THE UNIVERSITY of ADELAIDE

Epigenetic Regulation of Cells Involved in Periodontal Bone Destruction through Targeted Histone Deacetylase Inhibition

A thesis submitted in fulfilment for degree of

DOCTOR OF PHILOSOPHY

In

The Discipline of Anatomy and Pathology
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The University of Adelaide

By

Kent Algate

31.01.2018

Declaration

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Thesis Abstract

Periodontitis (PD) is one of the most common bone loss pathologies in adults and currently affects more than 60% of the population in its destructive form. Ineffective or surgically invasive treatment options can result in patient non-compliance, gingival recession, alveolar bone destruction and eventual loss of teeth. Aside from the psychosocial effects of poor dental health, PD has been associated with a variety of systemic conditions, such as rheumatoid arthritis and cardiovascular disease, exacerbating their onset and severity. Histone deacetylase (HDAC) enzymes are molecules that control cellular activity through modifications to gene expression and protein function at an epigenetic level. Alterations to HDAC expression or function can cause diminished physiological cell regulation, thought to be an essential factor in the pathogenesis of disease. The use of selective HDAC inhibitors (HDACi) targeting candidate HDACs may be an effective, non-invasive therapeutic tool to treat both inflammation and bone loss in PD. The aim of this research was to investigate the effects of novel HDACi designed to target individual HDAC isoforms as a PD treatment. *In vitro* investigations identified therapeutic potential for targeting HDAC 1 and HDAC 2 during the inflammatory response and catabolic actions of human monocytes and osteoclasts. Whereas HDAC 5 inhibition holds regenerative potential, as osteoblastogenesis and matrix mineralisation was induced by its suppression. With the additional identification and characterisation of candidate HDAC isoforms in an *in vivo* mouse model of PD, the results of this thesis will provide a strong foundation for future investigations and clinical translation of HDACi.

Table of Abbreviations

Activator protein 1	AP-1	Neurodegenerative diseases	NDs
Alkaline Phosphatase	ALP	Non-steroidal anti-inflammatories	NSAIDs
Baculoviral inhibitors of apopotosis proteins	C-IAPs	Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-KB
Bone marrow mesenchymal	BMM	Nuclear factor of activated T cells cytoplasmic 1	NFATc1
Bone morphogenic protein	BMP	Osteocalcin	OCN
C-jun N-terminal kinases	JNK	Osteoclast-associated immunoglobulin-like receptor	OSCAR
Calcitonin receptor	CTR	Osteopontin	OPN
Cathepsin K	CatK	Osteoprotegerin	OPG
Collagen type 1 α1	Col1α1	Periodontal ligament	PDL
Cylindromatosis	CYLD	Periodontitis	PD
Dental Pulp	DP	Peripheral Blood Mononuclear Cells	PBMC
Deoxyribonucleic Acid	DNA	Porphyromonas Gingivalis	P.ging
Dimethyl Sulfoxide	DMSO	Quantitative real time polymerase chain reaction	qRT-PCR
Fas-associated death domain	FADD	Receptor activator of NF-KB	RANK
Field emissions scanning electron microscope	FESEM	Receptor activator of NF-KB ligand	RANKL
Fusobacterium Nucleatum	F.nuc	Receptor-interacting serine-threonine kinase 1	RIP1
Histone Acetyltransferase	HAT	Recombinant human	rh
Histone Deacetylase	HDAC	Rheumatoid Arthritis	RA
Histone Deacetylase Inhibitor	HDACi	Runt-related transcription factor 2	Runx2
Human acid ribosomal protein	hARP	Sirtuins	SIRT
Inhibitor of kappa-B kinase	IKK	Sodium Butyrate	NaB
Inhibitor of kappa-Bα	IKBα	Stem Cells	SC
Insulin like growth factor-I	IGF-I	Stromal-derived factor-1	SDF-1
Interferon	IFN	Suberanilohydroxamic acid	SAHA
Interleukin	IL	Tartrate resistant acid phosphatase	TRAP
Intracellular adhesion molecule 1	ICAM1	Tissue-nonspecifiic alkaline phosphatase	TNAP
Lipopolysaccharide	LPS	TNF Receptor	TNFr
Macrophage colony stimulating factor	M-CSF	TNF receptor associated factor	TRAF
Macrophage colony stimulating factor receptor	M-CSFr	TNF receptor-associated death domain	TRADD
Macrophage inflammatory protein	MIP1α	Transforming growth factor-β1	TGF-β1
Matrix metalloproteinase	MMP	Tumour Necrosis Factor	TNF
Mesenchymal stem cell	MSC	Valproic Acid	VPA
Mitogen-acivated protein 3 kinases	MAP3K	Vorinostat	TSA
Monocyte chemoattractan protein 1	MCP1	Wingless-type MMTV integration site	Wnt
Neural Crest	NC	α-Minimum Essential Media	αMEM

Publications Arising From This Thesis

***K. Algate,** M.D. Cantley, R.C. Reid, D.P. Fairlie, P.M. Bartold, D.R. Haynes (2018) Histone Deacetylase Inhibitor NW-21 regulates TNFα Driven Human Osteoclast Activity. *Journal of Cellular Physiology*.

K. Algate, D.R. Haynes, P.M. Bartold, T.N. Crotti, M.D Cantley (2016) The effects of tumour necrosis factor-α on bone cells involved in periodontal alveolar bone loss; osteoclasts, osteoblasts and osteocytes. *Journal of Periodontal Research*. 51(5) 549-66

PDFs of manuscripts in press can be found in Appendix I

*****Denotes accepted for publication with revision; to be resubmitted

Additional Publications Arising From Outside This Thesis

During my PhD candidature, I was involved in several other published studies as both primary and co-author/investigator. These are as follows:

*B. Williams, H. Kamitakahara, F. Najimi, E. Tsangari, **K. Algate**, S. Chaudary, E. Perilli, T.N. Crotti, D.R. Haynes, A.A. Dharmapatni (2018) The effect of pharmacological modulation of autophagy and apoptosis in PBMC-derived osteoclasts and a mouse model of inflammatory arthritis. *Journal of inflammopharmacology*

#**K. Algate**, #A.A. Dharmapatni, R. Coleman, M. Lorimer, M.D. Cantley, M.D. Smith, M.D. Wechalekar, T.N. Crotti. (2017) Osteoclast-Associate receptor (OSCAR) Distribution in the Synovial Tissues of Patients with Active RA, and TNFα and RANKL Regulation of Expression by Osteoclasts In Vitro. *Inflammation*. 40(5)L1566-1575

M.D. Cantley, A.A. Dharmapatni, **K. Algate**, T.N. Crotti, P.M. Bartold. (2016) Class I and II histone deacetylase expression in human chronic periodontitis gingival tissue. *Journal of Periodontal Research*. 51(2)143-51

S. Saad, A.A. Dharmapatni, T.N. Crotti, M.D. Cantley, **K. Algate**, D.M. Findlay, G.J. Atkins, D.R. Haynes. (2016) Semaphorin-3a, neuropilin-1 and plexin-A1 in prosthetic-particle induced bone loss. *Acta Biomaterials* 30:311-318

PDFs of manuscripts in press can be found in Appendix II

#Denotes equal primary contributors to publication

*****Denotes accepted for publication with revision; to be resubmitted

Chapter 1: The Effects of Tumour Necrosis Factor-α (TNFα) on Alveolar Bone Destruction in Periodontitis and the Therapeutic Potential of Selective Histone Deacetylase Inhibitors

K. Algate, D.R. Haynes, P.M. Bartold, T.N. Crotti, M.D. Cantley

Chapter 1 is an introductory chapter, setting the premise of this thesis. It incorporates a published review paper: The effects of tumour necrosis factor-α on bone cells involved in periodontal alveolar bone loss; osteoclasts, osteoblasts, and osteocytes. **K Algate**, D.R. Haynes, P.M. Bartold, T.N. Crotti, M.D. Cantley. Journal of Periodontal Research 2016;51:549-566

1.1 Abstract

Periodontitis is the most common bone loss pathology in adults and if left untreated, can cause premature tooth loss. Cytokines, such as tumour necrosis factor-α (TNFα), involved in the chronic inflammatory response within the periodontal gingiva, significantly influences the physiological bone remodelling processes. In this chapter, the process of bone metabolism will be discussed whilst reviewing the current understanding of the effects of TNFα on cells in periodontitis (PD). Furthermore, an overview of the current literature supporting the use of epigenetic modulation by histone deacetylase (HDAC) inhibition as a treatment for inflammatory bone loss is discussed. Evidence published to date suggests a potent catabolic role for TNFα through the stimulation of osteoclastic bone resorption as well as the suppression of osteoblastic bone formation and osteocytic regulation and survival. However, the extent and timing of TNFα exposure *in vitro* and *in vivo* greatly influences its effects on skeletal cells, with contradictory anabolic activity observed with TNFα in a number of studies. Pharmacological inhibition of HDAC in inflammatory bone disease produces variable outcomes, and appears to be dependent on dose, timing and isoform specificity. Nonetheless, it is evident that managing the chronic inflammatory response, in addition to the altered bone remodelling process, is required to improve PD treatments.

1.2 Statement of Authorship

Statement of Authorship

Title of Paper	The effects of tumour necrosis factor-α on bone cells invovled in periodontal alveolar bone loss; osteoclasts, osteoblasts and osteocytes
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Kent Algate
Contribution to the Paper	First author and main contributor; Concept and methodological design, investigation, project administration, validation and visualisation, data curation and analysis, formulation of primary draft, in addition to reviewing and incorporating co-author comments and suggestions
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 25.1.18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	David Haynes
Contribution to the Paper	Investigation, conceptualisation, supervision and manuscript review
Signature	Date 29/1/18

Name of Co-Author	Mark Bartold
Contribution to the Paper	conceptualisation and manuscript review
Signature	Date 30.1.18

Name of Co-Author	Tania Crotti
Contribution to the Paper	conceptualisation and manuscript review
Signature	Date 23/1/18

Name of Co-Author	Melissa Cantley
Contribution to the Paper	Investigation, conceptualisation, supervision and manuscript review
Signature	Date 29/1/18

1.3 Introduction

Irreversible alveolar bone destruction is a common outcome of the chronic inflammatory disease known as periodontitis (PD). Alveolar bone is essential for maintaining structural support for the teeth, and its untreated destruction can lead to the loosening and eventual loss of teeth (1). Benign and non-destructive forms of periodontal disease, such as gingivitis, are highly prevalent globally, affecting more than 80% of children and adults in the United States, Canada, Australia and other regions of the world (2-4). In the absence of effective dental management or oral hygiene, this can develop into the more permanent and destructive forms of PD. Chronic PD affects up to 9% of adults aged 18-24 worldwide and more than 60% of those aged over 65 years. Early onset aggressive PD, a less common albeit severe form of PD that causes rapid degeneration of tooth support, affects up to 5% of the juvenile population (2, 4).

Considered the most common bone loss pathology in humans, the initiation and progression of alveolar bone destruction in PD is associated with the inflammatory reaction in the surrounding gingival tissue (5). The immune response is initiated by high numbers of anaerobic bacteria such as *Porphyromonas Gingivalis*, *Fusobacterium Nucleatum*, *Tannerella Forsythensis,* and *Actinobacillus Actinomycetemcomitans* contained within the subgingival biofilm (5-8). Environmental and host factors, such as cigarette smoking (9), genetics (10) and certain medications (11-13) have also been shown to influence the onset of PD and periodontal bone quality. The microbial foreign body challenge induced by lipopolysaccharides present in gram-negative bacterial membranes of the abovementioned species is a potent stimulus for the release of inflammatory molecules by the host, promoting the migration and activation of inflammatory cells (monocytes, macrophages, T-lymphocytes) (14-16). This, in turn, increases the numbers of pro-inflammatory cytokines such as tumour necrosis factor-α (TNFα) and a variety of interleukins (IL) being released into the surrounding tissue (15, 16). These factors, particularly TNFα, are essential to the inflammatory response, vital for pathogen elimination and resolution. However, excessive levels and/or prolonged production of TNFα is implicated in the pathogenesis of numerous autoimmune and inflammatory diseases, including PD.

This host response is now known to be a critical component in the associated bone destruction that occurs in PD. Historically considered two separate and distinct systems within the body, the skeletal and immune systems are now recognized as sharing intricate regulatory pathways, molecules, cytokines and receptors, which not only orchestrate the individual system, but influence the outcome of the other (17, 18). Therefore, effective management of PD should include therapies that target both the inflammation and bone cells involved in alveolar bone loss. This editorial will discuss the effects of inflammation, specifically by TNFα, on osteoclasts, osteoblasts and osteocytes. In addition, Histone Deacetylases (HDACs), a novel therapeutic target to treat the destructive mechanisms observed in PD will be introduced.

1.4 TNFα Signalling

Extensive research on this potent pro-inflammatory cytokine has demonstrated its pivotal role during the immune response and the development of chronic disorders (as reviewed in (19-22)). The protein was first successfully purified and cloned in the early 1980s (23, 24), allowing the systematic mapping of its cellular pathways and signalling mechanisms in a variety of cell types. TNFα is a biologically active protein, distributed as a bound transmembrane unit to its parent cell types, mainly monocytes and T-cells, or in its soluble form after the cleaving actions of TNFα converting enzymes (25, 26). TNFα binds to one of two cell surface receptors (TNFr) that initiate downstream cellular responses. Activation of TNFr1 (55 kDa) can induce cellular proliferation, activation and survival (27, 28), or conversely, it can trigger apoptotic signalling and cell death (28, 29) (Figure 1.1). The conducting pathways that determine survival or cell death through TNFα ligation is dependent on two unique complexes bound to the C-terminal end of the intracellular TNFr1 region. Signalling through complex I involves components that induce cell survival through the expression of anti-apoptotic proteins and the activation of transcription factors, activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (30-33). In contrast, the presence and activation of complex II initiates receptor internalisation, triggering

pathways responsible for cell death and apoptosis (27, 29). TNFr1 related cell survival is achieved through interactions with adaptor proteins, namely members of the TNF receptor associated factor (TRAF) family (TRAF 1, 2 and 3) and the signal mediator TNF receptor-associated death domain (TRADD) (34). TRADD directs signalling information from TNFr1 through TRAF 1 and 2, which leads to the rapid attachment of ubiquitin proteins to receptor-interacting serine-threonine kinase 1 (RIP1) (31, 35). This post-translational modification of RIP1 allows signalling to continue through mitogen-activated protein 3 kinases (MAP3K) and the activation of the inhibitor of kappa-B kinase (IKK) complex (30, 35). IKK complex activation initiates the phosphorylation of inhibitor of kappa-Bα (IκBα), allowing NF-κB to translocate into the nucleus, thus initiating the transcription of anti-apoptotic and proliferative genes (36, 37).

TNFα binding can alternatively induce a conformational change to the cytoplasmic motif of TNFr1, allowing the attachment of additional adaptor proteins to TRADD that constitutes complex II and cell death signalling (38). RIP1 ubiquitination, which is essential for NF-κB activation and cell survival, is reversed by the cylindromatosis (CYLD) enzyme (39-41). This enables the attachment of RIP1 kinase into complex II that consists of pro-caspase 8 and fas-associated death domain (FADD) bound to TRADD (39-41). In turn, signalling through this complex initiates the suppression of NF-κB transcription of anti-apoptotic signals, such as, baculoviral inhibitors of apoptosis proteins (C-IAPs), activating a caspase cascade of cell death through apoptosis. Investigations targeting this TNFα pathway in malignancies have identified CYLD as a key factor causing tumour cell death through inhibition of NF-κB transcription (39-41).

TNFα can alternatively bind to its second receptor TNFr2, which is structurally distinct from TNFr1, being larger (75 kDa) and consisting of a unique cytoplasmic motif absent of the death domain (27, 28). Despite the lower membrane expression of TNFr2 (42), it appears to maintain an important role in regulating and communicating with TNFr1. Studies focusing on the signal transduction pathways of TNFr2 have uncovered several similarities with the TNFr1 proliferation and survival system, in that associated adaptor proteins are required (TRAFs) to transmit signalling through to c-jun N-terminal kinases (JNK) and the IKK

complex (43, 44). These pathways lead to AP-1 and NF-κB activation as well as the transcription of anti-apoptotic signalling and cell survival. However, abnormal TNFr2 activity has been identified in autoimmune disorders and autoreactive T cells (44), where signalling pathways are shifted from proliferation and survival to cell death. It is hypothesized that interruption to this crosstalk between receptors results in signalling preferentially using the TRADD/FADD pathway of cell death.

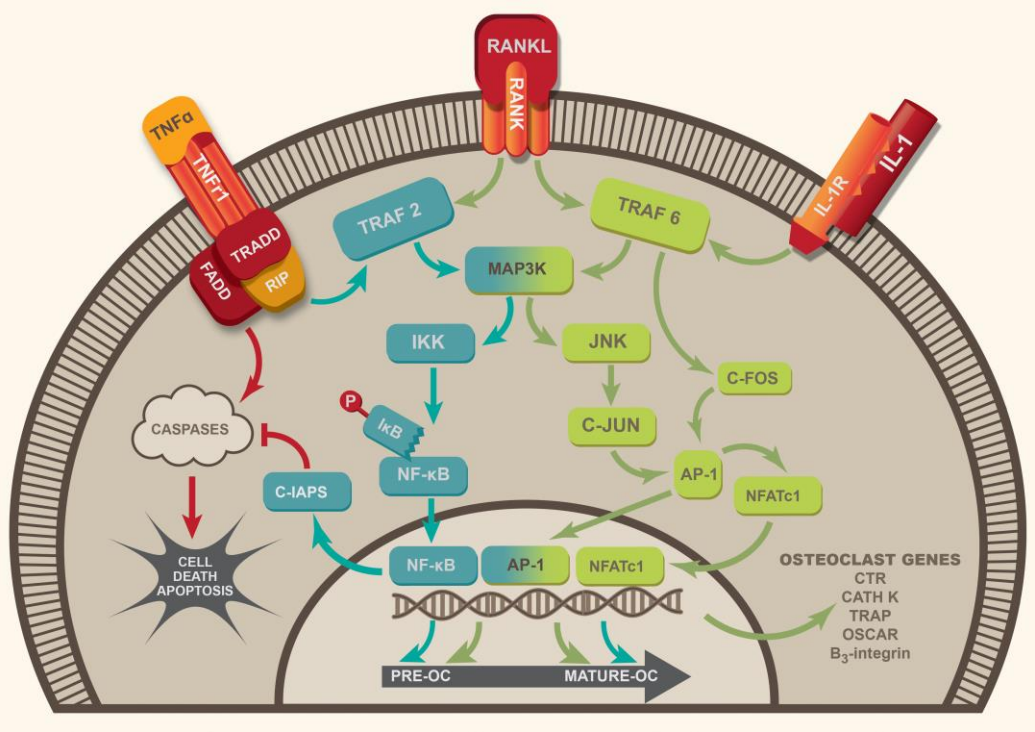

Figure 1.1 Influence of inflammatory cytokines, TNFα and IL-1 on OC signalling (45). Cell proliferation and survival can be activated via TNFα binding to TNFr1 on the cell surface and signalling through TRADD and RIP1 cytoplasmic motifs leading to TRAF 2/NF-κB activation. Conversely, the presence of FADD on the TNFr1 motif induces activation of caspases and cell death. IL-1R activates TRAF 6/NFATc1 signalling, which can be induced in the absence of RANKL interaction with its receptor RANK. AP-1, activator protein 1; CIAPs, inhibitors of apoptosis protein; FADD, fas-associated death domain; IκB, inhibitor of kappa-B; IκK, inhibitor of kappa-B kinase; IL-1, interleukin 1; JNK, c-jun N-terminal kinase; NF-κB, nuclear factor kappa-light chain enhancer of activated B cells; MAP3K, mitogen-activated protein 3 kinase; NFATc1, nuclear factor of activated T-cell cytoplasmic 1; OC, osteoclast; RANKL, receptor activator of nuclear factor B ligand; RIP, receptor interacting serine threonine kinase 1; TNFα, tumour necrosis factor-α; TNFr1, tumour necrosis factor receptor 1; TRADD, tumour necrosis factor receptor associated death domain; TRAF 6, tumour necrosis factor receptor associated factor 6.

1.5 TNFα in Periodontitis

In the human body, TNFα is responsible for mediating inflammatory responses and maintaining immune system activity, in addition to causing apoptosis of specific tumour cell populations (46, 47). Conversely, when there is sustained production of TNFα, a variety of unwanted pathological responses can occur. Studies evaluating PD in humans and experimental animal models have demonstrated significantly higher levels of TNFα in periodontal gingival tissue and crevicular fluid (48-51). These higher levels of TNFα are associated with increased osteoclast formation and activity, leading to reduced alveolar bone volume and tooth support (52, 53). The evaluation of TNFα receptors in periodontitis has demonstrated abundant receptor expression (both TNFr1 and TNFr2) by a variety of infiltrating cell types. These include pre-osteoclastic monocytes/macrophages, fibroblast like cells and endothelial cells (48). With increased production of both TNFα and its corresponding receptors in the inflamed gingiva, this potent pro-inflammatory cytokine promotes the tissue and bone destruction seen in PD (Figure 1.2). However, its direct effect on skeletal cells is still an area requiring further investigation.

Several studies have demonstrated the beneficial effect of reducing inflammatory cell infiltrate and cytokine expression on alveolar bone in periodontal animal models (54, 55). Specifically, reductions in TNFα were associated with decreased osteoclast formation and bone resorption in a ligature-induced PD rat model using the anti-inflammatory and anti-oxidative compound, Epigallocatechin-3-gallate (55). However, the use of common therapeutics that reduce inflammation in humans, such as non-steroidal anti-inflammatories (NSAIDs), have produced variable results on periodontal parameters as reviewed by Salvi and Lang (56). In addition to the broad molecular suppression of NSAIDs, they can produce undesirable gastrointestinal and renal effects (57, 58). Conversely, although no studies to our knowledge assess anti-TNFα therapy as a specific treatment for PD in humans, there are several comparative studies that assess the effects of TNFα antagonists in rheumatoid arthritis (RA; a pathology shown to be highly associated with and comparable to the PD pathogenesis (1, 59)) on periodontal disease markers (60-62). These reports indicate that the suppression of TNFα is positively

correlated with decreased PD parameters, such as probing index and depth, gingival index and bleeding, and attachment loss. In addition, several animal models analysing the effects of anti-TNFα therapy in PD have demonstrated favourable results. Targeted suppression of TNFα production with pentaxifylline and thalidomide in rats with PD resulted in reduced cellular infiltrate into gingival tissues and inhibited alveolar bone loss by almost 70% (63). Etanercept, an FDA approved anti-TNFα agent for RA, is also reported to suppress the development of PD and inhibited tissue destruction in an animal model (64). In the last decade, however, the use of anti-TNFα therapy has raised serious concerns with incidences of adverse effects. As TNFα is involved in pathogen elimination its therapeutic inhibition may increase the risk of opportunistic infections (65, 66). Several studies have revealed increased infections, such as histoplasmosis (67), tuberculosis (68), listeriosis (65) and coccidiomycosis (67) are associated with anti-TNFα therapy. Furthermore, as PD is associated with oral and gingival infections, the use of anti-TNFα treatments should be assessed with caution.

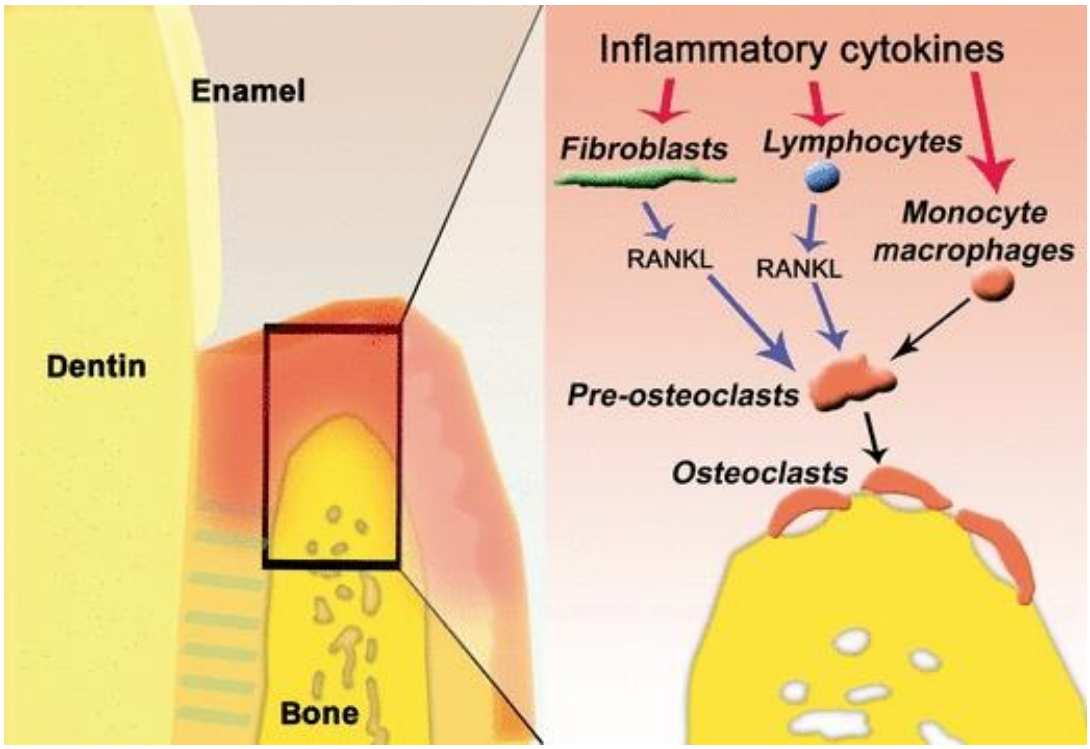

Figure 1.2 The effect of inflammatory cytokines on alveolar bone in PD (1). Cytokines such as tumour necrosis factor-α enhance osteoclast formation and bone resorption within the periodontium by directly stimulating precursor monocytes to differentiate and form active osteoclasts, or indirectly by inducing receptor activator of nuclear factor kappa-B ligand (RANKL) expression on local cell types such as gingival fibroblasts and lymphocytes, further inducing osteoclast formation and activity causing alveolar bone resorption and loss of tooth support.

1.6 Osteoclasts, Bone Resorption and the Effects of TNFα

1.6.1 Osteoclastogenesis

Osteoclasts are large multinucleated cells that are involved in physiological and pathological bone resorption as recently reviewed by Cappariello *et al* (69). The precursor cells arise from hematopoietic cells formed within the marrow of bone. Responding to osteoclastogenic factors, including a number of key cytokines and chemokines, precursors migrate from the marrow into the blood stream to the site of intended bone resorption where they differentiate into large multinucleated osteoclasts (17). It is well established that macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) are two key factors for osteoclastic differentiation (70, 71). M-CSF stimulation of precursor monocytes enhances cell survival, promotes commitment to the osteoclastic lineage and induces the expression of RANK (70, 72). Binding of RANKL to its receptor, RANK, initiates a series of intracellular pathways promoting the expression and transcription of a number of key osteoclast specific genes, such as calcitonin receptor (CTR), tartrate resistant acid phosphatase (TRAP), cathepsin K (CatK), Osteoclast-associated immunoglobulin-like receptor (OSCAR) and Beta 3 integrin (73, 74) (See Figure 1.1).

RANK is a member of the TNF receptor sub-family, and thus holds a variety of similarities to the TNF receptors discussed earlier (TNFr1 and TNFr2). RANK is membrane bound protein, comprised of an important cytoplasmic motif that requires the interactions with adaptor proteins from the TRAF family (namely TRAF 6) to transmit its signal downstream (75, 76). Several studies have highlighted the importance of these three factors with knockdowns of M-CSF (or its receptor M-CSFr), RANKL or RANK, or TRAF 6, all of which result in reduced production or complete lack of bone resorbing osteoclasts (71, 77-79). This results in increased bone volume and an osteopetrotic phenotype.

Downstream of TRAF interactions with RANK, several distinct pathways are activated which involve a variety of protein kinases, such as IKK, p38, and JNK (80). The conduction of these signalling cascades through the RANK pathway induce several well described transcriptive factors including nuclear factor of activated T cells cytoplasmic 1 (NFATc1), NF-κB and AP-1 (76, 80). Experimental genetic mutations of the NF-κB subunits p50 and p52 *in vivo* revealed similar osteopetrotic phenotypes to manipulations of the upstream RANK system (81, 82). This holds true for knockouts of c-Fos (83, 84), a main component of the AP-1 transcription complex. Conversely, studies investigating NFATc1 knockouts have demonstrated embryonic lethality due to its vital role in development (85, 86). However, conditional knockouts of NFATc1 in young and adult mice result in significant increases in bone volume compared to wild type controls due to deficient osteoclast presence and function (87-89).

1.6.2 The Effects of TNFα on Osteoclastogenesis and Bone Resorption

The role of RANK/RANKL signalling in osteoclasts is well known both *in vivo* and *in vitro*; however the influence TNFα has on this system remains an area of debate. As discussed above, increased production and exposure to TNFα is associated with bone loss conditions such as PD. A number of studies have attempted to unravel the mechanisms involved in TNFα mediated bone resorption. From this, TNFα has been demonstrated to promote the recruitment of osteoclasts and osteoclastic progenitors to the site of inflammation through several mechanisms (90-92). For example, Zhang *et al* (90) described TNFα regulation of stromal-derived factor-1 (SDF-1) synthesis. SDF-1 is produced by bone marrow derived cells and attracts osteoclast and inflammatory cell precursors. In physiological states, marrow SDF-1 concentrations are high, preserving precursors and the overall pool of potential osteoclasts. TNFα overexpression in transgenic mice, or TNFα injection into wild type rodents reduced SDF-1 concentrations in bone marrow, inducing mobilization and release of cells into circulation towards regions of higher SDF-1 concentration (90). Investigations using a rat model of PD that was also supported analysing human gingival crevicular fluid revealed increased concentrations of SDF-1 at the site of periodontal inflammation (93).

The suppressive effects of TNFα on SDF-1 production within the marrow may account for the increased osteoclastic cells elicited into the periodontal lesion.

With increased numbers of osteoclast precursors in the peripheral circulation and at the site periodontal inflammation, TNFα appears to prime and enhance differentiation into bone resorbing osteoclasts via RANK signalling. Like M-CSF, TNFα promotes the expression of RANK on the hematopoietic precursor monocyte/macrophages, increasing the availability of receptor activation via RANKL (94). In addition, this cytokine promotes RANKL expression in the inflamed region by gingival fibroblast and epithelial cells, T-cells, and osteoblasts (95, 96). Overall, this cytokine has the capacity to produce an environment rich in osteoclastic growth and activity factors, in addition to promoting the migration of their precursors to the site of bone destruction.

TNFα has also been shown to directly target osteoclastic differentiation through mechanisms independent of RANK signalling (Table 1.1). Initial studies using whole marrow or co-cultures with stromal cells and osteoclast precursors showed that TNFα stimulated increased numbers of multinucleated cells expressing TRAP, an enzymatic marker of cells devoted to the osteoclast lineage (97, 98). Although these experiments observed osteoclastogenesis *in vitro* with the addition of only TNFα and M-CSF as growth factors, it is possible that endogenous RANKL production by co-cultured stromal cells also stimulated osteoclast formation. However, the addition of osteoprotegerin (OPG; a natural soluble decoy receptor of RANKL) in supplemental studies did not suppress TRAP positive cell formation (97, 98). This observation suggests a direct effect of TNFα on the differentiation of osteoclasts. In line with this, other studies have demonstrated a concentration dependant increase in TRAP forming cells induced by TNFα in mouse bone marrow cultures being independent of RANKL stimulation *in vitro* (98). However, supplementary studies analysing the activity of TNFα mediated differentiation have produced conflicting results (summarised in Table 1.1).

Although some studies report observing osteoclastic resorption in response to TNFα alone (98), other studies were unable to identify any positive osteoclastic effect (99), concluding that additional factors may be required to promote their

formation and activity *in vitro*. TNFα administration with RANKL during osteoclastogenesis synergistically promotes increased osteoclast size and number (99, 100). Even with RANKL concentrations too low to produce reportable quantities of osteoclasts (RANKL: 0.1-1ng/ml) TNFα treatment enabled significant increases in the numbers of osteoclasts formed. Additional pro-inflammatory cytokines present during periodontal inflammation and bone loss such as IL-1 (101, 102), are also shown to enhance the stimulatory effects of TNFα on osteoclastogenesis in the absence of RANKL (97, 98, 103). Upon ligation of IL-1 with its receptor (Il-1R), TRAF 6 actively propagates its signalling downstream to NFATc1 and NF-κB (104-106) (See Figure 1.1). It is proposed that these cytokines bypass the need for RANK/RANKL interactions, allowing crosstalk between immune receptor pathways to induce transcription of osteoclast related genes. These findings demonstrate the idea of an inflammatory osteoclast capable of resorbing bone whilst avoiding inhibitory factors such as OPG, which are dependent on RANKL being the activating molecule (97, 101). Further evidence for a stimulatory role of TNFα in osteoclasts, has been demonstrated in TNFr1 knockouts or TNFα antagonist studies (Table 1.1), which result in reduced osteoclast formation (100, 107, 108).

It is evident from these studies that TNFα acts on osteoclasts during the proliferation and differentiation phases, stimulating their ability to respond to RANKL. This is likely to occur via TRAF 1, 2, and 3, and the activation of NF-κB and AP-1 resulting in the formation, differentiation and survival of osteoclastic cells. In contrast, the RANKL/IL-1/TRAF 6 pathway is necessary for the activation and hence resorption stages of osteoclasts (97, 99). These findings support a need for treatments that target both the inflammation along with bone resorption.

Table 1.1 (Continued): The Effects of TNFα on Osteoclasts (45)

Cell Source	Assay Design	Outcome	Effect on cells	Mechanism of Effect	Reference
Mouse Bone Marrow Macrophages (BMM)	a) RANKL (20ng/ml) for 4 days followed by TNFα (10ng/ml) alone for 1 day	a) Stimulation	a) Cell number and size	a) Enhanced TRAF2-MEKK1 signalling	(109)
Mouse BMM	a) TNFα (1-10ng/ml) with RANKL (50-100ng/ml) for 4 days	a) Stimulation	a) Cell number	a) TNFα (early) dose dependent increase in number of TRAP+ cells	(110)
	b) RANKL (50-100ng/ml) for 2 days followed by TNFα (5-1ng/ml) for 2 days	b) Inhibition	b) Cell number	b) TNFα (late) dose dependent decrease in number of TRAP+ cells	
Mouse BMM	a) TNFα (20ng/ml) alone for 3 days	a) Inhibition	a) Activity	a) Dose dependent increase in formation through TNFr1 & TNFr2. Suppression of resorption pits in absence of RANKL.	(97)
	b) TNFα (20ng/ml) with IL-1α (10ng/ml) for 3 days	b) Induction	b) No change	b) Bypassing RANK activation with TNFα & IL-1α to induce active osteoclasts	
Mouse BMM	a) After 3 days whole marrow culture, isolated macrophages treated with TNFα (0.1-1000ng/ml) alone, for 5 days	a) Stimulation	a) Cell number	a) Dose dependent increase in formation without RANKL. However, formation greater with added RANKL	(98)
Mouse Stromal Derived Macrophages	a) TNFα (10ng/ml) alone for 4 days	a) Stimulation	a) Cell number	a) Activity through TNFr1 & TNFr2	(108)
	b) TNFα (10ng/ml) with RANKL (50-100ng/ml) for 4 days	b) Stimulation	b) Cell number	b) Activity through TNFr1 & TNFr2	

Table 1.1 (Continued): The Effects of TNFα on Osteoclasts (45)

Cell Source	Assay Design	Outcome	Effect on cells	Mechanism of Effect	Reference
Mouse BMM	a) TNFα (1-160ng/ml) alone 5-9 days	a) Inhibition	a) Cell Number	a) No osteoclasts formed with TNFα alone	(99)
	b) 3 day whole marrow culture followed by TNFα (100ng/ml) 5-9 days	b) No change	b) Cell Number	b) Osteoclasts formed with TNFα alone, however, this was suppressed with addition of OPG during 3 day whole marrow culture	
	c) Suboptimal RANKL (1ng/ml) priming followed by TNFα (500pg/ml)	c) Stimulation	c) Cell Number	c) Synergistic increase with TNFα 2 days after RANKL priming through the Induction of SAPK/JNK and NF-κB	
Mouse Raw264.7 macrophages	a) TNFα (50ng/ml) alone for 3 days	a) Inhibition	a) Cell number	a) No osteoclasts formed with TNFα alone	(111)
	b) TNFα (50ng/ml) with RANKL (20ng/ml) for 3 days	b) Stimulation	b) Cell number	b) TNFα and RANKL (20ng/ml) formed more cells, although smaller, than RANKL (100ng/ml) alone.	
Mouse Raw264.7 macrophages	a) 24 hours of RANKL followed by 3 days of TNFα (10ng/ml)	a) Stimulation	a) Cell number	a) TNFα with RANKL (25ng/ml) priming increased the number of osteoclast through a TNFα induced double-stranded RNA-dependent protein kinase (PKR) expression increase. PKR inhibitor 2AP prevented PKR induction from TNFα.	(112)
Rat BMM	a) TNFα (4-20ng/ml) alone for 3 days	a) Stimulation	a) Cell number	a) TNFα alone stimulated differentiation of pre-osteoclast (mononuclear TRAP+ cells).	(113)
	b) TNFα (20ng/m) for 3 days followed by RANKL (30ng/ml) for days 1 day.	b) Stimulation	b) Cell number	b) The addition of RANKL stimulated multinucleation.	

Table 1.1 (Continued): The Effects of TNFα on Osteoclasts (45)

Cell Source	Assay Design	Outcome	Effect on cells	Mechanism of Effect	Reference
Rat BMM	a) TNFα (10ng/ml) with rat osteoblastic heat-treated conditioned media	a) Stimulation	a) Cell number and activity	a) TNFα with osteoblast conditioned media increased osteoclast formation and resorption	(114)
	b) TNFα (10ng/ml) and RANKL (10ng/ml) for 4 days	b) Stimulation	b) Cell number and activity	b) TNFα and RANKL synergistically increased formation and resorption area of osteoclasts	
Human CD11+ cells	a) TNFα (0.01-1ng/ml) with RANKL (0.1-1ng/ml) for 7 days	a) Stimulation	a) Cell number	a) Synergistic dose dependent increase in formation in multinucleated osteoclasts	(100)
Human PBMC	a) 14 day osteoclast culture with TNFα (0.1-10ng/ml) added from a variety of time points (on day 1, 4, 7, or 10)	a) Stimulation	a) Cell activity	a) A dose dependant synergistic increase in resorption with RANKL (5-30ng/ml). Timing of TNFα essential. TNFα day 1 alone generated the greatest resorption. TNFα required IL-1β (10ng/ml) to induce osteoclast function.	(101)

1.7 Osteoblasts, Bone Formation and the Effects of TNFα

1.7.1 Osteoblastogenesis

Osteoblasts play a critical role in the bone remodelling process by forming and depositing new bone material. In pathological conditions such as periodontitis, there is an imbalance in the resorption and formation processes resulting in an overall loss of bone. This imbalance is not only due to excessive osteoclastic bone resorption but also the suppression of osteoblast differentiation and activity (115).

The role of osteoblasts changes throughout their life cycle and depends on a number of factors, such as skeletal development, hormones, and inflammatory mediators that influence cell differentiation and function (116). Osteoblast precursors originate from pluripotent stem cells (SC) derived from the neural crest (NC) during embryonic development (117). Their differentiation into various cell types including skeletal, adipose and connective tissue cells is essential for the development of vertebrates (117, 118). Furthermore, NC-SC transient progression into the mesenchymal SC (MSC) lineage within bone marrow is needed for the development of oral structures such as the dental pulp (DP) and periodontal ligament (PDL) (119). These structures are reported to store additional MSCs for continued osteogenic cell differentiation, to repair damaged bone and tissue within the periodontium (120). TNFα has a diverse range of effects on the differentiation of these cells (discussed in detail below). Therefore, it is important to understand the effects associated with differentiation to the bone cell lineage, as these stem cells are widely investigated as regenerative cell based oral therapies (121, 122).

Osteoblastic commitment requires activation of the Wnt (wingless-type MMTV integration site) signalling pathway within the progenitor SC, in addition to a variety of other intracellular and secreted mediators (123, 124). Activation of the Wnt pathway results from the ligation of a Wnt protein (Wnt2a, Wnt5a, or Wnt10b) to its receptor, Frizzled, on the cell surface (Figure 1.3). In turn, a series of intracellular pathways leads to the stabilisation of β-catenin and the promotion of differentiation (125). Osteoblast formation and activity is achieved through increased intracellular calcium levels via the activation of G-proteins, JNKs, and

the activation of transcription factors, Runt-related transcription factor 2 (Runx2), NFAT, and NF-κB (124, 125).

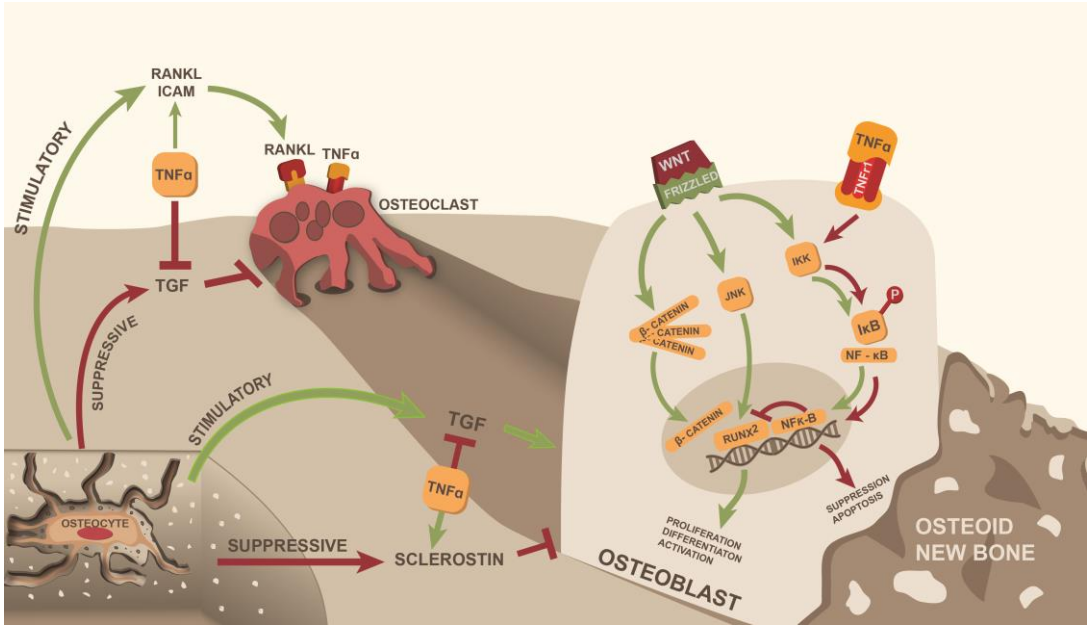

Figure 1.3 Proposed influence of TNFα on the overall bone microenvironment (45). TNFα binding to TNFr1 on the osteoblast membrane interferes with WNT signalling via the induction of NF-κB related suppression of RUNx2 transcription, leading to cell death. TNFα influences osteocytic regulation of bone cells through the promotion of factors, which stimulate osteoclasts (ICAM, RANKL) and suppress osteoblasts (sclerostin). ICAM, intracellular adhesion molecule; NF-κB, nuclear factor kappa-light chain enhancer of activated B cells; RANKL, receptor activator of nuclear factor B ligand; TNFα, tumour necrosis factor alpha; TNFr1, tumour necrosis factor receptor 1.

1.7.2 The Effects of TNFα on Osteoblastogenesis and Bone Formation

Numerous studies investigating the effects of TNFα on osteoblastogenesis have revealed suppression of bone formation (Table 1.2). This has been observed with both direct and indirect actions of TNFα disrupting osteoblast formation at various stages of their differentiation. At the early stages of stem cell division, paracrine factors are required to promote the cells ability to grow and differentiate into pre-osteoblasts (126, 127). Essential factors supporting this transition are insulin growth factor-I (IGF-I) and bone morphogenic proteins (BMPs; mainly -2, -4, and -6) (109, 128, 129). TNFα has been demonstrated to suppress osteoblast differentiation from isolated foetal calvaria precursors via reducing the expression of IGF-1 (130, 131). However, subsequent investigations that involved treating cells with additional IGF-1 were not successful in reversing the suppressive effects of TNFα (131), suggesting a more specific target downstream of IGF-1 activity.

Further analyses into the suppressive actions of TNFα on osteoblastogenesis have revealed additional targets where TNFα interferes with protein signalling and gene expression. Factors that regulate the synthesis of bone material are essential for functional osteoblasts to form (132). However, several experimental models have demonstrated that collagen type 1 α1 (Col1α1) and osteocalcin (OCN), both skeletal matrix proteins, are affected by TNFα (133, 134). The mechanisms behind decreased collagen production was identified by Mori *et al* (135) in a study using fibroblasts in which the Col1α1 gene promoter was shown to be suppressed in response to TNFα, abrogating transcription factor binding. In contrast, another study showed OCN production was suppressed via a developed resistance and decreased receptor expression to vitamin D *in vitro* (136, 137). Vitamin D receptor activation on osteoblastic cells appears to be required for the transcription and production of OCN (138).

In addition to bone formation, TNFα has been reported to affect factors associated with the formation and survival of osteoblasts through negative regulation of critical transcription factors, such as Runx2 and Osterix (130, 131). These factors are required for developing and maintaining the skeletal system, through the transcription of a variety of osteoblast specific genes, including OCN, Col1α1,

OPG, alkaline phosphatase (ALP) and osteopontin (OPN) (See Figure 1.3). Transgenic knockout mouse studies with deletions of either Runx2 or Osterix demonstrate their critical role, as mice develop with a cartilage rich skeleton (139, 140) due to a deficiency in osteoblast differentiation and an inability to form mineralized bone.

Furthermore, studies on the effects of TNFα on osteoblastogenesis, specifically from isolated DP or the PDL have reported conflicting findings (141-145). These differences appear to be related to the concentrations of TNFα used *in vitro*. Concentrations of TNFα above 10ng/ml have been reported to decrease osteogenic differentiation, down regulate BMPs and ALP activity, and reduce mineralisation (141, 142, 144, 145). Conversely, lower concentrations of TNFα, ranging from 0.01 to 10ng/ml, lead to more osteogenic characteristics (142-144). Xing *et al* (142) reported increased extracellular calcification and Runx2 protein expression in TNFα treated DP-SC. In addition, an earlier study showed NF-κB activation by low levels of TNFα enhanced osteogenic differentiation (141). However, these findings are not fully supported by a recent study using PDL-SC and bone marrow mesenchymal (BMM)-SC treated with low (10ng/ml) concentrations of TNFα resulted in decreased ALP activity, Runx2 expression and calcium deposition (146, 147).

Although a large number of studies demonstrated suppression of bone formation by osteoblasts exposed to TNFα, there is further evidence to support the contrary. Interestingly, Briolay *et al* (148) demonstrated that TNFα stimulated calcium deposits associated with increases in Wnt5a and Wnt10b expression in human MSC cultures. Conversely, decreases in the master transcription factor Runx2 were also observed in this study, indicating delayed or suppressed proliferation. Additional studies assessing TNFα effects on human MSC differentiation have observed a dose-dependent increase in osteogenic differentiation over a 3-day period (144). These positive results could be related to acute cytokine exposure, promoting the activity of TNAP (tissue-nonspecific alkaline phosphatase) and the synthesis of calcium, along with the rapid induction of NF-κB and expression of anti-apoptotic signals. However, TNFα is also reported to suppress collagen matrix production by early osteoblasts (149), in addition to preventing their

differentiation (150, 151). This may indicate that the observed increase in intracellular calcium and early proliferation *in vitro* may not be translatable to the pathological suppression of completed mineralized bone formation *in vivo*.

Osteoblasts are also important regulatory cells, which have the capacity to orchestrate osteoclastic bone resorption. This is achieved by producing pro-osteoclastic signalling molecules such as M-CSF, RANKL, and interleukins (152-154). Interestingly, TNFα increases the expression of these genes, in addition to promoting the production of proteolytic enzymes such as matrix metalloproteinases (MMP) (154, 155). MMP have been implicated in the induction of osteoclast differentiation and attachment to bone surface (156). This increase in osteoclastic promoting factors is observed occurring simultaneously with the suppression of the pro-osteoblastic factors mentioned above, indicating an essential role for TNFα in periodontal and inflammatory bone loss.

Overall these results demonstrate that TNFα negatively influences the activity of osteoblasts *in vitro* and *in vivo*. However, the timing and length of exposure appear to drastically impact the resulting outcome, thus these factors should be carefully considered, particularly *in vitro*, during the interpretation of results. Further analyses are needed to fully understand the mechanisms behind TNFα regulation of osteoblastogenesis at different stages of cell differentiation. This will aid in developing effective therapies, targeting specific pathways or factors depending on disease state and bone quality.

Table 1.2: The Effects of TNFα on Osteoblasts (45)

Cell Source	Assay Design	Outcome	Effect on cells	Mechanism of Effect	Reference
Mouse Myoblast C2C12 cells	a) 3 day osteoblast differentiation in the presence of TNFα (10-100pg/ml)	a) Inhibition	a) Cell number	a) Dose dependent reduction of ALP and inhibition of Runx2 and SMAD 1, 5, 8 key transcription regulators	(157)
Mouse Bone Marrow Stromal Cell (BMSC) & MC3T3 Osteoblast Cells	a) TNFα (0.1-20ng/ml) on day 1 or on day 7 of 14 day osteoblast differentiation assay	a) Inhibition	a) Cell number and activity	a) Dose dependent reduction of ALP, matrix deposition and Runx2 through TNFr1. Greatest suppression with day 1 TNFα (95%) vs. day 7 (60%)	(158)
Mouse MC3T3-E1 cells	a) 28 day osteoblast differentiation in the presence of TNFα (1ng/ml)	a) Inhibition	a) Cell number and activity	a) Inhibition of ALP, matrix deposition, Runx2 and Osterix	(159)
Mouse MC3T3-E1 cells	a) 24 hour osteoblast culture in the presence of TNFα (20ng/ml)	a) Inhibition	a) Cell number	a) Up to 6 hours of TNFα upregulated caspase -1, -7, -11, and -12, FAS and FAP and related TNF induced apoptotic factors.	(160)
a) Rat BMSC	a) 21 day osteoblast differentiation in the presence of TNFα (10-100ng/ml) from day 2, 7 or 14.	a) Inhibition	a) Cell number and activity	a) Dose dependent TNFα from day 2 or 7 suppressed confluence and mineralisation. OCN mRNA was also suppressed. Commencing TNFα from day 14 had no effect.	(130)
b) Mouse MC3T3-E1	b) 16 day osteoblast differentiation with TNFα (0-10ng/ml)	b) Inhibition	b) Cell number and activity	b) Dose dependent suppression of confluence and mineralisation.	

Table 1.2 (Continued): The Effects of TNFα on Osteoblasts (45)

Cell Source	Assay Design	Outcome	Effect on cells	Mechanism of Effect	Reference
Mouse BMSC ($TNFr1^{-/-}$, $TNFr2^{-/-}$ and Wild Type)	a) 14 day osteoblast differentiation in the presence of TNFα (10ng/ml)	a) Inhibition	a) Cell number and activity	a) Inhibition of differentiation and mineralisation through TNFr1	(161)
Mouse ST2 cells	a) 48 hour osteoblast differentiation with TNFα (0.01-100ng/ml)	a) Stimulation and Inhibition	a) Cell activity	a) Low dose TNFα (0.01 and 0.1ng/ml) induction of ALP, RUNx2, OCN, and OSX. High dose TNFα (1-100ng/ml) dependent suppression of ALP, RUNx2, OCN, and OSX	(144)
	b) 4 week osteoblast differentiation with TNFα (0-100ng/ml)	b) Inhibition	b) Cell activity	b) Dose dependent TNFα suppression of mineralisation	
Mouse C2C12 cells	a) 24 hours of osteoblast culture with TNFα (10ng/ml)	a) Inhibition	a) Cell number and activity	a) TNFα almost completely suppressed ALP activity and mRNA expression through induction of NF-κB and upregulation of Msx2 expression, in addition to Smurf1 induction and degradation of RUNx2.	(150)
Mouse BMSC (TNFα-Tg)	a) TNFα-Tg mice develop arthritis at 2-3-months old, and osteoporosis as 4 months.	a) Inhibition	a) Cell number and activity	a) TNFα-Tg mice - less stromal cell colonies than WT and decreased ALP and OCN gene expression and ALP activity in isolated BMCS.	(151)

Table 1.2 (Continued): The Effects of TNFα on Osteoblasts (45)

Cell Source	Assay Design	Outcome	Effect on cells	Mechanism of Effect	Reference
Rat BMSC	a) 16 day osteoblast differentiation on biodegradable polymer scaffolds with TNFα (50ng/ml); continuous (16 days); early (day 0-4); intermediate (day 4-8); late (day 8-12)	a) Stimulation and Inhibition	a) Cell number and activity	a) TNFα (50ng/ml) suppressed ALP activity. Cell proliferation was decreased with continuous and early TNFα at day 4 and day 8. Intermediate and late TNFα increased cellularity at day 16. Mineralisation was increased in all TNFα time courses.	(144)
Human BMSC	a) 14 day osteoblast differentiation in the presence of TNFα (1-10ng/ml)	a) Stimulation	a) Cell activity	a) Dose dependent stimulation of calcium deposits through increased TNAP activity.	(148)
Human BMSC	a) 14 day osteoblast differentiation with TNFα (0.1-10ng/ml) and/or IL-1β (0.1-1ng/ml) added from day 2	a) Stimulation and Inhibition	a) Cell activity	a) Dose dependent TNFα and/or IL-1β increased ALP activity and calcium deposits through increase in TNAP activity. However, RUNx2 mRNA and OCN secretion were suppressed by both TNFα and IL-1β.	(162)
Human BMSC	a) 21 day osteoblast differentiation with TNFα (20ng/ml)	a) Stimulation	a) Cell activity	a) TNFα increases calcium and phosphate deposition through induction of BMP2 and NF-κB.	(163)

Table 1.2 (Continued): The Effects of TNFα on Osteoblasts (45)

Cell Source	Assay Design	Outcome	Effect on cells	Mechanism of Effect	Reference
Human DPSC	a) 14 d osteoblast differentiation in the presence of TNFα (50ng/ml)	a) Inhibition	a) Cell activity	a) Dose dependent decrease in osteogenesis, calcium deposits, ALP, OPN, OCN, Osterix and Runx2 expression	(141)
Human PDLSC	a) 14 d osteoblast differentiation in the presence of TNFα (0-10ng/ml)	a) Stimulation and no effect	a) Cell number and activity	a) Low dose (2.5-5ng/ml) TNFα increased proliferation, osteogenesis and calcium deposits. No effect at high dose.	(145)
Human DPSC	a) 14 d osteoblast differentiation in the presence of TNFα (0-100ng/ml)	a) Inhibition	a) Cell activity	a) Dose dependent decrease in osteogenesis, calcium deposits, ALP and BMP expression	(142)
Human DPSC	a) 14 d osteoblast differentiation in the presence of TNFα (10ng/ml)	a) Stimulation	a) Cell activity	a) Increased osteogenesis, calcium deposits, ALP, BMP and Runx2 expression	(143)
Human PDLSC	a) 14 d osteoblast differentiation in the presence of TNFα (10ng/ml)	a) Inhibition	a) Cell activity	a) Decreased osteogenesis, calcium deposits, ALP and Runx2 expression	(146)
Human PDLSC	a) 21 d osteoblast differentiation in the presence of LPS-stimulated monocyte media with endogenous TNFα or TNFα (10ng/ml)	a) Inhibition	a) Cell number and activity	a) LPS stimulated monocyte TNFα-positive media and suppressed osteogenesis and calcium deposits. TNFα (10ng/ml) suppressed osteogenesis, calcium deposits, ALP, Runx2, OCN and osterix expression.	(147)

1.8 Osteocytes, Bone Cell Regulation and the Effects of TNFα

Osteocytes are regarded as terminally differentiated osteoblasts that have reached the end of their differentiation pathway and are embedded within the bone microstructure. They reside in microscopic cavities (lacunae), encased in a stationary position within bone. Cytoplasmic projections that extend from their cell body travel through bony networks, or canaliculi, to resident bone cells (164, 165). This enables a complex system of cell contact, communication and regulation. After developing their characteristic phenotype within the osteoid, they begin to produce factors that have varied effects on adjacent cell types, in particular osteoclasts and osteoblasts. These factors include RANKL, intracellular adhesion molecule 1 (ICAM1), transforming growth factor β1 (TGF-β1), and sclerostin (166) (See Figure 1.3). The expression and release of these factors can influence the rate of bone resorption or formation on the surface. Interestingly, the actions and outcomes of octeocytic activity, although not fully understood, appear to change in response to different stimuli, such as mechanical stress, micro-facture, or immune and inflammatory mediators such as TNFα (167, 168). Studies investigating the direct actions of TNFα on osteocytes *in vitro* are described in Table 1.3.

Osteocytes can promote osteoclastogenesis and osteoclast activity through a variety of mechanisms. For example, the death or targeted ablation of osteocytes results in severe bone loss (169). This is reported to be a result of increased RANKL expression by the dying osteocytes, inducing osteoclast formation and bone resorption on the surface. Additional studies identified apoptotic bodies released from dying osteocytes being the primary source of the increased RANKL concentrations (167, 170). In fact, the expression of RANKL by apoptotic osteocytes is reported to surpass levels produced by resident osteoblasts, as targeted mutations of osteoblasts within mice had no effect on overall RANKL expression (171, 172). Interestingly, several studies have shown that osteocytic apoptosis and RANKL production is induced by TNFα *in vitro* (See Table 1.3). Furthermore, TNFα also induces apoptotic osteocytes to express factors that promote osteoclast precursor adhesion to endothelial cells, such as ICAM1 (173).

This was observed using osteoclastic precursor RAW264.7 cell line and media from TNFα induced apoptotic MLO-Y4 osteocytes. Taken together, these findings suggest the essential role for osteocyte regulation of osteoclasts is highly influenced by inflammation and TNFα exposure.

Osteocytes have also been shown to influence the activity of osteoblasts through several mechanisms. For example, TGF-β1, which is synthesized by a variety of cell types including osteocytes, enhances proliferation of pre-osteoblasts, suppresses apoptotic signalling within osteoblasts and promotes their survival (160, 174). In addition, TGF-β1 has been reported to induce osteoblast migration to the bone surface for the synthesis of bone matrix proteins (174, 175). Interestingly, TGF-β1 has also been implicated in the suppression of osteoclastic bone resorption *in vitro*, indicating an important anabolic role for osteocytes during physiological bone growth and repair (176).

Osteocytes also produce sclerostin, an essential glycoprotein that orchestrates the activity of osteoblasts (177). Physiologically, sclerostin levels appear to alter in response to mechanical loading, with heavy loading or concentrated strain leading to the suppression of sclerostin levels (178). Decreased sclerostin enables osteoblastic function during stages of osteoblastogenesis, where cell activity is required for skeletal growth (179). Conversely, a reduction in mechanical stress, such as being sedentary for long periods, results in an increased production of sclerostin and the subsequent apoptosis of osteoblasts, preventing the formation of bone (178). Although the function of sclerostin suppression of osteoblasts is not entirely understood, it is thought to interfere with the Wnt signalling and stabilisation of β-catenin (180, 181).

In pathological conditions, sclerostin expression is altered in response to inflammatory molecules such as TNFα. Recently, a study using a rat model of ligature-induced PD revealed significant increases in sclerostin expression in osteocytes during the early and destructive phase of the disease (182, 183). Interestingly, the rise in sclerostin levels was associated with a similar increase in RANKL production. However, expression levels normalised during the recovery phase of the disease, suggesting that alveolar bone loss may be an outcome of

osteocytic suppression of osteoblasts and promotion of osteoclasts during the active phase of PD. In addition, reports identifying a role for sclerostin in the promotion of osteoclastic bone resorption (179, 184) are supported by studies that observed an increase in RANKL expression and decrease in OPG expression in the MLO-Y4 osteocyte cell line in response to sclerostin (185). Further evidence is demonstrated using a number of *in vivo* and *in vitro* models, where TNFα alone is found to directly induce the expression and increased synthesis of sclerostin in osteocytes, through the activation of TNFα mediated NF-κB binding to the sclerostin gene (SOST) promoter region (168). In addition, the administration of TNFα antagonists prevents this stimulation, further supporting this important relationship in inflammatory bone loss.

Table 1.3: The Effects of TNFα on Osteocytes (45)

Cell Source	Assay Design	Outcome	Effect on cells	Mechanism of Effect	Reference
Mouse MLO-Y4 cells	Cell cultured for 6 hours with TNFα (10ng/ml)	Inhibition	Cell number	Induction of osteocyte apoptosis. Apoptotic media promotes osteoclast adhesion to endothelial cells through high endothelial ICAM-1 expression.	(173)
Mouse MLO-Y4 cells	Cells were cultured with TNFα (10ng/ml) for 2 hours up to 5 days	Inhibition	Cell number	Increased sclerostin expression through NF-κB binding to SOST gene, which was most significant with TNFα for 2 hours and 5 days.	(168)
Mouse MLO-Y4 cells	Cells cultured for 30 minutes or 24 hours with TNFα (0.5-30ng/ml) and/or IL-1β (0.1-10ng/ml)	Inhibition	Cell number	TNFα and IL-1β suppress nitrous oxide production.	(186)
Mouse MLO-Y4 cells	Cells cultured for 6 hours with TNFα (0-20ng/ml)	Inhibition	Cell number	TNFα dose dependent increase in osteocyte apoptosis.	(187)

1.9 Histone Deacetylases as a Novel Therapeutic Target for Periodontitis

1.9.1 Current Therapies for Periodontitis

As PD is associated with pathogenic bacteria forming a subgingival biofilm, patient education and improving oral hygiene is the first line of defence to prevent or reduce disease progression. Clinicians will also use surgical and/or mechanical means to remove the infected tissue and biofilm with some success (188-192). However, there are several factors that influence homecare or the effectiveness of mechanical techniques. These include improper plaque control (193-195), increased pocket depth creating 'out-of-reach' areas for effective scaling and/or root planning (193). In addition to disease severity and aggressiveness of the individuals host response (1, 196). Further to this, recent advances in the understanding of the PD pathogenesis and the osteoimmunological relationship in this disease has prompted new therapeutic strategies to reduce the inflammation and target the bone cells concurrently (1, 17).

A considerable amount of literature suggests the use of antibiotic treatment alongside mechanical debridement provides beneficial outcomes as a periodontal therapy (197-199). However, a similar quantity of opposing evidence exists (199) as there may be some disadvantages of this approach. Firstly, prescribing antibiotic treatment for any illness carries the risk of developing further antibacterial resistant strains. A recent review of subgingival microflora identified a substantial 2-fold rise in tetracycline and penicillin resistant bacterial strains over the last decade in treated periodontal patients (200, 201). Furthermore, many of the pathogenic bacteria found to be associated with PD, such as *Porphyromonas Gingivalis*, *Fusobacterium Nucleatum* and *Actinobacillus Actinomycetemcomitans*, enter host cells, rendering antibiotic treatments useless (202, 203).

A novel therapeutic strategy gaining clinical relevance in a number of areas is the epigenetic control over cell differentiation and activity by targeting a group of enzymes called histone deacetylases (HDACs) (204, 205).

1.9.2 Histone Deacetylase Overview

Differentiation and cell function is dependent on the genetic expression and production of molecules that form the components of the target phenotype. These gene-mediated events can be controlled at the epigenetic level, where modifications to histone proteins and the resulting chromatin configuration tightly regulate the expression of specific genes (204, 206, 207). Histone deacetylases (HDACs) are a group of enzymes that are integral to this process. Within the cell nucleosome histone proteins organise and maintain deoxyribonucleic acid (DNA) in its chromatin state. In effect, HDACs coordinate the conformational changes chromatin to both protect genetic material from damage and to promote cellular changes such as mitosis or differentiation. HDACs remove acetyl groups that are located on lysine residues within the histone protein core, altering lysine charge and affinity for phosphate groups present within the DNA backbone (204, 206, 207). The subsequent interaction between lysine and phosphate increases the histone binding capacity for DNA, forming a tightly bound chromatin structure that results in gene silencing and/or repression (heterochromatin) (Figure 1.4). Conversely, histone acetyltransferase (HAT) enzymes counteract histone deacetylation by HDACs, prompting a euchromatic open state of DNA by returning the acetyl body to lysine (204, 206, 207). Furthermore, research has identified a post-translational mechanism of protein modifications for HDACs (206). Deacetylation of subsequent non-histone proteins also modifies their structure and charge, and in turn, function and interaction outcomes. As a result, the functional properties of cellular proteins are affected, altering intracellular processes, such as, signalling pathways and reactivity.

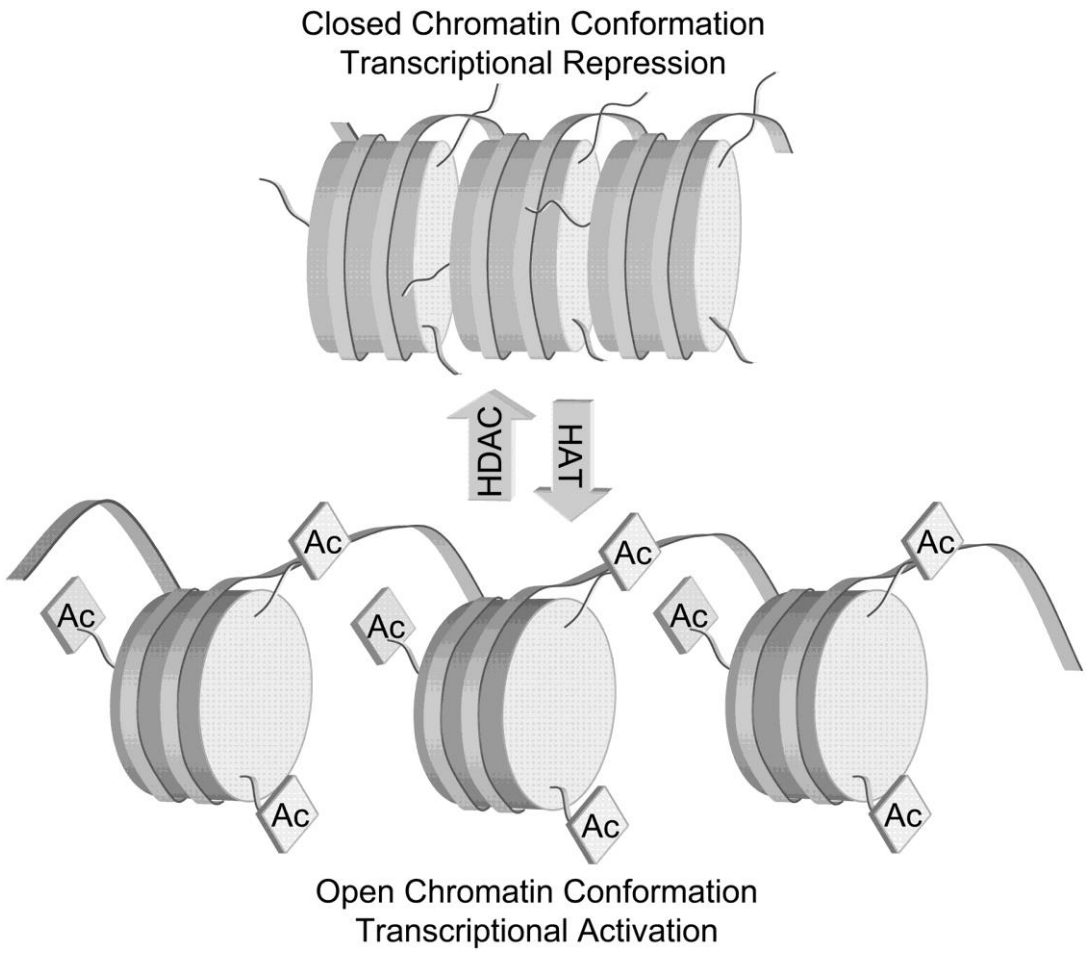

Figure 1.4 The outcome of epigenetic modulation on chromatin configuration upon Histone deacetylase (HDAC) and Histone acetyltransferase (HAT) activity (206). Deacetylation of lysine groups on histone proteins increases the affinity for phosphates in the DNA causing transcriptional repression. This process is reversed by the acetylating role of HAT enzymes.

HDACs comprise of 11 individual and structurally distinct enzymes that are categorized into 4 classes due to their cellular distribution and organisational status (208, 209) (Figure 1.5). Class I HDACs comprise of HDAC enzymes 1, 2, 3, and 8, and are primarily found within the nucleus of the cell. Class II HDACs, which are further subdivided into class IIa (HDACs 4, 5, 7, and 9) and IIb (HDACs 6, and 10) have the capacity to translocate between the nucleus and the cytoplasm. Class III HDACs work via different mechanisms to both class I and II, whereby they require the addition of an NAD+ co-factor for activation and catalytic activity. These enzymes are referred to as sirtuins (SIRT), and comprise of SIRT 1-7. Finally, the sole class IV HDAC, HDAC 11, is rarely reported on, though functions in a similar method to that of class I and II HDACs (208, 209).

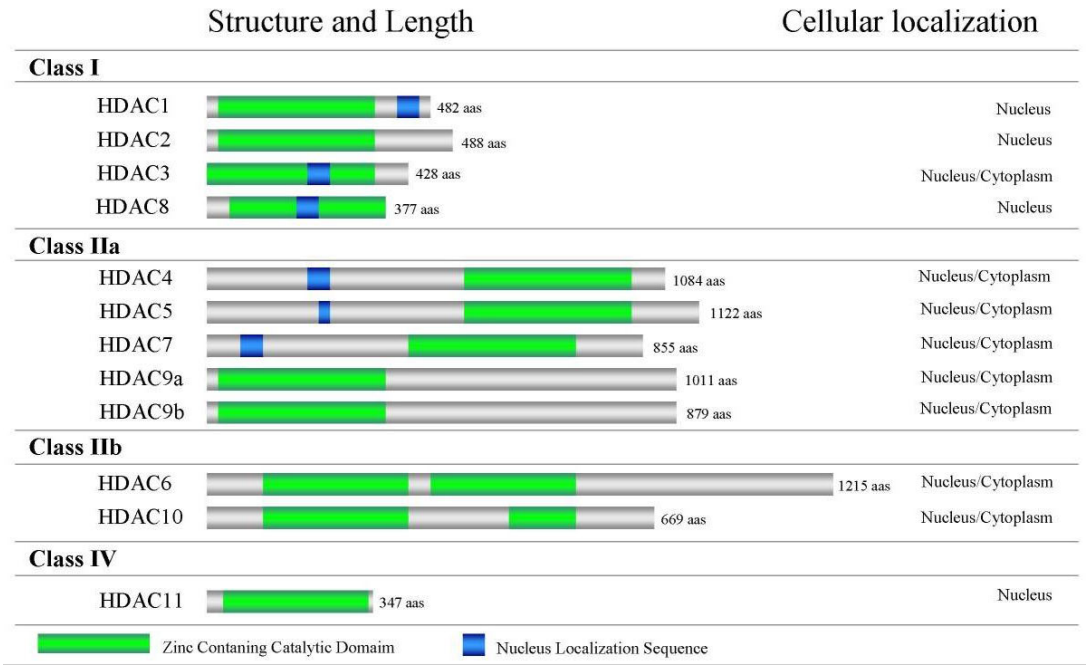

Figure 1.5: Classification of the 3 main Histone deacetylase (HDAC) classes (Class I, Class IIa and b, and Class IV) as determined by structure, length and cellular location (208).

1.9.3 Histone Deacetylases in Bone Loss Pathologies

Irregularities in the expression and function of HDACs have been implicated in several pathological processes, including malignant cell division, suppression of apoptosis (210-212), neuronal cell death in neurodegenerative diseases (NDs) (213) and more recently, chronic inflammation and associated bone loss (205, 207, 214). Studies investigating HDAC expression in RA demonstrated substantial modifications to the HDAC profile when comparing to patients with non-rheumatic, osteoarthritic lesions (215, 216). Specifically, the class I isoform, HDAC 1, is highly upregulated in synovial fluids and tissues from RA patients (215). Interestingly, it has also been shown to co-localise and correlate with increased levels of TNFα expression (216). More recently, analysis of gingival tissues from patients with PD identified a similar rise in HDAC 1 expression that was associated with both TNFα and osteoclasts near alveolar bone lesions (217). *In vitro* analyses of HDAC 1 expression in human osteoclasts have observed a substantial increase throughout osteoclastogenesis, particularly during the activation of the resorptive phase (218). HDAC 2 commonly associates with HDAC 1, forming a complex protein that interacts as one functional unit (219). They are both similar in size and structure, and it is thought that redundancies exist between these two HDAC isoforms. In addition to HDAC 1 and 2, HDAC 5 from class II is reported to be upregulated in PD patients (217). Interestingly, HDAC 5 function inversely correlates with Wnt-signalling and Runx2 transcription in isolated mice primary osteoblasts (220, 221) as discussed in detail in Chapter 5 and 6. HDAC 5 has also been identified as a locus for bone mineral density with rises in its expression (222), as seen in juvenile osteoporosis and PD (220), being associated with decreased bone integrity through diminished osteoblast formation and function.

The observation of altered HDAC expression and activity in disease has prompted researchers to develop HDAC inhibitors (HDACi) (205). HDACi are now used clinically as adjuvants to chemotherapy as anti-cancer agents (223), and are gaining interest as potential therapies for NDs (213), parasitic and viral infections (224-226), and importantly, inflammatory bone loss pathologies like RA and PD (214, 217, 227).

1.9.4 Histone Deacetylase Inhibitors for the Treatment of PD

HDACi have recently been investigated for treatment of inflammation and bone loss in several bone loss disease models and *in vitro* investigations (as recently reviewed (205)). Classical HDACi were designed as 'pan-inhibitors', as they target several HDACs due to their metal binding moiety that interacts with the catalytic domain of many HDACs across class I, II, and IV (228, 229). Interestingly, these broader acting compounds, such as Trichostatin A (TSA) (230-232), Sodium Butyrate (NaB) (231-233), Valproic acid (VPA) (232), Vorinostat (Suberanilohydroxamic acid; SAHA) (217, 234), MS-275 (31, 32, 232) and a novel investigative compound, 1179.4b (214, 218), have distinct effects on bone cells and inflammatory processes *in vitro* and *in vivo* . Specifically, results indicate prophylactic properties with reduced bone destruction and inflammation. Givinostat, another pan-inhibitor of HDACs, has progressed to clinical trials for the treatment of idiopathic juvenile arthritis (235). However, due to the broad nature of HDAC expression in human tissue, in addition to their specific isoform functionality (which is yet to be fully understood), future progression of pan-inhibitors into the clinic is unlikely (228, 229). At present, researchers are investigating the specific role of each individual HDAC isoform, and developing new compounds that selectively target individual HDACs that are important to the pathogenesis of disease. It is proposed that selective HDACi will improve therapeutic efficacy and reduce the risk of adverse outcomes from broad inhibition (228, 229).

1.10 Thesis Hypothesis and Aims

Two hypotheses were proposed. Firstly, *that HDAC 1, 2 and 5 are key modulators of inflammation and bone metabolism*. Secondly, *that specific HDAC inhibitors targeting HDAC 1, 2 and 5 will reduce inflammation and bone loss in inflammatory bone loss disease, such as PD.* Studies were carried out with human cells *in vitro* and *in vivo* using a mouse model of PD. For *in vitro* investigations, the addition of the inflammatory cytokine TNFα was used to represent an inflammatory stimulus.

These hypotheses led to the development of 4 main aims:

1. To determine if targeted inhibition of HDAC 1 and/or 2 would reduce human osteoclast formation and bone resorption *in vitro*.

2. To determine the effects of inhibitors targeting HDAC 1, 2 and/or 5 on cytokine and chemokine production by human monocytes *in vitro*.

3. To investigate the effects of inhibiting HDAC 5 during human osteoblastic bone formation *in vitro*.

4. To investigate and characterise specific HDAC expression in a mouse model of PD for future investigations using HDACi *in vivo*.

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Chapter 2: Histone Deacetylase Inhibitor NW-21 Regulates Tumour Necrosis Factor-α (TNFα) Driven Human Osteoclast Activity

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2.1 Abstract

Bone loss associated with inflammation is observed in a variety of pathologies. While the induction of bone resorbing osteoclasts during inflammation is not fully understood, it is apparent that cytokines, such as Tumour Necrosis Factor-α (TNFα), play an important role in promoting bone loss. A number of studies have shown elevated expression of histone deacetylase (HDAC) 1 during the inflammatory response in both rheumatoid arthritis and periodontitis. Therefore, this study tested the hypothesis that HDAC 1 elevation by TNFα enhanced osteoclast activity, and that the HDAC inhibitor (HDACi), NW-21, would suppress this TNFα induced osteoclastic bone resorption. In this study, human osteoclasts were formed over 17 days in the presence of receptor activator nuclear factor kappa B (RANKL) (0-50ng/ml) with and without TNFα (10ng/ml). Treatment with the HDACi, NW-21 that targets HDAC1 (20ng/ml), was also investigated. Assessment of important mediators of osteoclastogenesis, apoptosis, and key intracellular signalling factors were carried out at the mRNA and protein levels to investigate possible mechanisms involved. The findings of this study support our hypothesis that TNFα enhances HDAC 1 expression in osteoclasts ($p=0.002$) and that inhibitor NW-21 inhibited osteoclast activity ($p<0.005$). Two likely mechanisms were identified. Firstly, HDAC 1 activity appears to be important in the TNFα mediated mechanisms of NF-κB induction, resulting in excessive osteoclastic activity. Secondly, HDAC 1 regulation of osteoclast apoptosis may also be important through expression of the apoptosis inhibitor, survivin. This study demonstrates that compounds that suppress HDAC 1, such as NW-21, are likely to be useful in the treatment of inflammation mediated bone pathologies.

2.2 Statement of Authorship

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Kent Algate
Contribution to the Paper	First author and main contributor; Concept design, literature search, review and formulation of primary draft, in addition to reviewing and incorporating co-author comments and suggetsions.
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 25.1.18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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2.3 Introduction

Irreversible bone loss is a characteristic feature of several chronic inflammatory conditions including periodontitis (PD), rheumatoid arthritis (RA), and peri-prosthetic osteolysis (PPO). The loss of bone volume can be attributed to increased osteoclastic resorption on the bone surface closely associated with a localised chronic inflammatory reaction (1). Osteoclasts are large multinucleated cells uniquely designed to remove both the inorganic and organic components of bone that are then replaced by the bone forming osteoblasts (2, 3). In inflammatory states, however, the production of cytokines can result in elevated osteoclast differentiation and activity leading to increased bone resorption (3-5). Consequently, suppressing this inflammation-driven osteoclast activity is recognised as a key approach to reducing damaging bone loss in these conditions.

Osteoclasts originate from the monocyte/macrophage lineage with both macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB (RANK) ligand (RANKL) necessary to promote and sustain their differentiation into mature and active cells (6-8). RANKL interacts with its receptor RANK on pre-osteoclasts, initiating downstream signalling pathways involving the activation of TNF receptor-associated factors (TRAFs), along with transcription factors; nuclear factor of activated T-cells cytoplasmic-1 (NFATc1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (9-11). These proteins play an important role in the formation, activity, and survival of osteoclasts.

Numerous studies have demonstrated that the inflammatory cytokine, Tumour Necrosis Factor-α (TNFα) is a potent stimulator of bone loss through enhancing osteoclastic resorption (5). Indirectly, TNFα induces osteoclast formation and activity by increasing RANKL expression by several cell types. These include T and B-lymphocytes (12, 13), gingival epithelial cells (14, 15), arthritic synovial fibroblasts (16), osteoblasts and osteocytes (17, 18). Interestingly, TNFα has been shown to directly activate TRAF signalling independently of RANK/RANKL interactions (19, 20), promoting osteoclastogenesis through the induction of key factors regulating gene expression such as NF-κB (21). Phosphorylation of the

inhibitor of κB (IκB) allows translocation of NF-κB into the nucleus, and hence differentiation, activation, and survival of osteoclasts (22). TNFα can also initiate a secondary signalling cascade through intracellular death domains TNFRSF1A Associated Via Death Domain (TRADD) and Fas-associated with death domain (FADD), as well as the activation of several caspases leading to cell death (23). It is important to note that TNFα can promote cell survival and activity or induce cell death depending on the pathological condition. Despite these opposing effects it has proven to be an important cytokine in both inflammation and skeletal cell regulation in a variety of diseases.

Compounds that suppress osteoclast differentiation (such as the monoclonal antibody to RANKL, Denosumab) or resorptive activity (Bisphosphonates) can reduce systemic bone loss in conditions such as osteoporosis. However, long-term use of these compounds as therapies in high dosages is known to result in several adverse effects (reviewed in (24)). In addition, antagonists of the TNFα receptor have been shown to reduce bone resorption in mouse models of osteolysis (25, 26), however, these factors have been shown to be less effective in long term human trials (27, 28). We have previously demonstrated a method for regulating osteoclastogenesis and bone resorbing activity through targeted inhibition of histone deacetylase (HDAC) enzymes. HDAC inhibitors (HDACi) have been shown to reduce osteoclast resorption *in vitro* and bone loss *in vivo* (29-31). Furthermore, HDACi reduce the production of inflammatory cytokines and inflammation *in vivo* (29). Physiologically, HDAC enzymes are involved in the epigenetic regulation of gene expression and the post-translational modifications of proteins. These enzymes are required for the reversible removal of acetyl groups bound to proteins, altering their structure and function. HDAC regulation of gene expression is achieved through deacetylating lysine residues on core histone N-terminal tails (32). In addition, HDACs are involved in deacetylating and regulating non-histone proteins involved in a wide variety of cellular processes including production of inflammatory cytokines (33-35). There have been a total of 18 mammalian HDACs identified, 11 of which have been separated into 4 sub-classes based on their structure and cellular distribution: class I (HDAC 1, 2, 3, and 8), class IIa (HDAC 4, 5, 7, and 9), class IIb (HDAC 6, and 10) and class IV (HDAC 11). The remaining 7 atypical enzymes are classified as Sirtuins (class III)

due to their differing structure and co-factor activity (32). Dysregulation in the expression and activity of individual HDACs has been associated with the pathogenesis of a number of human diseases (36, 37), including inflammatory osteolysis (32, 38, 39).

HDACi have been used clinically as a treatment option for a range of malignancies both as mono- and combination therapies (40). In addition to other non-oncological applications (neurodegenerative, cardiac, and pulmonary disorders) their ability to attenuate inflammation and bone loss at doses substantially lower than their current clinical use for cancers is becoming more evident. Classical broad acting HDACi, that target a range of HDACs across both class I and II have been shown to reduce osteolysis in mouse models of RA and PD (29, 30, 41). In addition, more selective HDACi (class specific) have suppressive effects on bone resorption *in vitro* (31), along with reducing inflammation levels in animal models (42, 43). Variations in HDACi efficacy can possibly be attributed to their selective inhibition of specific HDACs. An understanding of the specific actions of individual HDACs will aid in identifying potential therapeutic targets for several diseases including inflammatory associated osteolysis.

We have recently identified elevated HDAC 1 expression, at both the mRNA and protein level, in gingival tissue excised from patients with chronic PD and alveolar bone loss (44). HDAC 1 protein localisation was observed in the nucleus and cytoplasm of large multinucleated osteoclast-like cells at sites of inflammatory osteolysis, with high levels of TNFα expression present. Therefore, we hypothesised that HDAC 1 elevation by TNFα would increase osteoclast activity and that the HDACi, NW-21, would reduce this activity *in vitro.* NW-21 was originally designed to target HDAC 1, but also has some affinity for HDAC 2, which is not surprising given the high sequence homology and apparent co-localisation of these two proteins (45, 46), hence redundancies may exist.

2.4 Methods and Materials

2.4.1 Histone Deacetylase Inhibitor (HDACi)

Compound NW-21 (29), a novel HDACi developed at the University of Queensland (also known as compound 51 (47) and ASU-13 (48)) designed to target the class I, HDAC 1 over other HDACs (IC_{50} = 0.021±0.02μM against HDAC 1 (Class I), 0.042±0.02μM for HDAC 2 (Class I), 0.3μM for HDAC 3 (Class I), >10μM for HDAC 4 (Class IIa) 0.88±0.06 for HDAC 6 (Class IIb), >1μM for HDAC 7 (Class IIa), >3μM for HDAC 8 (Class 1) (49)

2.4.2 Human In Vitro Osteoclast Assay

Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll-Paque separation media (Pharmacia Biotech, SWE) and differential centrifugation of human blood buffy coats obtained from the Australian Red Cross (Melbourne, AUS) as previously described (31). Once isolated, cells were suspended in an α-minimal essential media (α-MEM; Invitrogen, Melbourne AUS) with the supplements; 10% foetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine, 100nM dexamethasone (Invitrogen, Melbourne, AUS), 25ng/ml M-CSF (Chemicon International Inc., Millipore, MA), and seeded into wells of Falcon© *in vitro* assay plates at a concentration of $2x10^6$ cells/ml. After 24 hours, non-adherent cells were removed, leaving the remaining osteoclast precursors to culture for a further 16 days, maintained in complete media (changed every 3-4 days) at 37°C with 5% CO_2. From day 7, media was supplemented with recombinant human (rh) RANKL to promote the differentiation and activation of human osteoclasts as previously described (50). Subsequent assays involved supplementing media with rhTNFα (10ng/ml) commencing from various time points (days 1, 5, 7, 10, and/or 13) with RANKL (10, 20 or 50ng/ml). These doses of RANKL were chosen as 50ng/ml RANKL stimulates maximal osteoclast activity in the system, whereas 10 and 20ng/ml RANKL stimulate approximately 50% of the maximal stimulation of osteoclast formation and activity (50). Other than in the initial experiment, 10ng/ml RANKL was used, as this "sub-optimal dose" would allow us to identify both stimulatory and inhibitory effects of TNFα and/or HDACi treatments on the osteoclastogenic process. The HDACi NW-21 at

20nM in 0.01% dimethyl sulfoxide (DMSO) vehicle was used as previously described (29). The effects on osteoclast activity were assessed with treatments beginning on day 7 (early treatment) or on day 10 (late treatment). Control cells were treated with 0.01% DMSO.

2.4.3 Osteoclast Formation and Activity

Osteoclast formation was assessed via the quantification of multinucleated (>3 nuclei) tartrate resistant acid phosphatase (TRAP) positive cells (31) and compared between individual donors of each experimental treatment group. PBMC derived osteoclasts were also cultured on whale dentine (gift from Australian Customs and Border Protection Services) for the analysis of bone resorptive activity. Resorption pits formed on the dentine surface were photographed with the Philips XL30 Field Emission Scanning Electron Microscope (FESEM; 200x magnification) (Adelaide Microscopy, University of Australia), traced using Adobe Photoshop Elements 7.0 software (Adobe Systems, CAL), and quantified using Image J Software (1.47v, National Inst of Health, USA). The area of resorption within three representative images per dentine slice was used to calculate the average overall resorption per donor/treatment as previously described (31). Osteoclast activity was then estimated by calculating the percent area of dentine resorbed per number of TRAP positive multinucleated cells (MNCs) formed, and expressed as a percentage of resorption per osteoclast.

2.4.4 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using a TRIzol based method as per guidelines (Invitrogen Life Technologies, Carlsbad, USA) on day 14 of the human osteoclast assay, following RANKL, TNFα stimulation, and NW-21 treatment from either day 7 or day 10. Complimentary DNA (cDNA) was generated via reverse transcription using a Rotor-Gene Q (Qiagen, VIC, Australia) with 250ng of random hexamer and 200U of superscript III reverse transcriptase (Geneworks, Adelaide, SA, Australia). Amplification was conducted on samples in triplicate, using Platinum SYBR Green qPCR Supermix-UDG (Life Technologies, Pty, Ltd, VIC, Australia) as previously described (29). Gene expression was quantified using the comparative 2ΔΔCT method (51) and expressed as fold change in gene

expression which was normalized to an endogenous reference gene, human acidic ribosomal protein (hARP; (52)), and relative to the untreated control (in the absence of TNFα). Primer sequences used in this study were obtained based on previous publications; TNFr1 and TNFr2 (53), TRAF6 (31), TRAF2 (54), NFATc1, TRAP, Cathepsin K (CatK) and Calcitonin receptor (CTR) (10), Caspase 3, XIAP, and Survivin (55).

2.4.5 Extraction of Whole Cell Proteins and Western Immunoblots

Human Osteoclasts ($1x10^7$) were seeded into $75cm^2$ flasks supplemented with osteoclastogenic media as previously described, with RANKL (10ng/ml) addition commencing from day 7. TNFα (10ng/ml) with the presence or absence of HDACi NW-21 (20ng/ml) was added to cells from day 10, and left to incubate for 10 minutes followed by 2x ice cold washes with PBS, along with cell lysis with ice cold RIPA buffer (0.22% Beta glycerphosphate, 10% Tergitl-NP40, 0.18 Sodium orthovanadate, 5% Sodium deoxycholate, 0.38% EGTA, 1%SDS, 6.1 Tris, 0.29% EDTA, 8.8% Sodium chloride, 1.12% Sodium pyrophosphate decahydrate; diluted 10X in distilled water) (ab156034; Abcam). After 15 minutes incubation on ice, samples were centrifuged for collection of supernatants. Quantification of protein concentration in cell lysates was determined via BCA assay as per manufacturers' recommendations (Thermo Scientific BCA Protein Assay Kit).

Western immunoblots were conducted using a standard procedure. Briefly, 40 μg of whole cell proteins were loaded per lane on a 4-12% gradient Bis-Tris precast gel (NuPage, Invitrogen) at 200V for 60 minutes, followed by polyvinyliden fluoride (PVDF) membrane transfer for blotting using anti-IKB alpha (pospho S32+S36) mouse monoclonal antibody (1:500 dilution; abcam ab12135), anti-IKB alpha rabbit monoclonal antibody (1:1000 dilution; abcam ab32518), and anti-beta Actin mouse monoclonal antibody (1:1000 dilution; abcam ab8226) after blocking of non-specific binding sites (PBS/0.1% Tween/10%BSA). Immunoblots were scanned and visualized using the LI-COR Odyssey system (LI-COR Biotechnology, Lincoln, NE, USA). Quantification of extracted proteins was calculated using Image J software (1.47v; National Inst of Health, USA) and expressed as optical density ratio from beta Actin standard.

2.4.6 Immunofluorescent Labelling of HDAC 1 in Human Osteoclasts Exposed to TNFα

Control Cells (RANKL 10ng/ml with 0.01%DMSO) and cells exposed to TNFα from day 10 were fixed on day 14, with a 1:1 acetone/methanol solution for 5 minutes, followed by 5x PBS washes. Staining was conducted for polyclonal rabbit anti-human HDAC 1 (ab53091; Sapphire Bioscience Pty Ltd, NSW, Australia) with donkey anti-rabbit Cy5 (11175152; Jackson Immunoresearch Laboratories Inc., PA, USA) using standard procedures. Briefly, cells were submerged in 0.1% triton PBS for 5 minutes followed by overnight incubation at room temperature in primary HDAC 1 antibody (1:100), diluted in PBS 1% BSA. The following day, cells were rinsed in PBS (3x5 minutes) and incubated in conjugated secondary antibody (1:100), diluted in PBS 1% BSA, protected from light at room temperature. Cells were then PBS washed (3x5 minutes) and counterstained with DAPI for 5 minutes, washed, and mounted with Proscitech coverslips and SlowFade® Antifade Kit (33342; Life Technologies Pty Ltd, VIC, Australia) and left in dark.

2.4.7 Statistics

For analysis of osteoclast formation, activity, and mRNA gene expression, one-way ANOVA followed by Dunnett's multiple comparisons tests or students T-tests were used to identify changes between experimental groups and control cells. GraphPad Prism® for Windows, version 6.01, was used for the statistical analyses in this study.

2.5 Results

2.5.1 TNFα increases osteoclast activity in the presence of RANKL

RANKL induced a concentration dependent increase in osteoclast formation as indicated by numbers of TRAP expressing multinucleated cells (Figure 2.1). Interestingly, TNFα treatment reduced the numbers of osteoclasts in the presence of 10ng/ml RANKL by 2-fold ($p=0.0384$) but did not have this effect at higher doses of RANKL. Even though there were reduced numbers of osteoclasts there was not a statistically significant change in the area of dentine resorbed (Figure 2.1). This meant that, at 10ng/ml RANKL, TNFα reduced osteoclast numbers but the ones that formed had more than a 2-fold increase in bone resorbing activity ($p=0.0329$) (Figure 2.1).

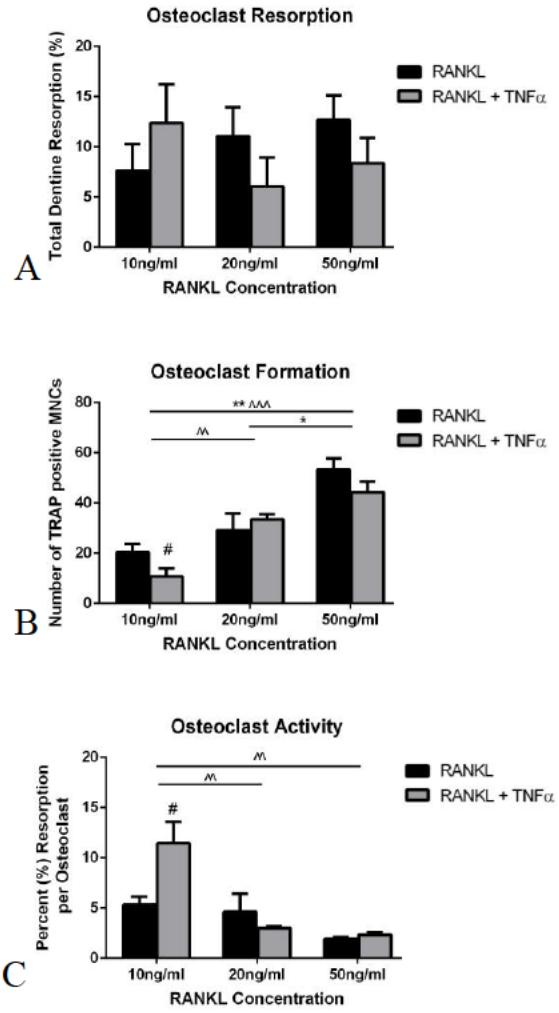

Figure 2.1 A) RANKL (dose-response) induced osteoclast formation as assessed by the quantification of large multinucleated cells expressing tartrate resistant acid phosphatase (TRAP) in the presence or absence of TNFα (10ng/ml) from day 10 **B)** Osteoclastic resorption pits measured in whale dentine in response to RANKL ± TNFα, presented as total dentine surface resorbed (%). **C)** Average osteoclast activity in the presence or absence of TNFα, as calculated by the number of cells per proportion of dentine surface resorption, and expressed as the percent resorption per cell. (* when comparing between RANKL concentrations; ^ when comparing between RANKL concentrations + TNFα; when # when comparing between RANKL and RANKL + TNFα within specified groups. (*,#,^ when $p<0.05$; **,##,^^ when $p<0.01$, ***,###,^^^ when $p<0.001$)).

2.5.2 TNFα induces HDAC 1 overexpression in osteoclasts

Based on the findings obtained with TNFα, further experiments were carried out to induce osteoclast formation using suboptimal doses of RANKL (10ng/ml). Osteoclasts were formed in the presence of RANKL (10ng/ml) from day 7, followed by the addition of TNFα (10ng/ml) from day 10. After 72 hours of TNFα exposure a significant 2-fold increase ($p=0.002$) in the mRNA expression of HDAC 1 was noted (Figure 2.2). Immunofluorescence staining of these cells demonstrated that HDAC 1 was also increased at the protein level (Figure 2.2). HDAC 1 expression was evident in both the nucleus and cytoplasm. Microscopic analysis of control cells (no TNFα addition) identified concentrated nuclear staining of HDAC 1. Interestingly, exposure to TNFα resulted in intense cytoplasmic staining of HDAC 1.

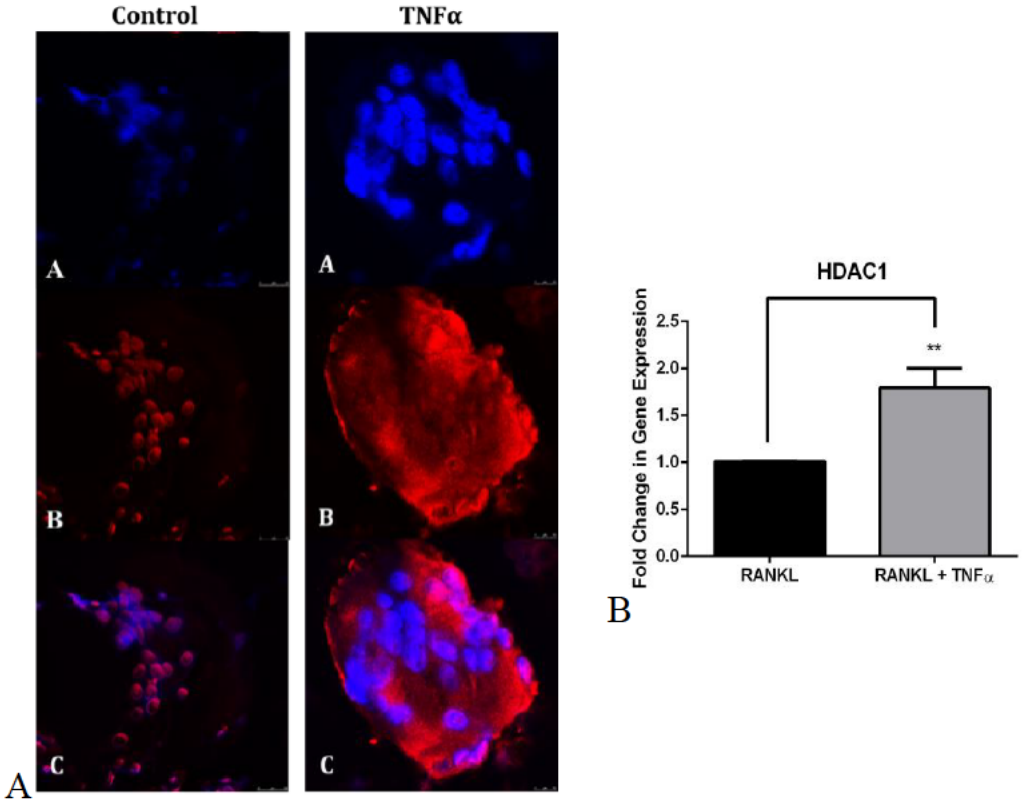

Figure 2.2 A) Representative Immunofluorescent staining of HDAC 1 protein localization in response to TNFα stimulation. *Left Column* RANKL (10ng/ml): a) DAPI in violet; b) HDAC 1 in red; c) overlay/co-localization in pink. *Right Column* TNFα + RANKL (10ng/ml): a) DAPI in violet; b) HDAC 1 in red; c) overlay/co-localization in pink. **B)** Effects of TNFα on HDAC 1 expression at mRNA level after 72 hours of cytokine exposure (** = $p<0.01$).

2.5.3 NW-21 supresses TNFα stimulated osteoclast formation and bone resorption

Given the increased levels of HDAC 1 in response to TNFα, HDACi NW-21 was tested to assess the effects on TNFα stimulated osteoclasts *in vitro* (Figure 2.3). Treatment with NW-21 significantly suppressed osteoclast formation and resorption ($p<0.001$ and $p<0.005$, respectively) confirming our previous findings in unstimulated cells (29). The suppressive effects appear to be dependent on timing, as treatments commencing from day 10, after the addition of RANKL, were less effective than treating from day 7, which potently inhibited osteoclastic formation and resorption in both the TNFα and unstimulated osteoclasts (Figure 2.3). However, it is important to note that inhibition of osteoclast numbers and activity were markedly suppressed by HDAC inhibition in the presence of TNFα when commenced from both the early and late time points ($p<0.005$).

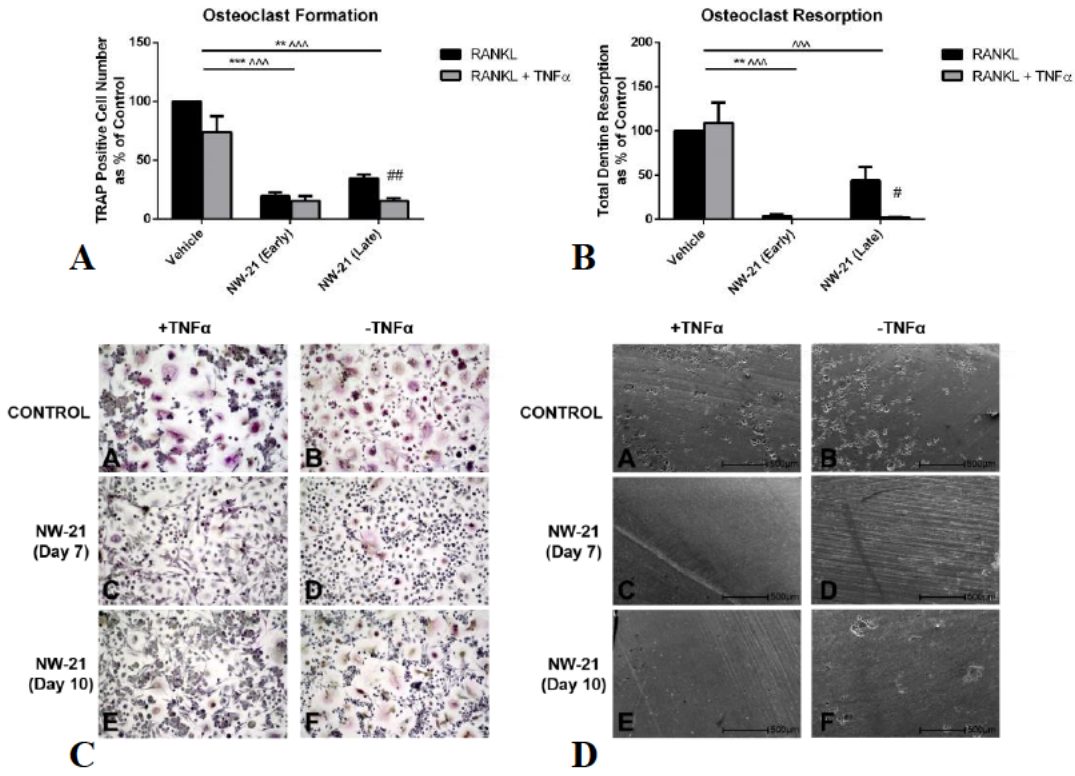

Figure 2.3 The effects of suppressing HDAC 1 with NW-21 (20nM) from either day 7 (Early) or day 10 (Late) on osteoclast in the presence or absence of TNFα (10ng/ml) **A)** Osteoclast Formation and **B)** Resorption. NW-21 significantly suppressed osteoclastic activity with early treatment in both RANKL mediated and TNFα stimulated cells. HDAC 1 inhibition also suppressed resorption from the later day 10 time point, however this was only statistically significant in the TNFα group. (* when comparing between RANKL concentrations; ^ when comparing between RANKL concentrations + TNFα; when # when comparing between RANKL and RANKL + TNFα within specified groups. (*,#,^ when $p<0.05$; **,##,^^ when $p<0.01$, ***,###,^^^ when $p<0.001$)). **C)** Representative osteoclast formation images of multinucleated cells expressing TRAP (violet). *Left Column* TNFα + RANKL (10ng/ml): a) Vehicle control; b) NW-21 (early) c) NW-21 (late). *Right Column* RANKL (10ng/ml): a) Vehicle control; b) NW-21 (early) c) NW-21 (late). **D)** Representative osteoclast resorption pits formed in dentine. *Left Column* TNFα + RANKL (10ng/ml): a) Vehicle control; b) NW-21 (early) c) NW-21 (late). *Right Column* RANKL (10ng/ml): a) Vehicle control; b) NW-21 (early) c) NW-21 (late).

2.5.4 NW-21 does not affect mRNA expression of RANK related signalling factors in TNFα stimulated osteoclasts

In order to investigate the mechanisms by which NW-21 reduced osteoclast activity in the presence of TNFα, we investigated the changes in expression of key factors involved in RANK signalling. NW-21 treatment had no effect on TRAF6 mRNA expression and NFATc1 mRNA expression was only slightly reduced (Figure 2.4). NFATc1 induced genes, other than CTR ($p=0.0394$) were also not significantly reduced following NW-21 treatment. Consistent with our formation analyses, the expression of a number of osteoclastogenic factors were reduced by TNFα treatment including CTR, TRAP and to a lesser extent the TNF receptors (Figure 2.4).

2.5.5 NW-21 inhibits TNFα stimulation of osteoclast activity, consistent with reduced cell survival

As the initial experiments indicated that the enhanced resorption by osteoclasts may be related to enhanced osteoclast survival we investigated the effect of TNFα and NW-21 treatment on factors relating to apoptosis. Caspase 3, a potential apoptotic factor executing TNF receptor death signalling, was not affected by TNFα or NW-21 treatment. However, NW-21, in absence of TNFα appeared to reduce Caspase 3 at both early and late treatment time-points ($p=0.0073$ and $p=0.0099$, respectively) (Figure 2.4). Interestingly, Survivin, a target gene of NF-κB activity induced by TNFα stimulation ($p=0.0425$), was also markedly suppressed (3 to 5-fold) by both early and late NW-21 treatments ($p=0.0040$ and $p=0.0015$, respectively). XIAP, another survival factor assessed was not affected by either TNFα or HDAC 1 inhibition (Figure 2.4).

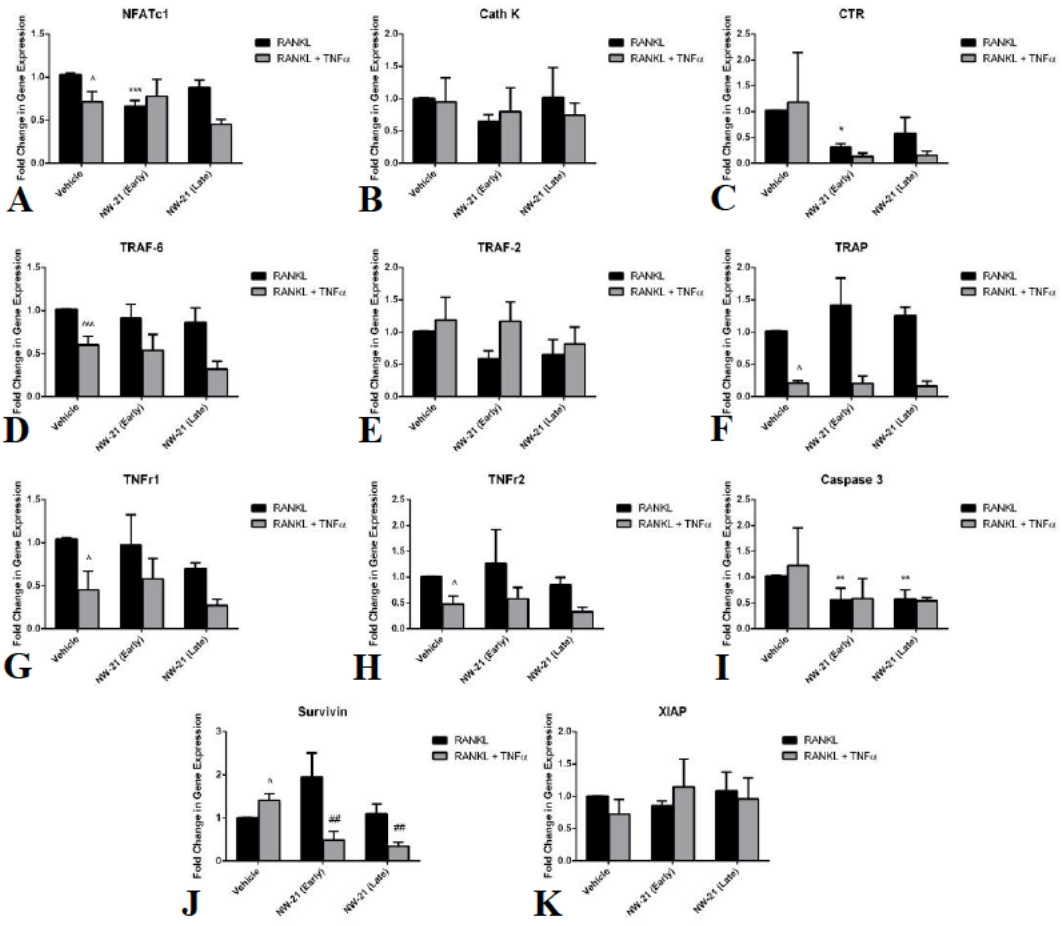

Figure 2.4 Effects of HDAC 1 inhibition with NW-21 (20nM) in the presence or absence of TNFα (10ng/ml) from day 10 on the fold change in mRNA expression after 72 hours of TNFα exposure. NW-21 treatment commenced in conjunction with RANKL (early) or TNFα (late). Results are expressed relative to vehicle (0.01% DMSO in regular osteoclastogenic media) **A)** NFATc1; **B)** Cathepsin K; **C)** CTR; **D)** TRAF 6; **E)** TRAF 2; **F)** TRAP; **G)** TNFr1; **H)** TNFr2; **I)** Caspase 3; **J)** Survivin; **K)** XIAP. (* when comparing treatments to vehicle in RANKL treated cells; # when comparing treatments to vehicle in RANKL + TNFα treated cells; ^ when comparing between RANKL and RANKL + TNFα vehicles; (*,#,^ when $p<0.05$; **,##,^^ when $p<0.01$, ***,###,^^^ when $p<0.005$))

2.5.6 NW-21 supresses TNFα activation of NF-κB

NF-κB is a specialized transcription factor stimulated by inflammatory cytokines such as TNFα. When IκB is phosphorylated its degradation results in NF-κB no longer bound within the cytoplasm, allowing its translocation into the nucleus and the transcription of inflammatory, proliferative, and survival factors. Acetylation of IKK has been reported to reduce IκB phosphorylation, resulting in NF-κB inactivation. As acetylation may have an important role in this process we investigated the effects of NW-21 on TNFα induced NF-κB activity. TNFα induction of IKK is consistent with the marked increase in phosphorylated IκB ($p=0.001$) (Figure 2.5) and the disappearance of non-phosphorylated IκB ($p<0.0001$) within 10 minutes of TNFα treatment (figure 5B). Interestingly, treatment with NW-21 significantly suppressed the induction of IκB phosphorylation ($p=0.002$) and increased levels of non-phosphorylated IκB ($p=0.0003$) (Figure 2.5).

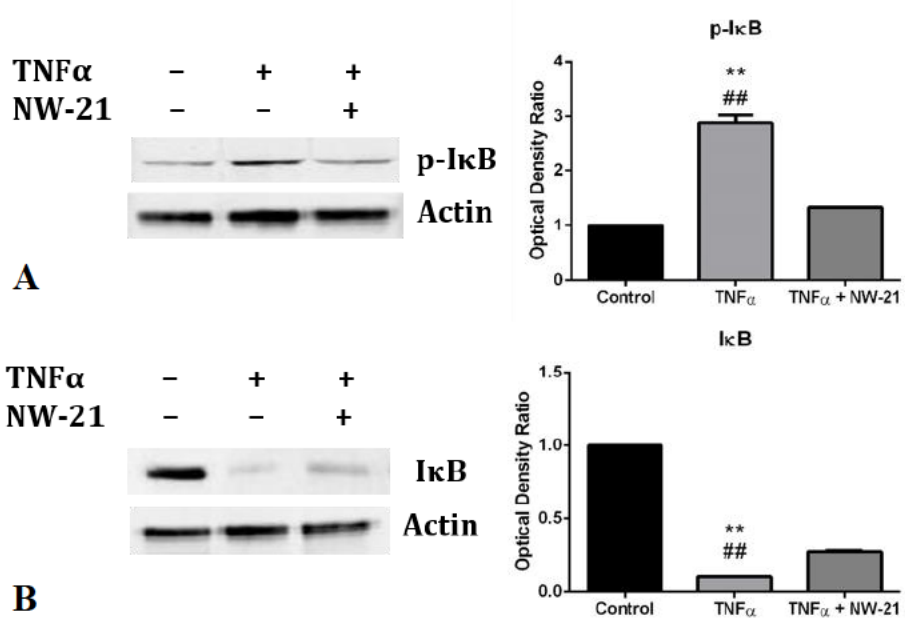

Figure 2.5 Effects of HDAC 1 inhibition with NW-21(20nM) in TNFα (10ng/ml) stimulated cells on **A)** the phosphorylation of IκB (p-IκB) and **B)** total non-phosphorylated IκB (IκB). * when comparing TNFα with control; # when comparing TNFα with TNFα + NW-21 (**,##,^^ when $p<0.01$; ***,### when $p<0.001$)

2.6 Discussion

The findings of this study support the hypothesis that HDAC 1 is a key factor in the formation and activity of osteoclasts in an inflammatory environment. Targeting HDAC 1, and to a lesser extent HDAC 2, with a novel HDACi, NW-21, significantly suppressed the formation and bone resorptive capabilities of human osteoclasts *in vitro*. The addition of TNFα resulted in less osteoclasts forming, but those that did were more active with increased areas of bone resorption present on the dentine surface. The addition of TNFα from day 10 with sub-optimal RANKL (10ng/ml) resulted in a two-fold increase in the mRNA expression of HDAC 1, suggesting a role for this HDAC in the enhanced osteoclast activity observed. Treatment of the cells with the HDACi, NW-21, resulted in a marked suppression of osteoclast formation in the presence of TNFα and this was observed when treatments commenced from both early (day 7) and late treatment time points (day 10). This suppression was not found to be due to reductions in key signalling molecules involved in the RANK/RANKL pathway as previously reported (29), but due to a suppression of the activation of NF-κB by TNFα. These results suggest that HDACi, NW-21, could be a possible option for targeting TNFα associated osteoclast bone loss.

It is well established that TNFα is a potent, pro-inflammatory cytokine in bone loss pathologies including PD, RA and PPO. Excessive and prolonged production of TNFα leads to tissue destruction, specifically in relation to skeletal integrity (5). We recently evaluated HDAC expression in the gingival tissue of patients with PD and found excessive levels of HDAC 1, corresponding with cells staining positive for TNFα (29, 44, 56). In addition, TRAP expression, a marker of cells devoted to the osteoclast lineage, is significantly elevated. This study further demonstrates an important relationship between HDAC 1 and TNFα, with TNFα markedly elevating HDAC 1 mRNA expression in osteoclasts.

An interesting finding of this study was the observation that the action of NW-21 on osteoclast development was different when TNFα was present. Within the RANKL system utilised, the timing of HDACi treatment was very important. Commencing NW-21 treatment with RANKL resulted in the greatest inhibition.

When NW-21 was present prior to RANKL induction (data not shown), there was no suppression of osteoclast differentiation or activity. This finding is supported by our previous studies *in vitro* assessing several broad and selective HDACi compounds (29).

This study shows that TNFα may mediate its effects through stimulation of the NF-κB pathway and may explain the sensitivity of TNFα treated cells to NW-21. The important role of NF-κB in osteoclasts and bone metabolism has been observed in a variety of studies where deletions in NF-κB subunits p50 and p52 (57,58), or the targeted deletion of IKK (59), result in severe osteopetrotic phenotypes in mice. Interestingly, several studies have identified NF-κB as a central factor regulating a variety of osteolytic inflammatory disorders, such as PD and RA (as reviewed in (60)). The role of NF-κB in inflammatory bone loss is further supported by studies blocking NF-κB signalling, which result in anti-osteoclastic and anti-inflammatory outcomes (61, 62).

During osteoclast development, deacetylated IKK is required for the phosphorylation of IκB that results in activation and translocation of NF-κB into the nucleus, thus stimulating osteoclastogenesis (60). Furthermore, we show that NW-21, can inhibit elevated phosphorylation of IκB in the presence of TNFα, and thus may regulate the translocation of NF-κB into the nucleus. This is consistent with TNFα induction of HDAC 1 in the cytoplasm causing non-histone protein deacetylation of IKK. This results in increased IκB phosphorylation and NF-κB activity that is greater than RANKL mediated NF-κB activation alone. This observation is supported by a report showing NF-κB suppression by a broad acting class I and class II HDACi, Trichostatin A (TSA) and sodium butyrate (NaB) (63). In addition, TSA and NaB reduced expression of NF-κB reporter genes, as well as decreasing levels of p65 in nuclear extracts (63). Our findings support the observation of an important role for HDACs in this NF-κB pathway.

Studies evaluating the effects of HDACi have observed similar bone benefits in a variety of disease models. We recently reported that a broad acting HDACi, 1179.4b, significantly reduced alveolar bone loss in a mouse model of PD consistent with reductions in osteoclasts formation adjacent to the bone surface

(30). Osteoclast inhibition was also evident in a number of *in vitro* assays of human osteoclasts (31)**.** Similarly, suppression of pro-inflammatory cytokines such as TNFα, interleukins, and MMPs have been reported through targeted suppression of HDAC enzymes (64, 65).

An apparent inconsistent finding of this study was the minimal effect on NFATc1 mRNA expression by NW-21, however this could be due to the adjusted signalling mechanisms in the presence of TNFα, or the suboptimal dose of RANKL used in this assay. A reduction of NFATc1 expression in human osteoclasts is observed by the broad acting HDACi, 1149.4b (31) and with HDACi NW-21 (44). The reduction of NFATc1 was consistent with decreased osteoclastic gene transcription, hence, suppression of osteoclastogenesis. In the presence of TNFα, however, this mechanism of action appeared to be less influenced by NFATc1 despite the potent inhibition of osteoclast activity with NW-21. NFATc1 is an essential transcription factor that is necessary for the differentiation and activation of osteoclasts (11) acting through the induction of osteoclasts specific factors such as TRAF6, CTR, Cathepsin K, and OSCAR. Experimental knockouts or antagonists of upstream signalling factors such as RANK or RANKL, or NFATc1 itself, results in severe osteoclast suppression *in vitro* and osteopetrotic phenotypes *in vivo* (66). Interestingly, in this study there were no differences in the mRNA expression of these factors following NW-21 treatment of TNFα stimulated osteoclasts. This finding indicates an additional post-transcriptional target or role for HDAC 1 during TNFα stimulated osteoclastogenesis.

Considering the fundamental role of HDAC 1 in the epigenetic regulation of multiple genes, it would not be surprising if more than one mechanism of action is involved. The expression of factors regulating apoptosis were interesting with the apoptotic inhibitor, survivin (55), being elevated by TNFα and markedly suppressed by NW-21. TNFα's elevation of survivin may explain the increase in resorption observed with decreased osteoclasts numbers, as prolonged survival may have accounted for the elevated activity. Furthermore, our group has shown increased levels of survivin in RA and periodontal disease in humans with active disease and bone destruction (22). These findings show further studies are required into how apoptosis may be regulated by HDAC 1 in bone pathologies.

2.7 Conclusion

In conclusion, the findings of this study indicate a significant role for HDAC 1 during inflammatory bone metabolism. HDAC 1 activity appears to be important in the TNFα mediated mechanisms of NF-κB induction, resulting in excessive osteoclastic activity *in vitro.* In addition, HDAC 1's regulation of apoptosis of osteoclasts may also be important. Suppressing HDAC 1 and 2 with NW-21 may change osteoclast activity in a number of ways, effectively changing them from an inflammatory driven catabolic phenotype to a less destructive phenotype. While HDACi were originally developed as chemotherapeutic/ anticancer compounds, the use of more specific targeting HDACi at significantly lower doses are now being investigated for the treatment of a variety of other diseases including inflammatory conditions. The present study has demonstrated that the class I HDAC inhibitors such as NW-21 are likely to be useful in the treatment of inflammatory bone pathologies.

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Chapter 3: Selective Histone Deacetylase Inhibition Supresses Cytokine Synthesis and Bone Resorption by Human Monocytes and Osteoclasts in vitro

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3.1 Abstract

Epigenetic regulation is an emerging therapeutic target of host immune regulation in a variety of conditions, including periodontitis (PD). Histone deacetylases (HDAC) are involved in the deacetylation of core histone proteins, regulating pre- and post- transcriptional activity. PD is due to the host immune response to infective pathogens, initiating catabolic processes, such as osteoclast driven alveolar bone loss. In this study, we investigate the activity of novel therapeutic agents targeting specific HDAC (HDAC 1, 2 and 5) shown to be upregulated in human periodontitis, in cytokine stimulated human monocytes and osteoclasts *in vitro*. Inhibition of both HDAC 1 and 2 significantly ($p<0.05$) reduced the expression of several inflammatory cytokines and chemokines at the mRNA and secreted protein level ($p<0.05$). The formation of tartrate resistant acid-phosphatase (TRAP)-positive osteoclasts and their bone resorptive capabilities *in vitro* were also significantly diminished through reduced Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) expression and osteoclast specific target genes ($p<0.05$). Similar trends were observed when inhibiting HDAC 1 and, to a lesser extent, HDAC 2 in isolation, however their combined inhibition had the greatest anti-inflammatory and anti-osteoclastic effects. Targeting HDAC 5 had minimal effects on the inflammatory or osteoclastic processes observed in this study, whereas a broad acting HDACi, 1179.4b, had profound effects on suppressing cellular function. This study shows that targeting the epigenetic factors, HDACs, is a potent and effective way of regulating the cellular response to inflammatory stimuli, important for the destructive processes in PD.

3.2 Statement of Authorship

Statement of Authorship

Title of Paper	Selective Histone Deacetylase Inhibition Supresses Cytokine Synthesis and Bone resorption by Human Osteoclastic cells *in vitro*
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Kent Algate
Contribution to the Paper	First author and main contributor; Concept and methodological design, investigation, project administration, validation and visualisation, data curation and analysis, formulation of primary draft, in addition to reviewing and incorporating co-author comments and suggestions
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date: 25.1.18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	David Haynes
Contribution to the Paper	Investigation, conceptualisation, supervision and manuscript review
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Name of Co-Author	Tracy Fitzsimmons
Contribution to the Paper	Investigation, methodology, conceptualisation, data interpretation and review of manuscript
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Co-Author Contributions

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i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Edward Holson		
Contribution to the Paper	Formulation, supply, and methodological advice for use of HDAC inhibitors		
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Signature		Date	29/1/18

3.3 Introduction

Periodontitis (PD) has been identified as one of the most common inflammatory bone loss diseases worldwide, with the most aggressive form affecting more than 11% of the adult population (1, 2). In addition to being the leading cause for tooth loss in humans, individuals with advanced PD have a high comorbidity with conditions such as rheumatoid arthritis, acute coronary syndrome and cognitive impairment (3). PD is characterised by the destruction of tooth supporting structures within the periodontium, such as alveolar bone. This is due, in part, to the host immune response to periodontal pathogens within the subgingival microbiome (4). Destruction of both hard and soft oral tissue in untreated PD may promote the progression of the local infection to the development of systemic reactions that induce the onset of associated conditions with high mortality rates (5, 6). Therefore, it is necessary to develop effective treatments that target both the inflammation and bone loss.

During the inflammatory reaction, activated immune cells have the capacity to stimulate bone resorbing osteoclasts by the synthesis of cytokines with pro-osteoclastic properties, such as tumour necrosis factor-α (TNFα) and receptor activator of nuclear factor-κB ligand (RANKL) (7, 8). Investigations assessing inflammatory related bone pathologies have utilized the pro-catabolic features of cytokines such as TNFα, to establish *in vitro* techniques that represent this stimulated environment and the formation of inflammatory osteoclasts (7, 9). Furthermore, the production of chemokines such monocyte chemoattractant protein-1 (MCP1) and macrophage inflammatory protein-1α (MIP1a) by immune cells have profound effects on exacerbating this response, whilst directly influencing the catabolic processes of bone metabolism (10-12). A number of studies have demonstrated increased expression and production of these molecules in inflamed periodontal gingival tissues and crevicular fluids (13, 14), supporting the need to reduce both the immune response and the activity of inflammatory osteoclast in PD.

As we now understand the relationship between the immune and skeletal system (12), novel therapeutic targets have been identified in a range of bone loss diseases.

Over the past decade, epigenetic regulation of cells has been established as a potent method for regulating cell phenotype and activity. Studies have revealed the anti-inflammatory and anti-osteoclastic activity of histone deacetylase (HDAC) inhibitors (HDACi) (15-17). HDACs are a group of 18 human enzymes that are involved in the epigenetic processes of gene regulation and post-transcriptional protein modification (18). These include class I HDACs (1, 2, 3, and 8), class IIa (HDAC 4, 5, 7, and 9), class IIb (HDAC 6, and 10) and class IV (HDAC 11). The remaining 7 atypical enzymes are classified as Sirtuins (class III) due to their differing structure and co-factor activity. Physiologically, the catalytic domain of HDACs prompts the removal of acetyl groups from histone lysine side chains to condense chromatin and repress gene expression; counteracting the acetylation capacity of histone acetyl transferase (HAT) enzymes (18). However, dysregulation of their expression or activity can lead to altered epigenetic modifications, potentially explaining the functional changes in cell cycle and immunoregulatory processes, perpetuating the development of disease. Our recent analysis of HDAC expression in human periodontal gingiva from patients with chronic PD identified significant variations in the physiological HDAC profile (19). Specifically, HDAC 1, 5, 8 and 9 were overexpressed in PD tissue, with HDAC 1 co-localising with TNFa and TRAP (tartrate resistant acid-phosphatase) positive osteoclastic cells.

To date, investigations into bone metabolism and inflammation with the use of HDACi have primarily focussed on broad acting compounds (pan inhibitors) that supress enzymes across the 4 classes. These include 1179.4b, Suberanilohydroxamic acid (SAHA), Trichostatin A (TSA), Romidepsin (FR901228), and Givinostat (ITF2357), as reviewed by Cantley *et al* (16). Despite reported anti-osteoclastic and anti-inflammatory activities in several *in vitro* and *in vivo* models, only Givinostat has progressed to phase 2 clinical trials for the treatment of juvenile arthritis with positive outcomes (20). As the enzyme suppression profile of these HDACi are broad, with variations in HDAC expression and activity between cell types in addition to redundancies between HDAC gene and protein targets (21), further progression of these compounds to clinical use may be challenging. Subsequently, more recent studies using HDACi have focused on characterising class specific inhibitors with high affinity for

individual isoforms, such as MS-275 and NW-21 (class I, HDAC 1) that present favourable anti-osteoclastic and anti-inflammatory properties (22-24).

We now have access to a range of compounds designed to inhibit individual or combinations of HDAC isoforms. Therefore, the current study aimed to suppress HDACs that are upregulated and potentially involved in the pathogenesis of chronic PD, HDACs 1, 2 and 5. The effects of selective HDAC inhibition on cytokine/chemokine production by TNFα-activated monocytes were investigated. In addition, these HDACi were assessed for their anti-osteoclastic effects during cytokine-induced osteoclastogenesis *in vitro*.

3.4 Methods

3.4.1 Histone Deacetylase Inhibitors (HDACi)

Selective HDACi for HDAC 1 (**BRD0302**) and HDAC 2 (**BRD6688**) (25) were designed and synthesized, along with **Merck60** (HDAC 1 and 2 inhibitor) being supplied by the Stanley Centre for Psychiatric Research at the Broad Institute of MIT and Harvard (Cambridge, USA) (25). Effects **Compound 39** (HDAC 5 inhibitor) and **1179.4b** (Class I and II inhibitor), supplied by the University of Queensland's Institute of Molecular Bioscience, were also investigated in this study (26, 27). HDACi used for *in vitro* treatments were suspended in a dimethyl sulfoxide (DMSO) vehicle at 0.01%.

3.4.2 In Vitro Human Monocyte/Macrophage and Osteoclast Assay

Isolation of peripheral blood mononuclear cells (PBMCs) from healthy human blood donors (Australian Red Cross, AUS) using a Ficoll-Paque media (Pharmacia Biotech, SWE) and differential centrifugation was conducted as previously described (23). PBMCs were then suspended in α-minimal essential media (α-MEM; Invitrogen, AUS) with 10% foetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine, 100nM dexamethasone (Invitrogen, AUS) and 25ng/ml recombinant human (rh)-macrophage stimulating factor (M-SCF; Millipore, USA) before being seeded into Falcon© assay plates at $2x10^6$

cells/ml. Non-adherent cells were removed after 24 hours at 37°C, 5% CO_2, leaving the remaining monocyte/osteoclast progenitors to culture for the remainder of experimental assays.

To assess the anti-inflammatory activity of HDAC suppression, isolated PBMCs from 4 donors were stimulated with the pro-inflammatory cytokine rh-TNFα (TNFα; 10ng/ml; R&D Systems, USA) for 24 hours to induce an activated inflammatory state in the presence or absence of HDACi: **BRD0302, BRD6688, Merck60, Compound 39,** and **1179.4b.** PBMCS (osteoclast progenitors) from 8 donors were left untreated (±24 hour stimulation of TNFα; 10ng/ml) for 7 days until induction of osteoclastic differentiation with rh-RANKL (RANKL; 10ng/ml; Millipore, USA). Treatments of HDACi targeting HDAC 1, HDAC 2, HDAC 1 and 2, and HDAC 5 were then analysed for their anti-osteoclast/anti-bone resorbing efficacy (**BRD0302, BRD6688, Merck60,** and **Compound 39**) over the remaining 10 days of the 17-day assay period, as previously established (23). Effects of HDACi on cell viability/proliferation was evaluated by colorimetric WST-1 based (Roche, DEU) assay as per manufacturer's instructions. All HDACi treatments were compared to 0.01% DMSO vehicle treated cells.

3.4.3 Assessment of HDACi Activity on Cytokine/Chemokine Production

TNFα (10ng/ml) activated monocyte/macrophages were treated with HDACi (10 and 100nM) for 24 hours as previously described. Cytokine and chemokine levels (Interleukin (IL)-1β, 1L-10, regulated on activation, normal T cell expressed and secreted (RANTES), TNFα, Interferon (IFN)-γ, MCP-1, and MIP-1α) in cell culture supernatants were determined by enzyme-linked immunosorbent assay or by multiplex immunoassay. Assays were performed in accordance with manufacturer's directions.

3.4.4 HDACi Effect on Osteoclast Formation and Bone Resorbing Activity

RANKL (10ng/ml) induced differentiation of TNFα (10ng/ml) stimulated pre-osteoclastic cells were treated with HDACi (10-1000nM) for 10 days as outlined above. After 1 week of treatment, osteoclastic cells cultured on 16 well Falcon© chamber slides were fixed for 10 minutes in 4% Glutaldehyde solution, followed by serial washes with distilled water. Cells were then stained for tartrate resistant acid phosphatase (TRAP) using an established method (23), and imaged by light microscopy (Nikon Microphoto FXA Photomicroscope, USA). Large TRAP-positive staining cells with more than 3 nuclei, characteristic of mature osteoclasts, were quantified for comparative analysis between treatments (23).

Remaining TNFα (10ng/ml) stimulated osteoclastic cells were cultured on whale dentine discs in the presence of HDACi (10nM). After a total of 17 days in culture, dentine was trypsinized and washed vigorously to remove adherent cells. Discs were then carbon coated for imaging under a Philips XL30 Field Emission Scanning Electron Microscope (FE-SEM) at 200x magnification (Adelaide Microscopy, University of Adelaide, AUS). Analysis of osteoclast activity was conducted by the measurement of osteoclastic resorption pits present on dentine surface using Adobe Photoshop Elements (7.0v, Adobe Systems Software Ireland Ltd) and Image J software (1.47v, National Inst of Health USA). The total area of resorption present within 3 representative images per dentine slice was used to quantify the average overall resorption per donor, and compared between treatment groups (23). Osteoclast activity was determined by calculating the percent area of dentine resorbed per number of TRAP positive multinucleated cells forming, and expressed as a percentage of resorption per osteoclast.

3.4.5 Analysis of Inflammatory and Osteoclastic Gene Expression with HDACi Treatment

Total RNA was extracted from TNFα (10ng/ml) stimulated PBMCs using TRIzol (Thermo Fisher Scientific; AUS) based methods as per manufacturers' guidelines after 24-hour incubation with HDACi, or on day 10 and 14 following osteoclast differentiation with RANKL (10ng/ml) at day 7 of the 17-day osteoclast assay. Complimentary DNA (cDNA) was generated using Rotor-Gene Q (Qiagen, AUS) reverse transcription with 250ng of random hexamer and 200U of superscript III reverse transcriptase (Geneworks, AUS), followed by amplification of samples in triplicate using a Platinum SYBR Green qPCR Supermix-UDG (Life Technologies, Pty, Ltd, AUS) as previously described (23). Gene expression was quantified using the $2^{-\Delta CT}$ method (28) and expressed relative to an endogenous reference gene, human acidic ribosomal protein (hARP) (29). Inflammatory cytokine/chemokine molecules investigated in this study included TNFα, IL-1β, 1L-10, IFN-γ, MCP-1, MIP-1α (24) and RANTES (37). Osteoclast related genes analysed were nuclear factor of activated T-cells 1 (NFATc1), TRAP, Cathepsin K (Cath K), Calcitonin receptor (CTR) (24, 30), TNF associated receptor activator factor-6 (TRAF6) (23), dendritic cell-specific transmembrane protein (DC-STAMP) and β3-integerin, in addition to HDACs 1, 2 and 5 (23).

3.4.6 Statistics

Statistical analysis of osteoclast formation and activity, along with gene and protein expression studies used one-way ANOVA followed by Dunnett's multiple comparisons tests to identify significant variations between experimental groups and control cells with statistical significance being accepted at $p<0.05$. GraphPad Prism® version 7 was used in this study.

3.5 Results

3.5.1 HDACi alter the cytokine profile of TNFα stimulated monocytes

As TNFα is a key molecule involved in a variety of cellular functions, including orchestrating the cytokine cascade during an immune response, it was used to induce and mimic inflammatory events *in vitro*. As expected, stimulating human PBMCs with TNFα (10ng/ml) for 24 hours prompted the robust production of pro-inflammatory cytokines and chemokines IL-1β, TNFα, MCP-1 and MIP-1α both at the mRNA (Figure 3.1) and protein level (Table 3.1) ($p<0.05$). Whereas production of RANTES, IFN-γ and the anti-inflammatory cytokine, IL-10, were increased in supernatant only ($p<0.05$). Interestingly, the assessment of HDAC 1, 2 and 5 identified the induction of only HDAC 1 and 2 expression by TNFα (Figure 3.2)

The HDAC 1 inhibitor (**BRD0302**) and HDAC 2 inhibitor (**BRD6688**) reduced the expression of IL-1β, TNFα, MIP-1α and MCP-1 at the mRNA level in a dose dependent mechanism (10-1000nM; Figure 3.1). However, only protein levels of IL-1β and MCP-1 were significantly reduced in cell supernatant with inhibition of HDAC 1 or HDAC 2 at doses investigated (10-100nM) ($p<0.05$) (Table 3.1). Secreted TNFα, MIP-1α, RANTES, IFN-γ, and IL-10 were not affected by selective suppression of HDAC 1 or 2.

Targeting HDAC 1 and 2 in combination with **Merck 60,** reduced both mRNA expression and protein secretions of IL-1β and MCP-1 ($p<0.05$) (Figure 3.1; Table 3.1). Gene expression of TNFα and MIP-1α, and IFN-γ protein were also suppressed ($p<0.05$). Although notable reductions in TNFα and MIP-1α protein secretions were observed with **Merck 60** treatment, these did not achieve statistical significance. Interestingly, IL-10 levels in cell supernatants were reduced at the highest dose (100nM) of **Merck 60.** Levels of RANTES were unchanged by HDAC 1 and 2 inhibition.

Compound 39 (HDAC 5 inhibitor) had minimal effect on the cytokine profile of activated monocyte/macrophages in these assays, with no significant variations in any factors investigated (Figure 3.1; Table 3.1).

Interestingly, broad suppression of class I and II HDACs with low doses (10nM) of **1179.4b** had a similar anti-inflammatory profile as the class 1 inhibitor Merck 60, with majority of pro-inflammatory factors evaluated in this study being significantly reduced both at the mRNA and protein level. These included IL-1β, IFN-γ, MCP-1, and MIP-1α gene expression and protein secretions. (Figure 3.1; Table 3.1).

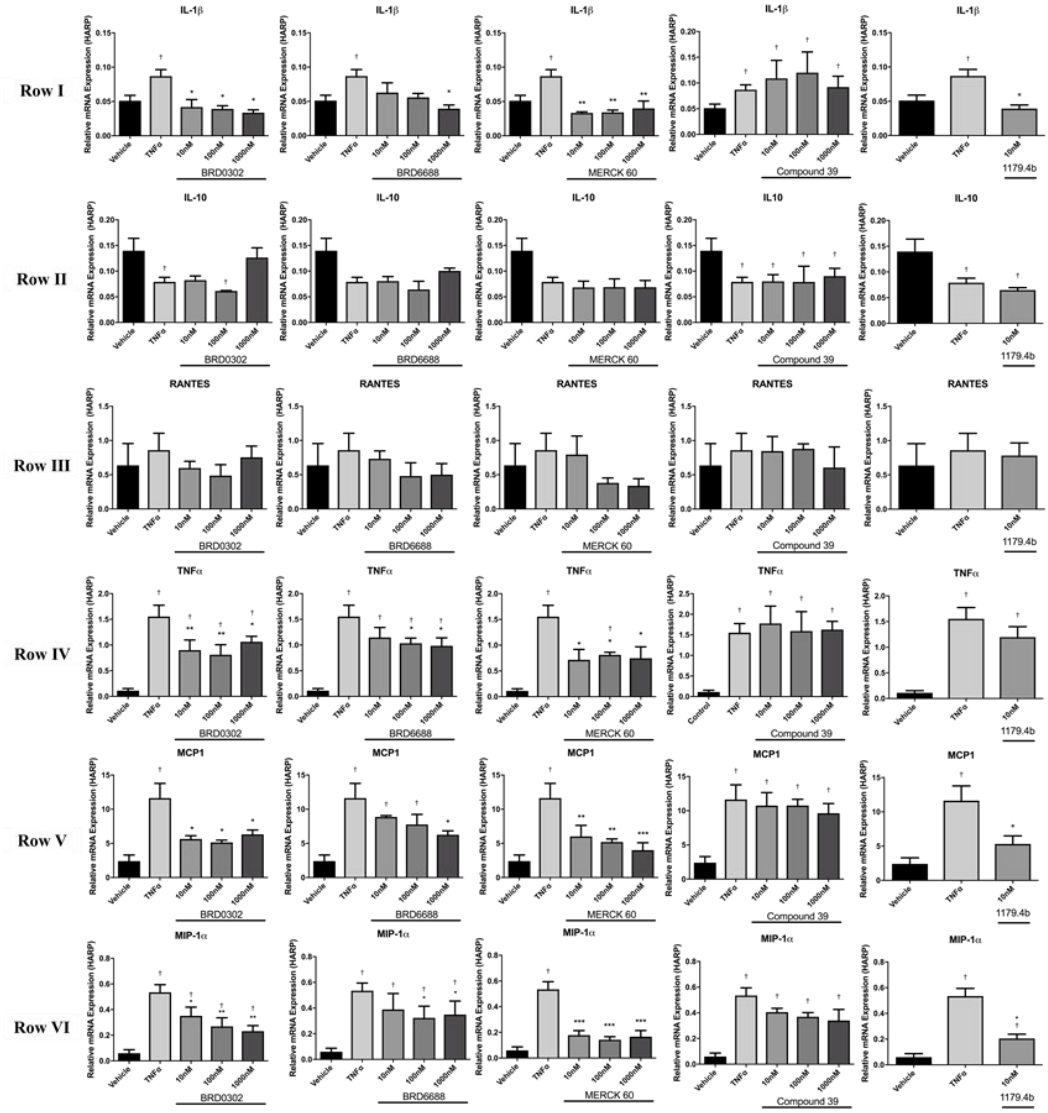

Figure 3.1 Effects of HDACi (10-1000nM) targeting HDAC 1 (**BRD0302**), HDAC 2 (**BRD6688**), HDAC 1 and 2 (**MERCK 60**), HDAC 5 (**Compound 39**) and broad suppression of class I and II HDACs (**1179.4b**) on mRNA expression of cytokines/chemokines in TNFα (10ng/ml) stimulated monocytes. **Row I)** Interleukin (Il)-1b; **Row II)** Il-10; **Row III)** Regulated on activation, normal T cell expressed and secreted (RANTES); **Row IV)** Tumour necrosis factor-α (TNFα); **Row V)** Monocyte chemotactic protein-1 (MCP-1); **Row VI)** macrophage inflammatory protein-1α (MIP-1α). Values expressed as relative to the housekeeping gene, human acidic ribosomal protein (hARP). * ($p<0.05$) Compared to TNFα stimulated control cells † ($p<0.05$) compared to vehicle control.

Table 3.1: HDACi effects on cytokine/chemokine production by TNF stimulated monocytes

Treatment	IL-1β	IL-10	TNFα	IFN-γ	MCP-1	MIP1a	RANTES
Vehicle	5.8±0.6	13.6±2.2	11.6±5.7	58.1±18.3	240.466±54.4	790.7±100.0	450.3±89.5
TNFα Stimulated	20.9±3.8†	57.6±9.1†	2675.0±213.9†	436.5±78.0†	1706.5±324.3†	2039.1±197.1†	2308.3±430.1†
BRD0302: 10nM	14.0±2.5	45.2±3.3†	2725.2±253.4†	388.0±50.1†	<5.5±0*	1954.3±98.7†	2077.9±266.6†
100nM	19.2±9.4	47.2±8.2†	2745.5±206.7†	393.5±48.3†	<5.5±0*	1959.3±116.7†	2119.5±270.6†
BRD6688: 10nM	19.5±7.6	46.9±5.0†	2644.6±229.2†	409.7±35.0†	23.5±9.0*	1985.7±69.3†	2126.0±182.5†
100nM	11.5±2.6	42.6±6.2†	2873.4±268.8†	404.4±49.1†	33.7±7.3*	1974.5±142.4†	2121.8±282.2†
MERCK 60: 10nM	17.8±6.9	44.6±6.6†	2539.6±228.6†	358.9±34.5†	<5.5±0*	1916.8±116.9†	1968.7±224.2†
100nM	8.2±2.1*	36.5±6.9*†	2545.6±213.9†	297.1±19.6*†	<5.5±0*	1887.8±158.4†	1924.9±338.7†
Compound 39:100nM	29.7±3.3†	58.1±10.4†	2749.1±214.4†	457.8±74.8†	907.4±321.0†	2348.3±295.0†	2329.6±485.1†
1179.4b: 10nM	4.7±0.0*	31.8±2.6*	2301.4±170.3†	266.7±21.3*†	<5.5±0*	1587.8±70.8*†	1419.1±125.3*†

Levels of cytokine/chemokines present in cell culture supernatant (pg/ml) produced by monocytes stimulated with TNFα (10ng/ml) as assessed by immunoassay (mean ± SD, pg/ml). Cells were treated with HDACi (10nM-100nM) targeting HDAC 1 (**BRD0302**), HDAC 2 (**BRD6688**), HDAC 1 and 2 (**MERCK 60**), HDAC 5 (**Compound 39**) and broad suppression of class I and II HDACs (**1179.4b**) for 24 hours. * ($p<0.05$) Compared to TNFα stimulated control cells † ($p<0.05$) compared to vehicle control.

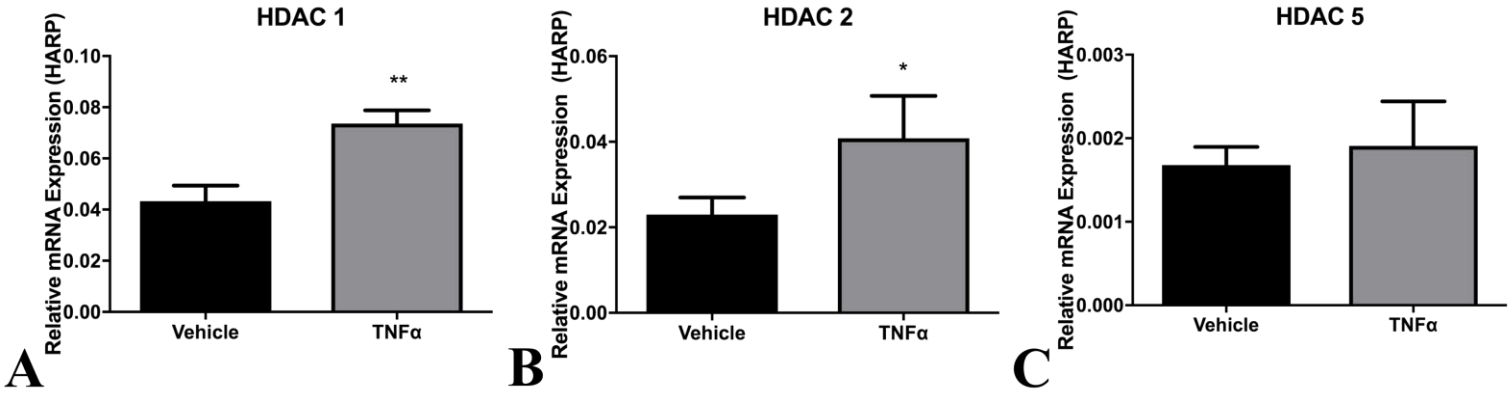

Figure 3.2: TNFα regulated HDAC expression in osteoclast progenitors. Relative mRNA gene expression of A) HDAC 1; B) HDAC 2; C) HDAC 5; +/- TNFα (10ng/ml) as outlined in methods. *$p < 0.05$

3.5.2 HDACi suppresses osteoclast formation and bone resorbing activity

Induction of inflammatory osteoclast formation and activity *in vitro* was achieved by treating osteoclastic progenitors with TNFα (10ng/ml) as outlined above. Larger, albeit fewer, TRAP-positive cells formed with TNFα stimulation (Figure 3.3; data not shown). Interestingly, these cells had significantly greater resorptive capacity than unstimulated vehicle control cells. This finding is consistent with previous investigations stimulating osteoclasts with TNFα (chapter 2).

HDAC 1 inhibition with **BRD0302** or HDAC 2 with **BRD6688** had minor effects on osteoclastic formation in the absence of TNFα, with numbers of TRAP-positive multinucleated cells being similar to untreated vehicle control (data not shown). However, TNFα (10ng/ml) stimulated cells were more sensitive to HDAC 1 suppression, with high doses of **BRD0302** (1000nM) reducing the number and size of osteoclastic cells forming *in vitro* (Figure 3.4). Interestingly, osteoclast activity was diminished in the presence of HDACi targeting either HDAC 1 or 2 with significant reduction in surface resorption being observed at doses 100-1000nM for **BRD0302** and 1000nM for **BRD6688** (Figure 3.4).

Suppressing both HDAC 1 and 2 with **Merck60** reduced TRAP-positive multinucleation at all HDACi concentrations investigated (10-1000nM), which translated to significant declines in bone resorptive capabilities at dose-dependent rates (Figure 3.4).

Conversely, **Compound 39** failed to have any substantial influence on osteoclastic formation or resorptive activity *in vitro*, with no statistical variations from the untreated control (Figure 3.3 & 3.4). Furthermore, combining **BRD0302, BRD6688 or Merck60** with **Compound 39** resulted in no alterations to targeting the class I HDACs alone, indicating no observable osteoclastic effect of the HDAC 5 inhibitor.

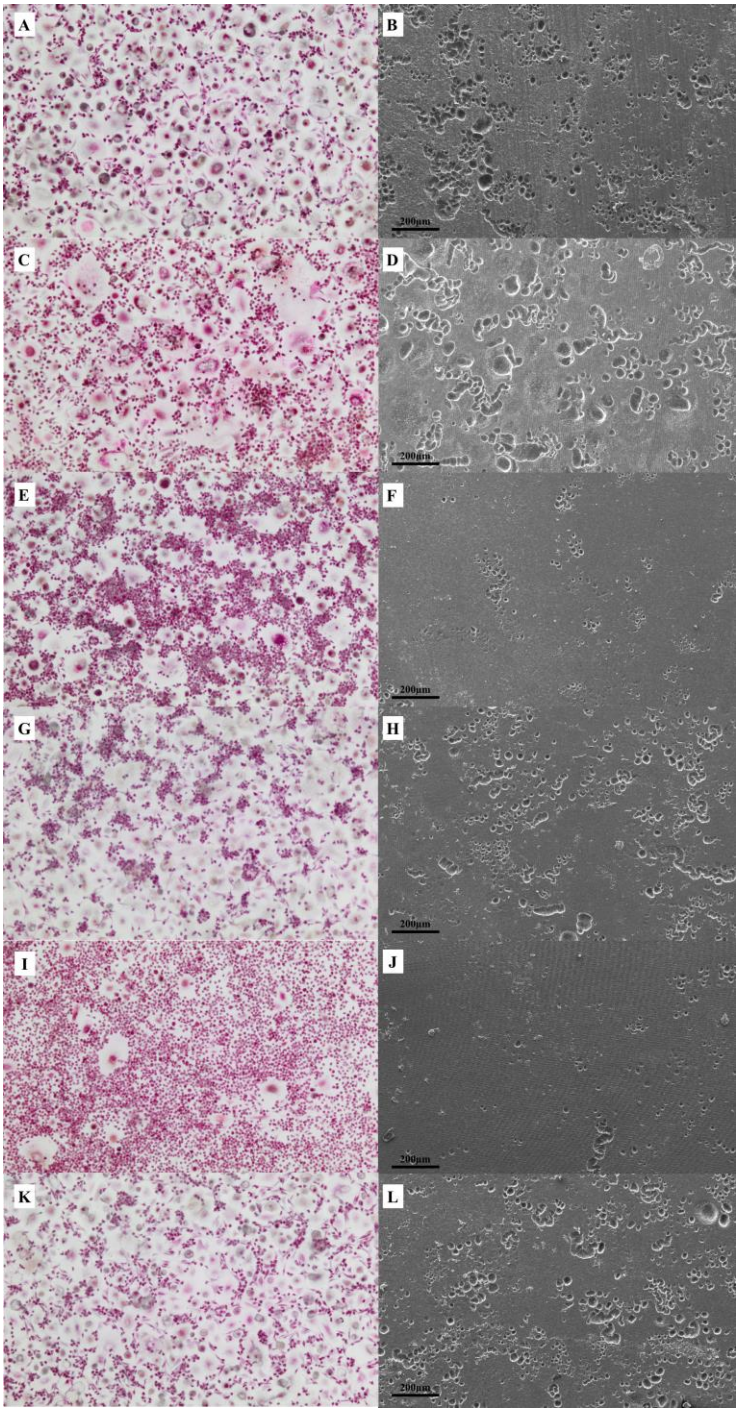

Figure 3.3 Representative images of TRAP-positive multinucleated osteoclastic cell formation (left) and resorption pits on dentine slices (right) in response to HDACi treatments (100nM). **A,B)** vehicle control; **C,D)** TNFα control; **E,F)** TNFα + BRD0302; **G,H)** TNFα + BRD6688; **I,J)** TNFα + MERK60; **K,L)** TNFα + Compound 39.

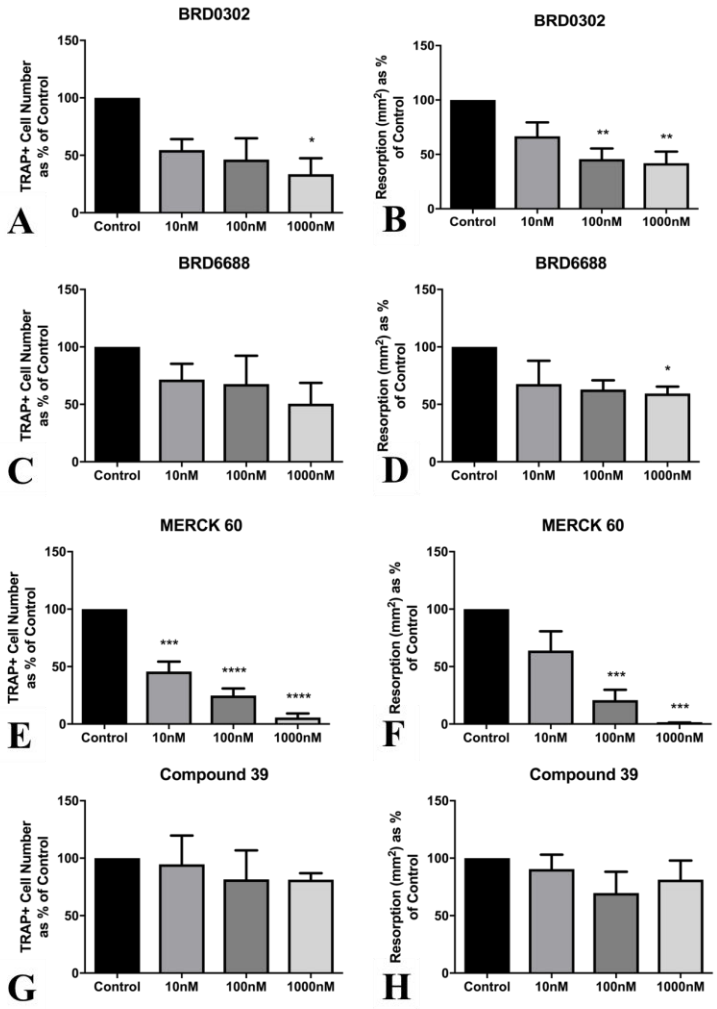

Figure 3.4 HDACi regulate human osteoclastic cell formation and bone resorptive activity in vitro**.** Fold change in the number of TRAP-positive multinucleated osteoclastic cell formation (left) and area (mm^2) of topographical resorption in dentine slices (right). **A,B) BRD0302** (HDACi 1); **C,D) BRD6688** (HDACi 2); **E,F**) **MERCK 60** (HDACi 1&2); **G,H) Compound 39** (HDACi 5). * ($p<0.05$) Compared to TNFα stimulated control cells

3.5.3 Suppression of human osteoclasts through decreased osteoclast signalling, activity and fusion factor expression with HDACi targeting HDAC 1 and HDAC 2

Gene expression via quantitative RT-PCR of essential osteoclast factors were analysed after TNFα stimulation and HDACi treatments, targeting HDAC 1 (**BRD0302**), HDAC 2 (**BRD6688**) or HDAC 1 and 2 (**Merck60**) at day 10 and 14 of the osteoclastic culture. No statistically relevant alterations were observed when measuring expression at day 10 (data not shown). However, levels of genes investigated were significantly affected by day 14 with HDAC inhibition (Figure 3.5). Both HDAC 1 and 2 inhibitors **BRD0302** and **BRD6688** (10nM) reduced levels of the signalling factor TRAF-6, activity markers TRAP and Cath K, and the fusion factor DC-Stamp.

Conversely, suppressing both HDAC 1 and 2 with **Merck60** (10nM) had profound effects on the expression of osteoclastic factors, with observable declines in NFATc1, TRAF-6, TRAP, Cath K, CTR, β3-integrin and DC-STAMP at day 14 (Figure 3.5).

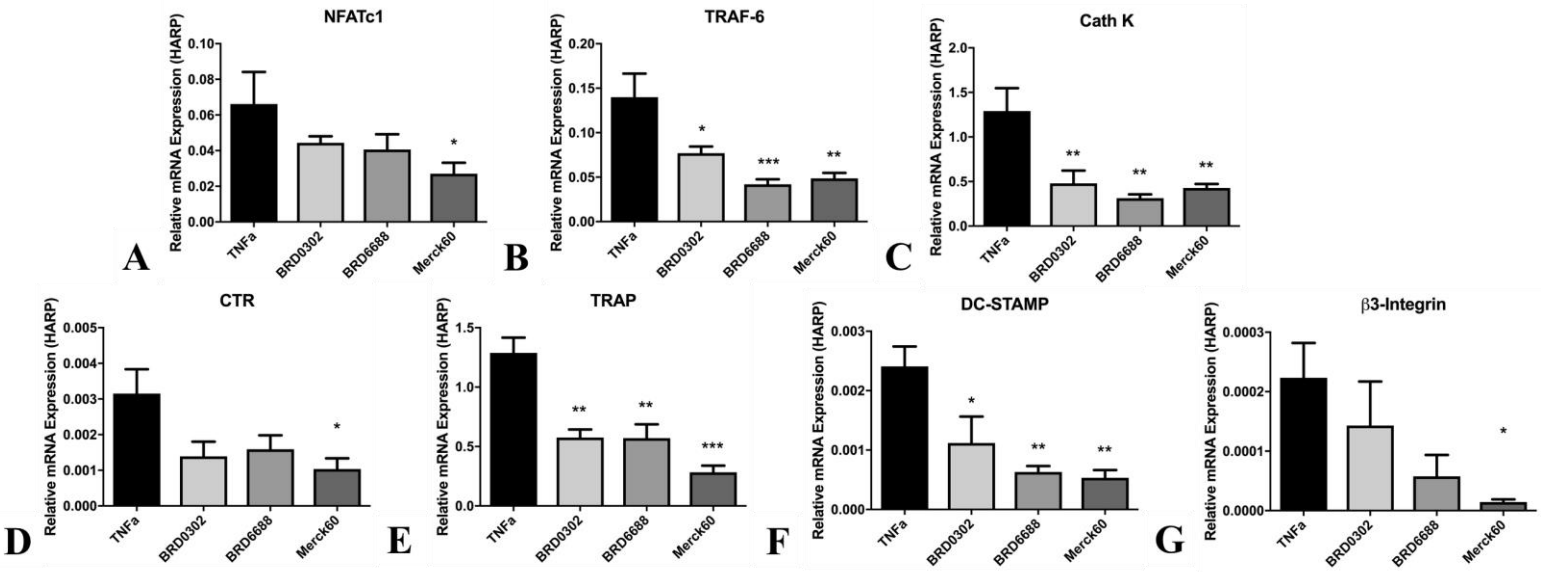

Figure 3.5 Regulation of osteoclast-related gene expression by HDACi. Relative mRNA gene expression of osteoclast-related genes in TNFα stimulated osteoclastic cells **A)** Nuclear factor of activated T-cells 1 (NFATc1) **B)** TNF associated receptor activator factor-6 (TRAF6); **C)** Cathepsin K (Cath K); **D)** Calcitonin receptor (CTR); **E)** Tartrate resistant acid phosphatase (TRAP); **F)** dendritic cell-specific transmembrane protein (DC-STAMP); **G)** and β3-integrin. * ($p<0.05$) Compared to TNFα stimulated control cells

3.6 Discussion

The pro-inflammatory effects of TNFα have been extensively researched over the past decade, with a wide range of systemic and cellular outcomes being reported (7). In chronic inflammatory conditions such as PD where levels of TNFα are substantially upregulated (19, 31), its destructive influence appears to be through the enhancement of cytokine/chemokine production leading to increased cellular infiltration (12), and both direct and indirect stimulation of osteoclastic processes (7). This study utilized the stimulatory effects of this cytokine to promote the pathological activity of human monocytes/macrophages and osteoclastic cells *in vitro* for the assessment of novel therapeutic compounds designed to suppress the actions of individual HDAC enzymes.

As expected, TNFα induced the synthesis of all inflammatory mediators investigated in this study by human monocytes (IL-1β, 1L-10, RANTES, TNFα, IFN-γ MCP-1, and MIP-1α). Broad suppression of HDAC activity by **1179.4b** (pan-inhibitor) reduced the stimulatory effect of TNFα, as concentrations of these inflammatory molecules in cell supernatants were drastically reduced. This anti-inflammatory result of broad HDAC suppression is consistent with the effects observed in a variety of inflammatory animal models as recently reviewed (16). Interestingly, **1179.4b** was used in a mouse model of PD where levels of inflammation were unaffected (24), despite positive outcomes on bone integrity. However, cytokine production was not evaluated in this study, as levels of inflammation were determined by histological assessment of cellular infiltrate in gingival tissue. Furthermore, doses used were substantially lower than other investigations using broad HDACi (1179.9; 1mg/kg compared with SAHA; 50mg/kg).

It is important to note that the anti-inflammatory cytokine IL-10 was markedly reduced by **1179.4b** in the present study. IL-10 is produced by stimulated monocytes and reported to modulate and suppress the pro-inflammatory cascade. The observation that broad inhibition of HDACs suppresses both anti- and pro-inflammatory molecules, further supports the necessity for characterizing the

actions of individual HDAC isoforms, and targeting those involved in promoting pathological conditions.

We observed the HDAC 1 isoform, and to a lesser extent HDAC 2, being actively involved in regulating individual molecules of the pro-inflammatory cascade. IL-1β and MCP-1 were reduced with HDAC 1 and/or 2 inhibition both at the mRNA and protein level by activated monocytes. IL-1β, like TNFα, is integral to the immune response, mediating cellular and cytokine networks. High levels of IL-1β have been observed in the serum of patients with chronic PD (32) and are substantially increased when patients suffer from additional systemic conditions, such as cardiovascular disease (33). Furthermore, MCP-1 is an essential chemotactic factor that is involved in promoting the migration and recruitment of mononuclear inflammatory cells. Recent research implies that levels of IL-1β, combined with the actions of MCP-1, could be a causal link to the development of chronic PD in addition to its contributing exacerbation of systemic conditions such as coronary heart disease (33).

This study further assessed the anti-osteoclastic actions of HDAC inhibition by targeting a range of HDAC isoforms previously identified as being dysregulated in human chronic periodontal tissue. It is clearly evident from the results of this study that the HDAC 1 and 2 complex is essential for inflammatory induced osteoclastogenesis. We observed slight reductions in osteoclast formation and activity when targeting HDAC 1 or 2 in isolation. However, when HDACi suppress both HDAC 1 and 2, bone resorptive capabilities were abolished. These results indicate that not only do redundancies in the actions of HDAC 1 and 2 exist (in respect to osteoclast activity), but compensatory mechanisms of these HDACs allow for fundamental cellular processes to continue in the absence of individual select HDAC isotypes.

An interesting finding of this study was the negative results observed from HDAC 5 suppression with **Compound 39**. The class II, HDAC 5, is overexpressed in chronic PD tissue (19) and upregulated during RANKL induced osteoclastogenesis *in vitro* (23), despite being reported to be involved with RANKL mediated NFATc1 stabilization (34), diminishing osteoclast

differentiation. Furthermore, a recent study utilizing selective shRNA-mediated suppression of HDAC 5 report increased osteoclastogenic activity in isolated primary bone macrophages (35). These studies may indicate an anti-catabolic role for HDAC 5, which acts to protect against and regulate osteoclastic bone resorption. Further analyses of the actions of HDAC 5 during inflammation and bone metabolism are required to elucidate its role during the destructive processes of PD.

3.7 Conclusion

In conclusion, the results of this study identified an essential role for the HDAC 1 and 2 complex during inflammatory and osteoclastogenic processes *in vitro*, as inhibition with HDACi reduced pro-inflammatory cytokine/chemokine production by activated monocytes, and the suppression of pathological bone resorption by TNFα stimulated osteoclasts. These findings further support the necessity for continued research into HDACi as potential therapeutic options for chronic inflammatory bone loss diseases, such as PD.

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Chapter 4: Histone Deacetylase 5 Inhibition Enhances Markers of Bone Formation by Human Osteoblasts *in vitro*

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4.1 Abstract

Osteoblasts are bone-forming cells that are required for normal skeletal growth and repair. In chronic inflammatory conditions such as periodontitis (PD), bone volume is reduced due to an imbalance between osteoblasts and bone resorbing osteoclasts. Histone Deacetylase (HDAC) 5 is upregulated in inflammatory bone loss conditions and it has shown to regulate osteoblastogenesis through the repression of osteoblast specific genes. Here, we show the induction of HDAC 5 by the inflammatory cytokine, tumour necrosis factor-α (TNFα), and the resulting inhibition of human osteoblast formation and mineralization *in vitro*. Using the novel HDAC 5 inhibitor, Compound 39, normal osteoblastogenic activity was restored via increased Runx2, osteocalcin and collagen type 1a expression. Taken together, these results highlight the necessity to target not only inflammation but also bone cells in PD and similar inflammatory bone loss diseases. This study also identifies HDAC 5 as a key target for increased bone formation and repair.

4.2 Statement of Authorship

Statement of Authorship

Title of Paper	Histone Deacetylase 5 Inhibition enhances markers of bone formation by human osteoblasts *in vitro*
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Kent Algate
Contribution to the Paper	First author and main contributor; Concept and methodological design, investigation, project administration, validation and visualisation, data curation and analysis, formulation of primary draft, in addition to reviewing and incorporating co-author comments and suggestions
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 25-1-18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	**Formulation, supply, and methodological advice for use of HDAC inhibitor.**		
Signature		Date	

22/1/2018

Name of Co-Author	David Fairlie		
Contribution to the Paper	Formulation, supply, and methodological advice for use of HDAC inhibitor.		
Signature		Date	22-1-18

Name of Co-Author	David Haynes		
Contribution to the Paper	Funding acquisition, investigation, conceptualisation, supervision, project administration and manuscript review		
Signature		Date	22/1/18

4.3 Introduction

Periodontitis (PD) is a disease that involves a chronic inflammatory reaction leading to the destruction of both hard and soft tissues of the periodontium (1). The immune response in PD is reported to cause disturbances within the bone remodelling process due to an imbalance between bone resorbing osteoclasts and bone forming osteoblasts (2, 3). If left untreated the integrity of alveolar bone is compromised and tooth support gradually diminished (1). PD is the leading cause for tooth loss in adults and currently affects up to 60% of the global population in its destructive form (4). Improving treatments that maintain or promote tissue regeneration is necessary to improve current periodontal therapy.

Over the past decade researchers have characterized the complex mechanism that coordinates the formation and activity of bone cells. These investigations have identified a plethora of growth factors, cytokines and chemokines that regulate this delicate process (2). Osteoblasts differentiate from mesenchymal stem cells (MSC) (5), creating highly specialized cells that synthesize and maintain bone matrix during bone growth or remodelling. This requires repression of proliferative signals in MSC and lineage dependent induction of the transcription factor, runt-related transcription factor-2 (Runx2). Runx2 activity is also essential for normal formation of osteoblasts and continuation of their anabolic processes (5, 6). However, in conditions, such as, PD where the immune system is highly active, cytokines, such as, tumour necrosis factor-α (TNFα) affect osteoblast function and bone maintenance (7). Many reports have shown inhibition of osteoblastic cells by TNFα, in part, through direct suppression of Runx2, in addition to key osteogenic factors such as Insulin like growth factor-1 (IGF-1) and Osterix (OSX) (8-10). Furthermore, TNFα stimulates the production of sclerostin (SOST) and Dickkopf-related protein 1 (DKK_1) in inflamed tissues (11-14). These factors potently inhibit Wnt-signalling and the induction of Runx2, inhibiting osteoblast differentiation and function (15-18). Treating both the inflammatory processes in PD and the destruction to alveolar bone is critical for the successful treatment of inflammatory bone loss. Pharmacological interventions that regulate

these processes through epigenetic mechanisms may be an effective adjuvant to current therapeutic strategies.

Epigenetic modulators that are involved in cellular differentiation and function are currently being investigated for their effects in bone loss disorders such as PD (19-21). Specifically, a group of enzymes called histone deacetylases (HDAC) regulate gene and protein expression by removing acetyl groups from histone and non-histone proteins (22). Their role in disease is of great interest due to growing evidence of altered function and/or expression in several pathologies (23-26). HDACs are a group of 18 mammalian enzymes that are divided into two distinct categories based on their catalytic domain and reactivity (27). HDAC 1 to 11 contain a zinc ion and the remaining seven (termed sirtuins 1 to 7) are NAD^+-dependent. HDAC 1 to 11 are further subdivided into 3 classes based on their cellular location and distribution. Class I HDAC 1, 2, 3, and 8 are located primarily within the cell nucleus, whereas class IIa HDAC 4, 5, 7, and 9 can migrate between the nucleus and cytoplasm. HDAC 6 and 10, which are only present within the cell cytoplasm, are from class IIb. The remaining HDAC 11 is in class IV. We have recently shown dysregulated HDAC expression in tissues affected by PD and rheumatoid arthritis, both at the clinical and experimental level (20, 23, 28). Furthermore, HDACs have been implicated in a variety of non-bone related disorders including malignancies (24), cardiac and neurodegenerative disease (26, 29). This has prompted interest in using HDAC as pharmacological targets.

Interestingly, the specific HDAC isoform from class IIa, HDAC 5, is reported to be a locus for bone mineral density and has been shown to be highly involved in modulating normal osteoblastogenesis (30, 31). Recent clinical investigations in juvenile osteoporosis identified a substantial rise in HDAC 5 expression in bone tissue, which was associated with low levels of Runx2 and diminished bone density (30, 31). HDAC 5 is also upregulated in human gingival tissue from patients with PD (23). Runx2 and upstream Wnt-signalling processes are negatively associated with HDAC 5 activity in mice primary osteoblastic cells (32) and targeted deletion of HDAC 5 by miRNA produces increases in osteoblast differentiation *in vitro* (33).

Based on this evidence we aimed to investigate the effects of a novel HDAC inhibitor (HDACi) designed to target the HDAC 5 isoform, Compound 39, during osteoblastogenesis and bone formation using human osteogenic cells *in vitro*. Supplementary experiments involved exposing cells to the cytokine TNFα to investigate the effects in a simulated inflammatory environment. We hypothesized that osteoblast activity would be stimulated by HDAC 5 inhibition, and that Compound 39 would ameliorate the negative effects on osteoblastogenesis associated with TNFα.

4.4 Materials and Methods

4.4.1 HDAC Inhibitor (HDACi), Compound 39

Compound 39, a HDAC 5 inhibitor, was designed and supplied by colleagues from the University of Queensland's institute of Molecular Bioscience (34, 35, 40). Compound 39 was suspended in a dimethyl sulfoxide (DMSO) vehicle at 0.01% for *in vitro* investigations in this study. Samples not receiving Compound 39 were treated with a 0.01% DMSO vehicle control.

4.4.2 Human Osteogenic Cell Isolation and Culture

Normal osteoblast donor (NOD) isolates were generously supplied by the Mesenchymal Stem Cell/Myeloma Research Laboratory of The University of Adelaide, South Australia. Samples were originally obtained and isolated from healthy donors (aged 20-35 years) with informed consent of the SA Pathology normal bone marrow donor program, Royal Adelaide hospital, as previously published (36). Briefly, bone marrow obtained from the posterior iliac crest was strained for bone chips created during the aspiration process using a 0.7μm strainer. The strainer was subsequently washed thoroughly in standard proliferative α-MEM media (supplemented with 20% foetal calf serum (FCS), 2mM-L-glutamine, 100μM L-ascorabte-2-phosphate, 1mM sodium pyruvate, 50U/ml penicillin and 50μg/ml streptomycin) and this media along with the microscopic bone chips were collected in a T-75 tissue culture flask. Primary

cultures of NODs were left to establish and grow for 10-14 days before being passaged and expanded.

4.4.3 Cell Proliferation and Viability Assay

Passage 5 human NODs (n=6) were grown ($1x10^5$ cells per cm^2) in standard proliferative media and culturing conditions outlined above for 7 days. Cells were treated with tumour necrosis factor-α (TNFα; 10μg/ml) and the HDAC 5 inhibitor, Compound 39 (0.1-1000nM). Throughout the 7-day incubation period NOD proliferation and viability was assessed via cell counts of trypan-blue negative cells under light microscopy, in addition to the use of the WST-1 cell proliferation assay (ab155902 Abcam, AUS) as per manufacturers' recommendations. Briefly, WST-1 reagent (10μl/well) was added to each well and incubated for 2 hours in the dark using experimental culture conditions. Absorbance of the resulting culture solution was measured on a micro plate reader at a 450nm wavelength.

4.4.4 Osteogenic Induction and Mineralization Assay

Human NODs (n=6) were cultured ($1x10^5$ cells per cm^2) under established conditions to promote osteogenic induction and mineralizing functionality as previously reported (36). Briefly, cells were cultured for 21 days in osteogenic inductive media, including α-MEM supplemented with 5% FCS, 100μM L-ascorbate-2-phosphate, 10^{-7}M dexamethasone and 3mM inorganic phosphate. Coinciding with the induction of osteogenic processes, media was supplemented with either TNFα (10μg/ml) and/or the HDAC 5 inhibitor, Compound 39 (1.0nM-1000nM). Experimental treatments were replenished with each media change (every 3-4 days) and continued throughout the 21-day assay. At weekly intervals, alizarin red S staining (1% [w/v] Alizarin Red S in distilled water, at a pH of 4.1-4.3 using ammonium hydroxide) was conducted and imaged by light microscopy to identify extracellular mineral deposits in 96-well plates. Synthesized calcium produced by NODs was quantified using a colourimetric calcium concentration assay as described by Granthos *et al* (36). Briefly, cells cultured in triplicate in 96-well plates were washed in a Ca^{+}/Mg^{2+} free phosphate-buffered saline solution, followed by an overnight incubation in 0.6M Hydrochloric acid (HCl). Solubilized

calcium matrix samples were supplemented with the metalochromic indicator, Arsenazo III reagent, and measured on a micro-plate reader at a 570nm wavelength. Calcium concentrations were then normalized to DNA content per well by incorporation of PicoGreen (Life Technologies) to produce a reportable value of cell activity.

4.4.5 Real-Time Polymerase Chain Reaction

Total RNA was extracted from cultured cells during MSC proliferation and throughout osteogenic induction of NODs, using a TRIzol (Thermo Fisher Scientific; AUS) based method as per manufacturers directions. Total RNA isolates were then used as a template for the formation of complementary DNA (cDNA) using the Rotor-Gene Q (Qiagen, AUS) reverse transcription method, with 250ng random hexamer and 200U of superscript III reverse transcriptase (Geneworks, AUS). Platinum SYBR Green qPCR Supermix-UDG (Lift Technologies, AUS) was used for the real-time polymerase chain reaction (PCR) amplification of samples in triplicate as previously described (37). Genes investigated in this study include HDAC 5 (37), Runx2, Osteocalcin, Osteopontin, Collagen type-1a, Receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG) (41, 42). Changes in gene expression were determined based on the $2^{-\Delta ct}$ method (38) relative to the housekeeping gene, GAPDH (41, 42).

4.4.6 Statistics

Statistical analysis of proliferation, mineralization and gene expression between treatments was determined using a one-way ANOVA followed by the Dunnett's post hoc test. Students T-test was used identify variations between standard culture conditions and the inflammatory cytokine, TNFα. Statistical significance was accepted at $p < 0.05$. GraphPad® Prism version 7 was used in this study.

4.5 Results

4.5.1 HDAC 5 inhibition has no effect on osteoblast proliferation or viability

In order to assess the effects of HDAC 5 on osteoblast proliferation and viability, cells were grown in normal proliferation media (described above). As shown in Figure 4.1, Compound 39 (1.0-1000nM) had no effect on proliferation, with consistent rates of proliferation at all doses investigated in the presence or absence of TNFα. Furthermore, cell numbers as assessed by trypan-blue exclusion identified no significant change in the number of viable cells (data not shown).

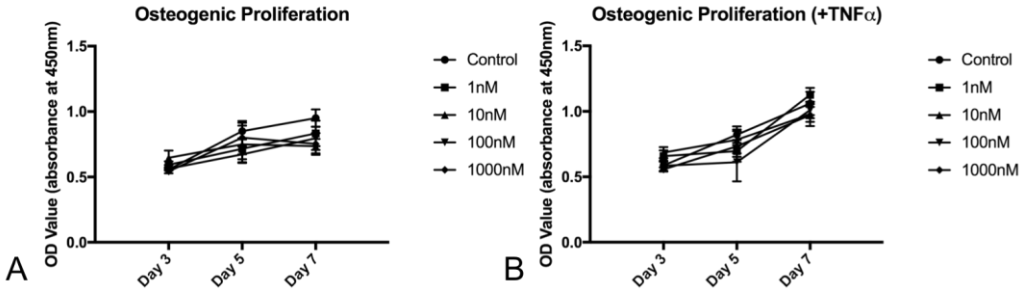

Figure 4.1 Effects of Histone Deacetylase 5 inhibition (Compound 39; 1-1000nM) on cell proliferation and viability over a 7-day period in the (A) presence and (B) absence of tumour necrosis factor-α (TNFα: 10μg/ml). Points indicate average rate of optical density (OD)/absorbance values ± SEM.

4.5.2 HDAC 5 expression during osteoblast induction

Throughout the induction of osteoblast mineralization, HDAC 5 expression declined and was significantly reduced ($p < 0.05$) at days 14 and 21 when compared to day 7 (Figure 4.2). A similar decline was noted in the cells exposed to TNFα ($p < 0.05$). However, TNFα significantly upregulated HDAC 5 expression in osteoblasts when compared to control cells (day 7: $p < 0.05$; day 14: $p < 0.05$)

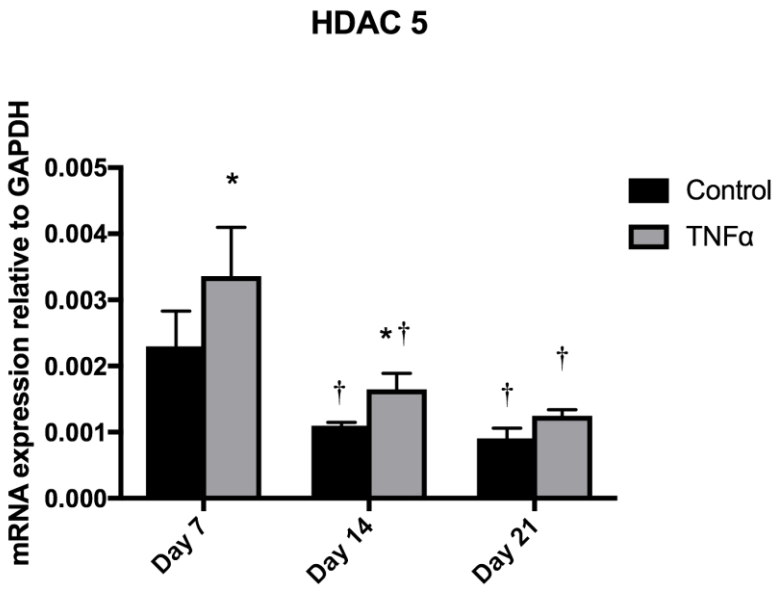

Figure 4.2 Histone Deacetylase (HDAC) 5 expression throughout induction of osteogenic cells (n=6) in the presence and absence of tumour necrosis factor-α (TNFα: 10μg/ml). Bars represent average mRNA expression relative to house-keeping gene, GAPDH ± SEM. * = $p < 0.05$ when compared between Control and TNFα at each individual day; † = $p < 0.05$ when compared to Day 7 in each corresponding experimental group.

4.5.3 Effects of HDAC 5 inhibition on osteoblast mineralization

After the supplementing media with essential factors to induce osteoblastic mineralization cells were treated with the HDAC 5 inhibitor, Compound 39, in the presence or absence of TNFα (10μg/ml) for 21 days. Quantification of mineral deposits, normalized to DNA content per well, resulted in a bell-shaped dose effect, as a low dose (1.0nM) or high dose (100-1000nM) had no effect on mineralization (data not shown). However, an increase in calcium deposition was identified at 10nM, albeit not statistically significant ($p > 0.05$; n=3). Additional experiments, using an additional 3 donors, were carried out using a narrowed dose range (2.5-20nM; 2-fold increments). Interestingly, a dose-specific increase in calcium concentration was identified which, when combined with identical data from primary experiments, resulted in levels of mineralisation statistically greater compared to controls at 10nM ($p < 0.05$; n=6) and 20nM ($p < 0.05$; n=3) (Figure 4.3).

The effects of TNFα were detrimental to the functional capacity of osteoblastic cells used in this study. Significantly less calcium concentrations per DNA content was measured in TNFα treated cells when compared to controls ($p < 0.05$). Interestingly, inhibition of HDAC 5 (10nM and 20nM) stimulated TNFα/NOD mineralization to levels statistically similar to control cells (Figure 4.3).

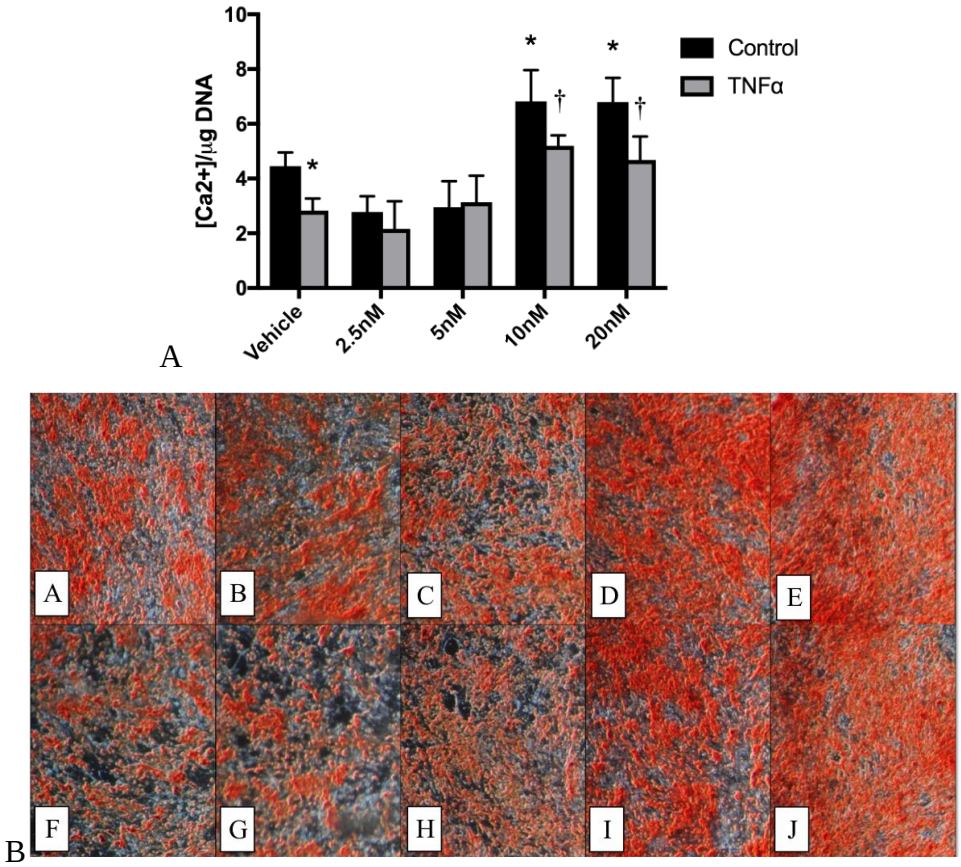

Figure 4.3 Effects of HDACi, Compound 39 in the presence and absence of tumour necrosis factor-α (TNFα: 10µg/ml) on osteoblast mineralization. A) Quantification of calcium content at day 21 with Compound 39 (1.5-20nM) and TNFα. B) Representative alizarin red S staining for detection of mineralized nodules at day 21 with Compound 39 (A: Vehicle; B: 2.5nM; C: 5mM; D: 10nM; E: 20nM; F: TNFα; G: TNFα + 2.5nM; H: TNFα + 5nM; I: TNFα + 10nM; J: TNFα + 20nM) * = $p < 0.05$ when compared to vehicle control; † = $p < 0.05$ when compared to TNF vehicle

4.5.4 HDAC 5 inhibition increases Runx2 expression and markers of bone formation in osteoblasts

Compound 39 treatment (10nM; n=6) was investigated for its effects on Runx2 expression and markers of bone formation, osteopontin, osteocalcin, and collagen type 1a. In addition, factors expressed by osteoblasts that are involved in osteoclastic signalling and bone metabolism, RANKL and Osteoprotegerin (OPG) were analysed. As identified in Figure 4.4, HDAC 5 inhibition stimulated the expression of Runx2 (Day 14 and 21; $p < 0.05$), osteocalcin (day 7; $p < 0.05$), and collagen type 1a (day 7; $p < 0.05$). At the dose investigated, no significant changes were observed in osteopontin expression.

The effects of TNFα on osteogenic gene expression were consistent with declines in the anabolic activity of the cells *in vitro*. Significant declines in Runx2 and osteocalcin were identified at various time-points throughout the assay. Interestingly, HDAC 5 inhibition had the opposite effect, stimulating these factors to levels equal or significantly greater than cells not exposed to TNFα. In addition, collagen type 1a was significantly upregulated by Compound 39 compared to both TNFα and control cells.

Interestingly, the stimulatory effect of TNFα on RANKL expression at day 21 was not affected by Compound 39, nor were any changes observed in OPG levels. HDAC 5 inhibition appears to have no direct influence on the crosstalk molecules between osteoclasts and osteoblasts.

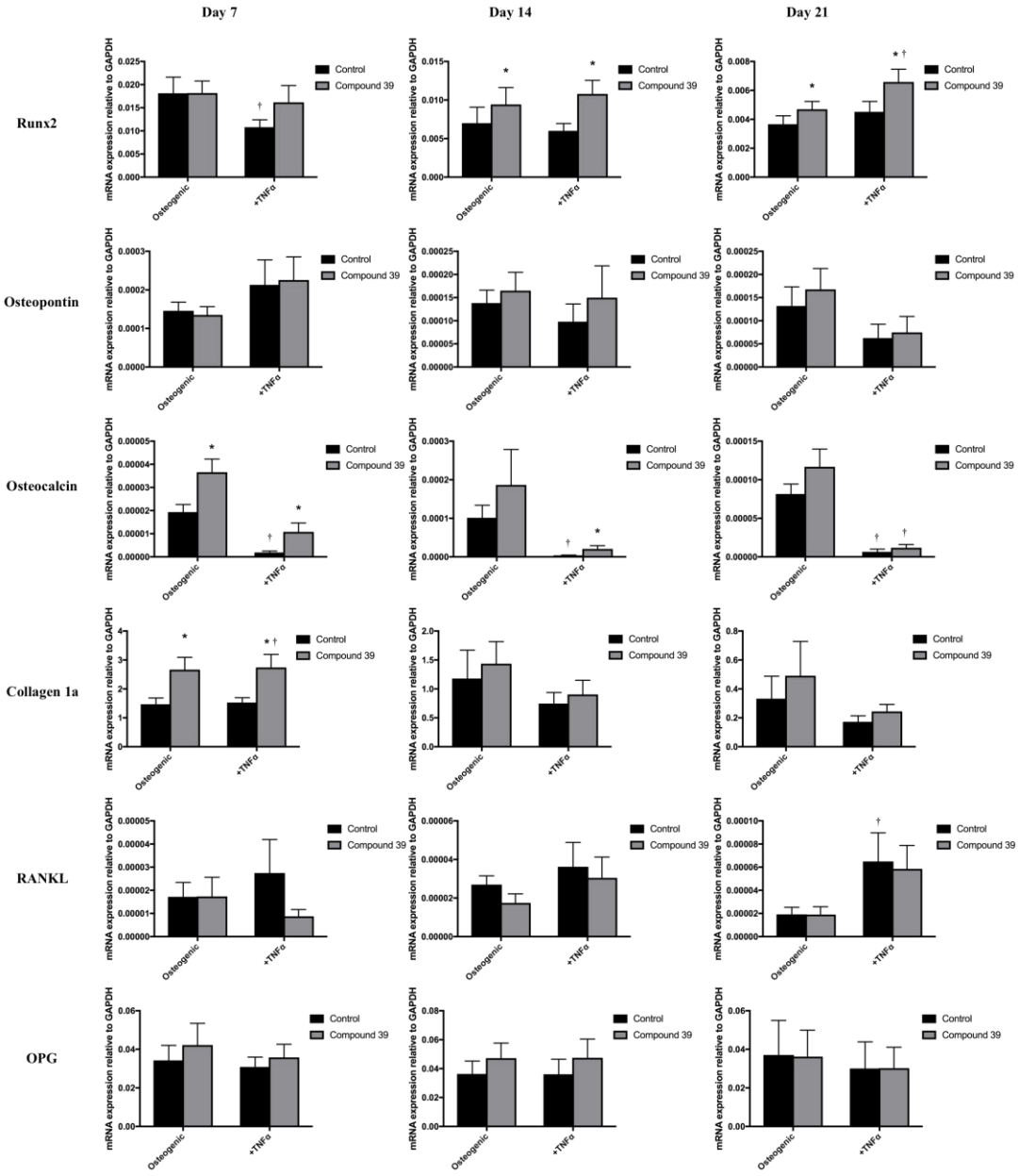

Figure 4.4: Expression of osteoblast related factors throughout induction of osteogenic cells (n=6) in the presence and absence of tumour necrosis factor-α (TNFα; 10nM) and HDAC 5 inhibitor, Compound 39 (10nM). Bars represent average mRNA expression relative to house-keeping gene, GAPDH ± SEM. * = $p < 0.05$ when compared to untreated control; † = $p < 0.05$ when compared to osteogenic control (-TNFα)

4.6 Discussion

The present study aimed to investigate the effects of a novel HDAC 5 inhibitor, Compound 39, on normal osteoblast donor cells (NODs). The cytokine TNFα was also included in investigations to represent an inflammatory environment *in vitro*. This was due to decreased bone formation and repair by osteoblasts in inflammatory conditions, such PD, where TNFα as a key molecule of its pathogenesis (3, 7). TNFα has also been shown to directly affect the formation and activity of osteoblasts (7).

Osteoblasts isolated from bone chips were used in this study to determine the direct effect of compound 39 on their proliferative ability and mineralizing capacity. We observed no variations in cell cycle arrest by Compound 39, nor were reduced viability or cell death direct outcomes of HDAC 5 inhibition. While this was not consistent with previous studies, these were carried out on osteoblast progenitor cells. Specifically, human mesenchymal stem cells (hMSCs) halt proliferative processes and begin to produce bone mineral matrix by forced expression of a specific gene that codes for long-nonprotein-coding RNAs (H19/miR-675). Importantly, this induction is achieved in the absence of osteogenic media (39). It was concluded that osteogenic differentiation by H19/miR-675 expressing hMSCs was due to downregulations of HDAC 4 and 5. HDAC 5 is reported to form a stable complex with HDAC 4 that interacts with the Runx2-DNA binding domain (39). As a result, expression of Runx2 and additional osteogenic factors such as osteocalcin are suppressed. Although we saw no observable change in osteoblast proliferation in the present study, the induction of anabolic processes were enhanced by HDAC 5 inhibition, with increased expression of Runx2 and osteocalcin by Compound 39.

Several studies have reported changes in HDAC expression during inflammation and infection causing alterations in histone modifications and resulting gene expression (23-26). We report here, a TNFα dependent increase in HDAC 5 expression in human osteoblasts. Furthermore, the induction of HDAC 5 was associated with declines in the expression of osteogenic genes Runx2 and

osteocalcin. This is consistent with findings describing the interaction with HDAC 5 and Runx2 transcriptive processes in osteoblastic cells (32). Consistent with these findings, HDAC 5 inhibition by Compound 39 in TNFα treated cells stimulates the induction of Runx2 and osteocalcin, leading to enhanced osteoblastic mineralization.

4.7 Conclusion

The findings of this study warrant further investigations into the role of HDAC 5 during osteoblastogenesis and bone formation. The effect of a novel HDAC 5 inhibitor, Compound 39, protects cells against the inhibitory actions of the inflammatory cytokine, TNFα. However, continued evaluation of these mechanisms are required to determine if these effects are due to epigenetic modifications or direct non-histone protein interactions by HDACi. In addition, the relationship between TNFα and the dysregulation of HDAC 5 requires further analysis to identify their interacting mechanisms. In conclusion, these results identify HDAC 5 as a potential target for treatments promoting bone repair in inflammatory bone loss pathologies, such as PD.

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Chapter 5: Histone Deacetylase Class I and Class II Expression in Experimental Periodontitis

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5.1 Abstract

Periodontitis (PD) is a chronic inflammatory disease within the oral gingiva that causes destruction of alveolar bone and tooth supporting structures. Histone deacetylases (HDACs) are a class of enzymes involved in regulating cell cycle, differentiation and other cellular activities through an epigenetic process. They are currently being investigated for the treatment of inflammatory bone loss diseases such as PD. The aim of this study was to assess the expression of HDACs from class I (HDAC 1 and 8) and class II (HDAC 5 and 9), known to be involved in skeletal and inflammatory processes, in a mouse model of PD. It is believed that these isoforms may be potential pharmacological targets for future therapeutics targeting specific HDACs. Mice (n=30) were inoculated with *Porphyromonas gingivalis* and *Fusobacterium nucleatum* via regular oral swabs to induce experimental PD. *In vivo* analysis of alveolar bone by microcomputed tomography (Micro-CT), and histological assessment of periodontal tissues identified reduced alveolar bone height and upregulated class I HDACs 1 and 8 in experimental PD. This correlated with increased levels of gingival inflammation, increased tartrate resistant acid phosphatase-positive cells (osteoclasts) and the loss of alveolar bone ($p < 0.05$). Class II HDACs 5 and 9 remained unchanged in both control and experimental PD, despite their reported physiological role the anabolic processes of bone production and repair. The findings of this study provide evidence for the development of compounds that target specific HDAC isoforms in PD and related inflammatory bone loss diseases.

5.2 Statement of Authorship

Statement of Authorship

Title of Paper	Histone Deacetylase Class I and Class II Expression in Experimental Periodontitis
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Kent Algate
Contribution to the Paper	First author and main contributor; Concept and methodological design, investigation, project administration, validation and visualisation, data curation and analysis, formulation of primary draft, in addition to reviewing and incorporating co-author comments and suggestions
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 25.1.18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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5.3 Introduction

Periodontitis (PD) is a disease of the oral tissues that support and surround the teeth. Its development and continued reactivity is influenced by the host response to pathological bacteria, causing a chronic inflammatory reaction within the gingival and subgingival microenvironment (1). The inflammatory process observed in PD alters bone turnover by both osteoclasts and osteoblasts (1, 2). As a result, loss of alveolar bone will often cause tooth loosening and eventual loss of teeth if untreated. Controlling the inflammation and bone destruction in PD is a continuing challenge for clinicians. The identification of novel targets that regulate the host response and cells involved in bone breakdown will enhance current treatment options.

Histone deacetylases (HDACs) are a group of enzymes that are involved in diverse cellular processes including cell differentiation and activity, in part, by epigenetic control over gene expression and protein function (3, 4). By catalytically removing acetyl groups from lysine residues on histone proteins, chromatin condensation occurs. As a result, tight DNA binding to the histone core blocks promoter region access and the subsequent machinery required for gene transcription. Furthermore, these enzymes are involved in non-histone, post-transcriptional deacetylation of proteins (5, 6). These include signalling molecules and transcription factors, by altering their overall reactivity, stability and function (6). Irregular overexpression of HDACs has been implicated in a variety of pathophysiological processes, including malignant cell division, the development of cardiovascular and neurodegenerative disease, and inflammatory bone loss disorders (7-13). Hence the development and use of HDAC inhibitors (HDACi) is a promising therapeutic strategy for treating such conditions. HDACi are clinically used alone or in combination with other therapies for the treatment of cancers (7-9). They are currently being investigated for their potential anti-inflammatory and anti-bone loss effects in rheumatoid arthritis and PD (11-13). Interestingly, these effects or seen at doses approximately 100x lower than that used for oncological purposes. However, understanding the isoform-specific role of each HDAC is necessary to improve treatment efficacy, reduce toxicity and prevent potential adverse effects from non-selective inhibition of HDACs.

In humans, there are 18 specific HDAC isoforms based on reactivity, cellular location, tissue distribution and homology to yeast HDACs (14). Class I HDACs (HDAC 1, 2, 3 and 8) are located primarily within the cell nucleus and are ubiquitously expressed in human tissue. Whereas class II HDACs are subdivided into class IIa (HDAC 4, 5, 7 and 9) and class IIb (HDAC 6 and 10). Class II HDACs differ due to their catalytic domain and ability to shuttle between the nucleus and cytoplasm. HDAC 11 is the sole isoform in class IV and is localised within the nucleus. The remaining 7 HDACs, termed Sirtuins (SIRTS 1-7), are atypical when compared to other HDACs due to their catalytic process, targets and distribution within the cytoplasm, mitochondria and nucleoli of the cell.

A recent study of the HDAC superfamily in PD gingival tissue from patients undergoing surgical therapy, identified consistent overexpression of HDACs 1, 5, 8 and 9 in chronic PD tissue with alveolar bone loss, when compared to patients not suffering from PD (15). Interestingly, HDAC 1 protein was co-localised within large multinucleated, tartrate-resistant acid phosphatase (TRAP)-positive cells (an indicator of osteoclastic cells). Investigations supressing HDAC 1 in osteoclasts *in vitro* (Chapters 2 and 3) and in models of inflammatory bone loss (16) have observed reductions in osteoclast activity and bone resorption. HDAC 1 is also associated with cytokine and chemokine production by inflammatory cells (12, 16). When selectively or broadly suppressed, markers of inflammation are reduced both *in vitro* and *in vivo* (12, 16)(Chapter 3). Conversely, HDAC 9 is reported to reduce osteoclast differentiation through negative regulation of receptor activator and nuclear factor kappa-B (RANK) and proliferator-activated receptor gamma (PPARγ) signalling loops that are essential for osteoclast formation (17). *Ex vivo* deletions of HDAC 9 or knockdown models in mice result in animals with osteoporotic features due to enhanced osteoclastic resorption (17). Alternatively, HDAC 5 activity has been shown to inhibit the major osteoblast transcription factor, Runx2, in addition to regulating the expression of its upstream Wnt-signalling mediators (18, 19). Preliminary studies by our lab have observed increased markers of bone turnover by human osteoblasts *in vitro* with pharmacological inhibition of HDAC 5 (Chapter 4). Similar to this, HDAC 8 is reported to supress osteogenesis through reduced histone H3 lysine 9 (H3K9)

acetylation and reduced osteogenic gene expression (20). Whereas short-interfering RNAs targeting HDAC 8 enhance acetylation of H3K9, allowing the osteogenic process to continue (20).

The overexpressed and unregulated control of these specific HDAC isoforms in PD could be a contributing factor to the imbalance in cellular activity observed between osteoclastic resorption and osteoblastic bone formation, leading to alveolar bone loss and eventual loss of teeth. Targeting these specific HDACs at the appropriate time may be an effective therapeutic option to prevent or reverse alveolar bone loss. However, analysis of these individual HDACs has yet to be characterised throughout the pathogenic process in PD, as human diagnosis is based on pre-established infection and bone destruction. Therefore, the aim of this study was to utilize an established experimental model of PD in mice to characterise HDAC 1, 5, 8 and 9 expression throughout the disease process. In addition, expression of these HDACs will be compared to levels of gingival inflammation, osteoclast formation and activity, and alveolar bone damage.

5.4 Materials and Methods

5.4.1 Animals and Housing

6-8-week-old female BALB/c mice (n=30) were obtained from the Laboratory Animal Services of the University of Adelaide and housed in PC2 animal facilities for a 5-day acclimatisation period prior to the induction of experimental PD (Ethics #M-2014-158B). Mice were kept in standard room conditions throughout the experimental model, which included an average room temperature of 22ºC with 12-hour light/dark cycles. Mice had access to non-acidic drinking water and non-granular foods, in addition to antibacterial free housing products to reduce the environmental impact on experimental PD. Daily assessment of general health parameters were conducted throughout the experimental period.

10 mice were randomly assigned to 1 of 3 experimental groups (n=10 mice/group)
Group 1: Control (No Periodontitis)
Group 2: PD/*Pg* (Periodontitis/*Porphyromonas gingivalis* alone)
Group 3: PD/*Pg+Fn* (Periodontitis/*Porphyromonas gingivalis* and *Fusobacterium nucleatum*)

5.4.2 Induction of experimental PD

Induction of experimental PD was conducted as previously described (21, 22). Briefly, all mice received antibiotic treated water (1mg/ml kanamycin; Sigma-Aldrich, St Louis, MO, USA) for 7 days to reduce native oral microbiota and to support the colonisation of pathogenic bacteria *Fusobacterium nucleatum* (*F. nucleatum*; ATCC® 25586) and *Porphyromonas gingivalis* (*P. gingivalis*; strain W50), which used and prepared as previously documented (21, 23). Mice were rested for 3 days prior to commencing the bacterial inoculation process, which consisted of 100µl of bacteria (either $2x10^{10}$ CFU/ml of *P. gingivalis* alone or 10^{10} CFU/ml of *P. gingivalis* and 10^{10} CFU/ml of *F. nucleatum*) suspended in 2% carboxymethyl cellulose (CMC). The inoculation procedure, as routinely practiced and documented (21-23), involved the molars of each mouse being swabbed with inoculant every 48 hours for 8 days, followed by twice weekly for a 2-week period to maintain established infection (*primary sequence*). Following this, the *secondary sequence* began which involved repeating the *primary sequence*, completing the PD model. Mice dedicated to the control group were swabbed with 2% CMC in accordance with experimental procedure outlined above. At the completion of each inoculation, mice were fasted (food and water) for 1 hour, to minimize the removal of inoculant from oral tissues. A timeline of the periodontal inoculation model is depicted in Figure 5.1.

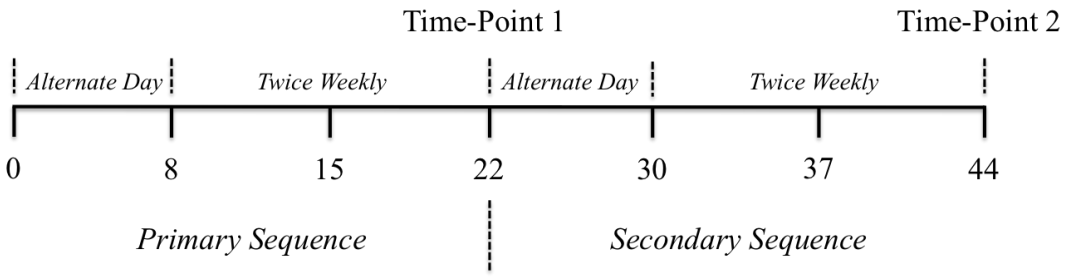

Figure 5.1 Experimental periodontitis inoculation timeline. Prior to day 0, mice were given antibiotic treated water (1mg/ml kanamycin) for 7 days. Values represent days.

5.4.3 Tissue Collection and Preparation

After completion of the *primary sequence* (Time point 1), 5 mice from each group were humanely culled via cervical dislocation under anaesthesia (xylazine; 20mg/kg and ketamine; 100mg/kg) for tissue processing and bone analysis. The remaining 5 mice from each group remained to complete the *secondary sequence* prior to being culled using the same technique at the end of the experiment (Time point 2).

Heads were removed and cleaned by dissection, followed by 24-hour fixation in a 10% buffered formalin solution. Samples were then washed in PBS and scanned for high resolution microcomputed tomography (Micro-CT) processing, described below. Post Micro-CT scanning, heads were decalcified in 10% EDTA for 2-4 weeks and embedded in paraffin for sectioning and histological analysis. Paws, internal organs, spines and serum were collected and stored for future investigations.

5.4.4 Microcomputed Tomography (Micro-CT)

To identify universal changes in alveolar bone throughout experimental PD, Micro-CT scans of mice heads were conducted after the primary inoculation sequence (Time point 1) and at the end of the experiment (Time point 2) using the Skyscan 1076 High Resolution Micro-CT Scanner (Skyscan, Bruker, Belgium). Acquisition of scan specifications included a pixel size of 8.5µm, scanned at 48kV/169µA using a 0.5mm aluminium filter.

5.4.5 Micro-CT Scan Reconstruction and Data Processing

Files from each scan were reconstructed for analysis of alveolar bone changes using a cone-beam algorithm with the following settings: smoothing = 3, ring artefact correction = 12 and beam hardening correction = 10% (NRecon Software, Version 1.6.8.0, Skyscan, Bruker, Belgium). Reconstructed images were aligned on the sagittal plane using Dataviewer software (Version 1.5.2.4, Skyscan) for the measurement of alveolar bone crest (ABC) height using CTAnalyser software (Version 1.15.4.0, Skyscan). For the measurement of bone changes, the ABC between the first and second maxillary molars was used as a point of reference. From the ABC, the distance to the cemento-enamal junction (CEJ) of the molars was measured to identify changes in alveolar bone height between experimental groups and disease progression. The CEJ-ABC distance was measured for each mouse from 5 sagittal scans on both left and right sides by two blinded observers, as previously described (31) (Figure 5.2).

Figure 5.2 Representative longitudinal Micro-CT images used for analysis of alveolar bone loss. Alveolar bone crest (ABC) to the cemento-enamel junction (CEJ) distance indicated by red bar. A) Control; B) PD/Pg; C) PD/Pg+Fn.

5.4.6 Haematoxylin and Eosin Staining

Paraffin embedded heads were sectioned and viewed to confirm precise longitudinal alignment of the first and second maxillary molars. Haematoxylin and Eosin staining was conducted as per standard protocol for histological analysis of inflammatory cellular infiltrated (neutrophils, macrophages, lymphocytes and/or plasma cells) as previously outlined (21-23). Briefly, slides imaged at 40x magnification using the NanoZoomer Digital Pathology System (NDP Hamamatsu, Hamamatsu City, Japan) were scored by two blinded observers using a 4-point system of inflammation severity within 4 subepithelial areas of $0.2mm^2$. Non-inflamed (normal) tissue was scored a 0 (<5% inflammatory cells), mild inflammation was scored a 1 (5-20% inflammatory cells), moderate inflammation was scored a 2 (20-50% inflammatory cells), and severe inflammation was scored a 3 (>50% inflammatory cells).

5.4.7 Tartrate-Resistant Acid Phosphatase (TRAP) Staining

Longitudinal sections of the maxillae were stained for Tartrate-resistant acid phosphatase (TRAP), a marker identifying cells of the osteoclastic lineage, using a modified procedure described by Udagawa *et al* (24). In summary, prepared TRAP stain (Sigma-Aldrich) was incubated with tissue for 15 minutes at 37ºC prior to be washed with PBS and haematoxylin counterstaining. Quantification of TRAP-positive cells with equal to or greater than 3 nuclei was conducted on the region of interest outlined above, between the first and second maxillary molars, using 40x magnified images on the NanoZoomer Digital Pathology System (NDP Hamamatsu, Hamamatsu City, Japan

5.4.8 Immunohistochemical Detection of HDAC 1, 5, 8 and 9

Protein distribution of HDAC 1, 5, 8, and 9 in the region of interest described above was detected using commercially available antibodies (anti-HDAC 1: ab53091, anti-HDAC 5: ab55403-100, anti-HDAC 8: ab39664, anti-HDAC 9: ab59718; Abcam). Negative controls for all sections were carried out using IgG1 kappa isotype control (Sigma-Aldrich) and a universal rabbit isotype control IgG (DAKO). Secondary antibody and amplification procedures were conducted using the VECTASTAIN ABC (Universal) kit (Vector Laboratories, CA, USA) as per manufacturers instruction as previously described (21). 10mM sodium citrate buffer (pH 6.0 at 80-90 ºC) antigen retrieval was required for HDAC 1 immunostaining, whereas HDAC 5, 8 and 9 required no additional antigen retrieval. Endogenous peroxide blocking was performed in a 0.3% v/v H_2O_2 methanol solution or phosphate-buffered saline (PBS)/0.1% sodium azide in 0.3% v/v H_2O_2 for 10-20 minutes. Normal horse serum supplied in ABC kit as blocking serum was finally applied to all sections and left to incubate for 30 minutes before applying primary antibody for overnight incubation at room temperature. The following morning, slides were washed in PBS and incubated for 30 minutes in ABC kit supplied biotinylated secondary antibody or avidin-biotin complex conjugated to horseradish peroxidase reagent followed by PBS washes and AEC Peroxidase Substrate Kit (Vector Laboratories) colour development in the dark. After MilliQ H_2O washing, haematoxylin counterstain was performed prior to mounting and imaging for analysis, described below.

5.4.9 Analysis of Immunohistochemistry

Sections were imaged using the NanoZoomer Digital Pathology System (NDP Hamamatsu, Hamamatsu City, Japan) for semiquantitative analysis at 40x magnification. 4 areas of 0.2mm^2, as outlined above, were assessed and scored by two blinded observers for the proportion of positive (red) staining cells, based on previously published methods (4). Briefly, a score of 0 was given when less than 5% of cells stained positive in a region; a score of 1 for 6-10% positive cells; 2 = 11-25%; 3 = 26-50%; 4 = <50%. Scores for each of the 0.2mm^2 regions per section were averaged, producing one score per sample. Final values were compared and

averaged between observers, creating a final score of each mouse that was used to calculate differences between experimental groups and time.

5.4.10 Statistics

All statistical analysis performed in this study utilized GraphPad Prism® software (V 7.0). Two-way repeated measures ANOVA was conducted using Sidak's post hoc analysis for comparison of variables from the same experimental group over time, whereas Tukey's post hoc test was used when comparing between experimental groups at the same time-point. Associations between HDAC expression and parameters of PD were determined using Pearson's Correlation coefficient. Statistical significance was determined when a p-value of 0.05 or less was identified.

5.5 Results

5.5.1 Animal general health

No adverse effects were observed in mice during the induction or maintenance of experimental PD. Mouse weights were consistent throughout the study with no differences observed between groups receiving bacteria or controls.

5.5.2 HDAC 1, 5, 8 and 9 protein expression in mice gingiva

Immunohistochemical analysis of HDAC 1, 5, 8 and 9 was conducted on the subepithelial gingival tissue between the 1st and 2nd maxillary molars. The proportion of cells expressing HDACs was determined after the primary inoculation sequence (Time-point 1) and at the end of the experiment (Time-point 2) to identify changes in HDAC expression over time and compared to the physiologically normal HDAC profile of unaffected control tissue. Over the course of the PD model, there were no variations in the HDAC profiles over time, with the proportion of cells staining positive for HDAC 1, 5, 8 and 9 not being significantly different between Time-point 1 and Time-point 2 in all of the experimental groups assessed.

There was, however, a significant increase in HDAC 1 expression in the PD disease Group 2 (PD/Pg) and Group 3 (PD/Pg+Fn) when compared to mice from the control group at both time points (Table 5.1; $p < 0.05$) (Figure 5.3). PD mice had an average of 6-25% of cells staining positive for HDAC 1 protein, whereas only 0-5% of cells expressed HDAC 1 in the control tissues. HDAC-5 protein, though highly expressed in approximately 50% of gingival cells, did not vary between the control or PD groups ($p > 0.05$).

Conversely, HDAC 8 expression was upregulated in the PD Group 3 (PD/Pg+Fn) at the end of the experiment (Table 5.1; $p = 0.010$) (Figure 5.3) with a proportion of 11-25% of cells staining positive when compared to 0-5% of cells in the tissues of control mice. An increase in HDAC 8 was also noted in mice from Group 2 (PD/Pg), with 6-10% of cells expressing the protein, although this rise was not statistically dissimilar to the control ($p = 0.602$). HDAC 9 expression did not significantly change despite a minor decline from 6-10% to 0-5% in PD tissues from Time-point 1 to Time-point 2 ($p > 0.05$), compared to a consistent 6-10% proportion of positive cells in healthy tissue from the control group.

Semi-quantitative analysis of HDAC protein expression in mouse subepithelial gingival tissue			
HDAC	Group 1 (Control)	Group 2 (PD/*P. gingivalis*)	Group 3 (PD/*P. gingivalis* + *F. nucleatum*)
Time-point 1			
HDAC-1	1.925 (0.30)	**2.68 (0.44)***	**2.60 (0.33)***
HDAC-5	4.00 (0.58)	3.83 (0.65)	3.88 (1.03)
HDAC-8	2.75 (0.35)	2.85 (0.86)	2.19 (0.21)
HDAC-9	2.69 (0.78)	2.70 (0.84)	2.65 (1.79)
Time-point 2			
HDAC-1	1.475 (0.09)	**2.20 (0.34) ***	**2.40 (0.41) ****
HDAC-5	4.15 (0.75)	3.68 (0.83)	4.18 (0.93)
HDAC-8	1.75 (0.96)	2.25 (0.59)	**3.4 (1.04) ***
HDAC-9	2.1 (0.75)	1.31 (0.67)	1.4 (0.80)

Table 5.1: HDAC, Histone deacetylase. PD, periodontitis. Semi-quantitative analysis score for proportion of positive stained cells within mouse subepithelial gingival tissue between the 1st and 2nd maxillary molars from the Time-point 1 and 2: 0 = <5% positive cells; 1 = 6-10%; 2 = 11-25%; 3 = 26-50%; 4 = <50%. Values represent Mean score ± SD. *$p < 0.05$ for HDAC expression in PD experimental groups vs. Control.

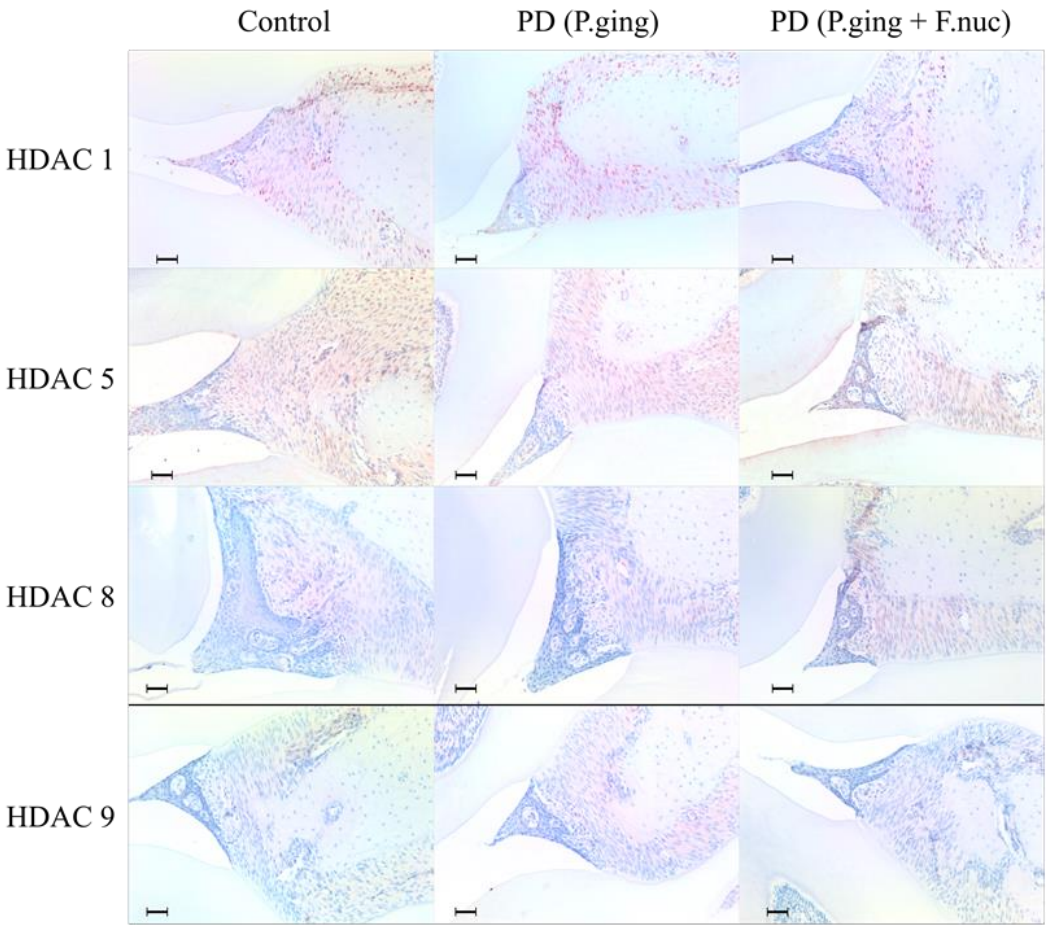

Figure 5.3 Representative immunohistochemical images of gingival tissue (40x magnification) from mice maxilla between the 1st and 2nd molars from Control (Left column), Periodontitis (P.ging; Middle column), and Periodontitis (P.ging+F.nuc; Right column); From top row down: HDAC 1, HDAC 5, HDAC 8 and HDAC 9. Scale bars represent 20μm.

5.5.3 Alveolar bone effects in experimental PD

Analysis of alveolar bone loss by Micro-CT (Figure 5.2 and Figure 5.4) and gingival inflammation by histology (Figure 5.5 and Figure 5.6) at time point 1 and 2 confirm established PD in both Groups 2 and Group 3 mice. PD mice from Group 2, inoculated with *P. gingivalis* only, had significant alveolar bone loss with reduced alveolar bone crest height, measuring an average of 70.58μm less than control mice at the end of the *primary inoculation sequence* (Time-point 1; $p < 0.0001$), and 96.86μm at the end of the experiment (Time-point 2; $p < 0.0001$). Mice from Group 3, inoculated with *P. gingivalis* and *F. nucleatum*, also had significant alveolar bone destruction, with an average loss of bone crest height of 89.42μm (Time-point 1; $p < 0.0001$) and 119.9μm (Time-point 2; $p < 0.0001$) compared to control mice. Further analysis of PD mice from Group 2 and 3 identified no significant variations in the amount of bone loss between Time-point 1 and Time-point 2 (Group 2: $p = 0.085$; Group 3: $p = 0.077$) or between PD groups assessed at the same time-point (Time-point 1: $p = 0.237$; Time-point 2: $p = 0.466$).

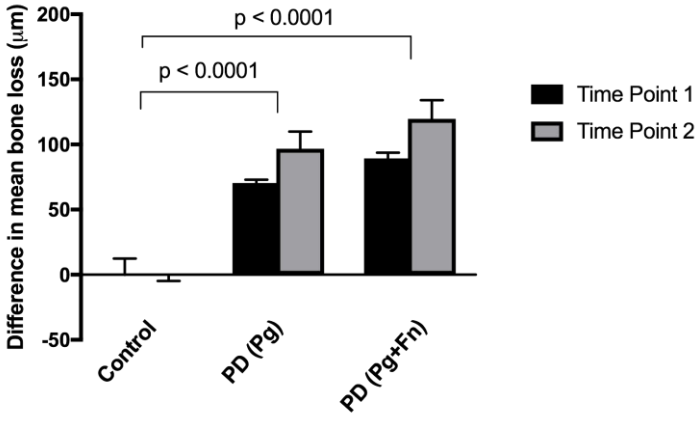

Figure 5.4 PD, Periodontal bone loss over time. Time-point 1 measurements conducted after completion of bacterial inoculations from the *primary sequence*; Time-point 2 measurements conducted after completion of *secondary sequence*; Control: normal mice; PD (Pg): Periodontitis mice inoculated with *P.gingivalis*; PD (Pg+Fn): Periodontitis mice inoculated with *P.gingivalis* and *F.nucleatum*. Values expressed as mean bone loss (μm) ± SEM.

5.5.4 Evaluation of gingival inflammation

Assessment of sub-epithelial gingival tissue in mice also supported the observation of established PD, with significantly greater numbers of inflammatory cells present in tissue (Figure 5.5 and Figure 5.6). Groups 2 and 3 had a marked increase in inflammatory cell infiltrate when compared to control mice across the two time-points (Group 2: $p = 0.006$ and 0.002, time-points respective; Group 3: $p = 0.0005$ and 0.0005 time-points respective; Figure 5.6). Similar to the bone changes observed between PD mice, levels of inflammation were slightly greater in Group 3 (PD/Pg+Fn) compared to Group 2 (PD/Pg), however these differences were not statistically significant at the two time-points analysed ($p > 0.05$).

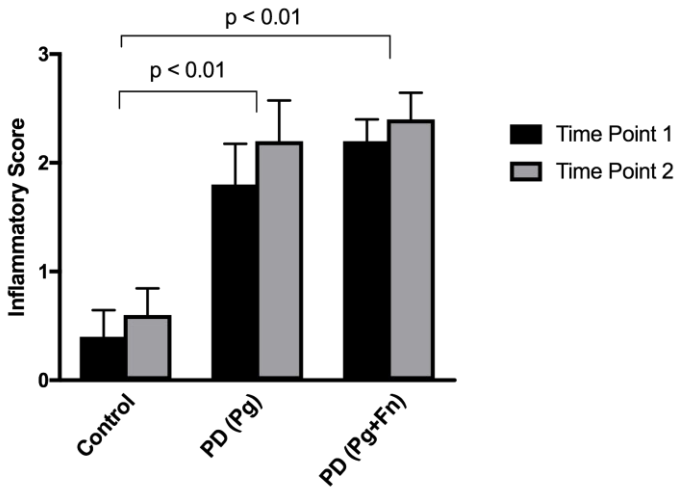

Figure 5.5 Semi-quantitative analysis of inflammatory cell infiltrate between the 1st and 2nd maxillary molars. Time-point 1 measurements conducted after completion of bacterial inoculations from the *primary sequence*, Time-point 2 measurements conducted after completion of *secondary sequence*; Control: normal mice; PD (Pg): Periodontitis mice inoculated with *P.gingivalis*; PD (Pg+Fn): Periodontitis mice inoculated with *P.gingivalis* and *F.nucleatum*. Values expressed as mean inflammatory score ± S

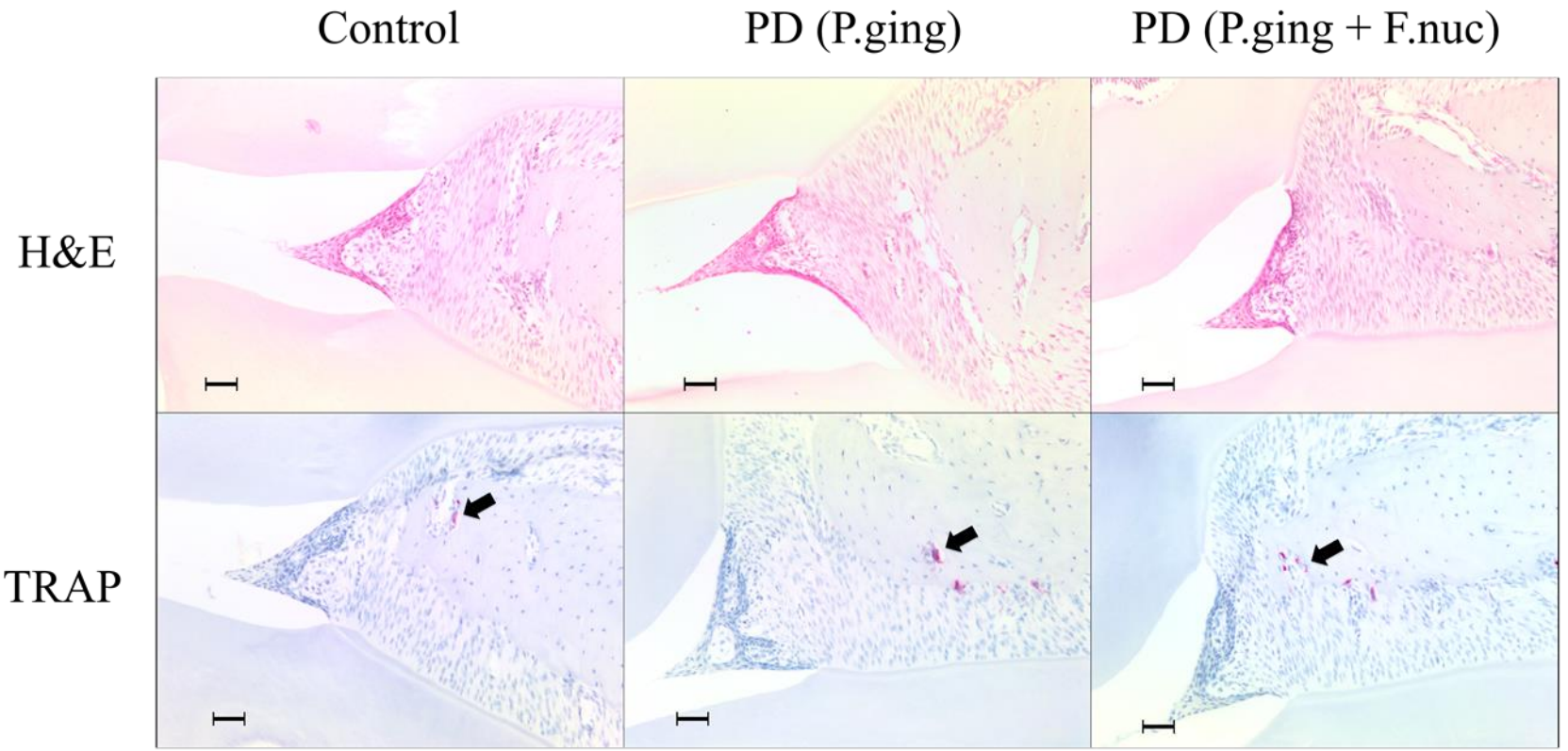

Figure 5.6 Representative immunohistochemical images of gingival tissue (40x magnification) from mice maxilla between the 1st and 2nd molars from the Control (Left column), Periodontitis (P.ging; Middle Column), and Periodontitis (P.ging + F.nuc; Right column); Top Row (Haematoxylin and Eosin; H&E); Bottom Row (Tartrate Resistant Acid Phosphatase; TRAP). Scale bars represent 20μm.

5.5.5 Increased osteoclastic cells experimental PD

Osteoclastic numbers in the tissues were elevated in mice with PD compared to control animals, with significantly ($p < 0.0001$) increased numbers of large, multinucleated, TRAP-positive staining cells present in the subepithelial gingival tissue and on the alveolar bone surface of mouse maxilla (Figure 5.6 and Figure 5.7). The presence of TRAP-positive cells within the tissue did not fluctuate between Time-point 1 or 2 in experimental PD, nor were there any observable differences in the number of osteoclasts in the tissue between Groups 2 or 3 ($p > 0.05$)

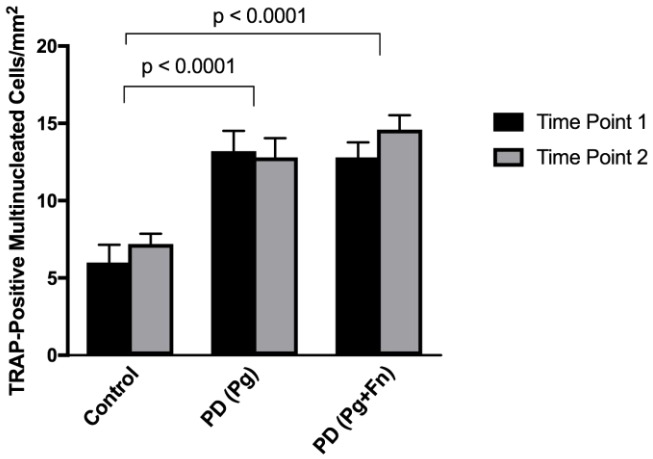

Figure 5.7 Quantitation of TRAP-positive multinucleated (3 or more nuclei) cells per mm^2 in mouse subepithelial gingival tissue between the 1st and 2nd maxillary molars. Time-point 1 measurements conducted after completion of bacterial inoculations from the *primary sequence*, Time-point 2 measurements conducted after completion of *secondary sequence*; Control: normal mice; PD (Pg): Periodontitis mice inoculated with *P.gingivalis*; PD (Pg+Fn): Periodontitis mice inoculated with *P.gingivalis* and *F.nucleatum*. Values expressed as mean inflammatory score ± SEM.

5.5.6 Relationship between HDAC expression and parameters of PD

A Pearson's correlation coefficient was used to identify any relationship between HDAC expression and inflammation, TRAP-positive osteoclast numbers, and bone loss. A strong positive relationship was observed between inflammatory scores, the number of osteoclasts present in the gingival tissue and the amount of alveolar bone damage ($p < 0.0001$; Table 5.2). Interestingly, these variables were also positively associated with an increase in the proportion of cells expressing HDAC 1 (HDAC 1: inflammation [$r = 0.573$, $p = 0.0009$], TRAP [$r = 0.385$, $p = 0.0357$], bone loss [$r = 0.423$, $p = 0.0198$]). Furthermore, there was a positive correlation between HDAC 8 expression and bone loss (HDAC 8: bone loss [$r = 0.383$, $p = 0.048$]), despite no observable relationship between HDAC 8 and TRAP-positive cell counts or levels of inflammation. No relationships were identified between parameters of PD and HDAC-5 or HDAC 9 expression ($p > 0.05$).

Table 2: Pearson's correlation results of HDAC expression, inflammation and parameters of bone loss

		Inflammation	TRAP	Bone Loss
	Pearson Correlation	**0.573**	**0.385**	**0.423**
	Sig. (2-tailed)	**0.0009*** **	**0.0357***	**0.0198***
HDAC-1	N	30	30	30
	Pearson Correlation	-0.101	-0.169	-0.0963
	Sig. (2-tailed)	0.596	0.372	0.612
HDAC-5	N	30	30	30
	Pearson Correlation	0.267	0.358	**0.383**
	Sig. (2-tailed)	0.179	0.067	**0.048***
HDAC-8	N	30	30	30
	Pearson Correlation	-0.145	-0.169	-0.289
	Sig. (2-tailed)	0.463	0.389	0.136
HDAC-9	N	30	30	30
	Pearson Correlation		**0.661**	**0.795**
	Sig. (2-tailed)		**< 0.0001**** **	**< 0.0001**** **
Inflammation	N		**30**	**30**
	Pearson Correlation			**0.829**
	Sig. (2-tailed)			**< 0.0001**** **
TRAP	N			30

Table 5.2: HDAC, Histone deacetylase, TRAP, tartrate resistant acid phosphatase staining positive multinucleated cells per mm^2, Bone Loss, measure of alveolar bone crest height. *$p < 0.05$, ***$p < 0.001$, **** $p < 0.0001$.

5.6 Discussion

The aim of this study was to assess the isoform specific HDACs from class I (HDAC 1 and 8) and class II (HDAC-5 and 9) throughout the pathogenesis of experimental PD. HDACs are integral for physiological processes during cell formation and activity, hence any variations in their distribution and/or function could regulate pathogenesis and the development of disease. Mice inoculated with pathogenic bacteria (*P. gingivalis* and *F. nucleatum*), present in the oral flora of human PD patients, developed characteristic gingival inflammation that lead to enhanced osteoclast formation and loss of alveolar bone. The results of this study show that class I HDACs 1 and 8 are significantly upregulated in PD and are associated with the severity of alveolar bone damage. A similar relationship was identified between the proportion of cells expressing HDAC 1 and the magnitude of gingival inflammation and the number of bone resorbing osteoclasts present in the tissue.

The characteristic bone loss observed in PD can be attributed to enhanced osteoclast formation and resorption, induced by inflammatory cytokines such as tumour necrosis factor-α (TNFα) and/or interleukin-1β (Il-1β) (1, 25). RANK ligand (RANKL), a vital inducer of osteoclastic differentiation and survival, is also upregulated in PD tissue (26, 27). As immune activity involves epigenetic modulation by HDACs (5), investigations into their pharmacological inhibition with HDACi has become of great interest over the past decade (13). We recently reported on the use of two HDACi (class I selective: MS-275, and broad acting 'pan'-inhibitor: 1179.4b) in a mouse model of PD (21), reducing levels of inflammation and bone loss. However, their efficacy was varied, possibly due to the broad nature of their inhibitory selectivity. The recent development of isoform specific HDACi has prompted further investigations into the individual histone deacetylase enzymes over the course of the cell cycle and disease. These studies indicate specific roles for individual HDAC isoforms.

The observation here that HDAC 1 is upregulated in experimental PD is consistent with findings in human gingiva (13). In addition, HDAC 1's association with inflammation and elevated osteoclastic activity is seen in two recent *in vitro* studies investigating HDAC 1 selective inhibitors (Chapter 3). These studies used osteoclasts derived from human monocytes and demonstrated that inhibition of HDAC 1 impaired the development and function of osteoclasts. Furthermore, NW-21, a novel HDACi designed by the University of Queensland to specifically target HDAC 1 and 2, repressed osteoclasts *in vitro* (4)(Chapter 2). NW-21 also protected the radiocarpal joint from destruction in an *in vivo* model of collagen antibody induced arthritis (CAIA) by reducing numbers of osteoclasts (12). Consistent with this, both broad class I or more specific HDAC 1 inhibitors are reported to reduce inflammation *in vivo* and the expression and synthesis of inflammatory mediators by monocytes stimulated with lipopolysaccharides or TNFα (28, 29). Similar to HDAC 1, another class I HDAC, HDAC 8, has shown been shown to regulate bone metabolism. As osteoclasts develop, HDAC 8 expression rises, particularly during the resorptive phase of the cell cycle (4). Furthermore, in osteoblasts there is a decline during osteoblastogenesis indicating elevated HDAC 8 is associated with bone loss (20). Enforced expression of HDAC 8 in bone marrow stromal cells represses transcriptional activity of Runx2 and osteogenic differentiation (20). The broad inhibition of HDACs including HDAC 8 by Valproic acid (VPA) or deletion of HDAC 8 expression restores osteogenesis, allowing bone formation to continue (20). To date, no selective HDAC 8 inhibitors have been investigated in studies with osteoclasts. However, taken together, these results indicate a rise HDAC 1 and HDAC 8, such as that observed in this study, is associated bone loss due to elevated osteoclastic bone resorption and reduced osteoblastogenesis.

The lack of change in HDAC-5 and HDAC 9 expression induced by experimental PD may indicate a lack of tissue repair occurring during disease progression. HDAC-5 has been shown to reduce bone growth, by inhibiting Wnt-signalling pathways and Runx2 transcriptive activity in osteoblasts (18, 19). Furthermore, adolescent patients suffering from primary osteoporosis express greater levels of HDAC-5, which correspond to declines in Runx2 (30, 31). Inhibiting HDAC-5

activity in mouse primary osteoblasts using miRNAs maintains Runx2 transcription and osteoblast differentiation (19). Hence, it can be deduced that reduced HDAC-5 activity stimulates osteoblastogenesis and the formation of new bone. As the mice used in the present study were of adult age, the observation of stable HDAC-5 expression in control mice was expected. The finding of constant HDAC-5 expression in PD mice, however, may suggest continued modulation over osteoblast activity and periodontal repair. The use of selective HDACi targeting HDAC-5 in experimental PD may promote tissue recovery by stimulating osteogenesis and bone deposition, counteracting the catabolic activity of osteoclasts. Interestingly, recent investigations into the class II, HDAC 9 isoform, have reported on its role as an osteoclast inhibitor (17); through negative regulation of RANK signalling molecules and the repression of osteoclast factors c-Fos, NFatc1, Dc-stamp and Cathepsin K. In the present study, HDAC 9 was expressed at the lower levels in mouse gingiva, and a decline over time and in PD tissue was noted (control = 11-25%, PD = 6-10%), although this did not achieve statistical significance. Similar to HDAC-5, the lack of change between control and disease groups in the expression of HDAC 9 may indicate an absence of reparative mechanisms being active during the pathogenesis of PD. This highlights another potential target for pharmacological intervention to promote repair of bone. Further analysis of these specific isoforms and their function promoting tissue repair will be necessary to elucidate their role in disease.

5.7 Conclusion

In conclusion, this study establishes the class I (HDAC 1 and 8) and class II (HDAC-5 and 9) HDAC profile in healthy and experimental PD mice; highlighting these specific HDACS as potential targets for future investigations. The uniformity of HDAC-5 and 9 in disease and the notable increases in the expression of HDAC 1 and 8 correlating with levels of gingival inflammation and alveolar bone damage, provide evidence to support their continued focus in future studies.

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Chapter 6: Thesis General Discussion and Future Considerations

6.1 Introduction

Alveolar bone breakdown continues to be a challenge for PD management due to the vast array of contributing factors that influence bone metabolism. In a healthy individual, the skeleton is continually turned over by bone resorbing osteoclasts and bone forming osteoblasts. The collaborative efforts by these cells in physiological conditions result no overall change in the mass or density of the skeleton (1) However, in PD, alveolar bone loss occurs due to an imbalance between these bone cells (2, 3). Osteoclasts are stimulated by the immune response to oral pathogens in PD (3-7). This in turn, promotes osteoclastogenesis and an increase in osteoclast numbers, activity levels and rates of survival. Osteoblasts are not able to compensate for the level of bone resorption taking place, and are also suppressed by inflammatory mediators, such as TNFα, present in PD tissues (7). This further exacerbates the gap between bone resorption and bone formation. Ultimately, if this process is left to continue without effective periodontal treatment, support for the teeth is removed with tooth loss being a permanent consequence (2).

Epigenetic regulation of disease by HDACs is currently being investigated as therapeutic target for inflammation and in bone loss pathologies, in addition to a variety of other illnesses (8-13). Initially, HDACi were used clinically to modulate the cell cycle processes in cancer (9, 10, 14). This has prompted their use in other systems that involve altered cellular mechanisms. Until recently, HDACi were commonly produced as broad acting compounds (pan-inhibitors) that target a large range of HDAC isoforms. This results in universal suppression of the HDAC family (15, 16). However, individual HDAC isoforms exist in different tissues, have distinct structures and vary in their deacetylating targets (15, 16). Although broad suppression of HDACs is effective when aiming to halt widespread

proliferation of rapidly dividing malignant cells (9, 10, 14), it may not be ideal as a mechanism for treating non-oncogenic pathologies. Therefore, the particular focus of this thesis involved investigating the therapeutic potential of targeting specific HDACs for PD and related inflammatory bone loss diseases. The overall body of this work was guided by two hypotheses. Firstly, *that HDAC 1, 2 and 5 are key modulators of inflammation and bone metabolism*. Secondly, *that specific HDAC inhibitors targeting HDAC 1, 2 and 5 will reduce inflammation and bone loss in inflammatory bone loss disease, such as PD.* Studies were carried out with human cells *in vitro* and *in vivo* using an animal model of disease. For *in vitro* investigations, the addition of the inflammatory cytokine TNFα was used to mimic inflammatory conditions.

6.2 Discussion and Future Considerations

6.2.1 Inflammation in PD and the anti-inflammatory effects of HDACi

It has been well established that the subgingival microflora is a major contributing factor for the initiation of PD. However, the individual host response to these oral pathogens is key to the progression and severity of this chronic disease (2, 3). As the rapidly evolving field of osteoimmunology defines the interaction between the immune system and the skeleton (17), modulating the inflammatory reaction in PD is a crucial aspect of effective therapy. HDACi are found to possess potent immuno-modulatory properties as they decrease the production of pro-inflammatory cytokines, both *in vitro* and *in vivo* (18-23). However, the majority of investigations to date have utilized broad acting inhibitors. For example, SAHA has been shown to reduce circulating levels of TNFα, IL-1β, IL-6 and IFN-γ in mice that have been subjected to LPS administration (18). Human PBMC stimulated with LPS are also reported to produce less TNFα, IL-1β and IFN-γ with SAHA treatment (18). It is important to note that this occurs at doses significantly less than those utilized in chemotherapy. Similar to this, TSA (22), NaB (16, 22) and VPA (20) have also been reported to supress pro-inflammatory cytokine production through dose dependant means. Investigations using mouse models of PD (24) and RA (13) have utilized the broad acting HDACi 1179.4b, the class I

inhibitor, MS-275, or the isoform HDAC 1/2 selective NW-21 compound. These studies reported reductions in the level of inflammation and expression of inflammatory mediators such TNFα, MCP-1 and MIP-1α (13). Importantly, this shows that broad suppression of all HDAC isoforms is not required to reduce inflammation, indicating a distinct role for individual HDACs during the inflammatory response.

This thesis (Chapter 3) further evaluated the role of individual HDACs in modulating the production of pro-inflammatory mediators by human cells. PBMC were stimulated with TNFα and treated with isoform specific inhibitors of HDAC 1, 2 and 5. Their effects were compared to that induced by the broad acting HDACi, 1179.4b. These findings demonstrated a similar pattern of reduced expression and synthesis of inflammatory mediators with selective HDAC 1 inhibition as that observed by 1179.4b. Interestingly, the anti-inflammatory properties of HDAC 1 inhibition were enhanced with concurrent HDAC 2 inhibition. This may be due to HDAC 1 and 2 being reported to contribute to similar tasks whilst associating as a single protein complex (25). Conversely, HDAC 5 inhibition had no anti-inflammatory effect in these assays.

The variations in the outcomes observed in investigations using HDACi may be a result of the doses used (18-23), or the specific deacetylating targets of each HDAC isoform, which is currently not well defined. Further to this, the isolated use of TNFα may not fully represent the clinical pathogenesis of PD in this *in vitro* model, with specific periodontal pathogens producing a plethora of inflammatory pathways and epigenetic process (3-7). However, a recent publication (13) using P.ging LPS demonstrated similar induction of inflammatory cytokines and chemokines by isolated monocytes as was observed with TNFα stimulation in Chapter 3. This supports the use of TNFα as an effective investigatory factor to induce the inflammatory cascade, representative of the inflammatory response to periodontal pathogens without the risk of cell culture infection with live microorganisms or LPS. Nevertheless, the results of this thesis suggest targeting the individual isoforms involved in similar outcomes, such as HDAC 1 and 2 to

suppress inflammation, without targeting additional HDACs involved in other processes.

6.2.2 Osteoclastic bone resorption and the effects of HDAC inhibition

In addition to modulating the immune response and inflammatory processes, HDACi are known to have anti-bone resorptive properties that are directed at inhibiting osteoclastogenesis (8). Osteoclasts differentiate from their precursor monocyte/macrophages in response to RANKL induced signalling (17). However, recent *in vitro* investigations have identified additional signalling pathways in osteoclasts that either support, enhance or bypass RANKL signalling all together (7). Despite this, RANKL and its downstream molecules remain an important component for osteoclast formation and activity *in vivo*. Interestingly, HDACi are reported to affect the major transcriptive factors NF-κB and NFATc1, in addition to their associated target genes that are responsible for osteoclastogenesis (8). As alveolar bone loss is caused by excessive osteoclast formation and activity within the inflamed periodontium, the use of HDACi with anti-inflammatory properties make them promising options as future therapies.

There have been numerous reports investigating the use pan-HDACi to suppress osteoclast driven bone destruction with overwhelmingly positive results (discussed in (8)). For example, TSA has been utilized in assays using primary bone marrow macrophages (26) or RAW-D macrophage cell lines (27). TSA was shown to suppress osteoclast differentiation through reduced NFATc1 activity and NF-κB translocation into the nucleus. SAHA was reported to suppress osteoclastogenesis from primary bone marrow macrophages in a co-culture system with osteoblasts through a similar mechanism as TSA that was associated with reduced NFATc1(26). It was later identified that NFATc1 expression was reduced due to the hyperacetylation of histone 3 (H3) due to TSA or SAHA inhibition of deacetylation (26). NaB has been investigated for its effects on osteoclast formation in mouse bone marrow macrophages and the RAW-D cell line (27). These assays resulted in dose dependent declines in osteoclast formation. In addition, 1179.4b significantly suppressed osteoclastogenesis from human PBMC through inhibition of RANKL signalling factors TRAF 6 and NFATc1 expression

(28). Despite the potent anti-osteoclastic effect of these HDACi, future progression of these HDACi into the clinic will benefit from further elucidation of the specific roles for each HDAC isoform to improve drug efficacy and safety.

Chapters 2 and 3 of this thesis involved assessing the effects of several novel HDACi that were designed to selectively target HDAC 1, 2 or 5, as preliminary and published investigations have highlighted these isoforms as key regulators of skeletal maintenance (8, 13, 24, 29). We have recently demonstrated higher levels of these HDACs in human PD gingival tissues (30). Specifically, HDAC 1 was observed to co-localise with TRAP-positive osteoclastic cells while being associated with a rise in TNFα expression. For this reason, along with the known impact of this cytokine on bone cells *in vitro* (discussed in Chapter 1) and significantly higher levels of its protein in human periodontitis gingival tissue (upwards of 20pg/ml) (49), TNFα at 10ng/ml successfully produced an experimental inflammatory representation of its effects on bone cells *in vitro*. As described in Chapter 2, the capacity for TNFα to stimulate osteoclasts was largely dependent on the timing and length of TNFα exposure, in addition to the dose of RANKL utilized. Several preliminary assays were conducted to design and refine an effective methodological approach to form a reproducible 'inflammatory osteoclasts' *in vitro*. Chronic exposure to TNFα over the course of full osteoclast assay (21 days) suppressed osteoclast differentiation from PBMCs. Their morphology remained macrophagic, being smaller in shape and consisting of a single nucleus. Furthermore, although expressing TRAP (a marker of osteoclast devoted cell-types) these cells failed to resorb any dentine *in vitro*. It was eventually determined that priming progenitors with TNFα increased the rate of osteoclast differentiation, which resulted in a greater resorptive capacity than RANKL-only activated cells.

The use of TNFα to mimic the inflammatory state proved to be productive, as an interesting finding of the assays described in Chapters 2 and 3 was the identification of the direct induction of HDAC 1 expression by TNFα. This observation supports the relationship identified with HDAC 1 expressing TRAP-positive cells and TNFα expression in PD gingival tissue (29). HDACi that target

HDAC 1 potently supressed osteoclast formation and activity through reduced NFATc1 and RANKL signalling factors affecting NF-κB translocation into the nucleus. Furthermore, the anti-osteoclastic effect of HDAC 1 inhibition was strengthened with concurrent inhibition of HDAC 2. These findings are fundamentally important for the future progression of selective HDACi into the clinic. As their mechanisms of suppression are similar to that of pan-inhibitors, selective inhibition does not bring the risk of targeting isoforms that may physiologically regulate the activity of osteoclast, or cause non-specific destructive outcomes *in vivo*. Further analyses of these compounds should consider their use in an inflammatory bone loss model of periodontitis and other inflammatory bone loss diseases.

6.2.3 Osteoblastic bone formation and the effects of selective HDAC inhibition

Reparative dentistry has become an important aspect of PD management, with aims to enhance bone strength and counteract the destructive outcomes of excessive bone resorption by osteoclasts. Recently, HDACi were demonstrated to induce osteogenic differentiation by stem cells, in addition to enhancing anabolic processes in osteoblasts. Mice MSC treated with TSA are reported to express greater levels of OPN in undifferentiated cells (31), whereas VPA has been shown to induce a dose dependent increase in osteogenic differentiation by human MSC. Similar to TSA, an induction of OPN was seen in response to VPA in human MSC, in addition to Osterix and Runx2 expression being promoted (32). Interestingly, VPA and NaB are reported to suspend the proliferative processes in human MSC *in vitro,* by inducing osteogenic differentiation (33). Primary mouse osteoblasts have been shown to respond to TSA, NaB and MS-275 at a dose dependent rate in a recent study (34). For example, accelerated matrix deposition was observed with TSA treatment through the stimulation of Runx2 transcriptional activity and the subsequent induction of key osteoblastic genes OPN, Osterix, OCN and type 1 collagen (34). Several other *in vitro* and *in vivo* findings report similar occurrences (35-38). Conversely, broad inhibition of HDACs in humans has been reported to produce negative outcomes on bone integrity. Systemic treatment for epilepsy with VPA has been shown to decrease bone mineral density after long-term use (39,

40), further supporting the continued investigation into the role of individual HDACs in osteoblasts. However, to date no selective inhibitors have been evaluated for osteogenic capacity.

The results of Chapter 4 in this thesis, using the novel HDAC 5 inhibitor, Compound 39, show that it accelerated matrix mineralisation in human osteoblasts by inducing the expression of Runx2, OCN and type 1 collagen. HDAC 5 was targeted in these investigations based on recent evidence of its specific involvement in bone repair and maintenance. Patients with osteoporotic phenotypes, such as that observed in juvenile osteoporosis, are reported to have diminished osteoblast activity that results in decreased bone volume. A study evaluating bone biopsies from juvenile osteoporotic patients identified a significant rise in HDAC 5 expression that was associated with decreased levels of Runx2 (41, 42). With specific reference to PD, our recent evaluation of human gingival samples also identified a significant rise in HDAC 5 in patients with alveolar bone loss and inflammation. Interestingly, TNFα increased HDAC 5 expression in human osteoblasts *in vitro* (Chapter 4), which was followed by diminished bone mineral production and suppression of Runx2 and OCN. The negative effects of TNFα on osteoblast are widely recorded, as recently reviewed (7). Inhibition of the elevated HDAC 5 with its inhibitor, Compound 39, restored the mineralising capacity and gene expression (to some extent) in cells exposed to TNFα. The fact that HDAC 5 inhibition alone did not return these cells to their full functioning capacity as those grown in the absence of TNFα indicates the need to investigate the additional HDAC isoforms as targets in conjunction with HDAC 5.

Future investigations should also analyse the specific mechanistic actions of HDAC 5 and its role during osteoblastogenesis. For example, a study evaluating the HDAC 8 isoform in bone marrow stromal cells reported on its physiological role negatively regulating osteoblast differentiation (43). Specifically, forced knockdown of HDAC 8 triggers acetylation of H3, which enhances osteogenic differentiation through the induction of Runx2 transcription (43). In addition, HDAC 4 forms a stable, complex molecule with HDAC 5 (much like the

associated relationship with HDAC 1 and 2 from Class I) (44). Declines in HDAC 4 and 5 are reported to occur during the induction of osteogenesis and Runx2 transcriptive activity (44). Interestingly, the opposite is associated with specific gene, H19, that codes for a group of long non-protein-coding RNAs (45), as its expression is induced during osteogenic differentiation in human MSC (44, 46). A recent study inducing forced expression of H19 in MSC cultured in non-osteogenic conditions, induced Runx2 transcription and osteogenic differentiation. It was reported that the induction of Runx2 was associated with a decline in HDAC 4 and 5 (44). Overall, these recent findings and those observed in this thesis identify HDAC 5 as an important regulator of osteoblastogenesis. Furthermore, pharmacological inhibition of HDAC 5 and possibly other recently identified HDAC isoforms (HDAC 4 and HDAC 8) could be an effective therapy for bone regeneration in PD.

6.2.4 A periodontitis model for future evaluation of HDACi in vivo

Progression of these compounds into the clinical setting will require further investigations into their specific mechanisms of action, in addition to characterising their broader effects *in vivo*. However, determination of their effects in humans remains a challenge. Although analysis of gingival tissue provides valuable insight into HDAC distribution and expression, we are unable to evaluate the effects of HDACi. To this end, using an animal model of periodontitis to characterise and investigate future therapies can be very useful.

Recently, a mouse model of PD was established in our laboratory for investigations into its pathogenesis and possible therapeutic options (47). With the induction of PD using pathogenic bacteria as the chronic inflammatory stimulus to propagate the immune response, a similar process of altered bone turnover occurs. Interestingly, this model induces a similar HDAC profile (Chapter 5) as that seen in human disease (29). Future investigations will utilize the results of this thesis to target individual HDACs and their effects on bone metabolism in this model. Further to this, isolation of pharmacological activity within the periodontal region to prevent off target effects could be achieved by using local local treatment techniques, such as the application of liposomal assisted drug delivery (50). This

process would involve HDACi being incorporated into ligand-targeted liposomes in an aqueous gingival paste. Upon administration, macrophagic cells of the ligand-targeted liposomes (osteoclast progenitors for example) would receive a local and specific dose of compound, improving efficacy and reducing the risk of off-target effects (50).

As outlined in Chapter 5, HDAC 1 and 8 expression is significantly induced in experimental PD and is associated with increased numbers of TRAP-positive osteoclasts and alveolar bone loss. This finding is also consistent with the observed profile in human PD (29). HDAC 5 was largely expressed in mouse gingiva and did not vary between PD and control mice. As previously discussed, HDAC 5 appears to negatively regulate osteoblastogenesis. Therefore, the observation of high and stable HDAC 5 expression indicates continued modulation over osteoblastic bone formation. As such, for reparative processes to commence, HDAC 5 repression may be required. This finding further supports the use of a HDAC 5 inhibitor as a pharmacological treatment for regenerative bone therapy. Conversely, HDAC 9 had minimal expression over the course of experimental PD. Recent investigations into the role of HDAC 9 in human osteoclasts identified it as a natural regulator and inhibitor of osteoclastogenesis (48). Therefore, it can be argued that osteoclastogenic processes are active in PD with low HDAC 9 expression. As such, although suppressing HDAC 9 is not a plausible option, the induction of this isoform in conjunction with inhibition of other individual HDACs (such as HDAC 1 and 5) may be a potential therapeutic cocktail for further investigations.

6.3 Thesis Conclusion

In summary, this thesis confirms the hypothesis that HDAC 1, 2 and 5 are key modulators of inflammation and bone loss, and that their selective inhibition may be an effective and well tolerated therapy targeting inflammatory induced bone loss. TNFα stimulated osteoclast activity, and this was suppressed by targeted inhibition of HDACs 1 and 2 (Chapter 2 and 3). In addition, this targeted inhibition suppressed the inflammatory cascade induced by TNFα in human monocytes (Chapter 3). HDAC 5 inhibition promotes mineralization by human osteoblasts by inducing the expression of important osteoblast genes, while opposing the suppressive effects of TNFα on osteoblastogenesis *in vitro* (Chapter 4). Furthermore, a mouse model of PD accurately represents the current understanding of HDAC activity in disease (Chapter 5). Therefore, future investigations should utilize this model in addition to the findings produced from this thesis, to further elucidate the role of HDAC 1, 2 and 5 as potential therapies in inflammatory bone loss.

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Appendix I: Published Manuscripts as PDFs Arising from This Thesis

Journal of
PERIODONTAL RESEARCH

J Periodont Res 2016; 51: 549–566

JOURNAL OF PERIODONTAL RESEARCH
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Review article

The effects of tumour necrosis factor-α on bone cells involved in periodontal alveolar bone loss; osteoclasts, osteoblasts and osteocytes

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Periodontitis is the most common bone loss pathology in adults and if left untreated is responsible for premature tooth loss. Cytokines, such as tumour necrosis factor-α (TNFα), involved in the chronic inflammatory response within the periodontal gingiva, significantly influence the normal bone remodelling processes. In this review, the effects of TNFα on bone metabolism in periodontitis are evaluated in relation to its direct and indirect actions on bone cells including osteoclasts, osteoblasts and osteocytes. Evidence published to date suggests a potent catabolic role for TNFα through the stimulation of osteoclastic bone resorption as well as the suppression of osteoblastic bone formation and osteocytic survival. However, the extent and timing of TNFα exposure *in vitro* and *in vivo* greatly influences its effect on skeletal cells, with contradictory anabolic activity observed with TNFα in a number of studies. None the less, it is evident that managing the chronic inflammatory response in addition to the deregulated bone metabolism is required to improve periodontal and inflammatory bone loss treatments.

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Irreversible alveolar bone destruction is a common outcome of the chronic inflammatory disease, periodontitis. Alveolar bone is essential for maintaining structural support for the teeth, and its untreated resorption can lead to the loosening and eventual loss of teeth (1). Benign and non-destructive forms of periodontal diseases, such as gingivitis, are highly prevalent globally, affecting more than 80% of children and adults in the United States, Canada, Australia and other regions of the world (2–4). In the absence of effective dental management or oral hygiene this can develop into the more permanent and destructive forms of periodontitis. Chronic periodontitis affects up to 9% of adults aged 18–24 worldwide and more than 60% of those aged over 65 years (2,4). Early onset aggressive periodontitis, a less common albeit severe form of periodontitis that causes rapid degeneration of tooth support, affects up to 5% of the juvenile population (2,4).

Considered the most common bone loss pathology in humans, the initiation and progression of alveolar bone destruction seen in periodontitis is related to the presence of a chronic inflammatory reaction within the

surrounding gingival tissue (5). This reaction is initiated in response to the development of a subgingival biofilm containing high levels of anaerobic bacteria, such as *Actinobacillus actinomycetemcomitans* (*Aggregatibacter actinomycetemcomitans*), *Porphyromonas gingivalis* and *Tannerella forsythensis* (5–8). Environmental and host factors, such as cigarette smoking (9), genetics (10) and certain medications (11–13) have also been shown to influence the onset of periodontitis and periodontal bone quality. The microbial foreign body challenge induced by lipopolysaccharides present in gram-negative bacterial membranes of the above-mentioned species is a potent stimulus for the release of inflammatory molecules by the host, promoting the migration and activation of inflammatory cells (monocytes, macrophages, T lymphocytes) (14–16). This, in turn, increases the numbers of proinflammatory cytokines, such as tumour necrosis factor-α (TNFα) and a variety of interleukins (IL), released into the surrounding tissue (15,16). These factors, particularly TNFα, are an essential component of the inflammatory response, vital for pathogen elimination and resolution. However, excessive levels or prolonged production of TNFα is implicated in the pathogenesis of numerous autoimmune and inflammatory diseases.

This host response is now known to be a critical component in the associated bone destruction that occurs in periodontitis. Historically considered two separate and distinct systems within the body, the skeletal and immune systems are now recognized as sharing intricate regulatory pathways, molecules, cytokines and receptors, which not only orchestrate the individual system, but influence the outcome of the other (17). This continually growing field of research is referred to as osteoimmunology (18). TNFα is a widely studied proinflammatory cytokine that has an important role in immune and skeletal cell regulation and plays a key role in the pathogenesis of a variety of inflammatory bone loss diseases, including periodontitis. Therefore, the aim of this review is to evaluate how TNFα affects the bone cells involved in periodontal alveolar bone loss; osteoclasts, osteoblasts and osteocytes.

Tumour necrosis factor-α signalling

Extensive research on this potent proinflammatory cytokine has demonstrated its role in the inflammatory response, and the development of chronic disorders (as reviewed in 19–22). The protein was first successfully purified and cloned in the early 1980s (23,24), allowing the systematic mapping of its cellular pathways and signalling mechanisms in a variety of cell types.

TNFα is a biologically active protein, distributed as a bound transmembrane unit to its parent cell types, mainly monocytes and T cells, or in its soluble form after the cleaving actions of TNFα converting enzyme (25,26). TNFα binds to one of two cell surface receptors (TNFr), initiating a cellular response. Activation of TNFr1 (55 kDa) can induce cellular proliferation, stimulation and survival (27,28), or conversely trigger apoptotic signalling and cell death (28,29) (see Fig. 1). The conducting pathways that determine survival or cell death through TNFα ligation is dependent on two unique complexes bound to the C-terminal end of the intracellular TNFr1 region. Complex I signalling involves components that induce cell survival, through the expression of anti-apoptotic proteins and the activation of transcription factors, activator protein 1 (AP-1) and nuclear factor kappa-light chain enhancer of activated B cells (NF-κB) (30–33). In contrast, the presence of

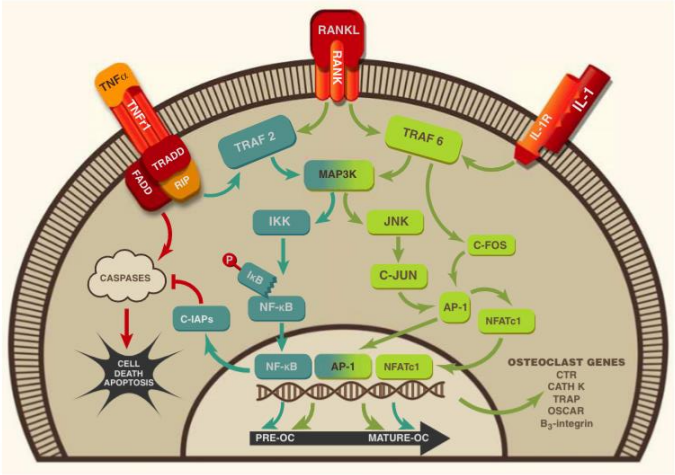

Fig. 1. Influence of inflammatory cytokines, TNFα and IL-1 on OC signalling. Cell proliferation and survival can be activated via TNFα binding to TNFr1 on the cell surface and signalling through TRADD and RIP1 cytoplasmic motifs leading to TRAF2/NF-κB activation. Conversely, the presence of FADD on the TNFr1 motif induces activation of caspases and cell death. IL-1r activates TRAF6/NFATc1 signalling, which can be induced in the absence of RANKL interaction with its receptor RANK. AP-1, activator protein 1; C-IAPsS, inhibitors of apoptosis protein; FADD, fas-associated death domain; IκB, inhibitor of kappa-B; IKK, inhibitor of kappa-B kinase; IL-1, interleukin 1; JNK, c-jun N-terminal kinase; NF-κB, nuclear factor kappa-light chain enhancer of activated B cells; MAP3K, mitogen-activated protein 3 kinase; NFATc1, nuclear factor of activated T-cell cytoplasmic 1; OC, osteoclast; RANKL, receptor activator of nuclear factor B ligand; RIP, receptor-interacting serine–threonine kinase 1; TNFα, tumour necrosis factor alpha; TNFr1, tumour necrosis factor receptor 1; TRADD, tumour necrosis factor receptor-associated death domain; TRAF 6, tumour necrosis factor receptor associated factor 6.

complex II initiates receptor internalization, triggering pathways responsible for cell death and apoptosis (27,29). TNFr1-related cell survival is achieved through interactions with adaptor proteins, namely members of the TNF receptor associated factor (TRAF) family (TRAF 1, 2 and 3), and the signal mediator TNF receptor-associated death domain (TRADD) (34). TRADD directs signalling information from TNFr1 through TRAF 1 and 2, which leads to the rapid attachment of ubiquitin protein to receptor-interacting serine–threonine kinase 1 (RIP1) (31,35). This post-translational modification of RIP1 allows signalling to continue through mitogen-activated protein 3 kinases, and the activation of the inhibitor of kappa-B kinase (IKK) complex (30,35). IKK complex activation initiates phosphorylation of the inhibitor of kappa-Bα (IκBα), allowing NF-κB to translocate into the nucleus, thus initiating the transcription of anti-apoptotic and proliferative genes (36,37).

TNFα binding can alternatively induce a conformational change to the cytoplasmic motif of TNFr1, allowing for the attachment of additional adaptor proteins to TRADD that constitutes complex II and cell death signalling (38). RIP1 ubiquitination, which is essential for NF-κB activation and cell survival, is reversed by an enzyme called CYLD (39–41). This enables the attachment of RIP1 kinase into complex II, consisting of pro-caspase 8 and fas-associated death domain (FADD) bound to TRADD (39–41). In turn, signalling through this complex initiates suppression of the NF-κB transcription of anti-apoptotic signals such as baculoviral inhibitors of apoptosis proteins (C-IAPs), and activates a caspase cascade of cell death through apoptosis (39–41). Investigations targeting this TNFα pathway in malignancies have identified CYLD as a key factor causing tumour cell death through inhibition of NF-κB transcription (39–41).

TNFα can alternatively bind to the second receptor TNFr2, which is structurally distinct from TNFr1, being larger (75 kDa) and consisting of a unique cytoplasmic motif absent of the death domain (27,28). Despite the lower membrane expression of TNFr2 (42), it appears to maintain an important role in regulating and communicating with TNFr1. Studies focusing on the signal transduction pathways of TNFr2 have uncovered several similarities with the TNFr1 proliferation and survival system, in that associated adaptor proteins are required (TRAFs) to transmit signalling through to c-jun N-terminal kinases (JNK) and the IKK complex (43,44). These pathways lead to AP-1 and NF-κB activation and transcription of anti-apoptotic signalling and cell survival. However, abnormal TNFr2 activity has been identified in autoimmune disorders and autoreactive T cells (44), where signalling pathways are shifted from proliferation and survival to cell death. It is hypothesized that interruption to this crosstalk between receptor results in signalling preferentially using the TRADD/FADD pathway of cell death.

Tumour necrosis factor-α and periodontitis

In the human body, TNFα is responsible for mediating inflammatory responses and maintaining immune system activity, in addition to causing apoptosis of specific tumour cell populations (45,46). Conversely, when there is sustained production of TNFα, a variety of unwanted pathological responses can occur. Studies evaluating chronic periodontitis in humans and experimental animal models have demonstrated significantly higher levels of TNFα in periodontal gingival tissue and crevicular fluid (47–50). These higher levels of TNFα are commonly associated with increases in large multinucleated osteoclasts and a reduction in alveolar bone volume and resulting tooth support (51,52). The evaluation of TNFα receptors in periodontitis has demonstrated abundant receptor expression (both TNFr1 and TNFr2) by a variety of infiltrating cell types, including pre-osteoclastic monocytes/macrophages, in addition to fibroblast-like cells, and endothelial cells (47). With increased production of both TNFα and its corresponding receptors in the inflamed gingiva, it is evident that this potent proinflammatory cytokine is associated with promoting the tissue destruction seen in this disease. However, its direct effect on skeletal cells is still an area requiring further investigation.

Several studies have demonstrated the beneficial outcome of reducing inflammatory cell infiltrate and cytokine expression on alveolar bone volume in periodontal animal models (53,54). Specifically, reductions in TNFα were associated with, and reported to be responsible for decreased osteoclast formation and bone resorption in a ligature-induced periodontitis rat model treated with an anti-inflammatory and anti-oxidative compound, epigallocatechin-3-gallate (54). However, the use of common therapeutics, which reduce inflammation in humans, such as non-steroidal anti-inflammatories, have produced variable results on periodontal parameters, as reviewed by Salvi and Lang (55).

In addition to their broad molecular suppression, they produce undesirable gastrointestinal and renal effects (56,57). Conversely, although to the best of our knowledge no studies assess anti-TNFα therapy as a specific treatment for periodontitis in humans, there are several comparative studies, which assess the effects of TNFα antagonists in rheumatoid arthritis on periodontal disease markers (58–60). These reports indicate that the suppression of TNFα is positively correlated with decreased markers of periodontitis, such as probing index and depth, gingival index and bleeding, and attachment loss. In addition, several animal models analysing the effects of anti-TNFα therapy in periodontitis have demonstrated favourable results. Targeted suppression of TNFα production with pentaxifylline and thalidomide in rats with periodontitis resulted in reduced cellular infiltrate into gingival tissues and inhibited alveolar bone loss by almost 70% in a dose-dependent nature (61). Etanercept, an FDA-approved anti-TNFα agent for rheumatoid arthritis, is also

reported to suppress effectively the development of, and tissue destruction in an animal model of periodontitis (62). In the last decade, however, the use of anti-TNFα therapy has raised serious concerns with incidences of adverse effects. As TNFα is involved in pathogen elimination and the development of granulomas, its therapeutic inhibition may increase the risk of opportunistic infections (63,64). Several studies have revealed increased infections, such as histoplasmosis (65), tuberculosis (66), listeriosis (63) and coccidiomycosis (65), are associated with anti-TNFα therapy. Furthermore, as periodontitis is associated with oral and gingival infections, the use of anti-TNFα treatments should be assessed with caution.

Effects of tumour necrosis factor-α on osteoclasts and bone resorption

Osteoclasts are large multinucleated cells involved in physiological and pathological bone resorption, as reviewed by Cappariello *et al.* (67). The precursor cells arise from the haematopoietic lineage formed within the bone marrow. Responding to osteoclastogenic factors, including a number of key cytokines and chemokines, precursors migrate from the marrow into the blood stream, to the site of intended bone resorption where they differentiate into large multinucleated osteoclasts (17). It is well established that macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) are two key factors for osteoclastic differentiation (68,69). M-CSF stimulation of precursor monocytes enhances cell survival, promotes commitment to the osteoclastic lineage and induces the expression of RANK (68,70). Binding of RANKL to its receptor RANK initiates a series of intracellular pathways promoting the expression and transcription of a number of key osteoclast-specific genes, such as calcitonin receptor, tartrate resistant acid phosphatase (TRAP), cathepsin K, osteoclast-associated immunoglobulin-like receptor and beta 3 integrin (Fig. 1) (71,72).

RANK is a member of the TNF receptor subfamily, and thus holds a variety of similarities to the TNF receptors discussed earlier (TNFr1 and TNFr2). RANK is membrane-bound protein, comprised of an important cytoplasmic motif that requires interactions with adaptor proteins from the TRAF family (namely TRAF 6) to transmit its signal downstream (73,74). Several studies have highlighted the importance of these three factors with knockdowns of M-CSF (or its receptor c-FMS), RANKL or RANK, or TRAF6, all resulting in reduced production or complete lack of bone resorbing osteoclasts (69,75–77). This leads to an increased bone volume and an osteopetrotic phenotype.

Downstream of TRAF interactions with RANK, several distinct pathways are activated, which involve a variety of protein kinases, such as IKK, p38 and JNK (78). The conduction of these signalling cascades through the RANK pathway induce several well-described transcriptive factors including nuclear factor of activated T-cell cytoplasmic 1 (NFATc1), NF-κB and AP-1 (74,78). Experimental genetic mutations of the NF-κB subunits p50 and p52 *in vivo* revealed similar osteopetrotic phenotypes to manipulations of the upstream RANK system (79,80). This holds true for knockouts of c-Fos (81,82), the main component of the AP-1 transcription complex. Conversely, studies investigating the outcome of NFATc1 knockouts have demonstrated embryonic lethality due to its vital role in development (83,84). However, conditional knockouts of NFATc1 in young and adult mice result in significant increases in bone volume compared to wild-type controls because of deficient osteoclast presence and function (85–87).

The role of RANK/RANKL signalling in osteoclasts is well known both *in vivo* and *in vitro*; however, the influence TNFα has on this system remains an area of debate. As discussed above, increased production and exposure to TNFα is associated with bone loss conditions such as periodontitis. A number of studies have attempted to unravel the mechanisms involved in TNFα-mediated bone resorption. TNFα has been demonstrated to promote the recruitment of osteoclasts and osteoclastic precursors to the site of inflammation through several mechanisms (88–90). Zhang *et al.* (88), identified a negative regulation for TNFα on specific chemokines, namely stromal-derived factor-1 (SDF-1), which maintains precursors in the bone marrow, thus increasing the overall pool of potential osteoclasts in circulation during chronic inflammation. TNFα overexpression in transgenic mice or TNFα injection into wild-type rodents suppressed the concentration of SDF-1 in bone marrow (88). SDF-1 is produced by bone marrow-derived cells, and mobilizes osteoclastic precursors and inflammatory cells towards a region of higher concentration. In addition, studies using a rat model of periodontitis, supported by a study of human gingival crevicular fluid, revealed increased concentrations of SDF-1 at the site of periodontal inflammation (91). The suppressive effects of TNFα on SDF-1 production within the marrow may account for the increased osteoclastic cells elicited into the periodontal lesion.

With increased numbers of osteoclast precursors in the peripheral circulation and at the site of periodontal inflammation, TNFα appears to prime and enhance differentiation into bone resorbing osteoclasts via RANK signalling. Like M-CSF, TNFα promotes the expression of RANK on the haematopoietic precursor monocyte/macrophage, increasing the availability of receptor activation via RANKL (92). In addition, this cytokine promotes RANKL expression in the inflamed region by gingival fibroblast and epithelial cells, T cells and osteoblasts (93,94). Overall, this cytokine has the capacity to produce an environment rich in osteoclastic growth and activity factors, in addition to promoting the migration of their precursors to the site of bone destruction.

TNFα has also been shown to target osteoclastic differentiation directly through mechanisms independent of RANK signalling (Table 1). Ini-

Table 1. Effects of TNFα on bone cells: osteoclasts, osteoblasts and osteocytes

	Cell source	Assay design	Effect (stimulation, inhibition or no change from control)	Effect on cells (cell number, size and activity)	Mechanism of effect	References
Osteoclasts	Mouse BMM	RANKL (20 ng/mL) for 4 d followed by TNFα (10 ng/mL) alone for 1 d	Stimulation	Cell number and size	Enhanced TRAF2-MEKK1 signalling	(105)
	Mouse BMM	a) TNFα (1–10 ng/mL) with RANKL (50–100 ng/mL) for 4 d b) RANKL (50–100 ng/mL) for 2 d followed by TNFα (5–1 ng/mL) for 2 d	a) Stimulation b) Inhibition	a) Cell number b) Cell number	a) TNFα (early) dose-dependent increase in number of TRAP+ cells b) TNFα (late) dose-dependent decrease in number of TRAP+ cells	(173)
	Mouse BMM	a) TNFα (20 ng/mL) alone for 3 d b) TNFα (20 ng/mL) with IL-1α (10 ng/mL) for 3 d	a) Inhibition b) Stimulation	a) No change on cell number or size. Inhibition of activity b) No change in cell number, size or activity	a) Dose-dependent increase in formation through TNFr1 & TNFr2. Suppression of resorption pits in absence of RANKL. b) Bypassing RANK activation with TNFα & IL-1α to induce active osteoclasts	(95)
	Mouse BMM	After 3 d whole marrow culture, isolated macrophages treated with TNFα (0.1–1000 ng/mL) alone, for 5 d	Stimulation	Cell number	Dose-dependent induction of formation without RANKL. However, formation was higher with equal RANKL concentration	(96)
	Mouse BMM	a) TNFα (1–160 ng/mL) alone 5–9 d b) 3 d whole marrow culture before macrophage isolation, followed by TNFα (100 ng/mL) 5–9 d c) Suboptimal RANKL (1 ng/mL) priming followed by TNFα (500 pg/mL)	a) Inhibition b) No change c) Stimulation	Cell number	a) No osteoclasts formed with TNFα alone b) Formation of osteoclasts formed with TNFα alone; however, this was suppressed with addition of OPG during 3 d whole marrow culture c) Synergistic increase with TNFα 2 d after RANKL priming through the induction of SAPK/JNK and NF-κB	(97)
	Mouse stromal derived macrophages	a) TNFα (10 ng/mL) alone for 4 d b) TNFα (10 ng/mL) with RANKL (50–100 ng/mL) for 4 d	a) Stimulation b) Stimulation	a) Cell number (mononuclear) b) Cell number (multinucleated)	Activity through TNFr1 & TNFr2	(106)
	Mouse RAW264.7 macrophages	a) TNFα (50 ng/mL) alone for 3 d b) TNFα (50 ng/mL) with RANKL (20 ng/mL) for 3 d	a) Inhibition b) Stimulation	a) Cell number b) Stimulation of cell number	a) No osteoclasts formed with TNFα alone b) TNFα and RANKL (20 ng/mL) formed more cells, although smaller, than RANKL (100 ng/mL) alone	(174)

Table 1. (continued)

	Cell source	Assay design	Effect (stimulation, inhibition or no change from control)	Effect on cells (cell number, size and activity)	Mechanism of effect	References
	Mouse RAW264.7 macrophages	24 h of RANKL followed by 3 d of TNFα (10 ng/mL)	Stimulation	Cell number	TNFα with RANKL (25 ng/mL) priming increased the number of osteoclast through a TNFα-induced double-stranded PKR expression increase. PKR inhibitor 2AP prevented PKR induction from TNFα	(175)
	Rat BMM	a) TNFα (4–20 ng/mL) alone for 3 d b) TNFα (20 ng/m) for 3 d followed by RANKL (30 ng/mL) for days 1 d	a) Stimulation b) Stimulation	a) Cell number (mononuclear) b) Cell number (multinucleated)	a) TNFα alone stimulated differentiation of pre-osteoclast (mononuclear TRAP+ cells) b) Addition of RANKL stimulated multinucleation	(176)
	Rat BMM	a) TNFα (10 ng/mL) with rat osteoblastic heat-treated conditioned media b) TNFα (10 ng/mL) and RANKL (10 ng/mL) for 4 d	a) Stimulation b) Stimulation	a) Cell number and activity b) Cell number and activity	a) TNFα with osteoblast conditioned media increased osteoclast formation and resorption b) TNFα and RANKL synergistically increased formation and resorption area of osteoclasts	(177)
	Human CD11+ cells	TNFα (0.01–1 ng/mL) with RANKL (0.1–1 ng/mL) for 7 d	Stimulation	Cell number	Synergistic dose-dependent increase in formation in multinucleated osteoclasts	(98)
	Human PBMC	14 d osteoclast culture with TNFα (0.1–10 ng/mL) added from a variety of time points (on day 1, 4, 7 or 10)	Stimulation	Cell activity	Dose-dependent synergistic increase in resorption with RANKL (5–30 ng/mL). Timing of TNFα essential. TNFα day 1 alone generated the greatest resorption. TNFα required IL-1β (10 ng/mL) to induce osteoclast function	(99)
Osteoblasts	Mouse myoblast C2C12 cells	3 d osteoblast differentiation in the presence of TNFα (10–100 pg/mL)	Inhibition	Cell number	Dose-dependent reduction of ALP expression and inhibition of Runx2 and Smad 1, 5, 8 key transcription regulators	(178)
	Mouse BMSC & MC3T3 osteoblast cells	TNFα (0.1–20 ng/mL) on day 1 or on day 7 of 14 d osteoblast differentiation assay	Inhibition	Cell number and activity	Dose-dependent inhibition of ALP expression and matrix deposition. Greatest suppression with day 1 TNFα (95%) vs. day 7 (60%). TNFα inhibits Runx2 DNA binding activity and transcription through TNFr1	(179)
	Mouse MC3T3-E1 cells	28 d osteoblast differentiation in the presence of TNFα (1 ng/mL)	Inhibition	Cell number and activity	TNFα suppressed ALP expression and calcium deposits through suppression of Runx2 & osterix transcription factors	(180)

Table 1. (continued)

Cell source	Assay design	Effect (stimulation, inhibition or no change from control)	Effect on cells (cell number, size and activity)	Mechanism of effect	References
Mouse MC3T3-E1 cells	24 h osteoblast culture in the presence of TNFα (20 ng/mL)	Inhibition	Cell number	Up to 6 h of TNFα upregulated caspase -1, -7, -11 and -12, FAS and FAP mRNA and related TNF-induced apoptotic factors. TUNEL staining was also significantly upregulated with TNFα treatment	(160)
a) Rat BMSC b) Mouse MC3T3-E1	a) 21 d osteoblast differentiation in the presence of TNFα (10–100 ng/mL) from day 2, 7 or 14 b) 16 d osteoblast differentiation with TNFα (0–10 ng/mL)	a) Inhibition b) Inhibition	a) Cell number and activity b) Cell number and activity	a) Dose-dependent TNFα from day 2 or 7 suppressed confluence and mineralization. OCN mRNA was also suppressed. Commencing TNFα from day 14 had no effect b) Dose-dependent suppression of confluence and mineralization	(123)
Mouse BMSC ($TNFr1^{-/-}$, $TNFr2^{-/-}$ and wild type)	14 d osteoblast differentiation in the presence of TNFα (10 ng/mL)	Inhibition	Cell number and activity	TNFα completely inhibited differentiation and mineralization through TNFr1 signalling as $TNFr1^{-/-}$ cells were completely unresponsive to TNFα treatment compared with $TNFr2^{-/-}$ cells	(181)
Mouse ST2 cells	a) 48 h osteoblast differentiation with TNFα (0.01–100 ng/mL) b) 4 wk osteoblast differentiation with TNFα (0–100 ng/mL)	a) Stimulation and Inhibition b) Inhibition	a) Cell activity b) Cell activity	a) Low-dose TNFα (0.01 and 0.1 ng/mL) upregulated ALP, Runx2, OCN and osterix. High-dose TNFα (1–100 ng/mL) dependent suppression of ALP, Runx2, OCN and osterix b) Dose-dependent TNFα suppression of mineralization	(138)
Mouse C2C12 cells	24 h of osteoblast culture with TNFα (10 ng/mL)	Inhibition	Cell number and activity	TNFα almost completely suppressed ALP activity and mRNA expression through induction of NF-κB and upregulation of Msx2 expression, in addition to Smurf1 induction and degradation of Runx2	(143)
Mouse BMSC (TNFα-Tg)	TNFα-Tg mice develop arthritis at 2–3 mo old, and osteoporosis as 4 mo	Inhibition	Cell number and activity	TNFα-Tg mice – less stromal cell colonies than wild-type and decreased ALP and OCN gene expression and ALP activity in isolated BMCS	(144)

Table 1. (continued)

Cell source	Assay design	Effect (stimulation, inhibition or no change from control)	Effect on cells (cell number, size and activity)	Mechanism of effect	References
Mouse PT67 cells	4 wk osteoblast culture with TNFα (0–100 ng/mL)	Inhibition	Cell number and activity	Dose-dependent decrease in osteogensis and proliferation, mineral nodule formation, and Runx2, osterix, ALP and OCN expression	(138)
Rat BMSC	16 d osteoblast differentiation on biodegradable polymer scaffolds with TNFα (50 ng/mL); continuous (16 d); early (days 0–4); intermediate (days 4–8); late (days 8–12)	Stimulation and inhibition	Cell number and activity	TNFα (50 ng/mL) suppressed ALP activity. Cell proliferation was decreased with continuous and early TNFα at days 4 and 8. Intermediate and late TNFα increased cellularity at day 16. Mineralization was increased in all TNFα time courses	(182)
Human BMSC	14 d osteoblast differentiation in the presence of TNFα (1–10 ng/mL)	Stimulation	Cell activity	Dose-dependent stimulation of calcium deposits through increased TNAP activity	(141)
Human BMSC	14 d osteoblast differentiation with TNFα (0.1–10 ng/mL) and/or IL-1β (0.1–1 ng/mL) added from day 2	Stimulation and inhibition	Cell activity	Dose-dependent TNFα and/or IL-1β increased ALP activity and calcium deposits through increase in TNAP activity. However, Runx2 mRNA and OCN secretion were suppressed by both TNFα and IL-1β	(183)
Human BMSC	21 d osteoblast differentiation with TNFα (20 ng/mL)	Stimulation	Cell activity	TNFα increases calcium and phosphate deposition through induction of BMP2 and NF-κB	(184)
Human DPSC	14 d osteoblast differentiation in the presence of TNFα (50 ng/mL)	Inhibition	Cell activity	Dose-dependent decrease in calcium deposits, ALP, OPN, OCN, osterix and Runx2 expression	(134)
Human PDLSC	14 d osteoblast differentiation in the presence of TNFα (0, 2.5, 5 or 10 ng/mL)	Stimulation	Cell number and activity	Low-dose (2.5–5 ng/mL) TNFα increased osteogenesis and calcium deposits. No change observed with 10 ng/mL TNFα	(137)
Human DPSC	14 d osteoblast differentiation in the presence of TNFα (0–100 ng/mL)	Stimulation and inhibition	Cell activity	Dose-dependent decrease in osteogenesis, calcium deposits, ALP and BMP expression	(135)
Human DPSC	14 d osteoblast differentiation in the presence of TNFα (10 ng/mL)	Stimulation	Cell activity	TNFα-stimulated osteogenesis, calcium deposits, ALP, BMP and Runx2 expression. No change in proliferation observed	(136)
Human PDLSC	14 d osteoblast differentiation in the presence of TNFα (10 ng/mL)	Inhibition	Cell activity	TNFα suppressed osteogenesis, calcium deposits, ALP and Runx2 expression.	(139)

Table 1. (continued)

	Cell source	Assay design	Effect (stimulation, inhibition or no change from control)	Effect on cells (cell number, size and activity)	Mechanism of effect	References
	Human PDLSC	21 d osteoblast differentiation in the presence of LPS-stimulated monocyte media with endogenous TNFα or TNFα (10 ng/mL)	Inhibition	Cell number and activity	LPS stimulated monocyte TNFα-positive media suppressed osteogenesis and biomineralization. TNFα (10 ng/mL) suppressed osteogenesis, calcum deposits, ALP, Runx2, OCN and osterix expression.	(140)
Osteocytes	Mouse MLO-Y4 cells	Cell cultured for 6 h with TNFα (10 ng/mL)	Inhibition	Cell number	TNFα suppressed osteocyte survival through TNFα-related apoptosis. Apoptotic media promotes osteoclast adhesion to endothelial cells through high endothelial ICAM-1 expression	(159)
	Mouse MLO-Y4 cells	Cells were cultured with TNFα (10 ng/mL) for 2 h up to 5 d	Inhibition	Cell number	TNFα increased sclerostin expression through NF-κB binding to SOST gene, which was most significant with TNFα for 2 h and 5 d	(154)
	Mouse MLO-Y4 cells	Cells cultured for 30 min or 24 h with TNFα (0.5–30 ng/mL) and/or IL-1β (0.1–10 ng/mL)	Inhibition	Cell number	TNFα and IL-1β suppress nitrous oxide production	(185)
	Mouse MLO-Y4 cells	Cells cultured for 6 h with TNFα (0–20 ng/mL)	Inhibition	Cell number	TNFα dose-dependent increase in osteocyte apoptosis	(186)

ALP, alkaline phosphatase; BMM, bone marrow macrophage; BMP2, bone morphogenic protein 2; BMSC, bone marrow stromal cell; DPSC, dental pulp stem cells; LPS, lipopolysaccharide; OCN, osteocalcin; OPG, osteoprotegerin; PDLSC, periodontal ligament stem cells; PKR, RNA-dependent protein kinase; RANKL, receptor activator of nuclear factor B ligand; TNAP, tissue-nonspecific alkaline phosphatase; TNFα, tumor necrosis factor.

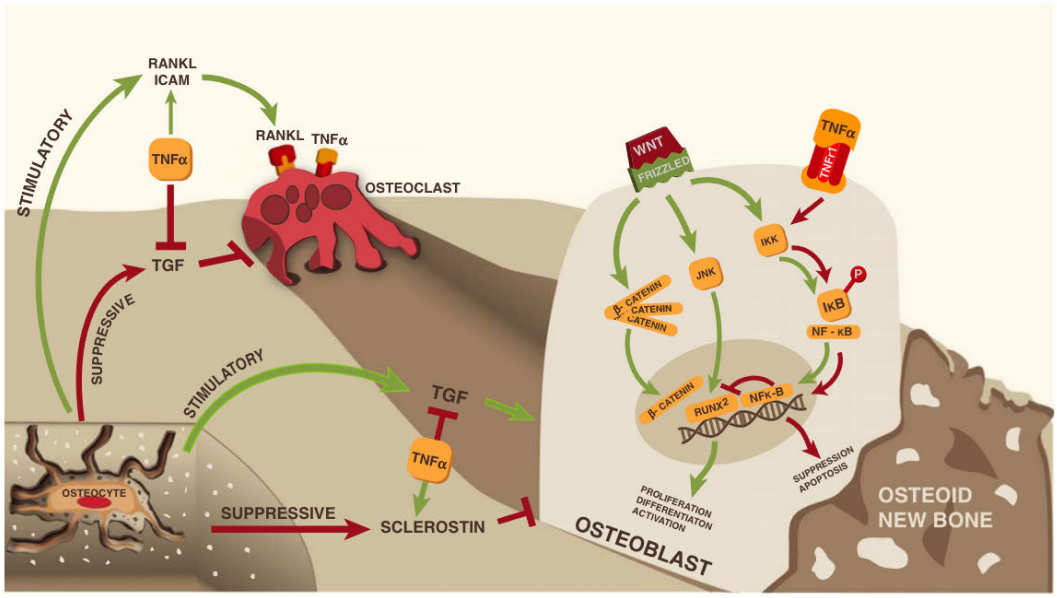

Fig. 2. Proposed influence of TNFα on the overall bone microenvironment. TNFα binding to TNFr1 on the osteoblast membrane interferes with WNT signalling via the induction of NF-κB related suppression of RUNx2 transcription, leading to cell death. TNFα influences osteocytic regulation of bone cells through the promotion of factors, which stimulate osteoclasts (ICAM, RANKL) and suppress osteoblasts (sclerostin). ICAM, intracellular adhesion molecule; NF-κB, nuclear factor kappa-light chain enhancer of activated B cells; RANKL, receptor activator of nuclear factor B ligand; TNFα, tumour necrosis factor alpha; TNFr1, tumour necrosis factor receptor 1.

tial studies, using whole marrow or co-cultures with stromal cells and osteoclast precursors, showed that TNFα resulted in increased numbers of multinucleated cells expressing TRAP, an enzymatic marker of cells devoted to the osteoclast lineage (95,96). Although these experiments observed osteoclastogenesis *in vitro* with the addition of only TNFα and M-CSF as growth factors, there was no evidence disputing endogenous RANKL production by co-cultured stromal cells as the osteoclastic stimulant. Interestingly, the addition of osteoprotegerin (OPG; a natural soluble decoy receptor of RANKL) in supplemental studies did not suppress TRAP-positive cell formation (95,96). This observation suggests a direct effect of TNFα on the differentiation of osteoclasts. In line with this, other studies have demonstrated a concentration-dependent increase in TRAP forming cells induced by TNFα in mouse bone marrow cultures (96), which was independent of RANKL stimulation *in vitro*. However, supplementary studies analysing the activity of TNFα-mediated differentiation have produced conflicting results (summarized in Table 1).

Although some studies report observing osteoclastic resorption in response to TNFα alone (96), other studies were unable to identify any positive osteoclastic effect (97), concluding that additional factors may be required to promote their formation and activity *in vitro*. TNFα coinciding with RANKL during osteoclastogenesis *in vitro* synergistically promotes increased osteoclast size and number (97,98). Even with RANKL concentrations too low to produce reportable quantities of osteoclasts (0.1–1 ng/mL), TNFα treatment enabled significant increases in the numbers of osteoclasts formed. Additional proinflammatory cytokines present during periodontal inflammation and bone loss, such as IL-1 (99,100), are shown to enhance the stimulatory effects of TNFα on osteoclastogenesis *in vitro* in the absence of RANKL (95,96,101). Upon ligation of IL-1 with its receptor (IL-1R), TRAF 6 actively propagates its signalling downstream to NFATc1 and NF-κB (102–104). It is proposed that these cytokines bypass the need for RANK/RANKL interactions, allowing cross-talk between immune receptor pathways to induce transcription of osteoclast-related genes. These findings demonstrate the idea of an inflammatory osteoclast capable of resorbing bone while avoiding inhibitory factors such as OPG, which are dependent on RANKL being the activating molecule (95,99). Further evidence for a stimulatory role of TNFα in osteoclasts, has been demonstrated in TNFr1 knockouts or TNFα antagonist studies (Table 1), which result in reduced osteoclast formation (98,105,106).

It is evident from these studies that TNFα acts on osteoclasts during the proliferation and differentiation phases, stimulating their ability to

respond to RANKL. This is likely to occur via TRAF 1, 2 and 3, and the activation of NF-κB and AP-1 resulting in the formation, differentiation and survival of osteoclastic cells. In contrast, the RANKL/IL-1/TRAF 6 pathway is necessary for the activation and hence resorption stages of osteoclasts (95,97). These findings support a need for treatments that target both the inflammation along with bone resorption.

Effects of tumour necrosis factor-α on osteoblasts and bone formation

Osteoblasts play a critical role in the bone remodelling process by forming and depositing new bone material. In pathological conditions such as periodontitis, there is an imbalance in the resorption and formation processes resulting in a net bone loss. This imbalance is due to not only excessive osteoclast bone resorption but also the suppression of osteoblast differentiation and activity (107).

The role of osteoblasts changes throughout their life cycle and depends on a number of factors, such as skeletal development, hormones and inflammatory mediators that influence cell differentiation and function (108). Precursors originate from pluripotent stem cells (SC) derived from the neural crest during embryonic development (109). Their differentiation into various cell types, including skeletal, adipose and connective tissue cells, is essential for the development of vertebrates (109,110). Furthermore, the transient progression of neural crest SCs into the mesenchymal SC lineage within bone marrow is needed for the development of oral structures such as the dental pulp (DP) and periodontal ligament (PDL) (111). These structures are reported to store additional mesenchymal SCs for continued osteogenic cell differentiation, to repair damaged bone and tissue within the periodontium (112). TNFα has a diverse range of effects on the differentiation of these cells (discussed in detail below). Therefore, it is important to understand the effects associated with differentiation to the bone cell lineage, as these SCs are widely investigated as regenerative cell based oral therapies (113,114).

Osteoblastic commitment requires activation of the Wnt (wingless-type MMTV integration site) signalling pathway within the progenitor SC in addition, to a variety of other intracellular and secreted mediators (115,116). Activation of the Wnt pathway results from the ligation of a Wnt protein (Wnt2a, Wnt5a or Wnt10b) to its receptor, Frizzled, on the cell surface (108) (Fig. 2). In turn, a series of intracellular pathways leads to the stabilization of β-catenin and the promotion of differentiation (117). Osteoblast formation and activity is achieved through increased intracellular calcium levels via the activation of G-proteins, JNKs and the activation of transcription factors, Runt-related transcription factor 2 (Runx2), NFAT and NF-κB (116,117).

Numerous studies investigating the effects of TNFα on osteoblastogenesis have revealed suppression of bone formation (see Table 1). This has been observed with both direct and indirect actions of TNFα disrupting osteoblast formation at various stages of their differentiation. At the early stages of SC division, paracrine factors are required to promote the cells' ability to grow and differentiate into pre-osteoblasts (118,119). Essential factors supporting this transition are insulin growth factor-I (IGF-I) and bone morphogenic proteins (BMPs; mainly -2, -4 and -6) (120–122). TNFα has been demonstrated to suppress osteoblast differentiation from isolated fetal calvaria precursors via reducing the expression of IGF-1 (123,124). However, subsequent assays that involved treating cells with additional IGF-1 were not successful in reversing the suppressive effects of TNFα (124), suggesting a more specific target downstream of IGF-1 activity.

Further analyses into the suppressive actions of TNFα on osteoblastogenesis have revealed additional targets where TNFα interferes with protein signalling and gene expression. Factors that regulate the synthesis of bone material are essential for functional osteoblasts to form (125). However, several experimental models have demonstrated that type 1 collagen (COL1a1) and osteocalcin, both skeletal matrix proteins, are affected by TNFα (126,127). The mechanism behind decreased collagen production was identified by Mori *et al.* (128) in a study using fibroblasts, in which the COL1a1 gene promoter was shown to be suppressed in response to TNFα, abrogating transcription factor binding. In contrast, another study showed osteocalcin production was suppressed via a developed resistance and decreased receptor expression to vitamin D *in vitro* (129,130). Vitamin D receptor activation on osteoblastic cells appears to be required for the transcription and production of osteocalcin (131).

In addition to bone formation, TNFα has been reported to affect factors associated with the formation and survival of osteoblasts through negative regulation of critical transcription factors, such as Runx2 and osterix (123,124). These factors are required for developing and maintaining the skeletal system, through the transcription of a variety of osteoblast-specific genes, including osteocalcin, COL1A, OPG, alkaline phosphatase (ALP) and osteopontin (Fig. 2). Transgenic knockout mouse studies with deletions of either Runx2 or osterix demonstrate their critical role, as mice develop with a cartilage-rich skeleton (132,133) due to a deficiency in osteoblast differentiation and an inability to form mineralized bone.

Furthermore, studies on the effect of TNFα on osteoblastogenesis, specifically from isolated dental SCs, derived from DP (DPSCs) or the PDL (PDLSCs) have reported conflicting findings (134–138). These differences appear to be related to the concentrations of TNFα used *in vitro*. Concentrations of TNFα above 10 ng/mL have been reported to decrease osteogenic differentiation, downregulate BMPs and ALP activity, and reduce mineralization (134,135,137,138). Conversely, lower concentrations of TNFα, ranging from 0.01 to 10 ng/

mL, lead to characteristics that are more osteogenic (135,136,138). Xing *et al.* (135) reported increased extracellular calcification and Runx2 protein expression with low concentrations of TNFα-treated DPSCs. In addition, an earlier study showed NF-κB activation by low levels of TNFα enhanced osteogenic differentiation (134). However, these findings are not fully supported by two recent studies using PDLSCs treated with low (10 ng/mL) concentrations of TNFα, which resulted in decreased osteogenesis, ALP activity, Runx2, osteocalcin and osterix expression, and calcium deposits (139,140).

Although a large number of studies demonstrated suppression of bone formation by osteoblasts exposed to TNFα, there is further evidence to support the contrary. Interestingly, Briolay *et al.* (141), demonstrated that TNFα promoted calcium deposits associated with increases in Wnt5a and Wnt10b expression in human mesenchymal SC cultures. Conversely, decreases in the master transcription factor Runx2 were also observed in this study, indicating delayed or suppressed proliferation. Additional studies assessing TNFα effects on human mesenchymal cell differentiation have observed a dose-dependent increase in osteogenic differentiation over a 3 d period (138). These positive results could be related to an acute cytokine exposure, promoting the activity of tissue non-specific ALP and the synthesis of calcium, along with the rapid induction of NF-κB and expression of anti-apoptotic signals. However, TNFα is also reported to suppress collagen matrix production by early osteoblasts (142), in addition to preventing their differentiation (143,144). This may indicate that the observed increase in intracellular calcium and early proliferation *in vitro* may not be translatable to the pathological suppression of completed mineralized bone formation *in vivo*.

Osteoblasts are also important regulatory cells, which have the capacity to orchestrate osteoclastic bone resorption. This is achieved by producing pro-osteoclastic signalling molecules such as M-CSF, RANKL and interleukins (145–147). Interestingly, TNFα increases the expression of these genes, in addition to promoting the production of proteolytic enzymes such as matrix metalloproteinases (147,148). Matrix metalloproteinases have been implicated in the induction of osteoclast differentiation and attachment to bone surface (149). This increase in osteoclastic promoting factors is observed occurring simultaneously with the suppression of the pro-osteoblastic factors mentioned above, indicating an essential role for TNFα in periodontal and inflammatory bone loss.

Overall, these results demonstrate that TNFα negatively influences the activity of osteoblasts *in vitro* and *in vivo*. However, the timing and length of exposure appear to drastically impact the resulting outcome, thus these factors should be carefully considered, particularly *in vitro*, during the interpretation of results. Further analyses are needed to understand fully the mechanisms behind TNFα regulation of osteoblastogenesis at different stages of the cell differentiation. This will aid in developing effective therapies, targeting specific pathways or factors depending on disease state and bone quality.

Effects of tumour necrosis factor-α on osteocytes and their regulation of the skeletal system

Osteocytes are regarded as terminally differentiated osteoblasts that have reached the end of their differentiation pathway and are embedded within the bone microstructure. They reside in microscopic cavities (lacunae), encased in a stationary position within bone with cytoplasmic projections that travel through networks or canaliculi to resident bone cells (150,151). This enables a complex system of cell contact, communication and regulation. After developing their characteristic phenotype within the osteoid, they begin to produce factors that have varied effects on adjacent cell types, in particular osteoclasts and osteoblasts. These factors include RANKL, intracellular adhesion molecule 1 (ICAM-1), transforming growth factor β1 (TGF-β1) and sclerostin (Fig. 2) (152). The expression and release of these factors can influence the rate of bone resorption or formation on the surface. Interestingly, the actions and outcomes of osteocytic activity, although not fully understood, appear to change in response to different stimuli, such as mechanical stress, microfracture, or immune and inflammatory mediators such as TNFα (153,154). Studies investigating the direct actions of TNFα on osteocytes *in vitro* are described in Table 1.

Osteocytes can promote osteoclastogenesis and osteoclast activity through a variety of mechanisms. For example, the death or targeted ablation of osteocytes results in severe bone loss (155). This is reported to be a result of increased RANKL expression by the dying osteocytes resulting in the development of osteoclastic cells at the site of bone loss. Additional studies identified apoptotic bodies released from dying osteocytes as the primary source of the increased RANKL concentrations (153,156). In fact, the expression of RANKL by apoptotic osteocytes is reported to surpass levels produced by resident osteoblasts, as targeted mutations of osteoblasts within mice had no effect on overall RANKL expression (157,158). Interestingly, several studies have shown that osteocytic apoptosis and RANKL production is induced by TNFα *in vitro* (summarized in Table 1). Furthermore, TNFα also induces apoptotic osteocytes to express factors that promote osteoclast precursor adhesion to endothelial cells, such as ICAM-1 (159). This was observed using osteoclastic precursor RAW264.7 cell line and media from TNFα-induced apoptotic MLO-Y4 osteocytes (159). Taken together, these findings suggest the essential role for osteocyte regulation of osteoclasts is highly influenced by inflammation and TNFα exposure.

Osteocytes have also been shown to influence the activity of osteoblasts through several means. For example, TGF-β1, which is synthesized by a variety of cell types, including

osteocytes, enhances proliferation of pre-osteoblasts, suppresses apoptotic signalling within osteoblasts and promotes their survival (160,161). In addition, TGF-β1 has been reported to promote osteoblast migration to the bone surface for the synthesis of bone matrix proteins (161,162). Interestingly, TGF-β1 has also been implicated in the suppression of osteoclastic bone resorption *in vitro*, indicating an important anabolic role for osteocytes in physiological bone growth and repair (163).

Osteocytes also produce sclerostin, an essential glycoprotein that orchestrates the activity of osteoblasts (164). Physiologically, sclerostin levels appear to alter in response to mechanical loading with heavy loading or concentrated strain leading to the suppression of sclerostin levels (165). Decreased sclerostin enables osteoblastic function during stages of osteogenesis, where cell activity is required for skeletal growth (166). Conversely, a reduction in mechanical stress, such as being sedentary for long periods, results in an increased production of sclerostin and the subsequent apoptosis of osteoblasts, preventing the formation of bone (165). Although the function of sclerostin suppression of osteoblasts is not entirely understood, it is thought to interfere with the Wnt signalling and stabilization of β-catenin (167,168).

In pathological conditions, sclerostin expression is altered in response to inflammatory molecules such as TNFα. Recently, a study using a rat model of ligature-induced periodontitis revealed significant increases in sclerostin expression in osteocytes during the early and destructive phase of the disease (169,170). Interestingly, the rise in sclerostin levels was associated with a similar increase in RANKL production. However, expression levels normalized during the recovery phase of the disease, suggesting that alveolar bone loss may be an outcome of osteocytic suppression of osteoblasts and promotion of osteoclasts during the active phase of periodontitis. In addition, reports identifying a role for sclerostin in the promotion of osteoclastic bone resorption (166,171) are supported by studies that observed an increase in RANKL expression and decrease in OPG mRNA in the MLO-Y4 osteocyte cell line in response to sclerostin (172). Further evidence is demonstrated using a number of *in vivo* and *in vitro* models, where TNFα alone is found to induce directly the expression and increased synthesis of sclerostin in osteocytes, through the activation of TNFα-mediated NF-κB binding to the sclerostin gene (SOST) promoter region (154). In addition, the administration of TNFα antagonists prevents this stimulation, further supporting this important relationship in inflammatory bone loss.

Conclusion

TNFα regulation of bone remodelling is complex with a large number of studies to date generally demonstrating an overall increase in bone resorption and hence a net bone loss. Numerous studies have shown that TNFα stimulates osteoclast proliferation, migration and formation in addition to synergistic increases in activity when acting concurrently with other factors such as RANKL and IL-1. TNFα is shown to suppress directly osteoblast activity, through the inhibition of osteoblast formation and differentiation, ultimately leading to inadequate or reduction levels of bone formation. Finally, there is evidence demonstrating TNFα stimulates osteocytic regulation of bone metabolism towards more bone resorption as observed via the increase in pro-osteoclast factors ICAM-1 and RANKL, decreases in pro-osteoblast and anti-osteoclast factor TGF-β1 and sclerostin. Treatments, which not only manage the effects of TNFα on the skeletal system, but also normalize skeletal cells activity may greatly benefit the prognosis of periodontal treatment and other related bone loss conditions.

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Appendix II: Published Manuscripts as PDFs Arising From Outside This Thesis

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ORIGINAL ARTICLE

Osteoclast-Associated Receptor (OSCAR) Distribution in the Synovial Tissues of Patients with Active RA and TNF-α and RANKL Regulation of Expression by Osteoclasts *In Vitro*

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Abstract—Osteoclast-associated receptor (OSCAR) is a co-stimulatory receptor in osteoclastogenesis. Synovial tissues from active rheumatoid arthritis (RA) patients express higher levels of OSCAR compared with osteoarthritic and normal patients; however, the comparison of OSCAR levels in different regions of active RA synovium has not been reported. The regulation of OSCAR by TNF-α and receptor activator of NF kappa β ligand (RANKL) in pre-osteoclasts/osteoclasts *in vitro* is unclear. OSCAR and tartrate-resistant acid phosphatase (TRAP) expression levels did not differ between the cartilage pannus junction (CPJ) and non-CPJ regions in active RA. We demonstrate a similar pattern of OSCAR expression in the CPJ and non-CPJ synovial tissue from patients with active RA. OSCAR was associated with mononuclear cells in both the lining and sub-lining and endothelial cells (von Willebrand factor positive). Pre-osteoclasts (TRAP-positive cells) were present in the lining and sub-lining of both regions. OSCAR messenger RNA (mRNA) expression and release by pre-oscteoclasts/osteoclasts was modulated by RANKL with/without TNF-α *in vitro*. Osteoclast resorption on dentine slices was significantly greater with TNF-α pre-treatment and RANKL (10 ng/ml) than RANKL 10 or 50 ng/ml alone or RANKL 10 ng/ml with TNF-α given from day 3 post-RANKL. The lower levels of OSCAR mRNA expression corresponded with high osteoclast activity levels.

KEY WORDS: rheumatoid arthritis; osteoclast; cartilage pannus junction; OSCAR.AASSK Dharmapatni and K. Algate contributed equally to this study.

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disorder affecting synovial joints [1] that exhibits inflammation, pannus formation, angiogenesis and bone loss, leading to joint destruction and dysfunction [2]. Focal bone erosion occurs in the area of contact between the pannus, cartilage and bone [3]. Cells isolated from rheumatoid synovium with phenotypic features of osteoclasts are involved in marginal bone erosion in RA [4–6] and cartilage destruction [6]. This is consistent with the identification of cells expressing the pre-osteoclast/osteoclast marker tartrate-resistant acid phosphatase (TRAP) *in vivo* [6, 7]. A limited number of reports

compare the histopathology, pre-osteoclast distribution or osteolytic factors between different areas within the same RA joint.

The increased presence and activity of osteoclasts in RA is attributed to an increased ratio of receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) to its decoy receptor, osteoprotegerin (OPG) [8–13]. This ratio is influenced by the presence of inflammatory cytokines such as TNF-α [14, 15]. Osteoclastogenesis is dependent on RANKL binding to its receptor RANK on monocytes/osteoclast precursors culminating in the de-phosphorylation and translocation to the nucleus of the transcription factor nuclear factor of activated T cells cytoplasmic 1 (NFATc1) [16, 17] in order to induce expression of osteoclast genes.

Optimal NFATc1 induction requires an increase in intracellular calcium, which occurs through the activation of a co-stimulatory pathway involving the immunoreceptor tyrosine-based activation motif (ITAM) [18]. ITAM signalling is induced *via* activation of a triggering receptor expressed on myeloid cells 2 (TREM2) [19] pairing with DAP12 or osteoclast-associated receptor (OSCAR) [20] *via* FcRgamma [21]. OSCAR is initially expressed in response to NFATc1 induction following RANKL/RANK signalling [22]. A positive feedback is then induced by the activation of OSCAR upon ligand interaction, followed by the initiation of calcium signalling, crucial to NFATc1 [22]. OSCAR is thus important for continual activity and late stage osteoclastogenesis.

OSCAR is detectable as a soluble (sOSCAR) protein in serum and synovial fluid [23–25]. Controversy exists as to how levels of sOSCAR in active RA influence bone erosion as reports indicate that sOSCAR in the serum of RA patients is increased [24, 25] or decreased [26] compared to that in healthy controls. Interestingly, RA patients with bone erosions showed significantly lower sOSCAR than RA patients without erosions [25]. sOSCAR has also been shown to inversely correlate to the Disease Activity Score 28 (DAS28) in RA [24, 25]. In a conflicting paper, levels in the plasma were found to be higher in erosive RA patients [26]. These findings suggest that a soluble form of OSCAR could act to regulate bone turnover but whether it acts as to prevent or induce bone resorption is not clear.

The ability of OSCAR-Fc to ablate osteoclastogenesis in a co-culture with osteoblasts initially identified the osteoblast as a putative ligand source [20]. Motifs of collagen types I, II and III [27] and oxidated low-density lipid [28] have since been identified as ligands. Degraded collagen fibres activate the OSCAR pathway *in vitro* [27, 29]. This interaction induces monocytes to release pro-inflammatory cytokines, TNF-α and IL-8 [29], and enhances osteoclastogenesis [27]. This is pertinent to diseases such as RA and to the cartilage pannus junction (CPJ) tissue, where degraded collagen is abundantly present. We thus propose that OSCAR expression would be greater in the CPJ of an RA joint compared to that in the synovial tissue region distant from the CPJ.

OSCAR expression in endothelial cells has been proposed to be involved with cell adhesion and activation [30]. *In vivo*, endothelial OSCAR expression is increased in active RA in comparison to OA and healthy synovial samples [23]. This could result in increased monocyte extravasation into affected joints, resulting in increased cells available for osteoclastogenesis. This is consistent with induction of membrane-bound and sOSCAR expression in endothelial cells *in vitro* by TNF-α [23]. Although monocytes have been shown to express OSCAR in response to TNF-α [24], the release of sOSCAR and pattern of expression in response to TNF-α in conjunction with RANKL have not been investigated.

Studies on OSCAR expression in synovial tissues have included patients with inactive RA, osteoarthritis [23] and active RA [23, 24] compared to healthy controls. The current study examines tissue expression of OSCAR and the pre-osteoclast marker TRAP to identify osteoclastic cells [7] in the CPJ and non-CPJ regions from the same patients with active RA. Serial sections were stained for von Willebrand factor (vWF) to identify endothelial cells. The presence of TNF-α was also verified. Regulation of sOSCAR and OSCAR gene expression by RANKL and TNF-α was examined *in vitro* in human peripheral blood mononuclear cells (PBMC)-derived osteoclast cultures. These analyses were designed to help us to better understand RA pathogenesis and possibly implicate OSCAR as a local indicator of bone erosion and marker for response to treatment.

Results

OSCAR and TRAP Expression in the Non-CPJ and CPJ Region of the Synovial Tissue from Joints with Active RA

Vessels were identified in serial sections by vWB staining (Fig. 1a, e). There was no significant difference in the expression of TRAP-positive cells between the lining ($p = 0.146$) or sub-lining ($p = 0.513$, Fig. 1b, f) in the synovial tissue of non-CPJ or CPJ regions from the same patient. A moderate and significant correlation ($r = 0.602$, $p = 0.023$) was observed in TRAP-positive cells between the lining and sub-lining regions of the non-CPJ tissues.

OSCAR-positive cells were present in the synovial lining cells of the majority of the samples and distributed sparsely in association with macrophages in the sub-lining

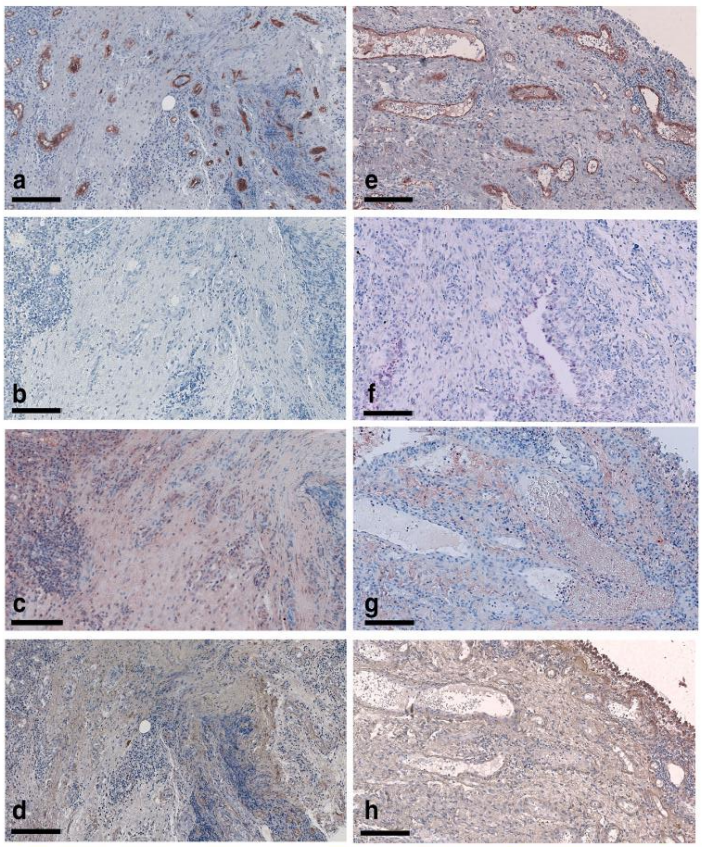

Fig. 1. Comparison of factor VIII, TRAP, OSCAR and TNF-α between CPJ and non-CPJ regions. Factor VIII **a**, **e** (Dako clone F8/86), TRAP (naphthol-based method) **b**, **f**, OSCAR **c**, **g** (S-16 [sc-34233], Santa Cruz Biotech Inc.) and TNF-α **d**, **h** (LS-B2123, LifeSpan Biosciences. Inc., Seattle, WA, USA) positive cells (*red*) in the non-CPJ **a**–**d** and CPJ **e**–**h** regions of synovial tissue within the same joint from 11 patients with active RA. Haematoxylin counterstaining. *Scale bar* 100 μm. Data are obtained from a single experiment for each type of staining and the results are representations of 11 pairs of samples obtained from 11 different patients.

or lymphoid aggregates in both non-CPJ tissues and CPJ tissues (Fig. 1c, g). Very mild staining associated with the vasculature and lumen was present in both non-CPJ and CPJ sites. The semi-quantitative (SQA) score for the proportion of positive cells in the sub-lining tissue was higher for OSCAR in non-CPJ tissues compared to CPJ from the same joints; however, this was not significant ($p = 0.307$, Fig. 1c, g). The presence of OSCAR in the lining region did not differ significantly between the non-CPJ and CPJ tissues ($p = 0.166$). There was a trend towards a higher OSCAR expression associated with vessels in non-CPJ compared with CPJ regions ($p = 0.058$). By evaluating vWB staining on serial tissue sections, it was confirmed that not all vWB-positive synovial vessels expressed OSCAR. TNF-α staining was performed to confirm the presence of this pro-inflammatory cytokine in tissues from the non-CPJ and the CPJ region (Fig. 1d, h).

Formation of Osteoclasts in Response to TNF-α and RANKL In Vitro

The effect of RANKL ± TNF-α addition, at different time points, on the induction of OSCAR and on the differentiation of PBMC into osteoclasts

was assessed. Multinucleated TRAP-positive cells and resorption pits were evident with all treatment combinations of RANKL ± TNF-α, confirming the presence of active osteoclasts (Fig. 2) at day 10 of differentiation. There was a significant increase in the number of multinucleated TRAP-positive cells with TNF-α pre-differentiation (day 0–1) when compared with RANKL alone at 10 ng/ml ($p = 0.0436$) and RANKL with TNF-α post-differentiation ($p = 0.0100$). The greatest percentage of dentine resorbed at day 10 differentiation also occurred when cells were treated with TNF-α pre-differentiation followed by RANKL at 10 ng/ml. This was significantly greater than RANKL 10 ng/ml ($p = 0.0063$), RANKL 50 ng/ml (0.0163) and RANKL 10 ng/ml with TNF-α from day 3 ($p = 0.0373$) (Fig. 2).

Levels of OSCAR Secreted During Osteoclast Differentiation in Response to RANKL With/Without TNF-α

Supernatants were collected from PBMC-derived osteoclasts after 7 days of differentiation with RANKL (with/without TNF-α). sOSCAR was produced by PBMC-derived osteoclasts cultured under all conditions. The highest levels of sOSCAR were detected when TNF-α was added pre-RANKL for 24 h and again on day 1 post-RANKL commencement (126 pg/ml). This was significantly higher than in cultures with RANKL 10 ng/ml alone ($p = 0.0108$), TNF-α pre-differentiation with RANKL 10 ng/ml ($p = 0.0007$) and RANKL with prolonged TNF-α exposure, commencing from day 3 (post-RANKL) ($p = 0.039$).

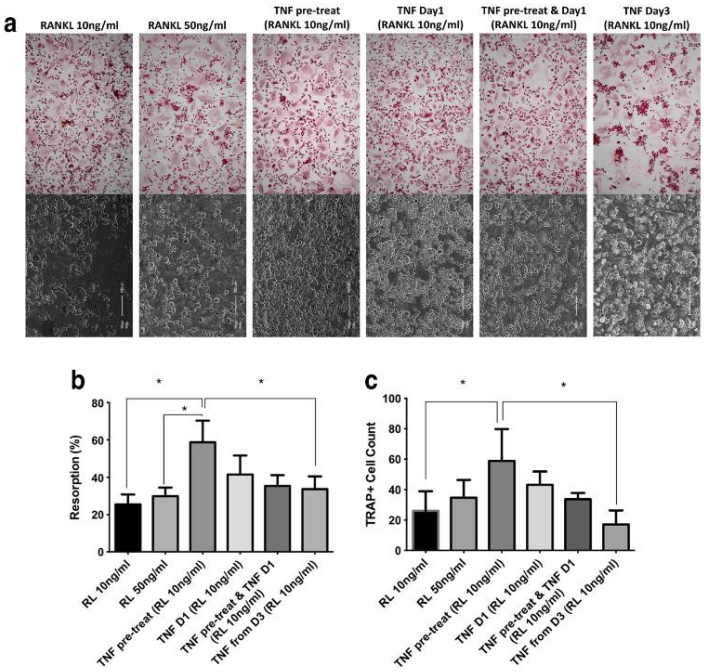

Fig. 2. TRAP staining and dentine resorption in PBMC-derived osteoclasts treated with TNF ± RANKL. PBMCs were pre-cultured for 6 days with MCSF. Osteoclastic differentiation was then initiated with RANKL at 10 or 50 ng/ml (day 0). TNF-α was added to relevant wells for 24 h pre-RANKL, after 1 or 3 days of RANKL treatment. After 7 days with RANKL ± TNF-α, cells were stained for TRAP (**a** *top row* ×75 total magnification) and those with >3 nuclei were counted as pre-osteoclasts **b**. After 10 days with RANKL ± TNF-α, dentine was visualized by scanning electron microscope (**a** *bottom row*, total magnification ×200) and resorption of dentine surface area was quantified as a percentage **c**. Data are presented as mean and standard error mean from three donors. Results presented include three donors from one representative experiment. The experiment was repeated four times. Statistical difference is determined using one-way ANOVA and Tukey's post hoc test. *Asterisk* indicates $p < 0.05$ and is considered as significant statistically.

OSCAR Gene Expression is Regulated by TNF-α and RANKL Treatment During Osteoclast Differentiation

OSCAR messenger RNA (mRNA) was expressed by PBMC-derived osteoclastic cells (differentiated with RANKL) in the presence and absence of TNF-α. The mRNA expression of OSCAR was not significantly different in any of the TNF-α treated cells when compared to 10 or 50 ng/ml RANKL alone. OSCAR mRNA expression was significantly higher when TNF-α was added pre-differentiation and from day 1 with RANKL ($p = 0.0266$) compared to when TNF-α was added pre-differentiation with RANKL 10 ng/ml, or when TNF-α was added from day 3 post-differentiation with RANKL ($p = 0.0319$). The lower levels of OSCAR mRNA expression were associated with high osteoclast activity levels (Figs. 2 and 3).

Discussion

The CPJ in RA affected synovial joints is the site of local erosive activity and the osteoclasts are the main cell responsible for this bone resorption. OSCAR is a co-stimulatory osteoclast molecule required for optimal osteoclast differentiation and activity [20]. OSCAR binds to collagen motifs exposed in the process of bone degradation resulting in activation of the ITAM signalling cascade [27]. The increased presence of unmasked motifs in inflamed tissue such as in RA might be crucial to osteoclast recruitment and activity *via* OSCAR. Additionally, the presence of sOSCAR in osteoclast-osteoblast cultures abrogates osteoclast differentiation suggesting a potential ligand is expressed by osteoblast cells [20]. Thus, the levels of sOSCAR present in serum and synovial fluid [23–25] potentially regulate osteoclast activity and the development of bone erosions by acting as a decoy receptor for a putative OSCAR ligand. In this study, we report high levels of OSCAR corresponding with TRAP and endothelial cells in non-CPJ and CPJ tissues within the same joint of patients with developed RA. Additionally, we demonstrate RANKL and TNF-α regulation of OSCAR secretion in human PBMC-derived osteoclast cultures and the concurrent gene expression.

vWF was used as a marker of endothelial cells. vWF has been found to play a role in angiogenesis [31] and regulates inflammation *via* modulation of leukocyte adhesion [32]. Endothelial expression of OSCAR is also believed to increase margination of mononuclear cells into the tissues. We observed OSCAR association with vessels in both non-CPJ and CPJ sites within the synovial tissue from the same joint; however, the extent of OSCAR staining in the vessels did not differ between sites.

There was no difference in TRAP-positive cells in the tissue from the non-CPJ compared with CPJ tissue from the same joint. This is in contrast to what was expected as

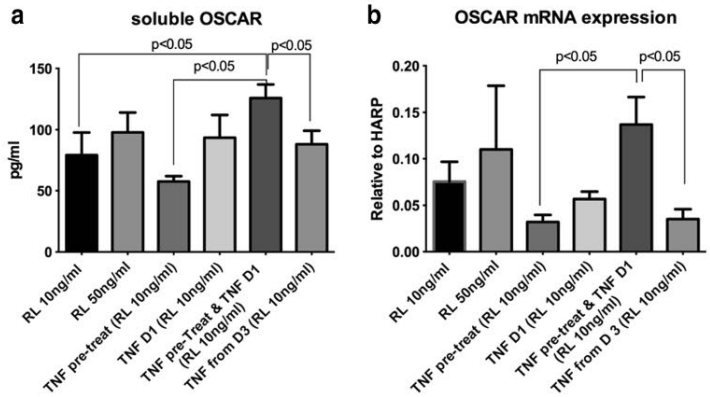

Fig. 3. Soluble OSCAR and OSCAR gene expression in PBMC-derived osteoclasts treated with TNF ± RANKL. Soluble OSCAR **a** and OSCAR **b** gene expression. PBMCs were pre-cultured for 7 days with MCSF. At day 0, cells were differentiated with RANKL at 10 or 50 ng/ml. TNF-α was added to relevant wells for 24 h pre-RANKL, after 1 or 3 days of RANKL treatment. Supernatants were collected during media changes after 7 days of differentiation by RANKL. Total RNA was isolated after 7 days with RANKL ± TNF. The delta cycle threshold (ΔC_t) values of OSCAR gene expression for each triplicate were normalized to the endogenous reference gene hARP, according to the formula C_t target gene − C_t hARP. Data are presented as mean and standard error mean from three donors of one representative experiment. The experiment was repeated four times. Statistical difference is determined using one-way ANOVA and Tukey's post hoc test and $p < 0.05$ and is considered as significant statistically.

previous reports suggest that osteoclasts in RA are usually localised around the CPJ or on the bone [44]. This may be as these patients have established RA and at this stage of disease all the areas of the synovium are affected. Of note, TRAP expression corresponded with positive OSCAR cells in the lining and sub-lining; however, the extent of OSCAR expression in these regions did not differ between sites.

TNF-α is a key inflammatory cytokine that plays a major role in inflammation in RA. Consistent with previous reports, we observed TNF-α-associated cells in the lining and sub-lining layers of the RA synovium, as well as at the CPJ [33, 34]. Consistent with previous reports, we observed TNF-α to be associated with lining cells, present in interaggregate areas and in a perivascular distribution. Some endothelial cells also express TNF-α. Cells containing TNF-α have also been implicated in affecting chondrocytes within the RA CPJ [33]. A previous study demonstrated that most TNF-α-secreting cells in the RA synovial membrane expressed the monocyte/macrophage marker antigens CD11b and CD14, and a few expressed the T cell marker CD3 [33]. Further to this, we have shown the markedly increased expression of OSCAR in active RA patients compared to that in healthy controls suggests that it is altered during progression of the disease in response to inflammatory mediators [23]. Further studies investigating expression of OSCAR in the synovial tissues of RA patients over time are warranted.

A putative binding motif for the OSCAR has been identified within the surfactant protein D (SP-D) collagenous domain [35]. The study reported TNF-α to be released when CCR2 (+) inflammatory monocytes were exposed to SP-D in an OSCAR-dependent fashion. This may be pertinent to chronic inflammatory diseases involving tissue accumulation of SP-D, infiltration of inflammatory monocytes and release of TNF-α and thus might be an area warranting further investigation.

The production of TNF-α has been demonstrated to directly enhance bone destruction by osteoclasts, as reviewed by [36] through progenitor recruitment and amplification of osteoclast signalling with permissive levels or independently of RANKL *in vitro* [37–39]. In such a case, the physiological regulator of RANKL and OPG may inadequately control the excessive osteoclastic differentiation. This has been demonstrated by TNF-α-induced osteoclast formation in the presence of recombinant OPG [37].

Herman et al. [24] observed OSCAR gene induction by TNF-α but not by RANKL and their study on PBMCs did not assess the combination nor alternate time course of addition of the two factors. We now provide evidence that OSCAR expression and release is mediated by RANKL alone and in combination with the inflammatory cytokine, TNF-α, in human PBMC-derived osteoclasts *in vitro*. This is consistent with our previous findings that OSCAR secretion and gene expression in human umbilical vein endothelial cells (HUVEC) is regulated by TNF-α *in vitro* [23]. Here, we also observed variations in OSCAR gene expression in response to RANKL and TNF-α from different stages of osteoclastogensis *in vitro*. Of interest, the lower levels of OSCAR mRNA expression were associated with high osteoclast activity levels. Perhaps, at late-stage osteoclast differentiation, the gene expression is then reduced.

Conclusions

OSCAR is expressed in the lining and sub-lining as well as in the microvasculature of non-CPJ and CPJ tissues in those with active RA. Similar levels of TRAP-positive cells were observed in the lining and sub-lining of the non-CPJ and CPJ regions. OSCAR gene and protein secretion by human pre-osteoclasts and osteoclasts is temporally regulated by both RANKL and TNF-α. Understanding of the pathogenesis of RA and the expression and release of modulators of the osteoclast cell such as OSCAR at different stages of the disease process will lead to better understanding in inflammation-driven bone loss diseases such as RA.

MATERIALS AND METHODS

Patient Criteria

Ethics approval was obtained from the Repatriation Hospital, Daw Park, SA (100911b) and the Southern Adelaide Clinical Human Research Ethics Committee (199.10) for synovial tissue collection. Ethics for obtaining human PBMCs from the Australian Red Cross Blood Service was through the University of Adelaide Human Ethics Committee (H-35-2001), in accordance with National Health and Medical Research Council of Australia guidelines. Informed written consent was obtained from all patients.

CPJ and non-CPJ tissue samples were collected from the synovium of the affected knees from 11 patients with active RA to compare expression between sites within an active joint. Each patient fulfilled 1987 American College of Rheumatology [40] for RA [41]. Synovial tissue was

then fixed in 10% buffered formalin and prepared using alcohol/isopropanol series and embedded in paraffin wax.

Immunohistochemistry

Antibodies

Primary antibodies included goat anti-human polyclonal OSCAR at (4 μg/ml) (S-16 [sc-34233], Santa Cruz Biotech Inc.); mouse anti-human vWF at (5 μg/ml) (Dako clone F8/86, M0616, Glostrup, Denmark) and mouse anti-human TNF-α (0.5 μg/ml) (LS-B2123, LifeSpan Biosciences Inc., Seattle, WA, USA).

Secondary antibody for vWF was provided in the ImmPRESS-AP-Anti-Mouse Ig Kit (Vector, MP-5402, Burlingame, CA, USA) and detection was using the Fast Red Alkaline Phosphatase Substrate Kit (SK-5100, Vector, Burlingame, CA, USA). For polyclonal goat anti-human OSCAR ImmPRESS goat Peroxidase kit (Vector Labs, Burlingame, CA, USA) was used with AEC as the substrate (SK 4200, Burlingame, CA). Isotype controls included the following at the same concentration as the primary: normal polyclonal goat IgG (AB-108-C, RD Systems Inc., MN, USA) for OSCAR and purified mouse IgG1k Isotype control (BD Biosciences, San Jose, CA, USA) for vWF.

Immunohistochemistry

Sections underwent antigen retrieval in 10 mM tris-(hydroxymethyl)-aminomethane ethylene-diamine-tetra-acetic acid (TRIS EDTA, pH 9.0) at 65–70 °C. Non-specific binding was blocked using horse serum 2.5% (provided in the ImmPRESS kit). Primary antibody or isotype control diluted in PBS/Tris PBS was placed on tissues overnight at room temperature.

Sections were incubated with anti-mouse Ig secondary antibody (for vWB) or anti-goat (for OSCAR) provided in ImmPRESS mouse AP kit or ImmPRESS goat peroxidase kit. The colour substrate fast red alkaline phosphate (for vWB) or AEC (for OSCAR) was added as per manufacturer's instructions. Sections were counterstained in Harris' haematoxylin/lithium carbonate.

TRAP Staining

TRAP staining was performed using the naphthol-based method [42], for both human tissues and cell culture to identify pre-osteoclasts/osteoclasts. The paraffin-embedded sections were de-waxed using histolene/alcohol series steps, then were placed in 0.1 M TRIS-HCl pH 9.0 overnight followed by 0.1 M sodium citrate buffer pH 5.2, for 3 h to pre-activate the enzyme [43]. The TRAP substrate was prepared according to previous validated methods [23, 42] and incubated with tissues at 37 °C. Tissues were counterstained in Harris' haematoxylin/lithium carbonate. Tissue retrieved from the acetabular capsule adjacent to osteolysis from a patient undergoing revision surgery was used as a positive control [44].

Immunohistochemical Analysis

Sections were scanned using NanoZoomer 2.0 (Hamamatsu Photonics, Japan) and four to six 2-mm^2 regions were selected on ×2.5 magnification of Hamamatsu software. OSCAR and TRAP staining were examined by two assessors (blinded to the patient information and synovial site). The presence of positive cells in the sublining and vasculature was assessed using a validated semi-quantitative (SQA) scoring system [23, 45]. The presence of positive cells in the lining was scored for presence (1) and absence (0).

Cell Culture Assays

PBMCs (collected by the Australian Red Cross Blood Service) were isolated by Ficoll-Paque (Pharmacia Biotech, Sweden) separation and gradient centrifugation [44, 46]. Results presented include three donors from one representative experiment. The experiment was repeated four times. Cells were suspended at 2×10^6 cells/ml in alpha-MEM (Invitrogen, Australia), with 10% foetal calf serum (Invitrogen, USA), 1% penicillin-streptomycin (Invitrogen, USA) and 1% L-glutamine (Invitrogen, USA). Cells were seeded into 96-, 48- and 16-well trays for resorption analysis on whale dentine (gift from Australian Customs Service, Canberra), RNA isolation and TRAP stain analysis, respectively.

After 24 h at 37 °C, 5% CO_2, media and non-adherent PBMCs were removed and replaced with alpha-MEM and accompanying components as previously described, supplemented with 10^{-8} mol/l dexamethasone (Fauldings, Australia) and 25 ng/ml recombinant human macrophage-colony stimulating factor (MCSF) (Chemicon Inc., USA) for 17 days (6 days pre-differentiation, 10 days differentiation; media replenished every 3–4 days). Recombinant human RANKL (10 or 50 ng/ml) (Chemicon Inc., USA) was added to induce differentiation and activation over the 10-day period. Recombinant TNF-α (10 ng/ml R&D Systems, MN, USA) was added to relevant wells of RANKL (10 ng/ml) at different periods to investigate its effect on OSCAR expression throughout the osteoclastogenic cell cycle. These time points include 24 h pre-RANKL (TNF pre-treat), 24 h post-RANKL

(day 1), both pre-treat and on day 1, or 3 days post-RANKL.

After 1 week with RANKL ± TNF-α, 300 µl TRIzol® was added to each well and total cellular RNA was isolated from duplicate wells, as per manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA, USA). Cells were also fixed and stained for TRAP after 7 days of differentiation as per the method above, and supernatants were collected for the assessment of sOSCAR levels.

Resorption Analysis

After 10 days with RANKL ± TNF-α, dentine slices were processed and imaged by scanning electron microscopy (SEM) and surface resorption was quantified to assess osteoclast activity [44] using ImageJ software.

Quantitative Real-time Polymerase Chain Reaction

Complementary DNA (cDNA) was prepared from 1 µg of total RNA using random hexamers (Geneworks Pty. Ltd., Adelaide, Australia) and 200 U Superscript™ III (Invitrogen) with Rotor-Gene™ 3000, software version 6.0.38 (Corbett Life Science, Mortlake, New South Wales, Australia), as per manufacturer's instructions.

Amplification of the genes of interest was performed using 1 µl of the pre-diluted (1/5) cDNA, 300 nM of each forward and reverse primer, Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) and DEPC H_2O. Primer pair sequences included human acidic ribosomal protein (hARP) (reference control gene) and OSCAR (designed using Primer3Plus) [44] (Geneworks, Adelaide, SA, Australia). Each donor sample was prepared in triplicate, including a no RNA RT control. Samples were placed in the Rotor-Gene™ 3000 and analysed using Rotor-Gene™ Series 1.7 software.

Detection of OSCAR by Enzyme-Linked Immunosorbent Assay

Human PBMC-derived osteoclast supernatants were collected during media changes after 7 days of differentiation by RANKL and assessed for sOSCAR using a human OSCAR enzyme-linked immunosorbent assay (ELISA; E9269Hu, USCN Life Science, Houston, TX, USA), according to manufacturer's instructions. Results were interpolated from a standard curve.

Statistical Analysis

The SQA of OSCAR and TRAP expression in the lining, sub-lining and vasculature of the synovial tissue at the CPJ *versus* non-CPJ within the same patient was compared using a paired *t* test. Significance was accepted when $p < 0.05$.

TRAP counts, surface dentine resorption and levels of sOSCAR *in vitro* data were calculated as mean ± standard error (SE) from three donors. To assess OSCAR gene expression, the delta cycle threshold (ΔC_t) values for each triplicate were normalized to the endogenous reference gene hARP, according to the formula C_t target gene − C_t hARP. The average of the triplicate ΔC_t was then calculated for each sample [47]. Statistical analysis of *in vitro* data involved one-way ANOVA for multiple comparison, with Tukey's post hoc test (GraphPad Prism 5 for Windows, Version 5.03, 2009).

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AUTHORS' CONTRIBUTIONS K.A. and M.C. designed and K.A. performed the *in vitro* experiments with peripheral blood mononuclear cells including analysis of gene expression, TRAP staining and resorption. T.C., A.D. and R.C. designed and performed experiments with the clinical samples. M.W. and M.S. performed clinical analyses and interpretation. M.L. performed and directed statistical analyses. All authors contributed to the writing of the manuscript and approved the final version.

COMPLIANCE WITH ETHICAL STANDARDS

Ethics approval was obtained from the Repatriation Hospital, Daw Park, SA (100911b) and the Southern Adelaide Clinical Human Research Ethics Committee (199.10) for synovial tissue collection. Ethics for obtaining human PBMCs from the Australian Red Cross Blood Service was through the University of Adelaide Human Ethics Committee (H-35-2001), in accordance with National Health and Medical Research Council of Australia guidelines. Informed written consent was obtained from all patients.

Conflict of Interest The authors declare that they have no conflict of interest.

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Class I and II histone deacetylase expression in human chronic periodontitis gingival tissue

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Background and Objective: Histone deacetylase inhibitors (HDACi) are being considered to treat chronic inflammatory diseases at low doses. Currently HDACi that are more specific are being developed to target particular HDACs; therefore, this study aimed to determine levels and distribution of class I and II HDAC in human gingival samples obtained from patients with chronic periodontitis.

Material and Methods: Gingival biopsies were obtained from patients with and without (mild inflammation, no bone loss) periodontitis. Total RNA was isolated for real-time quantitative polymerase chain reaction to determine expression of HDACs 1–10. Immunohistochemistry was used to determine protein distribution of HDACs 1, 5, 8 and 9. Factor VIII, CD3 and tartrate resistant acid phosphatase (TRAP) were detected in serial sections to identify blood vessels, lymphocytes, pre-osteoclasts and osteoclasts cells respectively. Tumour necrosis factor α (TNF-α) expression was also assessed.

Results: mRNA for HDAC 1, 5, 8 and 9 were significantly upregulated in chronic periodontitis gingival tissues compared to non-periodontitis samples ($p < 0.05$). Significantly higher HDAC 1 protein expression was observed in chronic periodontitis samples ($p < 0.05$), and was associated with CD3, TRAP and TNF-α-positive cells. HDAC 1, 5, 8 and 9 were expressed strongly by the factor VIII-positive microvasculature in the chronic periodontitis gingival tissues.

Conclusions: HDAC 1, 5, 8 and 9 expression was higher in gingival tissues from patients with chronic periodontitis compared to non-periodontitis samples. Results suggest that these HDACs could therefore be targeted with specific acting HDACi.

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Periodontitis is a chronic inflammatory disease affecting up to 60% of the world's population (1). It is characterized by chronic inflammation in gingival tissues and associated loss of the supporting structures, including the periodontal ligament and alveolar bone (2,3). Current treatments for this disease involve the use of a combination of antimicrobial treatments along with mechanical debridement of the affected sites. The effects of these treatments on the alveolar bone can be varied, and hence anti-resorptive medications (reviewed in reference 4) that can also reduce inflammation would be valuable adjuncts for treatment. Histone deacetylase inhibitors (HDACi) that regulate gene transcription and protein

acetylation are one such option. Histone deacetylase (HDAC) enzymes were initially found to act by counteracting the actions of histone acetyl transferase enzymes (HATs) and removing the acetyl groups from the lysine molecules of histone proteins. This results in a condensed chromatin structure leading to gene repression. More recently, HDACs have been shown to regulate expression of a number of key cytoplasmic proteins, including a range of inflammatory cytokines (5,6). There are two main classes of HDAC enzymes; class I includes HDACs 1, 2, 3 and 8 located within the nucleus of cells and class II that includes HDACs 4, 5, 7 and 9, which belong to class IIa HDACs, and HDACs 6 and 10 belonging to class IIb HDACs. The class II enzymes are able to shuttle between the nucleus and cytoplasm (7,8).

HDACi have been predominantly utilized to treat malignancies (9), but more recently, have been demonstrated to have the potential, at lower doses (10–100× lower), to treat a range of chronic inflammatory diseases, including rheumatoid arthritis (RA) and periodontitis (10–13). This could be related to their ability to alter the acetylation status of cytoplasmic proteins, including inflammatory cytokines, which might modulate and suppress inflammation and associated bone loss (5,6,14). A number of HDACi have also shown promise in suppressing osteoclast activity *in vitro* (13,15,16), as well as bone loss and inflammation *in vivo* (10,11,17). The majority of HDACi (trichostatin A, phenylbutyrate, vorinostat and givinostat) are broad acting as they target a range of HDAC enzymes across the two main classes. Recently, we have demonstrated in a mouse model of periodontitis that a novel HDACi targeting both class I and II HDACs may be a potential treatment for suppressing periodontal bone loss (12).

As newer and more selective acting HDACi are being developed, knowledge of the HDACs involved in human diseases is crucial to identifying which targeted treatments are likely to be most effective for treating different chronic diseases. At present, there is limited knowledge of which particular HDACs are expressed in various tissues and diseases. Studies in cancer have demonstrated elevated class I HDACs compared to class II in breast cancer tissues (18). In a range of cancers, such as in the stomach, oesophagus, colon, prostate, breast, ovary, lung, pancreas and thyroid, all class I HDACs were shown to be ubiquitously expressed (19). Expression of a selection of HDACs has also been shown in healthy tissues, including the brain, thymus, testis, pancreas, liver, skeletal muscle, uterine cervix, fallopian tube, bladder wall, lung and breast (20).

To date, the only investigations assessing HDAC expression in inflammatory diseases have been in RA tissues using osteoarthritic tissues as controls (21,22). It has been shown that HDAC 1 is highly expressed in synovial fibroblasts from patients with RA compared to osteoarthritis (21). High HDAC 1 levels were also shown to correlate with elevated tumour necrosis factor α (TNF-α) (expression in RA tissues) (22). In contrast, another study using synovial tissues from human RA joints demonstrated that HDAC activity was suppressed (22). Recently, mRNA expression of HDAC enzymes has been demonstrated in human osteoclasts cultured *in vitro,* with class I HDAC 8 and class II HDAC 5 found to be significantly upregulated during the later stages of osteoclast development (16). As increased pre-osteoclasts and osteoclasts are associated with bone erosion in periodontitis (23), it was hypothesized that HDAC 5 and 8 would be elevated in periodontitis tissues.

The development of isoform- or class-specific inhibitors enables the ability to target individual HDACs or classes that may offer therapeutic benefits with improved efficacy and reduced side effects compared to broad-acting inhibitors. To identify which treatment would be most beneficial, further elucidation of the expression and importance of particular HDACs in the inflammatory process and bone loss is needed.

In the current study, it was hypothesized that both HDAC classes I and II would be highly expressed in human periodontitis gingival tissues. Therefore, the aim was to determine the tissue expression and distribution of class I and II HDAC 1–10 in human gingival tissues obtained from patients with chronic periodontitis compared to non-periodontitis gingival tissues at the gene and protein level using real-time quantitative polymerase chain reaction (PCR) and immunohistochemistry.

Material and Methods

Patient samples

Human gingival tissues from patients with chronic periodontitis visiting the periodontal clinic of the Adelaide Dental Hospital were compared with non-periodontitis gingival tissues obtained during wisdom teeth removal and crown lengthening procedures. Informed consent was obtained from all patients. Details of the patient tissue samples collected are shown in Table 1. Different sample sets were used for PCR and immunohistochemistry due to limited amounts of gingival tissues obtained. For mRNA analysis using real-time PCR, nine samples of periodontitis and eight samples of non-periodontitis tissues were analysed. For immunohistochemical staining, 15 samples of periodontitis tissues and seven samples of non-periodontitis tissues were analysed for both HDAC 1 and 9 staining. For HDAC 5 and 8 staining, eight samples of periodontitis tissues and six non-periodontitis tissues were analysed. This study was approved by the University of Adelaide Human Ethics Committee (H-35-2001) and informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Periodontitis samples were all collected from patients diagnosed with chronic periodontitis with moderate to severe levels of inflammation assessed histologically and radiographic evidence of bone loss. The non-periodontitis samples were from patients undergoing crown lengthening procedures that demonstrated no or only mild levels of inflammation and had no evidence of bone loss.

Sample preparation

Half of each portion of gingival tissue collected was placed into 10% normal

Table 1. Patient data

	Gender	Age	Diagnosis/surgery	Inflammation	mRNA analysis	HDAC 1	HDAC 5	HDAC 8	HDAC 9
Periodontitis									
1	M	40	chronic periodontitis	Moderate to severe	X	X	X	X	X
2	M	45	chronic periodontitis	Severe	X		X	X	
3	M	61	chronic periodontitis	Moderate to severe	X		X	X	
4	M	43	chronic periodontitis	Moderate to severe	X		X		
5	F	39	chronic periodontitis	Moderate to severe	X		X	X	
6	F	45	chronic periodontitis	Severe	X	X	X	X	X
7*	F	29	chronic periodontitis	Severe	X	X	X	X	X
8	M	65	chronic periodontitis	Moderate to severe	X		X	X	
9	F	45	chronic periodontitis	Severe	X			X	
10	M	45	chronic periodontitis	Severe		X			X
11	M	Unknown	chronic periodontitis	Localized Severe		X			X
12	M	79	chronic periodontitis	Severe		X			X
13	F	72	chronic periodontitis	Severe		X			X
14	M	71	chronic periodontitis	Severe		X			X
15	M	52	chronic periodontitis	Severe		X			X
16	M	Unknown	chronic periodontitis	Severe		X			X
17	M	Unknown	chronic periodontitis	Moderate to Severe		X			X
18			chronic periodontitis	Severe		X			X
19	F	48	chronic periodontitis	Localized Severe		X			X
20	F	Unknown	chronic periodontitis	Severe		X			X
21			chronic periodontitis	Moderate-Severe		X			X
Non-periodontitis									
1	M	58	Crown lengthening	Minimal	X	X	X	X	
2	M	52	Crown lengthening	Minimal	X				
3	F	Unknown	Crown lengthening	Mild				X	
4	M	56	Crown lengthening	None	X				
5	M	56	Crown lengthening	None	X				
6	F	45	Healthy gingiva	None	X		X		
7	F	83		Mild gingivitis	X				
8	F	Unknown	Healthy gingiva	Mild inflammation	X		X		
9	F	Unknown	Healthy gingiva	Mild inflammation	X				
10	F	68	Gingivitis	Mild inflammation		X			X
11	F	Unknown	Crown lengthening	None		X			X
12	M	Unknown	Gingivitis			X			X
13	F	46	Healthy gingiva/ crown lengthening	None		X	X	X	X
15	M	35						X	
16	M	71	Crown lengthening	Mild gingivitis		X	X	X	X
17*	F	48	Crown lengthening	None		X	X	X	X
18	M	65	Healthy gingiva	None			X		
19	F	Unknown	Crown lengthening						X

HDAC, histone deacetylase.
*Sample for representative images in Figs 2 and 3.

buffered formalin overnight before processing and paraffin embedding. The remaining half was snap frozen in Tissue Tek OCT (Pro Sci Tech, Thuringowa, Qld, Australia) using liquid nitrogen and stored at –80°C before RNA isolation using Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA). Five micrometre sections were dewaxed for immunohistochemistry. Two of the paraffin-embedded sections were stained with haematoxylin and eosin to enable screening of tissue to ensure good tissue morphology. Stained sections were scored for histological severity of inflammatory cell infiltration in the soft tissues, as previously described (24).

mRNA expression of histone deacetylase 1–10

Frozen sections (×5 per sample at 20 μM) were cut using a cryostat and placed in Trizol (Invitrogen Life Technologies) for RNA extraction. Reverse transcription was conducted using the Corbett real-time PCR machine (Corbett Research Rotor Gene RG-3000; Corbett Life Science, Mortlake, NSW, Australia). The reverse transcription reaction to produce cDNA consisted of 1 μg RNA, 250 ng of random hexamer (Geneworks, Adelaide, SA, Australia) and 200 U of Superscript III Reverse Transcriptase, according to previously published methods (16). Real time quantitative PCR was conducted using Platinum SYBR Green

Table 2. HDAC primers used for quantitative polymerase chain reaction

HDAC gene	Primers
HDAC 1	Sense 5′-AGCCAAGAGAGTCAAAACAGA-3′ Antisense 5′-GGTCCATTCAGGCCAACT-3′
HDAC 2	Sense 5′-GCTCTCAACTGGCGGTTCAG-3′ Antisense 5′-AGCCCAATTAACAGCCATATCAG-3′
HDAC 3	Sense 5′-GAGAGTCAGCCCCACCAATA-3′ Antisense 5′-TGTGTAACGCGAGCAGAACT-3′
HDAC 4	Sense 5′-GACCTGACCGCCATTTGC-3′ Antisense 5′-GGGAGAGGATCAAGCTCGTTT-3′
HDAC 5	Sense 5′-CAACGAGTCGGATGGGATGT-3′ Antisense 5′-GGGATGCTGTGCAGAGAAGTC-3′
HDAC 6	Sense 5′-ACCTAATCGTGGGACTGCAAG-3′ Antisense 5′-GAAAGGACACGCAGCGATCT-3′
HDAC 7	Sense 5′-AGCAGCTTTTTGCCTCCTGTT-3′ Antisense 5′-TCTTGCGCAGAGGGAAGTG-3′
HDAC 8	Sense 5′-CGGCCAGACCGCAATG-3′ Antisense 5′-CACATGCTTCAGATTCCCTTT-3′
HDAC 9	Sense 5′-AGGCTCTCCTGCAGCATTTATT-3′ Antisense 5′-AAGGGAACTCCACCAGCTACAA-3′
HDAC 10	Sense 5′-ATGACCCCAGCGTCCTTTACT-3′ Antisense 5′-CGCAGGAAAGGCCAGAAG-3′

HDAC, histone deacetylase.

qPCR Supermix-UDG (Invitrogen Life Technologies, Mulgrave, VIC, Australia) according to the manufacturer's instructions. Each PCR reaction consisted of 1 μg cDNA, 2× supermix (containing SYBR Green 1 dye), 300 nM of forward and reverse primer and this was made up to a total volume of 15 μL with diethyl pyrocarbonate-treated ultrapure water. PCR was performed in triplicate for each sample. mRNA expression was determined relative to endogenous reference gene human acidic ribosomal protein (25). HDAC primers were as previously used (16) (as shown in Table 2). The relative quantification of the mRNA expression for each of the genes was then calculated using the comparative Ct method $2^{-\Delta\Delta Ct}$ (26).

Immunohistochemical detection of histone deacetylase 1, 5, 8 and 9

Based upon the mRNA expression data, the specific HDACs that were upregulated in periodontitis gingiva were then assessed at the protein level. Distribution of HDAC 1, 5, 8 and 9 proteins was detected in gingival tissues using immunohistochemistry with commercially available antibodies. Rabbit polyclonal antibodies raised against HDAC 1 (ab53091, at 20 μg/mL; Abcam, Cambridge, MA, USA), HDAC 5 (ab55403, at 1.25 μg/mL; Abcam), HDAC 8 (ab39664, at 1 μg/mL; Abcam) and HDAC 9 (ab59718, at 10 μg/mL; Abcam), factor VIII (M 0616 at 4.9 μg/mL; DAKO, Sydney, Australia), CD3 (ab5960 at 20 μg/mL; Abcam) and TNF-α (LS-B2123 at 0.5 μg/mL; LifeSpan Biosciences Inc, Seattle, WA, USA) were used. Positive controls included human breast and lung cancer tissues. Negative controls were carried out for all sections using universal rabbit isotype control IgG (DAKO) or IgG1 kappa isotype control (Sigma-Aldrich, Castle Hill, NSW, Australia) at equivalent concentrations. The secondary antibody and amplification method was performed using either VECTASTAIN Universal Quick kit, ready to use or VECTASTAIN Elite ABC (Universal) kit (Vector Laboratories, Burlingame, CA, USA) similar to previously published methods (27). Immunostaining of HDAC 1 and TNF-α required heat antigen epitope retrieval in 10 mM sodium citrate buffer (pH 6.0). Factor VIII and CD3 detection required 10 mM Tris EDTA buffer (pH 9.0) at 80–90°C for 10 min. Immunohistochemical detection of HDAC 5, 8 and 9 did not require antigen retrieval. Endogenous peroxide was then blocked with 0.3% v/v H_2O_2 in methanol or phosphate-buffered saline (PBS)/0.1% sodium azide in the presence of 0.3% v/v H_2O_2 for 10–20 min. Normal horse serum, supplied as blocking serum in the ready to use or ABC kit (Vector Laboratories), was then applied to all sections for 30 min. Without washing, sections were incubated with the primary antibody in PBS overnight at room temperature. Sections were washed in PBS then incubated with streptavidin or biotinylated secondary antibody (supplied in the ready to use or ABC kit), for 30–45 min. After washing in PBS, sections were incubated with streptavidin or avidin–biotin complex conjugated to horseradish peroxidase reagent for 30–45 min. Following washing in PBS, the colour reaction was developed using AEC (AEC Perox-

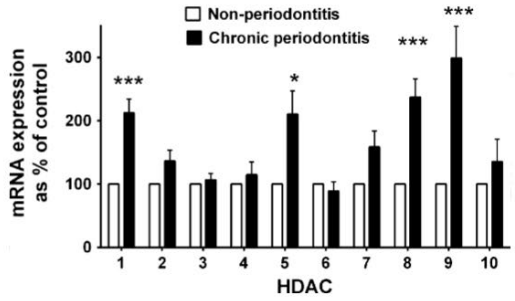

Fig. 1. Fold change in the mRNA expression of HDAC 1–10 relative to the non-periodontitis tissues. mRNA expression in both groups is relative to endogenous gene human acidic ribosomal protein. Bars represent mean ± SEM. ***$p < 0.001$, *$p < 0.05$ expression in chronic periodontitis samples relative to non-periodontitis gingival tissues. HDAC, histone deacetylase.

idase Substrate Kit; Vector Laboratories) for 30 min in the dark. The positive cells were stained red. Sections were washed in MilliQ H_2O, counterstained with haematoxylin and lithium carbonate then washed and mounted with Aquatex (Merck, Whitehouse Station, NJ, USA).

Tartrate resistant acid phosphatase staining

After dewaxing sections were immersed with 0.1 M Tris-HCl, pH 9.0 for 18 h, then transferred to 0.1 M sodium citrate buffer pH 5.2 for 3 h. Tartrate resistant acid phosphatase (TRAP) staining was performed according to previously published methods (28,29). Counterstaining was performed with haematoxylin and lithium carbonate.

Semiquantitative analysis of immunohistochemistry

Semiquantitative analysis was conducted on sections scanned using the Nanozoomer Digital Pathology imaging system (Hamamatsu, Shizouka, Japan). Up to six random areas (0.2 × 0.2 mm) were selected and assessed by two independent observers. Scoring was based on previously published methods and involved scoring the proportion of positive cells in the subepithelial region of the gingival tissues (30,31): a score of 1 = 0–5% of positive cells; 2 = 6–10%; 3 = 11–25%; 4 = 26–50%; 5 = > 50%. The scores for the six boxes were averaged thus one score represented each tissue section for each observer. The individual observer scores were then averaged to give a final score for each section. The mean scores for the periodontitis and non-periodontitis groups were then calculated along with the SD. To assess HDAC expression associated with the vasculature the level of staining intensity was assessed by two blinded observers. Staining was classified as negative, mild or intense and the proportion in each classification was compared between periodontitis and non-periodontitis samples. Representative images of scoring intensity, i.e. negative (no staining), mild (some light staining, but not all cells staining) and intense (dark staining of all endothelial cells for a particular vessel) were available to both scorers to use as a reference.

Statistics

For mRNA statistical analysis a Mann–Whitney *U*-test was conducted and significance accepted when $p < 0.05$. Statistical significance of the difference in HDAC 1, 5, 8 and 9 staining between groups was analysed using the Kruskal–Wallis followed by a Mann–Whitney test for comparing two groups. A value of $p < 0.05$ was considered statistically significant.

Results

mRNA expression of histone deacetylase 1–10

The expression of HDAC 1–10 mRNA was examined in chronic periodontitis gingival tissues and compared to non-periodontitis controls using real-time quantitative PCR. mRNA expression of HDAC 1, 5, 8 and 9 ($p < 0.05$) was significantly higher in chronic periodontitis samples compared to non-periodontitis samples (Fig. 1). Expression of HDAC 2, 3, 4, 7 and 10 was not statistically significant between the tissue types. HDAC 6 expression was not increased in periodontitis tissues. There was a twofold increase in class I HDAC 1 and 8 expression, a twofold increase in class II HDAC 5 and a threefold increase in HDAC 9 expression in chronic periodontitis samples compared to non-periodontitis gingiva samples (Fig. 1).

Table 3. Semiquantitative analysis of HDAC 1, 5, 8 and 9 protein expression in the subepithelial tissue

Protein	Non-periodontitis	Chronic periodontitis
HDAC 1 (mean ± SD)	1.94 (0.77)	3.20 (0.88)*
HDAC 5 (mean ± SD)	2.25 (1.07)	1.73 (0.77)
HDAC 8 (mean ± SD)	2.85 (0.47)	2.65 (0.68)
HDAC 9 (mean ± SD)	2.44 (0.90)	2.66 (0.93)

HDAC, histone deacetylase. Semiquantitative analysis grades for proportion of positive cells: score of 1 = 0–5% of positive cells; 2 = 6–10%; 3 = 11–25%; 4 = 26–50%; 5 = > 50%. Mean ± SD.
*$p < 0.05$ for HDAC 1 chronic periodontitis samples vs. non-periodontitis samples.

Table 4. Number of tissue samples in each scoring range

Score	Non-periodontitis (*n*)	Chronic periodontitis (*n*)
HDAC 1		
1	2	1
2	4	3
3	1	4
4	0	7
5	0	0
Total	7	15
HDAC 5		
1	2	4
2	2	3
3	1	1
4	1	0
5	0	0
Total	6	8
HDAC 8		
1	0	0
2	2	3
3	3	5
4	1	0
5	0	0
Total	6	8
HDAC 9		
1	1	2
2	4	4
3	1	6
4	1	3
5	0	0
Total	7	15

HDAC, histone deacetylase.
Semiquantitative analysis grades for proportion of positive cells: score of 1 = 0–5% of positive cells; 2 = 6–10%; 3 = 11–25%; 4 = 26–50%; 5 = > 50%. Mean ± SD.
Number of samples (*n*) in each scoring range.

Protein expression of histone deacetylase 1, 5, 8 and 9 in periodontitis and non-periodontitis gingiva

Based on the significant increase in HDAC 1, 5, 8 and 9 mRNA expression, and previous studies demonstrating high expression of HDAC 5 and 8 enzymes during the later stage of human osteoclast differentiation (16), immunohistochemistry was conducted to determine the protein distribution of these HDAC enzymes. The proportion of positively stained cells was assessed using previously published semiquantitative analysis methods (30,31). The proportion of HDAC 1-positive cells was significantly higher ($p < 0.05$) in the subepithelial cells between tissues from chronic periodontitis and non-periodontitis samples (Table 3). For the periodontitis group 11–25% of cells were positive whereas in the non-periodontitis group this was between 0% and 5%. There was no statistical variation in HDAC 5, 8 and 9-positive subepithelial cells between tissue groups. For HDAC 5 there were 0–5% positively stained cells in the periodontitis group and between 6% and 10% in the non-periodontitis groups. For both groups for HDAC 8 and 9 there was 6–10% positively stained cells in the subepithelial region. The number (*n*) of tissue sections in each scoring range are shown in table 4. Of particular note, HDAC 1, 5, 8 and 9 were all expressed strongly by the microvasculature in chronic periodontitis tissue. This intense staining for HDAC 5 and 8 was

Table 5. Proportion of samples with HDAC 1, 5 and 8 and 9 positive microvasculature

Protein	HDAC 1 (%)	HDAC 5 (%)	HDAC 8 (%)	HDAC 9 (%)
Non-periodontitis				
Negative	3/7 (43)	0/6 (0)	2/6 (33)	0/7 (0)
Mild	4/7 (57)	5/6 (83)	3/6 (50)	4/7 (57)
Intense	0/7 (0)	1/6 (17)	1/6 (17)	3/7 (43)
Chronic-periodontitis				
Negative	1/15 (7)	0/8 (0)	0/8 (0)	0/15 (0)
Mild	6/15 (40)	3/8 (38)	3/8 (38)	5/15 (33)
Intense	8/15 (53)	5/8 (63)	5/8 (63)	10/15 (67)

HDAC, histone deacetylase. Intensity of staining is represented as number of samples with the level of intensity out of the total number of tissue samples in that group and this is recorded as a percentage (brackets). Negative (no staining), mild (some light staining, but not all cells staining) and intense (dark staining of all endothelial cells for a particular vessel).

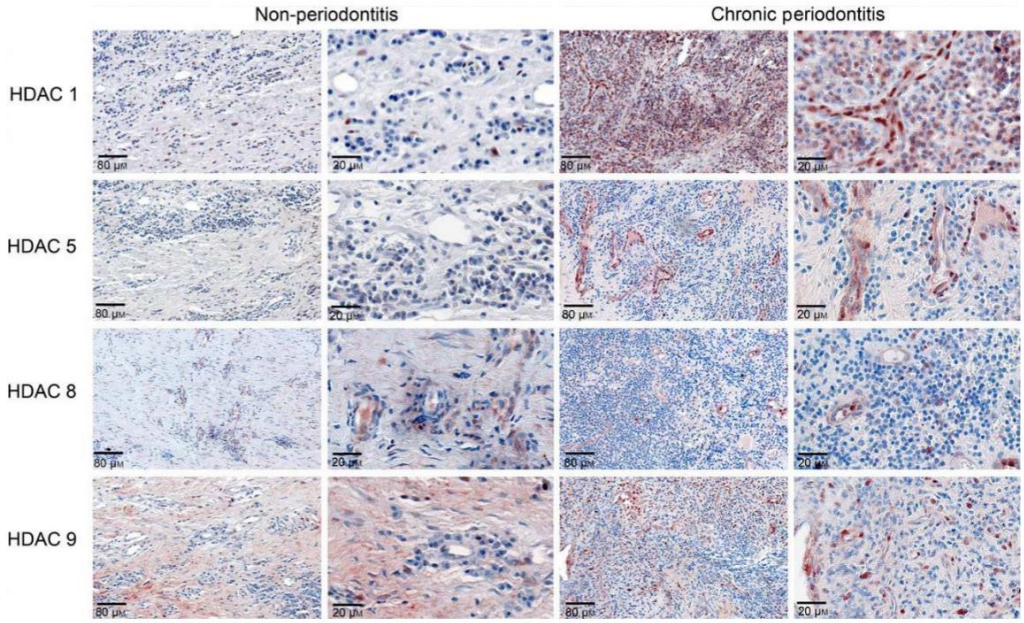

Fig. 2. Representative images of gingival tissues from non-periodontitis samples (no bone loss, healthy or mildly inflamed gingiva) or chronic periodontitis gingival tissues (taken from sites with both inflammation and bone loss). Immunohistochemistry detecting HDAC 1, 5, 8 and 9 in non-periodontitis (sample number 17; Table 1) and chronic periodontitis gingival tissues (sample number 7; Table 1). Cells positive for HDAC expression are seen as red with blue haematoxylin counterstaining. HDAC staining was imaged at 20× or 80× of Nanozoom scanned sections. HDAC, histone deacetylase.

observed in five of the eight chronic periodontitis tissue samples. In comparison, only one of the six samples assessed stained intensely for HDAC 5 and 8 in the non-periodontitis samples (Table 5). Intense HDAC 1 staining was detected in eight of 15 periodontitis samples, being compared to only negative to mild HDAC 1 staining in the seven non-periodontitis samples (Table 5). For HDAC 9, intense staining was detected in 10 of 15 periodontitis samples, being compared to only three of the seven non-periodontitis samples. Representative images in Fig. 2 demonstrate this intense staining by blood vessel lining cells in the chronic periodontitis tissues.

Cellular markers

To identify the association between HDAC 1, 5, 8 and 9 expression and specific cell lineages in chronic periodontitis gingival tissues, immunohistochemistry for different cell markers was conducted on serial sections. This included CD3 (detects lymphocytes) and factor VIII (detects endothelial cells of blood vessels) (see Fig. 3). Immunohistochemistry to detect TNF-α was also performed to identify whether HDAC expression is associated with cells expressing TNF-α. Analysis of serial sections found HDAC 1 corresponded with CD3 as well as TNF-α-positive cells. Vascular expression of HDAC 5, 8 and 9 corresponded with blood vessels expressing factor VIII. TRAP staining, indicating pre-osteoclasts/osteoclasts, was also associated with HDAC 1 in the gingival tissues (see Figs 2 and 3).

Discussion

The hypothesis that both HDAC classes I and II would be highly expressed in chronic periodontitis tissues was confirmed in this study. Class I HDACs 1 and 8 along with class II HDACs 5 and 9 were significantly elevated in human chronic periodontitis gingival tissues at the mRNA level. To date, studies assessing expression of HDACs in human inflammatory diseases have only been carried out in RA synovial tissues. In these studies, HDAC 1 was shown to be highly expressed in synovial fibroblasts from patients with RA compared to osteoarthritis controls (21,22) and to correlate with TNF-α expression (22). There are many similarities between periodontitis and RA (32–35) with high levels of inflammatory cytokines involved in the disease process of both conditions (4,36). The current results suggest that despite these similarities between periodontitis and RA there are differences in HDAC expression. HDAC 1 has been demonstrated in this current study as regulated in periodontitis and by other studies as regulated in RA (21,22). In periodontitis, we have also shown that other class I (HDAC 8) and class II HDACs (5 and 9) are also expressed.

In the present study, we demonstrate high expression of both HDAC classes in periodontitis. This is consistent with our previous study using a mouse model of periodontitis that demonstrated suppression of alveolar bone loss with a broad acting HDACi (12). Furthermore, the novel HDACi 1179.4b has been shown effectively to suppress bone loss, whereas MS-275, which is a HDAC 1 selective HDACi, had no effect (12). The alveolar bone loss was suppressed by 1179.4b despite no reduction in inflammation levels, whereas MS-275 did reduce inflammation but had little effect on

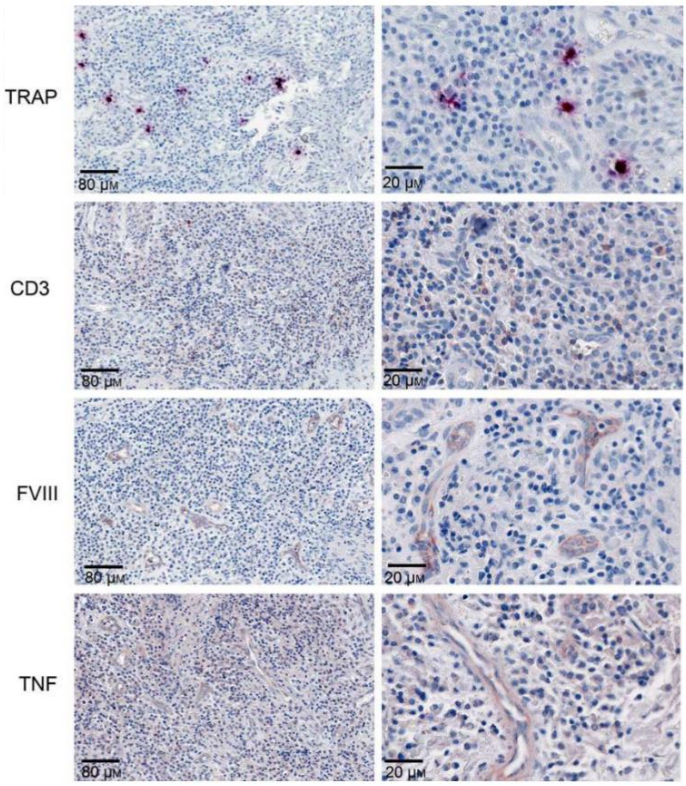

Fig. 3. Representative images of gingival tissues in serial sections of chronic periodontitis samples (sample number 7; Table 1). Cell markers used to identify corresponding cell types (TRAP, CD3, FVIII and TNF-α). Cells positive for cell marker expression are seen as red with blue haematoxylin counterstaining. Cell marker staining was imaged at 20× or 80× of Nanozoom scanned sections. FVIII, factor VIII; TNF, tumour necrosis factor; TRAP, tartrate resistant acid phosphatase.

bone erosion. This suggests that there may be some redundancy in the role of the specific HDACs in periodontitis, as more than one HDAC needs to be inhibited to observe positive effects.

Alveolar bone destruction is a characteristic feature of periodontitis, with high levels of inflammatory cytokines driving expression of the key osteoclast factor receptor activator of nuclear factor kappa B liagnd (RANKL) expression by inflammatory cells (30,37–39). The high RANKL expression results in increased formation and activity of osteoclasts leading to degradation of alveolar bone (30). It has been shown previously that HDAC 5 and 8 are highly expressed throughout human osteoclastogenesis *in vitro* and most significantly during the later stages (16). The gingival tissues used in this study were obtained from sites affected by periodontitis demonstrating evidence of associated bone loss. Macrophages and pre-osteoclasts cells, identified by TRAP staining in serial sections, were present in the gingival tissues of periodontitis tissues. The positive effects of 1179.4b in suppressing bone loss in a mouse periodontitis model (12) could be a result of targeting HDACs 5 and 8 as these specific HDACs appear important in osteoclast-mediated bone loss. A previous study demonstrated the role of HDAC 5 in bone, with its overexpression shown to reduce RANKL-mediated acetylation of NFATc1 (40). In addition, the HDAC 5 gene has been shown to be at a locus linked to bone mineral density in a genome-wide association study (41).

HDAC 8 expression has been shown by others in a range of normal tissues, including brain, thymus, testis, pancreas, liver, skeletal muscle, uterine cervix, fallopian tube, bladder wall and lung (20). It was concluded that HDAC 8 was expressed in tissues where smooth muscle differentiation occurs. Interestingly, immunohistochemical staining in the current study revealed HDAC 8-positive microvasculature in the gingival tissues, which lack smooth muscle (20). Although HDAC 8-positive microvasculature was seen in both periodontitis and non-periodontitis samples, the staining was more intense in the periodontitis samples. Class II HDAC 5 was also strongly expressed by microvasculature lining cells in both groups; however, this was more commonly seen in periodontitis samples. HDAC 1 was also strongly expressed by blood vessel lining cells, of note in the periodontitis tissues there was variation in expression, with not all cells staining positive. Blood vessel lining cells also stained strongly for HDAC 9. HDAC 9 is reported as important for the development and differentiation of a number of cell types, particularly regulatory T cells (42).

The implications of the higher levels of HDAC 1, 5, 8 and 9 expression by endothelial cells in periodontitis tissues is not clear. Of note, HDAC 5 has been shown to play a role in regulating immune cell adhesion (43). Consistent with this, a broad acting HDACi, trichostatin A, was shown to inhibit monocyte adhesion to the endothelium and this occurred via reducing expression of vascular cell adhesion molecule-1 (44). Although the proportion of positively stained cells was not statistically significantly elevated in the periodontitis tissues for HDAC 5 and 8, the RNA expression was found to be significantly higher. This is probably related to the more intense staining of the blood vessel lining cells that was noted in the periodontitis tissues. The observation that HDAC 1, 5, 8 and 9 were associated with vascular endothelium demonstrates that they are not just involved in osteoclast formation, but may also be involved with inflammation in periodontitis. Further studies are clearly necessary to elucidate whether HDAC 1, 5, 8 and 9 in the gingival vasculature play a role in the disease process.

The future use of isoform selective inhibitors will be vital to elucidate further the roles of individual HDACs in the pathogenesis of periodontitis. Research is now progressing with the development of isoform selective inhibitors, including those targeting HDAC 1 (45) and 8 (46,47) that could be considered as potential treatments for periodontitis.

This study has demonstrated expression of both class I (HDAC 1 and 8) and class II (HDAC 5 and 9) in human periodontitis gingival tissues. Given the expression of HDAC 1 by inflammatory cells (CD3- and TNF-α-positive cells) it is likely that this may be an important target along with the other HDACs to suppress inflammation. Further studies are needed to correlate expression of the HDACs demonstrated here with HDAC activity.

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Full length article

Semaphorin-3a, neuropilin-1 and plexin-A1 in prosthetic-particle induced bone loss

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ABSTRACT

Peri-prosthetic osteolysis (PPO) occurs in response to prosthetic wear particles causing an inflammatory reaction in the surrounding tissue that leads to subsequent bone loss. Semaphorin-3a (SEM3A), neuropilin-1 (NRP1) and plexin-A1 (PLEXA1) are axonal guidance molecules that have been recently implicated in regulating bone metabolism. This study investigated SEM3A, NRP1 and PLEXA1 protein and mRNA expression in human PPO tissue and polyethylene (PE) particle-stimulated human peripheral blood mononuclear cell (PBMC)-derived osteoclasts *in vitro*. In addition, the effects of tumour necrosis factor alpha (TNFα) on cultured osteoclasts was assessed. In PPO tissues, a granular staining pattern of SEM3A and NRP1 was observed within large multi-nucleated cells that contained prosthetic wear particles. Immunofluorescent staining confirmed the expression of SEM3A, NRP1 and PLEXA1 in large multi-nucleated human osteoclasts *in vitro*. Furthermore, *SEM3A*, *NRP1* and *PLEXA1* mRNA levels progressively increased throughout osteoclast differentiation induced by receptor activator of nuclear factor κB ligand (RANKL), and the presence of PE particles further increased mRNA expression of all three molecules. Soluble SEM3A was detected in human osteoclast culture supernatant at days 7 and 17 of culture, as assessed by ELISA. TNFα treatment for 72 h markedly decreased the mRNA expression of *SEM3A*, *NRP1* and *PLEXA1* by human osteoclasts *in vitro*. Our findings suggest that SEM3A, NRP1 and PLEXA1 may have important roles in PPO, and their interactions, alone or as a complex, may have a role in pathological bone loss progression.

Statement of Significance

Peri-prosthetic osteolysis occurs in response to prosthetic wear particles causing an inflammatory reaction in the surrounding tissue that leads to subsequent bone loss. The rate of hip and knee arthroplasty is increasing by at least 5% per year. However, these joint replacements have a finite lifespan, with data from the National Joint Replacement Registry (Australia) showing that the major cause of failure of total hip replacements is aseptic loosening. In aseptic loosening, wear particles liberated from prostheses are phagocytosed by macrophages, leading to release of inflammatory cytokines and up-regulation of osteoclast formation and activity. Semaphorin-3a, neuropilin-1 and plexin-A1 are axonal guidance molecules that have been recently implicated in regulating bone metabolism. This is the first report to show that these molecules may be involved in the implant failure.

1. Introduction

Total joint replacement is a procedure used to restore mobility to patients with end-stage arthritis and fractured neck of femur. The rate of hip and knee arthroplasty is increasing by at least 5% per year [1]. However, these joint replacements have a finite lifespan, with data from the National Joint Replacement Registry (Australia) showing that the major cause of failure of total hip replacements is aseptic loosening [2]. In aseptic loosening, wear particles liberated from prostheses are phagocytosed by macrophages, leading to release of inflammatory cytokines and up-regulation of osteoclast formation and activity [3–6].

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Osteoclasts are multi-nucleated cells, derived from precursor cells of the monocyte/macrophage lineage that have the specialised function of resorbing bone [7]. Osteoclast formation and activity is dependent upon macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) interaction with the receptors c-Fms and RANK, respectively [8]. Under physiological conditions, RANKL activity is regulated by its antagonist, osteoprotegerin (OPG), to maintain a balanced bone metabolism [9,10]. However, under pathological circumstances, such as chronic exposure to wear particles, elevation of RANKL occurs with associated excessive bone resorption [3].

Another key driver of osteoclast resorption and bone loss in peri-prosthetic osteolysis (PPO) is the release of tumour necrosis factor alpha (TNFα), a major cytokine of inflammation [6]. Markedly elevated levels of TNFα are present in areas surrounding pathological bone loss and correlate with elevated RANKL expression [11]. Additionally, in the presence of M-CSF, TNFα has been shown to be sufficient for inducing human macrophages, located adjacent to PPO, to differentiate into functional osteoclast independent of the RANK/RANKL signalling pathway [12].

Recently it has been recognised that, in addition to RANKL signalling, osteoclast differentiation also relies on the activation of the co-stimulatory immuno-receptor tyrosine-based activation motifs (ITAM) pathway [13]. This triggers calcium oscillation and induces the expression of the transcription factor nuclear factor of activated T cells (NFATc1) that is central to osteoclast formation [14]. Activation of the ITAM pathway occurs through the immunoglobulin-like receptors triggering receptor expressed in myeloid cells 2 (TREM2) [15] and osteoclast-associated receptor (OSCAR) [16] *via* the phosphorylation of their adaptor molecules DNAX-activating protein of molecular mass 12 kDa (DAP12) and Fc-receptor C-chain (FcRC) [14], respectively. Additionally, we have previously shown that polyethylene (PE) particles stimulate increased mRNA and protein expression of TREM2, DAP12, OSCAR, Cathepsin K and tartrate-resistant acid phosphatase (TRAP) leading to increased osteoclast activity *in vitro* [17]. It is unclear which ligand(s) stimulate the phosphorylation of the ITAM receptors (OSCAR and TREM-2), however recent findings provide insight that plexin-A1 (PLEXA1) is able to activate ITAM signalling *via* TREM2/DAP12 [18].

The semaphorin family are well established as neuro-immune molecules, however, an important role has now been identified in the skeletal system [19,20]. Semaphorin-3a (SEM3A) and its receptor neuropilin-1 (NRP1) have been shown to form a complex with PLEXA1 that is osteoprotective [21–23]. It has also been demonstrated that SEM3A secreted from neurons plays an important role in bone mass accrual [24]. SEM3A, secreted by neurons [24] and osteoblasts [21] associates with NRP1, and sequesters PLEXA1 from TREM2/DAP12, thus inhibiting ITAM signalling and suppressing osteoclast activity [21]. *SEM3A* gene expression has been demonstrated in osteoclast lineage cells, monocytes and macrophages, and increased mRNA expression of *NRP1* and *PLEXA1* was reported during macrophage differentiation under the influence of M-CSF [25]. *SEM3A* gene expression was, however, reduced or absent throughout osteoclast differentiation in mouse and rat calvarial cultures [21,24,26]. Little is known regarding the role of these molecules in human disease or during human osteoclast development *in vitro*. We hypothesise that these neuro-immune molecules are involved in the regulation of osteoclast development and are further implicated in the osteoclastic response to inflammation associated with PE particles. Therefore, this study aimed to assess mRNA and protein expression of SEM3A, NRP1 and PLEXA1 in human PPO tissues and human osteoclasts formed *in vitro*. In addition, the effect of PE particles and TNFα stimulation on expression of these factors was investigated.

2. Materials and methods

2.1. Antibodies

Primary antibodies used included polyclonal rabbit anti-human SEM3A (ab23393; Sapphire Bioscience Pty Ltd, NSW, Australia), monoclonal rabbit anti-human NRP-1 [EPR3113] (ab81321; Sapphire Bioscience Pty Ltd, NSW, Australia), monoclonal mouse anti-human NRP-1 (60067-1-Ig; Proteintech Group, Chicago, USA) and monoclonal mouse anti-human PLEXA1 (MAB6536; Bio-Scientific Pty Ltd, NSW, Australia). The relevant secondary antibodies used were donkey anti-rabbit Cy5 (11175152; Jackson Immunoresearch Laboratories Inc., PA, USA), donkey anti-mouse Cy3 (715166150; Jackson Immunoresearch Laboratories Inc., PA, USA) and goat anti-mouse Alexa 488 (115546003; Jackson Immunoresearch Laboratories Inc., PA, USA). Isotype control antibodies included universal rabbit Ig (N1699; Dako Australia Pty Ltd, NSW, Australia) and mouse IgG1κ (550878; BD Pharmingen, NSW, Australia).

2.2. Immunohistochemistry (IHC)

Tissue samples were obtained from 20 patients with PPO undergoing revision hip and knee surgeries, and osteoarthritic joint synovial tissue samples obtained from 12 patients undergoing primary hip/knee surgery at the Royal Adelaide Hospital. Tissue collection was approved by the Ethics Committee of the University of Adelaide (ethics number H-35-2001). Tissue samples were fixed in 10% normal buffered formalin, paraffin embedded and then 5 μM sections were cut and mounted on APTS-coated slides (Menzel-Gläser, Braunschweig, Germany).

IHC staining was performed on serial sections using a Universal Avidin/Biotinylated enzyme Complex (ABC) kit (PK-6200; Abacus, QLD, Australia) as reported previously [19]. Briefly, following de-waxing, slides underwent antigen retrieval for 10 min in 10 mM tris-(hydroxymethyl)-aminomethane ethylene-diamine-tetra-acetic acid (TRIS EDTA, pH 9.0) at 90 °C, and then cooled to room temperature (RT). Endogenous peroxidase activity was blocked using PBS/0.1% w/v sodium azide/0.3%v/v hydrogen peroxide at RT. Horse serum, as supplied, was applied to the sections and left for 40 min to prevent non-specific antibody binding. Sections were incubated with primary antibodies overnight; the following concentrations were used: 5 μg/ml polyclonal rabbit anti-human SEM3A, 1.9 μg/ml monoclonal rabbit anti-human NRP1, and 10 μg/ml monoclonal mouse anti-human PLEXA1. Rabbit isotype controls IgG or mouse isotype IgG1κ control were added at concentrations equivalent to those of the test antibodies. Following incubation with primary antibody, sections were incubated with biotinylated secondary antibody in the presence of normal horse serum for 45 min. Subsequently, horseradish peroxidase (HRP)-labelled ABC reagent (Abacus, QLD, Australia) was incubated on tissues as per manufacturer's instructions. An aminoethylcarbazole (AEC) kit (Abacus, QLD, Australia) was used for colour development for 20 min. Sections were counterstained with haematoxylin and lithium carbonate and slides were mounted with coverslips using GurrAquamount (Merck Millipore, VIC, Australia). Slides were photographed with Nikon FXA Research Microscope (Japan). Tissues used in this study are from the same cohort of samples previously published demonstrating expression of osteoclast-related proteins and genes, including OSCAR, Cathepsin K and TRAP [17].

2.3. Semi quantitative scoring analysis (SQA)

Stained sections were scanned with a Nano-Zoomer 2.0HT (Hamamatsu LTD, Japan). Five randomly selected areas (2 mm^2)

from each stained section were assessed in a blinded fashion by two observers, based on a 5-point scale scoring system (grade 0–4), as published previously [20]. Based on the number of positively stained cells: <5% = score 0, 6–10% = score 1, 11–25% = score 2, 26–50% = score 3 and > 50% = score 4.

2.4. Tartrate-resistant acid phosphatase (TRAP) staining

TRAP was used as a marker for the pre/osteoclast phenotype [27]. Sections were de-waxed and then underwent an activation step by immersion in 0.1 M Tris–HCl (pH 9.0) for 18 h at RT, followed by 0.1 M sodium citrate buffer (pH 5.2) for 3 h at ambient temperature [28]. The TRAP substrate was prepared using the napthol-based method [29], validated by us previously [17], and sections were incubated at 37 °C for 40 min. Sections were counterstained with haematoxylin and lithium carbonate; TRAP positive pre/osteoclasts containing more than 3 nuclei were counted in three random regions, which were photographed with 10x objective using a Nikon FXA Research Microscope (Japan).

2.5. In vitro osteoclast assay

Human peripheral blood mononuclear cells (PBMC) obtained from 12 healthy donors were isolated from whole blood buffy coats (Australian Red Cross Service; Ethics Committee of the University of Adelaide approval number H-35-2001) using ficoll-paque gradient (Lymphoprep™) as reported previously [11]. PBMC were resuspended in a complete medium: α-minimal essential medium (α-MEM); Life Technologies Pty Ltd, VIC, Australia) supplemented with 10% fetal calf serum (FCS; Life Technologies Pty Ltd, VIC, Australia), 1% penicillin–streptomycin (Life Technologies Pty Ltd, VIC, Australia), and 1% L-glutamine (Life Technologies Pty Ltd, VIC, Australia). PBMC were then seeded (5×10^5 cells/well) in duplicate into 16-well chamber slides for TRAP and protein staining (Thermo Fisher Scientific, VIC, Australia). Cells were also seeded onto 5 mm diameter slices of whale tooth dentine in 96 well trays (5×10^5cells /well) and into 48-well trays in for mRNA expression analysis in duplicate (1×10^6 cells/well) [11]. Cells were maintained at 37 °C with 5% CO_2 in complete medium with 100 nM 1α, 25$(OH)_2$ vitamin D3 (vitamin D3) (Novachem, VIC, Australia), 100 nM dexamethasone (Sigma–Aldrich Pty Ltd, NSW, Australia), and 25 ng/mL recombinant human M-CSF (Chemicon International Inc., USA) for 17 days. Adherent cells were differentiated into osteoclasts by the addition of human recombinant RANKL (50 ng/ml; Merck Millipore, VIC, Australia) from day 7 onwards [17]. Total RNA was isolated from PBMC cultures on days 0, 7, 10, 14 and 17 for qRT-PCR analysis.

Some subsets of cells were treated with Rh-TNFα (10 ng/ml) (210-TA-100; Sapphire Bioscience Pty Ltd, NSW, Australia) from day 10 in the presence of sub-optimal concentrations of Rh-RANKL (10 ng/ml) to highlight the effects of Rh-TNFα on RANKL-activated PBMCs based on previously optimised data (unpublished). RNA at day 14 was extracted for qRT-PCR. Coinciding with media changes, supernatants from days 5–7 (time before RANKL addition) and from days 14–17 (after RANKL and TNFα addition) were collected for ELISA analyses.

2.6. PE particle-stimulated PBMC-derived osteoclast culture

Ultra-high molecular weight PE (UHMWPE) particles (Ceridust 3615 PE powder, Clariant, Australia) within the biologically active size range 0.3–10 μm were prepared under sterile and endotoxin-free conditions, as described previously [11,30]. PBMC from 4 donors were prepared as above, and pre-cultured in a collagen type-1 gel (Cellmatrix Type 1-A; 637-00653, Novachem, VIC, Australia) in the presence or absence of 500 μg/ml PE particles, shown previously to be bioactive [11,30] and confirmed by preliminary experiments with phagocytosis of PE particles as an end-point, from day 1 to day 3 [17]. The collagen matrix allowed monocytes to better interact with the strongly hydrophobic PE particles to model their interaction in the tissues adjacent to implants. The concentration of particles was calculated using the TC20 cell counter device (BioRad, Cal, USA). It was calculated that there were approcimately 1000 particles for each cell. After 3 days of exposure to PE particles, the collagen matrix was digested using 0.2% w/v collagenase A (Roche Diagnostics Australia Pty Ltd, NSW, Australia) to enable the cells to be reseeded at 1×10^8 cells/well in a 12-well plate (Interpath, VIC, Australia). Non-adherent cells were removed by gentle washing as described previously [11], leaving the adherent cells with associated PE particles. After 17 days (14 days since cells were in collagen), total RNA was isolated for qRT-PCR analysis.

2.7. Analysis of osteoclast formation and activity using TRAP staining and dentine resorption assay

On day 14, cells were fixed with 4%v/v glutaraldehyde in Hanks buffered salt solution (HBSS) and stained as described [31] to assess the presence of TRAP-positive multi-nucleated cells. Characterisation of functional osteoclast activity was determined by assessing resorption pits on whale dentine slices, as described previously [31]. Briefly, after day 17 of culture, medium was removed and adherent cells detached by the addition of 0.05% (w/v) trypsin. Dentine slices were washed in pure water and air-dried prior to carbon coating. Visualisation of resorption pits by osteoclasts was conducted using a Phillips XL-30 scanning electron microscope (Adelaide Microscopy, Australia). Three images were taken at 150x magnification and the area of resorption was determined using Image J software, 1.47v.

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from PBMC cultures by the addition of 0.3 ml/well Trizol reagent (Life Technologies Pty Ltd, VIC, Australia), as per the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg RNA per reaction using Superscript III Reverse Transcriptase (Life Technologies Pty Ltd, VIC, Australia), as described previously [32]. Platinum SYBR Green qPCR Supermix-UDG (Life Technologies Pty Ltd, VIC, Australia) was used to perform qRT-PCR, as per the manufacturer's instructions. Amplification was carried out in a Rotor-Gene Q (Qiagen, VIC, Australia). All samples were investigated in triplicate and the specificity of each reaction was confirmed by melt curve analysis. Primer sequences for SEM3A, NRP1 and PLEXA1 were obtained based on previous publications [25,33]. The endogenous reference gene, human acidic ribosomal protein (hARP), was used to obtain the relative mRNA expression of each of the genes [34]. Gene expression was calculated as a fold increase from control cells using the $2^{-\Delta\Delta Ct}$ method [35].

2.9. Immunofluorescent labelling of SEM3A, NRP1 and PLEXA1 on PBMC-derived osteoclasts

On day 14, cells were fixed with 1:1 methanol:acetone solution for 5 min. Dual-staining was conducted for polyclonal rabbit anti-human SEM3A with monoclonal mouse anti-human NRP1 or monoclonal mouse anti-human PLEXA1, and for monoclonal rabbit anti-human NRP1 with monoclonal mouse anti-human PLEXA1, in addition to staining for each protein individually. The procedure was as follows: day one: 40 μg/ml anti-SEM3A (previously optimised) or 7.6 μg/ml rabbit anti-NRP1 was added to cells on slides, followed by overnight incubation in a wet chamber. Day two:

Anti-SEM3A and anti-NRP1 were tagged with donkey anti-rabbit Cy5 diluted in PBS/1% BSA, then left for 30 min at RT (in the dark), followed by incubation with the next primary antibody: anti-PLEXA1, mouse anti-NRP1. Negative controls, rabbit isotype control IgG and mouse isotype IgG control (at equal concentration to primary antibodies), were then added and slides were placed in a wet-chamber overnight at RT. Day three: anti-PLEXA1 and anti-NRP1 were tagged with goat anti-mouse Alexa488 diluted in PBS/1%BSA and controls were tagged with donkey anti-rabbit Cy5 and goat anti-mouse Alexa488, respectively. Counterstaining was carried out with Hoechst for nuclei labelling as per manufacturer's instructions (33342; Life Technologies Pty Ltd, VIC, Australia). Slides were mounted with SlowFade® Antifade Kit (536937; Life Technologies Pty Ltd, VIC, Australia) and left in the dark. The Nikon A1 Confocal microscope (NIE, Japan) was used for viewing the immunofluorescence staining.

2.10. Detection of soluble SEM3A in osteoclast culture supernatant

Levels of secreted SEM3A were detected in the supernatant of the osteoclast cultures collected at day 7 (pre-RANKL treatment) and 17 (post RANKL treatment) using a human SEM3A ELISA kit [MBS2020300; My BioSource, CA, USA], according to the manufacturer's instructions. Absorbance was measured with a Power-wave micro-plate reader (Biotek Instruments, Vermont, USA) with KC4 microplate data analysis software (Biotek Instruments) at a wavelength of 450 nm. A standard curve was generated using KC4 software and the concentration of SEM3A in each well was interpolated from the curve.

2.11. Statistical analysis

For IHC results (SQA data), a Mann Whitney-U test was used to determine differences between PPO and osteoarthritis (OA) groups. Differences between group treatments of PE/no PE and TNFα/no TNFα *in vitro*, and ELISA data, were analysed using an unpaired t-test performed with GraphPad Prism 5.01 software [CA, USA] expressed as mean ± standard error of the mean (SEM). Statistical analyses comparing qRT-PCR results from various time points for RANKL/no RANKL groups were conducted using a Dunnett's Multiple Comparison test ($n = 8$ or $n = 12$) [35]. Statistical significance was determined if $p < 0.05$.

3. Results

3.1. Increased expression of SEM3A and NRP1 in PPO tissues

Tissues used were taken from areas adjacent to PPO. The large multi-nucleated cells are likely to be foreign body giant cells (FBGC), however, they are present in close proximity to osteolysis of bone. It appears these cells may be closely related to osteoclasts as they readily become bone-resorbing cells [8,36]. Additionally, we have reported that these multinucleated cells in these same tissues express osteoclast-markers, TRAP, Cathepsin K and OSCAR [17]. Both SEM3A and NRP1 were highly expressed by the large multi-nucleated cells in PPO samples, compared with little or no expression in synovial tissues obtained at OA primary total hip replacement (Fig. 1). PLEXA1 immunostaining was conducted but very little or no staining was seen in any of the tissues (data not shown). Immunohistochemical detection of SEM3A

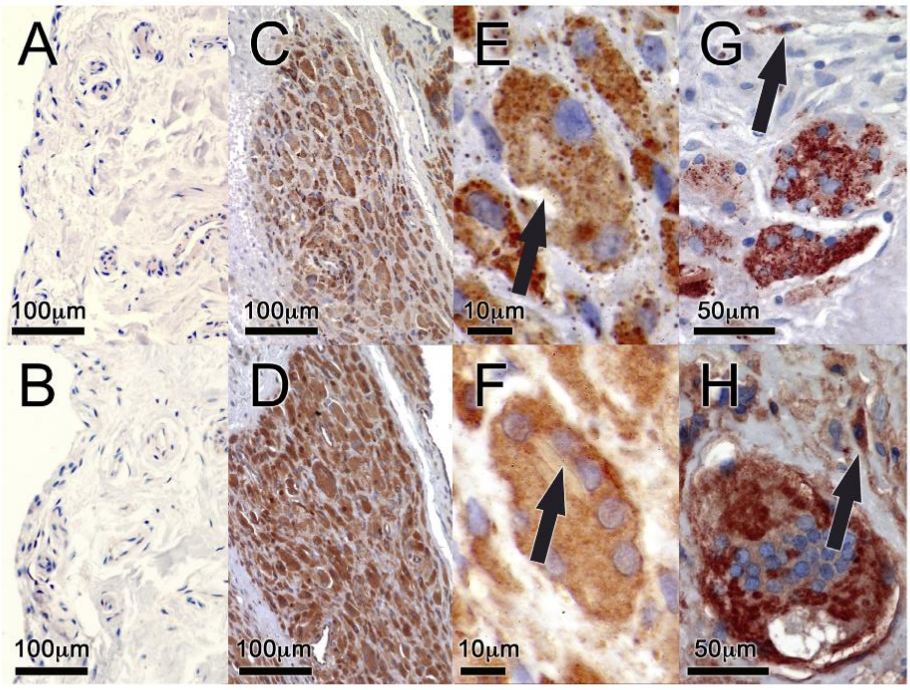

Fig. 1. SEM3A and NRP1 expression in OA and PPO tissues. Red staining represents SEM3A (A, C, E, and G) and NRP1 (B, D, F, and H) protein in OA (A and B) and PPO (C, D, E, F, G and H) tissues. Arrows in panels E and F point to positive cells for sem3A and NRP1 respectively, associated with PE particles. Arrows in G and H point to mononuclear positive SEM3A and NRP1 cells respectively.

Table 1
Semi-quantitative scoring analysis (SQA) of the immunohistochemical (IHC) results.

Proteins	PPO[a] ($n = 20$)	OA[a] ($n = 12$)	P value
SEM3A	2.62 ± 0.76	0.58 ± 0.24	0.006*
NRP1	2.86 ± 0.86	1.11 ± 0.37	0.017*

[a] Mean SQA ± SEM.
* $P < 0.05$ statistically significant.

(Fig. 1C and E) and NRP1 (Fig. 1D and F) in PPO tissue was carried out on serial sections from the same block (adjacent 5 μm sections), hence the cells shown in Fig. 1E and F are likely to be part of the same cell. Abundant PE particles were seen in these tissues, as indicated by the arrow in Fig. 1. SEM3A consistently appeared to be located in discrete cytoplasmic compartments (Fig. 1G). NRP1 expression had two distinct location patterns, either in discrete cytoplasmic compartments (similar to SEM3A) or diffusely in the cytoplasm with strong expression on the cell surface (Fig. 1H), consistent with its role as a cell surface receptor. The SEM3A and NRP1 expression, as assessed by SQA, was found to be significantly higher in PPO than OA tissues ($p < 0.05$; Table 1). Previous studies have indicated monocytes and macrophages also express mRNA for SEM3A, NRP1 and PLEXA1 [25]. Consistent with this we did note that some mononuclear cells express SEM3A and NRP1 (indicated in panels G and H of Fig. 1). However, these proteins were predominately found in large multi-nucleated cells.

3.2. SEM3A, NRP1 and PLEXA1 mRNA expression increase during human osteoclast differentiation in vitro

IHC detection of SEM3A and NRP1 in multi-nucleated osteoclasts in PPO tissues suggested that these molecules could be produced by osteoclasts. To investigate production of these molecules by osteoclasts, SEM3A, NRP1 and PLEXA1 mRNA expression was assessed in PBMC-derived human osteoclasts *in vitro*. All three genes were expressed during osteoclast development *in vitro* and expression was found to increase during the 17-day differentiation period, with significantly higher expression of *SEM3A* observed at day 17 compared to day 0. *NRP1* mRNA levels were higher at day 14 relative to day 0, *PLEXA1* mRNA levels were higher on days 10 and 17 compared to day 0 (Fig. 2).

3.3. PE particles in the presence of RANKL up-regulate mRNA expression of SEM3A, NRP1 and PLEXA1 in human osteoclasts in vitro

Given the observed expression of SEM3A and NRP1 protein in human PPO tissues (Fig. 1), *in vitro* differentiation of osteoclasts was stimulated in the added presence of PE particles. Significantly higher mRNA expression of *SEM3A* and *NRP1* was observed at day 14 in PE-stimulated osteoclast cultures compared to non-PE-stimulated cells ($p < 0.05$; Fig. 3). The apparent increase in *PLEXA1* mRNA in the presence of PE did not achieve significant difference possibly due to the high variation between donors and generally low levels expressed. This may be consistent with the low expression of PLEXA1 as seen in PPO tissues. Interestingly, PE particles in the absence of RANKL did not significantly alter expression of SEM3A, NRP1 or PLEXA1, other than SEM3A at day 7 (Supplementary data Table 1).

3.4. SEM3A, NRP1 and PLEXA1 show specific localisation in osteoclasts formed in vitro

Dual-immunofluorescence staining was conducted to investigate the detailed cellular localisation of SEM3A, NRP1 and PLEXA1 in osteoclasts formed *in vitro*. SEM3A (Fig. 4A and I) was found highly concentrated in the cytoplasm of large multi-nucleated cells. NRP1 (Fig. 4E and J) was dispersed throughout the cells and concentrated on the outer lining of the membrane. PLEXA1 (Fig. 4B and F) was detected throughout the cytoplasm and perinuclearly. In addition, dual-labelling between SEM3A or NRP1 with PLEXA1 and between SEM3A with NRP1 was conducted on osteoclasts *in vitro* to gain insight as to whether the molecules resided as a complex (Fig. 4C, G and K) as seen in other cell types [37]. Colocalisation of all proteins was found within the cell cytoplasm.

3.5. SEM3A is secreted by human PBMCs and osteoclasts in vitro

Since it has been suggested that secreted SEM3A by osteoblasts and neurons inhibits osteoclastic bone resorption [21,24], the possibility of osteoclast release of SEM3A was investigated *in vitro* during osteoclast differentiation. Soluble SEM3A was measured in supernatants from days 5–7 (prior to RANKL addition) and days 14–17 (following RANKL addition at day 7). Unlike the gene expression data, levels of soluble SEM3A were not different at the two time points, with SEM3A levels of 2.08 ± 0.56 ng/ml on day 5–7, comparable to 2.30 ± 0.72 ng/ml on day 17 ($p = 0.82$).

3.6. Influence of TNFα on SEM3A, NRP1 and PLEXA1 gene expression in osteoclasts in vitro

TNFα is an important inflammatory mediator in PPO [11]. Therefore, *SEM3A*, *NRP1* and *PLEXA1* gene expression was assessed in TNFα treated osteoclast cultures at day 14. TNFα decreased the expression of *SEM3A*, *NRP1* and *PLEXA1* mRNA expression in human osteoclasts differentiated *in vitro* (Fig. 5).

4. Discussion

SEM3A has been reported to support bone formation [24,26]. However, this study provides evidence that SEM3A, in association with its receptors, NRP1 and PLEXA1, may also have a role in osteoclast differentiation and function. Interestingly, our findings are in

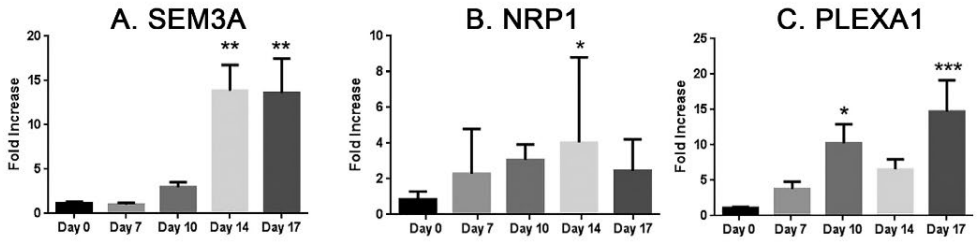

Fig. 2. *SEM3A*, *NRP1* and *PLEXA1* mRNA expression during osteoclast formation *in vitro*. Graphs represent mean ± standard error of the mean (SEM) of fold increase in gene expression (relative to hARP) in osteoclasts derived from 12 donors. Significance (*$p < 0.05$, **$p < 0.002$, **0.001) compared to day 0.

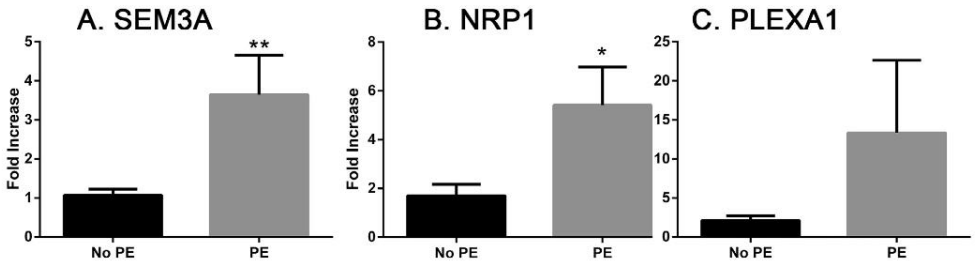

Fig. 3. *SEM3A*, *NRP1* and *PLEXA1* mRNA expression during osteoclast formation *in vitro* in the presence of PE particles. The mean fold increase in gene expression (relative to hARP reference gene) in day 14 osteoclasts exposed *in vitro* to PE particles, compared to controls. Shown is the mean fold increase ± SEM, in cells obtained from 4 donors. ($^{*}p < 0.05$, $^{**}p < 0.01$).

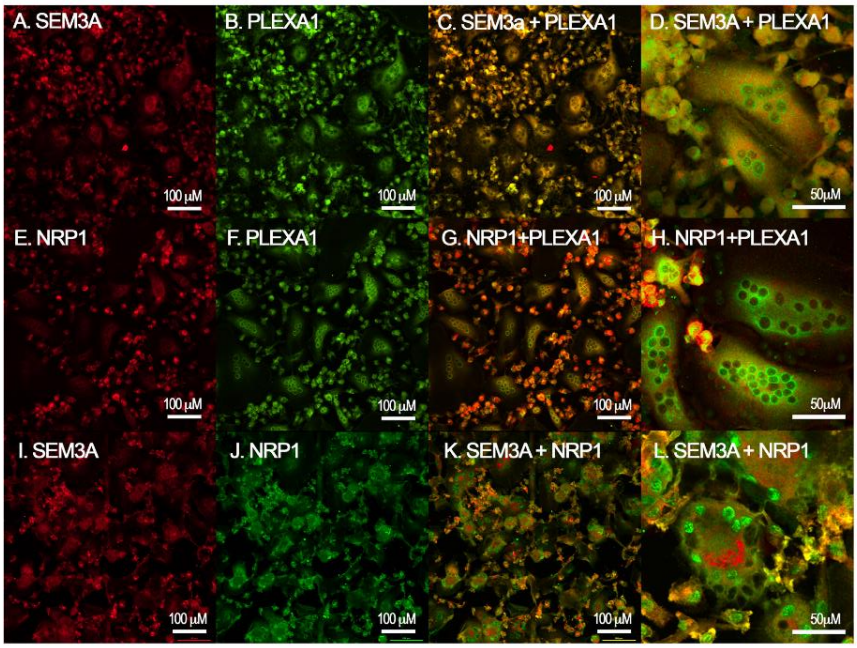

Fig. 4. SEM3A, NRP1 and PLEXA1 protein expression in human osteoclasts *in vitro*. Human osteoclasts differentiated *in vitro* with protein shown by immunofluorescence. Panel A: SEM3A shown in red, panel B: PLEXA1 shown in green and dual-staining of SEM3A and PLEXA1 in panels C and D. Panel E: NRP1 shown in red, panel F: PLEX1A shown in green and dual-staining for NRP1 and PLEXA1 in panels G and H. Panel I: Sem3A shown in green, panel J: Nrp1 shown in red and dual-staining for SEM3A and NRP1 in panels K and L. Areas of overlay are highlighted in yellow/orange. Dual-labelling (D, H and K) further displays co-localisation of the molecules.

contrast to the work of Hayashi et al., who reported that SEM3A had an osteoprotective effect via osteoblastic inhibition of osteoclastic resorptive activity [21]; however, analyses were conducted in murine systems, which could account for the lack of SEM3A expression by murine osteoclasts. In the present study, SEM3A expression was detected predominantly in cytoplasmic vesicles of the large multi-nucleated cells present in PPO tissues. A possible explanation for the sub-cellular localisation is that the SEM3A-NRP1-PLEXA1 complex may be internalised by osteoclasts [38], as has been shown in neuronal and endothelial cells [39,40]. This mechanism allows the receptor to select signals from multiple ligands and/or inhibit their effect on the cell [41,42]. Clathrin-mediated endocytosis is a cellular mechanism used for ligand/receptor recycling and selective signalling [43], and has been shown to be employed in osteoclasts [38]. However, dual-staining for SEM3A, NRP1 or PLEXA1, with Clathrin, did not result in obvious co-localisation (data not shown).

The SEM3A-NRP1-PLEXA1 complex has been reported to be associated with apoptosis [44]. Physiologically, osteoclasts are short-lived, surviving for about 2 weeks [45] before dying by apoptosis [46]. Increased gene expression of the SEM3A-NRP1-PLEXA1 complex has been observed in human monocytes when apoptosis

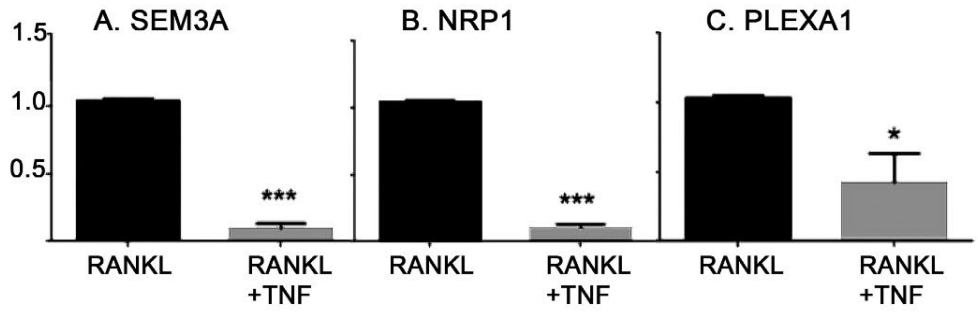

Fig. 5. *SEM3A*, *NRP1* and *PLEXA1* mRNA expression in osteoclasts *in vitro* following treatment with TNFα for 72 h. The mean fold in gene expression relative to hARP (reference gene) is shown as mean ± SEM in cells derived from 8 donors. Significance ($^{*}P < 0.05$; $^{***}p < 0.0001$) compared to no addition of TNFα (RANKL only; control).

is induced [25]. This may explain why the highest mRNA level of the three molecules was observed late in the human osteoclast *in vitro* cultures. It has been reported that there was no difference in the cell proliferation rate or the percentage of apoptotic cells among osteoclast precursors between SEM3A-treated and untreated cells [21], however, further studies investigating the role of these molecules in osteoclast apoptosis are needed.

We investigated the location of SEM3A and NRP1 within osteoclasts to investigate the complex formation seen in other cell types [37]. Dual-labelling of human osteoclasts *in vitro* suggested that SEM3A, NRP1 and PLEXA1 are concentrated within distinct regions of large multi-nucleated cells. SEM3A protein was highly concentrated within the cytoplasm, whereas, NRP1 was concentrated in the external borders of these cells. This localisation of NRP1 on or near the surface of large multi-nucleated cells was noted in both osteoclasts that formed *in vitro* and in pre/osteoclastic cells seen in the PPO tissues. This is consistent with the role of NRP1 as a receptor and as a highly conserved trans-membrane protein, originally identified as an adhesion molecule during neuronal development [47]. Finally, PLEXA1 had a peri-nuclear location, consistent with localisation to the Golgi apparatus. Plexins are crucial for actomyosin contraction and microtubule destabilisation, and plexin-mediated signalling has been implicated in the inhibition of integrin-mediated cellular adhesion and cytoskeletal remodelling functions important to osteoclast activity [48,49]. Some co-localisation was seen, predominantly in the cytoplasm, suggesting that some complex formation may exist within human osteoclasts.

We have described previously the 'hijacking' of physiological osteoclastogenesis in a number of pathological bone loss scenarios, including PPO, and shown that inflammatory cytokines may synergise with, or replace, RANKL in the presence of PE particles [11,50]. TNFα is an important inflammatory cytokine involved in stimulating osteoclast resorption in bone loss pathologies [11,51]. As SEM3A is also involved in immune reactions [52], we investigated the relationship between the two molecules. In this study, we found that *SEM3A*, *NRP1* and *PLEXA1* mRNA expressed by osteoclasts were all decreased in response to the addition of TNFα for 72 h *in vitro*. Recent studies have reported that the treatment of skin cells with TNFα does not affect either the mRNA or protein levels of SEM3A. However, there was a decrease in SEM3A expression in the presence of Interleukin-4 (IL-4; an inflammatory cytokine) and TNFα [53]. In allergic responses, SEM3A treatment decreased the serum levels of both TNFα and IL-4 [54], possibly suggesting each can inhibit the other. This is consistent with SEM3A suppression of immune reactions by inducing apoptosis in macrophages [25] and decreasing the production of both Th2-related cytokines and pro-inflammatory cytokines [54]. While we noted *SEM3A*, *NRP1* and *PLEXA1* mRNA expression are increased in response to PE particles, which are known to be associated with inflammation in PPO, this indicates that the role of SEM3A in regulating inflammation is complex. Its involvement in immune responses, particularly within bone, requires further investigation.

5. Conclusion

SEM3A, NRP1 and PLEXA1 are well-established molecules of the immune and nervous systems. This is the first study to demonstrate the expression of these axonal guidance molecules in the human bone pathology of PPO and human osteoclasts *in vitro*. While this report is largely descriptive, further studies of the molecular interactions will provide important information about bone diseases in general and may lead to novel therapies in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2015.11.025.

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