteoclast cells at 1nM concentration by 39% ($P<0.01$), at 10nM concentration by 53% ($P<0.01$) and at 100nM concentration by 45% ($P<0.01$) compared to control. Also, RvE1 significantly decreased number of MTT bromide reducing osteoclasts at the 10nM concentration compared to 1nM concentration ($P<0.01$) (Fig. 20B). In addition, U75302-cell cultures and U75302/RvE1-cell cultures did not show any decrease in the viability rate of osteoclast precursors and osteoclasts compared to control-cell cultures (Fig. 20D & E).

3.3.3. RvE1 increased osteoclast apoptosis through BLT1 receptor.

In osteoclast cell cultures, TUNEL assays were performed to assess cell apoptosis. The number of TUNEL stained cells is indicative of significant increase in cell apoptosis. RvE1 significantly increased the number of TUNEL stained osteoclasts in the RvE1 (1&2) group by 5-fold ($P<0.01$), in the RvE1(3&4) group by 7-folds ($P<0.01$), and in RvE1(5&6) group by 11-folds ($P<0.01$) compared to control (Fig. 21 A & B). Also, U75302-cell cultures and U75302/RvE1-cell cultures did not show any increase in apoptosis of osteoclasts compared to control-cell cultures (Fig. 21D & E). Western blot analysis showed that RvE1 increased levels of cleaved Caspase 3 protein by 3-folds ($P<0.01$) compared to no RvE1-osteoclast cultures (Fig. 21C).

Figure 18. Experimental design of MTT, BrdU, TUNEL and Western blot assays.

A



B

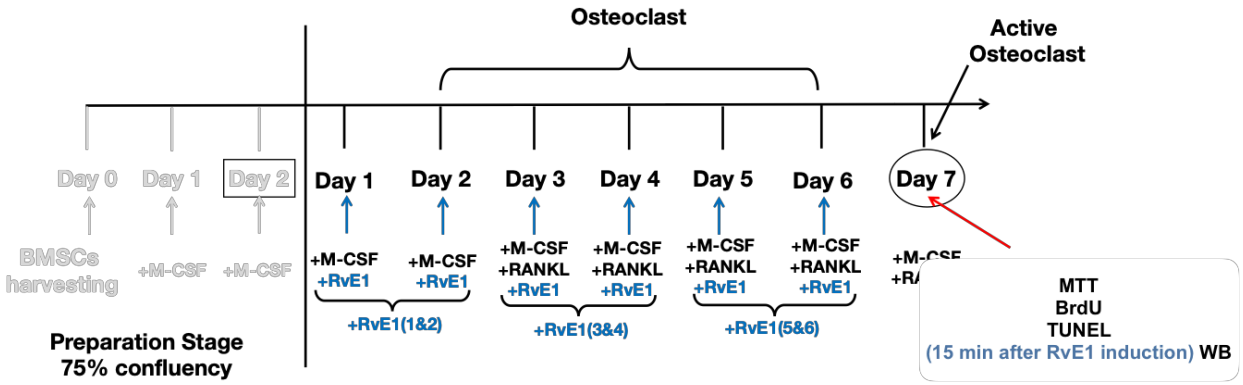
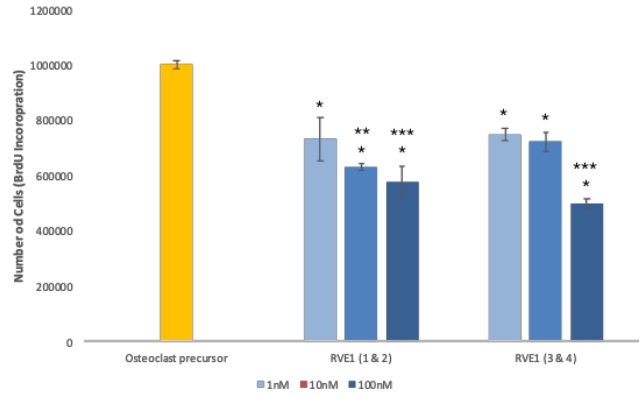


Figure 18. Experimental design of MTT, BrdU, TUNEL and Western blot assays. The time of RvE1 induction plays a critical role in detecting its hypothesized actions on osteoclast precursors and osteoclasts. Bone Marrow Derived macrophages cultures were exposed to M-CSF (50 ngm/L1) in 6-well plates for two days until 75% confluency is reached then cells are passed to 96-well plates ($2x10^5$ cells/well) for BrdU and MTT assays, and 24-well plates ($5x10^5$ cells/well) for TUNEL assay. Osteoclast precursor cultures were exposed to M-CSF (50 ngm/L1) at day 1 to induce osteoclast precursor differentiation and were replaced every day. RvE1 were delivered to the culture at two different time sets depending on the group; RvE1(1&2) group, where the time of delivery of RvE1 is at days 1 and 2 and RvE1(3&4) group, where the time of delivery of RvE1 is at days 3 and 4. BrdU, MTT and TUNEL assays were performed at day 5.

Osteoclast cultures were exposed to M-CSF (50 ngm/L1) at day 1 to induce osteoclast precursor differentiation and were replaced every day. RANKL (30 ngm/L1) introduced to cultures at day 3 to induce osteoclast differentiation and were replaced every day. RvE1 were delivered to the culture at three different time sets depending on the group; RvE1(1&2) group, where the time of delivery of RvE1 is at days 1 and 2 to investigate the RvE1 regulatory actions on osteoclast differentiation when cultures exposed to RvE1 at osteoclast precursor phase. RvE1(3&4) group was induced by RvE1 at days 3 and 4 and RvE1(5&6) group was induced by RvE1 at days 5 and 6. BrdU, MTT and TUNEL assays were performed at day 7. Also, whole-cell proteins were extracted 15 minutes after RvE1 induction at day 7 for western blot analysis.

Figure 19. RvE1 reduced osteoclast precursor and osteoclast proliferation in a dose-dependent manner through BLT1 receptor.

A



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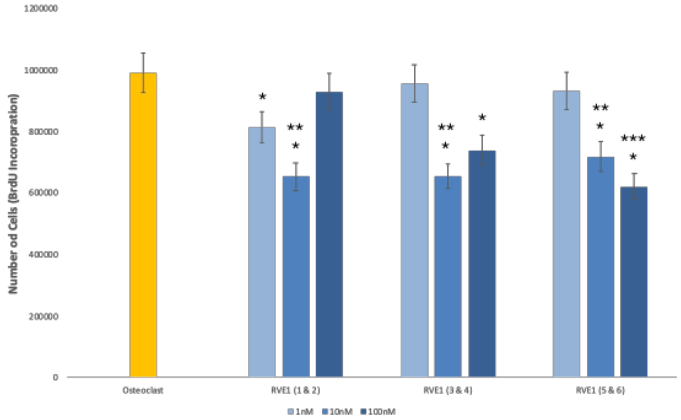

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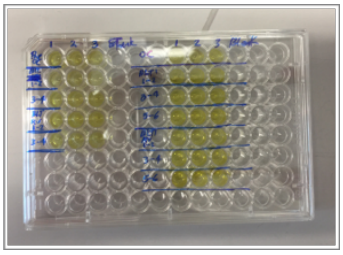
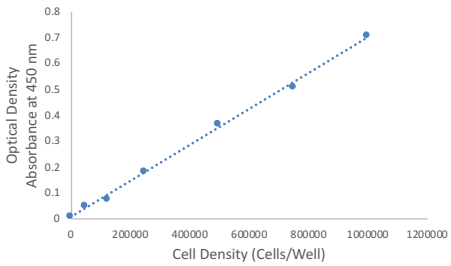




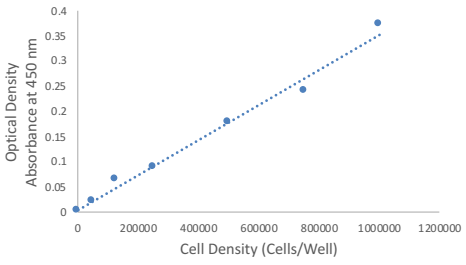
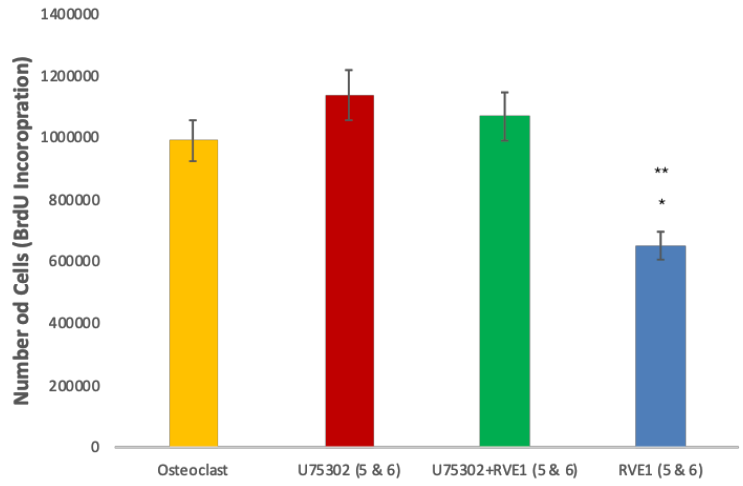


Figure 19. RvE1 reduced osteoclast precursor and osteoclast proliferation in a dose-dependent manner through BLT1 receptor.

D



E

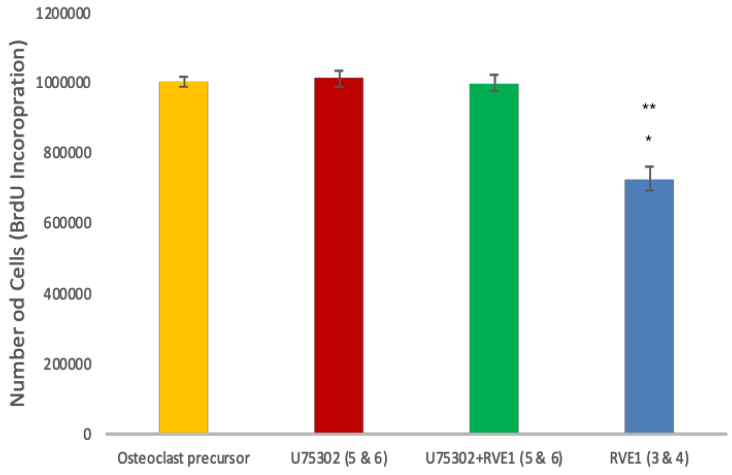


Figure 19. RvE1 reduced osteoclast precursor and osteoclast proliferation in a dose-dependent manner through BLT1 receptor. (A) In osteoclast precursor cell cultures RvE1(1&2) group, RvE1 significantly decreased number of BrdU incorporated osteoclast precursor cells at 1nM concentration by 27% ($P<0.01$), at 10nM concentration by 37% ($P<0.01$) and at 100nM concentration by 42% ($P<0.01$) compared to control. In RvE1(3&4) group, RvE1 significantly decreased number of BrdU incorporated osteoclast precursor cells at 1nM concentration by 25% ($P<0.01$), at 10nM concentration by 28% ($P<0.01$) and at 100nM concentration by 50% ($P<0.01$) compared to control. **(B)** In osteoclast cell cultures RvE1(1&2) group, RvE1 significantly decreased number of BrdU incorporated osteoclast cells at 1nM concentration by 18% ($P<0.01$) and at 10nM concentration by 34% ($P<0.01$) compared to control. In RvE1(3&4) group, RvE1 significantly decreased number of BrdU incorporated osteoclast cells at 10nM concentration by 34% ($P<0.01$) and at 100nM concentration by 25% ($P<0.01$) compared to control. In RvE1(5&6) group, RvE1 significantly decreased number of BrdU incorporated osteoclast cells at 10nM concentration by 27% ($P<0.01$) and at 100nM concentration by 37% ($P<0.01$) compared to control **(C)** Standard curves created to calculate number of BrdU incorporated cells from Optical density readings. **(D & E)** In addition, U75302-cell cultures and U75302/RvE1-cell cultures did not show any decrease in the proliferation rate of osteoclast precursors and osteoclasts compared to control-cell cultures. (Mean $\pm$ SD, ANOVA *, **, *** $P< 0.05$). * comparisons were made to the control group. (A &B) ** comparisons were made to 1nM RvE1. *** comparisons were made to 10nM RvE1. (D & E) ** comparisons were made to U75302+RvE1.

(OC precursors) RvE1 (1 & 2): +M-CSF (50 ngm/L1) at days 1-4. +RvE1(10nM) at days 1, and 2 only.

(OC precursors) RvE1 (3 & 4): +M-CSF (50 ngm/L1) at days 1-4. +RvE1(10nM) at days 3, and 4 only.

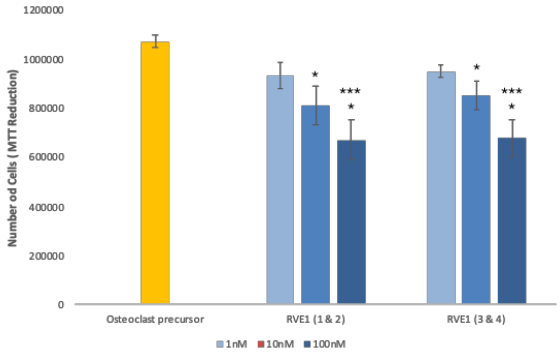
(OC) RvE1 (1 & 2): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(10nM) at days 1, and 2 only.

(OC) RvE1 (3 & 4): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(10nM) at days 3, and 4 only.

(OC) RvE1 (5 & 6): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(10nM) at days 5, and 6 only.

Figure 20. RvE1 reduced osteoclast precursor and osteoclast viability in a dose-dependent manner through BLT1 receptor.

A



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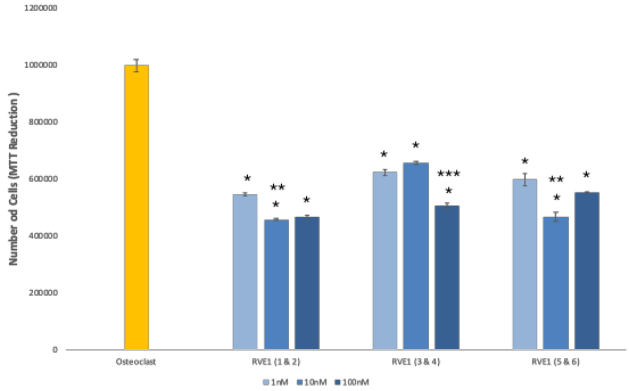

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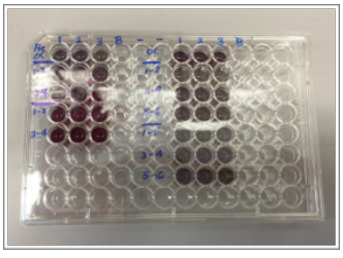
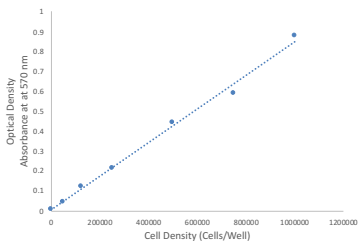




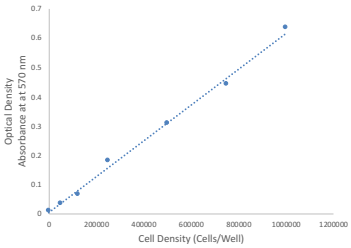
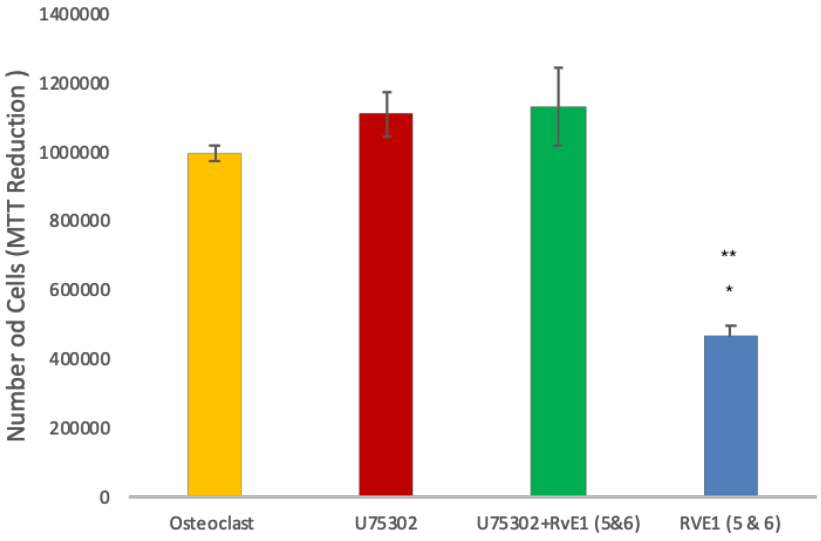


Figure 20. RvE1 reduced osteoclast precursor and osteoclast viability in a dose-dependent manner through BLT1 receptor.

D



E

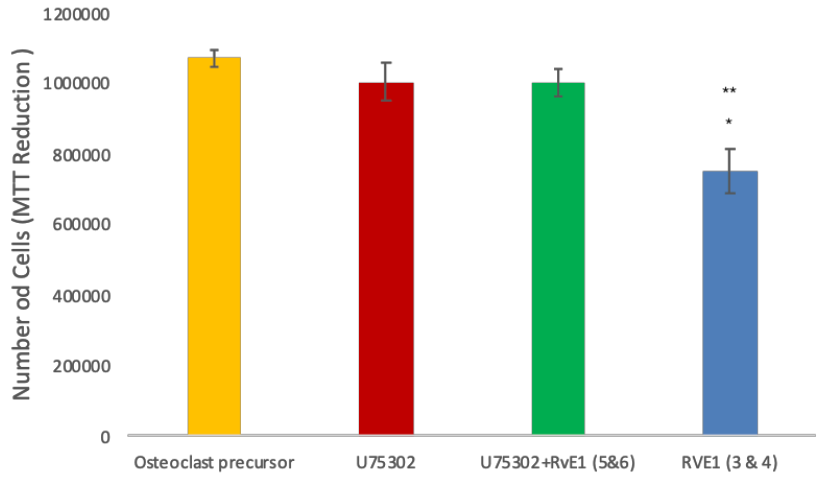


Figure 20. RvE1 reduced osteoclast precursor and osteoclast viability in a dose-dependent manner through BLT1 receptor. (A) In osteoclast precursor RvE1(1&2) group, RvE1 significantly decreased number of MTT reducing osteoclast precursor cells at 10nM concentration by 24% ($P<0.01$) and at 100nM concentration by 37% ($P<0.01$) compared to control. In RvE1(3&4) group, RvE1 significantly decreased number of MTT reducing osteoclast precursor cells at 10nM concentration by 20% ($P<0.01$) and at 100nM concentration by 37% ($P<0.01$) compared to control. **(B)** In osteoclast cell cultures RvE1(1&2) group, RvE1 significantly decreased number of MTT reducing osteoclast cells at 1nM concentration by 45% ($P<0.01$), at 10nM concentration by 54% ($P<0.01$) and at 100nM concentration by 53% ($P<0.01$) compared to control. In RvE1(3&4) group, RvE1 significantly decreased number of MTT reducing osteoclast cells at 1nM concentration by 37% ($P<0.01$), at 10nM concentration by 34% ($P<0.01$) and at 100nM concentration by 49% ($P<0.01$) compared to control. In RvE1(5&6) group, RvE1 significantly decreased number of MTT reducing osteoclast cells at 1nM concentration by 39% ($P<0.01$), at 10nM concentration by 53% ($P<0.01$) and at 100nM concentration by 45% ($P<0.01$) compared to control. **(C)** Standard curves created to calculate number of MTT bromide reducing cells from optical density readings. **(D & E)** U75302-cell cultures and U75302/RvE1-cell cultures did not show any decrease in the viability of osteoclast precursors and osteoclasts compared to control-cell cultures. (Mean $\pm$ SD, ANOVA *, **, *** $P< 0.05$). * comparisons were made to the control group. (A &B) ** comparisons were made to 1nM RvE1. *** comparisons were made to 10nM RvE1. (D & E) ** comparisons were made to U75302+RvE1.

(OC precursors) RvE1 (1 & 2): +M-CSF (50 ngm/L1) at days 1-4. +RvE1(10nM) at days 1, and 2 only.

(OC precursors) RvE1 (3 & 4): +M-CSF (50 ngm/L1) at days 1-4. +RvE1(10nM) at days 3, and 4 only.

(OC) RvE1 (1 & 2): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(10nM) at days 1, and 2 only.

(OC) RvE1 (3 & 4): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(10nM) at days 3, and 4 only.

(OC) RvE1 (5 & 6): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(10nM) at days 5, and 6 only.

Figure 21. RvE1 increased osteoclast apoptosis through BLT1 receptor.

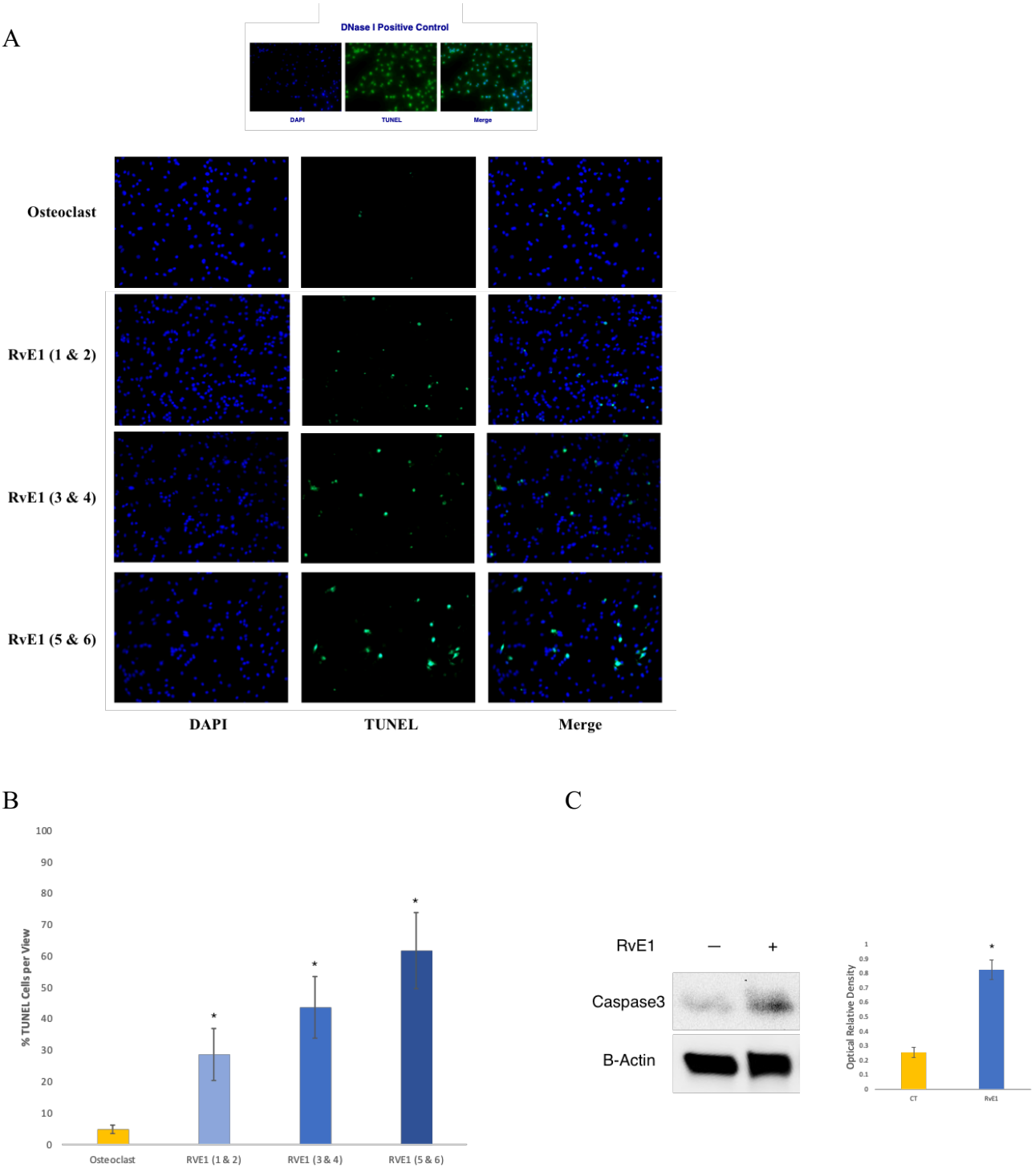
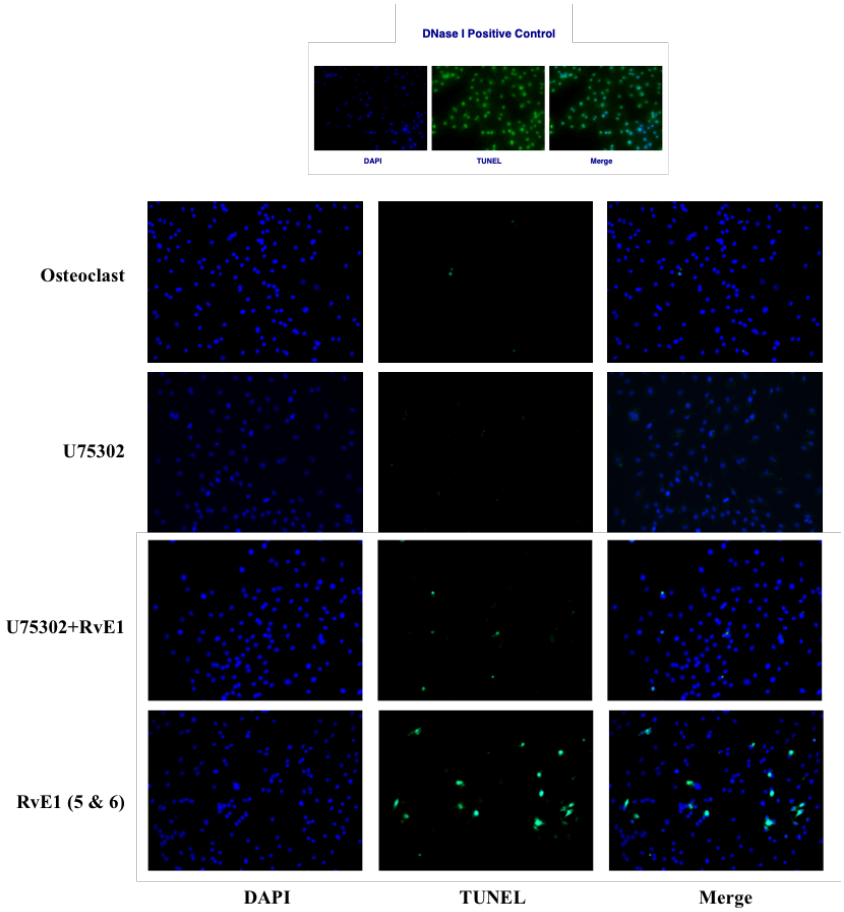


Figure 21. RvE1 increased osteoclast apoptosis through BLT1 receptor.

D



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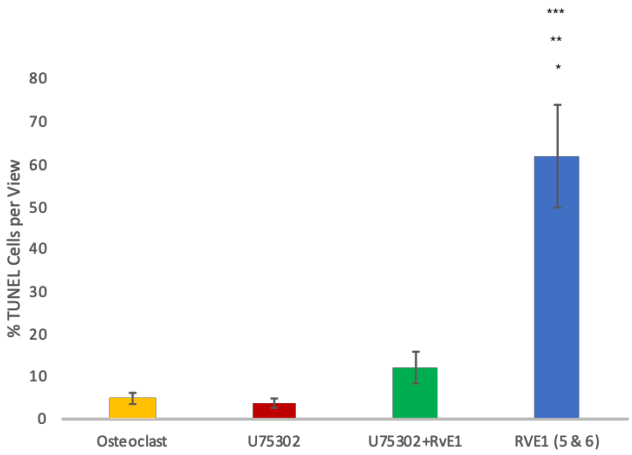


Figure 21. RvE1 increased osteoclast apoptosis through BLT1 receptor. (A & D) Immunofluorescence staining showing increased TUNEL (in green) stain in RvE1-induced osteoclast cultures. **(B)** RvE1 significantly increased number of TUNEL stained osteoclasts at in RvE1(1&2) group by 5-folds ($P<0.01$), in RvE1(3&4) group by 7-folds ($P<0.01$), and in RvE1(5&6) group by 11-folds ($P<0.01$) compared to control. **(C)** Western blot analysis showed that RvE1 increased levels of cleaved Caspase 3 protein by 3-folds ($P<0.01$) compared to no RvE1-osteoclast cultures. (**E)** U75302-cell cultures and U75302/RvE1-cell cultures did not show any increase in apoptosis of osteoclasts compared to control-cell cultures.

(Mean $\pm$ SD, ANOVA *, **, *** $P< 0.05$). * comparisons were made to the control group. ** comparisons were made to U75302+RvE1.

(OC precursors) RvE1 (1 & 2): +M-CSF (50 ngm/L1) at days 1-4. +RvE1(10nM) at days 1, and 2 only.

(OC precursors) RvE1 (3 & 4): +M-CSF (50 ngm/L1) at days 1-4. +RvE1(10nM) at days 3, and 4 only.

(OC) RvE1 (1 & 2): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(10nM) at days 1, and 2 only.

(OC) RvE1 (3 & 4): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(10nM) at days 3, and 4 only.

(OC) RvE1 (5 & 6): +M-CSF (50 ngm/L1) at days 1-6. +RANKL (30 ngm/L1) at days 3-6. +RvE1(10nM) at days 5, and 6 only.

3.4. Specific Aim 4: Determine the molecular signaling pathways involved in RvE1 regulation of bone marrow-derived osteoclast precursor and osteoclast differentiation, proliferation and survival.

Rationale: As shown from the previous experiments, RvE1 exerted direct pleomorphic anti-catabolic actions on osteoclasts in addition to preventing cell differentiation and fusion (Zhu et al., 2013) by reducing cell proliferation and survival. M-CSF- and RANKL-induced activation of bone marrow-derived macrophages c-Fms and RANK receptors results in increased osteoclast precursor and osteoclast proliferation and survival through the MAPK(ERK) and PI3K/Akt pathways (Cobrinik, 2005, Helgason et al., 1998, Takeshita et al., 2002, Funakoshi-Tago et al., 2003, Wong et al., 1999a, Xing et al., 2001). Therefore, we investigated these pathways to examine them as possible targets for RvE1 preventive regulation action on osteoclast precursor and osteoclast proliferation and survival (Fig. 4). We hypothesize that RvE1 interferes with the activation/deactivation of these signaling pathways through regulating the levels of phosphorylated/total signaling molecules.

Experimental approach: Different osteoclast precursor and osteoclast cultures were generated from bone marrow of 8- to 10-week-old mice for the different aims. Based on previous studies, the concentration of RvE1 used were 1.0, 10.0 and 100.0 nM concentrations. The control groups were identical culture medium supplemented with the corresponding amount of ethanol (vehicle). Bone Marrow Derived macrophage cultures were exposed to M-CSF (50 ng/L) in 6-well plates for two days until 75% confluency is reached then cells are passed to new 6-well plates (10^6 cells/well) for Western blot analysis.

Osteoclast precursor cultures were exposed to M-CSF (50 ng/L) at day 1 to induce osteoclast precursor differentiation and were replaced every day. RvE1 were delivered to the culture at day 5. Whole-cell proteins were extracted 15 minutes after RvE1 induction using CelLytic M solution (Sigma-Aldrich; Merck KGaA) with protease and phosphatase inhibitors for Western blot analysis (Fig. 22A).

Osteoclast cultures were exposed to M-CSF (50 ngm/L1) at day 1 to induce osteoclast precursor differentiation and were replaced every day. RANKL (30 ngm/L1) introduced to cultures at day 3 to induce osteoclast differentiation and were replaced every day. RvE1 were delivered into the cultures at day 7. Whole-cell proteins were extracted 15 minutes after RvE1 induction using CelLytic M solution (Sigma-Aldrich; Merck KGaA) with protease and phosphatase inhibitors for Western blot analysis (Fig. 22B).

LTB_4, U75302, Wortmannin (an Akt inhibitor) and PD98059 (an ERK inhibitor) were used in the osteoclast precursor and osteoclast cell cultures as previously described with and without RvE1 induction to validate RvE1-BLT1 signaling pathways using tartrate-resistant acid phosphatase (TRAP) staining and western blot analyses. LTB_4 and RvE1 were delivered into the cultures at day 7. U75302, Wortmannin and PD98059 were delivered 1 hour prior to RvE1 followed by whole-cell protein extraction 15 minutes after RvE1 induction using CelLytic M solution (Sigma-Aldrich; Merck KGaA) with phosphatase and protease inhibitors for Western blot analyses.

3.4.1. RvE1 diminishes PI3K/Akt & MAPK/ERK signaling pathways essential for osteoclast precursor and osteoclast proliferation, survival & differentiation.

In osteoclast precursor cell cultures, relative quantification of the bands from western blot were normalized to β-actin for phosphorylation level analysis to assess activation/deactivation of signaling pathway molecules during RvE1 exposure. The following antibodies were used to study the involved signaling pathways: Phospho-Akt (Ser473), Akt (pan), p44/42 MAPK (Erk1/2) and Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204).

Band density is indicative of level of phosphorylation/activation. RvE1 significantly decreased phosphorylation levels of Akt (Ser473) after 15 minutes of exposure by 69% ($P<0.01$). RvE1 significantly decreased phosphorylation levels of p44/42 MAPK (Erk1/2) (Thr202/Tyr204) after 15 minutes of exposure by 21% ($P<0.01$). Moreover, LTB_4-induced, U75302-induced and U75302/RvE1-induced osteoclast precursor cell cultures showed significantly higher phosphorylation levels of Akt (Ser473) ($P<0.01$) compared to RvE1-induced osteoclast precursor cell cultures. Conversely, LTB_4-induced, U75302-induced and U75302/RvE1-induced osteoclast precursor cell cultures showed significantly lower phosphorylation levels of p44/42 MAPK (Erk1/2) (Thr202/Tyr204) ($P<0.01$) after 15 minutes of exposure compared to RvE1-induced osteoclast precursor cell cultures (Fig. 23A, B & C).

In osteoclast cell cultures, relative quantification of the bands from western blot were normalized to β-actin for phosphorylation level analysis to assess activation/deactivation of signaling pathway molecules during RvE1 exposure. The following antibodies were used to study the involved signaling pathways: Phospho-Akt (Ser473), Akt (pan), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), p44/42 MAPK (Erk1/2), Phospho-NF-κB p65 (Ser536), NF-κB p65, Phospho-IκBα (Ser32), IκBα, Phospho-c-Jun (Ser63), c-Jun, Phospho-c-Fos (Ser32), c-Fos.

Band density is indicative of level of phosphorylation/activation. RvE1 significantly decreased phosphorylation levels of Akt (Ser473) after 15 minutes of exposure by 55% ($P<0.01$). RvE1 significantly decreased phosphorylation levels of p44/42 MAPK (Erk1/2) (Thr202/Tyr204) after 15 minutes of exposure by 45% ($P<0.01$). RvE1 did not affect the phosphorylation levels of NF-κB p65 (Ser536), IκBα (Ser32), c-Jun (Ser63) and c-Fos (Ser32). Also, LTB_4-induced, U75302-induced and U75302/RvE1-induced osteoclast cell cultures showed significantly higher phosphorylation levels of Akt (Ser473) ($P<0.01$) compared to RvE1-induced osteoclast cell cultures. Moreover, LTB_4-induced and U75302-induced osteoclast cell cultures showed significantly higher phosphorylation levels of p44/42 MAPK (Erk1/2) (Thr202/Tyr204) ($P<0.01$) after 15 minutes of exposure compared to RvE1-induced osteoclast cell cultures. However, U75302/RvE1-induced osteoclast cell cultures showed significantly lower phosphorylation levels of p44/42 MAPK (Erk1/2) (Thr202/Tyr204) ($P<0.01$) compared to RvE1-induced osteoclast cell cultures (Fig. 23D, E & F).

3.4.1. RvE1 signaling pathways cross-talk

To validate RvE1-BLT1 signaling pathways and investigate cross-talk between them, Wortmannin and PD98059 were used in the previously described osteoclast precursor and osteoclast cell cultures with and without RvE1 induction. Tartrate-resistant acid phosphatase (TRAP) staining and western blot analyses were performed.

In osteoclast precursor cell cultures, Wortmannin-induced and Wortmannin/RvE1-induced osteoclast precursor cell cultures showed significantly lower phosphorylation levels of p44/42 MAPK (Erk1/2) (Thr202/Tyr204) ($P<0.01$) compared to RvE1-induced osteoclast precursor cell cultures. However, PD98059-induced and PD98059/RvE1-induced osteoclast precursor cell

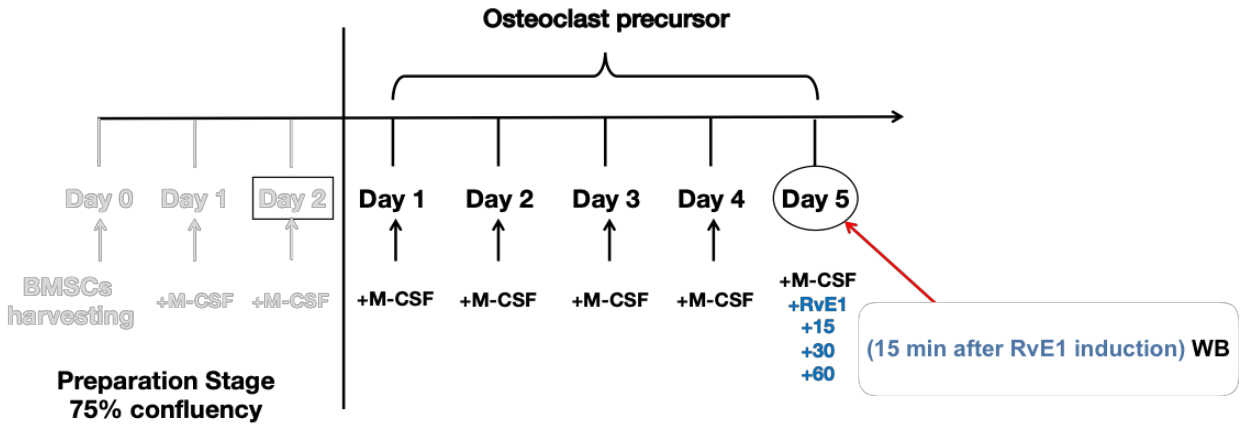
cultures showed significantly higher phosphorylation levels of Akt (Ser473) ($P<0.01$) compared to RvE1-induced osteoclast precursor cell cultures. This could indicate that PI3K/Akt is upstream to MAPK/ERK in RvE1 stimulated signaling in osteoclast precursor cell cultures. (Fig. 24A, B & C)

In osteoclast cell cultures, only Wortmannin-induced osteoclast cell cultures showed significantly lower phosphorylation levels of p44/42 MAPK (Erk1/2) (Thr202/Tyr204) ($P<0.01$) compared to RvE1-induced osteoclast cell cultures. Conversely, Wortmannin/RvE1-induced osteoclast cell cultures showed significantly lower phosphorylation levels of p44/42 MAPK (Erk1/2) (Thr202/Tyr204) ($P<0.01$) compared to RvE1-induced osteoclast cell cultures, indicating a possible alternative pathway through a different receptor. Moreover, PD98059-induced and PD98059/RvE1-induced osteoclast cell cultures showed significantly higher phosphorylation levels of Akt (Ser473) ($P<0.01$) compared to RvE1-induced osteoclast cell cultures, which solidify the possibility that PI3K/Akt is upstream to MAPK/ERK in RvE1 stimulated signaling in osteoclast precursor and osteoclast cell cultures. (Fig. 25A, B & C)

Measurements of osteoclast differentiation were made by counting the number of TRAP-stained multinucleated osteoclasts using 20X objective field of view images as previously described. Wortmannin-induced, Wortmannin/RvE1-induced, PD98059-induced and PD98059/RvE1-induced osteoclast precursor and osteoclast cell cultures all showed significant reduction in number of TRAP-stained multinucleated osteoclast compared to control osteoclast precursor and osteoclast cell cultures. This indicate a lack of osteoclastic phenotype related to inhibiting PI3K/Akt & MAPK/ERK signaling pathways. (Fig. 25 D, E & F)

Figure 22. Experimental design of Western blot and TRAP staining assays.

A



B

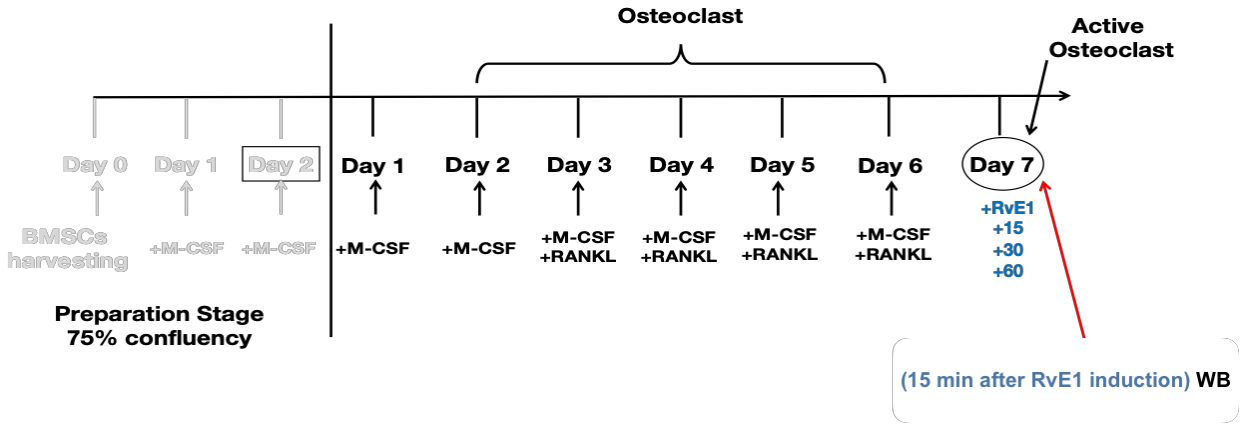


Figure 22. Experimental design of Western blot and TRAP staining assays. (A) Osteoclast precursor cultures were exposed to M-CSF (50 ngm/L1) at day 1 to induce osteoclast precursor differentiation and were replaced every day. RvE1 were delivered to the culture at day 5. Whole-cell proteins were extracted 15 minutes after RvE1 induction. **(B)** Osteoclast cultures were exposed to M-CSF (50 ngm/L1) at day 1 to induce osteoclast precursor differentiation and were replaced every day. RANKL (30 ngm/L1) introduced to cultures at day 3 to induce osteoclast differentiation and were replaced every day. RvE1 were delivered into the cultures at day 7. Whole-cell proteins were extracted 15 minutes after RvE1 induction. LTB_4, U75302, Wortmannin (an Akt inhibitor) and PD98059 (an ERK inhibitor) were used in the osteoclast precursor and osteoclast cell cultures as previously described with and without RvE1 induction to validate RvE1-BLT1 signaling pathways using tartrate-resistant acid phosphatase (TRAP) staining and western blot analyses at day 7. LTB_4 and RvE1 were delivered into the cultures at day 7. U75302, Wortmannin and PD98059 were delivered 1 hour prior to RvE1 followed by whole-cell proteins extraction 15 minutes after RvE1 induction.

Figure 23. RvE1 diminishes PI3K/Akt & MAPK/ERK signaling pathways in osteoclast precursors and osteoclasts.

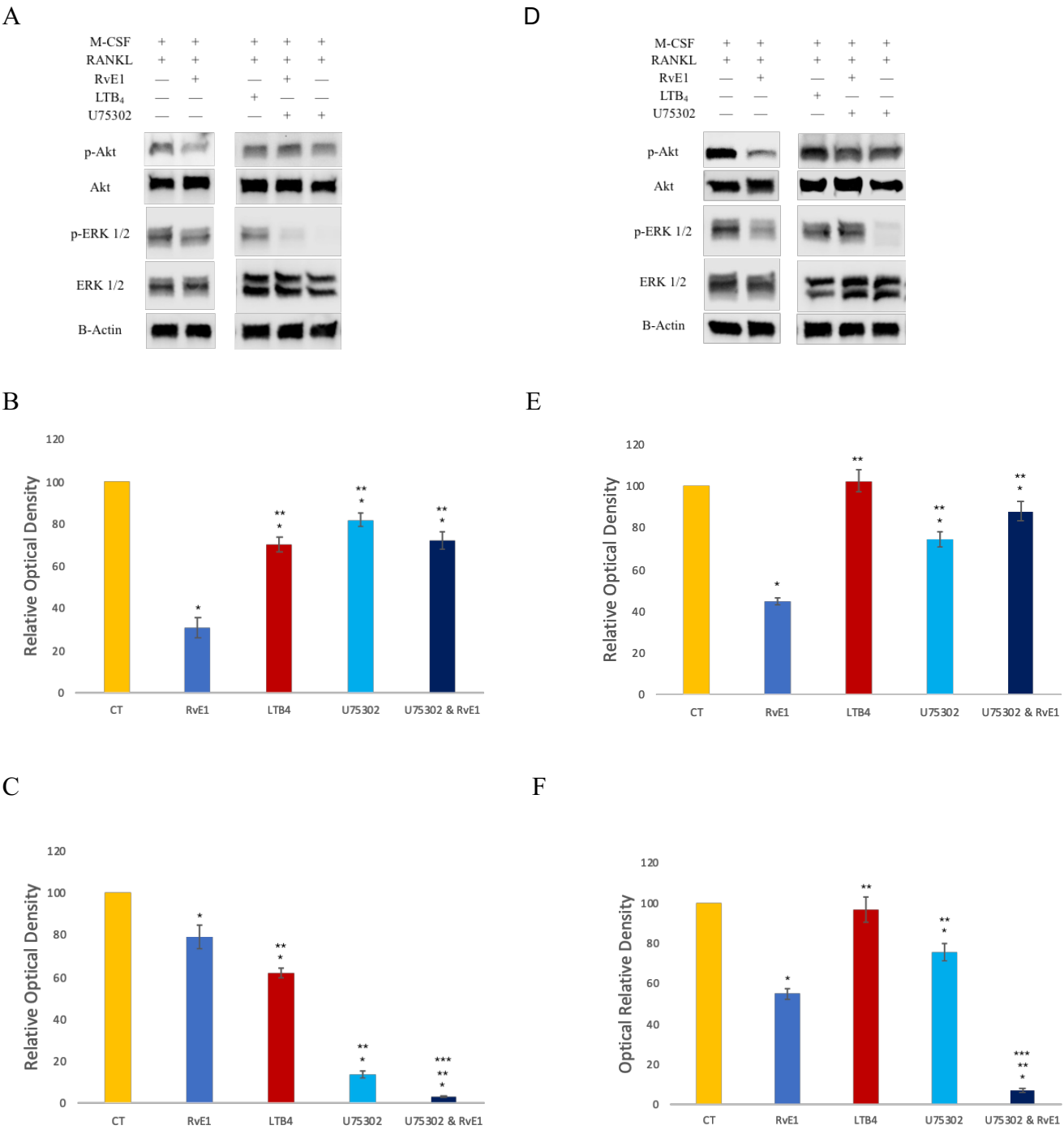
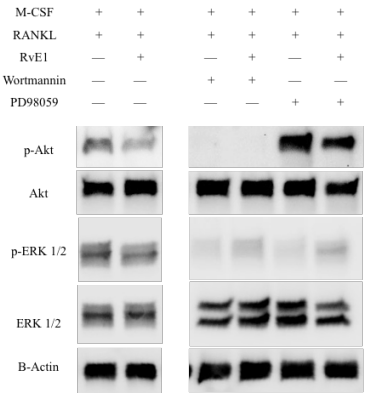


Figure 23. RvE1 diminishes PI3K/Akt & MAPK/ERK signaling pathways essential for osteoclast precursor and osteoclast proliferation, survival & differentiation. (A, B & C) In osteoclast precursor cell cultures, RvE1 significantly decreased phosphorylation levels of Akt (Ser473) by 69% ($P<0.01$) and significantly decreased phosphorylation levels of p44/42 MAPK (Erk1/2) (Thr202/Tyr204) by 21% ($P<0.01$) compared to control. Moreover, LTB_4-induced, U75302-induced and U75302/RvE1-induced osteoclast precursor cell cultures showed significantly higher phosphorylation levels of Akt (Ser473) ($P<0.01$) and significantly lower phosphorylation levels of p44/42 MAPK (Erk1/2) (Thr202/Tyr204) ($P<0.01$) compared to RvE1-induced osteoclast precursor cell cultures. **(D, E &F)** In osteoclast cell cultures, RvE1 significantly decreased phosphorylation levels of Akt (Ser473) by 55% ($P<0.01$) and significantly decreased phosphorylation levels of p44/42 MAPK (Erk1/2) (Thr202/Tyr204) by 45% ($P<0.01$). Also, LTB_4-induced, U75302-induced and U75302/RvE1-induced osteoclast precursor cell cultures showed significantly higher phosphorylation levels of Akt (Ser473) ($P<0.01$) and significantly higher phosphorylation levels of p44/42 MAPK (Erk1/2) (Thr202/Tyr204) ($P<0.01$) compared to RvE1-induced osteoclast precursor cell cultures. However, U75302/RvE1-induced osteoclast precursor cell cultures showed significantly lower phosphorylation levels of p44/42 MAPK (Erk1/2) (Thr202/Tyr204) ($P<0.01$) compared to RvE1-induced osteoclast precursor cell cultures.

(Mean + SD, ANOVA *, **, *** $P< 0.05$). * comparisons were made to the control group. ** comparisons were made to RvE1 group. *** comparisons were made to U75303 group.

Figure 24. Wortmannin and PD98059 inhibits RvE1 signaling in osteoclast precursors.

A



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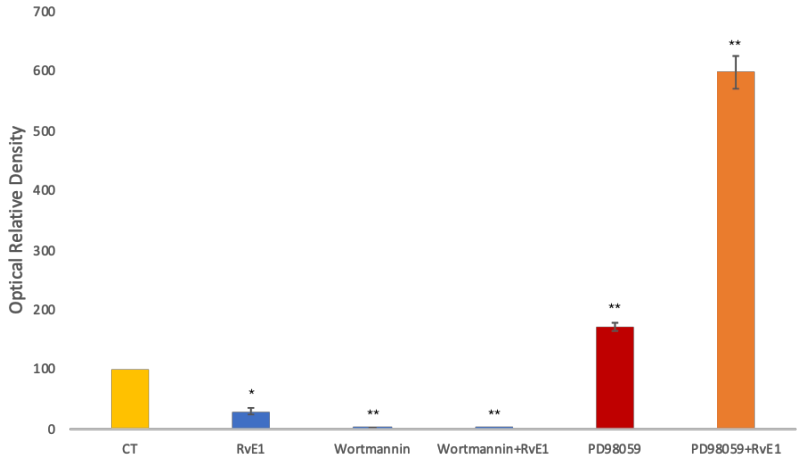


C

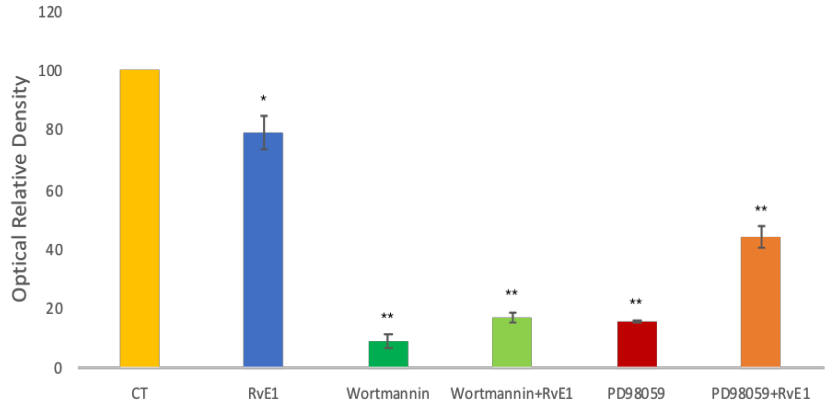
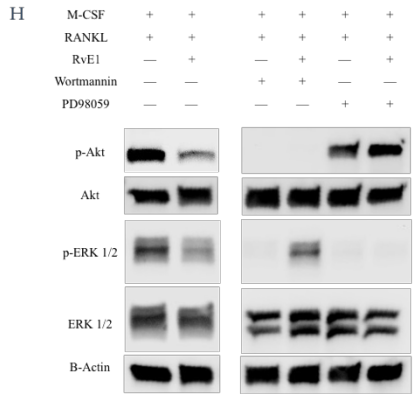


Figure 24. Wortmannin and PD98059 inhibits RvE1 signaling in osteoclast precursors. In osteoclast precursor cell cultures, PD98059-induced and PD98059/RvE1-induced osteoclast precursor cell cultures showed significantly higher phosphorylation levels of **(A & B)** Akt (Ser473) ($P<0.01$) compared to RvE1-induced osteoclast precursor cell cultures. However, Wortmannin-induced and Wortmannin/RvE1-induced osteoclast precursor cell cultures showed significantly lower phosphorylation levels of **(A & C)** p44/42 MAPK (Erk1/2) (Thr202/Tyr204) ($P<0.01$) compared to RvE1-induced osteoclast precursor cell cultures. This could indicate that PI3K/Akt is upstream to MAPK/ERK in RvE1 stimulated signaling in osteoclast precursor cell cultures.

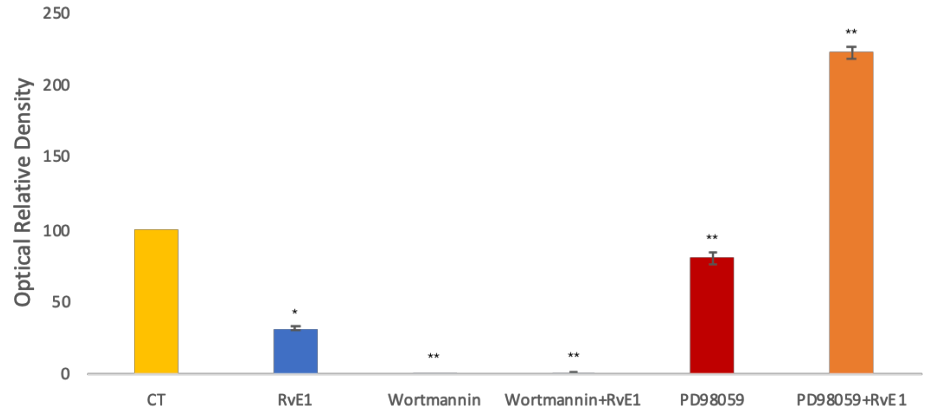
(Mean + SD, ANOVA *, **, *** $P< 0.05$). * comparisons were made to the control group. ** comparisons were made to RvE1 group.

Figure 25. Wortmannin and PD98059 inhibits RvE1 signaling in osteoclasts.

A



B



C

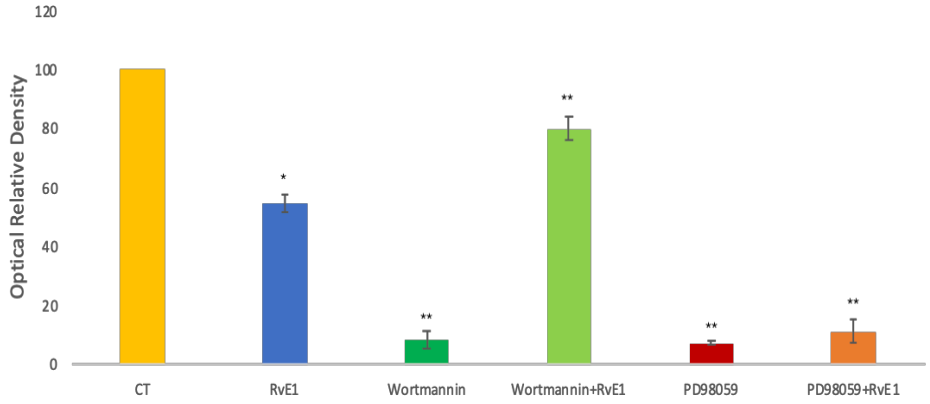
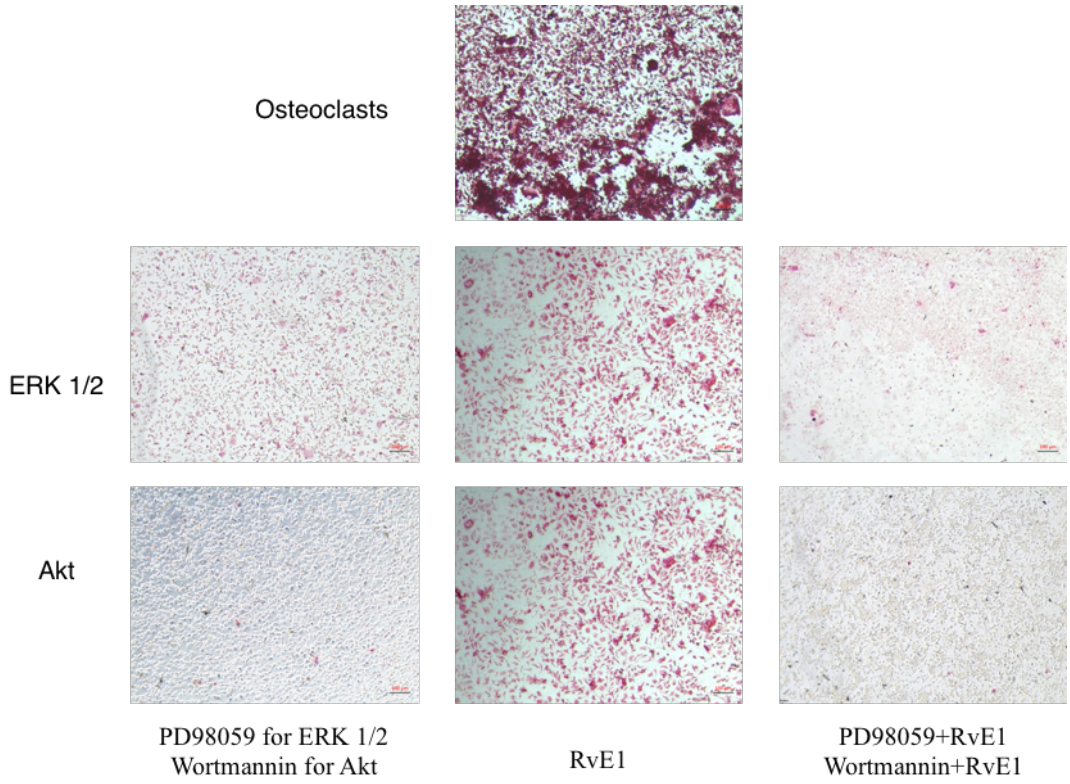
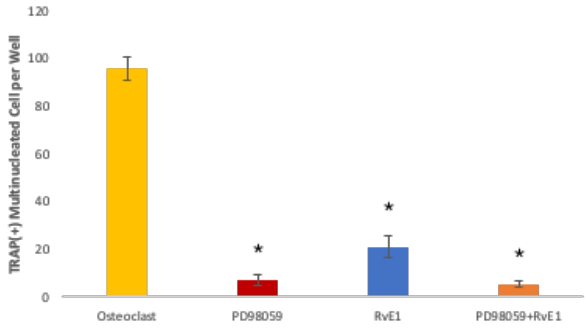


Figure 25. Wortmannin and PD98059 inhibits RvE1 signaling in osteoclasts.

D



E



F

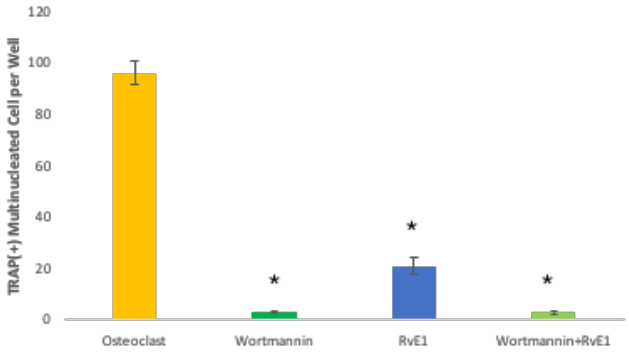


Figure 25. Wortmannin and PD98059 inhibits RvE1 signaling in osteoclasts. In osteoclast cell cultures, PD98059-induced and PD98059/RvE1-induced osteoclast cell cultures showed significantly higher phosphorylation levels of **(A & B)** Akt (Ser473) ($P<0.01$) compared to RvE1-induced osteoclast cell cultures. Only Wortmannin-induced osteoclast cell cultures showed significantly lower phosphorylation levels of **(A & C)** p44/42 MAPK (Erk1/2) (Thr202/Tyr204) ($P<0.01$) compared to RvE1-induced osteoclast cell cultures. Conversely, Wortmannin/RvE1-induced osteoclast cell cultures showed significantly lower phosphorylation levels of p44/42 MAPK (Erk1/2) (Thr202/Tyr204) ($P<0.01$) compared to RvE1-induced osteoclast cell cultures, indicating a possible alternative pathway through a different receptor. **(D)** Measurements of osteoclast differentiation were made by counting the number of TRAP-stained multinucleated osteoclast using 20X objectives field of view images as previously described. **(E & F)** Wortmannin-induced, Wortmannin/RvE1-induced, PD98059-induced and PD98059/RvE1-induced osteoclast cell cultures all showed significant reduction in number of TRAP-stained multinucleated osteoclast compared to control osteoclast precursor and osteoclast cell cultures. This indicate a lack of osteoclastic phenotype related to inhibiting PI3K/Akt & MAPK/ERK signaling pathways.

(Mean + SD, ANOVA *, **, *** $P< 0.05$). * comparisons were made to the control group. ** comparisons were made to RvE1 group.

CHAPTER FOUR
DISCUSSION

Osteoclastogenesis and inflammation interact through a complex network between bone and immune cells that exists as part of the osteoimmune system (Dar et al., 2018, Geusens and Lems, 2011, Nakashima and Takayanagi, 2009). Active inflammation resolution is part of this osteoimmune system and is mediated by several molecules systemically and locally (Savill et al., 1989a, Savill et al., 1989b, Serhan, 2007, Serhan, 2010, Serhan, 2011). These mediators activate macrophage non-phlogistic phagocytosis of neutrophils, normalize endothelial vascular permeability, reduce neutrophils transmigration and chemotaxis, attenuate the production of many local cytokines and chemokines and blocks platelet aggregation (Serhan, 2010). Previous studies have demonstrated that RvE1 is a key resolution mediator that is involved profoundly in all these active processes (Ariel et al., 2006, Bannenberg et al., 2005, Haworth et al., 2008). However, RvE1 was demonstrated to induce active direct actions on osteoclasts as well by inhibiting their differentiation and fusion *in vitro* (Herrera et al., 2008, Zhu et al., 2013).

RvE1 interactions are mediated through two receptors; ChemR23 (also known as ERV1) and BLT1. ChemR23 is expressed on monocytes, macrophages, dendritic cells, neutrophils and CD4+ T lymphocytes and osteoclasts, while BLT1 is expressed on polymorphonuclear leukocytes (PMN) and osteoclasts. (Arita et al., 2005, Yokomizo, 2011, Herrera et al., 2008). RvE1 down-regulates DC-STAMP and NFATc1 in osteoclasts by interacting with BLT1. (Zhu et al., 2013). However, there is limited data regarding RvE1 early cellular and molecular regulatory interactions and the pathways involved in signal transduction through the BLT1 receptor prior to NFATc1 inhibition. Hence, in this research project, we investigated RvE1 preventive regulation and direct

actions on osteoclasts *in vivo* using a ligature-induced alveolar bone loss disease model in mice and *in vitro* to elucidate its regulation of bone marrow-derived preosteoclast differentiation, proliferation, survival and apoptosis through the BLT1 receptor and the molecular signaling pathways involved.

4.1. RvE1 direct protective bone regulation *in vivo*

The development of a successful periodontal disease model in animals requires constructing standardized experimental methods. Previously, an experimental periodontal disease model in rabbits used ligature placement combined with topical application of *P. gingivalis*. also, the preventive regulation of RvE1 leading to diminished leukocyte infiltration and reduced alveolar bone loss progression were established in this experimental model (Hasturk et al., 2007, Hasturk et al., 2006). Therefore, a successful experimental periodontal disease model in mice was established to examine RvE1 protective regulation of alveolar bone and direct actions on osteoclasts. As shown in the presented data, ligature placement for one week resulted in significant alveolar bone loss compared to no ligature placement. In this *in vivo* model in mice, placing a ligature around maxillary second molars was enough to create alveolar bone loss similar to previous established models without the need for topical application of *P. gingivalis*. Also, RvE1 local application with ligature placement resulted in a significantly less alveolar bone loss compared ligature placement with vehicle, which is consistent with previously demonstrated protective actions of RvE1.

After establishing a successful periodontal disease model in mice and demonstrating RvE1 prevention of alveolar bone loss, RvE1 direct actions on osteoclasts were investigated. Histological analyses showed that ligature placement for one week resulted in significant increase in TRAP-stained multinucleated osteoclast cell counts compared to no ligature placement. Also, RvE1 local

application with ligature placement resulted in a significant decrease in TRAP-stained multinucleated osteoclast cell count compared to ligature placement with vehicle. These data suggested RvE1 induced protective actions to prevent alveolar bone loss through reducing the number of active multinucleated osteoclasts, which might indicate possible direct actions of RvE1 on osteoclasts. However, further investigations were needed since RvE1 could stimulates reduced osteoclasts count indirectly through inducing reduction of the release of osteoclastogenic pro-inflammatory mediators. Moreover, further analyses of the distribution and size of osteoclasts revealed two very critical findings. The first one was that RvE1 local application with ligature placement significantly decreased TRAP-stained mono- and multi-nucleated osteoclasts compared to ligature placement alone. This indicates that RvE1 inhibited both; multinucleated osteoclast differentiation and fusion and mono-nucleated osteoclasts proliferation and survival prior to their differentiation and fusion into multinucleated active osteoclasts, was augmenting the findings of (Zhu et al., 2013). The second one was that RvE1 local application with ligature placement significantly decreased TRAP-stained osteoclasts among the entire length of the alveolar bone adjacent to the maxillary second molar roots compared to ligature placement alone. This demonstrates the potent RvE1 pro-resolving actions in periodontitis extending all the way along the entire length of the tooth, which is similar to previous findings (Hasturk et al., 2007, Hasturk et al., 2006).

4.2. RvE1 targeting osteoclast precursor and osteoclast differentiation through BLT1 receptors

RvE1 bone protective actions during inflammation are complex, intricate and involve regulation of PMN, macrophages and several other cells. Previous studies from our group have shown that RvE1 has direct actions through the BLT1 receptor on osteoclasts leading to inhibition of osteoclast fusion and favoring bone preservation (Zhu et al., 2013). Also, *in vitro* inhibition of osteoclastogenesis by RvE1 has already been established previously (Herrera et al., 2008). However, no previous investigations were performed to examine RvE1 preventive regulatory actions on osteoclast precursor and osteoclast differentiation through the BLT1 receptor. Our *in vivo* prior data suggested possible direct actions of RvE1 on osteoclasts as well. Therefore, RvE1 actions were tested in a dose-dependent manner and through using a BLT1 inhibitor (U75302) on osteoclast precursor and osteoclast cell cultures to evaluate RvE1 direct actions on their differentiation. As shown in the results section, RvE1 reduced the expression of mRNA cell-specific markers of osteoclasts, but not osteoclast precursors indicating no possible regulatory effect exerted by RvE1 on the precursor cells at the gene expression level, which is consistent with a previous report (Zhu et al., 2013). However, TRAP staining, in a dose-dependent manner, and activity assays demonstrated RvE1 capability to reduce the number of mature osteoclasts when it is delivered in the tissue cultures only at the early osteoclast precursor phase. This indicates that RvE1 is negatively affecting osteoclast precursors yielding a smaller number of active osteoclasts in later stages. Moreover, immunohistochemistry analyses revealed that BLT1 receptor is expressed on the cell surface of mice alveolar bone osteoclasts and bone marrow-derived osteoclast precursors and osteoclasts. These data, together with U75302/RvE1-osteoclast cultures showing no decrease in number of TRAP-stained multinucleated osteoclasts compared to control-osteoclast

cultures demonstrate RvE1 preventive regulation of osteoclast precursor and osteoclast differentiation through BLT1 receptor

4.3. RvE1 direct pleomorphic anti-catabolic regulation of osteoclast precursors and osteoclasts

As previously mentioned, RvE1 reduces cell fusion in the late stages of osteoclast differentiation, which leads to reduced osteoclast formation through targeting DC-STAMP and NFATc1 (Zhu et al., 2013). However, our data suggested that RvE1 inhibited *in vivo* mono-nucleated osteoclast proliferation and survival prior to their differentiation and fusion into multinucleated active osteoclasts and induced direct preventive actions on osteoclasts when delivered in the tissue cultures only at the early osteoclast precursor phase. Therefore, investigations of RvE1 direct preventive regulation of osteoclast precursor and osteoclast proliferation and survival prior to cell-cell fusion were performed. RvE1 significantly reduced proliferation of osteoclast precursors and osteoclasts in a dose-dependent manner. Also, U75302/RvE1-cell cultures did not show any decrease in the proliferation rate of osteoclast precursors and osteoclasts compared to control-cell cultures solidifying the previous results that indicated RvE1 preventive regulation is through the BLT1 receptor. Moreover, RvE1 significantly reduced viability, in a dose-dependent manner, and increased apoptosis of osteoclast precursors and osteoclasts. Also, U75302/RvE1-cell cultures did not show any decrease in the viability or increase in apoptosis of osteoclast precursors and osteoclasts compared to control-cell cultures. A third test was performed to investigate changes in the levels of cleaved caspase 3 indicated higher quantities in RvE1-cell cultures compared to control-cells. Taken together, these data suggest that RvE1 decreased osteoclast precursor and

osteoclast proliferation and survival (decreased viability and increased apoptosis) through BLT1 receptor.

Previous reports regarding resolvin regulation of osteoclast proliferation, viability and apoptosis were conflicting (Benabdoun et al., 2019, Funaki et al., 2018, Herrera et al., 2008, Lee et al., 2016). A possible explanation for these inconsistent data could be attributed to the different experimental designs and RvE1 doses used in these reports compared to our study. Furthermore, as shown in our data, RvE1 preventive regulation of proliferation and survival at the 100nM dose were inconsistent, which indicate a possible feed-back mechanism to reduce excessive effects or other RvE1 targets activated only at higher doses, which may also explain some of the contradictory data as well.

4.4. RvE1/BLT1 signaling through PI3K/Akt

Our data demonstrated that RvE1 regulated osteoclasts directly preventing cell differentiation and reducing cell proliferation and survival. In leukocytes, RvE1 inhibits calcium mobilization stimulated by LTB4-BLT1 interaction and reduces the BLT1 intracellular signal activation of NF-kB. Also, RvE1, through BLT1, inhibits the essential osteoclast differentiation expression factor NFATc1 binding to the fusion protein DC-STAMP promoter leading to down-regulating its expression by 65.4%. (Zhu et al., 2013). However, there is limited data regarding RvE1 intracellular signaling events mediated via BLT1 prior to NFATc1 inhibition in osteoclasts. M-CSF- and RANKL-induced upstream activation of bone marrow-derived macrophages c-Fms and RANK receptors leads to increased osteoclast precursor and osteoclast proliferation, survival, anti-apoptosis and differentiation through the MAPK(ERK-JNK-P38) and PI3K/Akt pathways (Cobrinik, 2005, Helgason et al., 1998, Takeshita et al., 2002, Funakoshi-Tago et al., 2003, Wong

et al., 1999a, Xing et al., 2001). Therefore, we investigated these pathways to examine them as possible targets for the RvE1 preventive regulation action of osteoclast precursor and osteoclast proliferation, survival, anti-apoptosis and differentiation.

We showed in our data that RvE1 significantly decreased phosphorylation levels of Akt (Ser473) and p44/42 Erk1/2 (Thr202/Tyr204) in osteoclast precursor cultures. This indicates that both pathways are possible candidates for RvE1-BLT1 mediated signaling leading to reduced cell differentiation, proliferation and survival. However, the LTB_4-induced and U75302/RvE1-induced osteoclast precursor cell cultures showed significantly higher phosphorylation levels of Akt (Ser473) and significantly lower phosphorylation levels of p44/42 Erk1/2 (Thr202/Tyr204) compared to RvE1-induced osteoclast precursor cell cultures. These data validate PI3K/Akt signaling pathway over the MAPK(ERK) one via BLT1 receptor in osteoclast precursor cell cultures since LTB_4 and U75302 triggered, compared to RvE1 cultures, higher phosphorylation levels of Akt but not ERK.

Moreover, RvE1 significantly decreased phosphorylation levels of Akt (Ser473) and p44/42 Erk1/2 (Thr202/Tyr204) in osteoclast cultures but did not affect the phosphorylation levels of NF-κB p65 (Ser536), IκBα (Ser32), c-Jun (Ser63) and c-Fos (Ser32). Also, LTB_4-induced and U75302/RvE1-induced osteoclast cell cultures showed significantly higher phosphorylation levels of Akt (Ser473) but only LTB_4-induced cultures stimulated higher phosphorylation levels of p44/42 Erk1/2 (Thr202/Tyr204) compared to RvE1-induced. These data validate PI3K/Akt and MAPK(ERK) signaling pathways but suggests a possible alternative receptor for RvE1 triggering lower phosphorylation levels of ERK when BLT1 receptor is blocked by U75302.

Taken all together, our data suggests PI3K/Akt as a stronger candidate and more validated signaling pathway for the RvE1-BLT1 interactions compared to the ERK pathway. Several

previous studies support these findings as shown by Fukuda et al., that PI3K inhibitors prevented LTB_4-BLT1 signaling while Mek, of the ERK pathway, inhibitors did not, indicating a possible BLT1/PI3K/Akt pathway for RvE1-BLT1 interaction (Fukuda et al., 2005). Previous studies established that BLT1 transduces intracellular signals in osteoclasts rather through PTX-sensitive Gi protein and Rac1. The cell proliferation, survival and anti-apoptotic Akt pathway is activated either synergistically by Rac1 and PI3K or through a positive feedback loop between Rac1 and PI3K to control Akt activation (Hikiji et al., 2009). However, ERK signaling pathway cannot be ruled out completely since lower phosphorylation levels were induced by RvE1 induction. To further validate the PI3K/Akt pathway and provide explanations for the inconsistent ERK pathway findings, Wortmannin-induced, Wortmannin/RvE1-induced, PD98059-induced and PD98059/RvE1-induced osteoclast precursor and osteoclasts cell cultures were examined.

wortmannin-induced and wortmannin/RvE1-induced osteoclast precursor cell cultures showed significantly lower phosphorylation levels of Akt (Ser473) and p44/42 Erk1/2 (Thr202/Tyr204) compared to RvE1-induced ones while PD98059-induced and PD98059/RvE1-induced osteoclast precursor cell cultures showed significantly higher phosphorylation levels of Akt (Ser473) and lower phosphorylation levels of p44/42 Erk1/2 (Thr202/Tyr204) compared to RvE1-induced ones. This could indicate a cross-talk between PI3K/Akt and ERK pathways with PI3K/Akt being upstream in the RvE1 stimulated signaling in osteoclast precursor cell cultures.

Furthermore, wortmannin-induced osteoclast cell cultures showed significantly lower phosphorylation levels of Akt (Ser473) and p44/42 Erk1/2 (Thr202/Tyr204) while wortmannin/RvE1-induced ones showed significantly lower phosphorylation levels of Akt (Ser473) and higher phosphorylation levels of p44/42 Erk1/2 (Thr202/Tyr204) compared to RvE1-

induced cultures. This finding further drives the idea of a possible alternative pathway through a different receptor for RvE1 to induce the ERK pathway in osteoclast cell cultures.

Moreover, the significantly higher phosphorylation levels of Akt (Ser473) of PD98059-induced and PD98059/RvE1-induced osteoclast cell cultures compared to RvE1-induced ones promote the possibility that PI3K/Akt is upstream to MAPK/ERK in RvE1 stimulated signaling in osteoclast precursor and osteoclast cell cultures.

Finally, measurements of osteoclast differentiation showed significant reduction in number of TRAP-stained multinucleated osteoclast the wortmannin-induced, wortmannin/RvE1-induced, PD98059-induced and PD98059/RvE1-induced osteoclast precursor and osteoclast cell cultures compared to control one.

All the previous data and explanations provided validate the PI3K/Akt pathway and clarify some of the inconsistent results observed in the ERK pathway induced by RvE1. Previous reports demonstrated that RvE1 reduces the phosphorylation levels of Akt in osteoclasts in vitro. Also, they show that osteoclasts express both of RvE1 receptors; ChemR23 and BLT1. However, in vitro competitive radioligand binding with [H]-labeled RvE1suggested RvE1 to bind specifically to BLT-1 and not to ChemR23 (Herrera et al., 2008). Nevertheless, RvE1 binding to ChemR23 could provide an explanation for RvE1 stimulation of the ERK pathway in BLT1-inhibited cell cultures. Also, cross-talks between PI3K/Akt, ERK, JNK and P38 pathways in macrophages and osteoclasts is evident in the literature (Bode et al., 2012, McGuire et al., 2013, Meng et al., 2014, Xia et al., 2016), which support our finding of a possible cross talk between PI3K/Akt and ERK pathways with PI3K/Akt being upstream in the RvE1 stimulated signaling in osteoclast precursor and osteoclast cell cultures. A proposed signaling pathway for RvE1-BLT1 interaction was created based on the previous presented data showing the osteoclastogenesis signaling pathways of M-

CSF—c-fms and RANKL—RANK and the possible interaction points with the RvE1—BLT1 signaling pathway (Fig. 26).

4.5. Future directions

The osteoclastogenesis process is regulated by numerous pro- and anti-inflammatory mediators. RvE1 pro-resolving actions include regulation of several immune cells such as macrophages, neutrophils and Th17 subset of CD4+cells through controlling the production of their inflammatory mediators. Hence, exploring the possible indirect regulatory effects of RvE1 on osteoclastogenesis has great potentials in elucidating the full impact of RvE1 on the process of osteoclastogenesis.

Also, another possible pathway for transducing signals of RvE1-BLT1 interaction other than the previously mentioned ones exists through attenuating the activated pathway of phospholipase C (PLC_B) and reducing intracellular calcium flux mediated by calcium release–activated channel (CRAC) leading to inhibition of the essential transcription factor nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) (Dixit et al., 2014). Therefore, investigating this pathway is very critical since previous reports demonstrated that RvE1 down-regulates NFATc1 in osteoclasts by interacting with BLT1. (Zhu et al., 2013).

Figure 26. Proposed RvE1/BLT1 signaling pathway in osteoclast precursors and osteoclasts.

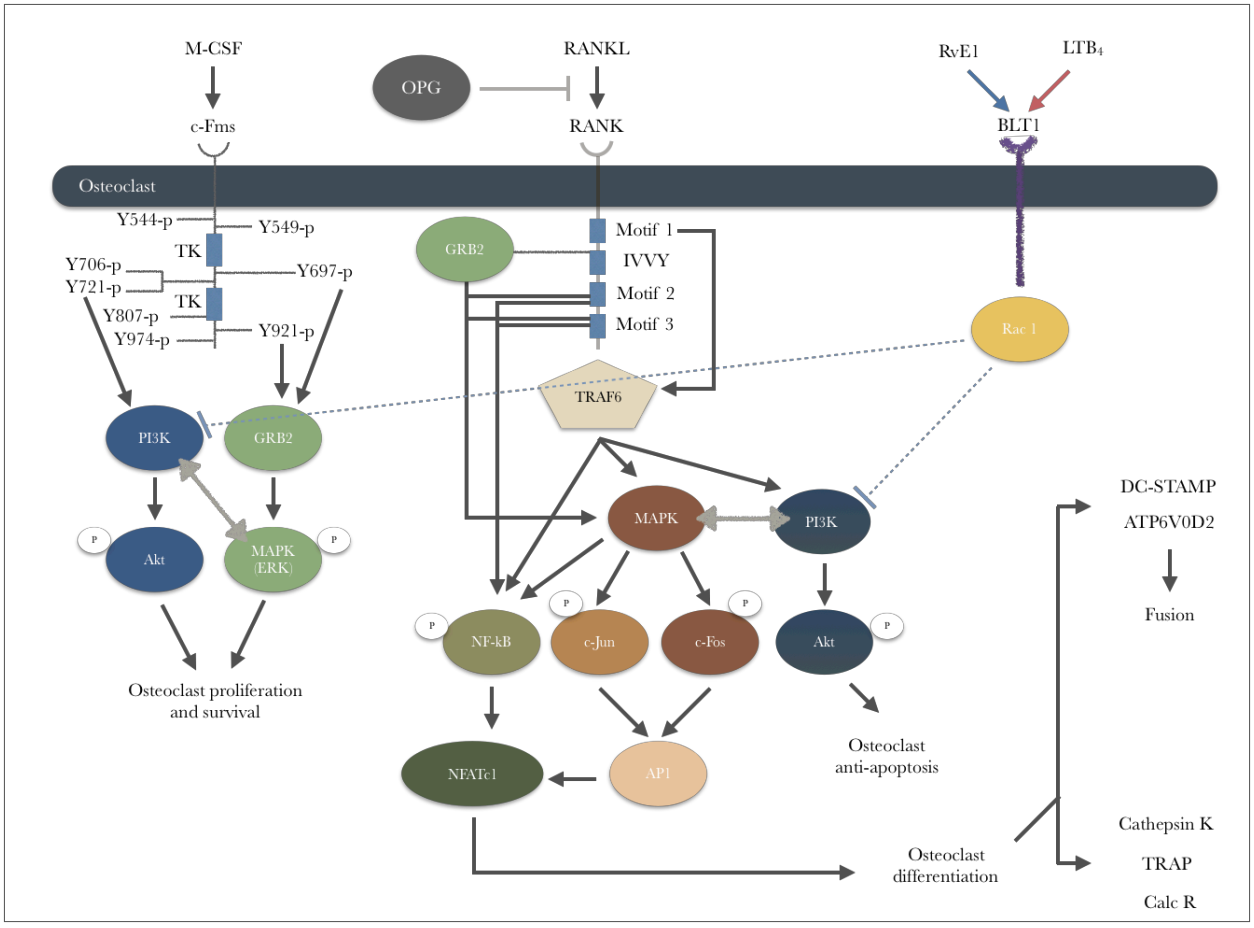


Figure 26. Proposed RvE1/BLT1 signaling pathway in osteoclast precursors and osteoclasts. PI3K/Akt and ERK pathways are responsible for regulating osteoclast proliferation, survival and anti-apoptosis. Several previous reports showed that LTB_4-BLT1/Rac1 interaction is transduced through the PI3K/Akt pathway rather than the ERK one indicating it is the pathway for RvE1-BLT1 interaction. Our data shows similar findings as BLT1/PI3K/Akt pathway proposed to be the pathway for RvE1-BLT1 signaling with possible cross-talks with the ERK pathway.

CHAPTER FIVE
SUMMARY AND CONCLUSIONS

Periodontitis, Rheumatoid arthritis and osteoporosis are all conditions where the balance of bone resorption and bone formation is deregulated. These inflammatory bone diseases typically occur when target tissues are infiltrated by macrophages, neutrophils, and T cells leading to a chronic inflammatory response. This is followed by the activation of osteoclasts leading to distorted bone turnover and gradual bone loss. Complete resolution of inflammation, defined as the complete removal of inflammatory cells with return to homeostasis, is considered the ideal and optimum outcome of acute inflammation. Tissue injury, scarring, and fibrosis are all other possible undesirable outcomes of inflammation when it doesn't resolve and becomes chronic. All of these unwanted consequences must be prevented to provide an optimum environment for tissue healing.

To control inflammation, endogenous specialized proresolving mediator (SPM) agonists that lead to inflammation resolution are considered a better approach than inflammatory inhibitors or immunosuppressors for treatment and prevention of inflammatory bone diseases. Eicosanoid-based lipid mediators (LM) have a significant impact on wound healing. There are numerous subclasses of eicosanoids, which include the classical proinflammatory prostaglandins (PG) and leukotrienes (LT), and the relatively newly described proresolution lipoxins (LX), resolvins (Rv) and protectins. These local mediators are highly efficient in treating a number of inflammatory diseases in which inflammation plays a significant role in animal models.

Previous studies have demonstrated that resolvins directly interact with bone cells to exert their activity. RvE1 treatment of isolated osteoclasts reduces cell fusion in the late stages of

osteoclast differentiation. The overall goal of these studies was to elucidate RvE1 preventive regulation of osteoclasts in vivo and in vitro and to investigate the molecular signaling pathways involved.

The hypotheses tested here in our studies were that RvE1 prevents bone loss in vivo in a ligature-induced periodontal disease model in mice and that RvE1 prevents bone marrow-derived osteoclast precursor and osteoclast in vitro differentiation and reduces their proliferation and survival through interfering with the activation/deactivation of the signaling pathways involved.

Four specific aims were proposed based on these hypotheses; 1) Establish RvE1 preventive regulation of bone loss in a ligature-induced periodontal alveolar bone loss in mice 2) Determine RvE1 regulation of osteoclast precursor and osteoclast differentiation through BLT1 receptors in bone marrow-derived cells 3) Determine RvE1 regulation of bone marrow-derived osteoclast precursor and osteoclast proliferation, viability and apoptosis 4) Determine the molecular signaling pathways involved in RvE1 regulation of bone marrow-derived osteoclast precursor and osteoclast differentiation, survival, proliferation and apoptosis.

Our investigations showed that RvE1 reduced bone loss area and volume at the maxillary second molar furcation and number of mono- and multi-nucleated TRAP-stained osteoclasts along the entire length of the tooth in ligature-induced periodontal disease model in mice. Also, RvE1 down-regulated gene expression of osteoclast markers and hindered their differentiation when induced at early preosteoclast and late phases in a dose-dependent manner.

Moreover, BLT1 was revealed to be expressed on the cell surface of mice alveolar bone osteoclasts and bone marrow-derived osteoclast precursors and osteoclasts. Also, U75302-osteoclast cultures and U75302/RvE1-osteoclast cultures did not show any decrease in number of TRAP-stained multinucleated osteoclasts compared to control ones.

Proliferation and survival experiments showed that RvE1 reduced osteoclast precursor and osteoclast proliferation and viability in a dose-dependent manner through BLT1 receptor. Also, RvE1 increased osteoclast precursor and osteoclast apoptosis through BLT1 receptor and induced higher cleaved caspase 3 levels.

Finally, RvE1 demonstrated to induce lower levels of phosphorylation of Akt and to some extent ERK pathways when compared to control groups. Also, results obtained through using LTB_4-, U75302-, wortmannin- and PD98059-induced osteoclast precursor and osteoclast cultures validated the Akt pathway for the RvE1-BLT1-Rac1 signaling and uncovered possible cross-talks between Akt and ERK as well as possible ERK inducing receptors and intracellular signaling molecules for RvE1 other than the BLT1-Rac1 pathway.

In conclusion, RvE1 decreases bone loss and number of osteoclasts *in vivo* in a ligature-induced periodontal disease model in mice. Also, RvE1 prevents bone marrow-derived osteoclast precursor and osteoclast in vitro differentiation, reduces their proliferation and viability and increases their apoptosis via BLT1 receptor. RvE1-BLT1 signaling is transduced through attenuating the activation of the PI3L/Akt signaling pathway.

LIST OF JOURNAL TITLES ABBREVIATIONS

Allergy	Allergy
Am J Pathol	The American journal of pathology
Am J Respir Crit Care Med	American journal of respiratory and critical care medicine
Ann Periodontol	Annals of periodontology
Annu Rev Immunol	Annual Review of Immunology
Arterioscler Thromb Vase Biol	Arteriosclerosis, thrombosis, and vascular biology
Arthritis Res Ther	Arthritis Research & Therapy
Biochem Biophys Res Commun	Biochemical and Biophysical Research Communications
Biochemistry	Biochemistry
Biochim Biophys Acta	Biochimica et biophysica acta
Blood	Blood
BMC Immunol	BioMed Central immunology
Bone	Bone
Br J Pharmacol	British journal of pharmacology
Cardiovasc Res	Cardiovascular research
Cell	Cell
Cell Differ	Cell differentiation
Circulation	Circulation
Curr Opin Pharmacol	Current opinion in pharmacology
Dev Cell	Developmental cell
EMBOJ	The EMBO journal

Endocr Rev	Endocrine reviews
Endocrinology	Endocrinology
Eur J Immunol	European journal of immunology
Exp Cell Res	Experimental cell research
FASEB J	The FASEB journal: official publication of the Federation of American Societies for Experimental Biology.
Front Biosci	Frontiers in bioscience: a journal and virtual library.
Gene	Gene
Genes Dev	Genes & development
Immunity	Immunity
Immunol Rev	Immunological reviews
Immunology	Immunology
Inflamm Bowel Dis	Inflammatory bowel diseases
J Biol Chem	The Journal of biological chemistry
J Bone Miner Res	Journal of bone and mineral research
J Cell Sci	Journal of cell science
J Clin Endocrinol Metab	The Journal of clinical endocrinology and metabolism
J Clin Invest	The Journal of clinical investigation
J Clin Periodontol	Journal of clinical periodontology
J Dent Res	Journal of dental research
J Exp Med	The Journal of experimental medicine
J Immunol	The Journal of immunology
J Ocul Pharmacol Ther	Journal of ocular pharmacology and therapeutics

J Pathol Bacteriol	The Journal of pathology and bacteriology
J Rheumatol	The Journal of rheumatology
J Steroid Biochem Mol Biol	The Journal of steroid biochemistry and molecular biology
Lancet	Lancet
Microbes Infect	Microbes and infection
N Engl J Med	The New England journal of medicine
Nat Immunol	Nature immunology
Nat Rev Cancer	Nature reviews. Cancer.
Nat Rev Drug Discov	Nature reviews. Drug discovery
Nat Rev Immunol	Nature reviews. Immunology
Oncogene	Oncogene
Periodontol 2000	Periodontology 2000
PLoS One	PloS one
Proc Natl Acad Sci U S A	Proceedings of the National Academy of Sciences of the United States of America
Prostaglandins Leukot Essent Fatty Acids	Prostaglandins, leukotrienes, and essential fatty acids
Respir Med	Respiratory medicine
Science	Science
Trends Cardiovasc Med	Trends in cardiovascular medicine
Trends Cell Biol	Trends in cell biology
Trends Mol Med	Trends in molecular medicine

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