OSTEOCLAST-ASSOCIATED INTRACELLULAR ITAM SIGNALLING MOLECULES IN HUMAN PERI-IMPLANT OSTEOLYSIS AND RHEUMATOID ARTHRITIS

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ABSTRACT

Peri-implant osteolysis (PO) and rheumatoid arthritis (RA) are examples of local inflammation-mediated bone loss, in which osteoclasts are believed to mediate the osteolysis. Besides the well-established OPG/RANK/RANKL system, ITAM-mediated signalling pathway has been found to be the co-stimulatory intracellular pathway mediating osteoclast differentiation and activity. TREM2, DAP12, FcRγ and OSCAR are components of the ITAM-mediated signalling pathway identified in osteoclasts. Another important molecule in the osteoclasts regulation is NFATc1, the key transcriptional factor mediating osteoclastogesis. Despite their known importance in the regulation of osteoclast and bone resorption, little is known if there any alteration in the expression of these molecules could be associated with the progression of bone loss in PO and RA.

In relation to study in context of PO, the expression of ITAM-related molecules, TREM2, DAP12, OSCAR and FcRγ, along with NFATc1 and osteoclast cell marker cathepsin K in PO tissues in comparison to OA tissues was examined at protein level through immunohistochemistry as well as at mRNA level using qRT-PCR. The effects of PE particles, a common PO-induced wear particles, on osteoclast formation and resorption activity as well as mRNA expression of NFATc1 and ITAM-associated molecules were studied *in vitro* using a novel collagen gel PBMC assay. As for studies on RA, the expression of all those molecules in RA (active and inactive) tissues was compared to OA and normal tissues. The levels of soluble OSCAR in synovial fluids from RA and OA patients was also measured through ELISA and compared. Following observation on immunostaining of RA tissues, the regulation on the expression of OSCAR in endothelial cells following TNFα and IL-1β stimulation was studied in BMEC culture *in vitro*. OSCAR protein expression was analysed through immunofluoresence and ELISA on the cell culture supernatants meanwhile mRNA level was measured using qRT-PCR.

Higher level of protein and mRNA for all those ITAM-associated molecules and cathepsin K was found in PO compared to OA tissues. Closer examination on tissue immunostaining found presence of PE particles inside and close to some cells positive for ITAM-related

molecules. Investigation on the effect of PE in culture of PBMC-derived osteoclast cells found that the particles promote more osteoclasts formed and higher resoprtion activity. The PE particles also appeared to stimulate the mRNA expression of cathepsin K and all ITAM-associated molecules studied. Examination on the immunostaining indicated that highest number of cells positive for NFATc1, TREM2, DAP12, OSCAR and FcRγ in active RA tissues compared to inactive RA, OA and normal tissues. High concentration of soluble OSCAR was found in synovial fluids of both RA and OA groups. Study on the expression OSCAR in BMEC demonstrated that TNFα and IL-1β could upregulate the expression of mRNA and protein in secreted form.

In general the expression of NFATc1, TREM2, DAP12, OSCAR and FcRγ was found high in PO and RA. Induction in expression of ITAM-associated molecules by PE particles and stimulation of OSCAR expression in endothelial cells by pro-inflammatory cytokines may suggest that these molecules may have role in the progression of PO and OA.

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ABBREVIATIONS

ACPA	anticitrullinated protein antibodies
ACR	American College of Rheumatology
AEC	3-amino-9-ethylcarbzole
AP1	activator protein-1
APTS	aminopropyltriethoxy-silane
Atpv0d2	ATPase V_0 domain
BMD	bone mass density
BMEC	bone marrow endothelial cell line
BMMs	bone marrow-derived monocyte/macrophage cells
BMUs	basic multicellular units
BSA	bovine serum albumin
Ca^{2+}	calcium
CCR	C-C chemokine receptor
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
CO_2	carbon dioxide gas
COCr	cobalt chromium
cpTi	commercially pure titanium
CRP	C-reactive protein
CsA	cyclosporin A
C_T	comparative threshold
CTR	calcitonin receptor
CVD	cardiovascular disease
DAP12	DNAx-protein 12kDa
DAPI	4', 6-Diamidino-2-phenylindole
DC-STAMP	dendritic-cell transmembrane protein
DEPC	diethylpyrocarbonate
DMARD	disease modifying antirheumatic drug
DNA	deoxyribonucleic acid
DPX	dibutyl phthalate xylene
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	endothelial cell growth factor
ELISA	enzyme-linked immunoabsorbant assay
ESR	erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FBGCs	foreign-body giant cells
FBLC	fibroblast-like cell
FBS	fetal bovine serum
FcRγ	Fc receptor common gamma-subunit chain
FCS	fetal calf serum
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

GM-CSF	granulocyte-macrophage colony-stimulating factor
H&E	hemotoxylin eosin
hARP	human acidic ribosomal protein
HBSS	Hank's balanced salt solution
HMVEC	human microvascular endothelial cells
HRP	horse radish peroxidase
HUVECs	human umbilical vein endothelial cells
ICAM	intracellular adhesion molecules
IgG	immunoglobulin G
IL	interleukin
IL-1R	IL-1 receptor
IL-1ra	IL-1 receptor antagonist
IP_3	inositol triphosphate
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
LDL	low density lipoprotein
LPS	lipopolysaccharide
M-CSF	macrophage colony stimulating factor
mAb	monoclonal antibody
MCP-1	monocyte chemoattractant protein-1
MDL-1	myeloid DAP12-associated lectin-1
mg	milligram
MIP-1	macrophage inflammatory protein 1
MIP-1α	macrophage inhibitory factor-1α
MIP1γ	macrophage inflammatory protein 1-gamma
MITF	microphthalmia transcription factor
ml	milliliter
mm	milimeter
MMPs	matrix metalloproteinases
MNC	multinucleated cell
mRNA	messenger ribosomal nucleic acid
MTX	methotrexate
NFATc1	nuclear factor activated T-cell 1
NFκB	nuclear factor-kappa-B
ng	nanogram
NK	natural killer
NO	nitric oxide
NRS	normal rabbit serum
NSAIDs	non-steroidal anti-inflammatory drugs
OA	osteoarthritis
OCT	Optimal Cutting Temperature medium
OPG	osteoprotogerin
OPG-Fc	OPG-fusion protein
ORO	Oil Red O
OSCAR	osteoclasts-associated receptor
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PE	polyethylene
PECAM-1	platelet-endothelial cell adhesion molecule 1

PGE_2	prostaglandin E_2
PIAS3	protein inhibitor of activated STAT 3
PIR-A	paired Ig receptor-A
PLCγ	phospholipase Cγ
PLOSL	polycystic lipomembranous osteodysplasia with sclerosis leukoencephalopathy
PMMA	polymethylmethacrylate
PO	peri-implant osteolysis
PP	peri-prosthetic
qRT-PCR	quantitive reverse-transcription polymerase chain reaction
RA	rheumatoid arthritis
RANK	receptor activator of NF kappa B
RANK-Fc	RANK fusion protein
RANKL	receptor activator of NF kappa B ligand
RANTES	regulated upon activation, normal T cell expressed and secreted
RF	rheumatoid factor
RGD	tripeptide arginine-glycine-aspartic acid
RNA	ribosomal nucleic acid
rpm	rotations per minute
RT	reverse-transcription
SDF-1	stromal-cell derived factor-1
SEM	standard error of mean
SH2	Src homology 2
SIRPβ	signal regulatory protein β
sOSCAR	soluble/ secreted form of OSCAR
SQA	semiquantitaive analysis
sRANKL	soluble RANKL
TACE	TNF-α converting enzyme
TMB	3, 3', 5, 5'-tetramethylbenzidine
TNF	tumor necrosis factor
TNFR	TNF receptor
TNFα	tumor necrosis factor-α
TRAF	TNF receptor activating factor
TRAP	tartrate-resistance acid phosphotase
TREM2	triggering receptor expressed by myeloid cells-2
USFs	upstream stimulating factors
V-ATPase	vacuolar (H^+) ATPase
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
ΔC_T	delta/difference in the comparative threshold

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1. LITERATURE REVIEW

1.1. Bone Remodeling

Bone is a very important structure that plays numerous crucial roles in human as well as animal biology. The bone plays essential roles in protecting the brain, lungs and bone marrow as well as storage site for calcium, phosphorus and quite number of proteins. In addition, it also functions as the site for harboring stem cells and commited hematopoietic and immune cells. Lastly, it is also well acknowledged that bone is a very important structure for mechanical support in the body (Parfitt 1987; Ross & Christiano 2006).

Bone is a dynamic tissue that undergoes physiological remodeling throughout life even though skeletal growth and modelling have been completed. Dynamic bone remodelling is essential for adaptation to mechanical constraints and to maintain appropriate levels of calcium, phosphorus and proteins stored in bone, as reviewed in Grimaud et al. (2003), besides its other roles in tooth eruption and removing fracture as well as renewing old bone (Vaananen et al. 2000).

Bone remodelling involves two tightly coupled events, which are bone resorption by osteoclasts and bone formation by osteoblasts. The remodeling of bone occurs in discrete small packets of cells called basic multicellular units (BMUs) (Frost 1964) throughout the skeleton. These BMUs consists of osteoclasts, osteoblasts and their precursors. Interaction between osteoclasts and osteoblasts either through cell-to-cell contact or paracrine method is in a bidirectional fashion (Matsuo & Irie 2008). Once the process of bone resorption is completely accomplished, it is believed that there are factors or “signals” sent and sensed by osteoblast to start bone formation process. Osteoblasts perform their function in bone formation by laying down unmineralized matrix composed of type I collagen and proteoglycans, which later become mineralised, onto the area where the bone has been resorbed. Besides, there are large amount of previous studies that also collectively indicate that osteoblasts regulate the differentiation and function of osteoclasts during normal bone turnover (Suda et al. 2001).

In healthy adult bone remodelling the processes of bone resorption and formation counter balances each other (reviewed in Grimaud et al. 2003) resulting in no net change in overall

bone volume. However in pathological bone loss diseases, the balance between bone formation and resorption is believed to be disrupted in favour of the latter, leading to excessive localised or net bone degradation. For instance, previous literature has indicated that there is an imbalance between bone formation and resorption in rheumatoid arthritis (RA) (as described in 1.3.3 below) and adjacent to prosthetic implants that leads to the bone loss and joint destruction or implant loosening (Goldring 2003; Gravallese et al. 2000; Redlich et al. 2002b).

1.2. Inflammation-Associated Bone Diseases

Bone loss is a serious public health problem in our growing, aging global population. In general, bone loss could be divided into two main categories; systemic and local bone loss. A well known example of systemic bone loss is osteoporosis, a disease characterised by decrease in bone mass and density which leads to increase in the bone fragility and hence susceptibility to bone fractures.

Besides systemic bone loss, diseases associated with localised bone loss also receive significant attention in orthopaedic research considering the high prevalence of the diseases and morbidity. Commonly described diseases associated with local bone loss include peri-implant osteolysis (PO), RA and periodontal disease. RA, for example, has been reported to affect about half million of the Australian population in 2007 (Economics 2007). However, in this review, focus will be placed on the pathology of PO and RA only.

There are quite a number of similarities shared between PO and RA. As the name of chronic inflammation-mediated local bone loss implies, in the pathology of these diseases, the bone loss occurred is highly associated with inflammation in the tissue microenvironment. Research over decades found that there is very close relationship between the bone loss and inflammation in these diseases. Due to this fact, a new term of osteoimmunology was first used to describe the close relation between bone homeostasis and immune systems (Arron & Choi 2000).

Osteoimmunology is becoming more relevant term in normal physiology and pathology as it demonstrates the complex crosstalk between immune system and bone remodeling unit

involving coordination of lymphocytes, mast cells and macrophages on osteoclasts, osteoblasts and osteocytes (Takayanagi 2007a; Walsh et al. 2006). This crosstalk becomes more obvious in the context of inflammation-mediated bone loss like particle-induced PO and RA. By virtue of this, it is interesting to look at the inflammation-mediated bone loss from the view point of osteoimmunology. Regulating immunomodulatory factors that play role in bone homeostasis will potentially be a therapeutical approach that will cover both bone and immunological sides in those diseases of inflammation-mediated bone loss.

1.2.1. Peri-implant Osteolysis

The invention and application of prostheses has been widely acknowledged as one of the most successful achievement in medical history. Nevertheless, implant failure is becoming a big problem and major concern haunting and shadowing this wonderful application. It was estimated that between 5 to 30% of total joint replacement require revision surgery within 15 to 20 years time after prostheses were primarily implanted (Allami et al. 2006; Callaghan et al. 1998; Fender et al. 1999; Graves et al. 2004). The loss in bone stock around the prostheses not only leads towards loss of fixation of the prostheses, but also revision surgery becomes more complicated and expensive with considerable risk of morbidity and even mortality. This problem becomes more significant when considering the community is aging due to medical advancement and improved lifestyle. Whilst the benefits offered by prostheses allow the application to become more widely accepted in the community, it is becoming a great concern that prostheses are being introduced to the younger community group. Data from Kavanagh et al. (1994) suggests that people from the younger group of age when the prostheses were first implanted have higher risk for implant failure (27% for < 59 years old, 13% for 59-65 years old and 7.5% for 65-70 years old). Together, all these factors contribute towards the increasing need for hip and knee replacement, reported to be between 5 to 10 % each year (Graves et al. 2004), leading to growing concern regarding implant failure as a critical health problem awaiting the community.

There is a number of factors identified as contributing towards aseptic prosthetic loosening such as fracture, infection, poor surgical technique, stress shielding and mechanical failure (Aspenberg & van der Vis 1998; Holding et al. 2006; Skripitz & Aspenberg 2000). However, studies over the few decades found that aseptic prosthetic loosening is the most common

reason for prosthetic implant joint failure requiring surgical revision (Graves et al. 2004; Kesteris et al. 1998; Malchau et al. 1993).

Aseptic loosening following PO is a complication that occurs as a result of granulomatous inflammation in the soft tissues in the vicinty of the prostheses, characterised by massive infiltration of inflammatory cells into the tissues. This lesion is characterised by the presence of inflammatory cells like macrophages, foreign-body giant cells, lymphocytes, fibroblasts and dendritic cells. The granulomatous lesion is also associated with the formation of synovial-like pseudomembrane predominated by macrophages at the interface of bone-cement around loosening implants (Goldring et al. 1983). The inflammation around the prostheses involves the release of inflammation-triggering mediators and chemoattractants for recruiting inflammatory cells by macrophages and other phagocytic cells following the phagocytosis of particle wear debris from the implanted prostheses (Baumann et al. 2004; Holding et al. 2006; Horowitz & Gonzales 1997; Matthews et al. 2001; Murray & Rushton 1990; Rader et al. 1999; Ren et al. 2008). This is most widely accepted theory for aseptic loosening by scientific community and this is further discussed in the following chapters. This inflammation condition, denoted as an increase in the release of pro-inflammatory cytokines within the tissue (Jiranek et al. 1993) will eventually in favour an increase in the bone resorption activity and lysis on bones around the prosthesis, a phenomenon known as PO (further details see section 1.3.3 below).

Studies over decades have suggested that there is an association between risk for aseptic loosening and implant wear rates. Patients with implant wear rates of more than 0.15mm/year are classified as having high risk of aseptic loosening (Sochart 1999). Meanwhile in general, normal wear rate for prostheses of well functioning prostheses is about 0.05mm/year (Ilchmann et al. 1998). It has been discovered from accumulating amount of studies that several types of materials liberated from components of prothesis have ability to regulate bioactivity of interacting cells and believed to play role in triggering particle-induced osteolysis. Those commonly-associated particles includes metals (Haynes et al. 1998; Haynes et al. 1993; Kaufman et al. 2008), polymethylmethacrylate (PMMA) cement (Jones et al. 2001) and polyethylene (PE) (Holding et al. 2006), a material usually used as lining in acetabular cup to reduce friction against the hard metal or ceramic femoral head. However, in this thesis, discussion is made to focus on PE only as it is seen as the most damaging particles being intimately associated with PO. This could be simply evaluated from the facts that

i) there is strong correlation between PE wear rates and the extent of osteolysis (Howie et al. 2007; Kadoya et al. 1998; Oparaugo et al. 2001; Orishimo et al. 2003),
and
ii) highest concentration of PE particles at the sites of osteolysis (Kobayashi et al. 1997).

The generation of PE particles is inevitable result from wear even in normal functioning prostheses, therefore the effect of PE particles on tissue and cellular activity should be considered as a major concern and this will discussed in the Chapter 2 and more thoroughly in the following Chapter 3. There are accumulating publications studying different materials as strategy to reduce the occurance of PO.

To date the diagnosis for assessing the extent of PO is only carried out through radiographic evaluation. Nevertheless, this approach is hindered by several limitations. Through this technique, the detection on the onset of osteolysis is limited by frequency of radiographs taken post-implantation. It is also a concern that standard anteroposterior radiographs oftenly undermine the extent of osteolysis lesion around metal-backed acetabular component. While there have been ideas related to detecting free particles liberated, such as PE, from implants as a strategy to detect the onset of PO, it seems to be not a practical approach due to the limitation in detecting the particles, which obviously be in very small amount in the early stage of wear and PO. Therefore, identifying biomarkers for diagnostic tool could provide better option for detecting and assessing early stage of PO. Studies relating to PO is the main theme covered in the following 2 chapters.

1.2.2. Rheumatoid Arthritis

RA is a systemic autoimmune disease with predominant clinical manifestation in the joint. Pathologies of RA joints includes chronic inflammation of the synovium (synovitis), thickening of the synovium and pannus formation which leads to cartilage and bone erosion. Hence RA is a typical example of chronic inflammation-induced localised bone loss.

It has been estimated that, in Northern American and North European regions, RA accounts for prevalence of 0.5-1% with a mean annual occurrance of 0.02 to 0.05% (Alamanos &

Drosos 2005). Despite the high number of patients having this disease, the main cause for the disease is still unknown and under study, however studies in the past have pointed out quite a number of factors associated with RA such as infection and autoimmunity (Weyand & Goronzy 1997), environment as well as genetics (Stahl & Raychaudhuri 2012).

The classification of acute RA has been widely made based on American College of Rheumatology (ACR) 1987 criteria (Arnett et al. 1988), as presented in the following Table 1.1. Patients are diagnosed as RA if at least 4 criteria listed are fulfilled, with the first four (1-4) criteria present for at least 6 weeks. Nonetheless, a much more recent criteria for RA (Aletaha et al. 2010), presented in the following Table 1.2, has been developed and becomes current guideline for identifying RA, with a total score of 6 out of 10 gives classification as RA (Table 1.2).

One of the hallmark clinical features of RA is the inflammation of synovium (or known as synovitis) associated with the infiltration of inflammatory cells into the synovium. The massive infiltration of inflammatory cells, which includes the likes of macrophages and T-cells, is believed to lead to the hyperproliferation observed in the synovial membrane. It is believed that the inflammatory mediators released by those cells contribute towards the destruction of bone and cartilage tissue, as reviewed in Walsh et al. (2005). This conclusion is based on the results shown in many studies, including in animal models (Ji et al. 2002; Shealy et al. 2002; van den Berg 2001; Zwerina et al. 2004).

Research in the past has also characterised angiogenesis as another common pathological feature of RA. Angiogenesis could be defined as the physiological process of the formation of new blood capillaries from the pre-existing vessels. In RA angiogenesis occurs in response to support and maintain the inflammation state by transporting inflammatory cells to the inflammation site and to accommodate demands nutrients and oxygen by the growing pannus.

Table 1.1: ACR 1987 Revised Criteria for RA

No.	Criterion	Definition
1	Morning stiffness	Morning stiffness in and around joints lasting at least 1 hour before maximal improvement
2	Arthritis of 3 or more joint areas	Soft tissue swelling of at least 3 joint areas observed by physician.
3	Arthritis of hand joints	At least 1 swollen area in wrist, proximal interphalangeal and metacarpophalangeal.
4	Symmetric swelling	Simultaneous swelling on joint areas refered in both sides of the body.
5	Rheumatoid nodules	Subcutaneous nodules over bony prominences or extensor surfaces, or in juxtaarticular regions, as observed by physician.
6	Rheumatoid Factor (RF)	Detection of abnormal level of serum RF.
7	Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints

Adapted from Arnett et al. (1988)

Table 1.2: The 2010 ACR-European League Against Rheumatism (EULAR) classification criteria for RA

No.	Criterion	Observations	Score
1	Joint Involvement	1 large joint	0
		2 to 10 large joints	1
		1-3 small joints (with or without involvement of large joints)	2
		4-10 small joints (with or without involvement of large joints)	3
		>10 joints (at least 1 small joint)	5
2	Serology	Negative RF and negative anticitrullinated protein antibodies (ACPA)	0
		Low-positive RF or low-positive ACPA	2
		High-positive RF or high-positive ACPA	3
	Acute-phase reactants	Normal C-reactive protein (CRP) and normal erythrocyte sedimentation rate (ESR)	0
		Abnormal CRP or abnormal ESR	1
	Duration of symptoms	< 6 weeks	0
		≥ 6 weeks	1

Adapted from Aletaha et al. (2010)

Bone loss in RA, another feature that may be observed in the disease, is targeted primarily to the diarthrodial joints (free-moving joints formed from two opposing bone surfaces with articular cartilage in the middle) and is characterised by progressive erosion of bone and cartilage extracellular matrices, which occasionally becomes apparent even in early stage of RA (Zhao et al. 2011). RA also has pathological characteristics of formation of pannus (thickened synovial tissue) resulting from the hyperproliferation of the synovial membrane. This structure of pannus is believed to "invade" bone and cartilage resulting the bone erosion following the release of pro-inflammatory cytokines and matrix metalloproteinases (MMPs). From the view point of bone erosion, there have been three forms of bone loss described in RA; focal bone erosions, periarticular bone loss and general osteopenia (Goldring 2009; Schett 2007; Walsh et al. 2005).

While those predominant features described above appear to be more associated with a localised disease, RA is nevertheless a systemic disease with the associated syptoms manifested throughout the body like rheumatoid nodules, vasculitis and Felty syndrome (Schneider et al. 1985). With the features such as synovitis and formation of pannus are considered as primary in RA, in general, this disease is also associated with secondary osteoporosis (Gough et al. 1998).

At cellular level, the initiation and progression of RA results from the interaction between cells such as T and B lymphocytes, cells from monocytes/macrophages lineage and both type A (macrophage-like) and B (fibroblast-like) synoviocytes, which produce and release products like chemokines and cytokines that recruit other cell types into tissues and enzymes such as MMPs, cathepsins and mast cell proteinases that directly cause damage on cartilage and bone. CD4-positive T-cells are thought to be the main player ochestrating the immune response in synovial inflammation (Rittner et al. 1997). There is also evidence that osteoclasts, the bone-resorbing cells, are involved in mediating the bone erosion observed in RA tissues (Bromley & Woolley 1984; Fujikawa et al. 1996; Gravallese et al. 1998; Shen et al. 2006) (to be described in 1.3.3 below).

1.2.3. Osteoarthitis

Osteoarthritis (OA) is the most common type of arthritis (Hedbom & Hauselmann 2002). Nonetheless like RA, the etiology is still unknown. The most common early symptom associated with osteoarthritis is pain at the affected joints, which can be worsened by load bearing and relieved following rest. Studies have pointed out quite a number of associated factors identified as contributing towards the onset of OA like aging, body weight load or obesity, mechanical stress on joints, probably due to misalignment of bones (Brandt et al. 2008) as well as genetics (Valdes & Spector 2008).

This type of arthritis is mainly characterized by slow but progressive degeneration of articular cartilage, which leads to pain and disability (Buckwalter et al. 2005). Unlike PO and RA as described earlier, this disease is not considered as a bone loss disease, indeed the subchondral bone underneath the degraded cartilage is generally observed in OA as thickening (Gevers et al. 1989). However, OA is reviewed in this thesis as the synovial tissues from OA patients were used in the study as controls (described further in Chapter 2 and 4). The primary process of cartilage degradation is believed to be due to increase in the synthesis and activity of extracellular degrading proteinases, mainly MMPs followed by secondary process of synovitis (inflammation of synovium). Due to that, OA is also considered to be an inflammatory disease following the release of pro-inflammatory cytokines found within the tissues (Haywood et al. 2003; Hussein et al. 2008; Nakamura et al. 1999).

Another hallmark feature that could only be uniquely found in OA is the formation of osteophytes (Felson et al. 2005), which is osteochondral nodule-like structure formed in the bone near chondro-synovial junction probably from progenitor cells inside the perichondrium (Matyas et al. 1997). The function of osteophytes still remains unknown, however they are believed to be associated with stabilizing the joints affected by OA.

Currently, the diagnosis criteria for OA widely used are based on the ACR criteria developed by Altman and colleagues (Altman et al. 1986). The classification for OA according to the guideline depends on the joint affected. Taking hip as example, considering tissues from OA

group used in this study were from hip, characterization for OA disease is based on the criteria listed in Table 1.3 below.

Table 1.3: Traditional format of ACR Classification Criteria for OA of the Hip

Main Criterion	**Additional criteria** with at least 2 must be fulfilled
Hip pain	1. ESR< 20mm/hour
	2.Radiographic femoral or acetabular osteophytes
	3.Narrowing of joint space on radigraphs

Adapted from Altman et al. (1986)

As mentioned above, previously OA is mainly viewed as a disease of cartilage. Cartilage is a structure cushioning between bones at joints for frictionless motion and resists compressive forces during loading. In terms of the structure composition, the matrix is mainly consisted of collagen (60%) and proteoglycan (25%) as well as non-collagenous proteins (Buckwalter et al. 2005). Chondrocytes are the only cell type exists in cartilage and only accounts for 2% of cartilage volume (Poole et al. 2001). Since there is no blood vessels or nerves in cartilage, synovial fluid is really crucial in providing them nutrition and oxygen. Chondrocytes are the key players for homeostasis or maintenance of cartilage as they produce collagen and proteoglycan during anabolic phase and metalloproteinases for catabolic phase of cartilage homeostasis.

Studies suggested that the degradation of cartilage has link to increase in cell death of chondrocytes in OA (Dai et al. 2006; Wei et al. 2006). Besides cell death, there are also thoughts that the reduction in chondrocytes has association with phenotype alteration of the cells into more fibroblast-like cells, and this is known as dedifferentiation (Aigner et al. 1993; Sandell & Aigner 2001). There is also evidence associating the initiation and progression of OA with the senescence activity of chondrocytes, which is closely linked to aging (Martin & Buckwalter 2001a, 2001b)

Studies on OA synovial tissues indicate that inflammation could be observed in all stages of OA (Smith et al. 1997). Synovitis, which means inflammation in the synovium, can be found

in the early and late stage OA, however it becomes more obvious in the later stages (Smith et al. 1997). This inflammation in tissues is characterized by thickening of lining layer, increased vascularity and massive infiltration of inflammatory cells into the thickening layer synovial membranes (Smith et al. 1997). Besides RA, OA also is another example of disease that could be characterised as having pathological feature of synovial angiogenesis (Walsh et al. 2007), as this could be seen from the high vascularity observed in OA synovial tissues (Smith et al. 1997). In comparison to late stage OA, it was found that there was more vascular formation and tissue infiltration of CD4 (T-cells) and CD68-positive cells (activated macrophages) as well as greater expression of vascular endothelial growth factor (VEGF) and intracellular adhesion molecules-1 (ICAM-1) in early OA (Benito et al. 2005).

1.3. Osteoclasts- The Bone Resorbing Cells

Osteoclasts are terminally differentiated multinucleated cells (normally ranged between 3 to 20 nuclei, as reviewed in Roodman and Windle (2005), formed from the fusion of marrow-derived mononucleated hemopoietic progenitor cells (Coccia et al. 1980; Walker 1975). Upon fusion, each nucleus within the multinucleated osteoclasts has been reported to have a limited lifespan of approximately 12.5 days or 2 weeks, therefore new osteoclast precursors need to be kept recruited and fused into the existing mature osteoclasts for longer resorption activity (Jaworski et al. 1981; Manolagas 2000).

As mentioned earlier, osteoclasts are cells uniquely specialized for resorbing bone. The significance of osteoclasts in resorbing bone could be seen from the occurrance of osteopetrosis (generally characterized as significantly higher bone mass and reduced bone marrow cavities) as well as defected hematopoietic and bone marrow cell development (Villa et al. 2006). From the physiological perspective, osteoclasts are very important in initiating bone remodeling and hence may affect other function of bone remodeling such as for calcium homeostasis.

The precursors to osteoclasts, referred to as pre-osteoclasts, are present in both peripheral circulation and bone marrow, however, they only differentiate into mature functional osteoclasts in the tissue in which they are recruited (Collin-Osdoby et al. 2001; Gravallese et al. 1998) and when in contact with bone (McHugh et al. 2007; Shen et al. 2006). Fully

differentiated osteoclasts are capable of causing bone resorption by forming bone resorption pits known as lacuna. The process of osteoclast formation is illustrated in Figure 1.1 below.

Osteoclasts are important to be investigated as it initiates bone remodeling and secondly they are dominant in pathological bone loss disorders like in RA and PO (further discussed in the following section 1.3.3 below). Discussions in the remainder of this review will be focused towards bone resorption and osteoclasts as well as both the intracellular and extracellular regulation of the bone resorbing cells.

1.3.1. Differentiation and Maturation of Osteoclasts

As briefly mentioned, osteoclasts have very short lifespan, therefore the development or differentiation process into mature osteoclasts is regarded as one of the main stages crucial to modulating bone resorption activity considering they are cells highly specialised for that function. There is accumulating evidence suggesting osteoclasts as among the key players mediating pathogenic bone loss like in PO and RA (to be discussed in section 1.3.3 below), further supports this area of study on osteoclast differentiation to become one of the principal areas of interest in relation to bone pathologies.

Figure 1.1 below illustrates the main stages in osteoclast life cycle involved, including the process of osteoclast differentiation. This differentiation process producing fully functional mature osteoclasts capable of resorbing bone from the precursor cells is also known as osteoclastogenesis. In general, osteoclast differentiation could be divided into 4 main stages. Typically, the development of osteoclasts begins with the commitment of precursor cells (from hematopoeitic lineage) towards osteoclastic lineage, and this is demonstrated by the expression of early osteoclast markers like tartrate-resistance acid phosphotase (TRAP) and cathepsin K. This is then followed by multinucleation, a fusion process between mononuclear pre-osteoclasts to form non-functional (due to inability to form ruffled borders) polykaryons. The significance of cell multinucleation for osteoclasts are further discussed extensively in 1.3.1.2 below. The formed polykaryons would then undergo maturation and activation to be functional osteoclasts capable of resorbing bone. In response to stimulating agent, the activation of mature osteoclasts involves in cytoskeletal rearrangement for the cell motility and attachment to the bone matrix. This two processes allow osteoclast to perform bone

resorption (detailed in section 1.3.2 below), and once the osteoclasts have performed their function, they will then undergo apoptosis.

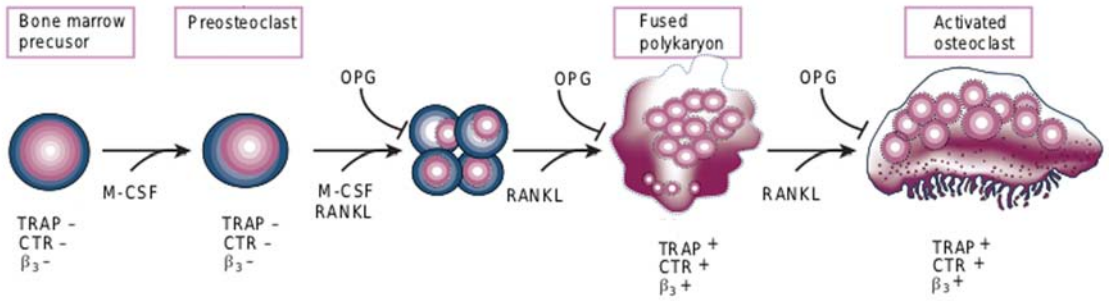

Figure 1.1: Main stages in osteoclast differentiation

Adapted from Boyle et al. (2003)

Osteoclastogenesis is believed to take place at or in close proximity to the site of bone resorption (Collin-Osdoby et al. 2001; McHugh et al. 2007). Generally, osteoclasts differentiate from monocytes, most likely to be CD14-positive cells (Atkins et al. 2006; Massey & Flanagan 1999; Nicholson et al. 2000; Nose et al. 2009; Shalhoub et al. 2000; Sorensen et al. 2007). This means that osteoclasts share similar progenitor to macrophages and dendritic cells, two types of immune cells derived from monocytes as well (Miyamoto et al. 2001). However, in some cases like in PO, in which there has been evidence that osteoclasts could be differentiated from macrophages (see section 1.3.3 below) as wells as from dendritic cells as indicated by a quite recent study (Gallois et al. 2010).

In general, the total of net bone resorption depends on two factors, which are the rate of cells recruitment and the bone resorbing capacity of each multinucleated cell (Zou et al. 2010). These two factors, with the latter is closely associated with multinucleation and cytoskeletal organisation (Teitelbaum 2011), are going to be discussed in detail below.

1.3.1.1. Recruitment and Targeting of Osteoclasts

The mechanism of how osteoclasts are recruited to specific site on the bone surfaces for resorption is not really clearly understood. However, it is suggested that the recruitment of pre-osteoclasts to site of bone resorption is on the basis of chemotaxis.

This is based on the initial work done by Malone et al. (1982), which showed that three constituents of bone matrix, collagen type I peptides, α2HS glycoprotein and osteocalcin appear to exhibit chemotactic effects on human monocytes, the cell type that osteoclasts are derived from. The chemotactic mobilization of osteoclasts could also be initiated by osteoblasts as the bone-forming cells are also known to produce collagen and osteocalcin (Malone et al. 1982). Osteoblasts also release stromal-cell derived factor-1 (SDF-1) that is thought to contribute in the migration and homing of osteoclasts (Wright et al. 2005; Yu et al. 2003).

It is also thought that osteocytes, the most abundant cells within bone matrix, may play a role in the recruitment of osteoclasts, especially in the context of initiating bone remodelling at a particular site of bone, for example at fractured site. It is hypothesized that dying osteocytes (by apoptosis) in the close proximity to the microcracks may transmit chemotactic signals to osteoclasts requesting for bone remodeling for fracture removal (Noble et al. 2003). Another good example of such signal transmission would be osteopontin, an osteoclast chemoattractant important for initiating bone remodeling following mechanical loading (Fujihara et al. 2006; Gross et al. 2005; Terai et al. 1999). It was found that the expression of osteopontin by osteocytes is upregulated in hypoxic condition (Gross et al. 2005). Indeed besides recruiting osteoclast, there is accumulating evidence pointing out that osteocytes are the cells that support osteoclast formation (Kurata et al. 2006; Xiong & O'Brien 2012; Zhao et al. 2002) (to be further discussed in section 1.3.5.1.4 below).

Besides those chemoattractants of osteoclasts described above, there is quite a number of other chemokines being associated with the recruitment of osteoclast precursor cells. Such common examples include monocyte chemoattractant protein-1 (MCP-1) (Hashimoto et al. 1998; Lu et al. 1998), C-C chemokine receptor 2 (CCR2) (Gao et al. 1997), macrophage inflammatory protein 1 (MIP-1) (Boring et al. 1997) and regulated upon activation, normal T cell expressed and secreted (RANTES) (Yano et al. 2005). Another example of osteoclast-associated chemokines is macrophage inhibitory factor-1α (MIP-1α)(Hashimoto et al. 1998), which has been shown to increase osteoclast motility and its receptor CCR1 expressed on osteoclast precursor cells (Yu et al. 2004).

1.3.1.2. Multinucleation

The multinucleated feature of osteoclasts is believed to be associated with optimisation of bone resorption capability and efficiency (Boissy et al. 2002). This cell-cell fusion process was found to be influenced by factors like tumor necrosis factor-α (TNFα) and lipopolysaccharide (LPS) (Lee et al. 2006b). It is hypothesized that multinucleation provides synergy in terms of resorption efficiency. Bone resorption by one multinucleated osteoclast is greater than the total bone resorption by the total number of mononuclear osteoclasts (equivalent to the number of nuclei in the single multinucleated osteoclast) (Bar-Shavit 2007; Boissy et al. 2002). However, findings by Lees et al. (2001) suggest that the total bone resorption is proportionate to the number of nuclei within osteoclasts. Inability to multinucleate in mutant mice-derived osteoclasts was found to result in less dentine resorption activity (Humphrey et al. 2004; Paloneva et al. 2003).

There is a number of proteins identified as crucially required in the process of multinucleation of osteoclasts and absence of those proteins severely affect the osteoclast resorption capacity. Such example is dendritic-cell transmembrane protein (DC-STAMP) (Yagi et al. 2005). Study by Yagi and co-workers (2005) demonstrated that cells deficient in DC-STAMP, had reduced resorption efficiency per nuclei.

Another protein that was found to be important in the process of cell multinucleation is the d2 isoform of vacuolar (H^+) ATPase (V-ATPase) V_0 domain (Atpv0d2) (Lee et al. 2006b). The multinucleated state of the osteoclasts is believed to enable osteoclasts to possess a high number of mitochondria, which is essential for the production of high amount of energy needed by the ATPase pump for the secretion of protons in the bone resorption as reviewed by Vaananen (2005).

The large size of osteoclasts in comparison to macrophage allows them to cover a large area on the bone surfaces, which further enhancing the efficiency of bone resorption (Bar-Shavit 2007). In addition, it was found that large sized osteoclasts are more active in comparison to the small sized ones (Lees et al. 2001). Examination at the protein level found that the expression levels of proteins that play crucial role in osteoclast signalling, attachment, cell fusion and matrix degradation (refer to following sections) are relatively higher in large sized osteoclasts than the small ones (Trebec et al. 2007).

1.3.1.3. Osteoclast-associated Cell Markers

As pre-osteoclasts diferentiate into mature osteoclasts, the pre-osteoclasts begin to express a number of proteins. The expression of these proteins is very useful as markers in determining or estimating the stage of the osteoclast differentiation (see Figure 1.1). Commonly used osteoclast gene markers are *ctsk* (encoding cathepsin K) (Drake et al. 1996), *Acp5* (encoding TRAP) (Janckila et al. 2005; Janckila et al. 2001), *calcr* (calcitonin receptor (Lee et al. 1995) and osteoclasts-associated receptor (OSCAR)-encoding gene (Kim et al. 2002).

Cathepsin K (catK) is a protein found to be expressed at high level in osteoclasts from all species studied thus far (Drake et al. 1996; Littlewood-Evans et al. 1997; Troen 2003; Yamaza et al. 1998). Cathepsin K appears to be a main bone-matrix degrading enzyme as mice deficient in cathepsin K exhibited osteopetrosis (Gowen et al. 1999; Saftig et al. 1998). This is further proven in a study in which a significant reduction in bone resorption activity following treatment with a cathepsin K inhibitor in a rat model was observed (Xia et al. 1999). Meanwhile in human, mutation on cathepsin K gene leads to pycnodysostosis disease (Gelb et al. 1996).

Cathepsin K has been found to be able to degrade collagen type I and II as well as bone matrix protein osteonectin (Bossard et al. 1996; Garnero et al. 1998; Kafienah et al. 1998). The unique feature of cathepsin K in comparison to other mamalian proteinase is that it cleaves collagen type I molecules both sides of in and outside helical region (Garnero et al. 1998). Cathepsin K is regulated by microphthalmia transcription factor (MITF) (Motyckova et al. 2001). As osteoclast differentiates, the amount of cathepsin K produced increases, which is believed to be as a result of receptor activator NF kappa B ligand (RANKL) stimulation (Corisdeo et al. 2001) (RANKL to be discussed in detail in section **1.3.5.1**).

TRAP is a widely used histochemical marker for the detection of osteoclasts as well as for bone resorption activity (Halleen 2003; Halleen et al. 2006; Rissanen et al. 2008). TRAP is an enzyme expressed by osteoclasts with a role in bone resorbing activity. In TRAP-knockout mice, osteoclasts were still found to be formed, however, accompanied by a reduction in bone resorptive activity (Hayman et al. 1996). Meanwhile, overexpression of TRAP was found to

lead to an increase in bone turnover (Angel et al. 2000). TRAP is found in various types of tissues, nevertheless high TRAP activity is found to be predominant in bone, spleen, liver and thymus as it is expressed abundantly by osteoclasts, macrophages, multinucleated foreign-body giant cells and dendritic cells (Chun et al. 1999; Halleen et al. 2006; Hayman & Cox 2003). TRAP is an early marker for the detection of cells committed towards osteoclast-lineage as it is expressed in the early stage of commitment towards osteoclast lineage (before mutinucleation of pre-osteoclasts takes place). Like cathepsin K, TRAP is also regulated by MITF (Luchin et al. 2000).

Even though cathepsin K and TRAP has been widely accepted as osteoclast cell markers, it appears that researchers need to be cautious over reliance on TRAP and cathepsin K as solely osteoclastic markers as these two markers are also found to be expressed in dendritic cells, another cell type sharing the same precursor with osteoclasts, even though in low quantity (Kim et al. 2002). Cathepsin K has also been found to be expressed in fibroblasts (Hou et al. 2002; Hou et al. 2001), macrophages and smooth muscle in atheroma (Sukhova et al. 1998) and a number of cell types in lung tissues (Buhling et al. 2000).

It was suggested that the newly discovered osteoclast-specific receptor named OSCAR, to be discussed in further detail in section **3.5.2**) to be more reliable as an osteoclast gene marker as this receptor was understood to be not expressed in both dendritic cell and monocyte-derived macrophage in mice (Kim et al. 2002). In addition, the expression of OSCAR was also detected in RAW264.7 macrophage cell line-derived osteoclast-like cells, with peak expression upon the completion of the osteoclast differentiation following four days of RANKL stimulation (Kim et al. 2002).

1.3.2. Mechanism of Bone Resorption

The resorption of bone by osteoclasts begins with the attachment of the mature osteoclast to the surface of the bone. The attachment of bone and osteoclasts is largely mediated by membrane-bound molecules known as integrins. To date, there are at least five types of integrins identidfied as being expressed in osteoclasts; α2β1, αvβ1, αvβ3, αvβ5 (Nesbitt et al. 1993) and the recently discovered α9β1 (Rao et al. 2006), however, only αvβ3 and αvβ5 integrins are of particular interesting in relation to osteoclast bone resorption activity as it

appears that these two integrins are differentially expressed as the pre-osteoclasts mature into active osteoclasts (Lane et al. 2005; McHugh et al. 2000).

αvβ3 seems to play a significant role in mediating attachment of osteoclasts for bone resorption (McHugh et al. 2000; Nakamura et al. 1996; Nesbitt et al. 1993; Reinholt et al. 1990; Zhao et al. 2005a). The binding of the integrin to extracellular proteins such as osteopontin at tripeptide arginine-glycine-aspartic acid (RGD) recognition site (Lakkakorpi et al. 1991) induces cell survival since unoccupied αvβ3 is believed to give a positive death signal to the corresponding osteoclasts for apoptosis via the expression of caspase 8 (Zhao et al. 2005b). The αvβ3 integrin is highly present at the ruffled membrane and has high affinity for adherence to denatured collagen (Nesbitt et al. 1993). Whereas, αvβ5 is mainly expressed in pre-osteclasts in prior to osteoclast differentiation (Inoue et al. 1998) and seems to play role in anti-osteoclastogenic activity (Lane et al. 2005).

On attachment the osteoclast forms an intimate contact with the bone surface known as the "sealing zone", which is a crucial requirement for bone resorption. The sealing zone of the osteoclast is rich in filamentous actin and surrounded by a ruffled membrane. Cytoskeletal reorganisation results in the polarisation of the nuclei to a site opposite the ruffled membrane (see Figure 1.2). This enables firm adhesion of the osteoclasts to the bone thereby preventing leakage of any resorption agents, such as protons and matrix degrading enzymes, released by the osteoclast (Abu-Amer et al. 2000). The release of such resorption agents and enzymes takes place across the ruffled border via exoytosis (refer Figure 1.2 below) (Teitelbaum 2011).

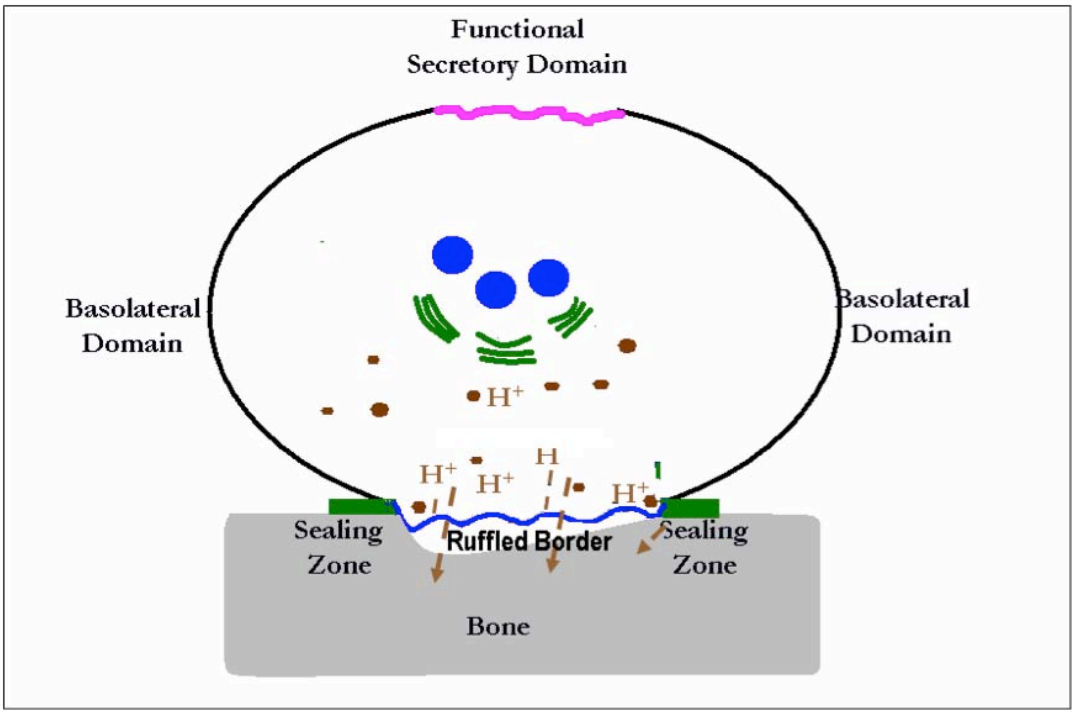

Figure 1.2: Transportation of bone resorbing agents through a trans-Golgi trafficking network in osteoclasts and release across the ruffle border via exocytosis

Adapted from Vaananen et al. (2000).

The bone degradation process could be divided into two main phases; inorganic (bone demineralisation) and organic (matrix degradation) phases (Abu-Amer et al. 2000). The demineralisation of bone occurs before the degradation of matrix protein. The process of demineralisation of bone occurs by the acidification of the sealed region and is mediated by the vacuolar H^+-ATPase pump located in the osteoclast ruffled membrane (Blair et al. 1989; Mattsson et al. 1994; Vaananen et al. 1990). Protons (H^+), produced by cytoplasmic carbonic anhydrase II within the osteoclasts (Gay & Mueller 1974), are pumped out of the osteoclasts into the area between the cells and the bone causing a drop in pH to ~4.5 (Schlesinger et al. 1997) that leads to the dissolution of bone mineral (the solid hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ to free Ca^{2+}, $HPO4^{2-}$ and water, H_2O). The build up of alkaline intracellular condition as a result of pumping protons out from the cells is neutralized by the chloride/bicarbonate exchange pump (Hall & Chambers 1989; Schlesinger et al. 1997; Teti et al. 1989).

The degradation of matrix protein occurs through the secretion of lysosomal proteases, MMPs and a variety of other matrix degrading enzymes, which are released from the osteoclasts by

the process of exocytosis. The high expression of cathepsin K (Drake et al. 1996), MMP9 (Tezuka et al. 1994) and type IV collagenase/gelatinase B (Wucherpfennig et al. 1994) in osteoclasts suggests that these enzymes play roles in the organic phase of the bone resorption. Screening on patients with pycnodysostosis, a rare disease which has a feature of increased bone density, showed that the disease is linked to mutation on cathepsin K gene, which may indicate that this enzyme may play important role in bone resorption (Gelb et al. 1996; Ho et al. 1999; Johnson et al. 1996; Motyckova & Fisher 2002). Meanwhile, knockout on MMPs results in defects in bone resorption (Delaisse et al. 2003). The cumulative effect of the bone demineralization and matrix degradation is the formation of lacuna pits on the surface of the bone.

Products produced from the degradation activity by the osteoclasts are removed from the sealing-zone confined microenvironment to the extracellular space via transcytosis through the osteoclast itself from the ruffled membrane to the opposite functional secretory domain (refer Figure 1.2) (Nesbitt & Horton 1997; Salo et al. 1997). A study by Halleen et al. (1999) found that TRAP is localised together with matrix degradation products in the transcytotic vesicles and may play a role in further fragmentation of the endocytosed materials by producing reactive oxygen species. The transcytotic secretory system of the matrix-degraded components in the osteoclasts seems to share similarity with the antigen presentation presentation mechanism in macrophage, the immune cell derived from the same progenitor cells for osteoclasts (Vaananen et al. 2000).

The mechanism by which osteoclast-mediated bone resorption is stopped or arrested is poorly understood. However, there are a couple of theories suggested. First, the bone resorption process could stop by itself considering osteoclasts have limited lifespan. Secondly, the resorption process might be arrested as a consequence of inhibition on the osteoclasts by the accumulated high concentration of calcium resulted from the bone mineral dissolution (Zaidi 1990) or TGF-β cytokine or other products released from the bone matrix (Pfeilschifter et al. 1990a; Pfeilschifter et al. 1990b).

1.3.3. Osteoclasts in Pathology of Inflammation-Mediated Bone Loss

As briefly mentioned earlier in this chapter, osteoclasts become one of the central theme discussed in this thesis since studies over decades found that they present in pathological bone disorders like PO and RA as well as being associated with the initiation and progression of those diseases.

In PO, it has been found that there is increased osteoclast bone resorbing activity that is partially compensated by high osteoblast activity (in terms of mineral apposition and bone formation rate), which was indicated by the increased remodelling of peri-implant bone and immature bone formation around loosened prosthetic hip implants (Takagi et al. 2001).

There is also accumulating evidence pointing out the increase in the recruitment and differentiation of osteoclast precursor cells in PO tissues as might be the main factor responsible for wear particle-induced osteolysis (Eftekhar et al. 1985; Greenfield et al. 2002; Kadoya et al. 1996). Examination on the interface tissues around failed prostheses found high level of cathepsin K, which is an osteoclast enzyme, in the PO tissues (Konttinen et al. 2001). A more detail investigation by Shen and colleagues (Shen et al. 2006) found cells with PE particles inside, both mononucleated and multinucleated, were positive for osteoclast-associated markers cathepsin K and TRAP. Previous work carried out in this laboratory found that RANKL, which has been demonstrated to increase osteoclast bone resorbing activity (see 1.3.5.1 below), to be significantly higher in PO tissues compared to OA tissues (Crotti et al. 2004). A recent study by Qin and coworkers (2012) demonstrated that treatment with an osteoclast vATPase inhibitor, saliphenylalamide was able to attenuate particle-induced osteolysis in a murine calvarial model.

As mentioned earlier, macrophages have been found to be a dominant player involved in the disease progression of PO (refer 1.2.1 above). Macrophages are also dominant in PO tissues in term of cell population, which has been indicated by higher level of macrophage cell marker, in PO tissues as compared to the controls (Kadoya et al. 1996; Sabokbar et al. 1997). Those high number of macrophages found in the tissues are also thought to be potential osteoclast precursor cells (Haynes et al. 2001b; Sabokbar et al. 1997). There has been evidence demonstrating PE-associated multinucleated osteoclast-like cells in PO tissues were found to be positive for macrophage cell marker CD68 (Shen et al. 2006). It has also been shown in the past that these cells (i.e macrophages) isolated from peri-prosthetic tissues

around loosening implants are capable of differentiating into bone-resorbing osteoclasts *in vitro* (Haynes et al. 2001b; Neale et al. 2000; Sabokbar et al. 1997). However, there has been contradiction underlying the capacity of those tissue-isolated cells to readily differentiate into osteoclasts as some studies reported that such *ex vivo* differentiation required presence of osteoblasts (Neale et al. 2000; Sabokbar et al. 1997), while another study (Haynes et al. 2001b) indicated presence of osteoblasts is not necessary for the differentiation process.

The thought that macrophages can be differentiated into osteoclasts is further supported by the data that reported expression of osteoclast cell markers by macrophages in the pseudomembrane surrounding the loose prostheses (Kadoya et al. 1994; Kadoya et al. 1996; Neale & Athanasou 1999). With the capability of macrophages to get differentiated into osteoclasts, there is possibility that the bone erosion occurring in PO results from the presence of massive number of osteoclasts derived from large population of macrophages dominating in the tissues.

In view of the increased recruitment of osteoclast precursors into PO tissues, it has been found that the presence of biomaterial debris has effect on the expression of osteoclasts-associated chemokines (refer 1.3.1.1 above). Stimulation with ultra-high molecular weight polyethylene (UHMWPE) particles *in vivo* was found to increase the expression level of MCP-1 (Rhodes et al. 1997). Meanwhile, stimulation by PMMA and titanium alloy in an *in vitro* monocyte/macrophage model demonstrated increased in the expression of MCP-1 and MIP-1α, but not RANTES (Nakashima et al. 1999a). Study on human PO tissues (Haynes et al. 2004) supported these earlier findings, in which there was higher level of MCP-1 and MIP-1α demonstrated in those tissues. It has been suggested that different types of biomaterial give different response in C-C chemokines expression (Nakashima et al. 1999a). Based on the expression profile of chemokines, biomaterial debris has also been found to affect cells activity. For instance, PE was suggested to have effect on the activation and migration of macrophages (Ishiguro et al. 1997).

Studies in the past has described the formation of osteoclasts as a key process in inflammatory bone erosion occuring in RA (Durand et al. 2011) besides imbalance in regulation of bone remodeling resulted from excessive bone resorption and decrease in bone formation (Goldring 2003; Gravallese et al. 2000; Redlich et al. 2002b). High disease activity, which is a common

marker for RA disease progression, was found to be correlated with osteoclastogenesis (Zhao et al. 2011).

Through examinations on the human tissue and the arthritic animal models, it appears osteoclasts are the cells mediating the pathological bone loss present in RA patients (Bromley & Woolley 1984; Fujikawa et al. 1996; Gravallese et al. 1998; Gravallese et al. 2000; Kuratani et al. 1998; Redlich et al. 2002b; Suzuki et al. 1998). Mature osteoclasts could be found in the close proximity to the bone surface in the joints of the RA patients (Bromley & Woolley 1984; Gravallese et al. 1998). In addtion, it has been found that monocytes/macrophages isolated from human rheumatoid arthritis synovial tissues could and is more potent to differentiate into bone resorbing osteoclasts *in vitro* (Fujikawa et al. 1996; Nose et al. 2009; Vandooren et al. 2009).

1.3.4. Regulation on Osteoclastogenesis and Bone Resorbing Activity by Cytokines

There is quite a number of factors that have been shown in the past as having influence on the regulation of osteoclast differentiation and bone resorbing activity. Age, genders (Jevon et al. 2002), hormones (Faucheux et al. 2002; Huang et al. 2004; Rodan & Martin 1981), cytokines (Fujikawa et al. 2001; Tsuboi et al. 1999; Tsuda et al. 1997) are among factors known to incluence bone loss. Of all those factors, release of cytokines appeared to be most studied, and since local inflammation-mediated bone loss becomes the scope of this study, this section of this thesis is going to discuss the influence of cytokines, especially the major pro-inflammatory cytokines in on the regulation of osteoclast differentiation and activity in inflammation mediated localised bone loss,.

Numerous cytokines have been identified and reported as capable of altering and modulating the rate of osteoclastogenesis and they are believed to play significant role in the pathogenesis of those diseases described above. However, in this review, the cytokines that are going to be discussed are mainly TNFα and interleukin-1 (IL-1), which are prominently and widely reported to be involved in local inflammation-mediated bone loss. The discussion in this thesis is limited to TNFα and IL-1 only since those two cytokines are involved in the study presented in this thesis (see Chapter 5).

It appears from previous literature that TNFα and IL-1 are the predominant cytokines as they are abundant in inflamed tissues as well as in the sera and synovial fluid in RA patients (Buchan et al. 1988; Feldmann & Maini 2001; Maini et al. 1995; Tak & Bresnihan 2000). Examination on tissues taken from arthritic patients, for instance, found that these two pro-inflammatory cytokines are mainly expressed by activated macrophages and activated synoviocytes in the lining layer (Danning et al. 2000; MacNaul et al. 1990) and the synovial fibroblasts (Farahat et al. 1993; Niki et al. 2001). It has also been found that endothelial cells also express TNFα in RA tissues (Farahat et al. 1993).

In the context of PO, receptors of TNFα and IL-1 are among cytokine receptors detected to be expressed by macrophages, foreign body giant cells and osteoclasts isolated from peri-prosthetic tissues (Merkel et al. 1999; Neale & Athanasou 1999; Xu et al. 1996) and interaction between these cytokine receptors and their ligands is suggested to play role in osteoclast formation and bone loss (Chiba et al. 1994b; Jiranek et al. 1993). The release of those two cytokines by activated macrophages could be stimulated by various types of prosthetic wear particles *in vitro* (Haynes et al. 1998; Merkel et al. 1999). TNFα and IL-1β does not only promote osteoclastogenesis by directly affect osteoclast precursors, but they also promote osteoclast formation through regulation on osteoblasts (Haynes et al. 1997).

Meanwhile, in OA the presence of TNFα and IL-1 has also been reported (Smith et al. 1997). A study comparing between stages of OA indicated that there was greater proportion of cells expressing TNFα and IL-1β in early OA than in late OA (Benito et al. 2005). It has also been reported that there was a pattern of lower level of IL-1 receptor antagonist (IL-1ra) and declining ratio IL-1ra/IL-1 as the disease progresses (Fernandes et al. 2002; Smith et al. 1997), and this may give a picture on the significance of IL-1 in OA progression. Meanwhile for TNFα, the significance of this cytokine towards OA progression could be seen from the fact that there is higher level of p55 TNFα receptor (refer section 1.3.4.1 below) being expressed in OA than in normal tissues (Alaaeddine et al. 1997; Fernandes et al. 2002; Smith et al. 1997).

It has been suggested that TNFα and IL-1 play role in the progression of OA by promoting the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) (Alaaeddine et al. 1997; Amin & Abramson 1998), which in turn stimulate other cellular changes in OA (Hedbom &

Hauselmann 2002) such as decline in collagen and proteoglycan production, increased apoptosis of cells including chondrocytes and inhibiton of β1 integrin-dependant adhesion to the extracellular matrix (Amin & Abramson 1998). Previous literature also indicates that TNFα and IL-1 significantly stimulate the expression of MMP such as MMP-3, which would obviously involved in the degradation of cartilage in OA (Fernandes et al. 2002). It appears that these two cytokines were likely to regulate inflammation in the tissue, as local injection with anti-TNFα and anti-IL-1 results in reduction in recruitment of inflammatory cells and in the number of osteoclasts by 80% and 67% respectively, as well as accompanied by a decrease in bone loss (60%) in comparison to the control (Assuma et al. 1998).

Considering TNFα and IL-1 as the most dominant pro-inflammatory cytokines in mediating the progression of these diseases, the regulation of bone resorption by these two cytokines will be discussed in more detail below. There is another osteoclast-regulating cytokine described in this section 1.3.4, which is macrophage-colony stimulating factor (M-CSF) as this cytokine was also used in study reported in this thesis (Chapter 3).

1.3.4.1. Tumor Necrosis Factor-Alpha (TNFα)

It has been long known that TNFα is a promoter for bone resorption and inhibitor for bone formation expressed and secreted by activated macrohages (Bertolini et al. 1986). Taking RA as an example of local inflammation-mediated bone loss, TNFα is reported to be the principal cytokine responsible for initiating bone loss in the disease (Shealy et al. 2002; Teitelbaum 2006).

Through histological examination on human RA tissues, it has been found that there is massive number of cells in the synovial membrane as well as in pannus-cartilage junction expressing TNF-α (Chu et al. 1991). The significant contribution TNFα has towards the pathogenesis of rheumatoid arthritis could be seen from *in vivo* study on a human (h) TNFα (h-TNFα) transgenic mouse (Tg197), which found out that the animal model developed severe progressive polyarthritis as well as histological features commonly found in RA (Keffer et al. 1991). Treatment in the same model using an anti-TNFα antibody allowed the repair of the joint damage and reduced the bone erosion in the transgenic mouse (Redlich et al. 2002a; Shealy et al. 2002). In occurance with this in a TNFα-transgenic serum transfer

arthritis-induced mouse, blockade of TNFα using infliximab (anti-mouse TNFα) results in a significant reduction in osteoclast number (Redlich et al. 2002a; Zwerina et al. 2004). Treatment with etanercept, which is a decoy recombinant human TNFα receptor, currently has been widely applied and has been reported to provide an effective therapy for RA (Gomez-Puerta et al. 2004; Wallenius et al. 2005).

As mentioned above, TNFα is also reported to be among the most important pro-inflammatory mediators in PO (Chiba et al. 1994b; Jiranek et al. 1993). Like in RA, TNFα was found to be expressed widely by high number of cells that include macrophages and foreign-body giant cells (FBGCs) isolated from PO tissues (Holding et al. 2006; Neale & Athanasou 1999; Xu et al. 1996). TNFα was also detected be expressed by fibroblasts, endothelial cells and other cells present in the pseudosynovial membrane (Xu et al. 1996). There is also data suggesting that TNFα could also be expressed by osteoclasts reported in a previous study carried out in the laboratory (Holding et al. 2006), in which TNFα expression was found to be strongly associated with multinucleated osteoclast-like cells containing PE particles.

TNFα has been shown to be important soluble mediator in murine *in vitro* model of PMMA-induced osteolysis as there was increase in gene and protein expression of TNFα upon stimulation by PMMA cement and knockout of TNFα receptors resulted inhibition on PMMA-induced osteolysis *in vivo* (Merkel et al. 1999). It has also been reported that TNFα was among increased cytokines following exposure to PE particles *in vitro* (Horowitz & Gonzales 1997).

TNFα can stimulate osteoclastogenesis either by directly on the pre-osteoclasts (Fuller et al. 2002; Kim et al. 2005c; Kobayashi et al. 2000; Komine et al. 2001; Lam et al. 2000; Zhang et al. 2001) or indirectly by upregulating the expression of RANKL and M-CSF in the interacting osteoblasts (Wei et al. 2005). TNFα is also thought to prolong the survival of osteoclasts, thereby leads to increasing the bone resorption volume (Holding et al. 2006). It has been shown that TNFα was able to inhibit the apoptotic effect of bisphophonates targeted on osteoclasts (Zhang et al. 2005)

TNF-α interact with osteoclasts via two membrane receptors, which are TNF receptor type 1 (p55r) and type 2 (p75r) (Abu-Amer et al. 2000; Suda et al. 2001). However, it is believed

that p55r, which is expressed by each of pre-osteoclasts and marrow stromal cells, (Wei et al. 2005; Zhang et al. 2001) is the one which mediates osteoclastogenic signalling by TNFα (Abu-Amer et al. 2000; Zhang et al. 2001). An examination of the expression Fas in human osteoblasts, the bone-forming cells, has suggested that TNFα does not only promote bone loss by inducing osteoclastogenesis, but also by stimulating apoptosis of the cells following stimulation on p55r (Tsuboi et al. 1999). This implies that TNFα does not only promote bone resorption by enhancing osteoclastogenesis, but might also by inducing the apoptosis of osteoblasts, which eventually leads to decrease in the rate of bone formation. Meanwhile, Abu-Amer et al. (2000) found that ligation of the TNF molecule on p75 on opposite appeared to result in reduction in the TRAP intensity indicating suppression of osteoclastogenesis, despite its well known role in inducing cell proliferation.

1.3.4.2. Interleukin-1 (IL-1)

IL-1 was the first cytokine (and even the first immune cell-derived product) recognized to be shown to have an effect on osteoclast activation (Gowen et al. 1985). In the presence of M-CSF, IL-1 was reported to be able to prevent the apoptosis, hence enhance the survival of purified osteoclasts isolated from osteoblasts/marrow stromal cells (Jimi et al. 1995) as well as involved in the resorptive function of osteoclasts (Kobayashi et al. 2000; Suda et al. 2001). IL-1 was also reported to directly induce the multinucleation and activation of osteoclasts (Jimi et al. 1999) and involves in a bidirectional interaction between pre-osteoclasts and osteoblastic cells during osteoclastogenesis process (Atkins et al. 2000; Haynes et al. 1999). Like TNFα, IL-1α has been shown to prolong the survival of osteoclasts (Lee et al. 2002).

The involvement of IL-1 in the pathogenesis of rheumatoid arthritis could be seen from a number of studies using IL-1 transgenic animals which have shown that the overexpression of IL-1α (Niki et al. 2004; Niki et al. 2001) and IL-1β (Ghivizzani, Kang et al. 1997) led to the onset of arthritic features of bone and cartilage degradation. This is further supported by findings from numerous studies investigating the blocking effect on IL-1 in animal models of arthritis using anti-IL-1 monoclonal antibodies, endogenous IL-1 receptor antagonist, or decoy soluble IL-1 type II receptors. Blocking IL-1 in all those animal models markedly reduced bone erosion and the cartilage degradation (Abramson & Amin 2002). Meanwhile knockout of IL-1 receptor diminished signs of arthritis in mice in which arthritogenic serum

was transferred (Ji et al. 2002). Like TNFα, IL-1 might also promote further bone destruction in RA by reducing the rate of bone formation by inducing Fas-mediated apoptosis of the bone-forming osteoblast cells (Tsuboi et al. 1999).

In PO IL-1 β is among important pro-inflammatory mediators involved in the progression of the pathology (Chiba et al. 1994b; Jiranek et al. 1993). Investigation on a murine model of prosthesis failure found that expression of IL-1β was common at the joints (Yang et al. 2007). Higher level of IL-1β was reported in synovial fluids from knees of patient with PO than in synovial fluids from patients with primary arthroplasty (Kovacik et al. 2000; Kovacik et al. 1997). Higher expression of IL-1, based on the level measured in the supernatant of subcultured medium, was also reported in PO tissues with evidence of femoral osteolysis than in PO tissues without sign of bone erosion (Chiba et al. 1994a).

Through immunohistochemistry, IL-1β was detected as being expressed by macrophages, FBGCs and osteoclasts isolated from PO tissues, besides TNFα (Neale & Athanasou 1999). Earlier study reported detection of IL-1β mRNA in macrophage-like cells in human PO tissues (Jiranek et al. 1993). Analysis on the cytokine expression profile found that there was more expression of cytokines including IL-1β by peripheral blood mononuclear cells (PBMCs) from patient undergoing implant revision surgery than cells from patient undergoing primary hip replacement (Waddell et al. 2005). Through an *in vitro* model of monocyte/macrophage culture, Kamikawa and colleagues (2001) demonstrated PE particles could cause the upregulation of IL-1β expression in PE-induced PO.

The increased expression of IL-1β in wear particle induced PO, as suggested from all those studies, may suggest that this cytokine plays important role in mediating pathogenesis of particle-induced PO. This is also further supported by other study showing knockout of IL-1 receptor in a murine model challenged with TiAlV particles caused reduction in the infllammatory response such decreased expression of IL-6 and MCP-1 besides diminshed formation of periprosthetic fibro-inflammatory membrane (Epstein et al. 2005). Introducing IL-1 receptor antagonist into a murine *in vivo* model was found to give protective effects from PE-particle induced osteoysis (Yang et al. 2004).

In terms of the role IL-1 has in the narrow context of mediating osteoclastogenesis, the significance of IL-1 could be seen from the study by Zwerina et al. (2004), which showed that

blockade of IL-1 using anakinra resulted in reduction in osteoclast number. IL-1 and TNFα seem to have an additive effect *in vivo* mouse model since the combination of anakinra (which blocks IL-1R) and infliximab (which have blocking effect on TNFα) resulted in a greater reduction in osteoclast number (almost complete suppression on osteoclastogenesis) as compared to treatment with single antibody to either IL-1 or TNFα (Zwerina et al. 2004). A later finding revealed that IL-1 is also involved in mediating TNFα-induced osteoclastogenesis through the action on the osteoblasts (Wei et al. 2005). This is demonstrated from the inhibition of TNFα-induced RANKL expression by osteoblasts as a result of IL-1 receptor (IL-1R) blockade or gene-knockout.

1.3.4.3. Macrophage-Colony Stimulating Factor (M-CSF)

Unlike TNFα and IL-1 that are classified as pro-inflammatory cytokines and very relevant to be discussed in the context of inflammation-associated bone diseases like PO and RA, M-CSF is not considered to be pro-inflammatory. However, this cytokine is a very important factor regulating osteoclast development and survival, therefore it has been considered to be necessary component in many osteoclast *in vitro* culture, including in an experimental setup for study presented in this thesis (see Chapter 3).

M-CSF appears to stimulate proliferation of the osteoclast pool via the binding of M-CSF expressed by stromal or osteoblastic cells to its receptor c-Fms on the surface of pre-osteoclasts (Fujikawa et al. 2001; Lee et al. 1994). It appears that the interaction between M-CSF and c-Fms is a crucial lineage-fate determining factor into osteoclastic lineage instead of dendritic cell (Lee et al. 1994; Miyamoto et al. 2001). M-CSF also exerts an autocrine effect by mediating RANKL upregulation, thereby further promoting osteoclastogenesis (Udagawa et al. 2002). The M-CSF expanded pool of pre-osteoclasts are then stimulated by subsequent RANKL-RANK interaction to differentiate into mature osteoclasts as reviewed by Teitelbaum (2007).

As the name of M-CSF (also known as colony-stimulating factor-1) implies, the main function of this cytokine is promoting cell proliferation. In *in vitro* cell culture, M-CSF is considered to be very important component to be included in an *in vitro* model of monocyte/macrophage-associated cells as it will promote cells to proliferate before

differentiate into the particular cell type to be studied, therefore allowing sufficient amount of biomolecules such as RNA, DNA and proteins to be harvested and measured in further tests. Besides cell proliferation, M-CSF has also been found to regulate cell spreading and migration (Faccio et al. 2003c). In context of osteoclast cell culture, M-CSF is usually expressed by osteoblasts to support the survival of pre-osteoclasts (Fuller et al. 1993; Jimi et al. 1995; Lagasse & Weissman 1997; Woo et al. 2002; Yoshida et al. 1990). However, in cell culture of RAW264.7, which is a cell line commonly used as an *in vitro* model of osteoclasts, M-CSF is not required as they are reported to produce M-CSF in autocrine manner (Humphrey et al. 2004)

The essential role M-CSF plays in osteoclastogenesis is exemplified by the osteopetrotic feature as a consequence of defect in the differentiation and survival in the M-CSF-mutated mice (Yoshida et al. 1990). Meanwhile in the context of bone pathology, M-CSF has been detected to be present in synovial-like membrane (Xu et al. 1997) and in synovial fluid taken from patients with aseptic loosening (Takei et al. 2000). Besides PO tissues, M-CSF was also detected in synovial fluids taken from RA and OA patients, even though at lower level (Takei et al. 2000). There is also evidence of elevation of M-CSF level in tissues of PO in comparison to the tissues taken from patients undergoing primary hip replacement (Xu et al. 1997) suggesting M-CSF might have influence in pathogenesis of peri-implant osteolysis. The significance of M-CSF has in PO could be seen from the excretion of M-CSF in co-cultures of arthroplasty-derived monocytes and osteoblastic cells (Neale & Athanasou 1999; Quinn et al. 1998). It seems activated macrophages are major source of M-CSF release in peri-prosthetic tissues (Haynes et al. 2001b; Xu et al. 1997).

1.3.5. Molecular Regulation and Intracellular Signalling in Osteoclastogenesis

1.3.5.1. RANK/RANKL-dependent Pathway in Osteoblast/Osteoclasts Interaction

The close interaction between osteoclasts and osteoblasts has been recognised for decades in terms of the regulation on osteoclastogenesis (or osteoclast differentiation). The inter-relationship between the osteoblasts and the pre-osteoclasts was first hypothesized in 1981 by Rodan and Martin, when it was proposed that the immature osteoblasts (Atkins et al. 2003)

express an important factor in mediating osteoclastogenesis. This was subsequently supported in work by Udagawa and colleagues (Takahashi et al. 1988; Udagawa et al. 1990) who showed that the differentiation of bone marrow-derived monocyte/macrophage cells (BMMs) into mature osteoclasts was achieveable *in vitro* in the presence of osteoblasts. However, the nature of the interaction between the osteoblasts and pre-osteoclasts was not understood until the discovery and identification of the OPG/RANK/RANKL system by a number of groups of researchers in the late 1990's (Lacey et al. 1998; Simonet et al. 1997; Tsuda et al. 1997; Wong et al. 1997b; Yasuda et al. 1999). The discovery of these molecules should be regarded as the starting point for osteoimmunology area as these molecules, whose significance was earlier identified in the immunology field, has also been shown to be very important in bone homeostasis, particularly in the regulation of osteoclast differentiation. Further literature review on this system of OPG/RANK/RANKL is as documented below.

1.3.5.1.1. RANKL

RANKL is a member of the TNF superfamily. RANKL is also known as osteaclast differentiation factor (ODF), OPG ligand (OPGL) and TNF-related activation-induced cytokine (TRANCE) (Anderson et al. 1997; Lacey et al. 1998; Wong et al. 1997a; Wong et al. 1997b; Yasuda et al. 1998b). The membrane-bound human RANKL is 317 amino acids in length and is in trimeric form as a type 2 transmembrane glycoprotein (carbox-terminus on the extracellular side) characterized by a long extracellular stalk region together with a receptor-binding core domain (Wong et al. 1997b). A smaller monomer soluble form, reported to be less efficient than membrane-bound RANKL at stimulating osteoclast formation, also exists and is obtained through the cleavage of membrane-bound RANKL at the Phe 139 site within the ectodomain by a metalloproteases called TNF-α converting enzyme (TACE) (Clohisy & Harris 1999; Nakashima et al. 2000). In PO tissues for instance, it has been found that expression of TACE colocalised on RANKL-positive cells (Horiki et al. 2004), and this might indicate the secretion of soluble form *in situ*.

In context of bone homeostasis, RANKL is expressed by stromal cells and osteoblasts (Lacey et al. 1998; Suda et al. 1999; Yasuda et al. 1998b) as well as in osteocytes (Atkins et al. 2009; Nakashima et al. 2011; Xiong & O'Brien 2012). RANKL has been shown to be highly expressed in bone, bone marrow and also lymphoid tissues (Lacey et al. 1998; Yasuda et al.

1998b) with fibroblasts (Mandelin et al. 2005; Quinn et al. 2000; Sakai et al. 2002), mammary epithelial cells (Theill et al. 2002) and lymphocytes (Horwood et al. 1999; Kong et al. 1999; Kotake et al. 2001; Wong et al. 1997b) including B-cells (Manabe et al. 2001) and activated T-cells (Anderson et al. 1997; Horwood et al. 1999) are among other cell types reported to express RANKL.

One of the major roles of RANKL is stimulation of osteoclast differentiation. The work by Kong et al. (1999) showed the importance of RANKL in osteoclastogenesis by demonstrating osteopetrosis and complete absence of osteoclasts in a RANKL-knockout mouse. *In vitro* studies have shown that in the presence of M-CSF, soluble RANKL is sufficient to induce the differentiation of the pre-osteoclast into an active osteoclast (Lacey et al. 1998; Yasuda et al. 1998b).

In addition to promoting the differentiation of the pre-osteoclast into a mature osteoclast, RANKL was also found to be involved in enhancing the resorptive activity of the mature osteoclasts (Faust et al. 1999; Lacey et al. 1998). The enhanced resorption activity induced by RANKL was reported to be due to induction of rapid reorganization of cytoskeleton of osteoclasts into actin rings (hence forming the sealing zone) and stimulation of mature osteoclasts to perform multiple cycles of resorption (Faust et al. 1999).

1.3.5.1.2. Receptor Activator of NF-κB (RANK)

The discovery of RANKL as a crucial factor in osteoclastogenesis enabled the identification of RANK as the receptor for RANKL on the pre-osteoclasts and osteoclasts (Abu-Amer et al. 2000; Khosla 2001; Yasuda et al. 1998a). Human RANK, a 616 amino acid long protein belonging to the TNF receptor (TNFR) family first discovered in dendritic cells, is composed of a 28 amino acid extracellular domain, followed by a 21 amino acid long transmembrane domain, with the remainder of the RANK protein occupying the large cytoplasm (Anderson et al. 1997). This large cytoplasmic domain is important in triggering the intracellular signalling cascade that occurs upon the binding of RANKL to the extracellular domain.

RANK, which is also known as TRANCE-receptor or osteoclast differentiation and activation receptor (ODAR), has also been identified as expressed in dendritic cells, endothelial cells,

fibroblasts and both T and B lymphocytes, besides in mature osteoclasts or precursors (Haynes et al. 2001b).

RANK-knockout mice was found to have severe osteopetrosis due to complete absence of osteoclasts (Dougall et al. 1999). Meanwhile in genetically modified mouse model the expression of RANK-Fc fusion protein (which blocks interaction between RANK and its ligand) was found to cause severe osteopetrosis, which is a phenotype usually characterized by deficiency in osteoclasts and greater bone volume than normal (Hsu et al. 1999).

1.3.5.1.3. Osteoprotegerin (OPG)

It is now well established that osteoprotegerin (OPG) is a decoy receptor of RANKL (Yasuda et al. 1998b). OPG is a 380 amino acid long protein (401 amino acid in inactive pro-peptide form with 21-residue signal sequence (Kwon et al. 1998) expressed by osteoblasts (Simonet et al. 1997). OPG has been found to be expressed in marrow stromal cells, monocytes/marophages, dendritic cells, fibroblasts, both T and B lymphocytes (Simonet et al. 1997; Tsuda et al. 1997), endothelial cells (Collin-Osdoby et al. 2001; Crotti et al. 2003; Crotti et al. 2004; Haynes et al. 2003) and most recently shown to be expressed in mesenchymal stem cells (Oshita et al. 2011).

OPG was discovered before RANKL, therefore RANKL was also known as OPG ligand (OPGL) (Simonet et al. 1997; Tsuda et al. 1997). OPG is a member of the TNF superfamily of proteins that are secreted by the cells. It lacks a transmembrane and cytoplasmic domain and is secreted as a soluble protein (Simonet et al. 1997; Yasuda et al. 1998a). OPG is identical to osteoclasts inhibitory factor (OCIF) (Simonet et al. 1997; Yasuda et al. 1998a; Yasuda et al. 1998b) and TR1 (Kwon et al. 1998; Tan et al. 1997). OPG-knockout mice were observed to develop osteoporosis as well as other negative effects on the circulation system (Bucay et al. 1998; Mizuno et al. 1998). Conversely, transgenic mice over-expressing OPG demonstrated signs of suppresion on bone resorption (Stolina et al. 2007). It was concluded from these studies that OPG plays a major role in bone metabolism because uncontrolled osteoclast activity occurred in its absence. The involvement of RANKL-RANK interaction in the osteoclastogenic process and the confirmation of OPG as an antagonist for their interaction have been demonstrated in numerous subsequent studies.

1.3.5.1.4. OPG/RANKL/RANK System

As mentioned earlier, the osteoblast indirectly regulates osteoclastogenesis by producing RANKL, OPG, and M-CSF. While M-CSF is seen to be more associated with the expansion of the pre-osteoclast pool (Kodama et al. 1991a; Kodama et al. 1991b) as well as essential for the survival of the cells (Felix et al. 1994; Jimi et al. 1995; Lagasse & Weissman 1997), the OPG/RANKL/RANK system appears to be more involved in driving the differentiation process of pre-osteoclasts into mature osteoclasts, as well as the activation of mature osteoclasts (Figure 1.3). Studies have shown that combination of M-CSF and RANKL is sufficient to induce and drive the formation of human, mouse and rat multinucleated osteoclasts *in vitro* in the absence of stromal cell line (Alias et al. 2012; Cantley et al. 2011; Gyda et al. 2001; Holding et al. 2006; Quinn et al. 1998).

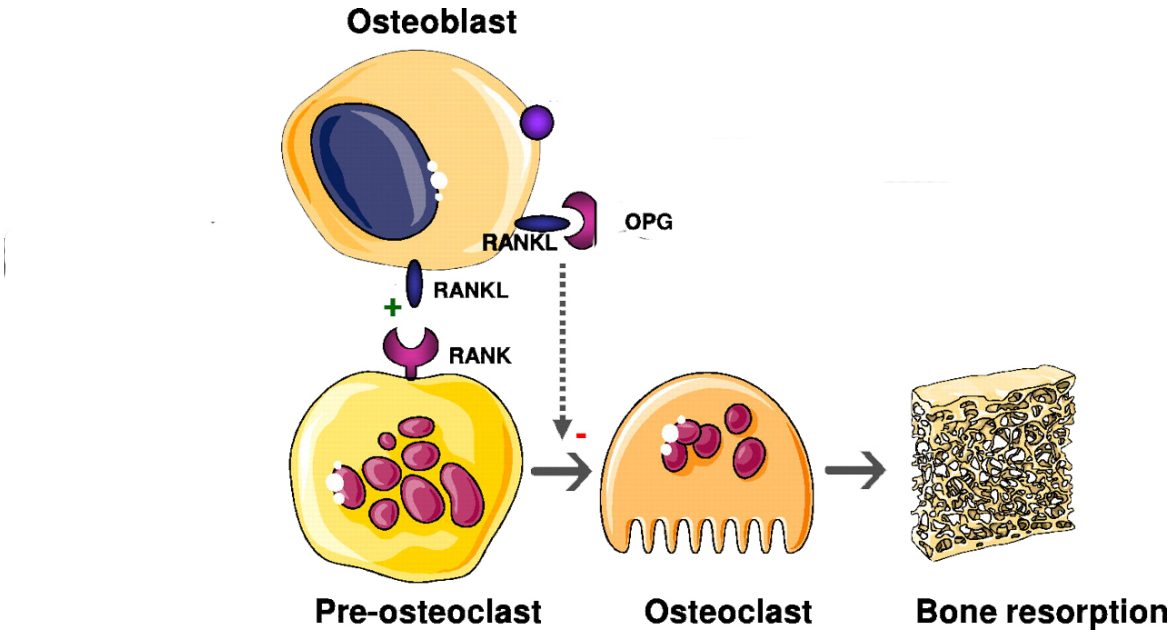

Figure 1.3: Overview on the interaction between pre-osteoclasts and osteoblasts, involving molecules like RANKL and OPG, which regulate the osteoclastogenesis process

Adapted from Lehouck et al. (2011)

Briefly, the regulation of osteoclastogenesis by OPG/RANKL/RANK system could be summarised in Figure 1.3 above. RANKL expressed by either osteoblasts or osteocytes binds to its natural receptor RANK (Anderson et al. 1997; Nakashima et al. 2011; Xiong & O'Brien 2012; Yasuda et al. 1998a) on the surface of the pre-osteoclast. There have been controversies over the cells expressing the RANKL supporting for osteoclast formation as there have been reports in the early days of the OPG/RANK/RANKL system discovery indicating osteoblasts or stromal cells are the cells providing such RANKL. However more recent studies indicating that osteocytes actually perform the function of releasing the RANKL for promoting osteoclastogenesis instead of osteoblasts (Kurata et al. 2006; Nakashima et al. 2011; Xiong & O'Brien 2012; Zhao et al. 2002).

The binding of RANKL to RANK triggers an intracellular signalling pathway, which begins with the activation of JNK, followed by recruitment and activation of a number of TNF receptor activating factor (TRAF) members, especially TRAF6 adaptor protein (Wong et al. 1998), which then activates nuclear factor-kappaB (NFκB). The activated NFκB plays an important role in inducing the activation of nuclear factor of activated T-cells 1 (NFATc1). The activated NFATc1 co-operates with other transcription factors to induce the transcription of osteoclastic-specific genes (Asagiri and Takayanagi 2007). The crucial role of NFATc1 in osteoclastogenesis process is presented in detail in section 1.3.5.1.6 below. The presence of OPG, the negative regulator of osteoclastogenesis, prevents such events from occurring by binding to RANKL, thereby preventing RANKL from interacting with RANK (Lacey et al. 1998; Yasuda et al. 1998b) (refer Figure 1.3).

1.3.5.1.5. Intracellular Signalling Post-RANK Activation

TRAF6 has been shown to play a crucial role in the intracellular signalling pathway of RANKL-induced osteoclast activation rather than differentiation. Even though osteoclastogenesis still occurs, TRAF6-knockout mice was found to have an osteopetrotic phenotype following defect in cytoskeletal organization (Armstrong et al. 2002; Lomaga et al. 1999). This is supported by another study by Naito and colleagues (1999) who demonstrated that lack of TRAF6 appeared to cause defective osteoclastogenesis. Subsequent work done by Gohda et al. (2005) suggested that, following induction on the RANK in RANKL-induced osteoclastogenesis, TRAF6 transduces the intrinsic signal which later lead to the increase in

the gene expression of the newly known key regulator of osteoclastogenesis, NFATc1. It has been suggested that activation of TRAF6 could lead to the dephosphorylation of NFATc1 mediated by JNK, p38 and NFκB1 (p50/p105) and NFκB2 (p52/p100) as the activation of those molecules could not be observed in TRAF6-deficient mouse (Hirotani et al. 2004). This is further supported by work done by Franzoso et al. (1997) and Iotsova et al. (1997) in which gene-knockout on both p50 and p52 was demonstrated to lead to osteopetrosis due to a lack of osteoclasts, suggesting an essential role of NFκB molecule in mediating osteoclastogenesis. Another pathway believed to relay the intracellular signal from RANKL stimulation besides TRAF6 is via the expression of c-fos, another crucial component of osteoclastogenesis (Grigoriadis et al. 1994). c-fos is a factor that heterodimerizes with c-Jun to form the another important transcription factor, activator protein-1 (AP-1).

1.3.5.1.6. NFATc1- The Key Transcriptional Factor in Osteoclastogenesis

NFATc1 has been discovered as the master transcriptional factor and key regulator involved in mediating the osteoclastogenesis process. The discovery of NFATc1 as a key regulator in osteoclastogenesis (Ishida et al. 2002; Takayanagi et al. 2002) has opened up a new era in the understanding of osteoclast cellular biology. Since this high impact discovery, there have been numerous studies, providing great insights into osteoclast biology and the field of osteoimmunology.

NFAT transcription factor family was first identified in T-cells (Shaw et al. 1988). Currently there are 5 members identified in this transcription factor family, which are NFAT1 (NFATc2), NFAT2 (NFATc1), NFAT3 (NFATc4), NFAT4 (NFATc3) and NFAT5 (Rao et al. 1997; Takayanagi 2007b). It seemed to be very challenging to study the function of NFATc1 *in vivo* in early days as targeted deletion on NFATc1 resulted in embryonic lethality as it is also important in cardiac morphological formation (de la Pompa et al. 1998; Ranger et al. 1998). The essential role NFATc1 plays in osteoclastogenesis could be seen from the retardation of osteoclastogenesis *in vitro* resulting from the suppression of NFATc1 (Ishida et al. 2002) and knockout of NFATc1 in embryonic stem (ES) cells (Takayanagi et al. 2002).

Dephosphorylation of the hyperphosphorylated cytoplasmic NFATc1 allows the transcription factor to be activated and translocated into the nucleus (Hirotani et al. 2004). The activation of phospholipase C by TRAF6 produces inositol triphosphate (IP_3) which mediates the increase in the intracellular calcium level that in turn leads to the activation of calcineurin, which is responsible for dephoshorylation of NFATc1 (Hirotani et al. 2004). Another mechanism that is thought to mediate the dephosphorylation of NFATc1 is through the inhibition of glycogen synthase kinase-3, the principal inhibitor for NFATc1 translocations into the nucleus (Beals et al. 1997), resulting from the activation of TRAF6.

NFATc1 expression was notably upregulated in response to RANKL stimulation during osteoclast differentiation period (Hirotani et al. 2004; Ishida et al. 2002; Takayanagi et al. 2002). Meanwhile, the over-expression (Takayanagi et al. 2002) or ectopic expression of constitutively active NFATc1 (Hirotani et al. 2004; Kim et al. 2005b) is sufficient to bypass the requirement of RANKL for osteoclastogenesis *in vitro* (as indicated by formation of TRAP-positive multinucleated cells), implying that up-regulation of NFATc1 expression is crucial to mediate the RANKL stimulation. In model of RAW264.7 cell line, stimulation by soluble RANKL protein was reported to increase the mRNA expression of NFATc1, NFATc2 and NFATc3. Nevertheless, only the induction of NFATc1 mRNA expression was found to be more than two-fold, occurring in less than 5 hours and remained high until 24 hour post RANKL stimulation and the nuclear localisation of NFATc1 was found to take place after 24 hour post- RANKL stimulation (Ishida et al. 2002).

NFATc1 has been reported to be able to auto-amplify its own expression following the induction for osteoclastogenesis and the auto-amplification lasts until the end of the differentiation process (Asagiri et al. 2005). Regulation at epigenetic level was believed to underlie this mechanism of NFATc1 auto-amplification (Asagiri et al. 2005). It was also found out that the process of auto-amplification of NFATc1 is initiated by temporal high expression NFATc2 occuring at the early stage following RANKL stimulation (Asagiri et al. 2005). An earlier study by Ikeda and co-workers (2004) indicated that overexpression of NFATc2 was able to enhance osteoclastogenesis. It appears that NFATc2 is involved in the mechanism by directly binds to the promoter of NFATc1, as evidenced from discovery of NFATc2 binding site on NFAc1 promoter region (Zhou et al. 2002), and upregulating the expression of NFATc1 (Asagiri et al. 2005). This NFATc1 auto-amplification during the osteoclastogenesis (Asagiri et al. 2005) supported by sustaining calcineurin-mediated Ca^{2+}

oscillation following RANKL stimulation (Takayanagi et al. 2002) (which promote nuclear localisation for its own and other gene transcription), indicates that NFATc1 actively plays a central role in the molecular mechanism underlying osteoclastogenesis, particularly on osteoclast cell fate determination in late stage of the process.

NFATc1 is believed to work with other transcription factors in synergy to initiate gene transcription (Kim et al. 2005d). NFATc1 is believed to co-operate with c-Fos, an upstream component of AP-1, in inducing osteoclast-specific genes (Takayanagi et al. 2002) by binding on the promoter regions of those genes. NFAT binding sites have been identified in the gene promoter regions of those osteoclast-specific markers such as *Acp5* (TRAP-encoding gene) (Takayanagi et al. 2002), *Calcr* (expressing calcitonin receptor) (Matsuo et al. 2004; Takayanagi et al. 2002), *Ctsk* (encoding cathepsin K) (Matsumoto et al. 2004; Takayanagi et al. 2002), and OSCAR-encoding gene (Kim et al. 2005b; Kim et al. 2005d) (OSCAR is going to be discussed in further detail in the following 1.3.5.2.2 section). Another such example of osteoclast-inducible gene targeted by NFATc1 is the β3-integrin encoding-gene (Crotti et al. 2006; Crotti et al. 2008), a cell attachment-facilitated receptor which facilitates the adhesion and motility of osteoclasts required for the osteoclast differentiation and bone resorption process (as described earlier in 1.3.2). NFATc1 cooperatively binds with PU.1, a member of the ETS family of transcription factors to induce the gene expression of the β3-integrin (Crotti et al. 2008).

NFATc1 was also found to be involved in osteoclast multinucleation process by directly regulates the mRNA expression of a couple of cell fusion-mediating molecules, which are dendritic-cell transmembrane protein (DC-STAMP) and Atp6vod2 (described earlier in section 1.3.1.2 above) with the discovery of the NFAT-binding sites within the promoter regions of those protein-encoding genes (Lee et al. 2006b). Inhibition of NFATc1 nuclear localisation by cyclosporin A (CsA) appeared to retard the multinucleation process in response to RANKL stimulation (Ishida et al. 2002). Overexpression of DC-STAMP and Atp6vod2 was found to drive and restore cell-cell fusion process even under inhibition by CsA (Lee et al. 2006b). Even though the intracellular localisation of NFAT family members is a very strictly controlled mechanism (Crabtree & Olson 2002; Macian et al. 2001), however excessive NFATc1 level is believed to be able to overcome the tight regulation (Takayanagi et al. 2002).

1.3.5.1.7. Pathological Relevance of OPG/RANKL/RANK System

In pathological bone loss it is believed that there is an alteration in bone metabolism resulting in excessive osteoclast formation and hence bone resorption following a shift in the RANKL/OPG ratio (Crotti et al. 2003; Crotti et al. 2004; Haynes et al. 2001a) as well as a possible suppression of osteoblast differentiation and activity (Schett 2009; Walsh & Gravallese 2010).

In the context of inflammation-mediated bone loss, the inflammation (presence of pro-inflammatory cytokines) within the tissue results in alteration in the expression level of RANKL and OPG on the osteoblasts towards in favour of increase in osteoclastogenesis, which eventually leads to excessive bone resorption (Boyle et al. 2003; Crotti et al. 2003; Crotti et al. 2004; Crotti et al. 2002; Haynes et al. 2003; Haynes et al. 2001a; Haynes et al. 2001b; Lerner 2006). This imbalance in RANKL and OPG does not only affect the osteoclastogenesis directly, however it could also be associated with the increase recruitment of inflammatory cells including osteoclast precursor cells through the regulation of associated chemokines. For instance, RANKL has been shown to upregulate the expression of MIP-1α, which is one of the chemokines shown to increase osteoclast motility and its receptor CCR1 expressed on osteoclast precursor cells (Yu et al. 2004). In addition, high titre of RANKL is also believed to enhance the osteoclast bone resorbing activity and this could be seen *in vitro* data indicated that higher dose of RANKL (50 instead of 10ng/ml) led to formation of more pits on dentine (Holding et al. 2006). The study also reported synergistic effect on the volume of dentine resorbed when combining RANKL and pro-inflammatory cytokine TNFα in the treatment on the culture osteoclast-like cells (Holding et al. 2006).

There is quite a number of convincing evidence indicating a shift in the RANKL/OPG ratio in tissues of local inflammation-mediated bone loss disease is associated with the severity of the diseases such as in peri-implant osteolysis (Gehrke et al. 2003; Haynes et al. 2001b; Haynes et al. 2004; Mandelin et al. 2003) and RA (Crotti et al. 2002; Haynes et al. 2003; Vanderborght et al. 2004). Some studies suggest that the shift in the RANKL/OPG ratio is resulted from the elevated expression of RANKL (Crotti et al. 2004; Crotti et al. 2002; Kawai et al. 2006; Wara-aswapati et al. 2007), while others indicate that the alteration in the ratio is

also accompanied with decrease in OPG expression as well (Crotti et al. 2003; Haynes et al. 2001b; Lu et al. 2006).

The sources of high RANKL levels in PO is believed to include osteoblasts (Pioletti & Kottelat 2004), fibroblasts (Horiki et al. 2004; Quinn et al. 2000), activated T-lymphocytes, giant cells and macrophages (Gehrke et al. 2003; Haynes et al. 2001b; Haynes et al. 2004; Mandelin et al. 2003). However, the main cell type thought to be responsible for the release of high level of RANKL in the tissue around the prostheses are macrophages following phagocytosis of wear particles (Crotti et al. 2004; Holding et al. 2006).

In the context of PO pathology, it has been reported that wear debris, even at low concentration, was shown to be able to increase the expression of RANKL and M-CSF by osteoblast culture (Pioletti & Kottelat 2004). Meanwhile *in vivo*, metals and PE wear debris has been shown to increase RANKL/OPG ratio in mouse calvarial model (Masui et al. 2005). Kim and colleagues (2001) found that there was lower level of OPG in synovial fluid of PO in comparison to OA. More interestingly, this low level of OPG could be associated with osteoclast formation and activity in PO as it was found that addition of the synovial fluid taken from PO patients into an osteoclast culture enhanced the formation of osteoclasts and addition of exogenous OPG could counteract the effect (Kim et al. 2001).

It is interesting to note that cells isolated PO tissues have the ability to readily form osteoclasts in the absence of RANKL as demonstrated by a number of studies (Haynes et al. 2001b; Neale & Athanasou 1999; Sabokbar et al. 2003). The ability of monocytes/macrophages isolated from PO tissues to readily differentiate into osteoclasts capable of resorbing bone in the absence of support from osteoblasts or RANKL supplementation (Haynes et al. 2001b; Sabokbar et al. 2003) might be explained by the ability of the cells to express RANKL and RANK besides abundance of TNFα themselves (Holding et al. 2006). This theory is supported by the immunohistochemistry data from the examination on the PO tissues, in which higher expression of RANKL and RANK in PO tissues than in OA and normal tissues was reported, with the expression to be mainly in cytoplasm of aggregated cells (Crotti et al. 2004; Haynes et al. 2001b). About half population of the cells expressing RANKL was reported to be positive for CD68, which is a cell marker for activated macrophages (Crotti et al. 2004; Sabokbar et al. 1997). It has also been noticed that in human

PO tissues, RANK was found to be expressed by similar cells that also expressing RANKL, including strongly in multinucleated osteoclast-like cells containing PE particles (Crotti et al. 2004; Holding et al. 2006). Addition of OPG was found able to inhibit osteoclast formation from cells isolated from around failed prostheses (Itonaga et al. 2000).

Similar to PO, in RA there have been numerous studies indicating shift in the expression of RANKL and OPG associated with the progression of the disease. A number of studies have demonstrated that RANKL was highly expressed, both at mRNA and protein level, in synovial tissues taken from RA patients (Crotti et al. 2002; Gravallese et al. 2000; Horwood et al. 1999; Kotake et al. 2001; Shigeyama et al. 2000). The data indicating higher expression of RANK in active RA tissues than in normal and OA groups (Haynes et al. 2001a) may further support the idea that the OPG/RANK/RANKL system has association with RA disease progression. This high expression of RANKL in the inflamed tissue in RA is believed to be one of the main factors that is responsible for the increase in osteoclastogenesis which eventually leads to excessive bone resorption (Haynes et al. 2001a). This is supported by the finding that there is positive correlation between severity of synovitis in the RA patients and the increase in RANKL (Crotti et al. 2002) accompanied with drop in the OPG expression (Haynes et al. 2003).

A study using an RANKL-knockout mouse model demonstrated the failure of getting any expected sign of arthritis following arthritic serum transfer (Pettit et al. 2001). Similarly, in a rat model of collagen-induced arthritis treatment with OPG-fusion protein (OPG-Fc) was able to arrest the bone erosion in the model by reducing the number of osteoclasts formed by over 75% (Romas et al. 2002). This is further supported by another *in vitro* study which demonstrated that treatment with OPG alone is sufficient to significantly arrest TNFα-mediated bone destruction by limiting the number osteoclasts formed (Redlich et al. 2002b).

There is accumulating evidence suggesting that activated T-cells (Haynes et al. 2003; Horwood et al. 1999; Kim et al. 2000; Kong et al. 1999; Kotake et al. 2001; Weitzmann et al. 2001) and synovial fibroblasts (Quinn et al. 2000; Takayanagi et al. 2000a) as might be the main source for the high expression of RANKL in the RA inflamed joints. Even though B lymphocytes are also associated with RANKL production and may play role in osteoclastogenesis (Manabe et al. 2001), this appeared to not to be in the context of RA tissues (Kotake et al. 2001).

Having established that the increase in RANKL plays a major role in RA bone resorption, it was suggested that treatment with OPG or molecules mimicking the protein might have potential to overcome or reduce the excessive bone erosion occurs in RA. The potential of this strategy appears to be promising as studies by Cheng et al. (2004), using a peptide that functions like OPG, and Onyia et al. (2004) who used a molecules that modulates endogenous OPG expression managed to demonstrate the ability of those tested molecules in reversing the bone loss in ovariectomised mouse as well as in amine-induced adjuvant arthritis (Cheng et al. 2004; Onyia et al. 2004).

Besides PO and RA, there are also other diseases being associated with alteration in expression of OPG/RANK/RANKL. Mutations in RANK is associated with excessive osteoclast activity resulting bone abnormality as been observed in familial expansile osteolysis (Hughes et al. 2000) and familial Paget disease (Whyte & Mumm 2004; Whyte et al. 2002). Meanwhile, diseases like idiopathic hyperphosphatasia (Cundy et al. 2002) and juvenile Paget disease (Whyte & Mumm 2004; Whyte et al. 2002) was reported to have relationship with functional mutation in OPG. On the other hand, OPG gene polymorhism seemed to be associated with inreased risk of osteoporotic fracture (Arko et al. 2005; Arko et al. 2002). The relevance RANK/RANKL/OPG system has in clinical sense has opened up windows for becoming good therapeutic target for the treatment of bone loss-associated diseases.

1.3.5.2. ITAM-mediated Signalling Pathway

It seems like there is another concurrent intracellular signalling pathway involved in driving osteoclastogenesis since it is less likely that the stimulation of the RANK receptor, which belongs to the tumor necrosis family receptor family (Anderson et al. 1997; Wong et al. 1997b), directly induce calcium signalling (Kim et al. 2005d). A number of publications have suggested that ITAM signalling might be involved co-stimulating osteoclastogenesis along together with RANK-RANKL pathway (Humphrey et al. 2006; Koga et al. 2004; Mocsai et al. 2004). The involvement of this co-stimulating pathway is thought to enable a fine-tune regulation of osteoclastogenesis (Herman et al. 2008).

The ITAM signalling system seems to be an active area for investigation in studying and understanding the regulation of osteclastogenesis because this signalling system is also reported to be crucial in the regulation of effector immune cells proliferation, survival and differentiation (Koga et al. 2004; Mocsai et al. 2004). The ITAM motif, whose sequence is well conserved YxxL/Ix6-8YxxL/I (where x represents any amino acid, see Table 1.4) suggesting its significance in mediating signal in a number of pathways in leukocytes (Humphrey et al. 2005), is usually located in the cytoplasmic domain of receptor or adaptor protein. Studies in past suggested that stimulation on this ITAM signalling pathway is stopped (as part negative feedback loop) by stimulation on the ITAM-counter immunoreceptor tyrosine-based inhibitory motif (ITIM)-mediated pathway (Barrow & Trowsdale 2006).

Table 1.4: Alignment of the conserved amino acids sequences of ITAM motifs in DAP12 and FcRγ between species

Species	Proteins	Protein Sequence
Zebrafish	DAP12	PESPYQELYGV---QSDIYSDLQ
	FcRγ	GEGVYEGLKPHET---DTYETIK
Pufferfish	FcRγ	DERIYMGLAPHE--QS-TYETIG
Frog	FcRγ	EEGEYTGLESVDKG---TYETIK
Mouse	DAP12	TESPYQELQGQR---PEVYSDLN
	FcRγ	ADAVYTGLNTRS---QETYETLK
Human	DAP12	TESPYQELQGQR---SDVYSDLN
	FcRγ	SDGVYTGLSTRH---QETYETLK

Adapted from Östergaard et al. (2009)

There are two ITAM-containing adaptor proteins identified as being expressed in myeloid (osteoclasts derived-lineage) cells, which are DNAX-activating protein 12kDa (DAP12) and

Fc receptor common gamma subunit (FcRγ) (Humphrey et al. 2005). The significance of ITAM-containing adaptor proteins in the development of functional osteoclasts appears to be equivalent to the essential role RANKL/RANK axis plays and this is shown in several studies (Humphrey et al. 2004; Koga et al. 2004) (to be discussed in further in the following section).

Due to lack or minimal extracellular domain for ligand binding and hence unlikely direct binding to the ligands (Abram & Lowell 2007; Lanier & Bakker 2000; Takai et al. 1994), DAP12 and FcRγ must pair to other receptors in order to sense and receive signals from the extracellular environment (Lanier & Bakker 2000; Takai et al. 1994). OSCAR and triggering receptor expressed by myeloid cells (TREM)-2 are examples of receptors that have been found to pair with FcRγ and DAP12 respectively via ionic interaction (positively charged transmembrane residue on receptor binds to the negative charge on adaptor proteins contributed from the amino acids) (Bouchon et al. 2001; Daws et al. 2001; Daws et al. 2003; Hamerman et al. 2006; Humphrey et al. 2005; Ishikawa et al. 2004; Lanier & Bakker 2000; Long & Humphrey 2012). A single mutation on any of those amino acids is sufficient to disrupt the ionic interaction (Lanier et al. 1998a; Wu et al. 2000). These transmembrane receptors, together with their corresponding ITAM-containing adaptor proteins, are going to be discussed further below.

The ITAM signalling system involves intracellular signal transduction following the stimulation on the ITAM-adaptor associated receptor, resulting phosphorylation of tyrosine within the ITAM on cytoplasmic domains of the adapter molecules (Humphrey et al. 2005). In osteoclast precursors, the phosphorylation of tyrosine within the ITAM allows the recruitment of Syk to the motif, which serves as the docking site (McVicar et al. 1998). This is then followed by the activation of the Syk kinase (Faccio et al. 2003c; Koga et al. 2004; Mocsai et al. 2004; Zou et al. 2007) through binding of the Src homology 2 (SH2) domain of the Syk's C-terminal to the ITAM motif (Futterer et al. 1998; Mocsai et al. 2004) and autophosphorylation of phospholipase Cγ (PLCγ) (Pitcher & van Oers 2003) that in turn mediates several other intracellular pathways that take place during osteoclastogenesis process. It has been reported that deficiency or inhibition of Syk tyrosine kinase resulted in defect in osteoclast differentiation (Koga et al. 2004; Mocsai et al. 2004) and function (Zou et al. 2007).

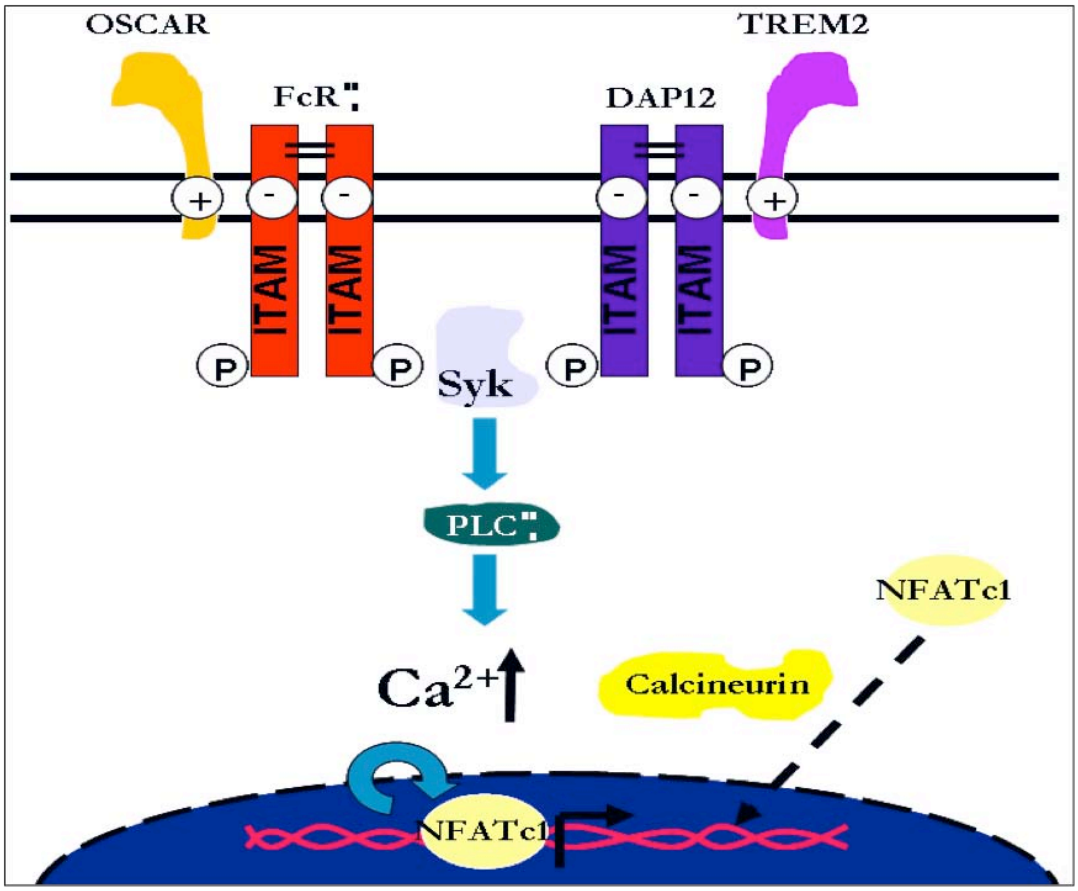

Figure 1.4: ITAM signalling plays role as costimulatory pathway involved in osteoclast differentiation

Adapted from Wu et al. (2008)

The activation of PLCγ results in increased intracellular calcium level, which then activates calcineurin to dephosphorylate NFATc1 to allow it to be translocated into nucleus. NFATc1 works together with other transcription factors (not shown) to activate the transcription of several osteoclast-specific genes. Auto-amplification of NFATc1 also occurs leading to rapid increase and high level of NFATc1 during the osteoclastogenesis process (Takayanagi et al. 2002).

This activated ITAM-signalling pathway led to oscillation of calcium level following activation of PLCγ is important for the expression and autoamplification of NFATc1 during osteoclast differentiation (Takayanagi et al. 2002). The RANKL-mediated NFATc1 stimulation has been proven to be dependant on ITAM-signalling pathway as double knockout of both DAP12 and FcRγ blocked such activation of NFATc1 and hence

osteoclastogenesis (Koga et al. 2004). The RANKL-induced osteoclastogenesis in the *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ model could be restored by ectopic expression of NFATc1 (Koga et al. 2004).

Not only important in mediating the process of osteoclast differentiation, ITAM signalling pathway has also been found to play significant role in influencing the activity or capacity of osteoclasts as well. Furthermore, both ITAM-mediated pathways in osteoclasts, which include OSCAR/FcRγ and TREM2/DAP12, seems to work in synergy and coherent manner in regulating the resorption activity or capacity of oscteoclasts. This could be seen from the mutation in both DAP12 and FcRγ that appeared to result in more severe osteopetrosis as well as reduction in bone resorption *in vivo* as well as more defect on osteoclast activity and formation *in vitro* than mutation in either DAP12 or FcRγ alone (Koga et al. 2004; Mocsai et al. 2004). The theory has also been supported by data from Kim et al. (2005) (Kim et al. 2005d), which indicated that the blockade of both TREM2 and OSCAR led to greater suppression on osteoclasts than the inhibition on either one of those receptors. This theory was also further supported by a recent study in mouse *in vivo* indicating double knockout on both OSCAR and DAP12 that led to reduction in TRAP-positive osteoclasts total number and bone loss by about a half as compared to cells lack in DAP12 or OSCAR alone (Barrow et al. 2011).

There are a few possibilities on the mechanism of regulation on osteoclast bone-resorbing capacity by the ITAM signalling pathway in osteoclasts. The first one would possibly be through the cell polarisation (during cell attachment to the bone, see section 1.3.2) as it was found that DAP12 and FcRγ appeared to mediate $\alpha_v\beta_3$-induced cytoskeletal organisation (Zou et al. 2007; Zou et al. 2010). The second mechanism ITAM signalling pathway might affect osteoclast activity or capacity is through the stage of cell multinucleation, which has been suggested based on data from several previous studies. *In vitro* examination on osteoclast-like cells derived from *DAP12* and *FcRγ*-deficient mice demonstrated a defect in multinucleation of osteoclasts as well as retardation in the expression of NFATc1 (Koga et al. 2004; Mocsai et al. 2004). The defect in the multinucleation of *DAP12* and *FcRγ*-deficient osteoclasts appears to be resulted from the failure in expressing NFATc1 as introduction and transduction of NFATc1 was able to restore the capability of the cell to differentiate appropriately (Koga et al. 2004). It is quite interesting to note that deficiency of DAP12 in macrophages (cell type sharing similar progenitor to osteoclasts) in DAP12-knockout mice also impaired the ability of the cells to fuse and form multinucleated giant cells (Helming et al. 2008). Considering

multinucleation is related to the degree of osteoclast bone resorbing activity (Boissy et al. 2002), in this study, it is generally hypothesized that regulation of ITAM-related molecules may have significant impact in the increase of bone resorption observed in PO and RA (see 1.4.1 below).

Collectively, all those studies described above suggested that there is crucial role ITAM signalling pathway has in mediating osteoclastogenesis beside RANKL-induced signalling pathway. Considering this, it would be interesting and worthwhile to study this signalling pathway by looking at the associated molecules such as TREM2 and OSCAR more closely. Those osteoclast ITAM-associated molecules are further discussed below.

1.3.5.2.1. TREM2 and DAP12

DAP12 is a membrane adaptor molecule containing ITAM identified as expressed in osteoclasts couples together with TREM2. DAP12 is also known as tyrosine kinase binding protein (TYROBP) or KARAP (Lanier et al. 1998b; Paloneva et al. 2000). Comparison on the homologs between species found DAP12 in human shares 75% sequence homology with the ones in mouse.

DAP12, which was first discrovered in natural killer (NK) cells as dimer of adaptor protein/receptor complex (covalently attached to NIC-receptor) (Lanier & Bakker 2000), is also identified as being expressed in many other myeloid cell types, such as macrophages, monocytes, dendritic cells, granulocytes as well as some T and B lymphocytes (Chen et al. 2009; Goronzy et al. 2005; Humphrey et al. 2004; Lanier & Bakker 2000; Tomasello et al. 1998).

DAP12 is a transmembrane protein identified as an important component in transducing activating signals in NK cells for targeting cells to be lysed (Campbell & Colonna 1999; Colonna 1998; Lanier et al. 1998b; McVicar et al. 1998). It seems like DAP12 play significant role in monocyte differentiation in general, as it appears that the adaptor protein was found to modulate and expressed during monocyte differentiation into macrophages (Aoki et al. 2000) and dendritic cells (Bouchon et al. 2001), other cell types sharing similar progenitor osteoclasts. In dendritic cells, DAP12 also appears to play important role in cell

maturation and prolonging cell survival by enhancing antigen presentation through the upregulation of CD40, CD86 and MHC class II (Bouchon et al. 2001).

Even though DAP12 has been reported to play various important roles in numerous cell types, the signficance of this molecule in bone homeostasis, in the context of osteoclasts in particular, should also be acknowledged. As briefly described earlier (see 1.3.5.2 above), DAP12 appears to play role in bone resorption, and this could be seen from DAP12-deleted mice exhibiting higher bone mass of femur and tibia in addition to increased trabecular thickness (Humphrey et al. 2004). As mentioned previously, it has been reported that DAP12-deficient mice developed severe osteopetrosis and *in vitro* works demonstrated that the osteoclast precursor cells isolated from the mice did not undergo differentiation (Koga et al. 2004; Mocsai et al. 2004; Takai et al. 2005). It was suggested that the suppression on the osteoclastogenesis in the $DAP12^{-/-}$ mice-derived cells occurred between the intermediate and late stage as mononuclear cells derived from these mice induced into osteoclastogenesis following RANKL stimulation *in vitro* were still found to be TRAP-positive and expressed osteoclast-specific marker like cathepsin K, β3-integrin, calcitonin receptor, RANK and OSCAR, nevertheless lost its capability of resorbing bone (Humphrey et al. 2004; Mocsai et al. 2004). Very recent study by Despars and coworkers (2013) found that DAP12 overexpression could promote osteoclastogenesis by increasing the proliferation rate for the osteoclast progenitors. It turns out that DAP12 is critical in osteoclastogenesis as retroviral introduction of DAP12 into $DAP12^{-/-}/FcR\gamma^{-/-}$ is able to restore the capability of the osteoclast precursor cells to get multinucleated and differentiated into mature functional osteoclasts (Humphrey et al. 2004; Mocsai et al. 2004; Paloneva et al. 2003).

DAP12 also has role in osteoclast bone resorbing activity through cyoskeletal reorganisation (Zou et al. 2010) triggered by M-CSF (Otero et al. 2009). This DAP12-induced cytoskeletal reorganisation was found to be mediated by Syk phosphotyrosine (Faccio et al. 2003c). Targeted deletion on Syk, which is downstream of DAP12 (Koga et al. 2004), led to the phenotype of failure in cytoskeletal dysfunction that led to inability to resorb mineralised substrate *in vitro* (Zou et al. 2007). Besides failure in actin ring and ruffle border formation, the dysfunction of cytoskeletal reorganization in $DAP12^{-/-}$ cells were further supported by inability of the mutant osteoclasts formed to transmigrate through osteoblast layer (Zou et al. 2010). The signficance of this TREM2/DAP12-mediated ITAM signalling pathway in osteoclast cytoskeletal reorganisation is redemonstrated by Faccio and colleagues (2005),

whom reported that deficiency in Vav3, another candidate target downstream of those ITAM molecules, led to failure in actin-cytoskeleton organisation and defect in resorption activity.

Besides TREM2, DAP12 could also couple with other receptors such as myeloid DAP12-associated lectin (MDL-1), TREM1, TREM3, NKGD2 and signal regulatory protein β (SIRPβ) (Daws et al. 2001; Humphrey et al. 2004; Joyce-Shaikh et al. ; Long & Humphrey 2012). However, considering TREM2 is the particular immunoreceptor that paired to DAP12 in osteoclasts (Daws et al. 2001; Humphrey et al. 2006), the scope of these studies will focus on TREM2. In addition, it has been suggested by quite a number of studies that TREM2 is linked to osteoclastogenesis.

TREM2 is a member the TREMs family (Bouchon et al. 2000) that also consists of TREM1, TREM3 and recently identified TREM-like 4 (Hemmi et al. 2009). TREM2 are reported to be widely expressed by cells from myeloid lineage such as dendritic cells, macrophages, microglial cells and osteoclasts (Bouchon et al. 2000; Daws et al. 2001; Paradowska-Gorycka & Jurkowska 2013). TREM2 was first discovered in human monocyte-derived dendritic cells and mouse macrophage RAW264.7 cell line (Bouchon et al. 2001; Daws et al. 2001). Of note, TREM2 expression level appears to be relatively constant regardless of stimulation by RANKL and regulation of the interaction seems to dominantly influenced by the expression of the ligand (Kim et al. 2005d). To date, the ligand for TREM2, including the one expressed in osteoclasts, is still unknown. Humphrey et al. (2006) (Humphrey et al. 2006) suggested that TREM2 signalling might be activated by negatively charged inorganic bone matrix, hence may play role in mediating osteoclast development in bone microenvironment. TREM2 has been shown to bind to anionic ligands possibly through pattern recognition (Daws et al. 2003; Roodman 1999). A more recent study suggested plexin A1, a receptor for semaphorins, as potential activating ligands in TREM2/DAP12-mediated signalling pathway during osteoclastogenesis as stimulation of osteoclast precursors with cells deficient in plexin A *in vitro* led to reduction in osteoclast formation and *in vivo* mouse model lack of plexin A1 manifested osteosclerosis together with increased bone mass (Takegahara et al. 2006). In the latest discovery on ligand of TREM2, TREM2 was reported to bind bacterial LPS (Quan et al. 2008) and has been suggested to function as phagocytic receptor for bacteria in macrophage (N'Diaye et al. 2009).

Deficiency in TREM2 was found to result in arrest in the differentiation of human pre-osteoclasts into functional osteoclasts (Cella et al. 2003; Paloneva et al. 2003). In the RAW264.7 cell line, blockade of TREM2 and its interaction with its ligand led to the inhibition of osteoclastogenesis including on the ability of osteoclasts to resorb bone efficiently as well as their capability to migrate (Humphrey et al. 2006). On the other hand, stimulation of TREM2 mouse pre-osteoclasts appeared to promote osteoclast multinucleation and migration/chemotaxis (Helming et al. 2008; Humphrey et al. 2006). However, in a study by Paloneva et al. (2003) it was reported that mutations in DAP12 and TREM2 did not lead to defect in the osteoclastogenesis, but only resulted in delay in osteoclasts differentiation and decrease in bone resorption assessed based on dentine pit analysis.

Experiments in TREM2/DAP12 non-knockout models also demonstrated the significance of TREM2 and DAP12 in mediating osteoclastogenesis. Addition of TREM2-Fc protein , which provided competition for binding to ligand, into an *in vitro* osteoclast-osteoblast co-culture seemed to give inhibitory effect on osteoclasts (Kim et al. 2005d). Suppression of TREM2 through RNA silencing was found to inhibit RANKL-driven osteoclastogenesis in RAW264.7 cell line and mature osteoclast formation and bone resorbing activity (Humphrey et al. 2006; Park-Min et al. 2009). Meanwhile stimulation of TREM2 in those cells and murine primary BMMs via antibody cross-linking promoted osteoclast-like cells formation by three-fold in the presence of RANKL and M-CSF with DAP12 appeared to be essential in mediating the process (Humphrey et al. 2006).

Unlike OSCAR (to be discussed in the following section), TREM2 appears not to be regulated by NFATc1, based on the findings that there is no putative NFAT binding site on *TREM2* promoter region and treatment with FK506 (which hence inhibits nuclear localization of NFATc1) does not significantly affect TREM-2 gene expression (Kim et al. 2005d). Indeed, there have been more recent studies using *in vitro* model of PBMC-derived osteoclast culture suggesting that TREM2 is upstream of NFATc1 expression in the RANKL-induced CaMK-MEK-ERK the cell signalling pathway (Kalliolias et al. 2010; Park-Min et al. 2009). They also demonstrated elevation in TREM2 mRNA expression level taking place during osteoclastogenesis period, in parallel to RANK mRNA expression on the human PBMC-derived pre-osteoclastic cells. Nonetheless, for mouse *TREM2* the gene seems to be regulated by MITF, a basic helix-loop-helix transcription factor and PU.1 as the promoter region of this gene contains the binding sites for these transcription factors (Kim et al. 2005d).

In the context of human pathology, DAP12 deficiency, as a result of genetic mutation on chromosome 19 leading to premature termination of the protein, is believed to be the cause of an uncommon (reported to be among Japanese, Finnish and Italian population thus far), genetically recessive human disease called polycystic lipomembranous osteodysplasia with sclerosis leukoencephalopathy (PLOSL), which also known as Nasu-Hakola disease (Kondo et al. 2002; Nakamagoe et al. 2011; Paloneva et al. 2000; Paloneva et al. 2003; Soragna et al. 2003; Thrash et al. 2009). There is also evidence of the genetic disease associated with mutation in TREM2 splicing (Numasawa et al. 2011; Thrash et al. 2009). Recent studies also pointed out TREM2 as strongly involved in Alzheimer's disease (Fenoglio et al. 2007; Neumann & Daly 2013).

PLOSL is characterized by osteopenia, which lead to early onset of osteoporosis, multiple lipid filled bony cysts restricted to wrists and ankles, trabecular bone loss and cortical bone demineralisation, loss of brain white matter leading to presenile dementia and premature death before age 50 (Paloneva et al. 2001; Verloes et al. 1997). There is also evidence associating the disease to the fragility of bone manifested by repeated bone fracture in a Japanese patient (Nakamagoe et al. 2011) believed to resulted from bone spongy texture following formation of multiple lipid-filled bone cysts. This disease was suggested to be associated with deficiency of DAP12, as this could be seen from *in vivo* data by Kaifu et al. (2003) indicating that DAP12-deleted mice were shown to manifest bone and brain abnormalities displayed in PLOSL. However a very recent data by Despars and colleagues (2013) indicated otherwise as the study reported that osteopenia, which could be seen in PLOSL, was manifested by transgenic mice overexpressing DAP12. Mutation on TREM2 in PLOSL is believed to cause brain abnormality in the disease by introducing defect on the neuronal apoptotic clearance by microglial as TREM2 is found to be important in the phagocytosis process by microglials (Hsieh et al. 2009; Prada et al. 2006). It is quite interesting to note that, even though the function of DAP12 is mainly described in NK cells as important component in mediating signals for targeting cells to be lysed (Campbell & Colonna 1999; Colonna 1998; Lanier et al. 1998b; McVicar et al. 1998), there was other study reported no defect on NK cell function in PLOSL patients (Paloneva et al. 2000), suggesting cellular function is affected by mutation in DAP12/TREM2 more obvious in monocyte/macrophage lineage (such as microglials (Cuadros & Navascues 1998) and osteoclasts).

Even though there are accumulating studies indicating the significance of TREM2 and DAP12 in bone homeostasis, there is controversy surrounding the specific or exact role the activation of these molecules play to be resolved. While majority of those studies above suggested TREM2/DAP12 is involved in co-stimulating osteoclast differentiation process, a very recent study by Otero et al. (2012), using a mouse model, in contrast found that deficiency in TREM2 leads to “accelarated” differentiation of osteoclast accompanied with earlier experssion of osteoclast-specific markers. Otero et. al. (2012) suggested that the activation of TREM2/DAP12 signalling pathway may play important role in early stage of osteoclast development, most likely during the stage of proliferation of precursor cells before differentiation. Based on the data obtained, they speculated that deletion of TREM2 led towards earlier cell cycle arrest for proliferation resulting earlier osteoclast differentiation (Otero et al. 2012). This is supported by earlier findings that reported expression of TREM2 in macrophages in culture containing M-CSF (supplement mainly for cell proliferation) only (Turnbull et al. 2006).

Such contradictions among all these findings might be attributed by differences in cell types and models used as well as cellular response in respond to range of stimulating ligands, avidity of ligands and spectrum of associated-receptors (related to DAP12) (Otero et al. 2012). Therefore, further studies should be carried out in the future on DAP12 and TREM2 molecules in order to gain better understanding on those molecules, not only in the context of cell and molecular biology, but also more importantly the signficance and relevance of the protein at higher level of physiology and pathology, if there any. Thus far, DAP12 and TREM2 are not explored yet in studies investigating local inflammation-mediated bone loss tissue such as in RA and PO.

1.3.5.2.2. OSCAR and FcRγ

Early in this century, an immunoglobulin-like receptor expressed in murine osteoclasts known as OSCAR was discovered, thanks to the work done by Kim et al. (2002). OSCAR discovered via the PCR-Subtraction, was found to play an important role in osteoclastogenesis (Kim et al. 2002). Human and mouse OSCAR share 73% sequence homology (Kim et al. 2002) and the expression of OSCAR in bone is highly conserved among various species (Nemeth et al.

2011), suggesting a significant role it plays in bone function or homeostasis. OSCAR, which is a novel member of leukocyte receptor complex (LRC)-encoded protein, was thought to be expressed at both gene and protein predominantly in mature osteoclasts only in murine bone tissues and RAW264.7 cells (Kim et al. 2002). However unlike in murine cells, OSCAR could also be found to be expressed in monocytes (the precursor cell of osteoclasts), dendritic cells (which is also derived from monocytes (Miyamoto et al. 2001)) in human tissues (Merck et al. 2005; Merck et al. 2004; Merck et al. 2006; Tenca et al. 2005) and very recently in endothelial cells (Goettsch et al. 2011; Nemeth et al. 2011).

OSCAR was also reported to be expressed in RAW264.7 cell line-derived osteoclast-like cells (common model or cell line of osteoclasts used for study purpose as it has been shown to differentiate into osteoclast-like cells *in vitro* following stimulation of RANKL and regulation of NFATc1 (Hirotani et al. 2004; Sinningen et al. 2013), making OSCAR as a good candidate of marker for osteoclasts (Humphrey et al. 2004; Kim et al. 2002). The expression of OSCAR is thought to start in the late stage of osteoclast differentiation after the formation of TRAP-positive pre-osteoclasts (Kim et al. 2002) and NFATc1 expression (Kim et al. 2005b).

OSCAR is a type I transmembrane protein with full length cDNA of 282 amino acids (Kim et al. 2002; Nemeth et al. 2011) and possesses a positively charged arginine residue in the transmembrane domain (Kim et al. 2002), which suggests that this immunoreceptor might be coupled with other transmembrane protein. Based on protein structure modeling, human OSCAR is expected to contain a signal peptide (Nemeth et al. 2011) (see Figure 1.5) speculating its secretory function and supporting the idea of soluble isoform (Herman et al. 2008; Khan 2007).

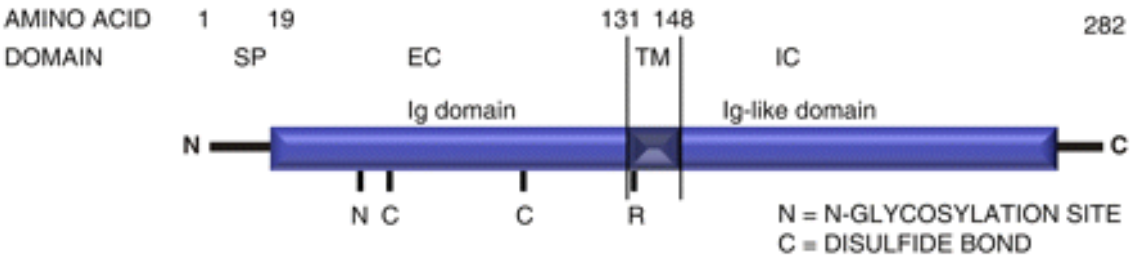

Figure 1.5: Polypeptide structure and domains of human OSCAR structure and domains

Taken from Nemeth et al. (2011)

Figure 1.5 above is a schematic diagram representing full-length encoded human OSCAR polypeptide. The polypeptide could be divided into four distinct domains, which include a signal peptide (**SP**), two Ig-like motifs and transmembrane domain (**TM**) in between those two Ig-like domains. Presence of **SP** supports the possibility of OSCAR present in secreted form. For the Ig-like motif, when the receptor is bound to the cell membrane, there is one located in extracellular (**EC**) and this domain spans from amino acid 38-121. This domain contains two cysteine residues (**C**) essential for disulphide bonds for protein stabilisation and a N-glycosylation site (**N**), which allows the protein to present as monomer (Merck et al. 2004). The other one located in intracelullar (**IC**) spans from amino acid 135 to 215 and is important for mediating signal transduction as it is connected to a short cytoplasmic tail. The transmembrane domain (**TM**) consists of 282 amino acids long, including an arginine residue (positively charged, represented as **R**) that is essential to get the receptor associated with FcRγ (Nemeth et al. 2011).

In its function as an immunoreceptor, OSCAR is physically associated with FcRγ chain, an adaptor protein for Fc receptors (Ishikawa et al. 2004; Koga et al. 2004). The FcRγ chain has been demonstrated to play a role in facilitating or upregulating the expression of OSCAR on the cell surface (Ishikawa et al. 2004) in addition to mediating signal transduction (Koga et al. 2004). Besides OSCAR, FcRγ has also been found to couple with other receptor such as FcRIII (Athanasou & Quinn 1990) and paired Ig receptor-A (PIR-A)(Maeda et al. 1998; Taylor & McVicar 1999).

A ligand for OSCAR has only recently been identified as an extracellular matrix collagen (Barrow et al. 2011). Earlier, it has been suggested that the ligand of OSCAR might be expressed by osteoblasts since it has been demonstrated by Kim and colleagues (2002) that osteoblasts stained well with OSCAR-Ig fusion protein. This data was also supported by later study (Kim et al. 2005d) which indicated detection of OSCAR ligand on osteoblast upon stimulation by osteotropic factors 1, 25-$(OH)_2$ vitamin D_3 and PGE_2 as well as RANKL. Herman et al. (2008) later reported that the introduction of OSCAR-Fc protein competed for OSCAR ligand in homogenous cell culture that eventually led to decrease in osteoclast formation. This suggested that ligand of OSCAR is expressed in PBMC-cell culture, hence may further speculate role in cellular amplifying response. Nonetheless, the finding from Barrow et al. (2011) does not contradict data provided by earlier studies (Kim et al. 2002;

Koga et al. 2004) as extracellular matrix (ECM) collagen are expressed by osteoblastic bone-lining cells (Andersen et al. 2009; Hauge et al. 2001; Miller & Jee 1987). Indeed in his study, Barrow and colleagues (2011) demonstrated interaction between mononucleated osteoclast-expressing OSCAR and collagen expressed by bone-lining osteoblasts. The finding of collagen type I as the ligand for OSCAR may suggest that this receptor might be responsible for osteoclast differentiation process in close proximity to bone instead in tissues and circulation.

OSCAR has been shown to play role in immune system (both innate and adaptive) through dendritic cell maturation and activation (Merck et al. 2005; Merck et al. 2004), MHC class II-mediated antigen presentation for adaptive immune system (Merck et al. 2004) as well as in augmenting pro-inflammatory reaction in monocytes and neutrophils by inducing the secretion of cytokines like granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 that sustain T-cell proliferation (Merck et al. 2006; Tenca et al. 2005). Besides, OSCAR also prolongs cell survival through inhibition of apoptosis in dendritic cells, denoted by reduction in numbers of apoptotic cells and increase in the expression of anti-apoptotic molecule Bcl-2 (Merck et al. 2005; Tenca et al. 2005). Unlike in osteoclasts, the expression of OSCAR in dendritic cells is independent of the cell differentiation stage (Merck et al. 2004).

It was suggested that OSCAR has a more specific role in co-stimulating osteoclastogenesis process based on data documented by a number of studies (Kim et al. 2002; Koga et al. 2004). An upregulation in the expression of OSCAR has been observed during osteoclastogenesis (Ishikawa et al. 2004) and downregulation in the gene expression is resulted following inhibition of the calcineurin-mediated pathway (which also regulates NFATc1 nuclear translocation) using FK506 (Kim et al. 2005d). Recently, Barrow et al. (2011) demonstrated that interaction between OSCAR and ligand (collagen) enhanced osteoclastogenesis *in vitro* and blocking the interaction would inhibit such enhancement in osteoclastogenesis. More interestingly, activation of OSCAR- FcRγ was also found to inhibit apoptosis in M-CSF and RANKL-induced osteoclastogenesis (Zou et al. 2010).

As mentioned earlier, OSCAR gene expression could be directly regulated by NFATc1 like other genes of osteoclast-specific marker as this has been demonstrated through direct chromatin immunoprecipitation (ChIP) assay and gene reporter assay (Kim et al. 2005b; Kim et al. 2005d). Constitutively active NFATc1 was found to promote the expression of OSCAR

(Kim et al. 2005b). There are two NFATc1-binding sites identified on mouse OSCAR promoter region, however the regulation of OSCAR expression occurred mostly on only one of those binding sites (Kim et al. 2005b). While NFATc1 also has the capability of directly inducing OSCAR gene expression (Kim et al. 2005d), at the same time NFATc1 has also been reported to be upregulated downstream of calcium signalling mediated by OSCAR (Koga et al. 2004). This implies that a positive feedback loop may be involved in the interaction between OSCAR and NFATc1 (refer Figure 1.6).

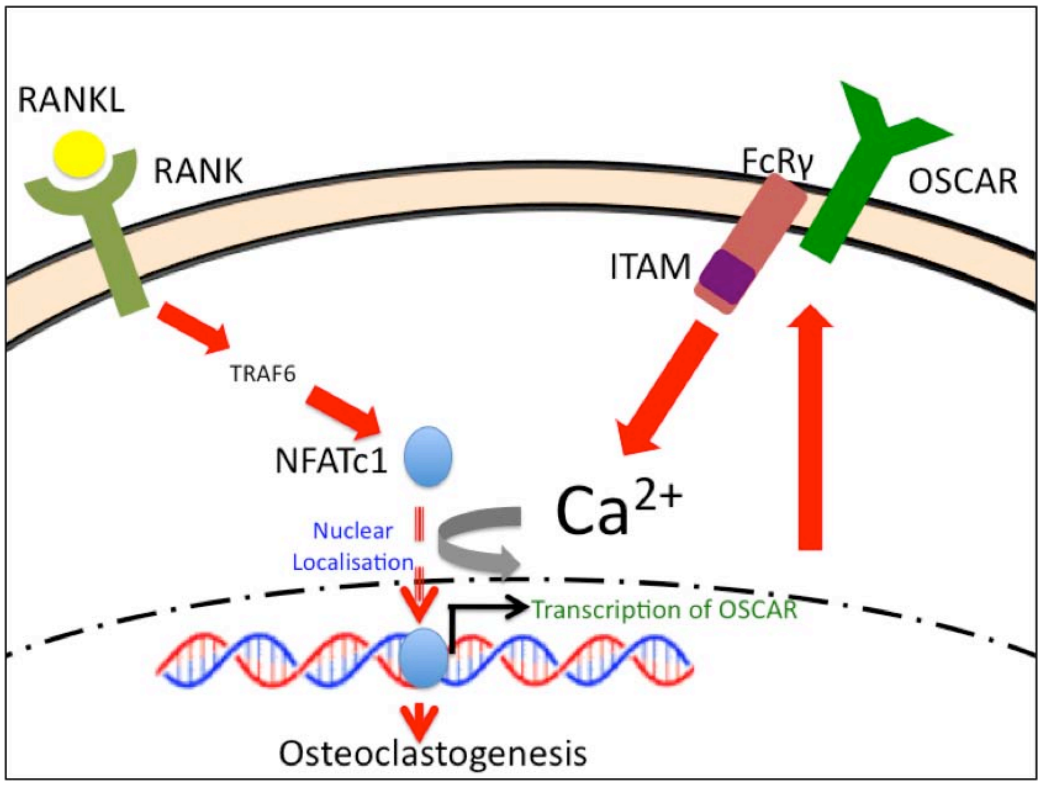

Figure 1.6: Positive feedback loop between NFATc1 and OSCAR expression and activity

Adapted from Kim et al. (2005b), Kim et al. (2005d) and Koga et al. (2004)

The ligation of RANK to its ligand RANKL triggers a cascade of intracellular signalling pathway that eventually produces NFATc1, the key transcriptional factor for switching on the gene expression of a number of osteoclast-specific cells, including OSCAR (Kim et al. 2005b; Kim et al. 2005d). The transcription of those osteoclast-specific genes by NFATc1 is important in allowing the osteoclastogenesis process to progress. Meanwhile, the stimulation of OSCAR by its ligand causes the activation of ITAM in the associated adapter molecules FcRγ, which eventually leads to the activation of PLCγ and increase in intracellular calcium level. This increase in calcium level is important for the nuclear localisation of NFATc1 (Koga et al. 2004) and hence promoting further transcription of osteoclast-specific genes, including OSCAR. Therefore, this interaction between NFATc1 and OSCAR based on their regulation of expression and activity forms a positive feedback loop.

Studies on the mouse promoter region of the OSCAR gene indicate that transcription factors MITF and PU.1 also play a role in regulating transcription of the *OSCAR* gene (So et al. 2003), like TREM2 gene (Kim et al. 2005d). It was reported that the stimulation of OSCAR gene expression by NFATc1, like TRAP (Kim et al. 2005d) and cathepsin K (Matsumoto et al. 2004), could be further enhanced in synergy with a combination of MITF and PU.1 transcription factors (Kim et al. 2005d; So et al. 2003). Other transcription factors also known to regulate OSCAR expression include USFs (promote OSCAR gene expression) (Kim et al. 2008) (Figure 1.7) and inihibitory transcription factors inhibitor of differentiation (ID) 2 (Lee et al. 2006a) and MafB (Kim et al. 2007a). Figure 1.7 represents a schematic diagram of illustrating the loci on human gene where the transcription factors are bound for initiating gene transcription. There is a spelling error in the diagram, where MIFT is supposed to be corrected to MITF.

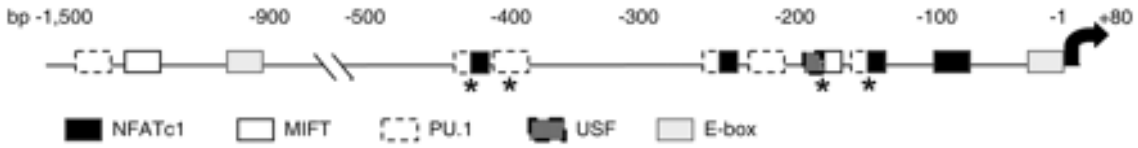

Figure 1.7: Binding sites for transcription factors to bind on human OSCAR gene

Taken from Nemeth et al. (2011)

Besides transcription factor, there is quite a number of other factors identified to date as able to regulate the transcription of OSCAR gene, which are compiled and listed in Table 1.5 below.

Further evidence of the significance of OSCAR in mediating osteoclastogenesis could be observed from the retardation of the osteoclast differentiation process as a result of down-regulation in the expression of OSCAR and NFATc1 as a consequence of the over-expression of protein inhibitor of activated STAT 3 (PIAS3) (see Table 1.5) (Kim et al. 2007b). PIAS3 is postulated to mediate the inhibitory effect on the OSCAR expression by recruiting histone deacetylase 1 to the promoter of OSCAR (Kim et al. 2007b).

Table 1.5: Factors regulating OSCAR mRNA expression in osteoclasts

Category	Factors	Function
Inducer	RANKL	Cytokine
	IL-1	Cytokine
	FcRγ	Adaptor molecule
	NFATc1	Transcription factor
	MITF	Transcription factor
	PU.1	Transcription factor
	USFs	Transcription factor
Repressor	MafB	Transcription factor
	ID	Transcription factor
	PIAS3	Repressor of transcription
	KR62776	PPAR-γ
	Silibinin	Polyphenolic flavonoid
	MHC CIITA	Non-DNA binding coactivator

Adapted from Nemeth et al. (2011)

Surprisingly, mice deficient in FcRγ, the OSCAR-associated ITAM-containing adaptor protein, do not exhibit signs of osteopetrosis, suggesting that the role of FcRγ chain could be compensated by other mechanism or pathway *in vivo* (Ishikawa et al. 2004; Koga et al. 2004; Mocsai et al. 2004). The significance of the contribution FcRγ gives towards mediating bone loss, however, still should not be undermined since mice deficient in both DAP12 and FcRγ showed more severe osteopetrosis as compared to those which lacked of DAP12 alone even though *in vitro* examination demonstrated that either deficiency of DAP12 alone and double knockout of both DAP12 and FcRγ led to failure for multinucleation (Koga et al. 2004; Mocsai et al. 2004; Takai et al. 2005). The significant role of FcRγ in compensating the osteoclastogenesis in DAP12-deficient cells (Kim et al. 2002; Koga et al. 2004; Mocsai et al. 2004) could also be seen from the partial increase in the size of DAP12-deficient osteoclasts formed, besides increasing osteoclast formation following the activation of OSCAR-FcRγ (Zou et al. 2010). Recently Barrow et al. (2011) demonstrated that stimulation of OSCAR or FcRγ-mediated pathway was able to compensate the reduction in osteoclastogenesis in

DAP12-deficient cells. Together findings from all these studies imply that the contribution in osteoclast development or bone resorption by FcRγ should not be neglected.

Studies on the ITAM-containing adaptor proteins also suggest that DAP12 and FcRγ are cooperatively involved in transducing signals for osteoclastogenesis (Kim et al. 2005d). This can be seen from studies in which pre-osteoclasts with double mutations in both *DAP12* and *FcRγ* could not differentiate into multinucleated osteoclasts even in the presence of osteoblasts, however mutation on *DAP12* alone still enabled the ability of the pre-osteoclasts to multinucleate provided the differentiation process took place in the presence of osteoblasts (Koga et al. 2004; Mocsai et al. 2004). This fits well in the context of human pathology of PLOSL (described in previous section 1.3.5.2.1 above), which is also characterised by trabecular bone loss besides bone cysts (Paloneva et al. 2000), suggesting that dysfunction in TREM2-DAP12 pathway still allows osteoclastogenesis to occur (Chouery et al. 2008). And recently, it has been shown that the interaction of OSCAR, the FcRγ-associated transmembrane receptor, with its ligand, provide the compensatory mechanism for the impairment in osteoclastogenesis resulted from deficiency in DAP12 or TREM2 observed in both *in vivo* and *in vitro* studies in mouse cells and human cells from PLOSL patients (Barrow et al. 2011; Koga et al. 2004; Mocsai et al. 2004).

Bringing OSCAR and FcRγ into the perspective of bone physiology and pathology, currently there has been a few studies investigating the expression of these ITAM-associated molecules in those contexts. Recently Herman et al. (2008) revealed that the gene and protein expression of OSCAR is upregulated in synovial tissues of rheumatoid arthritis patients. It is interesting to note that the upregulation of OSCAR expression in rheumatoid arthritis occurs not only locally in mononuclear monocytes prior to differentiation into osteoclasts at the inflamed synovium, but also in the circulating monocytes before the entry of the cells into synovium (Herman et al. 2008). Thus, it is thought that the increase in the expression of OSCAR could also be associated with systemic inflammation (Herman et al. 2008).

Besides RA, OSCAR has been found to be associated with the pathology of OA and other bone diseases. Incubation of cells with synovial fluid taken from OA patients *in vitro* was found to give increase in the mRNA expression of OSCAR and NFATc1, suggesting that there are unkown factors in those synovial fluids that stimulate the expression of OSCAR. Study on a Korean population found that a single nucleotide polymorphism (OSCAR-

2322A>G that affect CREB binding site in *OSCAR* promoter region) is associated with low bone mass density (BMD) (Kim et al. 2005a). In another more recent study, Wu et al. (2007) demonstrated deletion in both FcRγ and DAP12 in mice leads to reduction in bone loss resulted from acute estrogen deficiency-induced osteoporosis (following ovariactomised).

Considering the OSCAR-encoded gene is a newly-discovered target of NFATc1 and OSCAR itself is a more recently discovered osteoclast-specific protein, little is known about its regulation and the role it plays in osteoclastogenesis, therefore this area need to be explored further, particularly in correlation to the context of pathological bone loss such as the ones associated with localised inflammation. Scientific exploration on OSCAR and FcRγ in this area would be worthwhile as it could lead towards interesting finding in field of osteoimmunology.

1.4. Study Outline

In this thesis, those osteoclast ITAM-associated molecules discussed above were studied in the context of pathology of PO and RA. This study is novel in looking at the ITAM-associated molecules from the pathological angle of view on bone loss, particularly in PO that has not been looked at before.

There are four main chapters included in this thesis. Following this Chapter 1, Chapter 2 describes about the examination on the protein and mRNA expression of TREM2, DAP12, OSCAR and FcRγ in human tissues of PO and compared to OA tissues. In connection to the data presented in Chapter 2 which indicate that the expression of all those ITAM-related molecules in PE-containing cells, including osteoclast-like cells in the tissues, Chapter 3 investigates closely on the effect of PE particles might have on the mRNA expression of ITAM-related molecules and on the formation and activity of osteoclasts in the *in vitro* cell culture.

Quite similar to Chapter 2, the Chapter 4 is about examination on the expression of those ITAM-related molecules in RA in comparison to OA and normal human synovial tissues. Besides tissues, study was also carried out on samples of synovial fluid taken from RA and OA for measuring and comparing the levels of soluble OSCAR. In Chapter 5, following observation of OSCAR immunostaining in blood vessels, the expression of OSCAR was investigated closely in endothelial cell lines *in vitro*.

1.4.1. Hypotheses

The expression of NFATc1 and osteoclast ITAM-signalling associated molecules is upregulated in PO and RA and play role in the pathogenesis of bone loss in those diseases by modulating the human osteoclast activity

1.4.2. General Aims

The hypothesis will be investigated by addressing the following four general aims and each chapter in this thesis is organised according to the aims to be achieved:

1.4.2.1. Aim 1

To investigate protein and gene expression of ITAM receptors and adaptor molecules and NFATc1 in soft tissue retrieved from osteolytic sites adjacent to PE-implant in comparison with synovial tissues obtained from OA patients.

1.4.2.2. Aim 2

To investigate the effect of PE particles have on the bone resorption and gene expression of ITAM factors and NFATc1 in an *in vitro* model of human osteoclasts.

1.4.2.3. Aim 3

To investigate and compare the protein and mRNA expression of ITAM receptors and adaptor molecules and NFATc1 in synovial tissues from patients with active, inactive RA, OA and healthy controls.

1.4.2.4. Aim 4

To investigate if the expression and regulation of OSCAR in endothelial cell *in vitro* is regulated by pro-inflammatory cytokines TNF-α and IL-1β.

1.4.3. Significance of the Study

While the relevance of OPG/RANKL/RANK system in the context of inflammation-mediated bone loss is documented here (see section 1.3.5.1.7), there are very limited studies on ITAM-associated molecules in human tissues associated with PO and RA.

Current pharmacological treatments for bone diseases, usually osteoperosis in adults, using bisphosphonates seems to effectively regulate down bone resorption activity, mainly by inducing the apoptosis of osteoclasts (Marini 2003). However, by taking a case documented by Whyte et al. (2003), in which reported that a boy treated with high doses of pamidronate (a common example of bisphosphonates) exhibited signs of osteopetrosis (Odvina et al. 2005), as an example, it appears that alternative strategies addressing bone loss should be looked forward. In addition, there is accumulating evidence that bisphosphonate treatment in children with high bone fragility would introduce the risk of bone becoming brittle rather than better (Marini 2003). It also appears that administration of bisphosphonates might not be effective in controlling local bone loss like they do in systemic bone loss. In a study on rheumatoid arthritis by Eggelmeijer et al. (1996), even though there was increase in bone mass density, the treatment failed to inhibit bone focal erosion. Even though the application of bisphosphonates has already been established as treatment drugs in systemic bone loss like osteoperosis and Paget's disease, the effectiveness of the drugs in treating arthritis is still controversial and questionable (Herrak et al. 2004; Redlich et al. 2002a).

At the moment, in treating inflammation-associated bone diseases like RA many drugs widely used are mainly anti-inflammatory agents aiming to abrogate the inflammation within the tissues. Even though this treatment option in a certain extent is regarded to provide successful therapeutical outcome, there have been reports indicating that some of the existing drugs also provide unwanted side effects. For instance, etanercept (an antagonist to TNFα, see section 1.3.4.1 above) even though regarded as an efficient drug for RA treatment (Gomez-Puerta et al. 2004; Wallenius et al. 2005), there have been studies reporting the side effects associated with this drug such as development of tuberculosis (Gomez-Reino et al. 2007), psoriasis (Sfikakis et al. 2005), interstatial granulomatous dermatitis (Deng et al. 2006) and glomerulonephritis (Stokes et al. 2005).

Therefore, further research with the goal of achieving a complete understanding on the pathology of bone loss in addition to those existing studies needs to be carried on in order to identify better strategies and treatment options for the inflammation-associated localised bone loss. To date, investigation on the regulation of osteoclastogenesis still remains as an essential field in this bone research and looking at the signalling pathway and protein level as well as gene modulation in osteoclasts in the context of osteoimmunology appears to be a promising approach. Based on the literature discussed above, this will not only give insights into the regulation of osteoclastogenesis, but also more importantly provide strategies aimed at suppressing both the bone loss as well as inflammation, especially in the context of inflammation-related localised bone loss.

2. EXPRESSION OF NFATc1, OSTEOCLAST-RELATED ITAM-RELATED MOLECULES AND MARKERS IN HUMAN IN TISSUES ADJACENT TO PERI-IMPLANT OSTEOLYSIS

Some of the data presented in this chapter has been published in the following journal article:

Alias E, Dharmapatni ASSK, Holding AC, Atkins GJ, Findlay DM, Howie DW, Crotti TN, Haynes DR. Polyethylene particles stimulate expression of ITAM-related molecules in peri-implant tissues and when stimulating osteoclastogenesis in vitro. Acta Biomater 2012; 8:3104-12

2.1. Introduction

PO, as mentioned earlier in 1.2.1 above, has been found to be the most common cause for implants failure that leads to the need for revision surgery (Graves et al. 2004; Kesteris et al. 1998; Malchau et al. 1993). PO not only causes implant loosening and loss of fixation, but also makes the revision surgery more complicated and expensive (Harris 1995) due to loss of bone stock around the loosening prostheses.

PO is an example of local inflammation-mediated bone pathology that is oftenly associated with increase in osteoclast formation and activity that eventually results in excessive bone loss (Goldring et al. 1983; Greenfield et al. 2002; Haynes et al. 2004). This can be seen on radiological examination in the form of radiolucent zones, a radiographic hallmark features of aseptic loosening, at the interface between loosened implant and adjacent bone (Clohisy & Harris 1999; Gruen et al. 1979; Santavirta et al. 1990; Zicat et al. 1995).

Immunohistochemical examination on pseudomembrane surrounding the failed protheses indicated that the PO tissues were heavily populated with cells expressing osteoclast markers (Kadoya et al. 1994; Konttinen et al. 2001; Neale & Athanasou 1999). In other study, the progression of bone erosion in this disease appears to be dominated by macrophages with large numbers (identified through CD11b cell marker) found at the site of osteolysis in PO tissues (Kadoya et al. 1996). Many of these macrophages are believed to be the progenitors for osteoclasts seen in the tissues (Haynes et al. 2001b; Sabokbar et al. 1997). In consistent with fact, macrophages isolated from the PO tissues were found to have capability to become osteoclasts *ex vivo* either with (Neale et al. 2000; Sabokbar et al. 1997) or without the support of osteoblasts or stromal cells (Haynes et al. 2001b).

The most widely accepted theory for the initiation of PO suggests that the pathogenesis of the disease is due to chronic inflammation within the tissues triggered by activated macrophages and FBGCs (Haynes et al. 2001b; Holding et al. 2006; Howie 1990; Shen et al. 2006). The inflammatory reaction in response to particle wear debris is initiated from the phagocytosis of the particles by macrophages, most likely infiltrating macrophages (Ren et al. 2010), leading

to release of pro-inflammatory mediators that stimulate osteoclast differentiation amongst the macrophages (Holding et al. 2006; Horowitz & Gonzales 1997; Murray & Rushton 1990).

The pro-inflammatory mediators released by macrophages and foreign-body giant cells following phagocytosis of wear debris during inflammation within the tissues do not just only lead to increased recruitment of osteoclast precursor cells into tissues (as evident from the accumulation of pre-osteoclast in the form of macrophages in the granulomatous lesions between bone and loosened implant (Goldring et al. 1983; Santavirta et al. 1990) and release of osteoclast-associated chemokines (Nakashima et al. 1999a; Rhodes et al. 1997), see section 1.3.1.1 above), but also increases osteoclast differentiation process (Eftekhar et al. 1985; Greenfield et al. 2002; Kadoya et al. 1996). Furthermore, such released cytokines following the phagocytosis of wear debris includes RANKL, the crucial factor for osteoclastogenesis, besides TNFα and IL-1 (Baumann et al. 2004; Ishiguro et al. 1997; Matthews et al. 2001; Rader et al. 1999).

Through either one or both mechanisms described above (increased differentiation of osteoclast precursors within the tissue into mature functional osteoclasts and more recruitment of osteoclast precursor cells from blood circulation into the tissues), the outcome of increased in osteoclast differentation in the PO tissues appears to be definite (Purdue et al. 2006). Therefore, looking at the molecules that might be involved or regulated during osteoclastogenesis might be a good approach in gaining a better understanding on the pathology of PO.

In summary, the initiation process of particle-induced PO could be divided into 3 stages (Zhu et al. 2001), which are:

i) generation of particles
This will vary depending on the types and composition of biomaterials used.

ii) migration of particles into tissues
The presence of liberated particles is not only detected in the joint capsule, but also in all tissues surrounding the joint. Taking PE as an example, oftenly the particles were found to migrate to the cement-bone interface (Schmalzried et al. 1992b).

iii) cellular response to foreign-body particles

This is the area where most research on biomaterial is carried out. The ultimate goal to achieve in this research area is to reduce foreign-body particle-induced bone lysis. This area becomes the main emphasis covered in Chapter 3 of this thesis.

There is accumulating evidence indicating that PE wear debris is the major and most common factor associated with the pathogenesis of peri-implant osteolysis, particularly around hip prostheses. There has been correlation between the prevalence of osteolysis and PE wear rate described in the past (Holding et al. 2006; Looney et al. 2002; Orishimo et al. 2003; Wilkinson et al. 2005). This is further supported by the fact that there is higher incidence of peri-implant osteolysis in patients with PE-containing prostheses than those with metal-on-metal hip arthroplasty (Migaud et al. 2004; Migaud et al. 2011). Introduction of PE particle into *in vivo* model led to increase in osteoclast-like formation and activity in a particle concentration-dependent manner (Brooks et al. 2000; Kim et al. 1998). Another reason of why PE becomes important biomaterial to be studied is because PE was found to be the most abundant wear debris in PO tissues (Campbell et al. 1995; Hirakawa et al. 1996; Shanbhag et al. 1994b; Willert et al. 1990) (section 3.1 below for more information associating PE particles and osteolysis).

There have been quite a number of studies that suggests direct association between PE particles and the regulation on osteoclast-like cells in human PO tissues. Shen and colleagues (2006) demonstrated presence of PE particles within and in close proximity of multinucleated osteoclast-like cells expressing osteoclast phenotypic markers such as TRAP, cathepsin K and calcitonin receptor (CTR). Presence of PE particles was also detected in multinucleated cells positive for RANK (Holding et al. 2006), supporting an earlier finding of RANK mRNA expression in cells that had ingested PE particles (Crotti et al. 2004). This indicates that the expression of RANK in osteoclastic or/and pre-osteoclastic cells in response to PE might stimulate formation of osteoclasts in PO tissues. While study by Holding and colleagues (2006) demonstrated direct association between PE particles and RANK expression on osteoclasts in PO tissues, to date, there has been no study investigating the connection between PE and other osteoclast co-stimulatory receptors, such as TREM2 and OSCAR, reported. Indeed, thus far, there is very limited number of studies investigating the expression

of osteoclast ITAM-associated molecules in the context of human localized bone loss, including PO.

Considering the significance of ITAM signalling during osteoclast development as described in 1.3.5.2 above, it is surprising that more is not known about the role of these molecules play in pathogenic bone loss. Like OPG/RANK/RANKL system, the role of these molecules may be important. Two chapters in this thesis will cover the regulation of ITAM-related molecules in association with osteoclast formation in the context of PE-induced PO. This chapter investigates the expression of osteoclast ITAM-associated molecules and NFATc1 in human PO tissues from patients undergoing revision surgery. PO tissues were compared to OA tissues taken from those undergoing primary surgery for prostheses implantation. Expression of these molecules was investigated at both protein (through immunohistochemistry, see 2.2.2 below) and mRNA level (via real-time RT-PCR, see 2.2.3 below). In regard to the analysis on the protein expression, particular attention was given to multinucleated osteoclast-like cells positive for NFATc1 and ITAM-associated molecules in the PO tissues and observations were made as to the presence of PE particles in relation to levels and proximity of expression. In addressing the interest on the expression of these molecules in osteoclasts on the tissues, the immunostaining for those investigated molecules was compared with immunostaining for osteoclast phenotypic cell markers.

2.1.1. Hypothesis

NFATc1 and osteoclast ITAM-related molecules are expressed in human tissues of peri-implant osteolysis induced by PE and associated with the disease progression.

2.1.2. Aims

- To compare protein expression of NFATc1 and osteoclast ITAM-related molecules in human PO and OA tissues
- To identify TREM2 and OSCAR-expressing multinucleated cells in human PO tissues as osteoclastic cells
- To compare the mRNA level of NFATc1 and those ITAM-associated molecules between human PO and OA tissues

2.2. Methods

2.2.1. Subjects

Tissue samples used in the study were obtained from Department of Orthopaedics and Trauma of Royal Adelaide Hospital, South Australia. The study was granted ethical approval from the Royal Adelaide Hospital Human Ethics Committee, in accordance with the National Health and Medical Research Council of Australia guidelines. Informed consent was obtained from patient prior to each tissue collection.

For PO tissue group, a total of 12 (4 males and 8 females) soft tissue samples were taken from around sites adjacent to osteolysis (refer Holding et al. (2006) for illustration of the site where tissues were collected). Patients undergoing surgery for removal or replacement of failed hip (majority samples) or knee prostheses had age ranged between 56 to 83 years old with mean of 70.67 ± 10.99 years. Material composition of the failed prostheses was recorded and verified for PE-containing material for the purpose of the study designed here. All PO tissue samples were screened for presence of PE using polarized filter under which PE particles are viewed as birefringence in the dark polarized view under light microscope.

Controls included 12 synovial tissue samples (2 male and 10 female) obtained from OA joints of patients undergoing primary hip or knee replacement surgery. OA subjects were within age range between 60 to 81 years at the time of procedure. All samples from OA group fulfilled published criteria for the classification of OA (Altman et al. 1986). Recorded data incated that these OA tissues showed no radiological evidence of osteolytic lesions. Details of patient's demographics are summarised as in Table 2.1.

Table 2.1: Demographic details of PO and OA patients for immunohistochemistry study

Groups	PO		OA	
Mean Age (± SEM)	70.67 (10.99)		71.54 (7.75)	
Gender (Male/ Total)	4/8		2/10	
Site	Hip	11	Hip	7
	Knee	1	Knee	5
Tissue Type	Acetabular Membrane	4	Joint Capsule	3
	Femoral Membrane	2	Synovium	9
	Joint Capsule	6		

PO- peri-implant osteolysis tissues, OA- osteoarthritis tissues, M- male, F-female, PE- polyethylene, cpTi- commercially pure titanium, COCr- cobalt chromium, PMMA- polymethylmethacrylate

Tissue selection was based on histological assessment following routine hemotoxylin eosin (H&E) staining, which is described below.

2.2.2. Immunohistochemistry and Reagents

Tissues were fixed in 10% buffered formalin and embedded in paraffin. Five-micrometre thick sections were cut using a microtome and stored at room temperature mounting on 3-aminopropyltriethoxy-silane (APTS) (Sigma, St. Louis, MO)-coated glass slides. Prior to beginning of any histochemical work, tissue sections were de-waxed in 2 changes of histolene (Fronine Laboratory Supplies, Taren Point, NSW, Australia) for 10 minutes and 2 changes of 100% ethanol before being washed with MilliQ water and phosphate buffer saline (PBS).

As for H&E staining (for histological assessment as mentioned earlier), de-waxed tissue sections were immersed in hematoxylin solutions for 5 minutes before being dipped in acid alcohol 5 to 7 times. This was followed by immersion of the tissue sections into saturated lithium carbonate solutions for 2 minutes and eosin for 3 minutes with washing in MilliQ in between each step. Finally, after being washed in MilliQ, tissue sections were again immersed in 2 changes of alcohol followed by 2 changes of histolene before being mounted with

coverslips with dibutyl phthalate xylene (DPX) (Labchem, PA, USA) as the mounting medium.

For immunohistochemistry work, following de-waxing tissue sections were processed for antigen retrieval by heating up in either 10mM Tris-EDTA buffer pH 9.0 or 10mM sodium citrate pH 6.0 (refer Table 2.2) at 92-95^{o}C for 10-20 minutes. Selection of antigen retrieval buffer was based on the preliminary work on antibody optimisation prior to the actual immunostaining. In this thesis, immunohistochemical staining was detected based on three-step immunoperoxidase method previously published (Crotti et al. 2002; Kraan et al. 2000; Tak et al. 1995) with minor modifications, as described below. Since immunoperoxidase method was employed in the work, in order to prevent unspecific staining, endogenous peroxidase activity was inhibited by 0.1% sodium azide (NaN_3) and 0.3% (w/v) hydrogen peroxidase (H_2O_2) (Merck, Victoria, Australia).

The primary antibodies diluted in PBS and 1% bovine serum albumin (BSA) were applied at optimized final concentrations overnight in wet chamber at room temperature. The antibody working concentrations (see Table 2.3) were determined by applying a range of concentrations, with manufacturer recommended concentration as start points followed by series of 1 in 2 serial dilutions. Sections were then incubated with a horse-radish peroxidase (HRP)-conjugated secondary antibody diluted in PBS containing 1%BSA normal human serum for 30 minutes, followed by incubation with an HRP-conjugated tertiary antibody against the secondary antibody. HRP activity was detected using hydrogen peroxide as the substrate and 3-amino-9-ethylcarbzole (AEC) as the dye. There was a minimum three washes with PBS between each step to wash off any unbound antibody or protein for avoiding unspecific binding and hence staining. Sections were counterstained in Harris hematoxylin for 10 seconds, and then washed with MilliQ before immediately immersed in saturated lithium carbonate and mounted with aquamount (Microscopy Aquatex, Darmstadt, Germany).

Negative controls included tissue incubated with an isotype specific antibody for the monoclonal antibody or serum of corresponding species for polyclonal antibodies at equivalent concentrations to the primary antibody. Staining for each antibody was carried on all tissue samples involved at the same time to reduce staining variability due to experimental setup.

2.2.2.1. Antibodies and Reagents

The following antibodies used for the immunohistochemistry, which are polyclonal goat anti-human OSCAR (catalogue no. SC34233), polyclonal rabbit anti-human DAP12 (SC20783) and monoclonal antibody mouse anti-human NFATc1 (clone 7A6, SC7294) were purchased from Santa Cruz Biotechnology Inc. (CA, USA). Polyclonal rabbit anti-human FC€R1G (LS-B2169), which was chosen based on personal communication as FC€R1γ is equivalent to FcRγ (Ishikawa et al. 2004; Zou et al. 2010), was purchased from Lifespan Biosciences Inc. (Seattle, WA, USA). For immunostaining of TREM2, polyclonal rabbit anti human TREM2 (HPA010917) purchased from Sigma-Aldrich Pty. Ltd (Castle Hill, NSW, Australia) was used for the study. For the detection of osteoclast cells in the tissues, one of the cell markers used was cathepsin K and the antibody used for the immunostaining was a monoclonal antibody mouse anti-human Cathepsin K (MAB3324 clone 182-12G5) purchased from Millipore (Billerica, MA, USA).

Table 2.2: Secondary and tertiary antibodies as well antigen retrieval buffer corresponding to the primary antibodies used in immunohistochemistry

Primary antibody	Secondary antibody	Tertiary antibody	Antigen Retrieval Buffer
Mouse anti human - NFATc1	Goat anti-mouse	Swine anti-goat	EDTA
Goat anti human - OSCAR	Swine anti-goat	Rabbit anti-swine	Sodium citrate
Rabbit anti human - FcRγ	Goat anti-rabbit	Swine anti-goat	Sodium citrate
Rabbit anti human - TREM2	Goat anti-rabbit	Swine anti-goat	EDTA
Rabbit anti human-DAP12	Goat anti-rabbit	Swine anti-goat	Sodium citrate
mouse anti human - cathepsin K	Goat anti-mouse	Swine anti-goat	EDTA

EDTA- ethylenediaminetetraacetic acid

Table 2.3: Primary antibodies used for immunohistochemistry

Antibody	Isotype	Working Concentration (µg/mL)	Stock Concentration (µg/mL)
Mouse anti human -NFATc1	IgG_1	2	200
Goat anti human -OSCAR	Polyclonal	10	200
Rabbit anti human -FcRγ	Polyclonal	0.625	1000
Goat anti human -TREM2	Polyclonal	0.2	80
Rabbit anti human-DAP12	Polyclonal	0.4	200
Mouse anti human -cathepsin K	IgG_1	0.5	100

IgG_1- immunoglobulin subtype 1

Either affinity purified, HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (depends on the species in which primary antibodies were raised) was used as secondary antibodies (DakoCytomation, Glostrup, Denmark). For the tertiary antibodies, either HRP-conjugated swine-anti-goat or rabbit anti-swine IgG purchased from Biosource (Camerillo, CA, USA) were applied to the tissues before staining was carried out using AEC that was purchased from Sigma -Aldrich (St Louis, MO, USA).

2.2.2.2. TRAP Staining and Serial Labeling of Osteoclast Cell Markers

TRAP staining was performed on tissue sections using a commercial kit (386A, Sigma, St. Louis, MO, USA) as per manufacturer's instructions. Tissue sections on APTS-coated slides were incubated at 37°C for 2 hours in dark conditions with the filtered kit solution containing 0.5% Fast Green Garnet salt and 4% of each acetate, tartrate solution and Naphtol AS-BI Phosphate. As per the immunostaining procedure described earlier, tissue sections were counterstained with Methyl Green and mounted with aquamount before being viewed under microscopy for further analysis.

Serial sections of tissues, which had been immunostained for TREM2, OSCAR and osteoclast marker cathepsin K were compared with TRAP staining on tissue sections from corresponding patients. Particular attention was given to multinucleated cells for the identification of positively stained osteoclasts.

2.2.2.3. Scoring of Immunostaining Results

The immunohistochemically stained slides were scanned using Nanozoomer Digital Pathology (Hamamatsu, Shizouka, Japan). In order to evaluate the extent of positive staining on the tissues, three random areas (2mm by 2mm squares) were drawn in each section, in which semiquantitative analysis (SQA) scoring was carried out by two-blinded independent observer. The proportion of positive-stained cells was graded using a scale of 5 ranging from 0 to 4 adapted from previously published studies (Crotti et al. 2004; Kraan et al. 1999; Tak et al. 1995). A minimal grade of 0 is indicated by 5% of positive cells or less, 1 for 6 to 10%, 2 for cell immunostaining positivity between 11 to 25%, 3 for 26 to 50% and 4 for more than 50% positively stained cells.

2.2.3. Quantitative Real Time Reverse Transcription Polymerase Chain Reaction

Quantitive reverse-transcription polymerase chain reaction (qRT-PCR) was employed in this study to measure the level of mRNA expression of the investigated genes. This is described in detail below.

2.2.3.1. RNA Extraction from Frozen Tissues and Spectrophotometry

Tissues from some patients were snap-frozen in Optimal Cutting Temperature (OCT) medium (Tissue Tek^{R} Zoeterwoude, The Netherlands) using liquid nitrogen and stored in -80°C before used. A total of 12 PO and 6 OA frozen tissues, which was a subset of corresponding patient groups whom the tissue samples were selected for immunohistochemical study described earlier (see in the beginning of section 2.2.2), were selected for mRNA isolation and qRT-PCR analysis (samples are detailed as in Table 2.4 below). Those frozen tissues were chosen for RT-PCR based on histological assessment screening for multinucleated cells for PO and high cellularity OA tissues. Tissues were sectioned at a minimum of 20μm thickness in total using a cryostat (Leica Microsystems, Weitzlar, Germany).

Table 2.4: Demographic details of patients in which PO and OA mRNA samples taken from

Patient	Gender (M/F)	Age (years)	Site	Tissue Type	Composition of Prostheses
PP1	F	56	Hip	Joint capsule	PE, CoCr
PP2	M	68	Hip	Acetabular Membrane	cpTi, titanium, PE, CoCr
PP3	F	70	Hip	Femoral Membrane	cpTi, titanium, PE, CoCr
PP4	F	75	Knee	Joint capsule	no history
PP5	F	45	Hip	Femoral membrane	CoCr, PE, PMMA
PP6	F	78	Hip	Joint capsule	cpTi, titanium, CoCr, PE, stainless steel, PMMA
PP7	F	71	Hip	Joint capsule	cpTi, titanium, PE, CoCr
PP8	F	82	Hip	Acetabular membrane	PMMA, PE, stainless steel
PP9	M	74	Hip	Acetabular membrane	PE, CoCr
PP10	M	67	Hip	Joint capsule	PE, CoCr
PP11	F	79	Hip	Joint capsule	PE, PMMA, stainless steel
PP12	M	83	Hip	Acetabular membrane	cpTi, titanium, PE, CoCr
OA1	F	35	Wrist	Synovium	
OA2	F	79	Hip	Joint capsule	Not applicable
OA3	F	68	Hip	Synovium	
OA4	F	79	Knee	Synovium	
OA5	F	73	Knee	Synovium	
OA6	M	82	Knee	Synovium	
OA7	F	76	Hip	Synovium	
OA8	F	61	Hip	Synovium	

PP- Peri-prosthetic/peri-implant osteolysis tissues, OA- osteoarthritis tissues, M- Male, F-Female, PE- polyethylene, cpTi- commercially pure titanium, COCr- cobalt chromium, PMMA- polymethylmethacrylate

Total RNA was isolated from minimum of 5 pieces of 20μM frozen tissue sections and placed into 300μL TRIzol, as per manufacturers instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). Total RNA was isolated according to manufacturer instructions and a previously published method (Chomczynski 1993; Chomczynski & Sacchi 1987).

A one in five volume of chloroform to total TRIzol volume was added, then the tubes were shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes. Aqueous and organic phases were separated via centrifugation at 13000 rpm for 15 minutes in 4˚C. The aqueous layer containing total RNA was then transferred into fresh RNAse-free tubes. RNA was precipitated by adding an equal volume of ice-cold isopropanol containing 1μL of glycogen (to increase precipitation yield). Samples were then left at room temperature for 10 minutes before RNA pelleting through centrifugation at 13 000 rpm for 15 minutes at 4˚C

followed by aspiration of supernatant. The RNA pellet was washed in 500µL of 70% ethanol and centrifuged at 7500 rpm for 5 minutes at 4˚C. Supernatant was discarded and pellets were allowed to air dry. The obtained RNA pellet was resuspended in 20µL of diethyl pyrocarbonate (DEPC) (Sigma-Aldrich, St Louis, MO, USA)-treated water (DEPC water) and heated to 65˚C for 5 minutes.

RNA concentration was determined using a UV spectrophotometer (Shimadzu Corporation, Kyoto, Japan). RNA Concentraion was calculated according to the formula:

RNA Concentration (ng/ml) = OD_{260} x Dilution Factor x RNA Constant

with RNA constant = 40ng/µl

RNA isolated was kept in -80°C until needed for cDNA synthesis.

2.2.3.2. Reverse Transcription

One microgram of total RNA was combined with 500µM dNTPs (Bioline, London, United Kingdom), 1µg random hexamer (GeneWorks, Adelaide, SA, Australia) and DEPC water to a final volume of 14.75µl in a 0.2mL flat cap tube. Tubes were then heated to 65˚C for 5 minutes before being briefly cooled to 4˚C. This pre-reverse-transcription mix was then added with 200 units SuperScript™ III Reverse Transcriptase, 1x First Strand Buffer and 1.25mM dithiothreitol (DTT) (all those three reagents from Invitrogen Life Technologies, Carlsbad, CA, USA) for a final volume of 20µL. The reverse transcription process consisted of incubation at 25˚C for 5 minutes, followed by 30 minutes at 50˚C and finally 70˚C for 5 minutes. The step of reverse transcription (RT) for complementary deoxyribonucleic acid (cDNA) sysnthesis was performed using a Rotor-Gene 3000 (Corbett Life Science, Mortlake, NSW, Australia). All synthesized cDNA was stored at -80˚C until use.

2.2.3.3. Real Time Polymerase Chain Reaction

Similar to the reverse transcription process, the cDNA amplification via polymerase chain reaction (PCR) was carried out in the same machine Rotor-Gene 3000 (Corbett Life Science). Reaction mixtures contained 1µl of 1 in 5 pre-diluted cDNA, 7.5µl Platinum SYBR® Green qPCR Supermix-UDG, 300nM each of forward and reverse primer and DEPC-treated water to a final volume of 15µl.

In every PCR performed, samples were analysed in triplicate to obtain consistency in data and to eliminate any false results obtained through inaccurate pipetting. The PCR procedure began with an incubation at 50˚C for 2 minutes to activate UDG, which is an enzyme in the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen Inc.) used to destroy potential carry-over PCR contamination (Longo et al. 1990). Each cycle of amplification involved a three-step thermocycling procedure with DNA denaturation, annealing and elongation occurring at 95˚C, 60˚C and 72˚C respectively, with each step held for 20 seconds, for a total of 40 cycles. The SYBR® Green fluorescence measurement was taken at the completion of each elongation step.

All PCR reactions were validated by the presence of a single peak in the melt curve analysis, which was obtained from PCR melting protocol upon completion 40 cycles of PCR amplification. This involves heating the PCR product at the end of the procedure at a rate of 1˚C every 5 seconds from 72˚C to 95˚C and collecting fluorescence readings at every degree.

Primer3Plus online application (Untergasser et al. 2007) was used to design oligonucleotide primers to human OSCAR, FcRγ TREM2 and DAP12 in house, aimed to flank intron-exon boundaries. Primer sequences for NFATc1 were obtained based on a previous publication (Granfar et al. 2005). The endogenous reference gene human acidic ribosomal protein (hARP) was used to normalize the PCR data, with the primer sequence was taken from a published work (Franssen et al. 2005). Primers were purchased from Geneworks (Thebarton, SA, Australia). Primer sequences used for each gene in this study are as shown in Table 2.5.

Table 2.5: **Primer sequences of each gene investigated**

Target Gene	Sense	Primer Sequence (5'→3')	T_m (°C)	Product Length (bp)
NFATc1	S	GCA TCA CAG GGA AGA CCG TGT C	60	153
	AS	GAA GTT CAA TGT CGG AGT TTC TGA G	60	
TREM2	S	TCT CCG GCT GCT CAT CTT AC	60	112
	AS	GGA GTC ATA GGG GCA AGA CA	60	
DAP12	S	CCG AGT CGC CTT ATC AGG A	60	100
	AS	CAC TGT CAT TCG GGC T	60	
OSCAR	S	CCC AGC TTC ATA CCA CCC TA	59	151
	AS	GAA GAG AAG GGG AGC GAT CT	59	
FcRγ	S	ATT CCA GCA GTG GTC TTG CT	60	182
	AS	CCT TTC GCA CTT GGA TCT TC	60	
Cathepsin K	S	CAC TGG ATA ATT AAA AAC AGC TGG G	60	100
	AS	CCA GGT TGG CAA TGC CAC	60	
hARP	S	CAC CAT TGA AAT CCT GAG TGA TGT	60	116
	AS	TGA CCA GCC CAA AGG AGA AG	60	

hARP- human acidic ribosomal protein, S- sense, AS- anti-sense, T_m- melting temperature, bp- base pair

Relative expression of the target genes to the housekeeping gene (hARP) in the studied samples of *in vitro* assay was obtained using the difference in the comparative threshold (ΔC_T) method, which is a real time PCR calculation and analysis method documented by Livak and Schmittgen (2001). The levels of expression of the target genes in the studied samples as compared with the reference gene were calculated as 2 power-ΔC_T (Livak & Schmittgen 2001).

2.2.4. Statistical Analysis

Non-parametric tests were employed to analyse and compare the mean ranks of the SQA's data between the two groups. Kruskal-Wallis test was used to find any significant difference between any of the groups before performing Mann-Whitney U test to identify groups with statistical significant data, with a $p<0.05$ was considered to be statistically different.

As for the measurement of gene expression, differences in relative mRNA levels between two groups was also analysed using student t-test and $p<0.05$ was considered significant statistically. All statistical analysis in this study was performed using SPSS 11 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism (Graphpad Software, CA, USA).

2.3. Results

2.3.1. Histological Features of Peri-Implant Osteolysis and Osteoarthritis Tissues

Prior to any immunostaining, routine tissue staining using H&E was carried for the histological assessment as described in the Methods 2.2.1 above. Acellular tissues consisting mainly of connective tissues were avoided to ensure that data generated (cell immunostaining) would be meaningful and could be interpretable in context of cellular and molecular biology. By far the majority of tissues were largely cellular and tested. High cellularity in PO tissues also gives indication of the tissues are in active inflammatory stage of disease progression rather than scar tissues. In a number of PO tissue samples studied, there was mix of very cellular and fibrous tissues (that may give indication those tissues in later stage of PO progression).

As predicted in the PO group, all tissues displayed signs of granulomatous inflammatory reaction (see Figure 2.1A) that is commonly observed in PO tissues (see 1.2.1 above). All PO tissues were dominated by high number of multinucleated cells, possibly could be osteoclasts and foreign body giant cells. All samples demonstrated the presence of PE particles to some extent. These particles were observed as birefringent material under polarized microscopic view (arrowed in Figure 2.1A) and ranged in size, from just visible under microscope to approximately a milimeter in size. In addition, in a few tissues we noted presence of metal particles, seen as black debris on the tissues. It was also noted that multinucleated osteoclast-like cells were commonly associated with or in close proximity to smaller sized PE particles, whereas foreign body giant like cells appear associated with larger PE debris.

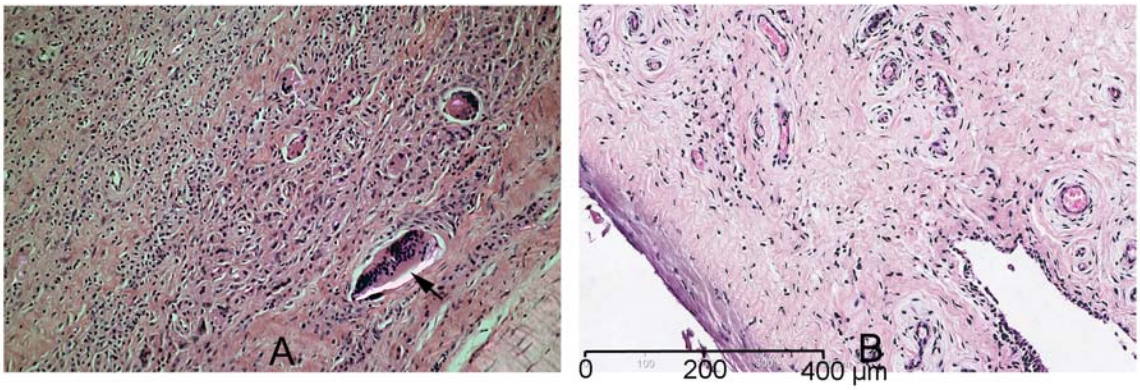

Figure 2.1: Representative histology of tissues from PO and OA groups following H&E staining.

Representative histology of PO (**A**) and OA (**B**) tissues following routine H&E staining viewed at 100X magnification. Presence of PE particles, indicated by birefringence (arrowed in **A**) under polarized view of light microscopy, was detected in all PO tissues studied.

Generally, the histology of OA tissues showed majority cells were in the lining layer of the synovial tissues. Presence of vasculature was occasionally seen in the OA synovial tissues. Some of the OA tissue samples demonstrated very mild infiltration of inflammatory cells composed of mononuclear, probably macrophage and lymphocyte-like, cells particularly around blood vessels. Overall there was minimal inflammatory reaction seen. Multinucleated cells were not detected in any of these sections.

2.3.2. Expression of NFATc1 and osteoclast ITAM-associated molecules in peri-implant osteolysis tissues in comparison to osteoarthritis tissues

SQA was carried out, as described earlier in section 2.2.2.3 above. The SQA scores for immunostaining of NFATc1 and each ITAM-associated molecules are summarised as in Table 2.6 below.

Table 2.6: SQA scores for immnostaining of NFATc1 and ITAM-related molecules in PO and OA tissues

Molecules	Mean (SEM)		*p*-value
	PO	OA	
NFATc1	2.25 (0.39)	1.27 (0.33)	0.091
OSCAR	3.25 (0.28)	1.33 (0.44)	0.002
FcRγ	3.73 (0.14)	1.25 (0.35)	0.000
TREM2	3.73 (0.19)	2.00 (0.29)	0.000
DAP12	3.80 (0.13)	1.27 (0.27)	0.000

significantly higher in PO than in OA if $p<0.05$

Observations on detection of each molecule are detailed in the following sections below.

2.3.2.1. NFATc1

In PO tissues, strong NFATc1 immunostaining was predominantly observed in cells with a lymphocyte morphology and only faint staining noted in a few number multinucleated cells (Figure 2.2A). Interestingly, NFATc1 was expressed in nuclei and peri-nuclei on

multinucleated cells, with stronger or more intensed red colour staining was observed on nuclei than in the cytoplasmic region (Figure 2.2D).

In OA, the majority of NFATc1 immunostaining was detected in mononuclear cells with lymphocyte-like morphology located mainly perivascular layer (Figure 2.2B). There was no positive staining found on the blood vessels.

There was no significant difference ($p>0.05$) in the proportion of cells expressing NFATc1 between in PO and OA tissues, as shown in Table 2.6 above.

There was no positive immunostaining found in peri-prosthetic tissues incubated with primary antibody isotype (mouse IgG_1 as negative control) (Figure 2.2C) indicating no unspecific or false positive NFATc1 immmunostaining on the tissues.

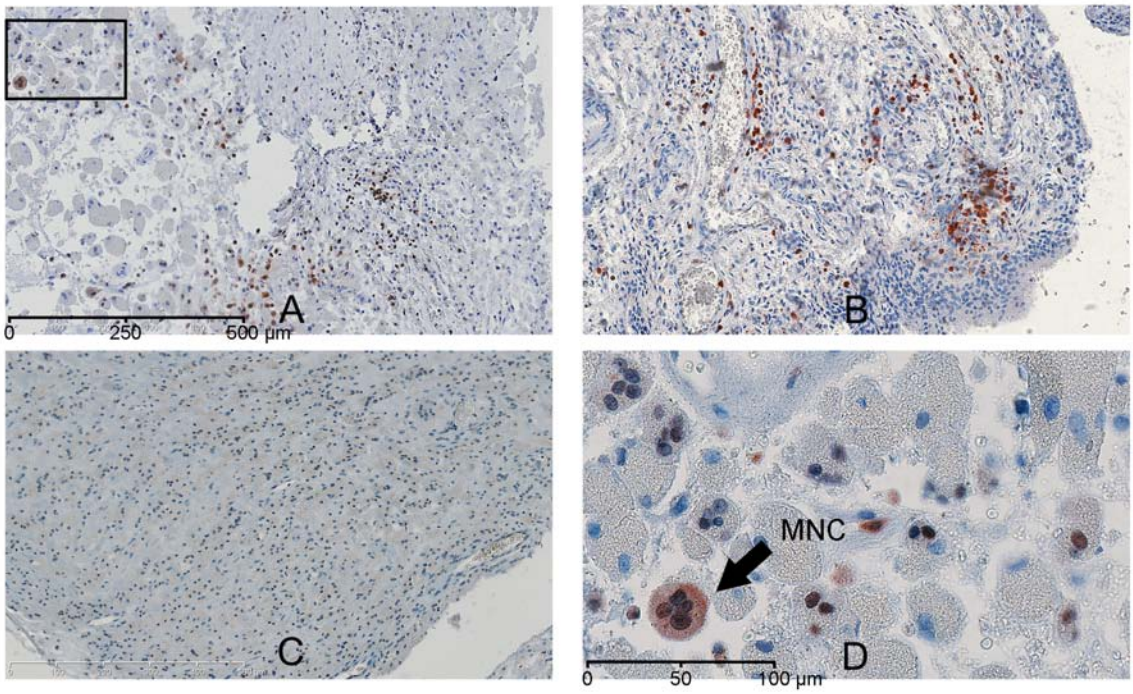

Figure 2.2: NFATc1 expression in human peri-implant osteolysis and osteoarthritis tissues

Representative pictures of NFATc1 immunostaining (red colour) in tissues from PO (**A**) and OA (**B**) groups viewed at a 50X magnification. **C** represents negative control of NFATc1 immunostaining on PO tissues, in which NFATc1 antibody was substituted with negative control IgG_1 isotype. **D** represents positive immunostaining of NFATc1 detected on a multinucleated cell (MNC, arrowed) in a PO tissue (400X, framed in **A**).

2.3.2.2. TREM2 and DAP12

TREM2 was found to be expressed at high levels in PO tissues compared to OA tissues (Figure 2.3A and B). The majority of TREM2 positive cells were multinucleated cells present in PO tissue (Figure 2.3D). TREM2 expression was also observed in macrophage and monocyte-like cells, mainly in the cytoplasm of cells. There was no staining found to be associated with or inside blood vessels was observed in the PO tissue group.

In OA tissues, TREM2 was expressed by few of the single nuclei cells with macrophage and occasionally lymphocyte morphology. These cells were located mainly in lining layer and peri-vascular region (Figure 2.3B). There was also staining of TREM2 associated with and inside blood vessel observed in nearly all OA tissues studied (7 out of 8 that were managed to be examined). There was no staining in PO tissues in the absence of TREM2 antibody (negative control) indicating the specificity of TREM2 staining on the tissues (Figure 2.3C).

DAP12 was expressed in similar pattern to TREM2 in PO tissue and OA tissues (Figure 2.4A and B), with more positive immunostained cells in PO tissues. Indeed in some of PO tissue samples studied, DAP12 was positive on nearly all cells present in those tissues. DAP12 expression appeared strongest in the multinucleated and monocyte-like cells (Figure 2.4D), but was also seen occasionally on fibroblast-like cells (not shown in picture). There was no staining on vasculatures, which was similar to TREM2 immunostaining in PO tissues.

In comparison to PO tissues, the total amount of positive DAP12 immunostaining was found to be far less in OA tissues (Figure 2.4B). Most of the immunostained lymphoid-like cells in OA were located peri-vascular and in the lining layer of the tissues. Occasionally DAP12 positive immunostaining was seen on fibroblast-like cells in OA tissues like in PO tissues, however most of the positive stained cells have morphology of monocyte-like cells.

No positive immunostaining was detected following incubation with rabbit normal serum as negative control for DAP12 antibody (Figure 2.4C), demonstrating specificity of the immunostaining observed in other tissues studied.

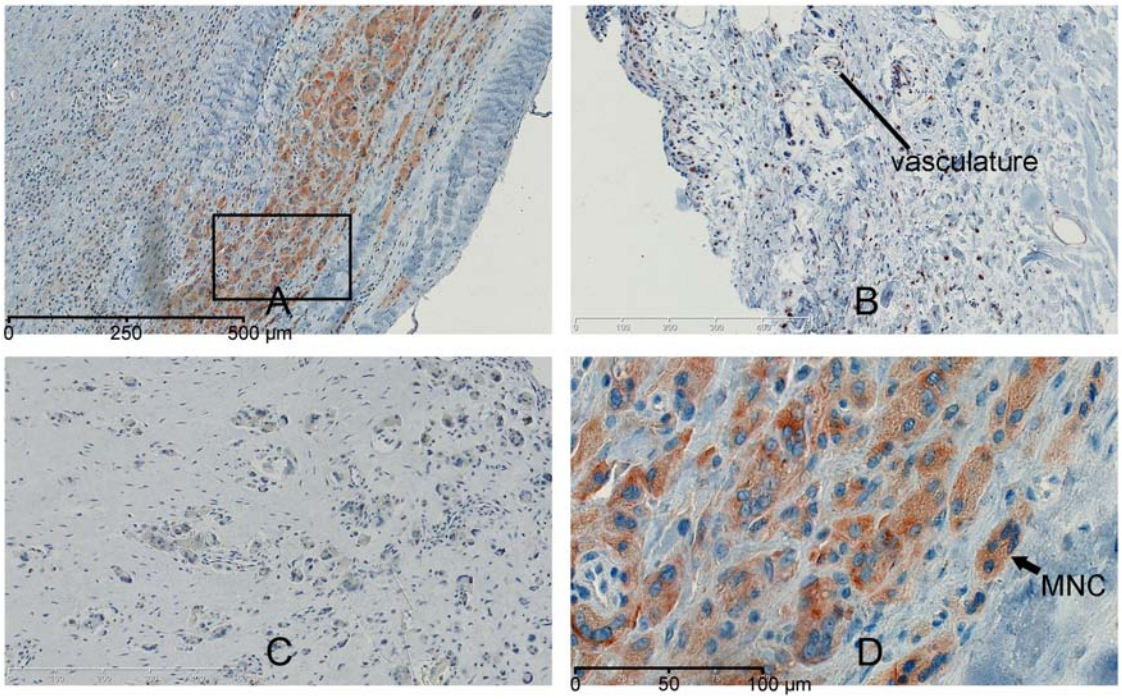

Figure 2.3: TREM2 expression in human peri-implant osteolysis and osteoarthritis tissues

Representative pictures of TREM2 immunostaining (red colour) in tissues from PO (**A**) and OA (**B**) groups viewed at a 100X magnification. **C** represents negative control of TREM2 immunostaining on PO tissues, in which TREM2 antibody was substituted with negative control antibody isotype. **D** represents positive immunostaining of TREM2 detected on a multinucleated cell (MNC, arrowed) in a PO tissue (400X, zoomed in from **A**).

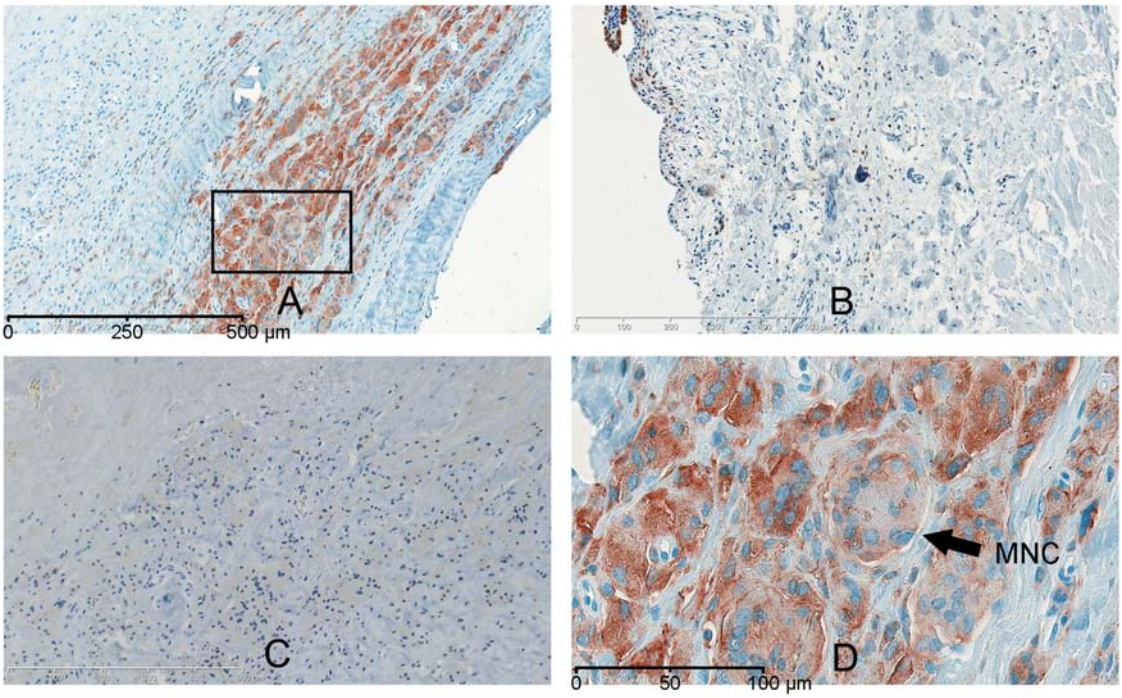

Figure 2.4: Representative pictures of DAP12 immunostaining in human peri-prosthetic and osteoarthritis tissues studied

Representative pictures of DAP12 immunostaining (red colour) in tissues from PO (**A**) and OA (**B**) groups viewed at a 100X magnification. **C** represents negative control of DAP12 immunostaining on PO tissues, in which DAP12 antibody was substituted with negative control antibody isotype. **D** represents positive immunostaining of DAP12 detected on a multinucleated cell in a PO tissue (400X, zoomed in from **A**).

SQA data of immunostaining of both TREM2 and its adaptor molecule, DAP12, demonstrated a higher proportion of positive cells in PO tissues compared to OA tissues (Table 2.6), which further supported the observations of tissue staining between those two groups.

2.3.2.3. OSCAR and FcRγ

There were notably more OSCAR positively stained cells in PO tissues in comparison to OA tissues (Figure 2.5A-B). In PO tissues, OSCAR immunostaining was clearly visible in majority of the multinucleated cells (Figure 2.5A and D), regardless of their proximity to wear particles (discussed later in 2.3.4 below). Staining was mostly confined to the cytoplasm although some cells occasionally demonstrated nuclear staining (Figure 2.5D for closer view). There was also some staining on fibroblasts occasionally found on some of the tissues (not shown). There was no or minimal positive OSCAR staining associated with vasculature.

In about half of the OA tissues studied, apart from positive staining seen mainly on mononuclear cells, OSCAR immunostaining was also observed on the blood vessel walls as well as inside the lumens (arrowed in Figure 2.5B). The majority of the OSCAR-positive cells in the OA tissue group were found to be distributed in the lining layer, though in some OA tissues, stained cells were also found to be scattered in the tissues.

Similar to OSCAR, the FcRγ-positively stained cells appeared more in PO tissues compared to in OA tissues (see Figure 2.6A-B). FcRγ was highly expressed by monocyte-like cells on cell membrane rather than in the cytoplasm (shown clearly in Figure 2.6D). FcRγ was also detected in the cytoplasm of multinucleated cells (arrowed in Figure 2.6D).

In some OA tissues, majority of the FcRγ positive-immunostained cells had monocyte and lymphocyte-like morphology. Generally, strong expression of FcRγ was seen in cells scattered on tissues (Figure 2.6B), however in some of the OA tissues, expression in the lining layer of the tissues as well as in perivascular areas was also noted. However, unlike OSCAR immunostaining described above, there was no staining for FcRγ associated with vasculature.

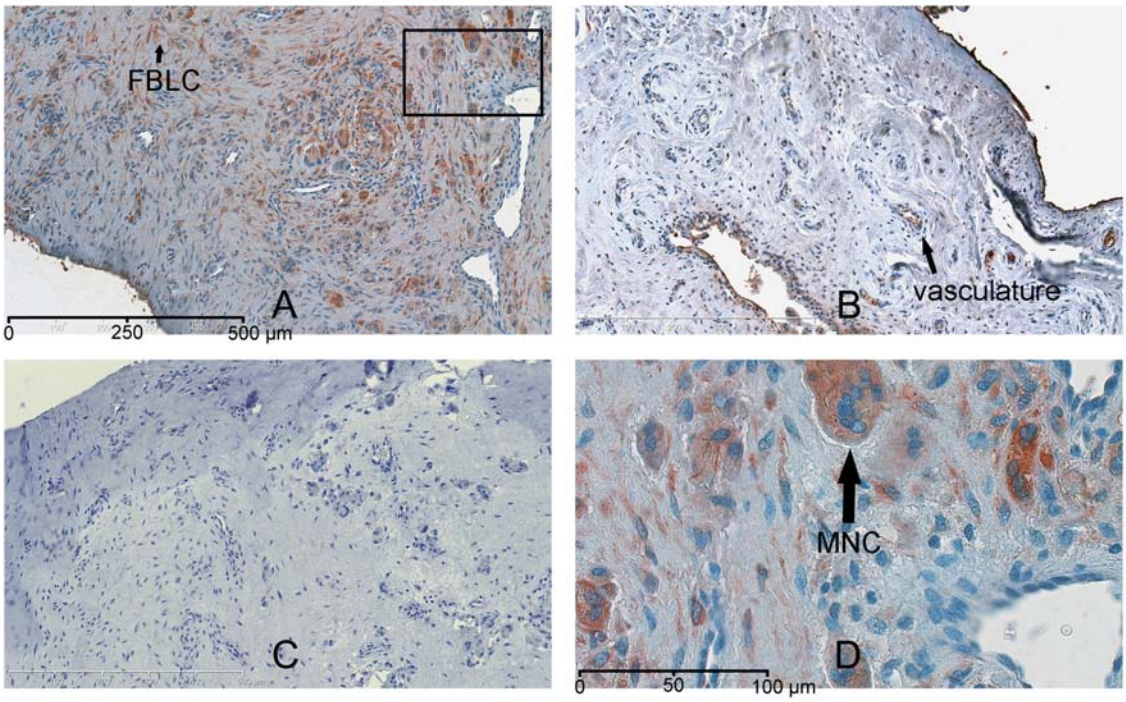

Figure 2.5: OSCAR expression in human peri-prosthetic and osteoarthritis tissues

Representative pictures of OSCAR immunostaining (red colour) in tissues from PO (**A**) and OA (**B**) groups viewed at a 100X magnification. Positive staining was seen on fibroblast-like cells (FBLC, arrowed) in OA tissues. **C** represents negative control of OSCAR immunostaining on PO tissues, in which OSCAR antibody was substituted with negative control antibody isotype. **D** represents positive immunostaining of OSCAR detected on a multinucleated cell (MNC, arrowed) in a PO tissue (400X, zoomed in from **A**).

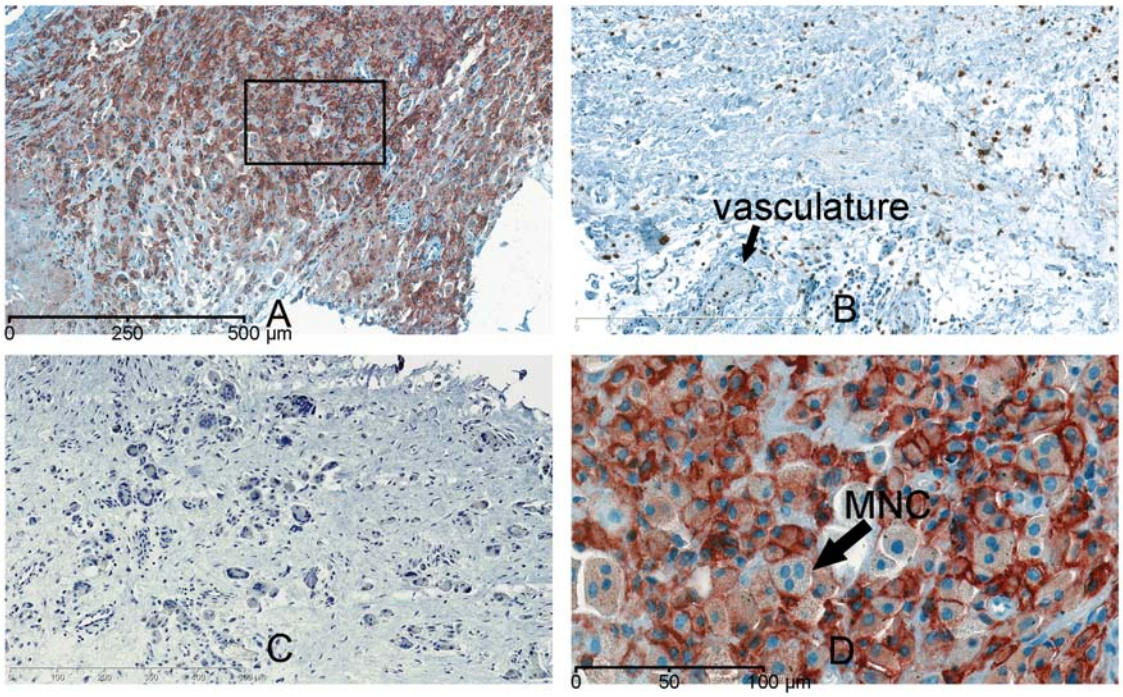

Figure 2.6: FcRγ expression in human peri-implant osteolysis and osteoarthritis tissues

Representative pictures of FcRγ immunostaining (red colour) in tissues from PO (**A**) and OA (**B**) groups viewed at a 100X magnification. **C** represents negative control of FcRγ immunostaining on PO tissues, in which FcRγ antibody was substituted with negative control antibody isotype. **D** represents positive immunostaining of FcRγ detected on a multinucleated cell in a PO tissue (400X).

Observation on PO tissues, which were incubated with normal rabbit serum (NRS) as equivalent negative control for the polyclonal antibody found no red staining, demonstrating the specificity of positive staining for FcRγ (Figure 2.6C). Compared to OSCAR staining in the serial sections from same patients, FcRγ staining was observed to be more widespread.

In general, the obtained observations, in which more cells positively-immunostained for OSCAR and FcRγ in PO tissues than OA tissues seen, were in agreement with the SQA data in Table 2.6. However it was interesting to note that the statistical difference in the SQA data between PO and OA tissue groups were more significant for FcRγ (p=0.0001, p<0.05) than for OSCAR immunostaining (p=0.002).

2.3.3. Detection of osteoclast-cell lineage expressing TREM2 and OSCAR in peri-prosthetic tissues with TRAP and cathepsin K

Analysis of the cells expressing osteoclast markers allowed further investigation of the types of cells expressing TREM2 and OSCAR. Two commonly used osteoclast markers, cathepsin K (Drake et al. 1996; Mandelin et al. 2006) and TRAP (Janckila et al. 2005) were used in labeling of serial sections to identify whether OSCAR and TREM2-immunostained multinucleated cells were osteoclasts.

In addition to multinucleated osteoclast-like cells, cathepsin K immunostaining was also observed in fibroblast-like cells in PO tissues (not shown in picture), which was in agreement with the literature reported (Hou et al. 2001; Mandelin et al. 2005). Cathepsin K immmunostaining on fibroblast-like cells was also seen in OA tissues. Meanwhile for TRAP histochemical staining, TRAP was predominantly expressed by multinucleated cells as well as a few single nucleated monocyte/macrophages.

Examination and comparison of staining of serial tissue sections for those four molecules (TREM2, OSCAR, cathepsin K and TRAP) suggested that those OSCAR and TREM2-expressing multinucleated cells also expressed TRAP and cathepsin K. This is strong evidence that these cells are osteoclasts.

SQA was also performed on cathepsin K and TRAP. Based on the comparison of the SQA data, TRAP was expressed more widespread than the cathepsin K and the two osteoclast-ITAM immunoreceptors (refer Figure 2.7 and Table 2.7).

Table 2.7: SQA scores for immunostaining of TREM2, OSCAR, cathepsin K and TRAP in PO and OA tissues

Molecules	Mean (SEM)		*p*-value
	PO	OA	
TREM2	3.73 (0.19)	2.00 (0.29)	0.000
OSCAR	3.25 (0.28)	1.33 (0.44)	0.002
CatK	3.55 (0.21)	2.33 (0.33)	0.010
TRAP	3.67 (0.65)	0.00 (0.00)	0.000

significantly higher in PO than in OA if $p<0.05$

Close examination found that not all cathepsin K or TRAP-expressing cells were also positive for TREM2 and OSCAR. This might indicate that those two immunoreceptors are expressed by only subsets of osteoclasts or only during a brief time during the development.

Table 2.7 showed that in PO tissues, OSCAR staining gave the lowest SQA, which could suggest OSCAR is a more specific marker for osteoclasts than the others investigated considering OSCAR was dominantly expressed by multinucleated osteoclast-like cells. This is also supported by closer observation, which indicated that there were subsets of multinucleated cells positive for TREM2, cathepsin K and TRAP but not for OSCAR. It was also noted that generally OSCAR-positive multinucleated cells displayed lower staining intensity in comparison to immunostaining for the other molecules.

Stronger and more diverse expression of TREM2 in comparison to OSCAR was noted. Such difference was consistent with a wider variety of cells, such as mononuclear macrophages expressing TREM2, but not OSCAR. On that basis, OSCAR could be regarded as a marker more selectively expressed by osteoclasts.

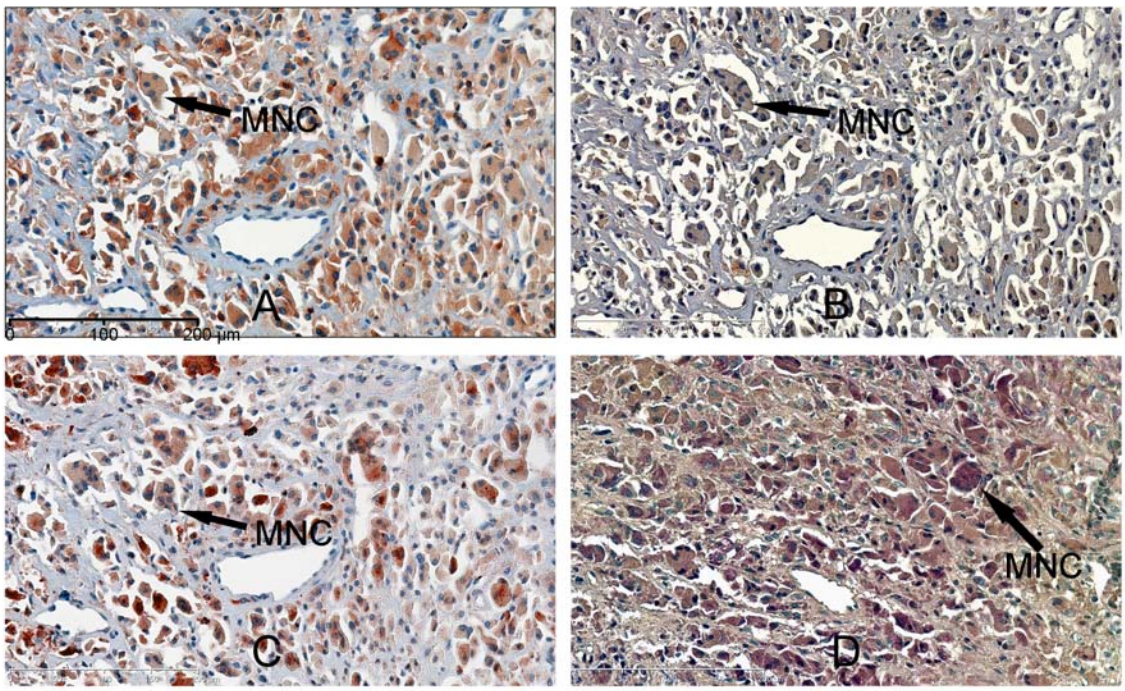

Figure 2.7: Serial tissue immunolabeling for TREM2 and OSCAR with osteoclast cell markers cathepsin K and TRAP

Serial tissue immunolabeling for TREM2 (**A**) and OSCAR (**B**) with osteoclast cell markers cathepsin K (**C**) and TRAP (**D**) on a tissue section from a PO patient. Positive staining of those osteoclast cell markers was detected on many multinucleated cell (MNC, arrowed) on the tissues. Photos were captured at original magnification of 200X.

2.3.4. Detection of polyethylene particles within immunostained multinucleated cells

By taking the fact that polyethylene particles were detected in all studied PO tissues into account, it was of particular interest to investigate if the expression of NFATc1 and ITAM-associated molecules studied in multinucleated osteoclast-like cells could be associated with the presence of polyethylene particles.

Generally, PE particles in varying sizes were observed as scattered throughout the PO tissues with the majority were in close proximity to the cells positively stained for all those ITAM-related molecules. Indeed in some cases, as shown in Figure 2.8 above, presence of PE particles was detected within multinucleated cells expressing TREM2, DAP12, OSCAR and FcRγ.

Examination on tissue sections stained for common osteoclast cell markers, cathepsin K and TRAP (Figure 2.9) showed some of the immunostained multinucleated cells were associated with or in close proximity of the polyethylene particles. Those multinucleated cells could possibly be pre-osteoclasts, as indicated from the work by Shen and colleagues (2006) (Shen et al. 2006).

Generally, it was found that multinucleated cells associated with PE particles displayed weak cathepsin K staining, as shown in Figure 2.9A, even though occasionally there were some PE-associated multinucleated cells showing strong cathepsin K immunostaining observed. For TRAP histochemical staining, there was no noticeable difference in the intensity of positive staining found between PE-associated and non-associated positive multinucleated cells.

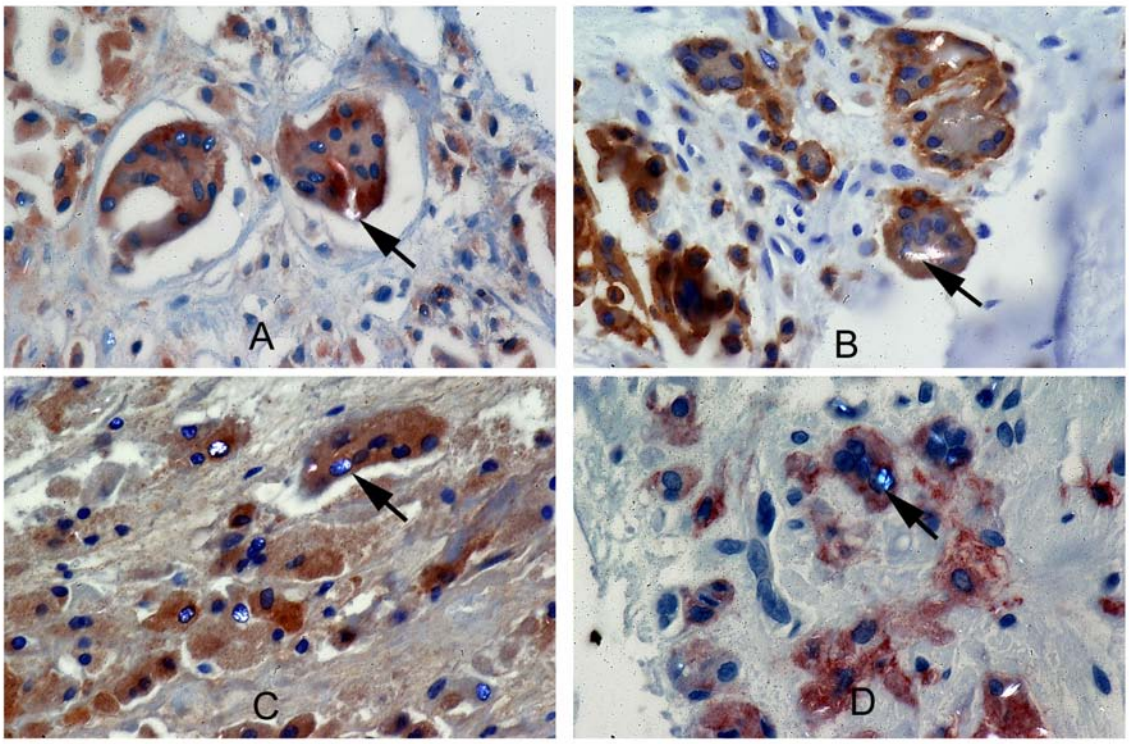

Figure 2.8: Detection of polyethylene particles within multinucleated cells positively stained for osteoclasts-associated ITAM related molecules in human peri-prosthetic tissues

PE particles (as indicated by birefringence in the dark view, pointed by arrows) within multinucleated cells (>3 nuclei counter stained, blue colour) positively-stained (red) for TREM2 (**A**), DAP12 (**B**), OSCAR (**C**) and FcRγ (**D**) under view of polarized microscopic lens (400X).

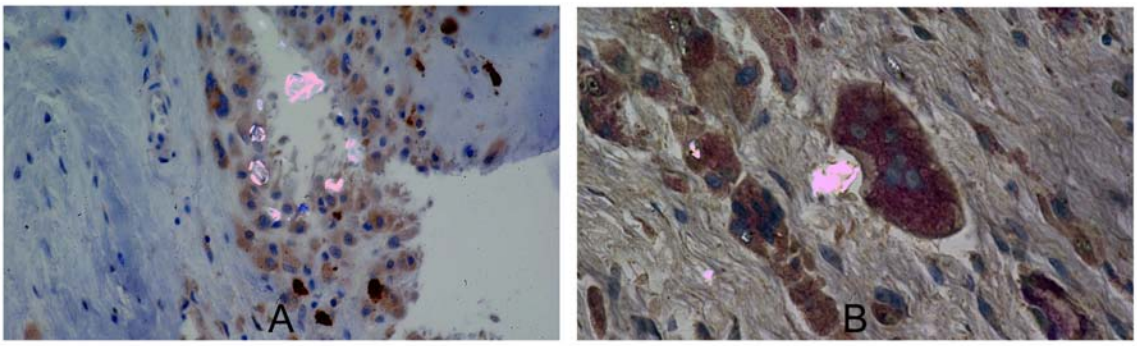

Figure 2.9: Detection of PE particles in close proximity of multinucleated cells expressing cathepsin K (A**) and TRAP (**B**).**

A Cathepsin K-positive cells, including multinucleated cells, were found to be in close proximity of PE particles (200X magnification). **B** PE particles (which appear as birefringence under the polarized view) were also detected to be within some multinucleated cells positively stained for TRAP (viewed at 400X magnification), which may suggest that uptake of PE particles by the multinucleated cells at mature or precursor stage.

2.3.5. mRNA expression of osteoclast ITAM-associated molecules in peri-implant osteolysis and osteoarthritic tissues

Quantitative real-time RT-PCR was performed to investigate the expression of NFATc1 and the ITAM-associated molecules at the mRNA level. In agreement with the immunohistochemical observations, there was no statistical difference in NFATc1 mRNA level between the two groups, even though it seemed there was a trend of higher NFATc1 mRNA expression in PO than in OA. Like NFATc1, there was a pattern towards higher levels of mRNAs of ITAM immunoreceptor OSCAR in PO compared to OA supporting the immunohistochemical findings, surprisingly there was no significant difference in the relative mRNA level achieved ($p>0.05$, see Table 2.8). The mRNA expression of TREM2, DAP12 and FcRγ was statistically higher in PO than in OA tissue ($p<0.05$) consistent with the immunohistochemical results. The relative levels of genes investigated in PO compared to OA tissues are summarized as in Figure 2.10**.**

The relative mRNA expression of the osteoclast cell markers, TRAP and cathepsin K, was significantly higher in PO tissue group than OA (Table 2.8 below), in agreement with the immunohistochemical SQA data presented ealier (Table 2.6). This is also in consistent with other published data (Koulouvaris et al. 2008) that showed higher mRNA level of cathepsin K in PO than in OA tissues. The relative mRNA expression of these two osteoclast-associated cell markers generally appeared higher than OSCAR. This agreed with the SQA data of protein expression obtained.

Table 2.8: Mean of relative mRNA expression ($-\Delta C_T$) of TREM2, OSCAR, TRAP and cathepsin K in PO and OA tissue groups

Molecules	Mean (SEM)		*p*-value
	PO	OA	
TREM2	0.137 (0.062)	0.015 (0.006)	0.072
OSCAR	0.044 (0.008)	0.026 (0.010)	0.084
TRAP	0.701 (0.181)	0.023 (0.007)	0.005
Cathepsin K	1.424 (0.422)	0.211 (0.068)	0.021

significantly higher in PO than in OA if $p<0.05$

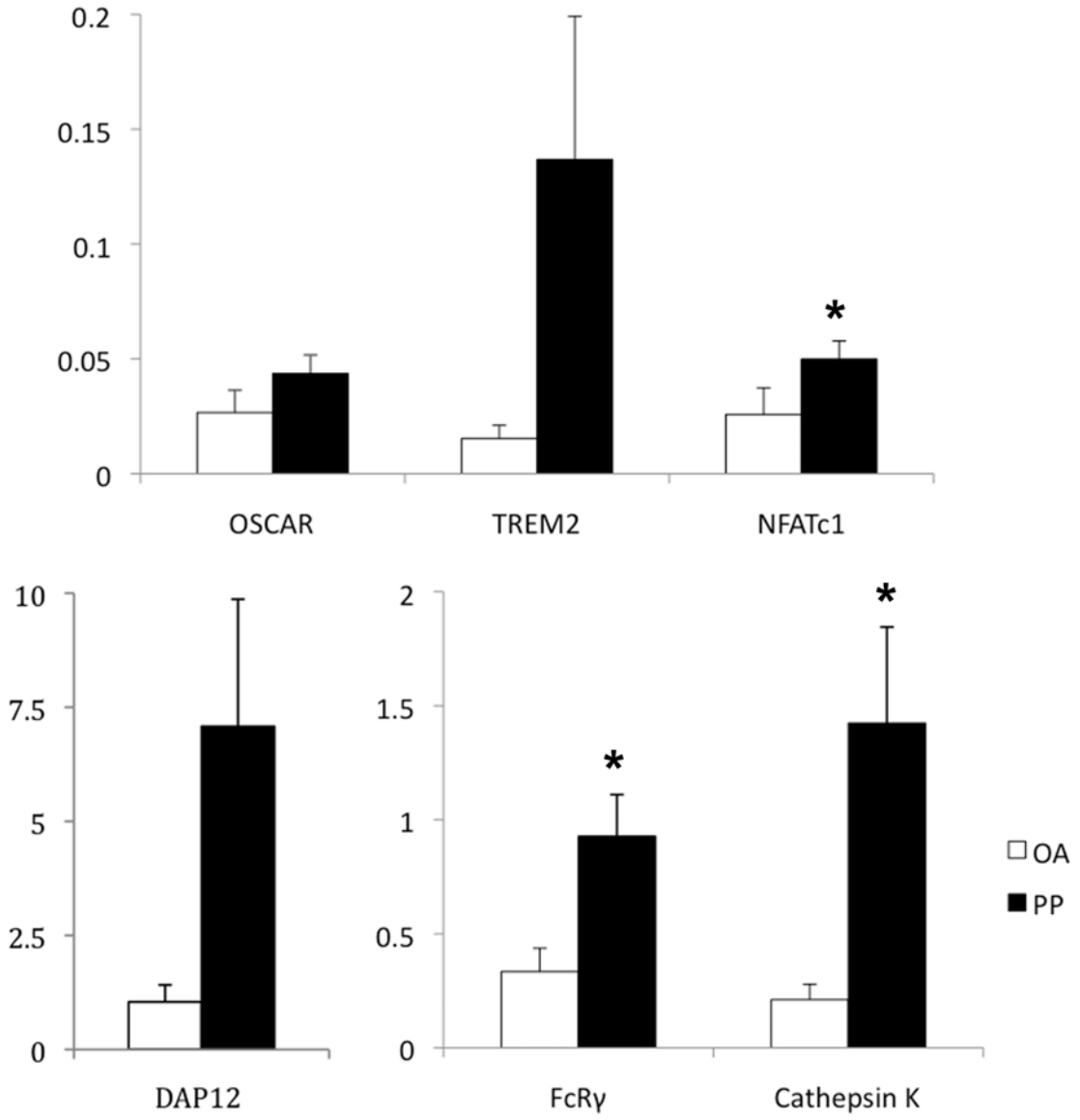

Figure 2.10: Relative mRNA expression for NFATc1, TREM2, OSCAR, DAP12 and FcRγ in PO and OA tissues

Mean mRNA expression in relative to hARP mRNA level ($-\Delta C_T$, see 2.2.3.3 above) (represented by the y-axis) of NFATc1, ITAM immunoreceptors, TREM2 and OSCAR (top), and corresponding adaptor molecules, DAP12 and FcRγ (bottom). The error bars shown on the graphs represent the standard error of mean (SEM) for the collective relative mRNA expression in those two tissue groups.

2.4. Discussions

This and the following chapter of this thesis are studies on the regulation of osteoclast formation in the context of PO pathology by looking at the molecules reported by literature as mediating osteoclastogenesis. The findings, for the first time, show several important molecules could be associated with excessive osteoclast formation and expression of osteoclast-associated cell markers (Kadoya et al. 1996; Neale & Athanasou 1999) in PO tissues. The findings support the contention that pre-osteoclasts and cells that have commited into osteoclastic lineage are among those recruited to form the granulomatous tissues (Haynes et al. 2001a; Neale et al. 2000; Sabokbar et al. 1997). This contention is further supported by the notion that the cells isolated from revision tissue readily form bone resorbing osteoclasts *in vitro* were found to have higher resorbing capability (Haynes et al. 2001b; Roato et al. 2010; Sabokbar et al. 1997).

Studies in this chapter were designed to investigate the expression of NFATc1 and osteoclast ITAM-related molecules in human PO tissues and compare to in OA tissues. As NFATc1 and ITAM-associated molecules have been shown to be crucial for intracelullar signalling in osteoclast differentiation, it was hypothesized that these molecules are expressed in PO tissues and involved in the excessive formation of osteoclasts. It was observed that there were changes in the expression profile towards higher level of those molecules at both protein and mRNA (or gene) level in PO compared to OA. The higher expression of these molecules in PO group suggests they play role in increased osteoclast number and activity in tissues and hence the progression of PO.

One of the strength of this study was that the samples used in the qRT-PCR were taken from the same group of patients used for the tissue immunostaining. In addition considering all PO tissues included in this study were taken from patients undergoing revision surgery who had primary hip or knee replacement following OA, the OA tissues could be regarded as an ideal control group to be compared with. Differences in expression profile observed between those two tissue groups could be associated with progression of PO.

The original hypothesis that NFATc1 and osteoclast ITAM-related molecules are expressed in human tissues of peri-implant osteolysis induced by PE and associated with the diseases was supported by the data presented in this Chapter 2. Being osteoclast-associated immunoreceptors that mediate the co-stimulatory signalling for osteoclast development and activity (Humphrey et al. 2005; Koga et al. 2004; Mocsai et al. 2004), TREM2 and OSCAR, like RANK (Crotti et al. 2004), alongside with the other ITAM-associated adaptor molecules, were found to be strongly expressed in PE-associated multinucleated cells in PO tissues in this study. While the presence of these factors here is largely observational and descriptive research the mechanisms by which these factors induce PO needs to be established. This data may suggest their role in the formation of osteoclasts in PO tissues (see 2.3.1 above) and this is going to be further investigated and discussed in following Chapter 3 below.

Before proceeding to the examination on the expression of those molecules through immunohistochemistry work, it is necessary to discuss the histology of the tissues used. The fact that OA tissues were used as the control tissue group was largely due to unavailability of normal tissues. Besides, OA tissue group is also an appropriate control to be compared with PO tissues as majority of patients from whom the PO tissues were collected had primary surgery for joint replacement and implanting the prostheses following OA. Hence, differences between those two tissue groups observed are appropriate to be considered as attributed by peri-implant osteolysis pathology and disease progression.

The most obvious histological difference between the OA and PO tissues was the presence of granulomatous lesion dominated by inflammatory cells, particularly macrophages, and foreign-body giant cells (Santavirta et al. 1990). Indeed, examination over PO tissues suggested that most of the PO tissues in this study were taken at the granulomatous stage, where numerous cells infiltrated. This granulomatous lesion in PO often leads to the formation of pseudosynovium-like structure, in which cells are organized into lining layer, in the membranous tissues adjacent to the failed implant surface (Goldring et al. 1983). Nevertheless, there were also parts of tissues that were more fibrous and collagenous, possibly scar tissues that gave indication for late stage of PO. It is believed that an inflammatory reaction in this granulomatous lesion is initiated in attempt for the particle clearance within the tissues. This inflammatory reaction within the tissues is also identified as factor contributing towards the promotion of osteoclast formation (Greenfield et al. 2002; Holding et al. 2006; Murray & Rushton 1990).

Throughout the immunohistochemistry work done, there were quite a number of immunostained tissue sections that could not be analysed due to damage of the tissues. This damage was mainly caused by treatment for retrieval of antigen epitopes that relied on heated non-neutral buffers (sodium citrate and Tris-EDTA, see Table 2.2). Unfortunately, this could not be avoided since the antigen epitope retrieval treatment was found to be necessary for reducing background staining while enhancing more specific staining.

Besides the osteoclast ITAM-related molecules another focus in this study was NFATc1 as it has been described by the literature as the key trascription factor that mediate the osteocastogenesis process (refer section 1.3.5.1.6). Therefore, if PO progression has been associated with excessive osteoclast formation, it is likely that this is associated with upregulation of this transcription factor. Thus, in the beginning of this project, it was hypothesized that there was significantly more cells expressing NFATc1 in PO tissues than in OA. However the SQA data of NFATc1 did not support this. In fact, the smaller proportion of cells expressing NFATc1 in PO tissues than predicted might suggest that the expression of the key transcriptional factor for osteoclastogenesis is tightly regulated, which is very important towards well-balanced bone homeostasis. In addition, it is possible that high levels of NFATc1 are only expressed briefly during the late stages of osteoclast formation, a stage most cells in the PO have passed or yet to encounter. This may be consistent with the fact that many of the NFATc1-immunostained mononuclear cells observed were single nucleated, which could be precursor cells of osteoclasts such as macrophages (Minematsu et al. 2007). It was interesting to note that NFATc1 immunostaining could also be detected in some multinucleated osteoclast-like cells. In these cells strong intensed red colour staining was localized on nuclei and peri-nuclei rather than in the cytoplasmic region (Figure 2.2D). This might suggest that in those cells, most of NFATc1 has been activated and recruited into nucleus to activate transcriptions of other genes.

Nonetheless, the majority of cells detected as expressing NFATc1 were observed as to have lymphocyte-like morphology. Such NFATc1 expression by lymphocytes has been reported in other conditions (Takayanagi 2005). However few of these cells were seen in the PO tissues consistent with the fact that lymphocyte infiltrates are not normally associated with PE-related PO (Crotti et al. 2004; Jiranek et al. 1993; Sandhu et al. 1998). Crotti et al. (2004) showed very few CD3-T cells in PO tissues and earlier Jiranek and colleagues (1993)

demonstrated T-cells make up less than 10% of total cell population. This may provide another explanation for lower NFATc1 expression, both at protein and mRNA, than predicted found in PO tissues.

Despite the small proportion of T-lymphocytes among the total cell population, the significance this cell type might have in PO progression should not be underestimated. Previous studies have reported detection of T-cells infiltrating membrane vessels and in close proximity to osteolcast-like cells, as well as higher T cell population in PO tissues than in the normal tissues (Hercus & Revell 2001; Roato et al. 2010; Sandhu et al. 1998). Other study found an increased ratio of CD8 to CD4-positive T-lymphocytes in human PO tissues containing PE and metal particles in comparison to OA tissues (Landgraeber et al. 2009). It is believed that those NFATc1-positive mononuclear cells observed in PO tissues are activated T-cells, considering most of NFATc1 positive staining localized mainly in nucleus (Figure 2.2). It is thought that these cells may play role in promoting osteoclastogenesis in early stage of PO possibly through RANK/RANKL pathway (Roato et al. 2010). Literature has suggested that activated T-cells have elevated expression of membrane-bound RANKL in the cells (Kiesel et al. 2009; Roato et al. 2010). This is commonly seen in RA (Haynes et al. 2003; Horwood et al. 1999; Kim et al. 2000; Kong et al. 1999; Kotake et al. 2001; Weitzmann et al. 2001) and will be further discussed in Chapter 4.

The significance of activated T-cells towards promoting osteoclastogenesis is still arguable as it has also been found that activated T-cells could have suppressive effect on osteoclastogenesis by secreting IFNγ (Takayanagi et al. 2000b) and IL-4 (Mirosavljevic et al. 2003). Data by Sandhu and colleagues (1998) suggested that T-cells may play role in modulating inflammation in the tissues only and are not directly regulated by UHMWPE particles.

TREM2 and DAP12 are thought to be important in mediating osteoclast formation and resorption activity through several mechanisms such as cell multinucleation (Helming et al. 2008; Humphrey et al. 2006), cytoskeltal organisation and hence migration/chemotaxis (Humphrey et al. 2006) and promoting cell proliferation by regulating cell cycle (Otero et al. 2012). In the context of human pathology TREM2 or DAP12 has only begun to be studied in diseased tissues other than in Nasu-Hakola disease (Numasawa et al. 2011; Paloneva et al.

2003) and very recently in Alzheimer's disease (Melchior et al. 2010). Therefore, investigating these two molecules in PO tissues as well as in OA tissues is novel.

It is quite interesting to note that TREM2 was expressed in multinucleated cells (section 2.3.2.2 above) that could be either osteoclasts or FBGCs, in PO tissues. It is difficult to differentiate between these cell types and, at time, their functions may overlap. If FBGCs were among cell types expressing TREM2, then this would be a novel finding since this has not been reported before. However it is more likely that those TREM2-positive multinucleated cells were osteoclasts or pre-osteoclasts in the late stages of differentiation in PO tissues. The strong expression of TREM2 in multinucleated osteoclasts could indicate that this immunoreceptor has an important role in osteoclast formation or/and activity. In addition to multinucleated cells, TREM2 expression was also observed strongly in macrophages and monocyte-like cells in the PO tissues. Expression of TREM2 in macrophages has been well documented and is closely associated in the activation of these cells (Turnbull et al. 2006). With TREM2 has also been suggested to be macrophage phagocytic receptor for bacterial products (N'Diaye et al. 2009; Quan et al. 2008), it would be interesting to know if TREM2 is directly or associated with the phagocytic receptor for biomaterials, such as PE or other types of biomaterial debris in PO. Phagocytosis of biomaterials in mononuclear cells has been shown to promote osteoclast formation (Sabokbar et al. 1998). Furthermore macrophages associated with biomaterial were able to directly resorb bones (Quinn et al. 1992) and this was further supported by the discovery of degradative enzyme for biomaterial substrate, such as cholesterol esterase, within macrophages (Al-Saffar et al. 1997; Kadoya et al. 1994; Labow et al. 1998).

In OA tissues, TREM2 immunostaining was noticed in monocyte/macrophage-like cells mainly around peri-vascular areas and on blood vessels. The positive TREM2 immunostaining on the vasculature is consistent with the finding on expression of TREM2 in endothelial cells that has been documented earlier (Chen et al. 2008b). TREM2 immunostaining was also occasionally spotted on lymphocyte-like cells in some OA tissues, however to date there has been no study indicating the expression of TREM2 in lymphocytes, further investigation needs to be carried out for confirmation.

It is interesting to note that the immunostaining for TREM2-associated adaptor molecules, DAP12 in PO tissues was similar as TREM2 immunostaining, in which the cells expressing

the molecules dominated by the likes of multinucleated cells and macrophages. In macrophages DAP12 is thought to be associated with cell fusion for the formation of multinucleated cells (Helming et al. 2008; Humphrey et al. 2004). In explaining the findings demonstrating the ability of cells isolated from PO tissues to readily form osteoclast-like cells (Haynes et al. 2001b; Neale et al. 2000; Sabokbar et al. 1997), the demonstration of DAP12 expression in mononuclear macrophages cells reported in this study may suggest that the DAP12 expression could have role in promoting osteoclastogenesis in PO tissues.

DAP12 was mainly expressed in mononuclear cells that were mostly located in lining layer of the OA tissues. Such observation raised up possibility of DAP12 might be expressed by type A synoviocyte, a cell type derived from macrophage lineage and usually dominate lining layer of synovial tissues. Expression of DAP12 in cells, majority with mononuclear monocyte and macrophage morphology, might indicate that this molecule is also involved in the activation of those cells (Turnbull et al. 2005) and hence in the progression of OA.

Although DAP12 appeared to be expressed mostly by monocyte/macrophage-like cells in both PO and OA tissues, DAP12 immunostaining was also noted on fibroblast-like cells (with elongated morphology). To date, there has been no literature reporting DAP12 expression in fibroblasts and immunostaining of DAP12 along with fibroblast cell markers should be done in future to confirm this observation. Overall the findings of differences in TREM2 and DAP12 expression between PO and OA tissue groups could suggest the intracellular TREM2/DAP12-mediated signal may have role in the initiation and progression of PO at the molecular level.

Another novel finding in this study is the demonstration of OSCAR expression in multinucleated osteoclast-like cells in PO tissues. Previously expression of OSCAR in specifically osteoclast-like cells has been shown in human RA (Herman et al. 2008; Herman et al. 2007) suggesting its role in RA progression. As discussed earlier in the previous Chapter 1, OSCAR has been suggested to be another co-stimulatory receptor mediating osteoclastogenesis (Kim et al. 2002; Koga et al. 2004). In the pathology of PLOSL, the intracellular signal through OSCAR/FcRγ is also thought to provide compensatory pathway for bone loss resulting the formation of bone cysts and trabecular bone loss observed in the TREM2/DAP12–deficient disease (Barrow et al. 2011; Chouery et al. 2008).

In both PO and OA tissue groups, there were several cell types expressing OSCAR. Unlike in murine bone tissues and RAW264.7 lineage, in which the expression of OSCAR is only limited to the osteoclasts (Kim et al. 2002) the cell types expressing the immunoreceptor also include monocyte/macrophages (Merck et al. 2005), dendritic (Merck et al. 2004; Tenca et al. 2005), and most recently shown in endothelial cells (Goettsch et al. 2011) in human tissues. OSCAR immunostaining in the control OA tissues was similar to TREM2 as it was mainly detected in mononuclear cells in lining layer, which could be synoviocytes type 1 (macrophage-derived). It was also noted occasionally on blood vessels in some OA tissues consistent with the recent reports that endothelial cells express the ITAM immunoreceptor (Goettsch et al. 2011).

Meanwhile in PO tissues, OSCAR was expressed by multinucleated osteoclast-like cells as expected and interestingly occasionally in fibroblast-like cells. There has been no literature on the expression of OSCAR by fibroblast being reported to date. It is quite interesting to find OSCAR immunostaining on vessels in some OA tissues, but not in PO tissues. Andersson et al. (2007) found that synovial fluid from 5 of 6 patients with OA stimulated the mRNA expression of OSCAR and NFATc1 in mouse calvarial implants *in vitro*, while mRNA expressions of DAP12 and FcRγ were not affected by synovial fluid from either revision or OA patient groups. The authors suggested that perhaps OSCAR and NFATc1 mRNA might be regulated by soluble factors present in OA synovial fluid, however DAP12 and FcRγ were not regulated in the same way. Synovial fluids from peri-implant patients are known to produce many inflammatory factors that stimulate bone resorption, such as IL-1α, IL-1β, IL-6, IL-8, IL-11, oncostatin M, TNF-α and M-CSF (Clarke et al. 2001; Nivbrant et al. 1999; Sypniewska et al. 2002). However in this study it should be noted that such factors in the synovial fluid were human proteins that were tested on mouse *in vivo*. In addition, it should be noted that there are differences in cell types expressing OSCAR between species as indicated earlier.

FcRγ adaptor molecule was mainly expressed by monocyte-like cells in PO and type 1 synoviocytes (which are related to monocyte/macrophage lineage) in OA tissues. Similar to OSCAR, FcRγ was also expressed in multinucleated cells in PO tissues. However larger numbes of FcRγ-positive cells were seen compared to OSCAR in PO tissues (as reflected in the SQA score, Table 2.6). Although expressed by similar cells the higher expression of FcRγ

compared to OSCAR could raise possibility of OSCAR might be, in part, explained by the fact that FcRγ has been shown to be able to stimulate the expression of OSCAR later (Ishikawa et al. 2004).

In general the differences in mRNA expression of the ITAM-related molecules between PO and OA (2.3.5 above) supported the data from immunohistochemical studies of protein expression, even though no significant difference was obtained in OSCAR mRNA levels between the two tissue groups. As SQA data indicates proportion number of positive cells rather than amount of protein expression level, the non-significant higher OSCAR mRNA level in PO tissues could be explained by generally low intensity of immunostaining observed in PO tissues. Nonetheless, the data of OSCAR protein and mRNA expression presented in this study is in agreement with Kim et al. (2002) that reported detection of OSCAR mRNA in osteoclast-rich tissues, hence supporting the hypothesis.

Cells markers are widely used in identifying cell types in both *in vitro* and *in vivo* studies as it is more reliable than looking at the cell morphology alone. For osteoclasts, cell markers such as cathepsin K and TRAP have widely been used to identify osteoclasts in past (refer section 1.3.1.3 above), including on PO tissues (Crotti et al. 2004; Kadoya et al. 1994; Shen et al. 2006).

While dual immunolabeling with cell markers is an ideal approach to identify those expressing multinucleated cells as osteoclasts, there was difficulty in obtaining clean positive dual-immunolabeling encountered on paraffin-embeded tissues using this method in the study. While the use of serial immunostaining, although is not equivalent, is the best alternative.

Immunostaining for cathepsin K and TRAP demonstrated large number of osteoclasts and pre-osteoclasts in PO tissues compared to OA (see Table 2.7). The SQA data for *in situ* TRAP histochemical staining comparing PO and control tissues was consistent with previous publications (Crotti et al. 2004; Kadoya et al. 1994). While Hansen et al. (2001) reported an absence of cathepsin K staining in tissues obtained during primary hip surgery, in this study staining of cathepsin K was observed in OA tissues mainly on fibroblast-like cells, which has been previously shown to express the enzyme (Hou et al. 2001; Mandelin et al. 2005), although in much less extent than in PO tissues.

The serial section analyses indicated multinucleated cells expressing TREM2 and OSCAR were also positive for cathepsin K and TRAP, which indicates that those two immunoreceptors were expressed in osteoclasts, rather than FBGCs in PO tissues. While other cell population may express cathepsin K and TRAP those cells were very likely to be osteoclasts as they were multinucleated cells. Cathepsin K has also been shown to be expressed in osteoblasts (Mandelin et al. 2006) and fibroblasts (Hou et al. 2001; Mandelin et al. 2005). This expression of cathepsin K by fibroblasts is consistent with these cells ability to resorb bone independent of osteoclasts (Pap et al. 2003) and stimulate osteoclastogenesis in PO by providing RANKL (Koreny et al. 2006; Sakai et al. 2002). TRAP, on the other hand, is thought to be expressed by foreign-body giant cells in PO tissues (Kadoya et al. 1994) besides in osteoclasts-closely related cells, macrophages and monocytes (Efstratiadis & Moss 1985; Takeshita et al. 2000).

There have been several reports indicating the expression of these osteoclast cell markers along with the ITAM-related molecules. To date this study is the first to demonstrate expression of TREM2 in osteoclast-like cells in human tissues. Deletion in DAP12 was reported as not affecting expression of known osteoclast cell markers like TRAP, CTR and cathepsin K (Faccio et al. 2003c; Humphrey et al. 2004), suggesting that the expression of those osteoclast markers is independent of DAP12 or the expression of those molecules downstream of those osteoclast markers. Nonetheless, besides significant higher level of osteoclast markers in PO tissues as compared to in OA tissues shown here, the expression of DAP12 and other studied ITAM-related molecules were also demonstrated to be more in PO tissues than in the OA tissues. Recently, Barrow et al. (2011) demonstrated the expression of OSCAR in not just only multinucleated cells, but also in TRAP-positive mononuclear osteoclasts in healthy human biopsies.

As described in Chapter 1, the expression of TREM2 and OSCAR has been demonstrated in multinucleated osteoclast-like cells in a number of publications (Barrow et al. 2011; Herman et al. 2008; Humphrey et al. 2006). However this is the first study to propose TREM2 is closely associated with the osteoclast phenotype in context of human tissue and pathology. OSCAR has been regarded as osteoclast markers as documented in a number of publications (Heinemann et al. 2011; Humphrey et al. 2004; Mabilleau et al. 2011). Indeed OSCAR was proposed to be a more specific osteoclast marker than cathepsin K and TRAP as the latter two

had mRNA detected in other tissues not containing in mouse (Kim et al. 2002), whereas OSCAR mRNA was reported to be detected in osteoclast-rich tissues only. Through the present study, TREM2 and OSCAR appeared to be expressed by subsets of multinucleated cells expressing cathepsin K and TRAP, and this could speculate that OSCAR and TREM2 are only expressed at certain stages of osteoclast development. Data from Kim et al. (2002) indicated that OSCAR expression was possibly more in mature osteoclasts and it was suggested that OSCAR is expressed mainly at the later stages of osteoclastogenesis (Kim et al. 2002). Future studies in assessing OSCAR potential to be accepted as late stage-osteoclast marker (Kim et al. 2005d) should include double label studies using between OSCAR and other more definitive osteoclast marker for more developed osteoclasts such as CTR (Hattersley & Chambers 1989; Takeshita et al. 2000).

There has been a large number of studies carried out on the cellular responses following the exposure and interaction with variety types of biomaterials. Throughout the literature, it has been found that different types of particles induce different responses on osteoclasts and related cells (Haynes et al. 1998; Haynes et al. 1993; Shanbhag et al. 1994a, 1995). Therefore, this study should be interpreted only in the context of the major biomaterial present in the PO tissues, which is PE.

It is suggested that osteolysis could be triggered by a threshold number of particles approximately 1×10^{10} particles per gram of tissues (Revell et al. 1997). This may suggest that 1mg of PE wear, for instance, could cause osteolysis considering that amount of PE wear could generate number of particles estimated about 1.3×10^{10} (Tipper et al. 2000). There has been compelling evidence indicating strong correlation between PE wear and PO particularly around hip prostheses, described in past (Brooks et al. 2000; Eftekhar et al. 1985; Holding et al. 2006; Kim et al. 1998; Looney et al. 2002; Orishimo et al. 2003; von Knoch et al. 2005; Wilkinson et al. 2005) (to be further discussed in detail in the following chapter 3.1 below). This is further supported by the fact that there is generally a higher incidence of peri-implant osteolysis in patients with PE-containing prostheses than those with metal-on-metal hip arthroplasty (Migaud et al. 2004; Migaud et al. 2011). For that reason, the study documented in this thesis placed PE as the chosen biomaterial to be investigated. It needs to be acknowledged that other biomaterials were also present on the tissues, besides PE particles. Besides PE, metal particles, which is the second most abundant wear debris type commonly

found in PO tissues (Hirakawa et al. 1996), could also be observed (seen as black particles, not shown in photo) in some of the tissue.

There is a number of previous studies that have characterized the PE particles isolated from human PO tissues (Campbell et al. 1996; Mabrey et al. 2002; Mabrey et al. 2001; Schmalzried et al. 1997; Wirth et al. 1999). Data from a number of studies indicates that there were differences in morphology and size of particles isolated from different sites depending on whether they were isolated from hip, knee tissue specimen and synovial fluid (Mabrey et al. 2002; Mabrey et al. 2001; Schmalzried et al. 1997; Wirth et al. 1999). For instance, PE particles isolated from hip tissues appeared to be smaller and more globular compared to the particles for the hip. There was also differerence in concentration of PE particles between in tissues and in synovial fluid (Mabrey et al. 2001). A study analyzing the presence of PE particles in peri-implant tissues indicated that the mean number of particles is 1.4×10^9 per one-gram tissue (ranging from 7.5×10^7 to 1.1×10^{10} particles per gram tissue) (Koseki et al. 2005). Besides PE presence in tissues around failed prostheses, there is also concern that PE particles could also be detected in other organs such liver and spleen, however it is currently non-achievable to investigate this due to limitation on sensitivity and specificity of detecting devices available (Urban et al. 2000).

The presence of PE particles on the human PO tissues was detected using polarized lens placed on the light microscope. Visualising using polarized light is a common technique to detect and view PE debris. With the aid of polarized light, PE debris could be visualized taking advantage of the birefringency of the crystalline components within the particle (Hansen et al. 2001). PE particles in paraffin embedded tissues could also be detected using Oil Red O (ORO) stain (Hansen et al. 2001; Holding et al. 2006; Schmalzried et al. 1993), which is able to detect small sized PE particles that this study may not have detected using conventional polarized light. Even though Hansen and colleagues (2001) argued ORO staining is more sensitive than by birefringence of polarized lens only because common immunostaining dye may diminish the brightness of tiny birefringence, there is also other study suggesting otherwise (Schmalzried et al. 1993).

PE particles were detected not only around, but also more interestingly within mononuclear and multinucleated cells. PE seen within mononuclear cells believed to be macrophages suggested that they actively phagocytose PE particles in the PO tissues adjacent to osteolytic

sites (Schmalzried et al. 1992a). The concept of macrophages engulfing foreign-body particles has been well established (Nakashima et al. 1999b; Voronov et al. 1998; Xing et al. 2002).

The observations obtained in this study agree with a previous study carried out in the same laboratories, which indicated the presence of PE particles associated with macrophages and multinucleated cells (Holding et al. 2006). Early studies believed that osteoclasts do not necessarily phagocytose wear particles in PO tissues (Athanasou 1996), even though more recent ones suggested the vice versa (Wang et al. 1997a, 1997b). There is also speculation that osteoclasts could phagocytose biomaterial particles while maintaining the capability of resorbing bone (Crotti et al. 2004; Wang et al. 1997a). It is also possible that the multinucleated osteoclast-like cells containing PE particles originated from macrophages that had earlier ingested those wear particles (Athanasou 1996; Pandey et al. 1996; Purdue et al. 2006; Sabokbar et al. 1998; Voronov et al. 1998). Previous studies had demonstrated that cells associated with PE particles in PO tissues were positive for CD68, a cell marker for tissue macrophage as well as osteoclasts (Hansen et al. 2001; Shen et al. 2006). The presence of PE particles (characterized as predominantly in submicron size (Benz et al. 2001; Campbell et al. 1996; Campbell et al. 1995) within the cells seemed to be non-toxic (Benz et al. 2001; Santerre et al. 2000; Xing et al. 2002) unlike some metal particles. It is possible that macrophages are unable to degrade the particles resulting a “frustrated phagocytosis” baby macrophages that then differentiate into osteoclasts with capability of resorbing bone (Pandey et al. 1996; Sabokbar et al. 1998; Wang et al. 1997a, 1997b). There is a further discussion on this in the following chapter).

Shen and colleagues (2006) demonstrated presence of PE particles around and within multinucleated cells expressing osteoclast phenotypic markers such as TRAP, cathepsin K and CTR. The expression of cathepsin K and TRAP in PE-associated multinucleated cells demonstrated in this study is in agreement with the findings in Shen et al. (2006). Similar to the previous studies (Hansen et al. 2001; Shen et al. 2006), this study also found PE-associated multinucleated cells in general displayed weaker immunostaining for cathepsin K than in non-PE containng cells. This may suggest the expression of cathepsin K is lower in PE-containing multinucleated cells than in the one without PE. It is thought that PE-containing cells undergo cell reprogramming rather than performing osteoclastic bone

resorbing function through reduction in expression of bone degrading enzyme such as cathepsin K (Catelas et al. 1998; Catelas et al. 1999b).

Besides osteoclast cell markers, the expression of important molecules regulating osteoclastogenesis such as RANK has also been studied in PE-containing multinucleated cells on PO tissues. Using immunohistochemistry, Holding et al. (2006) showed multinucleated cells containing PE particles were positive for RANK, supporting the earlier finding of RANK mRNA expression in cells that had engulfed PE particles (Crotti et al. 2004). This may indicate that the expression of RANK in macrophages or/and pre-osteoclasts is in response to direct stimulation from PE coming into direct contact with cells or following particle engulfment by the cells. This suggests PE might stimulate the expression of the key osteoclastogenesis-mediating receptor, which then promotes formation of osteoclasts in PO tissues.

The stimulation of RANK expression in macrophages in contact with PE particles in PO tissues (Holding et al. 2006) could be consistent with this occuring similarly for TREM2 and OSCAR in this current study. Upregulation of these co-stimulatory receptors would exacerbate the pro-osteoclastogenic effects of the upregulation of RANK and stimulation of the RANK/RANKL pathway. Based on suggestion by Crotti and colleagues (2004), it is believed that the high expression of RANK could be the mechanism underlying the ability of cells isolated from PO tissues to readily form osteoclasts (Sabokbar et al. 1997). Shen and colleagues (2006) suggested that osteoclast cell markers expressed in later stage of osteoclastogenesis are not expressed in cells associated with PE. However the observation of OSCAR and TREM2, along with their associated adaptor molecules shown here could suggest that these molecules might be regulated by PE particles. This will be will be covered in the following Chapter 3.

2.5. Conclusions

Generally there was consistent pattern of higher expression tissues of NFATc1 and all those ITAM-associated molecules at both protein and gene level in PO than OA. This may suggest that these molecules may play role in the pathogenesis of bone erosion in PO. This also implies that the hypothesis outlined in this chapter was supported by the data. These findings

raise interest in the regulation of those molecules by PE particles and led to more functional studies that will be discussed more in the following Chapter 3.

While the signifcance of ITAM-associated molecules has been largely established in the context of bone biology and an immunological point of view, the uniqueness of the study documented here is that those osteoclast-ITAM related molecules were studied from the point of view of human bone pathology of peri-implant osteolysis. In addition this study is a comprehensive investigation of all known molecules of ITAM-related pathway and NFATc1. Following all this meaningful data obtained from study documented in this chapter pointing towards the signficance ITAM-associated molecules might have in the pathology of PO, new target for drug therapy could be proposed in effort of developing better treatment for PO. To date, there have been few drugs designed to target the ITAM-associated components of osteoclast described in literature.

3. EFFECT OF POLYETHYLENE ON THE EXPRESSION OF ITAM-RELATED MOLECULES IN OSTEOCLASTS *IN VITRO*

Some of the data presented in this chapter has been published in the following journal article:

Alias E, Dharmapatni ASSK, Holding AC, Atkins GJ, Findlay DM, Howie DW, Crotti TN, Haynes DR. Polyethylene particles stimulate expression of ITAM-related molecules in peri-implant tissues and when stimulating osteoclastogenesis in vitro. Acta Biomater 2012; 8:3104-12

3.1. Introduction

PE is a common biomaterial used in either knee or hip prostheses. There is a number of physical properties of PE giving an advantage over other materials. Such physical properties of PE include low coefficient friction, good wettability and excellent energy absorption (Purdue et al. 2006).

Nevertheless, despite all these great properties for implant designing, there is growing concern over the application of PE as an increase in osteoclast formation and activity following inflammation triggered by exposure to PE particles has been reported. Besides, PE is also actively liberated from prostheses, as PE has been identified as the most abundant wear debris in PO tissues (Campbell et al. 1995; Hirakawa et al. 1996; Shanbhag et al. 1994b; Willert et al. 1990). A previous study has indicated that the penetration rate of femoral head into the PE-lining acetabular cup is 0.2mm/year on average and a small increase to more than 0.3mm/year is associated with osteolysis after 10 years post-implantation (Dowd et al. 2000). However, it is more concerning that metal head–on-PE prostheses has the highest wear rate as compared to other types of implants with wear rate reported to be between 0.075 to 0.4 mm/year (Sochart 1999).

Due to the undesired effects by PE particles, alternative biomaterials have been looked for and there have been move towards using PE-free prostheses, such as ceramic-on-ceramic and metal-on-metal prostheses, or modifying the PE to reduce wear. However, the move for alternative types of prostheses will not necessarily improve the situation. For example, alumina-on-alumina bearing (which is an example of ceramic-on-ceramic implant) have shown no significant difference with cobalt chromium-on polyethylene bearing in clinical performance in terms of wear rates (Capello et al. 2005). The move for using ceramic-on-ceramic implant is also over-shadowed by other complications it may bring (Barrack et al. 2004). Such concerns include chipping of the liner and the fragilty of ceramic for fracture. In addition, there is also concern about microseparation of ceramic-on-ceramic prostheses that may result a unique pattern stripe of wear. From the economic point of view, revision could become more difficult and costly since ceramic heads can not be used for second time and

ceramic prostheses are 20% more expensive than metal-on polyethylene prostheses (Purdue 2006).

As for the metal-on-metal prostheses, there are concerns on such implants like more expensive, higher risk of instability and metal hypersensitivity of patients (MacDonald 2004). There is also evidence that use of such implants is accompanied with systemic release of cobalt ions (Brodner et al. 1997; MacDonald et al. 2003). This raises concerns about the risks of the implants being carcinogenic even though this is not proven yet (Cuckler 2005). In addition, metal particles are generally smaller and present in higher numbers in tissues, therefore raising a concern that they may trigger stronger cellular responses. However, there is no evidence supporting such concern in particular to date (MacDonald 2004).

In conclusion the use of PE-containing prostheses in the community is still common, and as a consequence more studies need to be carried out to investigate the cellular response following the liberation and exposure to PE particles and their roles or involvement in the progression of PO.

There are several factors that may influence PE wear or particle generation from prostheses. Those factors include the implant design, such as modularity, geometry and implant fixation (Zhu et al. 2001), the femoral head size and the composition of the PE itself. PE volumetric wear increases as the head diameter increases. In addition to that, the femoral head size need to acccomodate a minimum 6mm (or ideally 8mm) thickness of PE liner (Bartel et al. 1986). As for the composition of the PE particles, highly crosslinked PE has been produced since it is more wear resistant (Muratoglu et al. 2001). However the highly crosslinked PE was found to still show signs of wear, therefore the problem may still present in patients (Engh et al. 2006; Leung et al. 2007; Schroder et al. 2011), even though at reduced rate of early incidence following prostheses implantation (Glyn-Jones et al. 2008; Thomas et al. 2011). Interestingly, a study by Endo and colleagues (2002) found that even though crosslinked PE particles demonstrated 30 percent lower rate of wear, there was higher percentage of small PE particles ranging from 0.1 to 1μm size, which were more biologically active and could be phagocytosed. This was further supported by a finding of elevated secretion of TNFα by macrophages following phagocytosis crosslinked PE as compared to non-crosslinked PE (Illgen et al. 2008), suggesting that crosslinked PE is more potent than the non-crosslinked PE in promoting inflammation.

Different types of prostheses wear debris were found to give different responses in cultures of macrophages or osteoclasts (Haynes et al. 1998; Haynes et al. 1993; Shanbhag et al. 1994a, 1995). For example, a previous study carried out in this laboratory indicated that there were differences in the level and pattern of mRNA and protein expression of osteoclastogenic molecules in response to stimulation by a number of types of wear particles (Haynes et al. 2001b). Even small differences in chemical composition could make differences in biological response. For instance, as shown by Rogers and coworkers (1997) titanium alloy containing vanadium instead of niobium stimulated higher levels of secretion of osteolytic agents like IL-1, IL-6 and prostaglandins E2. As mentioned earlier (refer section 1.2.1), studies have looked at quite numbers of different types of materials used in prostheses including PE (Holding et al. 2006), metals (Haynes et al. 1998; Haynes et al. 1993), ceramics (Kaufman et al. 2008) and PMMA cement (Jones et al. 2001).

However, this present study focused on PE since PE is considered to elicit damaging osteolytic effects and there is a strong correlation between PE wear rates and the extent of osteolysis (Howie et al. 2007; Kadoya et al. 1998; Looney et al. 2002; Oparaugo et al. 2001; Orishimo et al. 2003). This is further supported by the finding of higher numbers of PE particles detected in peri-implant tissues with osteolysis than ones without osteolysis (Kobayashi et al. 1997; Koseki et al. 2005). The significance of PE particles as the causative agent for osteolysis in PO has been demonstrated in *in vitro* (Baumann et al. 2004; Horowitz & Gonzales 1997; Matthews et al. 2001; Rader et al. 1999) as well as *in vivo* animal models (Barrow et al. 2011; Howie et al. 1988; Kim et al. 1998; Ren et al. 2004). A recent study performed in this laboratory indicated that there was a correlation between “defect size” (volume of peri-implant osteolysis lesions) and the amount of PE debris (based on numbers of PE debris present on PO tissues) (Holding et al. 2006). Other study using an *in vivo* murine model demonstrated in the number of cells infiltrating the air pouch in response to UHMWPE was higher than other biomaterials TiAlV, CoCr and PMMA (Wooley et al. 2002).

There is accumulating evidence suggesting the modulation of PE-induced osteolysis is through osteoclast-associated cells. Study by Shen and colleagues (2006) reported cells containing PE wear particles found on human peri-implant tissues expressed osteoclast phenotypic markers, such as tartrate resistant acid phosphatase (TRAP), cathepsin K and CTR. In addition these genes are further induced by interaction between osteoclasts and the

bone matrix (Shen et al. 2006). I*n vitro* studies have shown RANK mRNA expression in human monocytic (the cell lineage from which osteoclasts are derived) cell line was upregulated in response to stimulation by PE particles (Baumann et al. 2004). PE was also found to activate and stimulate cytokine release by monocyte/macrophage-lineage cells. A study by Chiba and colleagues (2001) showed that PE stimulated monocytes from PO tissues to release cytokines like IL-1β, IL-6 and TNF-α. In macrophages, PE was reported to be able to activate the cells (Boynton et al. 1991; Horowitz & Gonzales 1997; Willert et al. 1990) and stimulate TNFα release in a macrophage cell line (Catelas et al. 1998; Catelas et al. 1999a). Dendritic cells, a related cell type that has been shown to be able to differentiate into osteoclasts (Gallois et al. 2010), was demonstrated as could be activated by UHMWPE particles to increase secretion of IL-1β and IL-6 (Pal et al. 2011).

There is evidence that PE may also promote bone loss through regulation of osteoblasts, either by suppressing bone formation (Sacomen et al. 1998), like ALP activity (Atkins et al. 2009; Chiu et al. 2009; Dean et al. 1999; Pal et al. 2011) or promoting osteoclastogenesis through the release of increased pro-osteoclastogenesis mediators such as RANKL (Granchi et al. 2004; Pal et al. 2011). Nonetheless, Pal et al. (2011) indicated that the effect of PE particles was less on osteoblasts and bone formation compared to the effect on promoting osteoclastogenesis. This ground further supports approach of this thesis to choose osteoclast as model to study PE particles *in vitro*.

The focus on osteoclast effects is further supported by the fact that a number of treatments have been suggested to reduce PE-induced PO by targeting osteoclasts. Several types of bisphosphonates (which target osteoclast bone resorbing activity), such as alendronate (Millett et al. 2002; Shanbhag et al. 1997), TRK-530 (Iwase et al. 2002) and pamidronate (Horowitz & Gonzales 1997; O'Hara et al. 2004) have been used to treat the bone loss in *in vivo* PE-induced PO models with some success. Treatment with the RANKL inhibitor, OPG, reduced osteolysis in a mouse calvarial model using PE (von Knoch et al. 2005). Similarly, treatment with RANK-Fc (RANK-fusion protein, antagonist to RANKL composed of extracellular region of RANK with Fc portion of human IgG_1) also showed promise in a particle-induced PO model (Childs et al. 2002).

The discovery of the presence of TREM2 and OSCAR-expressing multinucleated cells associated with PE particles as described in Chapter 2 has prompted studies in this chapter to

investigate on the direct association between PE particles and the expression of those immunoreceptors as other ITAM-related molecules. As discussed in the previous chapter, the observation of PE-associated multinucleated cells positive for TREM2 and OSCAR was similar to the observation described in Holding et al (2006) for RANK expression and earlier studies has also indicated direct link between PE and expression of RANK (Baumann et al. 2004; Crotti et al. 2004). Therefore, this present study was aimed at investigating the direct effect of PE on osteoclastogenesis and the expression of co-stimulatory ITAM-associated molecules as well as the osteoclast markers. There has been no study investigating on the effect of PE on the mRNA expression of those molecules in human osteoclast assays being reported. The *in vitro* model used in this study was adapted from a novel 3D-collagen model described by Atkins and colleagues (2009).

3.1.1. Hypothesis

There is increase in osteoclast formation and bone resorbing activity as well as the mRNA expression of ITAM-associated factors in response to the stimulation by PE particles.

3.1.2. Aims

- To investigate the effect of PE particles on the formation of multinucleated osteoclast-like cells derived from PBMC
- To study the effect of PE particles on bone-resorption activity of PBMC-derived osteoclasts
- To investigate the effect of PE particles on the mRNA expression of ITAM-associated molecules and osteoclast markers in the PBMC-derived osteoclasts

3.2. Methods

3.2.1. Preparation of PE Particles

PE particles used in this *in* vitro study were Ceridust 3615 polyethylene powder (Clariant, Victoria, Australia), which have been previously characterized to be in size between 0.3 to 10μm (Matsusaki et al. 2007; von Knoch et al. 2004). This falls within the biologically active size range to stimulate macrophage (Green et al. 1998; Pal et al. 2011).

The particles were washed and sterilized by resuspending those particles at a concentration of 1mg/mL in 70% ethanol and then left incubated at room temperature for 48 hours (Hitchins & Merritt 1999). To remove the ethanol but yet maintaining that the PE particles to be introduced into the *in vitro* cell cuture system endotoxin-free, the particles were washed with endotoxin-free PBS (SA Pathology, SA, Australia). The particles were also sonicated for 30 minutes prior to addition into the collagen gel to avoid any clumps in the collagen gel.

3.2.2. Cell Culture

Studies indicate that cells recruited to peri-prosthetic tissues in response to wear particles *in vivo* are monocyte/macrophage lineage. Therefore, adherent PBMCs, in which monocytes make up the main cell population, were used in these *in vitro* studies to investigate the direct effect of PE particles on osteoclast formation and function. Osteoclast culture assays and analysis, including TRAP staining and resorption pit assays, were originally carried out by a research assistant in the laboratory (Christopher Holding). For the purpose of my Ph.D. I assessed gene expression of ITAM molecules in these cells.

PBMCs were extracted from human buffy coats obtained from three healthy donors at the Red Cross Transfusion Service (Adelaide, South Australia) as described previously (Holding et al. 2006). Buffy coats were diluted one in four with Hank's balanced salt solution (HBSS) (Gibco, BRL). PBMCs were then isolated through centrifugation at 1500rpm for 30 minutes using Ficoll hypaque separation gradient (Pharmacia Biotech, Uppsala, Sweden) and washed

three times with HBSS. PBMCs were then resuspended and seeded at 1 X 10^8 cells/mL into 12-well culture dishes (Nalge Nunc International, Rochester, NY) in 1ml volume per well with complete medium composed of α-MEM (Invitrogen, Melbourne, Victoria, Australia), 10% fetal calf serum (FCS) (Gibco, BRL) and 1% of each penicillin, streptomycin (Invitrogen) and L-glutamine (Invitrogen). The cell culture was incubated with complete medium containing 25ng/ml M-CSF (Chemicon, Millipore, Victoria, Australia) and soluble RANKL (50ng/ml, 10ng/mL or none accordingly) (Chemicon, Milipore, Victoria, Australia) in type I collagen gel (Cellmatrix Type 1-A, Nitta, Tokyo, Japan) with or without 500 μg/mL PE particles for 3 days in 5% of carbon dioxide (CO_2) (BOC Gas and Gear, South Australia, Australia) at 37°C in humidified condition. The protocols for preparation of the collagen gels and adding the PBMCs were as described in Atkins et al. (2009).

After 3 days of cells being exposed to the particles, the collagen in the cell culture system was digested using 0.2% w/v collagenase A (Roche, NSW, Australia) for 1 hour at 37°C in 5% CO_2 (with occasional mixing to aid digestion every 5 to 10 minutes) to allow the cells to be reseeded onto new plates for the remaining non-gel culture period. Following the digestion of the collagen gels, cells were pelleted and then reseeded at concentration of 1 X 10^6 cells/mL Cells were plated on 12-well plates (Falcon; Becton Dickenson Labware, New Jersey) for mRNA extraction in duplicate, or seeded onto coverslips for TRAP staining and dentine slices for resorption assay. M-CSF and soluble RANKL (sRANKL) was included in the subsequent medium changes up to day 17. Medium was changed every 2 to 3 days. Experiments were performed from 3 patients in duplicate. After 13, 15 and 17 days of growth (10, 12 and 14 days since in collagen) cells were fixed for TRAP staining and dentine was processed for resorption analysis. TRIZol was added and cellular RNA was isolated using manufacturers instructions for qRT-PCR assessment.

3.2.3. TRAP Staining

To confirm the presence of osteoclast cell lineage in the cell culture system, a portion of PBMCs were also cultured on 8-well chamber slides (Nalge Nunc International, Rochester, NY) for TRAP staining. Cells were fixed after 13, 15 and 17 days of cells culture using ice-cold acetone/methanol (1:1 ratio). TRAP staining was performed in single run using the standard TRAP staining kit described above (see section 2.2.2.2 above). Cells were left

incubated with 1mL volume per well of TRAP staining solutions (containing acetate, naphthol AS-BI phosphate and tartaric acid solution, see section 2.2.2.2 above) for one hour. Following several washes with distilled water, cells were counter stained with the nuclear stain Methyl Green. Excess Methyl Green stain was removed by washing with distilled water three times before air-dried for overnight. Finally stained cells were mounted with Aquatex mounting medium (Merck, KGaA, Germany).

Photos of the TRAP-stained cell culture were captured at magnification of 100X using Nikon FXA Research Light Microscope. 2 or 3 photos were captured for each samples and the total number of purple-coloured TRAP-stained multinucleated cells with more than 3 nuclei were counted as osteoclast-like cells formed in each photos before being averaged out to be the final data for TRAP staining.

3.2.4. Dentine Pit Resorption Assay

To confirm the formation of functional osteoclast and to assess the effect of exposure to PE particles on the bone resorption activity cells were seeded onto dentine slices of whale tooth. Culture medium and adherent cells were washed with HBSS and completely removedby trypsinisation (0.1% v/v in PBS/EDTA) for 1 hour (37°C, 5% CO_2) at day 17. After washing with distilled water and left air-dried overnight, dentine slices were mounted onto stubs and then carbon gold-coated for SEM viewing using tungsten filament Phillips XL20 Scanning Electron Microscopy (Philips, Amsterdam, Netherlands). Quantitation of the area of resorption was performed using Adobe Photoshop CS4 (Adobe, San Jose, CA) and Image J (National Institutes of Health, Bethesda, Maryland) software analysis. Three random fields (viewed at objective 100X magnification on scanning electron microscopy) were chosen for the analysis and percentage area of resorption was calculated.

3.2.5. qRT- PCR

Assessment of the change in the mRNA expression of NFATc1 and osteoclast ITAM-related molecules was performed using quantitative real-time RT-PCR as described in 2.2.3 previously.

3.2.5.1. RNA Extraction

To isolate RNA from PBMC-derived osteoclast culture 600µl of TRIzol was added to each duplicate samples. The duplicates were then pooled. Similar protocols for RNA isolation and quantitation as described in section 2.2.3.1 were employed.

3.2.5.2. Reverse-Transcription

The protocol for generating cDNA from RNA extracted was carried out as described in the previous chapter. All reagents and protocols were as detailed in section 2.2.3.2. 1µg of RNA was reverse transcribed for each samples.

3.2.5.3. Real Time PCR

Real-time PCR was as described earlier in Chapter 2 shown in section 2.2.3.3. The primers are shown in Table 2.5 above. As described in the previous chapter, the mRNA levels for all genes investigated were normalized to hARP mRNA. To reduce variations between samples and runs, the threshold was set to be at 0.1 and the C_T values (discussed in section 2.2.3.3) for each sample were used based on the threshold.

3.3. Results

To assess the effect of PE particles on expression of NFATc1and ITAM-associated molecules, PBMC-derived osteoclasts were cultured in the presence and absence of PE particles. The effect of PE on ITAM-associated molecules was assessed during the later stages of culture (13, 15 and 17 days with the first three days in collagen together with the PE particles).

3.3.1. Effect of PE particles exposure on osteoclast formation

Based on the immunohistochemistry data as presented earlier in 2.3.4 showing PE particles within multinucleated osteoclast-like cells (Figure 2.9), this section of the study was carried out to investigate whether these PE particles can stimulate more osteoclast formation *in vitro* similar to that observed on PO tissues. To demonstrate the formation of osteoclasts upon exposure to PE *in vitro*, TRAP staining was performed at the time points indicated.

Examination of the cell cultures from the all three donors included in this study showed that PE particles increased in the number of TRAP-positive multinucleated cells. In particular, it appears that the largest increase was observed at 14 days post collagen gel (Figure 3.1). For the purpose of consistency, the remaining panel of representative pictures presented below would be on cell culture at 14 days post collagen gel.

The TRAP staining at 14 days post collagen gel from each donor were compared and presented in the panel below (Figure 3.2). As indicated in the panel, there was difference in response between donors, however all donors showed increase in the number of osteoclast-like cells formed following exposure to PE particles.

The TRAP-stained culture viewed under polarised lens verified the presence of PE particles associated with the TRAP-positive osteoclast-like cells (Figure 3.3). In addition, the particles also appeared to be within the cells consistent with their phagocytosis.

Quantitative analysis on the number of TRAP-positive multinucleated cells revealed that there were more TRAP positive cells in cultures with PE than without PE on Day 17 (Figure 3.4).

All results described above were from cell culture system with an optimized dose of RANKL at 50ng/mL. This was determined from experiments of cell culture with and without PE particles grown in the presence of routine dose (50ng/mL), low dose (10ng/mL) or no RANKL (Figure 3.5).

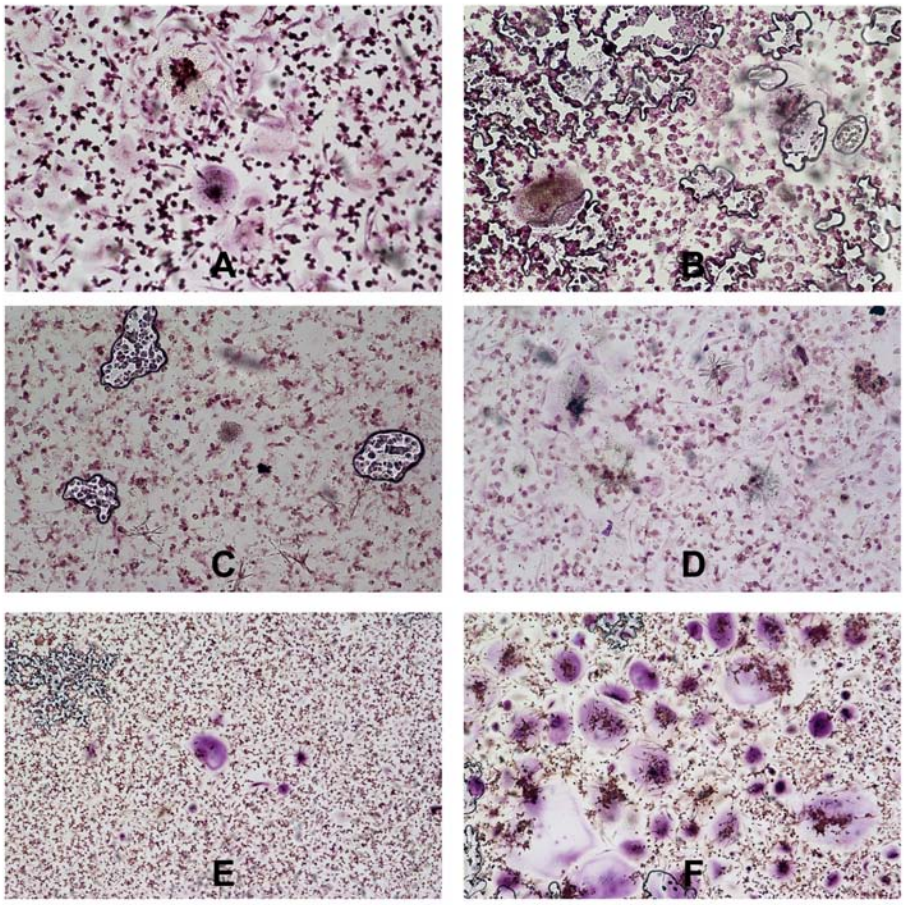

Figure 3.1: TRAP staining of PBMC-derived osteoclast cell culture at different time points following 3-days exposure with and without PE particles (between columns) in collagen gel.

Representative pictures of TRAP staining of PBMC-derived osteoclasts (Donor 2) at 10 (**A** and **B**), 12 (**C** and **D**) and 14 (**E** and **F**) days post culture in collagen gels (between rows) in the presence with (right column) or without PE particles (left column) (captured by Christopher Holding). All the cell cultures shown above were grown with 50ng/mL RANKL and 25ng/mL M-CSF. Only TRAP-positive cells with 3 nuclei or more were considered as multinucleated osteoclast-like cells. Photos were captured at 100X magnification.

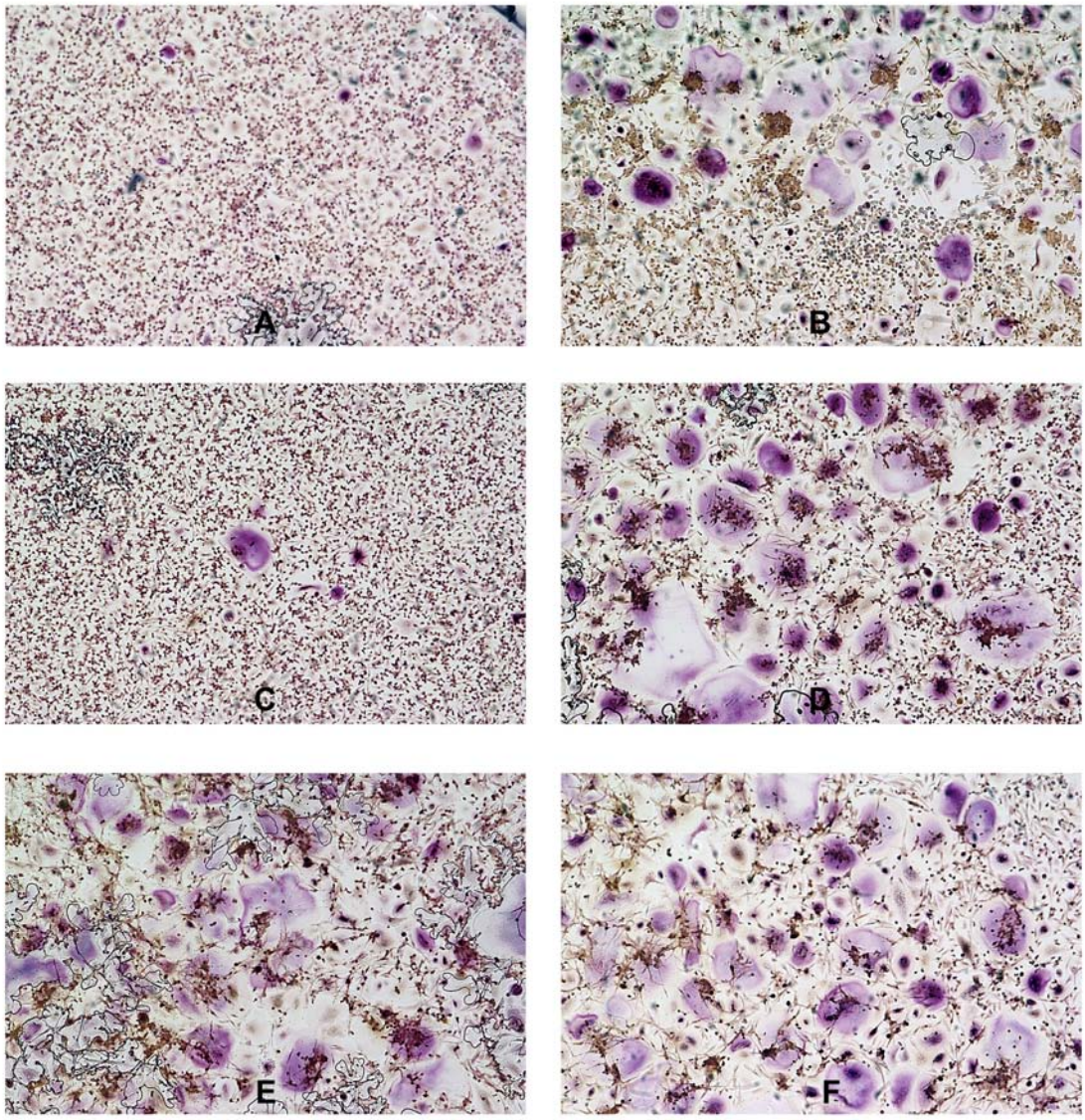

Figure 3.2: TRAP staining of PBMC-derived osteoclast culture from each donor (between rows) at 14 days post 3-days collagen gel with and without PE particles (between columns)

Representative pictures of TRAP staining of PBMC-derived osteoclasts from Donor 1 (**A** and **B**), 2 (**C** and **D**) and 3 (**E** and **F**) at 14 days post culture in collagen gels in the presence with (right column) or without PE particles (left column) (captured by Christopher Holding). All the cell culture shown above were grown with 50ng/mL RANKL and 25ng/mL M-CSF. Only TRAP-positive cells with 3 nuclei or more were considered as multinucleated osteoclast-like cells. Photos were captured at 100X magnification.

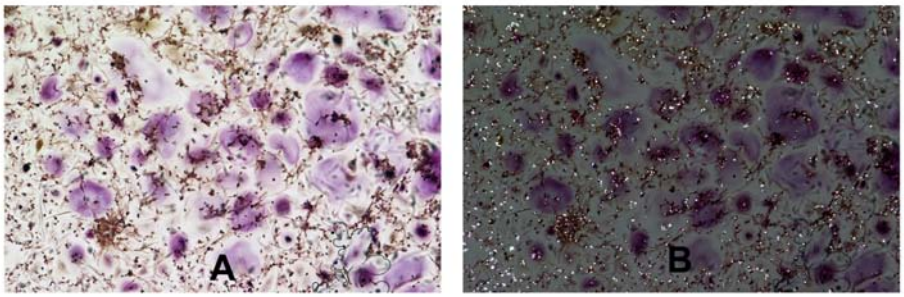

Figure 3.3: Detection of PE particles within and in close proximity to TRAP-positive mutlinucleated PBMC-derived osteoclast-like cells at 14 days post 3-days exposure to PE in collagen gel

Photos **A** and **B** (which are identical) were taken from cell culture at 14 days post 3-day in collagen from Donor 2 (captured by Christopher Holding). View under polarized lens (**B**) verified the presence of PE particles, indicated as birefringence, in the cell culture including within the multinucleated TRAP-positive cells. Photos were captured at 100X magnification.

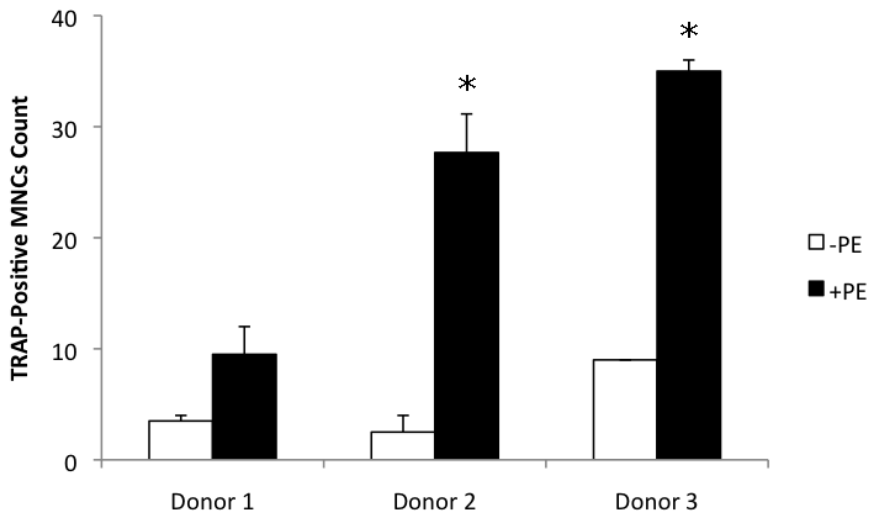

Figure 3.4: Counting of TRAP-positive multinucleated osteoclast-like cells following 14 days post 3-day culture in collagen gel

Mean number of TRAP-positive multinucleated cells formed counted in randomly chosen field of view (represented by the y-axis) on the cell culture following 3-day growth in collagen gel with (filled) or without PE (blank) for each all donors (on x-axis). The error bars shown on the graphs represent the SEM of cell numbers from 3 areas for each group. * indicates significant difference, where $p<0.005$.

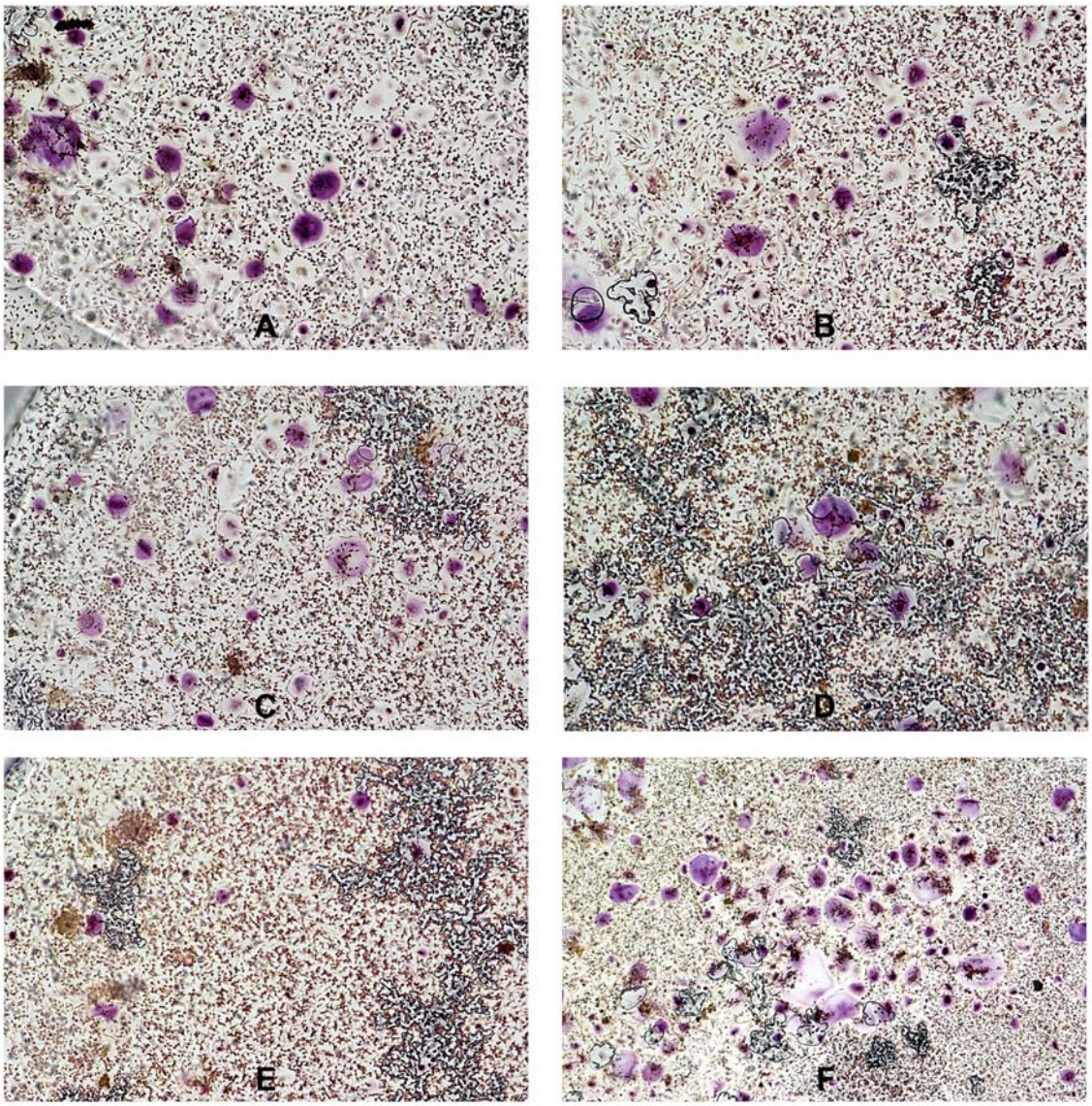

Figure 3.5: Representative pictures of TRAP staining in response to different doses of RANKL

Representative pictures of TRAP staining of PBMC-derived osteoclasts from Donor 2 at 14 days post culture in collagen gels in the presence with (right column) or without PE particles (left column) following growth with different doses of RANKL, which were no RANKL at all (**A** and **B**), 10ng/mL (**C** and **D**) and 50ng/mL (**E** and **F**) (captured by Christopher Holding). 25ng/mL M-CSF was also included in all the cell culture in the above panel. Photos were captured at 100X magnification.

From the panel in Figure 3.5 above, it appeared to be clear that the dose of RANKL had influence on PE-induced formation of osteoclast-like cells from PBMC, with 50ng/mL became the optimum dose required.

3.3.2. Effect of PE particles exposure on the osteoclast resorption activity

Representative pictures captured from scanning electron microscope scans are arranged in panels in the following figures for comparison on the effect of PE particles on osteoclast resorption function (Figure 3.6 and Figure 3.7).

Quantitative analysis of pit resorption indicates that there was greater resorption of dentine discs occurred in cells with PE exposure compared to dentine discs with no PE particles (Figure 3.8). It also appeared that RANKL dose had big influence on the total resorption activity looking at the minimal amount of total resorption on the dentine discs for low dose and no RANKL samples as compared the 50ng/mL RANKL (Figure 3.9).

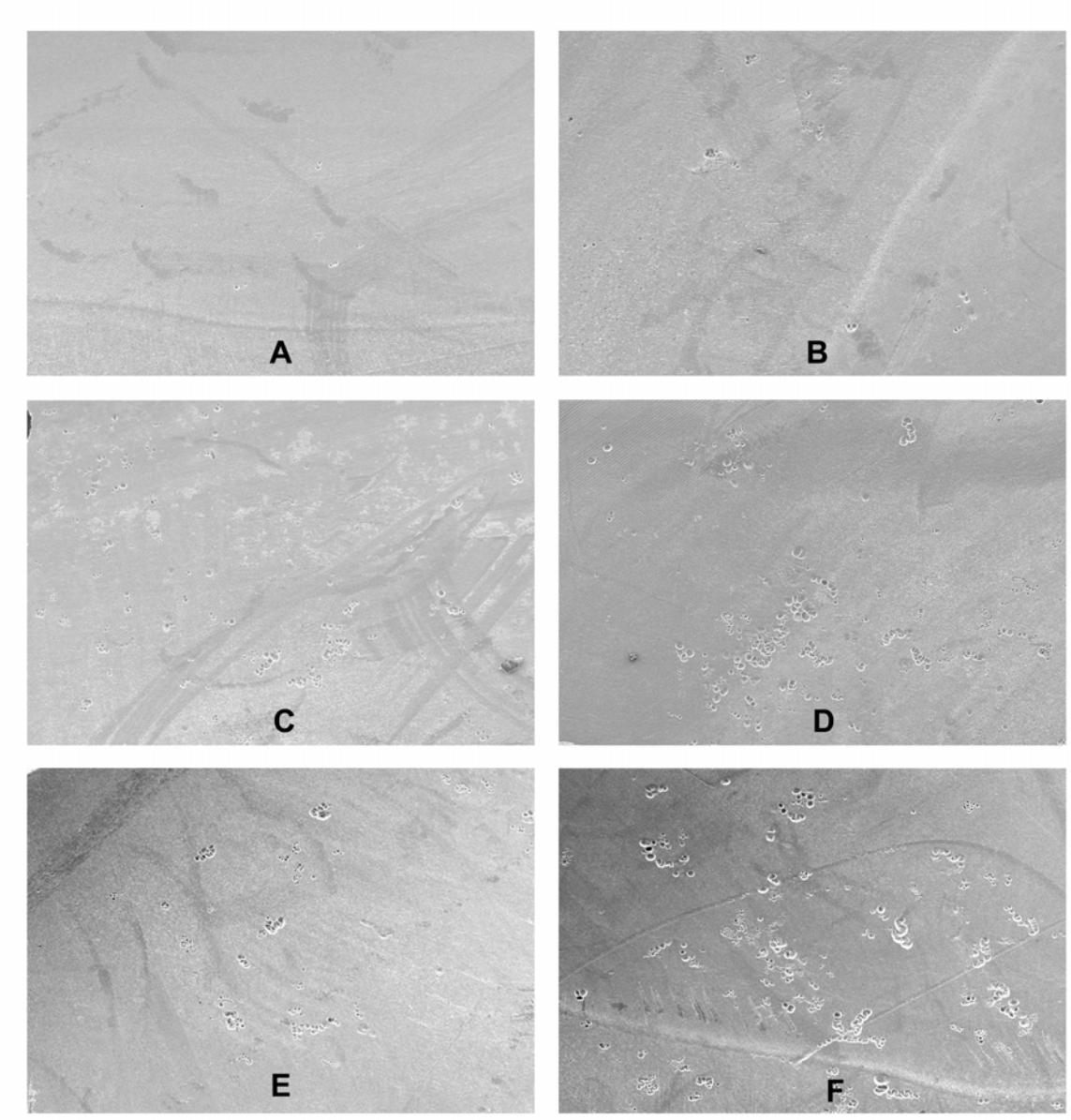

Figure 3.6: Dentine resorption pit assay of PBMC-derived osteoclast cell culture at different time points following 3-day exposure (beteen rows) with and without PE particles (between columns) in collagen gel

Representative pictures of resorption pits on dentine slices by PBMC-derived osteoclasts (from Donor 2 only) at 10 (**A** and **B**), 12 (**C** and **D**) and 14 (**E** and **F**) days post culture in collagen gels (between rows) in the presence with (right column) or without PE particles (left column) (scanning electron microscopic scans by Christopher Holding). All the cell cultures shown above were grown with 50ng/mL RANKL and 25ng/mL M-CSF. Photos were captured at 100X magnification.

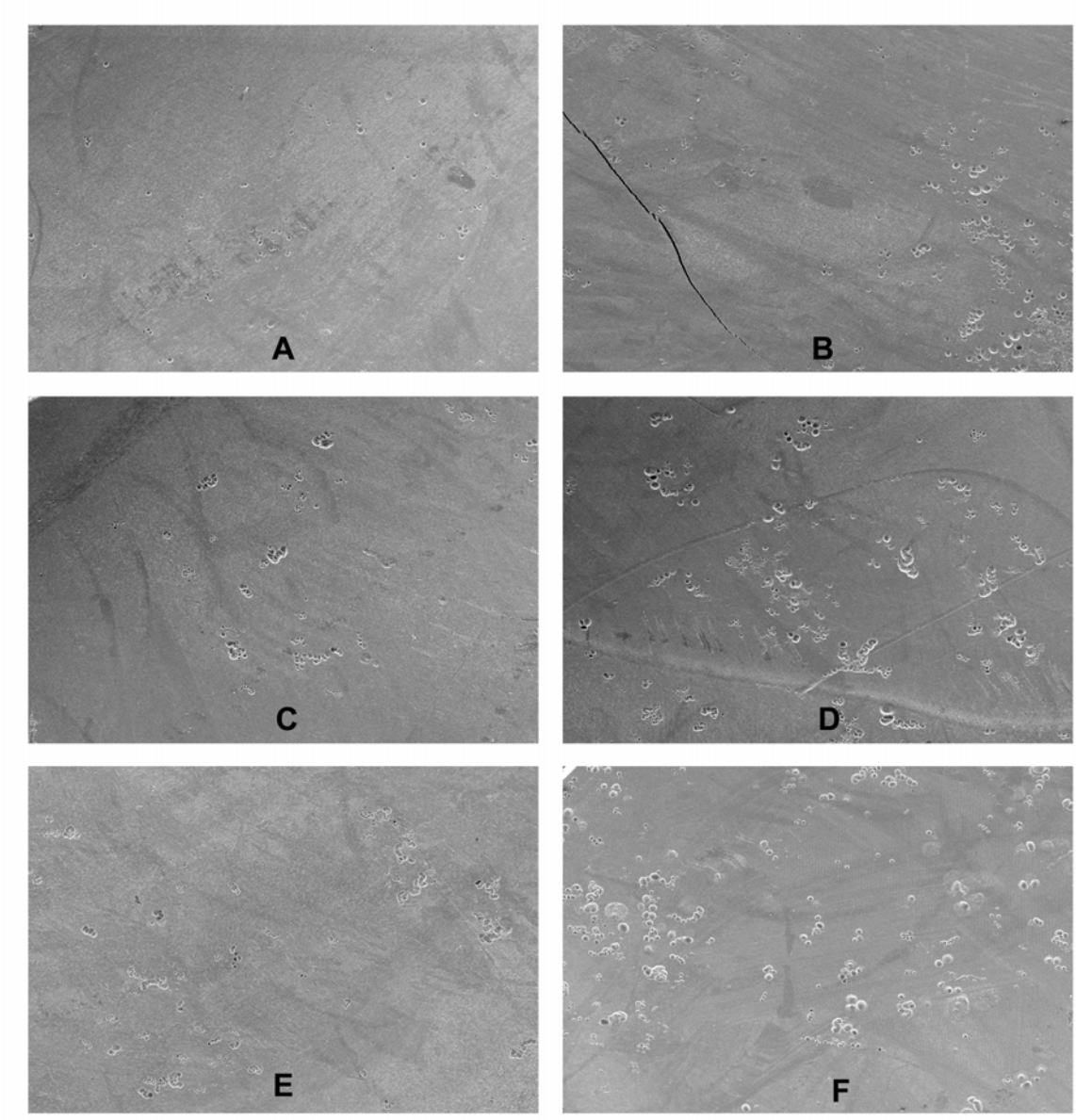

Figure 3.7: Dentine resorption pit assay of PBMC-derived osteoclast culture from each donor (between rows) at 14 days post collagen gel with and without PE particles (between columns)

Representative pictures of resorption pits on dentine slices by PBMC-derived osteoclasts from Donor 1 (**A** and **B**), 2 (**C** and **D**) and 3 (**E** and **F**) at 14 days post culture in collagen gels in the presence with (right column) or without PE particles (left column) (scanning electron microscopic scans by Christopher Holding). All the cell cultures shown above were grown with 50ng/mL RANKL and 25ng/mL M-CSF. Photos were captured at 100X magnification.

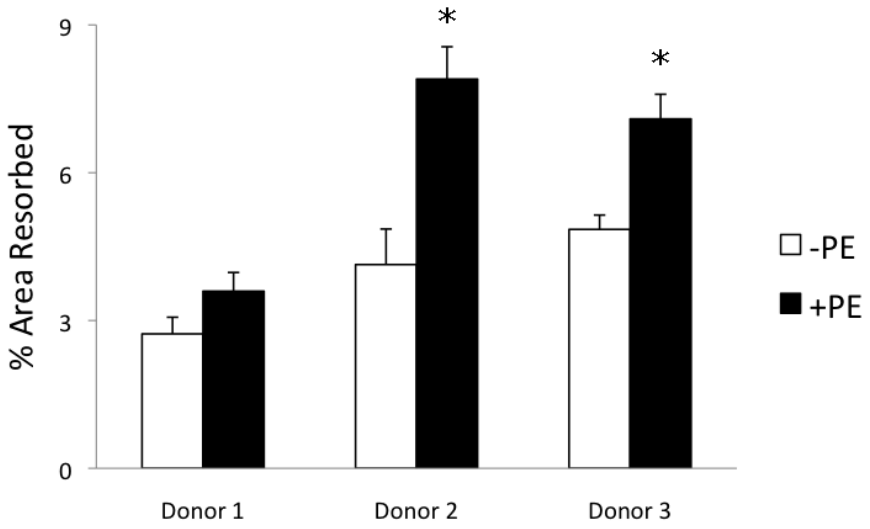

Figure 3.8: Total resorption areas on dentine assay by PBMC-derived osteoclast-like cells following 14 days post 3-day culture in collagen gel in response to stimulation with and without PE

The graph shows mean percentage of areas on dentine slices resorbed by osteoclast-like cells in randomly chosen field of view on the cell culture (represented by the y-axis) following 3-day growth in collagen gel with (filled) or without (blank) for each all donors (on x-axis). The error bars shown on the graphs represent the SEM of resorption areas from 3 fields for each donor.

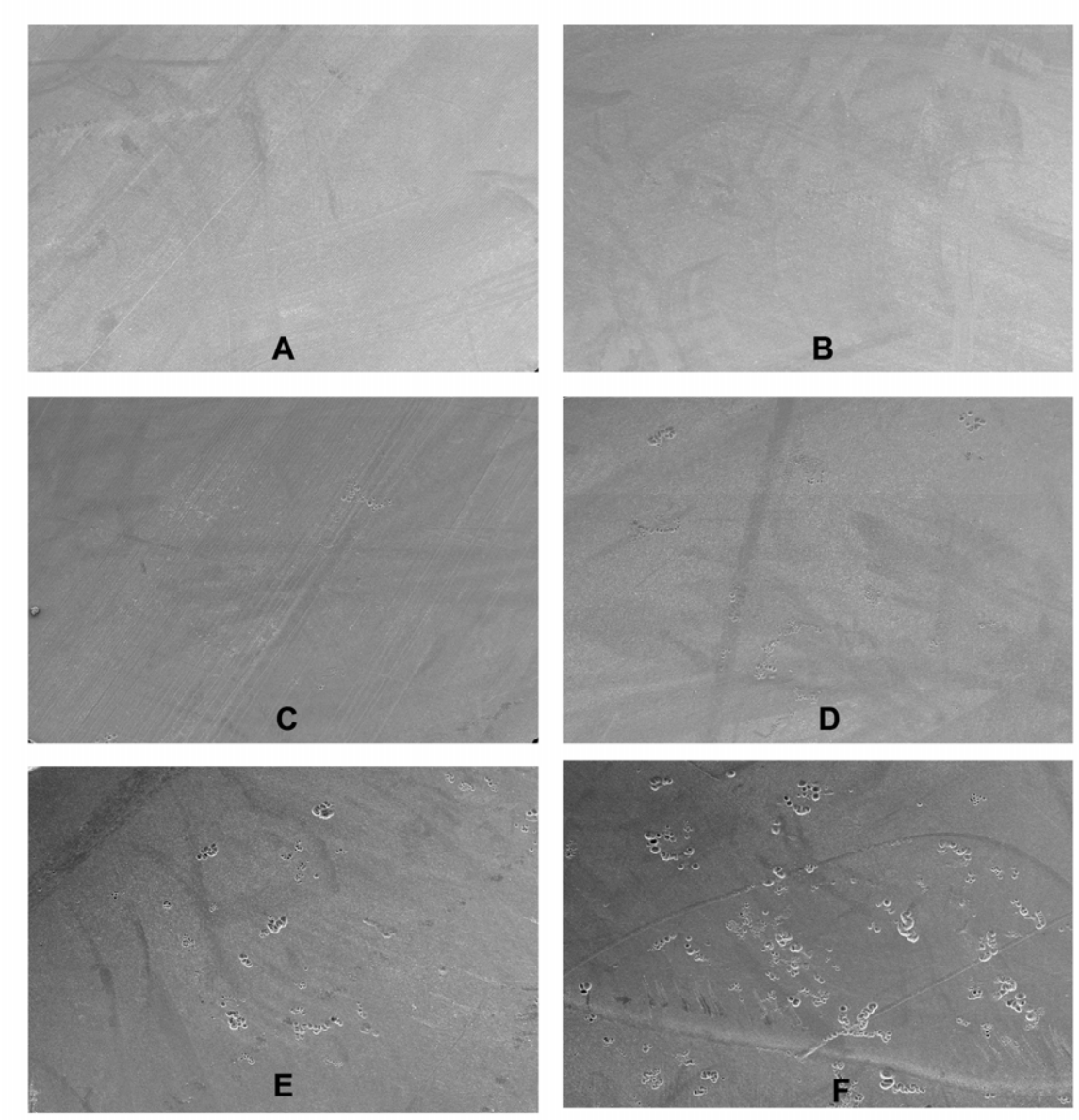

Figure 3.9: Representative picture of dentine resorption pits in response to different doses of RANKL

Representative pictures of resorption pits on dentine slices by PBMC-derived osteoclasts from Donor 2 at 14 days post culture in collagen gels in the presence with (right column) or without PE particles (left column) following growth with different doses of RANKL, which were no RANKL at all (**A** and **B**), 10ng/mL (**C** and **D**) and 50ng/mL (**E** and **F**). 25ng/mL M-CSF was also included in all the cell culture in the above panel (scanning electron microscopic scans by Christopher Holding). Photos were captured at 100X magnification.

3.3.3. Effect PE particles on gene expression of osteoclast ITAM-related molecules in PBMC-derived osteoclasts

The expression levels of ITAM-related molecules and NFATc1 in response to exposure to PE particles were studied at the gene level. The mRNA expression levels of these molecules were also compared and the expression of cathepsin K was used as the osteoclast marker. The results are shown in Figure 3.10 and Figure 3.11.

There was an inconsistent response to PE in the earlier time points (Day 10 and 12 post collagen gel) between donors (Figure 3.10). However, at 10 days following 3 days of collagen gel there was a trend for lower mRNA expression of ITAM-associated molecules, TREM2, OSCAR and FcRγ, in cells exposed to PE particles in the collagen gel in comparison to the controls. However, it seemed to be a turnover in the mRNA expression as there was a trend towards higher mRNA level of OSCAR, TREM2 and FcRγ in samples incubated with polyethylene particles at Day 14 post collagen gel and this was consistently observed in three donors assessed in this study (Figure 3.11)**.**

Changes in NFATc1 mRNA expression seemed to follow no common trend between donors. While a decrease in the gene expression over the time in response to PE particle exposure in Donor 1 and Donor 2 was observed the opposite occurred with Donor 3. The relative mRNA expression of cathepsin K appeared to be higher from Day 12 to Day 14 (even though not statistically significant) in cells exposed to PE than the controls in every donor. As an osteoclast cell marker, this higher mRNA expression of cathepsin K in cells mixed with PE particles, particularly at 14 days post collagen gel was consistent with the data for TRAP staining (Figure 3.2 and Figure 3.4) and dentine pits resorption assay (Figure 3.7 and Figure 3.8) presented earlier in this Chapter 3.

The mRNA expression level (in relative to hARP mRNA) of ITAM-related molecules (TREM2, DAP12, OSCAR and FcRγ) together with transcription factor NFATc1 and osteoclast cell marker cathepsin K (between rows) in PBMC-derived osteoclast-like cells in response to exposure to PE particles (filled) as compared to the controls (blank). All the cell culture shown above were grown with 50ng/mL RANKL and 25ng/mL M-CSF. The graphs were sorted according to the time point post 3-day collagen gel (in columns). Statistical difference, $p<0.05$ is indicated by * on the graphs.

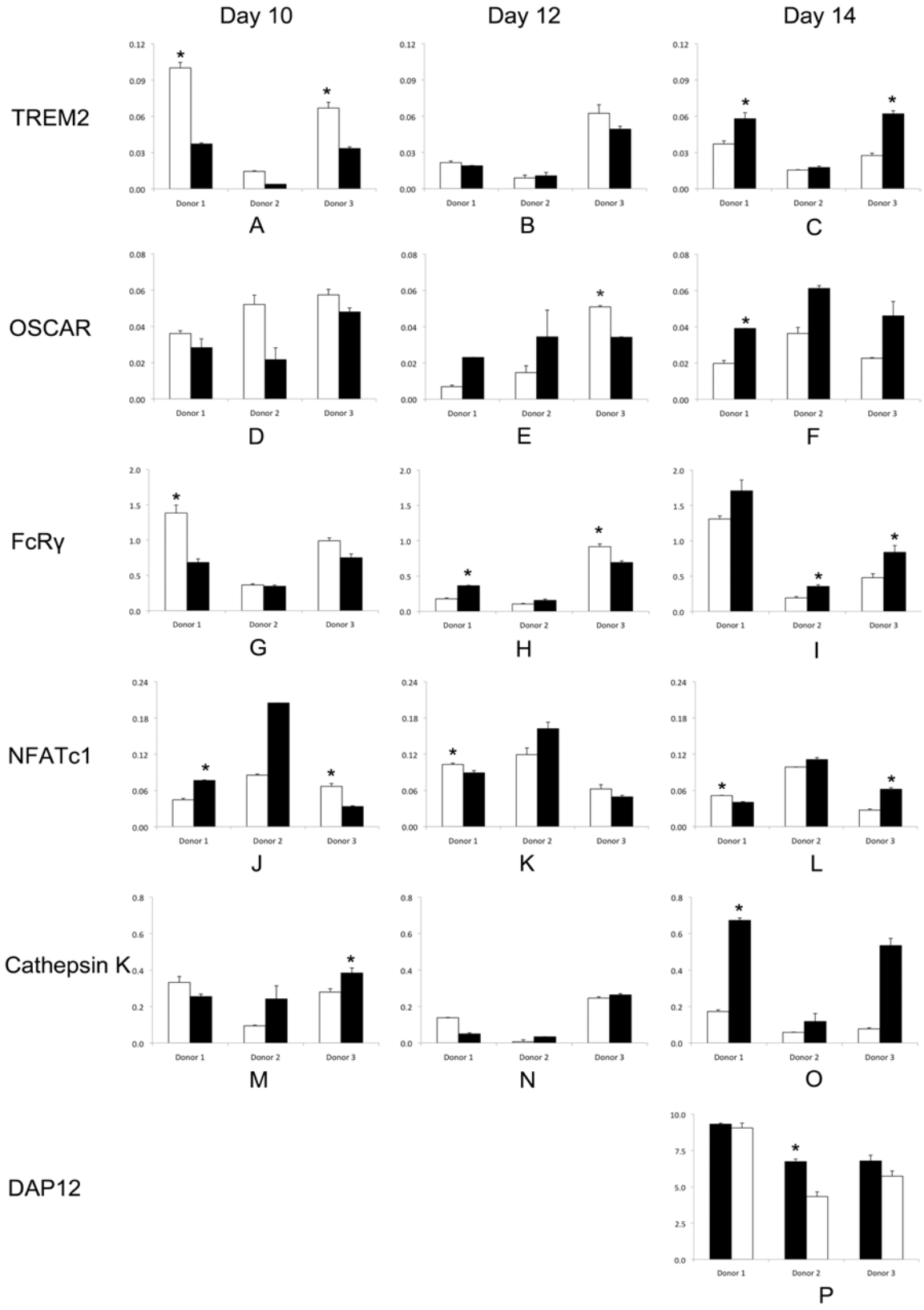

Figure 3.10: Expression level of genes investigated in three donors at 10, 12 and 14 days post collagen gel

The mRNA expression level (in relative to hARP mRNA) of ITAM-related molecules (TREM2, DAP12, OSCAR and FcRγ) together with transcription factor NFATc1 and osteoclast cell marker cathepsin K (between rows) in PBMC-derived osteoclast-like cells in response to exposure to PE particles (filled) as compared to the controls (blank). All the cell culture shown above were grown with 50ng/mL RANKL and 25ng/mL M-CSF. The graphs were sorted according to the donors the cells from (in columns). Statistical difference, $p<0.05$ is represented as * on the graphs.

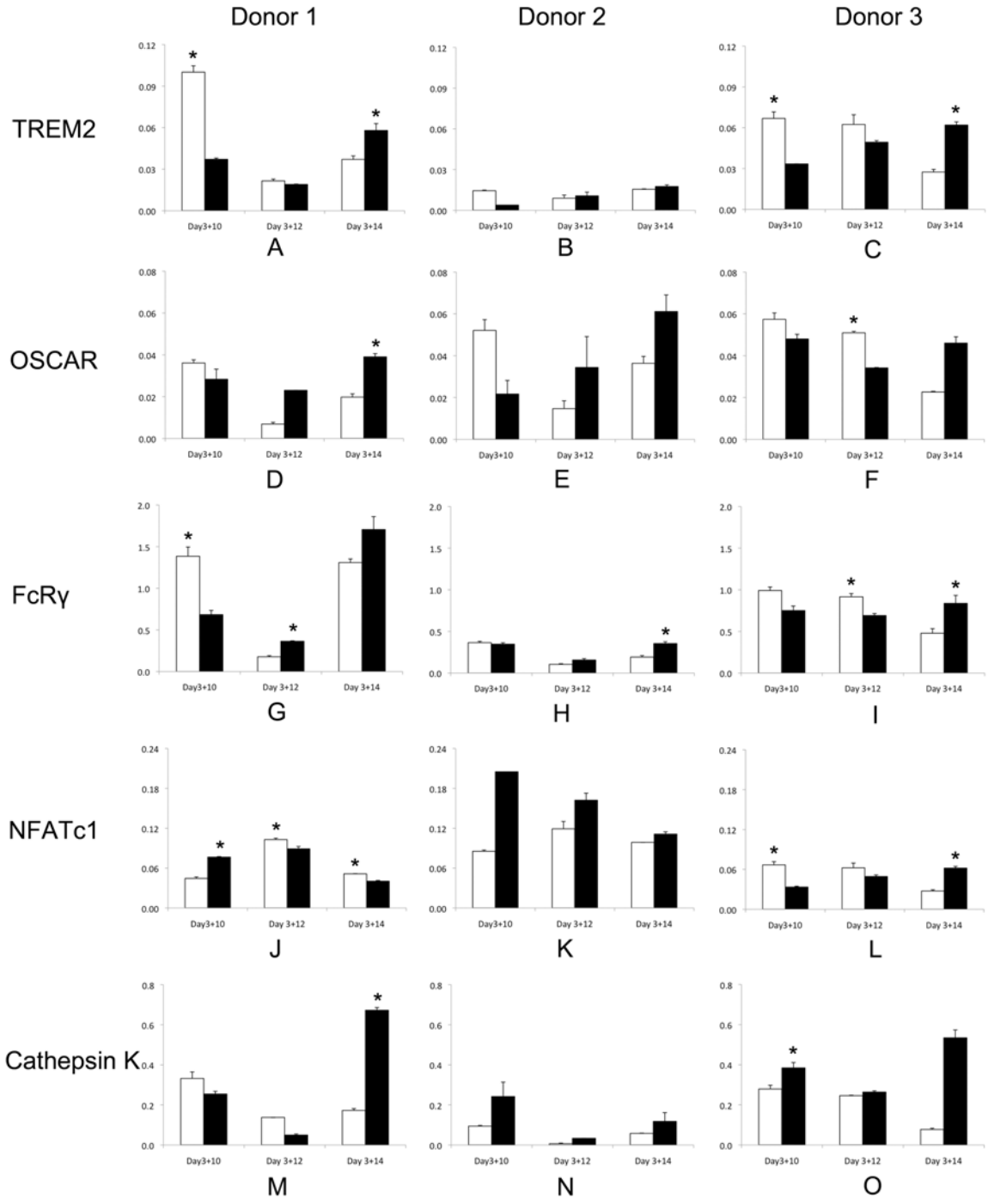

Figure 3.11: Expression level of genes investigated at 10, 12 and 14 days post collagen gel in three donors

The mRNA expression level (in relative to hARP mRNA) of ITAM-related molecules (TREM2, OSCAR and FcRγ) together with transcription factor NFATc1 and osteoclast cell marker cathepsin K (between rows) in PBMC-derived osteoclast-like cells (both exposed to PE particles (filled) and the controls (blank) in all donors studied. All the cell culture shown above were grown with 25ng/mL M-CSF, but differently in term of the doses of RANKL (between columns), which included no RANKL, 10 and 50ng/mL concentration. Statistical difference, $p<0.05$ is represented as * on the graphs.

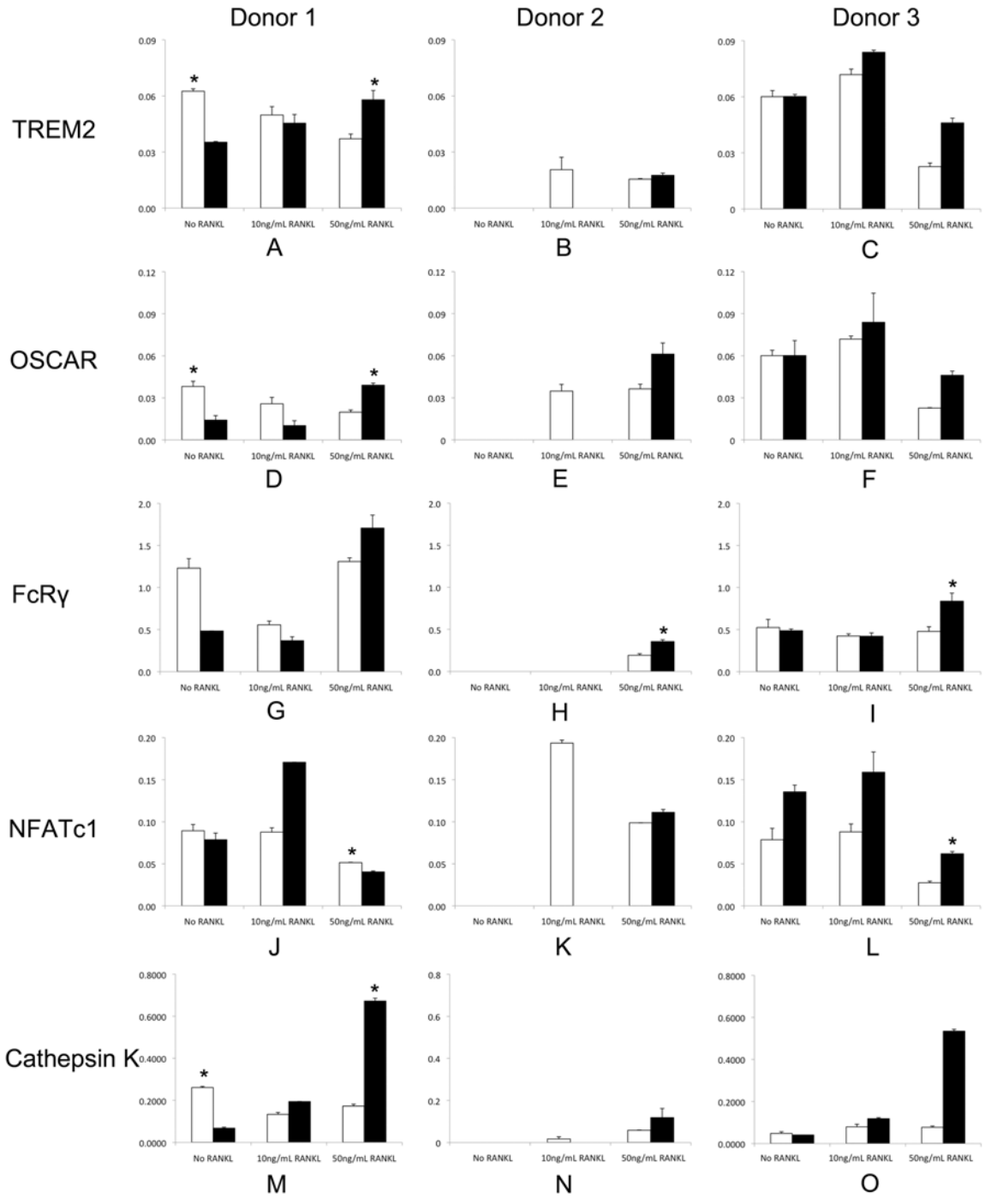

Figure 3.12: Expression level of genes investigated in three donors at 14 days post collagen gel in response to different dose of RANKL

The mRNA levels of each individual donor across the time point are shown in Figure 3.11. In general, there was no consistent trend in mRNA expression between donors, which may be due to variability between donors.

The data of mRNA expression in response to different dose of RANKL was presented in Figure 3.12 below. For this particular pool of data, comparison between sample groups was difficult to low and no RANKL samples groups (below the level of detection), which often seemed with Donor 2. For the other two donors, there was no particular trend on the regulation of mRNA expression in response to PE particles seen in the 50ng/mL RANKL group. Nevertheless, it was quite interesting to note a similar pattern of mRNA expression level of between molecules except NFATc1 (between rows) in each sample group in both Donor 1 and 3.

3.4. Discussions

The work presented in this Chapter 3 was carried out as a functional study to better understand the findings in the previous Chapter 2, which suggested that the expression of osteoclast ITAM-related molecules (TREM2, DAP12, OSCAR and FcRγ) may be involved in PE-induced osteoclastogenesis.

The novel aspect of this study is not just the *in vitro* model setup for osteoclast culture with particles, but also the investigation of osteoclast ITAM-related molecule expression upon stimulation of particles like PE. The rationale of having collagen gel to overcome hydrophobicity of the PE particles solved the problem of low density of PE particles that normally results in particles floating in the culture media (Atkins et al. 2009; Voronov et al. 1998). Collagen was included in the *in vitro* model to take advantage of collagen trapping the hydrophobic and low-densed PE particles and hence promote more contact between those particles and the cells seeded at the bottom of the culture plate wells (Voronov et al. 1998). The collagen gel also allows to better present presence of PE in PO tissues. In the tissues collagen type I is found in abundance in bone microenvironment and in inflammatory membrane (Voronov et al. 1998). This collagen gel model has allowed us to investigate the effect of PE on osteoclastogenesis *in vitro*.

There has been quite a number of earlier studies employing various methods to overcome those limitations of PE properties in aquoes media in order to optimize the interation between PE particles and the seeded cells (Voronov et al. 1998). Voronov and colleagues (1998) introduced PE in their *in vitro* cell culture system by coating the particles onto coverslips, which were then placed at the bottom of wells of cell culture plates. Meanwhile Rao and co-workers (1999) used a different approach by applying inverted cell culture system. However the best way to mimic th*e in vivo* condition *in vitro* could be the collagen model used here and the demonstration that cells were able to phagocytose PE particles is a good indication of this (Voronov et al. 1998). The method used here resulted in harvesting of large numbers of healthy cells containing particles which is consistent with work by Santerre and colleagues (2000), who reported on monocytes exposed to PE had markedly increased the cell life span from 10 days to 30 days (Santerre et al. 2000).

For this novel *in vitro* model of osteoclasts, PBMC–derived osteoclast-like cells were chosen since they are a very well established *in vitro* model for osteoclasts, in which the expression of osteoclast markers have been described earlier (Faust et al. 1999). It is believed that CD14-positive cells are the most likely the precursor cells for osteoclasts (Atkins et al. 2006; Massey & Flanagan 1999; Nicholson et al. 2000; Nose et al. 2009; Shalhoub et al. 2000; Sorensen et al. 2007). However, other study suggested CD11b-positive cells as among those that can serve as osteoclast precursors (Li et al. 2004).

The main focus of this chapter is on the effects of PE on osteoclastogenesis. It is often misunderstood that PE is a less damaging biomaterial. From the literature, it was found that the uptake of PE by macrophage cells did not cause any cytotoxicity to the engulfing cells, but indeed prolonged the survival of the cells (Benz et al. 2001; Santerre et al. 2000; Xing et al. 2002). However, a number of previous studies reported significant increase in the release of proinflammatory cytokines TNFα, IL-1 and IL-8 by macrophages after being challenged with PE particles (Boynton et al. 2000; Rader et al. 1999).

It has been shown that size and shape might influence the biological effects of PE particles (Ren et al. 2003). For instance, in a recent study Pal et al. (2011) had found that the size of PE particles liberated could affect the degree of osteoclast formation (Pal et al. 2011). The study indicated that PE particle of size 0.2μm or smaller induced more osteoclast formation and if

particles of similar size were used in the present study, it might have given more significant differences.

In this study Ceridust 3615 of a size range of within 0.3 to 10μm was used (Matsusaki et al. 2007; von Knoch et al. 2004). Studies using smaller size ranged PE particles will be irrelevant because the particles present in tissues surrounding failed prostheses are mostly in the size range of 0.5-3μm (Hirakawa et al. 1996; Howie 1990; Lee et al. 1992; Shanbhag et al. 1994b), with nearly all PE particles detected in PO tissues are reported to be less than 2μm (Jacobs et al. 2008; Koseki et al. 2005; Shanbhag et al. 1995; Shanbhag et al. 1994b; Visentin et al. 2004). This is further supported by more recent studies (Jacobs et al. 2008; Pal et al. 2011), which indicated that about 90% of PE particles liberated are less than 1μm size. Other studies have confirmed that very small size are outside of the range of optimal biological active size of polyethylene (Green et al. 1998; Matthews et al. 2000). Furthermore more recently Pal et al. (2011) found murine macrophages did not respond to PE particles with size less than 0.2μm. It is important to make sure that the size of the particles introduced was less than 10μm as those are phagocytosed by macrophages (osteoclast precursors) while the larger ones are surrounded by the FBGCs derived from macrophages (Ingham & Fisher 2005; Santerre et al. 2000; Voronov et al. 1998).

Ceridust 3615 has been widely used in other previous studies (Elfick et al. 2004; Green et al. 1998; Guo et al. 2012; Matsusaki et al. 2007). Ceridust 3615 is used for *in vitro* or *in vivo* studies as they are commercially available, cost effective and are of a size comparable to particles isolated from tissues (von Knoch et al. 2004). Regarding the physical properties, Ceridust 3615 has been shown to have a rougher surface, shorter length and more consistent size than PE particles isolated from the tissues (von Knoch et al. 2004). Previous studies have indicated that rough surface PE particles may stimulate stronger pro-inflammatory effect (represented by TNFα and IL-1β expression in murine model) in comparison to a smoother surface particles (Sieving et al. 2003; Yang et al. 2002b). Interestingly Pal et al. (2011) showed that exposure to PE particles with wider range of sizes resulted in higher number of osteoclast-like cells formed compared to exposure to PE particle of a more homogenous size.

500μg/mL was the chosen concentration Ceridust 3615 PE particles to be used in this experiment as it was optimised in a previous study (Matsusaki et al. 2007). This concentration

of PE particles in the *in vitro* system was also been optimised in preliminary work carried out in the laboratory (by Christopher Holding).

RANKL is another important component of the model that should be discussed in this Chapter 3. The hypothesis proposed by Rodan and Martin (1981), and later supported by Udagawa and colleagues (Takahashi et al. 1988; Udagawa et al. 1990) has indicated that the differentiation of BMMs into mature osteoclasts could be achieved *in vitro* in the presence of osteoblasts. Further studies revealed that supplementation of RANKL and M-CSF (Kong et al. 1999; Lacey et al. 1998; Nicholson et al. 2000; Quinn et al. 1998; Yasuda et al. 1998b), which are expressed by osteoblasts, is sufficient to compensate the need for the presence of osteoblasts in driving osteoclastogenesis *in vitro*. Matsuzaki et al. (1998) also described that addition of soluble RANKL, along with M-CSF could compensate absence of stromal cells for formation of osteoclast-like cells *in vitro.*

From previous studies, it appears that RANKL plays an essential role in PE-induced osteolysis as of inhibition of PE-induced osteolysis *in vivo* was achieved following therapy with anti-RANKL (Childs et al. 2002; Tsutsumi et al. 2008). The importance of RANKL in osteoclastogenesis *in vitro* stimulation of ITAM-pathway in the absence of RANKL did not induce osteoclast formation (Koga et al. 2004). RANKL is also important in promoting the survival and motility of osteoclasts (Lacey et al. 1998). It would be a particular interest to study cells derived from PO tissues in this model, as it has been shown that cells derived from PO tissues were able to differentiate into osteoclasts in absence of RANKL (Sabokbar et al. 2003). Nonetheless, considering the large number of sample groups required to fulfil the objectives of the study, the use of human PBMCs became the more realistic option for this study.

In this *in vitro* study, M-CSF was also used as it serves quite a number of important roles for the osteoclast cell culture. Based on previous studies, it has been known that M-CSF, released by osteoblasts, prolongs survival of osteoclasts and induces osteoclastic activity *in vitro* (Fuller et al. 1993; Lagasse & Weissman 1997; Yoshida et al. 1990). It is also needed for the cell spreading, migration and proliferation of the osteoclasts cultured (Faccio et al. 2003c). Supplementation of RANKL alone without M-CSF may not be sufficient to promote osteoclastogenesis *in vitro* (Quinn et al. 1998) as M-CSF is required to stimulate the expression of RANK in monocytes (progenitor for osteoclasts)(Arai et al. 1999). In the

context of PO pathogenesis, it has been found that RANK, possibly induced by M-CSF, is elevated in PO pseudosynovial membranes (Holding et al. 2006; Xu et al. 1997) suggesting the importance of M-CSF in this model. In PO tissues, a study by Neale and colleagues (1999) suggested that M-CSF was able to stimulate osteoclast formation and resorption activity in the tissues (Neale et al. 1999). There is also data indicating that M-CSF is particularly important for cell multinucleation (Faccio et al. 2003c). It is widely accepted that the optimal concentration of RANKL and M-CSF in an *in vitro* model of osteoclast is around 50ng/ml and 25ng/ml respectively. This concentration of RANKL and M-CSF has been used in several numbers of studies involving osteoclast assay (Baumann et al. 2004; Herman et al. 2008), including this study.

Atkins et al. (2009) have shown, in similar 3D collagen gel model, that osteoblast cells exposed to PE particles have striking effect in increasing expression of osteoclastogenic genes including for M-CSF and RANKL. This study here indicates PE elevated mRNA expression of osteoclast ITAM-related molecules, osteoclast formation and resorption activity in the absence of osteoblasts. It might be interesting to investigate if co-culturing osteoblasts-osteoclasts would have an additive effect towards osteoclastogenesis.

Based on the data documented in this chapter (in both sections 3.3.1 and 3.3.2), it was clearly demonstrated that the PE particles (Ceridust 3615) directly increased the formation of multinucleated PBMC-derived osteoclast-like cells and their bone resorption activity. Together this data was in consistent with other previous studies reported (Anderson et al. 2001; Granchi et al. 2004; Holding et al. 2006; Pal et al. 2011) even though the experimental setup was different between studies. It is quite interesting to note that the PE-induced increase in the formation of TRAP-positive osteoclasts seemed to require RANKL (refer to Figure 3.5).

While the study by Anderson et al. (2001) reported increase in both bone resorption activity and TRAP-positive cell formation from macrophages in response to biomaterials including PE, the study used animal systems, which is different from this current work that used human PBMCs. Even though there are close similarities with the work by Pal and co-workers (2011), which have already shown increase in the formation of TRAP-positive cells (viewed in 2 fields of visions at 4X magnification for SQA by two independent observers) upon exposure to PE particle *in vitro*, this study took a more extensive approach looking at the gene

expression of osteoclasts, particularly ITAM-associated molecules to illustrate the role they play in osteoclastogenesis in the context of peri-implant osteolysis. Besides, unlike the study that used PE wear debris isolated from PO tissues, the current presented work instead applied commercially available Ceridust 3615, which is physically more homogenous particles and hence could promisingly yield reproducible data.

In relation to the formation of TRAP-positive cells following stimulation by the PE particles, Pal et al. (2011) reported that even three days post-treatment with RANKL, giant (with size of more than 200μm) TRAP-positive multinucleated cells started to be observed as already being formed in response to the presence of PE particles. This increase in PE-induced osteoclast formation was also reported as becoming greater up to nearly five times after 10 day of cell culture than the control, which is quite in agreement with the data obtained from this study. It is important to note that, in applying TRAP as an osteoclast cell marker, counting the osteoclast-like cells should be only on multinucleated osteoclast-like cells only as TRAP has also been reported as could be expressed in mononuclear cells as well (Humphrey et al. 2004).

It was particularly interesting to note the detection of PE particles within the multinucleated TRAP-positive osteoclast-like cells formed in the cell culture (refer Figure 3.3). Such PE phagocytosis-like phenomenon has also been described earlier in a culture of macrophage cell-line by Matsusaki and colleagues (2007), who used similar PE particles (Ceridust 3615) in their work. While majority believes macrophages that have phagocytosed PE particles play role in promoting more osteoclast formation in the tissues by secreting pro-osteoclastogenic cytokines (Hirashima et al. 2001; Voronov et al. 1998), there is another line of opinion suggesting that those macrophages become the precursor cells for the multinucleated cells formed (Pandey et al. 1996; Sabokbar et al. 1998; Wang et al. 1997a, 1997b) (refer section 2.3.4 above). Indeed, the data of this study, including ones presented in the previous chapter (refer section 2.3.4 and the corresponding discussion 2.4), is in favour of the latter theory.

Another important component of this current study that distinguishes it from the work by Pal et al. (2011) is the addition of dentine pits resorption assay as a mean of looking on the activity of osteoclasts formed on top of total osteoclasts formed in response to exposure to PE particles. Inclusion of dentine pits resorption assay into an osteoclast *in vitro* assay should be regarded as important as it could give more meaningful data that would represent the extent of

bone resorption compared to counting of osteoclasts formed. Besides this assay also provides important information in assessing osteoclast functionality and activity. Indeed, having a mineralised substrate such as pieces of bone or dentine intact to the osteoclasts formed could influence regulation of the activity of the cells. It has been shown that interaction between osteoclast and bone substrate could affect "genetic programming", phenotype and activity of osteoclast formed (McHugh et al. 2000; McHugh et al. 2007; McHugh et al. 2010; Shen et al. 2006), for instance higher expression of osteoclasts cell markers such as TRAP and cathepsin K as this has been shown earlier by Shen and colleagues (2006). It would also be interesting to investigate the expression of other ITAM-related molecules, considering ITAM-related molecules such as OSCAR is believed to be expressed in late stage of osteoclastogenesis (Kim et al. 2002; Kim et al. 2005d).

Based on the data obtained, which clearly showed that the presence of PE particles would result in significant increase in the percentage of resorption pits areas particularly after two weeks post-exposure to PE, it could be concluded that PE has effect in enhancing bone resorption activity (refer to Figure 3.6). Nonetheless, the amount of RANKL appeared to have significant influence on the PE-induced increase in resorption pits. Data on the PE-induced increase in resorption pits in response to different dose of RANKL presented in this chapter was consistent with the earlier study carried out in this lab (Holding et al. 2006), which has demonstrated that 50ng/mL RANKL produced more pits on dentine than 10ng/mL dose. This gives indication that high amount of RANKL along with PE particles in the tissues could enhance the activity of osteoclasts. It is also important to note in Figure 3.9 that there was no (or very minimal numbers of) resorption pit formed on the dentine in the absence of RANKL even at two weeks post exposure to PE, and this may indicate that RANKL is an essential component for the bone resorption activity of the multinucleated cells formed in the model.

As indicated earlier, the investigation on the mRNA expression of ITAM-related molecules, as well as NFATc1 and cathepsin K, in response to PE stimulation becomes the main focus being looked at in this chapter. This focus of the study provide novel discovery as there has been no literature testing on the effect of biomaterials on the regulation of mRNA expression of ITAM –related molecules, either through *in vitro* or *in vivo*, being reported to date.

In comparison to the data from the TRAP staining and dentine pits resorption assay, the analysis on the mRNA expression was more difficult due to inconsistent trend of expression

between donors, as this could be seen from the graphs in the Results 3.3.3 (refer to Figure 3.11). It has been well acknowledged in studies in the past that there were differences in cellular response to foreign-body particles between donors or patients, including in PBMC *in vitro* model (Sethi et al. 2003; Yagil-Kelmer et al. 2004). However as being mentioned earlier in the Results (section 3.3.3 above), yet still there are some similar trends that could be deduced, which included in the observation that PE-induced change in mRNA expression level was in similar fashion between TREM2, OSCAR, FcRγ and cathepsin K (refer to Figure 3.11). This also may suggest that the PE-induced change in mRNA expression of TREM2, OSCAR, FcRγ and cathepsin K was in similar fashion, which would further speculate that the regulation of these molecules mRNA expression could share a common pathway. Such similar regulation pattern of mRNA expression between these molecules may also indicate that the induction of the mRNA expression of these molecules by PE occurred in similar stage during osteoclastogenesis.

While there has been very limited amount of studies investigating on NFATc1 in the context of peri-implant osteolysis, this thesis (Chapter 2 and 3), to the author's knowledge, is the first one to explore on NFATc1 in conjunction to PE-induced osteolysis in particular.

While Yamanaka and coworkers (Yamanaka et al. 2008; Yamanaka et al. 2013) demonstrated that NFATc1 is upregulated in PMMA-induced osteoclastogenesis data from this study was unable to show any marked pattern of regulation (either suppression or induction) by PE particles. Differences in cell types, biomaterial and size of particles tested might provide an explanation for the discrepancies in the response observed (Horowitz & Gonzales 1996). It could also be that high levels of NFATc1 expression during PE-induced osteoclast formation are transient with respect to the stage of osteoclast development.

Unlike the other molecules where their mRNA expression had common pattern of response following PE stimulation, there was no consistent in the change of NFATc1 mRNA expression. Response time for regulation of mRNA expression following PE stimulation or earlier expression could provide an explanation for such observation. Being a master transcriptional factor, change in the mRNA expression of NFATc1 is expected to take place earlier than the other molecules.

As for the ITAM-associated molecules, based on Figure 3.10, it appeared that at both 10 and 12 days post-collagen gel there was lower expression of TREM2 (even though only some were statistically different) in PE-exposed sample groups compared to non-PE ones consistently between donors. Interestingly it seemed that there was turnover in the mRNA expression as there was trend of higher level in PE-containing samples, which might give indication that PE-induced TREM2 mRNA expression took place after 14 days post-collagen gel. For DAP12, there was only data for the mRNA expression at 14 days post-collagen gel in the presence of 50ng/mL RANKL (in Figure 3.10). The real time PCR was not carried out for the other sample groups (earlier time points and samples with less than 50ng/mL RANKL) due to limitation on the amount of cDNA remaining. Nevertheless, at 14 days post-collagen gel, like TREM2, generally there appeared to be increase in the mRNA expression following exposure to PE in the gel. The increase in mRNA expression of TREM2 and DAP12 with higher number of TRAP-positive cells and resorption pits on dentine may suggest that these ITAM-related molecules might mediate those two PE-induced response.

As discussed earlier, findings from previous studies that reported the higher number of multinucleated cells formed in response to exposure to PE particles could possibly be in connection to higher mRNA expression of DAP12 in those samples. Previous studies have suggested that ITAM-related molecules including TREM2 and DAP12 are involved in osteoclastogenesis (Humphrey et al. 2006; Koga et al. 2004; Otero et al. 2012; Paloneva et al. 2003). However, this study on these molecules *in vitro* documented in this thesis is the first reported in the context of PO. Humphrey and colleagues (2006) reported that the addition of both M-CSF and RANKL resulted in increase in the expression of TREM2 accompanied with increase in the formation of osteoclast–like cells from RAW264.7 cells. Their data also suggested that there was a positive correlation between the expression of surface TREM2 and the potency of RAW264.7 cells for differentiation into osteoclasts. There is also evidence indicating that activation on TREM2 appeared to enhance M-CSF-promoted cell migration and spreading (Humphrey et al. 2006), and hence possibly on the resorption activity of the osteoclasts. There have also been studies reporting on increase in TREM2 mRNA expression in *in vitro* model of human PBMC-derived osteoclasts, therefore the data obtained (increasing TREM2 mRNA level throughout the osteoclastogenesis period) was an expected study outcome (Kalliolias et al. 2010; Park-Min et al. 2009). Park-Min and colleagues (2009) through their findings in human PBMC-derived osteoclast culture had suggested that elevation of TREM2 mRNA expression could take place quite quickly after addition of

RANKL. Inhibition on osteoclastogenesis in human CD14-positive PBMC also showed decreased mRNA expression of TREM2, besides NFATc1, and this may suggest that increase in TREM2 mRNA expression is necessary during osteoclastogenesis.

There have been debates as whether lack of DAP12 could lead to arrest in osteoclast formation or not. DAP12 has been shown to be upregulated by PU.1 transcription factor, a crucial transcription factor in osteoclast differentiation (Henkel et al. 2002). Humphrey and coworkers (2004) reported that deletion in DAP12 as not affecting expression of known osteoclast cell markers like TRAP, CTR and cathepsin K, suggesting there is no direct connection between DAP12 and the expression of those cell markers. Nonetheless through this study it was shown that increase in cathepsin K mRNA expression, alongside with great increase in osteoclast formation (from TRAP staining) at 14 days post-exposure to PE, is accompanied with a trend of higher level of DAP12 mRNA expression, therefore this could still leave room of connection between DAP12 and osteoclastogenesis (and expression of osteoclast cell markers) open.

There has been evidence suggesting that DAP12 deficiency could arrest osteoclastogenesis induced by RANKL and M-CSF (Koga et al. 2004; Mocsai et al. 2004). Data from Humphrey et al. (2004) suggested that DAP12 plays important in osteoclast multinucleation during differentiation process. It was reported that pre-osteoclasts from DAP12-deficient mice were unable to multinucleate *in vitro*, nonetheless they still possessed dentine-resorptive capacity even though at a reduced extend. Together this data suggests the trend of increase of DAP12 mRNA expression in response to exposure to PE particles is more relevant to the increased formation of multinucleated cells than the resorption activity. Humphrey et al. (2004) indicated that the activation of DAP12 was also able to increase the size of the multinucleated cells formed. However, such increase in multinucleated osteoclast formation and size appeared to require RANKL since Humphrey et al. (2004) reported that there was no multinucleated TRAP-positive cells formed following DAP12 activation in absence of RANKL.

Meanwhile, there is also evidence suggesting that DAP12 is more associated to the regulation of osteoclast bone resorbing activity than in the osteoclast formation itself. For instance, Kaifu et al. (2003) reported reduced resorption activity in DAP12-deficient cells, yet those cells were still able to multinucleate even though at much lesser extend. Zou et al. (2010)

found that osteoclastogenesis still occured in presence of M-CSF and RANKL despite the lack of DAP12, nevertheless there was defect in cytoskeletal organization that is important in ruffled border and actin ring formation, which subsequently affect osteoclast bone-resorbing capacity (Boissy et al. 2002; Faccio et al. 2003c; Humphrey et al. 2004; Paloneva et al. 2003; Teitelbaum 2007; Zou et al. 2010), therefore the increase in the DAP12 mRNA expression in PE-containing samples could also be associated with the higher area of dentine pits. DAP12 is also believed to mediate the β3 (interaction with bone substrate)-induced osteoclast cytoskeletal organization (Faccio et al. 2003b; McHugh et al. 2000).

For OSCAR and FcRγ, as what could be seen from Figure 3.10 and Figure 3.11, there was much more consistent trend of mRNA expression between between all donors across the three time points. At 10 days post-exposure to PE particles, the mRNA expression of the samples was generally lower than their corresponding controls (without PE). However, on the following time point (12 days post-collagen gel), there appeared to be a turnover in the mRNA expression and by 14 days post-PE stimulation, the mRNA expression of OSCAR and FcRγ in all donors appeared to be generally higher than the controls (even though not all statistically different). Like for TREM2 and DAP12, those higher trends of mRNA level of OSCAR and FcRγ at 14 days post-exposure to PE compared to the controls could be connected to the high PE-induced increase in the formation of TRAP-positive multinucleated osteoclast-like cells and total area of resorption pits.

To date, there has been very minimal information on the influence of OSCAR and FcRγ towards the bone resorbing activity of the osteoclasts. Another interesting data regarding the significance of OSCAR/ FcRγ in osteoclast function would be from Zou et al. (2010), which indicated that activation of OSCAR/FcRγ in osteoclasts grown on bone could partially compensate for the defect in cytoskeletal reorganisation in DAP12-deficient cells. In a previous study carried out in this laboratory, it was found that reduction in the resorption activity of PBMC-derived osteoclasts following inhibition by drugs *in vitro* was also accompanied by lower expression of OSCAR mRNA at a late time point (Cantley et al. 2011). These studies may suggest that obtaining study outcomes of higher in both total area of resorption pits (Figure 3.8) and mRNA level for OSCAR and FcRγ in PE-exposed cells at 14 days post-collagen gel (Figure 3.10) was not a coincidence, indeed may lead to hint of a possible connection.

Based on literature, the expression of OSCAR is seen to be more associated with further differentiation of monocyte/macrophage cell lineage in the development of dendritic cells (Merck et al. 2005; Merck et al. 2004) as well as osteoclastogenesis (Herman et al. 2008) in human. For instance, there has been a study reported that human PBMC-derived osteoclasts formed following LPS-induced osteoclastogenesis as expressing OSCAR and other osteoclast markers, however unable to produce resorption lacunar (Mabilleau et al. 2011), suggesting that the expression of OSCAR has more connection to the capability of forming the bone resorbing cells.

There are more studies that may provide better link between higher OSCAR and FcRγ expression (Figure 3.10) and more osteoclast formation *in vitro* (Figure 3.4). For example, on its discovery, it was found that inhibition of OSCAR by the addition of soluble form of OSCAR in a co-culture with osteoblasts reduced the formation of osteoclasts (Kim et al. 2002). Recently, Barrow et al. (2011) demonstrated that interaction between OSCAR and the ligand (collagen) enhanced osteoclastogenesis *in vitro* and blocking on the interaction would inhibit such enhancement in osteoclastogenesis. Barrow et al. (2011) also discovered reduction in the mRNA expression of NFATc1 and osteoclast markers like TRAP, cathepsin K and calcitonin receptor in mouse BMMs lacking either OSCAR or FcRγ. In DAP12-deficient cells (in the context of PLOSL), OSCAR and FcRγ has significance in the osteoclastogenesis, and this could be seen from the activation of these molecules, which was reported as able to partially increase the size of osteoclasts formed (Zou et al. 2010).

In contrast to the majority studies, Ishikawa et al. (2004) suggested FcRγ is not really crucial in osteoclast formation following their observation of no difference in the number of TRAP-positive cells between FcRγ-deficient (with low level of OSCAR detected) and the wild type cells. Nonetheless, there is possibiliity of the absence of FcRγ being compensated by another pathway in mediating the osteoclastogenesis.

In relation to the point in which OSCAR being nominated as a good candidate for osteoclast cell marker, it is quite interesting to note that the induction of the mRNA expression of cathepsin K by PE exposure in Donor 2 was very small, particularly at 14 days post PE exposure in comparison to the other two donors (refer Figure 3.11), where as the data from TRAP staining and dentine resorption pits assay indicated the increase in the number of osteoclast-like cells formed (refer Figure 3.4) and the functionality of osteoclast formed

resulted from 3-day exposure to PE (refer Figure 3.8) was huge. When comparing the data on mRNA expression against TRAP staining and resorption assay, it seemed that OSCAR mRNA expression was more consistent with the trend of data from those two assays as compared to cathepsin K (refer Figure 3.11). This may put on another credit for considering OSCAR as a good osteoclast cell marker (see section 2.4).

In corresponding to the investigation on whether the PE-induced elevation in OSCAR and FcRγ mRNA expression is RANKL-dependent or not, it appeared that such of increase could be consistently (between donors) observed only at the dose 50ng/mL RANKL (as partly discussed in this section). Herman et al. (2008) suggested these two pathways are independent of each other (M-CSF and RANKL did not increase OSCAR mRNA expression), however indeed, they together contribute towards osteoclastogenesis process in an additive manner.

However, there are some supports from other earlier studies that these intracellular pathways may be interconnected, even though to date there is quite limited amount of information directly on the relationship between OSCAR/FcRγ and RANKL-mediated pathway. There are quite numbers of studies which have shown RANKL to mediate increase in mRNA expression. For instance, the study by Kim et al. (2005d) reported increase in OSCAR mRNA expression up to 1200-fold following 3 days-growth of murine BMMs in the presence of RANKL and M-CSF. Indeed, OSCAR was discovered following its marked increase in expression post RANKL stimulation (Kim et al. 2002). The RANKL-mediated increase in OSCAR mRNA expression was reported to be upregulated by USFs (Kim et al. 2008) but negatively regulated by MafB (Kim et al. 2007a) and PIAS3 (Kim et al. 2007b) (refer Table 1.5).

As it applies to TREM2 and DAP12 (see Discussions 3.4 earlier) there was issue of very low gene transcription for OSCAR, FcRγ, NFATc1 and cathepsin K (some below detection limit) in some sample groups with low and no RANKL, particularly in Donor 2 (refer Figure 3.12). There are questions as whether cells for those groups actually viable at 14 days post collagen gel as they might required RANKL to allow them to differentiate, which then allow them to survive at that time point. In this *in vitro* model, in the presence of M-CSF but without RANKL, the cells will keep proliferating as monocytes without really going committed into osteoclast lineage. As a result of that, considering the time point of the extraction of cell

culture (13, 15 and 17 days) and short lifespan of monocytes/macrophages than osteoclasts, the cells were probably already undergoing apoptosis.

3.5. Conclusions

In summary, through this 3D collagen gel model all PE-induced stimulation for osteoclast-like formation (based on TRAP staining), bone resorbing activity (as indicated from dentine pits resorption assay) and more importantly mRNA expression of ITAM-related molecules and cathepsin K generally took place at 14 days after being exposed to the biomaterial. With all the data indicating the increase in response to PE occurring at 14 days post-collagen gels documented in this chapter, supported by higher expression of those molecules in peri-implant tissues as discussed earlier in Chapter 2, together they may also suggest that the investigated ITAM-related molecules could be involved in mediating the PE-induced increase in osteoclastogenesis and bone resorption.

Therefore, in addition to the improvements on the implant design, therapies targeting osteoclastogenic molecules like osteoclast ITAM-osteoclast associated molecules, which may be involved in the regulation peri-implant osteolysis as described here, could provide an excellent approach of treatment for the disease. There have been examples of studies in the past that showed targeting osteoclastogenic molecules, such as RANK/RANKL/OPG axis (Childs et al. 2002; Goater et al. 2002; Ulrich-Vinther et al. 2002; von Knoch et al. 2005; Yang et al. 2002a) could offer promising therapy potential in the treatment of peri-implant osteolysis.

Despite the great advantages in this novel *in vitro* model of osteoclasts, there are unavoidable limitations noticed throughout the study. In order to keep supplementing the cells with fresh media to avoid depletion of cell growth and differentiation factors over the long period of culture (up to 17 days), the collagen gel has to be digested to allow for media change, leaving the exposure of the cells to PE particles lasted for 3 days only. Improvement on the model in the future should consider for possible options of longer particles-containing collagen gel.

Another aspect that could be improved for future studies would be on the effort to have a better *in vitro* model of osteoclastogenesis mimicking the *in vivo* microenvironment. For

example, it is believed that osteoclastogenesis occurs in close proximity to bone microenvironment, which could be explained by the feedback provided from the interaction between β3 integrin and the substrate (McHugh et al. 2000; McHugh et al. 2007; McHugh et al. 2010; Shen et al. 2006). This aspect should be highly taken into account, particularly in the investigation on molecules like late stage osteoclast markers like cathepsin K (Shen et al. 2006) and ITAM-related molecules as there have been studies suggesting the significance of ITAM-associated molecules on such interaction between the cells and bone or mineralised substrates. For instance, β3-induced osteoclast cytoskeletal organization (McHugh et al. 2000) (following the interaction with the bone substrate) is believed to be mediated by DAP12 (Faccio et al. 2003a; Faccio et al. 2003c). Therefore, an example of improvement that could be done on the model would be inclusion of bone or other similar mineralized substrate for all sample groups of the cell culture including for the TRAP staining and RNA extraction.

The *in vitro* model wil be also better if it is not just a homogenous culture of osteoclasts only, but instead a co-culture of osteoblast and osteoclasts, which will better reflect BMU in bone microenvironment in *in vitro* model. Pal et al. (2011) found that non-significant inhibition on osteoblast proliferation following exposure to PE become significant in context of co-culture of osteoblast-osteoclast. In addition, there appeared to be a close interaction between osteoclast and osteoblast following exposure to PE particles as it has been observed that, in co-culture of those two cells, osteoclasts had been observed to be present around osteoblasts (Pal et al. 2011).

A possible future work that could complement and support the conclusion made from the outcome of this study would be investigation on the effect of OSCAR and TREM2 over-expression or activation on PE-induced osteoclastogenesis. Other possible future studies should also look on the combination of materials as combination of biomaterials may act synergistically in amplifying cellular response, cytokine release and osteclast formation and activity since oftenly there are more than one type of biomaterials found in PO tissues, like metals beside PE as documented in Chapter 2 here. While this study has used homogenous commercially available Ceridust 3615 PE particles, it also leaves a window for other interesting potential future studies to use PE particles isolated from PO tissues. It has been reported that PE particles retrieved from PO tissues could results in greater cell response or stimulation in comparison to Ceridust 3615 PE particles (Matsusaki et al. 2007). It would also be a particular interest to study cells derived from PO tissues in this model, which is more

appropriate to the context of PO as it has been shown that those cells derived from PO tissues were able to get differentiated into osteoclasts in absence of RANKL (Sabokbar et al. 2003).

4. DETECTION OF NFATc1 AND OSTEOCLAST ITAM-RELATED MOLECULES IN HUMAN RHEUMATOID ARTHRITIS

Some of the data presented in this chapter has been published in the following journal article:

Crotti TN, Dharmapatni ASSK, **Alias E**, Zannettino ACW, Smith MD, Haynes DR. The immunoreceptor tyrosine-based activation motif (ITAM)-related factors are increased in synovial tissue and vasculature of rheumatoid arthritic joints. Arthritis Research and Therapy 2012; 14:R245

4.1. Introduction

RA is a systemic autoimmune disease with predominant clinical manifestation in the joint. The etiology of rheumatoid arthritis is not exactly identified yet, however it is believed to be due to combinations of factors such as an autoimmune response of the body, genetics and environmental and biological triggers like infection and hormonal change (Firestein 2009; Jefferies 1998; McInnes & Schett 2011).

With synovium becoming the primary target organ for therapy in RA (Vieira-Sousa et al. 2011), studies of the synovial tissues are considered to be important in gaining better understanding of RA. The synovial tissues in RA have a massive infiltration of inflammatory cells thought responsible for many of the pathologic features of RA joints (Kraan et al. 1999; Tak et al. 1997). With regard to soluble factors in the development of joint pathology in RA, the synovial fluid is thought to provide better indication of the disease process rather than serum that reflect the systemic condition of the subjects (Lettesjo et al. 1998).

It is interesting to note that there is a number of similar pathologic features or changes seen in OA and RA synovial tissues. Such examples include increase in number of blood vessels formed (angiogenesis) and thickening in the lining layer due to infiltration of inflammatory cells into tissues. Therefore, given these similarities between RA and OA, it would be of interest to study the difference at the molecular level that may explain the difference in the disease outcome. This thesis studies the regulation of ITAM-associated molecules in the context of pathology and this becomes one of the main objectives in this Chapter 4. How RA and OA tissues differ from the normal tissues in regard to expression of those molecules will give us a further insight into the pathology of both diseases.

In regard to bone loss in RA (refer 1.2.2), previous literature has indicated that the imbalance between bone formation and resorption in rheumatoid arthritis leads to the bone loss (Goldring 2003; Gravallese et al. 2000; Redlich et al. 2002b). The role of osteoclasts in mediating the bone loss in the disease has long been elucidated (Gravallese et al. 1998) (refer 1.3.3). With mature osteoclasts present only close to bone surface of joint in RA patients (Gravallese et al. 1998) and the ligand of OSCAR has recently been identified collagen

(Barrow et al. 2011), there is possibility that OSCAR may play significant role towards the late stage of osteoclast formation in RA. This is supported by work carried out by Herman and colleagues (2008), who also demonstrated high expression of OSCAR in osteoclast-like cells close to bone microenvironment, where abundance of fibrillar collagen is expressed both in the soft tissues and on the bone surfaces (Chow & Chambers 1992; Miller et al. 1980). This concept is supported by Barrow et al. (2011), who also reported the interaction between mononucleated osteoclast-expressing OSCAR and collagen on bone surface using immunohistochemistry. Herman et al. (2008) also found higher expression of OSCAR in the mononuclear cells from RA patients than in controls and associated high expression of OSCAR with more potent precursor osteoclast cells.

While the expression of OSCAR has been shown to be higher in RA tissues and lower in serum compared to the normal (Herman et al. 2008), further more extensive studies should also be carried out to gain better understanding on the role of the immunoreceptor in the disease. As indicated in Chapter 1, aside from OSCAR, there has been no study reporting about any other osteoclast ITAM-associated molecules (TREM2, DAP12 and FcRγ) in association to bone loss in RA. However in the wider context of RA particularly on aspect of inflammation, DAP12 is acknowledged as component mediating signal for positive regulation for cytotoxic activity in NK cells (Snyder et al. 2003) and T-cell activation (Snyder et al. 2004).

Unlike osteoclast ITAM-related molecules, there are many studies investigating NFATc1 in relation to RA. For examples, there is a number of studies reporting the reduction of arthritic signs observed in animal arthritis models following treatment with a range of compounds was also accompanied with decrease in expression of NFATc1 (Hsu et al. 2011; Kubota et al. 2007; Li et al. 2012). However, even though disease progression of RA appears to be strongly associated with the regulation of NFATc1 (Miyazaki et al. 2007; Urushibara et al. 2004), until to date there has been no study reporting on the expression of NFATc1 in RA tissues (Sitara & Aliprantis 2010).

RA is a much more complex disease that provide a wider scope to look at the role of inflammation and the immune system in bone disease. The complexity of RA pathogenesis should be seen from wider scope than just bone loss as it is a systemic autoimmune disease. Cells such as endothelial cells, are important in regulating cell infiltration into the tissues and

immune cells, including T-cells, known to regulate both inflammatory cells and osteoclasts, are vital to the complex pathology of RA. Therefore, the experimental design for tissue analysis aims to take these features into account and investigate not just RA and OA tissues but also include the study of normal and inactive RA synovial tissues (see 4.2.1 below). In addition, the levels of OSCAR in the synovial fluid will also be analysed for the first time.

4.1.1. Hypothesis

NFATc1 and osteoclast ITAM-related molecules are highly expressed in human RA tissues and associated with the progression of the disease.

4.1.2. Aims

- To compare protein expression of NFATc1 and osteoclast ITAM-related molecules in tissues from active RA, inactive RA, OA and normal
- To investigate the level of soluble OSCAR in the synovial fluids of RA and OA patients

4.2. Methods

4.2.1. Subjects

Synovial tissue samples for immunostaining were collected from patients undergoing knee arthroscopy or total knee replacement (for OA group) at the Rheumatology unit in the Repatriation General Hospital, Daw Park, South Australia. Informed consent was obtained from the patients in prior to the procedures and the ethical approval was given by the Repatriation General Hospital Human Ethics Committee.

All patients diagnosed with RA fulfilled the ACR criteria for RA (Arnett et al. 1988), while for OA patients, the criteria is based on the one outlined by Altman and colleagues (1986). Patients diagnosed with active RA exhibited presence of active joint inflammation. Patients classified as inactive RA were in remission after successful treatment for active RA with

disease modifying antirheumatic drug (DMARD) and were undergoing routine follow-up knee joint arthroscopy. Normal tissues were obtained from patients at sports medicine clinics with unexplained knee pain (Smith et al. 2003). There was no sign of pathology on both cartilage and synovial membrane at the time of arthroscopy carried out in this group of patients.

Table 4.1: **Summary of the demographic information of patients where the synovial tissues obtained from for tissue immunostaining**

Groups	Active RA	Inactive RA	OA	Normal
Age (years)	62.5 ± 19.28	72.33 ± 7.07	69.22 ± 7.98	36.3 ± 10.39
Gender (Male/ Total)	2/10	6/9	6/9	6/10
CRP (IU/ml)	83.90± 83.78	9.78 ± 8.21	NA	NA
RF (mg/L)	19.40 ± 44.10	1.44 ± 0.53	<20	NA
Erosion Positivity	2	2	0	0
DMARDS	NSAIDs 9 Predinos 1	SSZ 1 im Gold 5 MTX 2 Plaqueni 1	No NSAIDs 5 Panadein 1 NSAIDs 3	None 9 Allopurinol 1

CRP = C-reactive protein; DMARD = disease modifying antirheumatic drug; IM Gold = intramuscular sodium aurothiomalate; MTX = methotrexate; NSAIDs = nonsteroidal anti-inflammatory drugs; Predinos = prednisolone; RF = rheumatoid factor; SSZ = sulphasalazine; NA = not applicable

A total of 38 paraffin-embedded synovial tissues were included in this study and the clinical and demographic features are summarised in Table 4.1. The information on the duration of the diseases was not included into the table as it was not regarded as significant since

previous study has shown that it does not give significant differences in the histology of RA tissues (Baeten et al. 2000; Tak et al. 1997).

Due to the volume of synovial fluid required for the enzyme-linked immunosorbent assay (ELISA) only a total 20 samples of synovial fluids were included in the study. There were only two disease groups of synovial fluids used for the OSCAR ELISA, active RA and OA (Table 4.2). All synovial fluids were also obtained from the Rheumatology unit in the Repatriation General Hospital, Daw Park, South Australia.

Table 4.2: Demographic details of active RA and OA patients where synovial fluids were used in OSCAR ELISA.

Patient	Gender (M/F)	Age (years)	CRP (mg/mL)	RF (IU/mL)	CCP (EU)	Treatment	Disease Duration
RA1	F	36	72	129	>100	CsA + Prednisolone	12 years
RA2	F	51	146	20	ND	Prednisolone	12 years
RA3	F	54	77	41	47	Plaquenil + Prednisolone	1 year
RA4	M	63	16	100	ND	NSAID	38 years
RA5	F	66	202	20	2	MTX	1 year
RA6	M	67	74	<20	<2	MTX	15 years
RA7	M	71	3	140	32	IM Gold + MTX	2 years
RA8	M	71	85	1390	59	MTX	3 months
RA9	F	79	14	20	ND	MTX + Prednisolone	5 years
RA10	M	80	44	56	ND	IM Gold	3 years
RA11	M	81	43	20	ND	MTX	3 months
RA12	M	82	33	20	ND	MTX + Prednisolone	Unrecorded
0A1	F	86					
0A2	F	59					
0A3	M	59					
0A4	M	61					
0A5	M	65					
0A6	F	71					
0A7	F	73					
0A8	F	85					

M = male; F = female; CRP = C-reactive protein; RF = rheumatoid factor; CCP = cyclic citrullinated peptide; CsA = cyclosporine A; IM Gold = intramuscular sodium aurothiomalate; MTX = methotrexate; NSAIDs = non-steroidal anti-inflammatory drugs; ND = not done

Collectively, there was little difference in the mean age between active RA and OA (66.75 and 69.88 years old respectively). For the RA groups, the patients had mean disease duration of 8.14 years (wide range between 3 months to 38 years), mean serum CRP level of 67.46gm/mL and RF of 177.82IU. Since the anti-CCP measurement is a recent parameter used in diagnosing for RA (Aletaha et al. 2010; Jung et al. 2012), not all patients in the study had anti-CCP levels measured.

4.2.2. Immunohistochemistry

All the immunohistochemical methods used in this chapter were similar to that described in the previous Chapter 2 (refer 2.2.2). Briefly, 5µm thick tissue sections were mounted on APTS-coated glass slides. Tissue sections were deparafinised using two changes of histolene followed by two changes of 95 to 100% alcohol, then washing with MiliQ water. The dewaxed tissue sections were pre-treated with either sodium citrate pH 6.0 or ethylenediamine-tetraacetic acid (EDTA) pH 9.0 (refer Table 4.3) at 90-95˚C for 20 minutes as procedure for antigen retrieval before subsequent immunohistochemistry performed. Tissue slides were immersed in PBS as washing protocol between the immunohistochemical steps. To block unspecific staining due to endogenous peroxidase activity, tissues were treated with 0.1% sodium azide and 1% hydrogen peroxide in PBS buffer. Three-step peroxidase-based immunostaining technique, as described in Haynes et al. (2003), was employed. Counter staining using Harris haemotoxylin (Scharlau Chemie, Sentmenat, Spain) and saturated lithium carbonate were applied on tissues before mounted in Gurr Aquamount (BDH, Poole, UK). The staining colour was allowed to develop for 20 minutes using AEC (K3469, Dako, CA, USA).

Staining for all groups of tissue samples was performed at the same time for each antibody to avoid any differences between groups due variability in the immunohistochemistry procedure. Negative controls used were isotype-matched antibody controls for monoclonal antibodies (mouse IgG_{1kappa} for mouse IgG_1) and equivalent antibody-raised serum for polyclonal antibodies (either normal rabbit or goat serum).

4.2.2.1. Antibodies and Reagents

All antibodies used for tissue immnostaining work have been described in Chapter 2 (presented in Table 2.3 on page 78). The optimal working concentrations were determined in prior to the tissue immunostaining. Three primary antibodies purchased from the Santa Cruz Biotechnology Inc. (CA, USA) were rabbit polyclonal anti-DAP12 (sc-20783, used at 2µg/mL), goat polyclonal anti-OSCAR (sc-34233, used at 10 µg/mL) and mouse monoclonal IgG_1 anti-NFATc1 (sc-7294 used at 4µg/mL).

TREM2 was detected using a rabbit polyclonal antibody (HPA012571, Sigma Life Sciences) at a working concentration 0.8μg/mL. The antibody used to detect FcRγ was a rabbit polyclonal (LS-B2169, Lifespan Bioscience, chosen from personal communication) was used at 1.25μg/mL. A mouse monoclonal IgG_1 anti-cathepsin K (MAB3324, Milipore) was used at a working concentration of 2μg/mL to detect any presence of osteoclasts.

There were four different polyclonal HRP-conjugated immunoglobulins used as either secondary or tertiary appropriate antibodies (Table 4.3). Swine anti-goat immunoglobulins (ACI3404), used at working concentration of 7 μg/mL, was purchased from Invitrogen Life Technology (CA, USA). The other antibodies were purchased from Dako Cytomation (Glostrup, Denmark); goat anti-rabbit immunoglobulins (P0448), goat anti-mouse immunoglobulins (P0447) and rabbit anti-swine immunoglobulins (P0164) were used at working concentrations of 3, 10 and 13 μg/mL respectively.

Table 4.3: List of antibodies and antigen retrieval buffer used for paraffin-embedded tissue immunostaining

Primary antibody	Secondary antibody	Tertiary antibody	Antigen Retrieval Buffer
Anti-NFATc1	Goat anti-mouse	Swine anti-goat	EDTA
Anti-OSCAR	Swine anti-goat	Rabbit anti-swine	Sodium citrate
Anti-FcRγ	Goat anti-rabbit	Swine anti-goat	Sodium citrate
Anti-TREM2	Goat anti-rabbit	Swine anti-goat	EDTA
Anti-DAP12	Goat anti-rabbit	Swine anti-goat	Sodium citrate
Anti-cathepsin K	Goat anti-mouse	Swine anti-goat	EDTA

EDTA- ethylenediaminetetraacetic acid

4.2.2.2. Scoring of tissue immunostaining

For consistency, the SQA scoring system for the measurement of immunohistochemistry results was performed by two “blinded” observers (EA and AASSK for NFATc1 immunostaining and EA and TNC for the immunostaining of other molecules) using a 5-scale score ranging from 0 to 4 described previously in Chapter 2 (refer 2.2.2.3) (exception for NFATc1 immunostaining). To aid the semiquantitative quantitation as well as for the purpose

consistency in the view field chosen, the SQA were carried on the scanned image of the tissues captured using the Nanozoomer Digital Pathology (Hamamatsu, Shizouka, Japan) technology.

To assess the amount of immunostaining of the ITAM-associated molecules and cathepsin K (in $2mm^2$ view area on tissues as well, counted as the percentage of positive stained cells), a score of 0 represented 0 to 5% positive stained cells, 6 to 10% of positive cells were indicated by a score of 1, 2 for 11 to 25% positive cells, 3 indicated positive cells between 26 and 50% of total cell population viewed and finally, a score of 4 indicated more than 50% of positive cells (adapted from Tak et al. (1995)).

Different SQA scoring method was placed on the NFATc1 immunostaining, in which the number of positive stained cell aggregations (defined as more than 25 cells) was used as the parameter for grading (adapted from Kraan et al. (1999)). In a viewed $2mm^2$ area, a score of 0 represented no positive stained cell aggregation, score of 1 indicated 1 to 3 positive cell aggregations, score of 2 indicated total positive cell aggregation between 4 to 6, 7 to 9 positive cell aggregations gave score of 3 and score of 4 indicated more than 9 positive cell aggregations presented.

4.2.3. ELISA of OSCAR in Synovial Fluids from RA and OA Patients

The patient details of the synovial fluid used in this study (for both RA and OA groups) are as displayed in Table 4.2 above. The level of soluble OSCAR in the synovial fluid was measured using the ELISA Kit for human OSCAR purchased from USCN Life Science Incorporation (Wuhan, China). The detection limit for the OSCAR ELISA kit assay was 0.108ng/mL.

Prior to assay all samples of synovial fluid were clarified through centrifugation at 13 000 rpm at 4°C for five minutes. Generally the supernatants of the samples were pre-diluted 1 in 5 using the sample diluent from the ELISA kit. This pre-dilution of the sample was performed as a measure to avoid any outcome of obtaining readings beyond the concentration range of the provided protein standards (0 to 20ng/mL). Sample pre-dilution was also carried out for the purpose of reducing the thickness of the synovial fluids, which may affect the accuracy of pipetting during the sample loading for the ELISA.

The OSCAR ELISA was performed according to the manufacturer's instructions. Briefly, a volume of 100µL of the pre-diluted synovial fluids was loaded into each well along with the standards and then left incubated for two hours. This was then followed by one-hour incubations with the provided reagent A and 30 minutes with reagent B, with washing steps done in between those two procedures and afterwards. All incubation took place at 37°C. Finally, 90µL volume of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution in the kit was added to each well and the colour development (from blue to yellow) was monitored every five minutes. Stop solution (containing sulphuric acid) was added after 30 minutes. The absorbance reading at the 450nm wavelength was measured using an ELISA plate reader. The levels of soluble OSCAR in the synovial fluids were determined based on a standard curve generated.

4.2.4. Statistical Analysis

Statistical analysis was performed using SPSS version 11.5 (Chicago, IL, USA). Kruskal Wallis analysis was performed to compare the mean of the SQA scores between groups. Comparison was made between each two groups using Mann Whitney analysis. The significant difference and a *p*-value< 0.05 was accepted as statistically significant.

For the data from the OSCAR ELISA work, the level of soluble OSCAR in the synovial fluid was compared between the two groups (RA and OA) through Student *t*-test using GraphPad Prism version 5.0 (GraphPad Software Inc. La Jolla, SA, USA). A *p*-value <0.05 was considered to be significantly different.

4.3. Results

Unlike in Chapter 2, there was no histological assessment carried out on every tissue in the collection due to limited amount sections available. The limited number of sections for every tissue samples also became the ground for not having negative control for each immunostaining procedure.

4.3.1. Immunostaining of cathepsin K

Similar to the data presented in Chapter 2, immunostaining of cathepsin K was performed to identify osteoclast-like cells on the tissues. Surprisingly the data indicated that the highest mean SQA score for the immunostaining of cathepsin K was obtained for the normal tissue group, followed by OA and RA tissues on the lowest score. The mean SQA score for each tissue group is presented in Table 4.4 below.

Table 4.4: SQA scores of cathepsin K immunostaining in human active, inactive RA, OA and normal tissues

Groups	n	Mean Score	(±SEM)	$p<0.05$
Active RA	10	0.60	0.22	■
Inactive RA	8	1.12	0.35	
OA	9	1.33	0.41	
Normal	9	1.89	0.35	◆

• if significantly different with the active RA group ($p<0.05$)

❖ if significantly different with the inactive RA group ($p<0.05$)

⊙ if significantly different with the OA group ($p<0.05$)

■ if significantly different with the normal group ($p<0.05$)

There was little positive staining in active RA tissues. Indeed about half of the RA samples showed minimal cathepsin K immunostaining. In the active RA group, the majority of the positive cathepsin K immunostained cells were mononucleated, with only very occasionally multinucleated cells could be found (on two tissues only, not shown in picture). Staining was also associated with blood vessels (Figure 4.2A) and a substantial amount of positively stained cells were observed in the lining layer of the tissues (Figure 4.1A). Staining was also detected on synoviocytes with fibroblast-like morphology occasionally (Figure 4.2A).

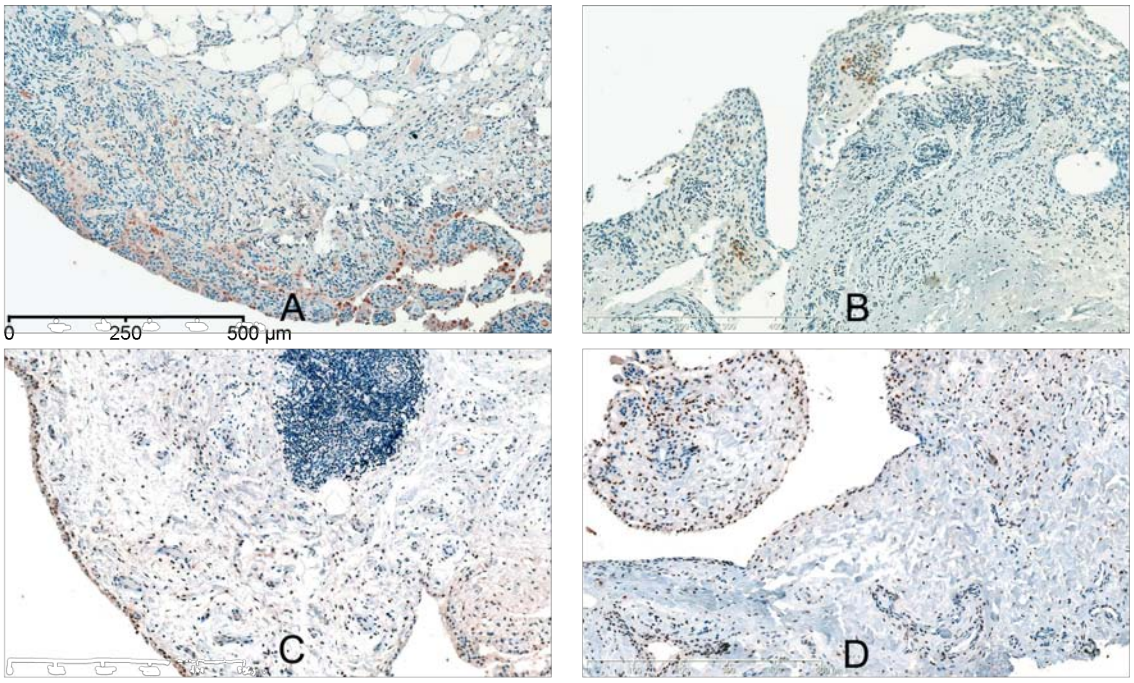

Figure 4.1: Cathepsin K expression in human RA, OA and normal tissues

Representative pictures of cathepsin K immunostaining in active (**A**) and inactive RA tissues (**B**) as well as OA (**C**) and normal (**D**) tissues viewed at a 100X magnification. Positive immunostaining was indicated by the red dye and tissue sections were counterstained with H&E (blue dye).

In the inactive RA and OA tissue groups, positive cathepsin K immunostaining was detected particularly in lining cells and blood vessels. In these two tissue groups, positive cathepsin K immunostaining was also occasionally detected in fibroblast-like synoviocytes (refer Figure 4.2B-C).

Surprisingly, the normal tissues exhibited a moderate amount of positve cathepsin K expressing cells (see Table 4.4). A big portion of the immunostained cells appeared to have fibroblast-like morphology (Figure 4.2D, see Discussion below).

Positive cathepsin K immunostaining in multinucleated cells was rarely detected in any of these tissue groups. Indeed, there were some tissues clean from any positive cathepsin K immunostaining at all. Those cells in aggregations, appeared to have lymphocyte-like morphology (refer to the following section 4.3.2 below), were not positive for cathepsin K immunostaining.

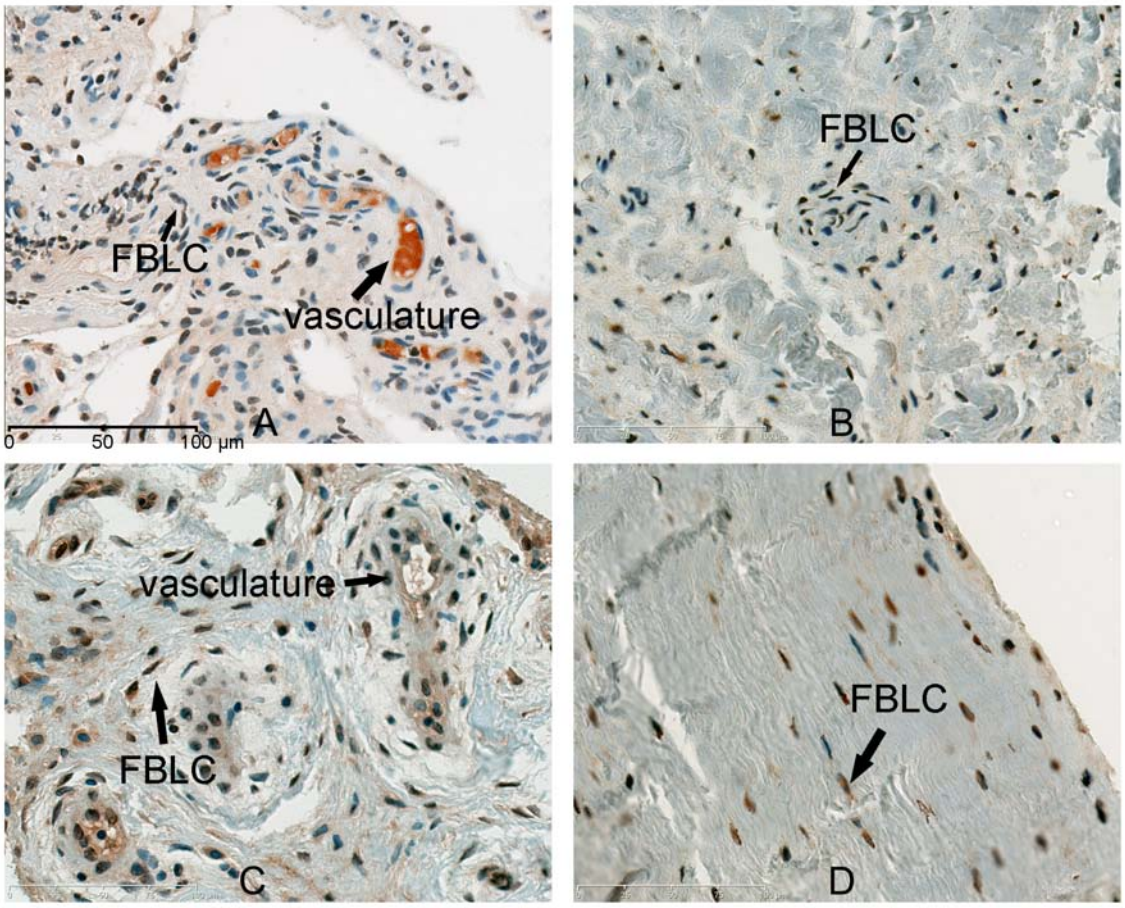

Figure 4.2: Panel of cathepsin K immunostaining in fibroblast-like synoviocytes in human active (A**) and inactive RA (**B**), OA (**C**) and normal tissues (**D**).**

Representative pictures of cathepsin K immunostaining in synoviocytes with fibroblast-like morphology (FBLC, arrowed) in active (**A**) and inactive RA tissues (**B**) as well as the OA (**C**) and normal (**D**) tissues viewed at 400X magnification. Positive immunostaining was indicated by the red dye and tissue sections were counterstained with H&E (blue dye).

4.3.2. Expression of NFATc1 by immunohistochemistry in RA and control tissues

Overall NFATc1 immunostaining positivity was highest in active RA tissues compared to other tissue groups. This is shown in the SQA scores for the NFATc1 immunostaining displayed in Table 4.5.

Table 4.5: SQA scores of NFATc1 immunostaining in human active, inactive RA, OA and normal tissues

Groups	n	Mean Score	(±SEM)	$p<0.05$
Active RA	10	2.50	0.21	❖⊙■
Inactive RA	8	0.75	0.23	•
OA	9	0.77	0.24	•
Normal	7	0.28	0.21	•

• if significantly different with the active RA group ($p<0.05$)

❖ if significantly different with the inactive RA group ($p<0.05$)

⊙ if significantly different with the OA group ($p<0.05$)

■ if significantly different with the normal group ($p<0.05$)

Majority cells expressing NFATc1 had a T-lymphoyte-like morphology (small-sized cells with large nuclei (Grossi et al. 1978), particularly in RA tissues. NFATc1 immunostaining was seen mainly in the nuclei of cells presented in aggregates of inflammatory-like cells, particularly in RA tissues. Due to the patchy nature of the staining pattern, the SQA scoring method was modified to be different from the one that has been reported (Kraan et al. 1999). This method was based on the number of aggregations of cells immunostained.

In active RA tissues, large numbers of positively stained cell aggregations were seen, generally more than 4 cell aggregations per $2mm^2$ view field. In some tissues most of the NFATc1-immunostained cells appeared to be synoviocytes in the lining layer of the tissues. No NFATc1 immunostaining was seen associated with the blood vasculature. Despite indication of very minimal amount of osteoclast-like cells in the tissues (see 4.3.1 above),

positive immunostaining for NFATc1 was also spotted on a multinucleated cell in a tissue (not shown in picture).

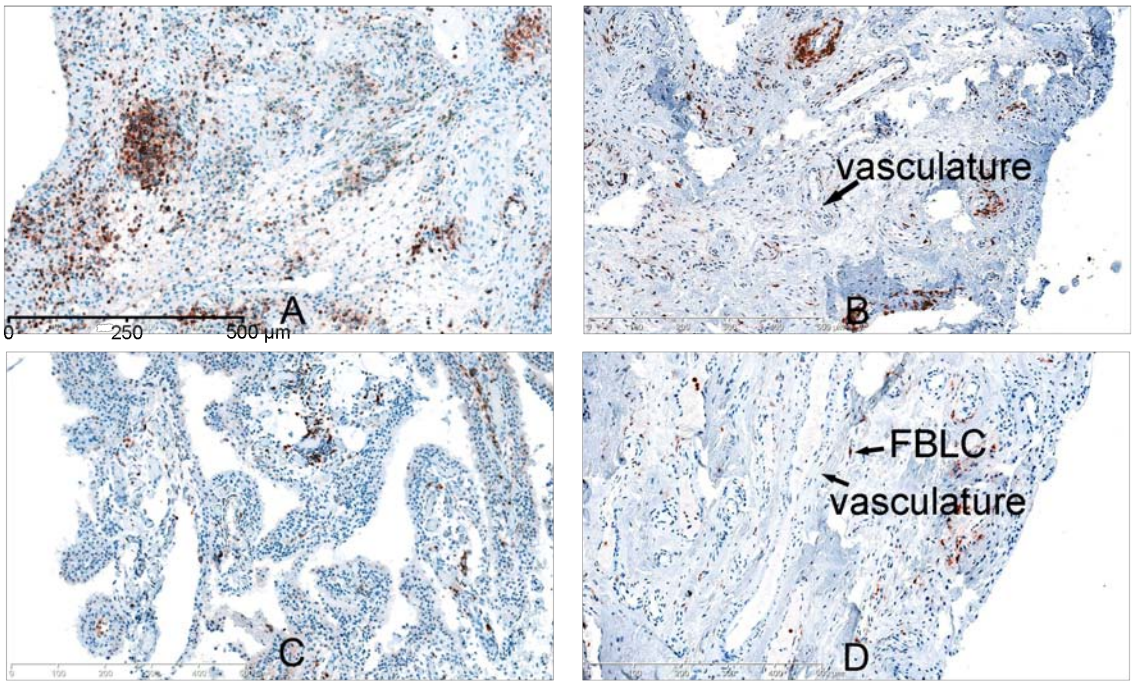

Figure 4.3: NFATc1 expression in human RA, OA and normal tissues

Representative pictures of NFATc1 immunostaining in active (**A**) and inactive RA tissues (**B**) as well as OA (**C**) and normal (**D**) tissuess viewed at a 50X magnification. Positive staining was detected on fibroblast-like cells (FBLC, arrowed) in normal tissue. Positive immunostaining was indicated by the red dye and tissue sections were counterstained with H&E (blue dye).

In the inactive RA group, a few cell aggregates expressed NFATc1, however these were fewer than in active RA. Nearly all of the tissue samples within the inactive RA group give score of 0 or 1. The NFATc1 immunostaining pattern generally appears to be similar between active and inactive RA tissues. In these tissues NFATc1 immunostaining was not seen in the vasculature meanwhile positive immunostaining was mainly observed in synoviocytes located in the tissue lining layers. Nonetheless, there was no NFATc1 immunostaining on multinucleated-like cell was observed.

OA tissues, like the inactive RA tissues, gives low number of cell aggregations. Unlike in RA tissue, OA tissues appear to have more scattered distribution of NFATc1 positive cells. Strong immunostaining in the OA tissues could be seen mostly on cells in the lining layer. Quite surprisingly, positive NFATc1 immunostaining was seen in quite a number of cells with multinucleated appearance (not shown in picture). No positive immunostaining was seen in the vasculature in OA tissues.

Finally, the normal tissue generally had minimal positive NFATc1 immunostaining. Indeed, the normal tissue section shown in the picture (Figure 4.3D) has the highest score of SQA among other normal tissues. In majority of the normal tissues, NFATc1 immunostaining was seen in tissue lining synoviocytes though the intensity of the staining was weak. Fibroblast-like synoviocytes scattered on the tissues were other examples of NFATc1-immunostained seen in a one or two normal tissues. Generally there was no immunostaining observed in the vasculature in the tissue group.

4.3.3. Expression of ITAM-related molecules in RA synovial tissues

4.3.3.1. TREM2 and DAP12

Immunohistochemistry staining for TREM2 and DAP12 demonstrated that both molecules could be detected in all tissue groups. Generally it was found that there was more positive immunostaining (statistically different) in the active RA tissues compared to the other tissue groups (refer Table 4.6 and Table 4.7 below). Interestingly there were also significant differences in the mean SQA score of TREM2 and DAP12 immunostaining obtained between

OA and the normal tissues. However, there was no significant difference in the SQA scores for both TREM2 and DAP12 immunostaining seen between the inactive RA and OA tissue groups.

Table 4.6: SQA scores of TREM2 immunostaining in human active, inactive RA, OA and normal tissues

Groups	n	Mean Score	(±SEM)	$p<0.05$
Active RA	9	3.44	0.17	❖⊙■
Inactive RA	9	2.55	0.34	•
OA	9	2.44	0.24	•■
Normal	9	1.77	0.22	•⊙

• if significantly different with the active RA group ($p<0.05$)

❖ if significantly different with the inactive RA group ($p<0.05$)

⊙ if significantly different with the OA group ($p<0.05$)

■ if significantly different with the normal group ($p<0.05$)

TREM2 immunostaining was observed on massive amount of cells in all tissues in the active RA tissues. TREM2 immunostaining was detected mainly in synoviocyte-like cells in the lining layer and in aggregations (with T-lymphocytes morphology as being mentioned in 4.3.2 above) in the active RA group. TREM2-immunostained mononucleated cells were also seen scattered on the tissues. Positive TREM2 immunostaining was seen in the multinucleated cells (similar outcome in 4.3.1 earlier) in some of the active RA tissues (not shown in picture).

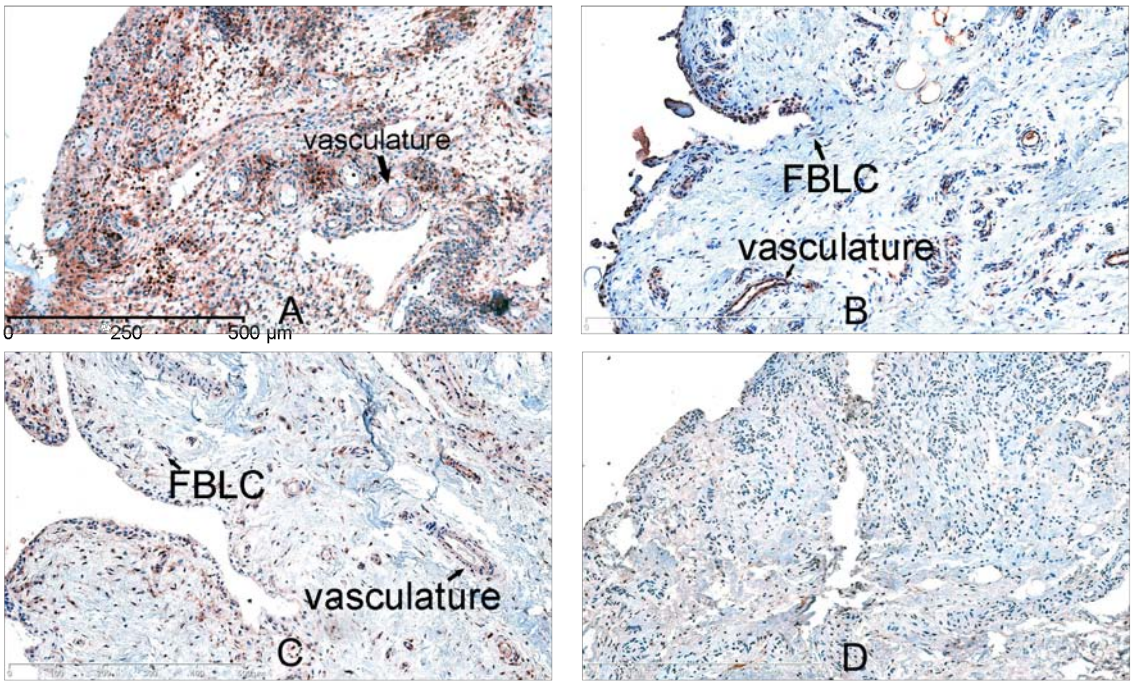

Figure 4.4: TREM2 expression in human RA, OA and normal tissues

Representative pictures of TREM2 immunostaining in active (**A**), inactive RA (**B**), OA (**C**) and normal tissues (**D**) viewed at a 100X magnification. Fibroblast-like cells (FBLC, arrowed) were among cells observed positive for TREM2. Positive immunostaining was indicated by the red dye and tissue sections were counterstained with H&E (blue dye).

In the inactive RA and OA tissue groups, the staining was seen more on the lining layer of the tissues. Strong TREM2 immunostaining was observed in these tissue groups on cells in the lining layer. TREM2 immunostaining was also seen associated with vasculature and fibroblast-like synoviocytes. Similar to the active RA group, multinucleated cells in the inactive and OA tissues were also positive for TREM2. In the inactive RA tissues, TREM2 was also expressed in a subpopulation cells in the lymphocyte aggregations.

In the normal tissues the expression of TREM2 was weak (Figure 4.4D). Most of the positive TREM2 immunostaining was observed in fibroblast-like synoviocytes. TREM2 immunostaining was also seen associated with the vasculature in some tissues in the group (not shown in picture). Unlike the other tissue groups, generally there was no obvious TREM2 immunostaining seen in cells in the tissue-lining layer.

Table 4.7: SQA scores of DAP12 immunostaining in human active, inactive RA, OA and normal tissues

Groups	n	Mean Score	(±SEM)	$p<0.05$
Active RA	10	1.70	0.21	❖⊙■
Inactive RA	9	0.67	0.29	•
OA	9	0.89	0.11	•■
Normal	10	0.40	0.22	•⊙

• if significantly different with the active RA group ($p<0.05$)

❖ if significantly different with the inactive RA group ($p<0.05$)

⊙ if significantly different with the OA group ($p<0.05$)

■ if significantly different with the normal group ($p<0.05$)

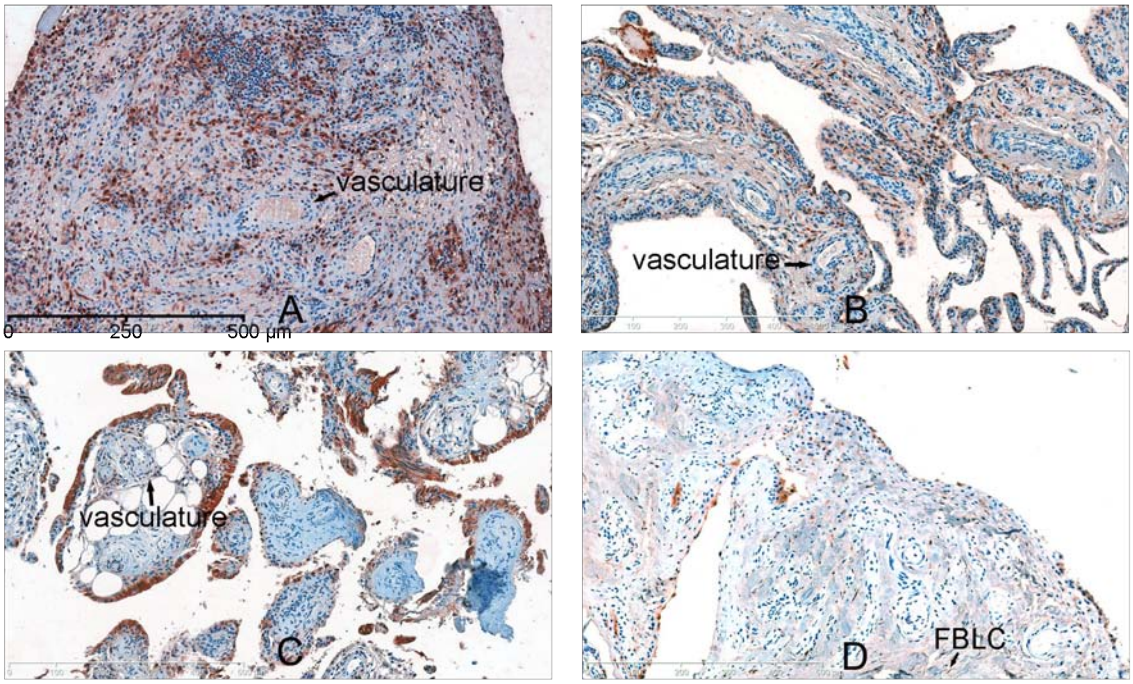

Figure 4.5: DAP12 expression in human RA, OA and normal tissues

Representative pictures of DAP12 immunostaining in active (**A**), inactive RA (**B**), OA (**C**) and normal (**D**) tissues viewed at a 100X magnification. Fibroblast-like cells (FBLC, arrowed) were among cells positive for DAP12-immunostained. Positve immunostaining was indicated by the red dye and tissue sections were counterstained with H&E (blue dye).

Generally there appeared to be a similar pattern of immunostaining for DAP12 and TREM2. However the SQA score for DAP12 immunostaining was markedly lower than for TREM2 (compare Table 4.6 and Table 4.7). DAP12-immunostained cells appeared to be tissue-lining synoviocytes and perivascular cells, however, there was no DAP12 immunostaining seen associated with the vasculature in this tissue group. In some of the active RA tissues, in which there appeared to be massive infiltration of T-lymphocyte-like cells (refer 4.3.2 above), most cells were negative for DAP12 resulting in a lower SQA score than it first appeared.

Similar observations were seen in both inactive RA and OA tissues, however DAP12 immunostaining was reduced (see Table 4.7). While a significant number of DAP12-immunostained cells were scattered throughout tissues in those two groups, most of the stained cells appeared to be in the tissue lining layer. Similar to that seen in the active RA tissues, multinucleated cells immunostained for DAP12 were seen in a number of inactive RA and OA tissues (not shown). However, there was no sign of DAP12 immunostaining on vasculature in both tissue groups. In some OA tissues, DAP12 immunostaining was also detected in synovial fibroblast-like cells (not shown in picture).

In normal tissues, few DAP12-immunostained cells were seen, however the staining intensity appeared to be quite weak. Fibroblast-like and tissue-lining synoviocytes were among the cells expressing DAP12 (Figure 4.5D). DAP12 immunostaining associated with the vasculature was also seen in a normal tissue (not shown in picture).

4.3.3.2. OSCAR and FcRγ

Generally the extent of positive immunostaining of OSCAR (in all tissue groups) was less than the other molecules investigated, as shown in Table 4.8 below. From the comparison on the mean SQA score for OSCAR immunostaining between tissue groups, significant difference could only be seen between the normal control group against both active RA and OA tissues.

Table 4.8: SQA scores of OSCAR immunostaining in human active, inactive RA, OA and normal tissues

Groups	n	Mean Score	(±SEM)	$p<0.05$
Active RA	9	1.44	0.24	■
Inactive RA	9	0.89	0.39	•
OA	9	0.89	0.26	■
Normal	8	0.00	0.00	•⊙ ❖

• if significantly different with the active RA group ($p<0.05$)

❖ if significantly different with the inactive RA group ($p<0.05$)

⊙ if significantly different with the OA group ($p<0.05$)

■ if significantly different with the normal group ($p<0.05$)

OSCAR expression was strongest in the active RA group. Many mononuclear cells scattered on the tissues displayed varying staining intensity with a proportion very strongly expressing OSCAR (refer Figure 4.6A). OSCAR immunostaining was also detected in some cells in the lymphocyte aggregations (not shown in picture). In some of the active RA tissues, OSCAR immunostaining was also detected in cells on the lining layer (refer Figure 4.10**A**). Multinucleated cells detected in active RA tissues (see 4.3.1 above), were also positive for the OSCAR. In nearly all active RA tissues immunostaining was associated with the vasculature but not in the surrounding cells on the tissues, which appeared to be a consistent obvious feature in OSCAR immunostaining in active RA tissue group (detailed in following section 4.3.3.3.2 below).

A similar pattern of OSCAR immunostaining was noted for the inactive RA tissues, even though the proportion of immunostained cells was less than in the active RA tissues. Scattered mononuclear cells and tissue-lining synoviocytes were among the cells expressing OSCAR in the inactive RA tissues. Interestingly, in most inactive RA tissues, there was little immunostaining associated with the vasculature but stronger staining inside the vessels. It is not clear if this staining is associated with cells inside the vessels or if this could be soluble OSCAR (discussed in 4.3.3.3.2 below).

Through this study expression of OSCAR was detected in OA tissues, which is in agreement with the observations documented earlier in Chapter 2 (see section **2.3.1.3**). However, OA

tissues displayed only mild to weak positive immunostaining. Generally strongest staining could be seen mainly in synoviocytes in the lining layer in OA tissues. Most tissues were negative for OSCAR immunostaining on blood vessels, with only a few were mildly positive. OA tissues that showed vasculature-associated immunostaining also displayed weak staining inside the blood vessels (Figure 4.5C).

OSCAR immunostaining in the normal tissues generally were as expected with very minimal positive immunostaining. However, similar to the OA group, there were a few tissues with no positive immunostaining at all. In some normal tissues, in addition to very few number of immunostained resident synoviocytes, positive immunostaining was also detected associated with and around vasculature (further discussed in section 4.3.3.3.2). There was also notable feature of immunostaining inside the vasculature in some of the normal tissues, which could further support the idea of soluble OSCAR in the blood serum (Herman et al. 2008).

While the pattern of DAP12 SQA score between goups followed TREM2, the trend for FcRγ mean SQA score between tissue groups (Table 4.9) was different when compared to OSCAR (Table 4.8). This was demonstrated by the data indicating statistically higher mean SQA score for FcRγ in active RA and OA tissue groups than in the inactive and normal ones. When compared to the OSCAR mean SQA score, it seemed that there were more FcRγ-immunostained cells in all tissue groups than cells positive for OSCAR.

Table 4.9: SQA scores of FcRγ immunostaining in human active, inactive RA, OA and normal tissues

Groups	n	Mean Score	(±SEM)	$p<0.05$
Active RA	10	2.50	0.31	❖■
Inactive RA	8	0.88	0.40	•⊙
OA	9	2.22	0.40	❖■
Normal	10	0.70	0.26	•⊙

• if significantly different with the active RA group ($p<0.05$)

❖ if significantly different with the inactive RA group ($p<0.05$)

⊙ if significantly different with the OA group ($p<0.05$)

■ if significantly different with the normal group ($p<0.05$)

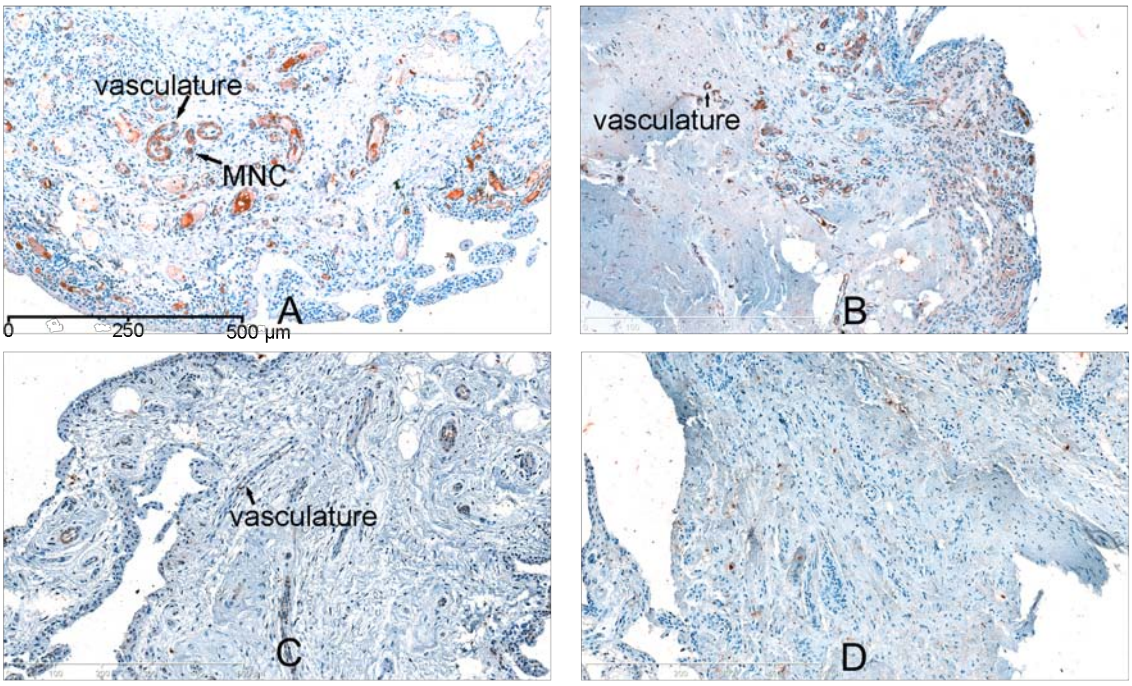

Figure 4.6: OSCAR expression in human RA, OA and normal tissues

Representative pictures of OSCAR immunostaining in active (**A**) and inactive RA tissues (**B**) as well as OA (**C**) and normal (**D**) tissues viewed at a 100X magnification. Multinucleated cell (MNC, arrowed) was observed to positively immunostained for OSCAR in an active RA tissue. Positive immunostaining was indicated by the red dye and tissue sections were counterstained with H&E (blue dye).

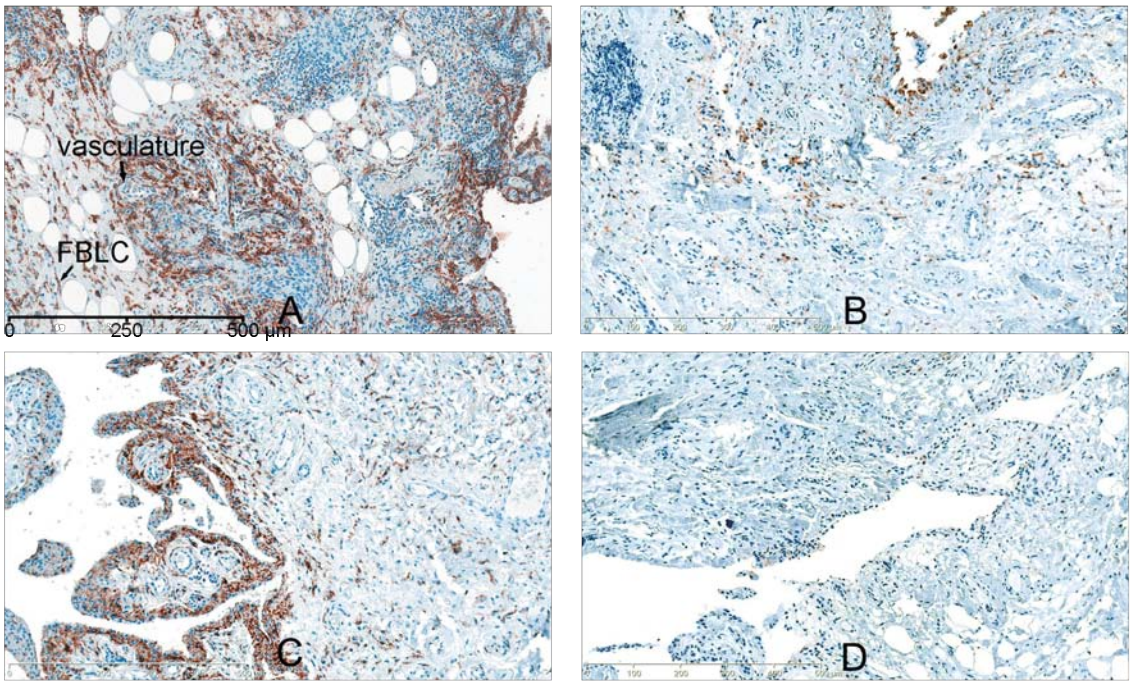

Figure 4.7: Panel of FcRγ immunostaining in human RA, OA and normal tissues

Representative pictures of FcRγ immunostaining in active (**A**) and inactive RA tissues (**B**) as well as OA (**C**) and control normal (**D**) groups viewed at a 100X magnification. Fibroblast-like cells (FBLC, arrowed) were among cells positively stained for FcRγ. Positive immunostaining was indicated by the red dye and tissue sections were counterstained with H&E (blue dye).

In the active RA tissues, unlike OSCAR, there was no staining for FcRγ associated with the vasculature structures. Nevertheless FcRγ-immunostained cells were detected in the perivascular areas (Figure 4.7A). Most of the FcRγ immunostaining were seen in synoviocytes residing in the tissue-lining layer. Most of these cells had the morphology of fibroblast-like cells. There were also some cells in aggregations expressing FcRγ.

There was not as many positively immunostained cells appeared in the inactive RA tissues. The majority of the immunostaining was seen in the tissue-lining synoviocytes in some of the inactive RA tissues. FcRγ-positive cells were also sparsely distributed in the tissues. There was no positive immunostaining in the cells in the lymphocyte aggregations in the inactive RA tissues.

In the OA tissues the pattern of FcRγ immunostaining was quite similar to the active RA tissues, in which strongest staining was seen concentrating in cells in the tissue lining layer. In the normal tissues, FcRγ immunostaining was detected not only in the cells in the tissue-lining layer but also resident synoviocytes sparsely distributed on the tissues. Similar to the other tissue groups, there was no positive immunostaining seen in those cells in aggregations (believed to be T-lymphocytes) in both OA and normal tissue groups.

4.3.3.3. Immunostaining of OSCAR and TREM2 Associated with Vasculature

Among all the ITAM-associated molecules investigated in this study, the expression of TREM2 and OSCAR was given more attention considering that they are the immunoreceptors that may be responsive in the regulation of cellular response. Interestingly, it was noted that for TREM2 and OSCAR expression, there was feature of positive immunostaining in and around blood vessel structures (refer Figure 4.8).

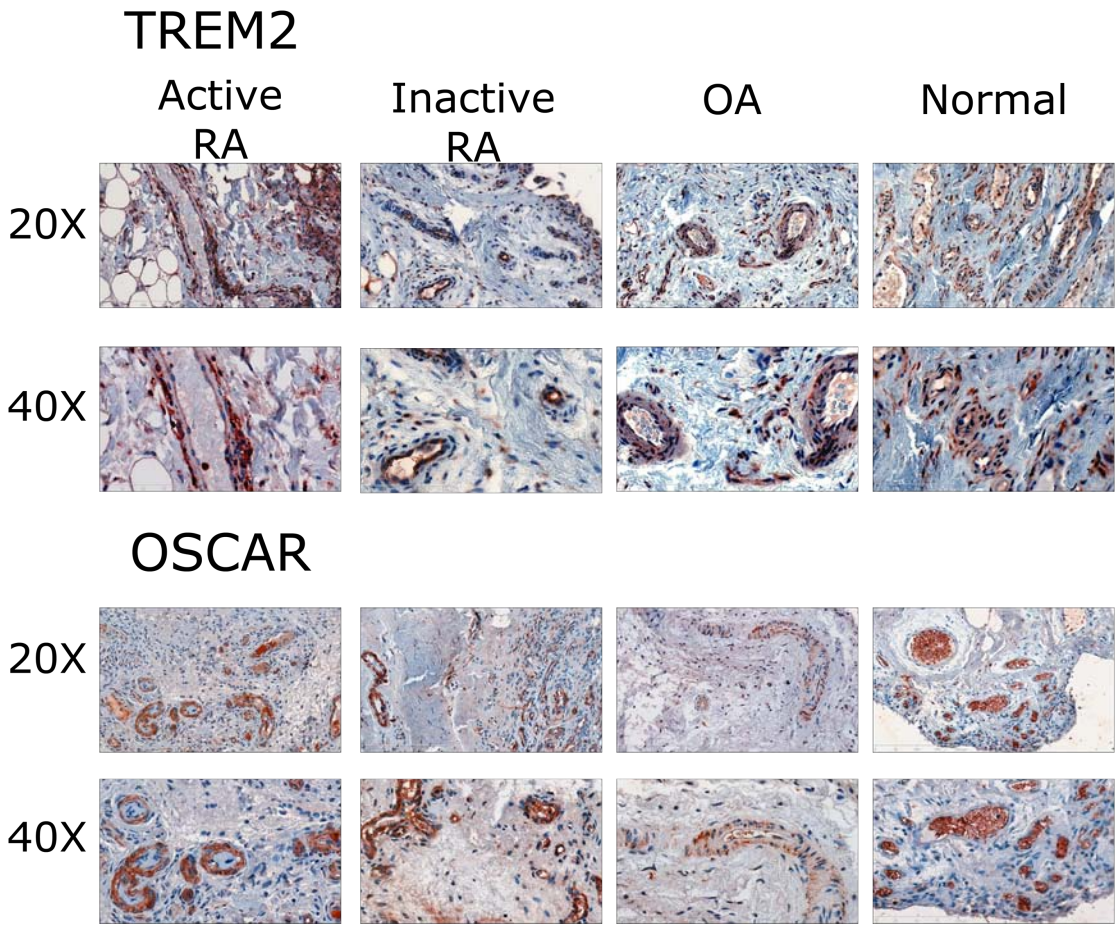

Figure 4.8: Immunostaining of TREM2 and OSCAR associated with blood vessel structures across different groups of human tissues

Representative pictures showing the TREM2 (top two rows) and OSCAR (bottom two rows) immunostaining associated with and around vessel-like structures on human active and inactive RA, OA and normal tissues (in columns from left to right). Images were captured at different magnifications, as indicated on the left side of the panel. Positive immunostaining was indicated by the red dye and tissue sections were counterstained with H&E (blue dye).

4.3.3.3.1. TREM2

Despite the lowest mean SQA score for TREM2 immunostaining was obtained for the normal tissue group, it was interesting to note that eight out of nine tissues studied demonstrated positive immunostaining in vasculature-like structures. Examination of tissues from other groups also found TREM2 immunostaining associated with the vasculature, however in smaller proportion number of tissues within each group. Only one tissue sample with active RA group demonstrated positive immunostaining, though mildly (refer Figure 4.9A). Meanwhile in the inactive RA tissue group, three tissues from total of 10 were found to have TREM2 expression associated with the vasculature. As for the OA group, TREM2 immunostaining in vasculature was seen in about half of total tissues in the group (4/9).

TREM2 immunostaining of the vasculature was generally quite mild in intensity, especially in tissues of the non-normal groups. Interestingly, in most RA and OA tissues displaying positive immunostaining on the vasculature, it seemed that most of the staining was more on the inner side of the vessel walls (see Figure 4.9F).

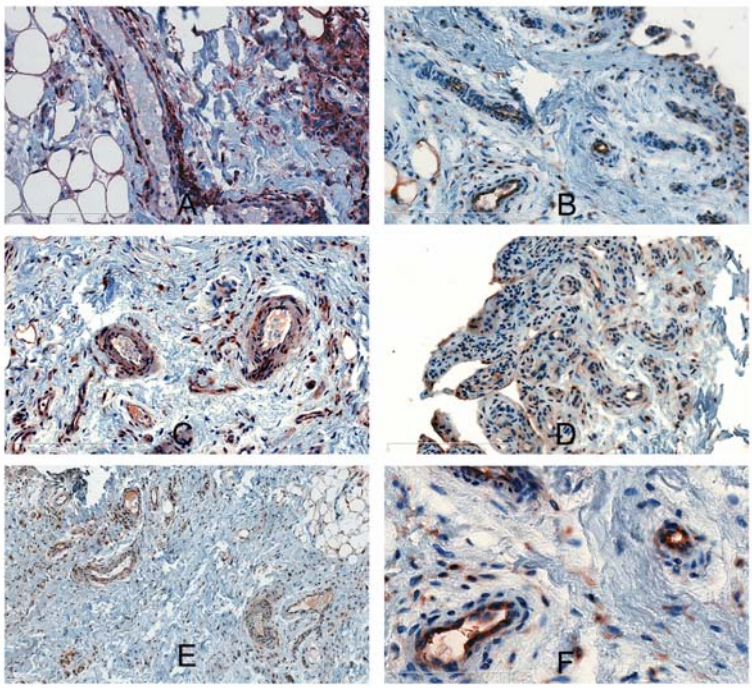

Figure 4.9: TREM immunostaining associated with vasculature

Representative pictures showing the TREM2 immunostaining associated with vasculature in active (**A**), inactive RA tissues (**B**) as well as by in OA (**C**) and normal tissues (**D**) captured at 200X magnification. **E** represents TREM2 immunostaining in an OA tissue at lower magnification (100X) to show notable specific staining on vasculature on tissues. **F** demonstrates TREM2 immunostaining in vasculature was more in the inner side of the vasculature (400X). TREM2 immunostaining was indicated by the red dye and tissue sections were counterstained with H&E (blue dye).

4.3.3.3.2. OSCAR

In comparison to TREM2, positive immunostaining associated with blood vessels was more notable for OSCAR, particularly in the RA tissues. In both active and inactive RA groups, OSCAR immunostaining was detected in vasculature-like structures in nearly all (8/9) tissues in those groups. The intensity of the immunostaining in the vessel-like structures varied between tissues, with majority of tissues in active RA group in particular displaying strong staining. In addition, there appeared to be positive staining in cells inside blood vessels (refer Figure 4.10**C**) in one tissue in the active RA. This sort of observation is in agreement with the findings of Herman and colleagues (2008), who showed that circulating monocytes can express OSCAR prior to entry into tissues. This could be further supported by an observation that cells appeared to be marginalising from the vessels displaying OSCAR immunostaining (refer Figure 4.10**D**).

For the OA tissues, only few tissues within the group (4/9) showed positive OSCAR immunostaining associated with blood vessel-like structures. Furthermore, the OSCAR immunostaining associated with the blood vessels was very mild in those tissues. Like in RA tissues, there was also staining inside blood vessels observed in most OA tissues (7/9).

OSCAR immunostaining for the normal tissues in and around blood vessel-like structures was quite similar to the OA group. Nearly all normal tissues (8/9) showed OSCAR immunostaining inside the blood vessel-like structure, however only 2 of those tissues displayed staining in wall of the vasculature. In most of the normal tissues immunostained for OSCAR, the staining inside the vasculature appeared to be stronger than in the other tissue groups.

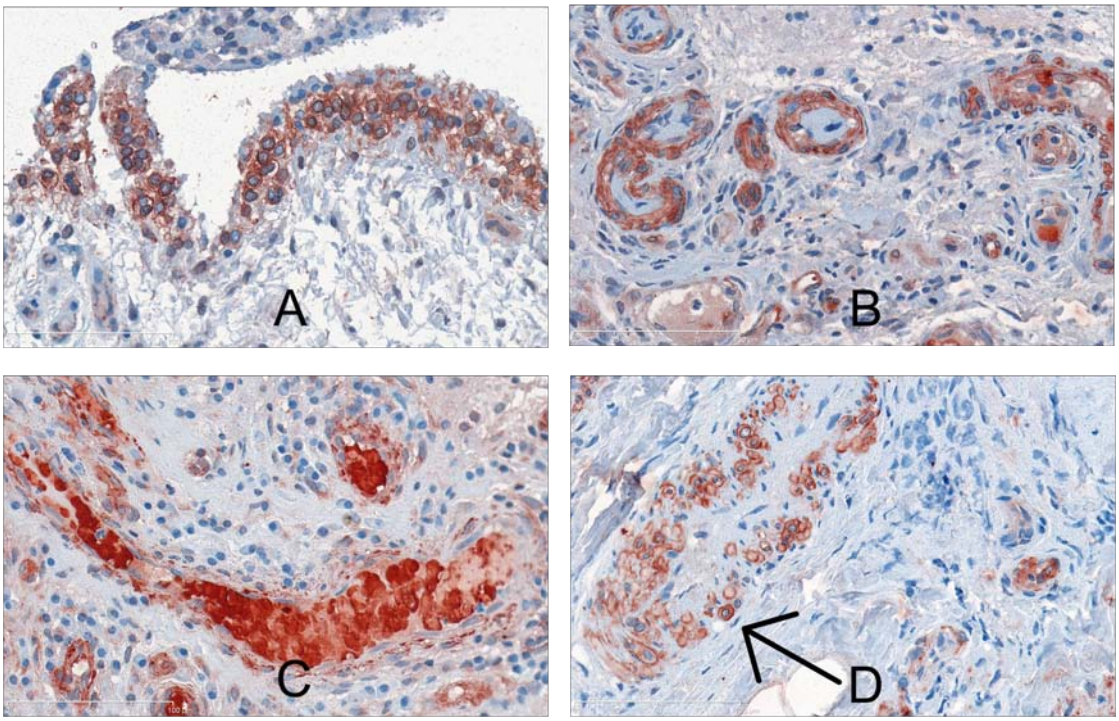

Figure 4.10: Immunostaining of OSCAR in human tissues of RA and OA

OSCAR-positive immunostaining was seen in lining cells in OA tissues (**A**). OSCAR immunostaining was detected associated with vasculuture in active RA tissues, as clearly shown in **B**. **C** shows positive immunostaining of OSCAR was also detected in cells within the blood vessels. **D** Examination on other part of the tissues shown in **B** showed appearance of migrated OSCAR-positive cells from blood vessel (shown by arrow in panel **D**). All pictures were captured at 400X magnification.

4.3.4. Level of soluble OSCAR levels in synovial fluids of RA and OA

The observation of OSCAR immunostaining within and inside the blood vessel walls in RA tissues particularly could also suggest the presence of soluble OSCAR in the serum, as this has been demonstrated in previous studies (Herman et al. 2008; Zhao et al. 2011). As level of soluble OSCAR in serum has been reported in previous studies (Herman et al. 2008; Zhao et al. 2011), synovial fluid was tested for soluble OSCAR for the first time. Due to the unavailability of synovial fluid from normal subjects, synovial fluids from OA patients were used as the controls. The concentrations of soluble OSCAR in the synovial fluids of RA, alongside with OA sample group, are presented in Figure 4.11 below.

Interestingly the levels of soluble OSCAR in serum of RA shown in previous studies (Herman et al. 2008; Zhao et al. 2011) appeared to be markedly lower than in the synovial fluids in RA patients used in this study. Surprisingly, it appeared that soluble OSCAR was not only detectable in synovial fluid from OA patients, but the level measured (mean 130.73ng/ml) was slightly higher than in RA samples (mean 111.74ng/mL). Moreover, the level of soluble OSCAR in synovial fluids from OA group was more consistent than in the RA groups, which include one outlier.

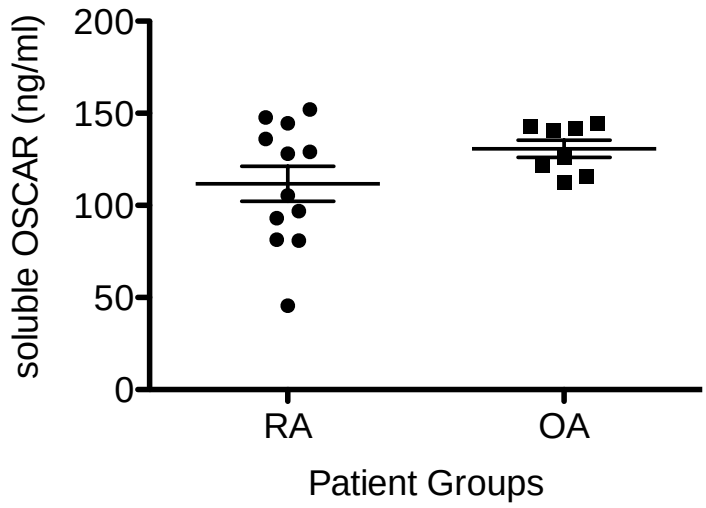

Figure 4.11: Level of soluble OSCAR in synovial fluid of patients with RA and OA

4.4. Discusssion

In contrast to the previous two chapters that are themed on peri-implant osteolysis, this chapter, together with the following Chapter 5, investigates rheumatoid arthritis. It is interesting to note the differences in ITAM-associated molecules involved in promoting osteoclast formation in peri-implant osteolysis and rheumatoid arthritis. The study presented in this chapter can be compared to Chapter 2 as it investigated tissue expression of ITAM-related molecules (including TREM2, DAP12, OSCAR and FcRγ), alongside with the osteoclast key transcriptional factor NFATc1 and cell marker cathepsin K, in RA tissues (and synovial fluid for OSCAR only) in comparison to the other groups.

The synovial membrane is believed to be an active site for osteoclastogenesis in RA (Gravallese et al. 2000). Those tissue samples used in this study were obtained from a well-characterised tissue collection, which is a unique feature of this study in comparison to the others. Another aspect that made the tissue collection unique was the presence of an inactive RA tissue group from patients in remission after successful treatment with DMARD. Since OA and normal tissues were also included in this study it made multi-groups comparisons possible rather than just only 2 groups comparison as in Chapter 2. The inclusion of OA and RA tissues allowed comparison to see how these different diseases varied. Tissues were collected through biopsy performed by a rheumatologist (Professor Malcolm D. Smith) and therefore consistent procedures and tissue site sampling were the key factors in obtaining this homogenous tissue library. In addition, this tissue collection has also been used in many published studies (Crotti et al. 2003; Crotti et al. 2002; Haynes et al. 2003; Smith et al. 2003).

The inclusion of inactive RA tissue group in the study has made information such as history of medication relevant to be recorded (see Table 4.1). The type of medication could be a confounding factor that may influence protein expression in the tissues (Vieira-Sousa et al. 2011). Unfortunately, complete information on the ESR and DAS28 was not recorded for all patients. Therefore the findings in this thesis could not be directly compared to the study by Herman and colleagues (2008) since these subject parameters could become factors that may give different outcome between studies.

Unlike in Chapter 2, there was no assessment on the tissue histology using H&E staining due to limited number of tissue sections from the small tissue samples. In addition, there was also no examination on tissue mRNA level carried out due to the very limited amount of frozen tissue (from the same collection) available for RNA extraction.

Similar to Chapter 2, dual immunolabeling was not feasible to be performed due to technical limitation associated with paraffin-embedded tissue sections. In addition serial immunolabeling with cell markers was not possible due to the limited number of tissue sections available. As a consequence, the tissue immunostaining described in this chapter could only interpreted based on the cell morphology alone.

Based on the SQA score obtained for cathepsin K immunostaining in this study (refer Table 4.4), surprisingly it appeared that the number of cells expressing cathepsin K was significantly lower in active RA tissues in comparison to the normal tissues. In addition there was no significant difference in the SQA score between RA and OA tissue groups. Both of these outcomes were in contradiction to the earlier findings made by Hou and co-workers (2002), which showed more cathepsin K-positive cells in RA than in OA tissues. This difference could probably be explained by variabilities such as tissue sites sampled and the progression of the disease of individual patients. Nonetheless, there was consistency between these two studies in terms of the phenotype of cells expressing cathepsin K seen in all tissue groups.

The immunostaining for cathepsin K did not reveal the presence of significant numbers of multinucleated osteoclast-like cells in the RA tissues (see Figure 4.1A and B). The presence of osteoclasts in RA tissues is believed to lead to bone erosion in the disease (Gravallese et al. 1998). However the fact that only 2 active RA tissues that showed positive signs of bone erosion (refer Table 4.1) (Crotti et al. 2012), in addition to the very rare detection of osteoclast-like cells in the active RA tissues found in this study, could suggest that most RA tissues studied were not from chronic RA patients. Another possibility is the tissues were collected from the synovial membranes that were not juxtaposing bone where osteoclasts would be present (Shen et al. 2006). It would be expected that collection of pannus would reveal presence of larger number of osteoclast-like cells. Future work using tissues sampled from bone-juxtaposed tissues or pannus in chronic RA patients may give more meaningful

outcome and allow the original research question of the study (in context of osteoclast) to be properly addressed.

In addition to the RA tissues, multinucleated osteoclast-like cells were also detected in an OA synovial tissue. This observation is consistent with several previous findings (Dodds et al. 1999; Hou et al. 2002). Even though there was minimal number of multinucleated osteoclast-like cells that could be seen, there are still possibility that the other cathepsin K-positive cells were macrophage-like cells (Haywood et al. 2003; Hummel et al. 1998), which could become osteoclast precursors (Dodds et al. 1999; Gravallese et al. 1998; Hou et al. 2002). Dodds and colleagues (1999) through their work found that mononuclear cells expressing cathepsin K in OA tissues were also positive for TRAP, suggesting that they were possibly osteoclast precursor cells.

It is also possible that cathepsin K is expressed by non-myeloid cell types in RA synovial tissues. For example, the observation in which cathepsin K-positive cells were among cells in lymphocyte aggregates seen in this study was consistent with a previous report that found cells containing cathepsin K mRNA transcripts in the areas of lymphocyte infiltration in RA tissues (Hummel et al. 1998).

Cathepsin K immunostaining in the diseased and normal tissues could also be seen in cells with fibroblast-like morphology (see Figure 4.2). This was consistent with previous literature reporting on the expression of cathepsin K in synovial fibroblasts, detected at both mRNA and protein level (Bottini & Firestein 2013; Hou et al. 2002; Hou et al. 2001). Through their observation in the normal tissues, Hou and colleagues (2002) suggested that cathepsin K is constitutively expressed by synovial fibroblasts.

Hummel and coworkers (1998) reported that cathepsin K-positive cells found at cartilage-pannus junction in RA tissues appeared to have macrophage or fibroblast-like morphology, leaving speculation of cathepsin K role in cartilage invasion and erosion (Li et al. 2000). This is because cathepsin K has been identified as able to degrade both types I (in bone) and II collagens (in cartilage) (Bossard et al. 1996; Kafienah et al. 1998). In addition, an earlier study found that synovial fibroblasts from human RA tissues (as well as from OA tissues) were able to cause cartilage degradation (Scott et al. 1997). The examination on tissues from a human fetus with pycnodysostosis (resulted from cathepsin K deficiency, refer section

1.3.1.3), which found presence of undigested collagen fibrils accumulated in lysosomal vacuoles of fibroblast (Everts et al. 2003), provides further evidence of role of cathepsin K in fibroblastic cells in collagen degradation. In an *in vivo* animal model, treatment with a cathepsin K inhibitor managed to reduce the extent of bone and cartilage erosion in mice subjected to collagen-induced arthritis (Svelander et al. 2009).

In RA synovial fibroblasts may play several roles in the disease progression. The pannus is made up from an over-abundance of syovial fibroblasts (Bottini & Firestein 2013). These cells can also produce a range of molecules that are able to modulate cell growth, angiogenesis, synovitis and stimulate production inflammatory mediators in other immune cells. In addition, they can also express proteolytic enzymes, including cathepsin K and MMPs that are important in both bone and cartilage erosion, and release cytokines that promote and support osteoclastogenesis in RA (Gravallese et al. 1998; Hase et al. 2008; Hwang et al. 2012b; Kim et al. 2012).

In comparison to RA, much less is known about the role of fibroblastic synoviocytes in OA. However, there is evidence associating the formation of fibroblast-like synoviocytes occurs at the expense of chondrocyes in OA (Aigner et al. 1993; Sandell & Aigner 2001) (see cell phenotypic alteration in section 1.2.3). A recent study by Zhang and colleagues (2012) reported that the inhibition on cathepsin K expression could slow down the process.

As stated earlier in the Introduction (section 4.1) even though a number of studies suggesting NFATc1 as might have role in RA disease progression and could be a good therapeutical target (Miyazaki et al. 2007; Urushibara et al. 2004), it appeared that there has been no study on NFATc1 expression in RA tissues being reported so far (Sitara & Aliprantis 2010). Indeed to the author's knowledge, there has been only one previous study demonstrating *in* vivo model of arthritis in NFAT-deficient mice (Gerth et al. 2004). Therefore, the data presented in this section is the first to report expression of NFATc1 protein in human RA tissues.

Interestingly as indicated in the results section, most of the positive NFATc1-immunostained cells were seen to be in aggregations with closer inspection suggesting they were infiltrating T-lymphocytes. Detection of NFATc1 on T-lymphocyte-like cells, particlarly in RA tissues is not an unexpected observation considering NFATc1 is a transcription factor expressed, and indeed was first discovered (Shaw et al. 1988), in the T-cells. NFATc1 in the activated T-

lymphocytes regulates the cell differentiation and activation (Pan et al. 2007) and may further stimulate osteoclastogenesis by stimulating RANKL expression in T-cells (Kotake et al. 2001). Previous work carried out in this laboratory has found high level of RANKL in human RA tissues (Crotti et al. 2002), therefore high NFATc1 expression in RA tissues (compared to other tissue groups) is not unexpected since NFATc1 has been shown to be induced following RANKL stimulation (Ishida et al. 2002; Kim et al. 2012; Takayanagi et al. 2002). Lower SQA score for NFATc1 in the inactive as compared to the active RA tissues could probably be explained by study by Urushibara and colleagues (2004) demonstrating that treatment with DMARD could reduce the expression of NFATc1.

Similar to RA, there has been no study investigating NFATc1 in the context of OA (Sitara & Aliprantis 2010). However recently, there are few studies suggesting a connection between OA disease progression and another NFAT family member, which is NFAT1 (distinct from NFATc1). Wang and colleagues (2009) found that reduced expression of NFAT1 in chondrocytes could result in development of OA. Further studies by that particular group (Rodova et al. 2011) suggested that NFAT1 may regulate cartilage homeostasis and could be associated with epigenetic modifications that could only occur in elderly or adult population.

While OA has traditionally been considered as a non-inflammatory disease, there is accumulating evidence indicating T–lymphocytes are involved and contribute to the disease progression. The presence of T-lymphocytes in OA has actually been reported long ago (Kuryliszyn-Moskal 1995; Lindblad & Hedfors 1987; Reidbord & Osial 1987; Revell et al. 1988) and recently the role of lymphocytes in OA disease has been actively discussed. Similar to RA, the detection of CD3, CD4 and CD8-positive T-cells in lining, sublining and occasionally in deep layer of OA synovial tissues has been demonstrated (Ishii et al. 2002; Nakamura et al. 1999; Sakkas et al. 1998). There are also other similarities with RA observed in OA tissues such as accumulation of activated T-cells (CD45RO-positive) (Ezawa et al. 1997) and increase in the CD4/CD8 T-cell ratio (Kuryliszyn-Moskal 1995). Indeed Leheita and colleagues (2005) reported that there was no significant difference in the CD4/CD8 cell ratio between in OA and RA. This alteration in T-cell populations in OA tissues suggests such cell populations have a role in the pathogenesis of OA (Hussein et al. 2008). Several mechanisms relating to the involvement of T-lymphocytes have been suggested (Sakkas & Platsoucas 2007). For example, CD4-positive T-helper cells promote osteoclast formation in

OA tissues by expressing cytokine macrophage inflammatory protein 1-gamma (MIP1γ) (Shen et al. 2011).

Even though it has been a while since TREM2 and DAP12 was discovered and believed to be involved as co-stimulatory receptor (and adaptor molecule) for promoting osteoclastogenesis (Humphrey et al. 2006; Humphrey et al. 2004; Otero et al. 2012), the significance of these molecules in bone-associated diseases has only been seen the pathogenesis of PLOSL (Cella et al. 2003; Numasawa et al. 2011; Soragna et al. 2003) and osteoporosis (Chouery et al. 2008; Hopwood et al. 2009). The author is unaware of any study investigating TREM2 (or DAP12) in the context arthritis being reported to date, therefore the findings discussed in this section could be regarded as novel.

It appeared that nearly all cells positive for TREM2 were mononucleated cells (see Figure 4.4). However positive immunostaining was also detected occasionally in multinucleated-like cells in some tissues. Like some other molecules studied here, it is possible that among the TREM2-stained mononuclear cells there were osteoclasts precursors as TREM2 might play a significant role during the cell proliferation stage of pre-osteoclastic cells that occurs before switching to osteoclast differentiation (Otero et al. 2012).

It would be an interesting topic to study TREM2 expression since besides in co-stimulation for osteoclastogenesis, the significance of TREM2 is also widely discussed in the context of inflammation as it also appears to play important role in a range of immune cell types (refer earlier sections 1.3.5.2.1). Recently there was evidence suggesting that TREM2 is involved in mediating inflammation, based on the evidence from TREM2-knockout mice had attenuated inflammatory response following stroke (Sieber et al. 2013). TREM2 is reported to induce cytokine production in macrophage (Hamerman et al. 2005) and involved in type II hypersensitivity-associated inflammation (Turnbull et al. 2006). In microglias (macrophages in brain), TREM2 is important in regulating phagocytosis mechanism for clearance of apoptotic neurons (Takahashi et al. 2005).

Recently there are studies linking RA with high expression of another TREM family member, TREM1 (Chen et al. 2008a; Collins et al. 2009; Kuai et al. 2009; Murakami et al. 2009). It has been reported that there was higher level of TREM1 being expressed in RA tissues as well as in the synovial fluids of RA patients as compared to the OA (Collins et al. 2009; Kuai

et al. 2009). However, it is unlikely to be associated with bone loss in RA since TREM1 is not expressed by osteoclasts (Humphrey et al. 2004).

DAP12 immunostaining followed the same pattern as for the TREM2, however the SQA scores for DAP12 immunostaining (for all tissue groups) were generally lower compared to TREM2 (compare Table 4.6 and Table 4.7). Probably one good explanation for such outcome would be DAP12 are expressed in narrower range of cell types than TREM2 in synovial tissues. DAP12 has been well known expressed in NK cells besides T-cells (Chen et al. 2009; Goronzy et al. 2005) (as discussed in detail earlier in section 1.3.5.2.1). DAP12 is a transmembrane protein identified as important component in transducing activating signals in NK cells for targeting cells to be lysed (Colonna 1998; McVicar et al. 1998). Deletion of *dap12*or blockade of either TREM2 using sTREM2 suppressed dendritic cell-mediated activation of NK cells (Terme et al. 2004).

The discovery of OSCAR (Kim et al. 2002) and its role as co-stimulatory immunoreceptor in osteoclastogenesis has prompted several research groups to study this molecule in bone-associated diseased tissues, including in RA (Herman et al. 2008; Zhao et al. 2011). These studies demonstrated that the level of OSCAR expression in RA tissues was higher than in the control groups. The data obtained from this presented study is in agreement with these reports (see Table 4.8).

This study surprisingly found that there were not as many OSCAR-positive cells in RA tissues as expected (where mean SQA score was 1.44 ± 0.24, see Table 4.8). One possible reason for this is that the active RA tissues in this study were from early stage of RA in which bone erosion may not yet be detected (radiographically seen bone erosion could be detected in only two active RA patients) and low expression of OSCAR may be expected since OSCAR was reported to be expressed in late stage of osteoclast formation (Kim et al. 2005d). This is unlike the study by Herman and colleagues (2008) in which all RA tissues used were collected from patients with chronic and longstanding RA.

There are several other differences from the study reported by Herman et al. (2008) that make this study unique and novel. Here there is a wider comparison of tissue groups that included inactive RA and normal tissues that may be a more appropriate control, as opposed to Herman et al. (2008) that used OA tissues. In addition, the inclusion of an inactive RA group in this

study could provide information on whether successful DMARD treatment leads to differences in the expression of OSCAR and hence indicate that OSCAR is a marker for RA disease progression. Earlier, Zhao and colleagues (2011) reported higher levels of soluble OSCAR in patient sera in RA controlled stage than ones in the active stage. This observation was taken to indicate a relationship between OSCAR levels and successful treatment. Herman et al. (2008), found that an increased level of soluble OSCAR in RA patient sera coincided with therapy with TNFα blocker.

This presented study also provides other additional insights. For example, it could be deduced that the expression of OSCAR in RA tissues is not only limited to osteoclasts but also in other cell types (very limited number of osteoclasts detected in early stage RA tissues). RA is an autoimmune disease that involves interaction between various immune cells. Unlike in mouse, OSCAR has been reported to be reported to be expressed by other cell types besides osteoclasts in human tissues (Merck et al. 2005; Merck et al. 2004; Merck et al. 2006), therefore discovery of OSCAR immunostaining in cells with no osteoclast-like morphology was not something unexpected. The data presented in this chapter here is also in agreement with findings from earlier unpublished work carried out in the laboratory using other IgG_{2A} OSCAR antibody (O7) (Ali Shah's summer vacation scholarship 2006/07), which found that OSCAR staining was mainly associated with cells of the monocyte lineage, with cell morphology of macrophages and/or dendritic cells. Nevertheless, unlike in NFATc1 immunostaining, in which majority of the staining was observed as from T-lymphocyte-like cells, OSCAR was not expressed by lymphocytes (see Figure 4.6), which is in agreement with an earlier report (Merck et al. 2004). Based on previous literature, OSCAR may have role in inflammation and this is consistent with its expression on inflammatory-like cells noted in RA and OA tissues. For instance, Merck and colleagues (2006) demonstrated that cross-linking of OSCAR activated monocytes and neutrophils to release numerous pro-inflammatory cytokines. Earlier, the same group suggested OSCAR is an endocytic receptor involved with MHC class II antigen presentation on dendritic cells (Merck et al. 2004) and may play role in the maturation and prolonging survival of the dendritic cells (Merck et al. 2005) and monocytes (Merck et al. 2006).

The work on the immunostaining of FcRγ and the comparison between RA, OA and normal tissue groups in this current study is novel. Like DAP12, the discovery of FcRγ as part ITAM-mediated signalling pathway in osteoclastogenesis as well as in some inflammatory

cells have made it quite interesting to be studied in inflammation-mediated bone disease like RA. FcRγ is known to be involved in mediating cell signalling in numerous inflammatory cell types including T-lymphocytes (Juvet et al. 2013), NK cells (Hwang et al. 2012a), neutrophils (Mocsai et al. 2006) and macrophages (Ito et al. 2012). The wide range of cell types expressing FcRγ compared to OSCAR contributed to the higher mean SQA score for FcRγ immunostaining compared to OSCAR.

Another significant novel finding of this study is the positive immunostaining of TREM2 and OSCAR associated with vasculature in human synovial tissues (see Figure 4.8). This sort of observation has not been reported in the study by Herman and colleagues (2008), which limited their findings of OSCAR-positive immunostaining to cells with monocyte-like morphology only in close proximity to synovial vessels.

Due to technical limitations relating to dual immunolabeling on paraffin-embedded tissues, vasculature-like structures could no be dual-immunostained with the vasculature marker Factor VIII (Haynes et al. 2003), making it difficult to perform SQA scoring of TREM2/OSCAR-immunostained blood vessels observers usually can easily identify vasculature-like structures in tissues.

It is quite interesting to note more normal tissues displayed TREM2 immunostaining associated with vasculature-like structures compared to the more inflamed tissue groups, particularly the active RA (mentioned in 4.3.3.3.1). There are previous literatures suggesting TREM2 is more associated with anti-inflammatory function (Hamerman et al. 2006; Klesney-Tait et al. 2006). This is quite different to another member in TREM family, TREM1 that was found to be associated with inflammation in arthritis (Chen et al. 2008a; Kuai et al. 2009; Murakami et al. 2009). There is a suggestion that the expression of TREM2 could be down-regulated and this could be supported by a study investigating TREM mRNA expression in mice. In this study hepatic endothelial cells were treated with TNFα and IL-1β resulting in a reduction in TREM2 mRNA levels (Chen et al. 2008b).

In the early time during the experiments, the observation of OSCAR immunostaining associated with vasculature, particularly in RA tissues (see Figure 4.10B), was unexpected finding as there has been no previous study reporting OSCAR expression in endothelial cells until it quite recently in the context of vascular system and atherosclerosis (Goettsch et al.

2011; Nemeth et al. 2011). Nonetheless, the observation obtained from this presented study is novel in demonstrating *in situ* OSCAR expression in human tissues.

It is interesting to note that a number of OSCAR-immunostained cells were seen around the vasculature, and among these cells expressing OSCAR there were some cells that seemed to be infiltrating into the tissues from the vasculature (refer Figure 4.10D). This might be consistent with the findings of Herman and co-workers (2008) reporting monocytes in the peripheral circulation, particularly those that can differentiate to become osteoclasts, express high levels of OSCAR (Herman et al. 2008). It may be these cells expressing OSCAR observed in close proximity to the vasculature are osteoclast precursors.

It is also interesting to note that OSCAR immunostaining associated with the vasculature was observed in nearly all RA tissues (both active and inactive ones) as well as in some OA tissues studied. However, this was very rarely seen in normal tissue group (mentioned in 4.3.3.3.2). This sort of observation has prompted further investigation documented in Chapter 5 of thesis. In Chapter 5 the expression of OSCAR in endothelial cells was investigated in regard to inflammation such that seen in RA and OA. The very recent work by Goettsch and colleagues (2013) suggests that in endothelial cells, OSCAR may play a role in inflammation by inducing the expression of monocyte adhesion molecules such as ICAM-1 and thus promoting the recruitment of inflammatory cells into tissues.

In comparison to the RA and OA tissues, OSCAR immunostaining around the vasculatures in normal tissues appeared to show more OSCAR immunostaining inside blood vessels but not in the wall of blood vessels as seen in the other tissues (see in Figure 4.8). This could indicate OSCAR, possibly in secreted form, is present in serum. This observation is in agreement with previous findings reporting higher levels of soluble OSCAR in normal sera compared to sera from RA patients (Herman et al. 2008; Zhao et al. 2011). The findings in regard to OSCAR immunostaining on vasculature in this study is similar to previous work in the laboratory on OPG, which showed OPG expression associated with vasculature in PO, OA and normal tissues (Crotti et al. 2004; Haynes et al. 2003).

To date levels of soluble OSCAR have not been studied in human disease except in the sera of RA patients and normal healthy donors (Herman et al. 2008; Zhao et al. 2011). In this chapter synovial fluids from OA patients were used as the control as synovial fluids from

normal healthy individuals could not be obtained due to ethical concerns. The levels of factors such as OSCAR in synovial fluid is believed to be important in promoting osteoclastogenesis and underlying bone erosion in joints (Adamopoulos et al. 2006a). It is interesting to note that macrophages in synovial fluid, which is more in RA, are able to differentiate into fully functional osteoclasts (Adamopoulos et al. 2006b).

It is quite interesting to note that the levels of soluble OSCAR reported in RA and normal serum by two different studies (Herman et al. 2008; Zhao et al. 2011) differed much. The mean level of soluble OSCAR in RA versus normal serum reported by Zhao *et* al. (2011) was 1.259 ± 0.450ng/mL and 1.754 ± 0.426ng/mL respectively, however the earlier study by Herman and colleagues (2008) found a much higher level at 21 ± 12ng/mL for RA and 112 ± 56ng/mL for normal. Here through this study the OSCAR concentrations in both RA and OA synovial fluids were more than 100ng/mL (refer Figure 4.11). Examination on soluble OSCAR in serum was not carried out in this study as this would only be a repeatition to the previous works (Herman et al. 2008; Zhao et al. 2011).

OSCAR was discovered as exists in soluble form about half decade after the discovery of OSCAR (Khan 2007; Kim et al. 2002). The presence of a soluble form of OSCAR is supported by the discovery of signal peptide as part of protein structure of human OSCAR (Nemeth et al. 2011) (see Figure 1.5). Following this, researchers such as Herman and co-workers interested in bone biology and pathology have included soluble OSCAR as part of their investigation. Further studies on the origin and function of soluble OSCAR would be interesting. The possibility of relating levels of soluble OSCAR in the synovial fluid and disease activity might prove valuable in predicting disease outcomes.

4.5. Conclusion

Chapter 4 of this thesis presents investigation on the expression of osteoclast ITAM-related molecules as well as NFATc1 and cathepsin K in RA (both active and inactive classes), OA and control normal synovial membrane. These studies were complemented by novel investigations into the level of soluble OSCAR in synovial fluids from RA and OA patients.

Importantly it was found that there were markedly more cells positive for all ITAM-associated molecules (TREM2, DAP12, OSCAR and FcRγ) in active RA and OA tissue groups compared to the normal tissues. Statistically higher mean SQA scores in active compared to the inactive RA tissues were noted for TREM2, DAP12 and FcRγ immunostaining. NFATc1 was associated with lymphoid aggregates and markedly more aggregations of NFATc1-positive cells were seen in active RA tissues than the other groups.

The osteoclast marker, cathepsin K was highly expressed in normal tissues in comparison to the other tissue groups. As cathepsin K was associated with synovial fibroblast-like cells this expression is unlikely to be associated with osteoclasts in this instance. This is further supported by the fact that multinucleated osteoclast-like cells were only occasionally detected in a few tissues from RA and OA groups. This indicates that this marker is not a valid indicator of osteoclastogenesis in synovial tissues.

The novel findings in regard to TREM2 and OSCAR detection in vasculature were interesting. While TREM2 immunostaining associated with vasculature was observed more in the normal tissue groups, the opposite trend applied for OSCAR immunostaining with more positive vessel staining was seen in both the active and inactive RA tissues. The observations of positive immunostaining associated with vasculature, particularly OSCAR in RA tissues, suggests that the expression of the ITAM-associated immunoreceptor in endothelial cells could be associated with a role in the progression of inflammation-mediated diseases like arthritis. This speculation is also based on finding on recent studies proposing OSCAR expression in endothelial cells is associated with the inflammation during the development of artherosclerosis (Goettsch et al. 2012; Goettsch et al. 2011).

Complementary work investigating on the level of soluble OSCAR in synovial fluids taken from patients demonstrated high level of the secreted-form OSCAR in both RA and OA samples. Since this was the first study to measure soluble OSCAR in synovial fluid, comparison and analysis was only possible to be drawn between data from this work and findings on soluble OSCAR level in sera of RA patients from previous studies (Herman et al. 2008; Zhao et al. 2011). Unfortunately level of soluble TREM2 that has been discovered to exist in secreted form (Piccio et al. 2008) in those synovial fluids was not studied due to the limited volume of the samples. Another limitation that may confound the investigations on

soluble OSCAR levels in those synovial fluids could be related to the accuracy of titration during pipetting the “very thick” synovial fluids.

Overall this chapter illustrates expression of all investigated molecules (NFATc1, TREM2, DAP12, OSCAR and FcRγ) between patient groups, which could provide insights on the basis of underlying progression of inflammation-associated bone diseases like RA and OA. Higher expression of NFATc1 and ITAM-associated molecules in RA tissues for instance indicates that these molecules could have role in the progression of the disease. These factors may have important role in the progression of RA (as well as OA) and could become promising therapeutical targets for better treatment of these diseases in the future.

5. REGULATION OF OSCAR EXPRESSION IN ENDOTHELIAL CELLS

Some of the data presented in this chapter has been published in the following journal article:

Crotti TN, Dharmapatni ASSK, **Alias E**, Zannettino ACW, Smith MD, Haynes DR. The immunoreceptor tyrosine-based activation motif (ITAM)-related factors are increased in synovial tissue and vasculature of rheumatoid arthritic joints. Arthritis Research and Therapy 2012; 14:R245

5.1. Introduction

The vasculature and endothelial cells play an important role in the pathogenesis of RA. Endothelial cells regulate the migration of inflammatory cells from the circulation into specific sites like synovial fluid and synovial membrane (Middleton et al. 2004). Previous research has reported endothelial cells in RA patients become activated and more permeable (Middleton et al. 2004; Szekanecz et al. 2010). The activated endothelial cells phenotype is also associated with the expression of chemokines and adhesion molecules that enhances leukocytes recruitment and extravasation into the inflamed tissue sites. Infiltration of leukocytes into synovial tissue is critical for the initiation and progression of RA resulting in changes in the sublining layer of the tissues, which are among the earliest types of changes observed in RA.

The findings from the previous Chapter 4, in which OSCAR was seen associated with the vasculature in tissues from OA and more obviously in RA patients, had prompted study in this chapter to investigation on the expression of this ITAM-associated immunoreceptor in endothelial cells *in vitro*. This approach is justified and supported by other recent discovery of detection of OSCAR expression in endothelial cells *in vitro* (Goettsch et al. 2011). While the work conducted by Goettsch and colleagues (2011) was on OSCAR expression in response to oxidised low density lipoprotein (LDL) in arthrogenic endothelial cells, the studies presented here explore the response on OSCAR expression following treatment with pro-inflammatory cytokines TNFα and IL-1β (a model that better fits with RA). To date, there are very few studies showing the association between pro-inflammatory cytokines (such as TNFα and IL-1β) and the expression of OSCAR (Herman et al. 2008; Kim et al. 2009), however none has been done in endothelial cells. Findings from previous studies (Goettsch et al. 2012; Goettsch et al. 2011; Herman et al. 2008) have given strong justification for furthering investigation on the influence of pro-inflammatory cytokines, like TNFα, on OSCAR expression in endothelial as documented in this Chapter 5.

The significance of pro-inflammatory cytokines TNFα and IL-1β in RA has been well documented in the literature (refer section 1.3.4). There have been many studies reporting these two cytokines as the key cytokines expressed in tissues, sera and synovial fluids of RA

patients (Altomonte et al. 1992; Buchan et al. 1988; Feldmann & Maini 2001; Maini et al. 1995; Tak & Bresnihan 2000). TNFα is considered as a significant cytokine in RA pathology since it has been found to act as a key inflammatory regulator and could induce the release or production of other pro-inflammatory cytokine. This was demonstrated in studies which found out that therapy with anti-TNFα, such as infliximab, in RA patients reduced the levels of IL-1β and IL-6 in the sera (Musacchio et al. 2009; Popa et al. 2009).

TNFα and IL-1β play significant roles in propagating inflammation in RA either through induction of other chemokines, such as MCP-1 (Hachicha et al. 1993; Lisignoli et al. 1999; Schmidt et al. 2013; Taylor et al. 2000) or directly affecting the vasculature causing leukocyte extravasation into the inflammatory sites (Issekutz et al. 1994). While the main focus of this particular chapter is on endothelial cells, the role of these pro-inflammatory cytokines in promoting leukocyte recruitment into tissues in RA appears to be relevant to be discussed in more detail. The possible connection between OSCAR and atherosclerosis-associated inflammation suggested following the discovery of the regulation of OSCAR expression in endothelial cells by oxidised LDL (Goettsch et al. 2012; Goettsch et al. 2011) could provides another good justification for investigation on the regulation of the expression of the immunoreceptor by pro-inflammatory cytokines. Previous reports on the human endothelial cell culture *in vitro* found that there was increase in the expression of cell adhesion molecules such as E-selectin, P-selectin, ICAM-1, ICAM-2, vascular cell adhesion molecule 1 (VCAM-1), platelet-endothelial cell adhesion molecule 1 (PECAM-1) in response to TNFα treatment (Gerritsen et al. 1993; To et al. 1996). In addition, in an *in vivo* mouse model, in which synovial tissues taken from RA patients was transplanted, injection of TNFα was found to stimulate the expression of cell adhesion molecules (Proudman et al. 1999; Wahid et al. 2000). This induced expression of cell adhesion molecules subsequently increases the retention of mononuclear cells and promotes the recruitment of the cells from blood circulation into the tissues (Jorgensen et al. 1996).

As indicated earlier, the expression of OSCAR in vasculature in arthiritic tissues appears similar to the observation in a previous work carried out in the laboratory on OPG expression in vasculature in human tissues (Haynes et al. 2003). OPG shares few common similarities with OSCAR, such as expression in endothelial cells (Collin-Osdoby et al. 2001) and existence in secreted form, apart from its well-known role in bone homeostasis. Early studies on OPG-knockout mice not only found symptoms of osteoporosis indicating OPG role in

protecting bone, but also identified signs of arterial calcification, demonstrating its significance in vascular disease (Bucay et al. 1998; Min et al. 2000). However, a vascular protecting role of OPG might also be argued in human (Venuraju et al. 2010) since OPG level appears to be associated with the progression of several diseases. OPG was upregulated in patients with arthrosclerosis and is associated with the stability of the arthrosclerotic plaques (Golledge et al. 2004). Previous studies have reported higher levels of serum OPG in patients with diabetes and hypertension-associated cardiovascular diseases (CVD) as well as renal failure (Blazquez-Medela et al. 2012; Eleftheriadis et al. 2013; Golledge et al. 2004) (Knudsen et al. 2003). A more recent study has also suggested serum OPG can be used as a biomarker for arthrosclerosis in diabetes (Augoulea et al. 2013). High levels of OPG in serum have also been reported in patients with renal failure and undergoing hemodialysis (Gonnelli et al. 2005; Kazama et al. 2002).

Considering OPG expression in endothelial cells has been well established in the literature, the expression (mRNA and protein) of OPG in the *in vitro* endothelial cell culture model was also examined besides OSCAR. This allows comparison on the validity of cell culture model used with others that have been reported could be made. There have been several numbers in the past narrating OPG expression in endothelial cells. Haynes and colleagues (2003) were among the first to report OPG immunostaining associated with vasculature in synovial tissues of patients with arthritis (Haynes et al. 2003). In the context of regulation by pro-inflammatory cytokines TNFα and IL-1β for instance, stimulation with those cytokines in human umbilical vein endothelial cells (HUVECs) was found to promote increased OPG expression at both mRNA and protein levels. Similarly in human microvascular endothelial cells (HMVEC) Collin-Osbody et al. (2001) (Collin-Osdoby et al. 2001) also found induction in OPG mRNA expression following TNFα treatment.

5.1.1. Hypothesis

The expression of OSCAR in endothelial cells is upregulated by treatment with pro-inflammatory cytokines TNFα and IL-1β *in vitro*

5.1.2. Aims

- To investigate changes in OSCAR mRNA expression following treatment with either TNFα or IL-1β in *in vitro* culture of HUVECs
- To investigate changes in OSCAR mRNA expression following treatment with either TNFα or IL-1β in *in vitro* culture of BMEC
- To measure the level of soluble OSCAR in the supernatants of TNFα or IL-1β-treated and untreated BMEC culture
- To investigate changes in OSCAR protein expression *in situ* in TNFα or IL-1β treated and untreated BMEC through immunofluoresence

5.2. Methods

5.2.1. Cell Culture

In this chapter the investigation on the expression of OSCAR in endothelial cells was carried out in two *in vitro* models, which were primary HUVECs and human bone marrow endothelial cell (BMEC) line. Prior to the commencement of the experiment those cells were grown up in culture flasks (pre-coated with gelatine for better adherence of cells on the flask walls), with passaging to further grow the total number required for the experiments (Maciag et al. 1981).

Endothelial cells were grown in media known as HUVE media consisting of M199 media (JRH, Brooklyn, Vic, Australia) and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Scoresby, Vic, Australia) (Maciag et al. 1982). The media was supplemented with additional components, all purchased from Invitrogen Life Technologies (Carlsbad, CA, USA) that included HEPES buffer solution (0.02M), L-Glutamine (200mM), non-essential amino acids (0.1mM), sodium pyruvate (1mM), 7.5% sodium bicarbonate, penicillin and streptomycin. Cells were also fed with endothelial cell growth factor (EGF) (BD Biosciences) and heparin sodium salt (Sigma Aldrich, St. Louis, MO), which were freshly added to the media before used.

During the stage of rapid cell proliferation cells were only passaged once or twice depending on the total number of cells required, therefore the cells used in the experiment were from passage 3 or 4. During cell passaging the old media was removed and remaining cells were washed with PBS containing 0.01M EDTA before cells were subjected to incubation with trypsin at 37°C for 1 minute. The trypsin was later neutralised by applying the HUVE media (enzyme inactivation by FBS in HUVE media). The harvested cell suspension (following trypsinisation) was washed through centrifugation at 1500rpm for 5 minutes at 20°C. The cell pellet was resuspended with fresh HUVE medium to be ready for reseeding or splitting into new gelatine pre-coated flasks.

HUVECs cells were seeded at concentration 2 X 10^5cells per well (with 100µL media) in triplicates into 12-well plates (Falcon; Becton Dickenson Labware, New Jersey). Cells were cultured over a period of 48 hours for HUVECs in the presence and absence of 5ng/mL of human recombinant pro-inflammatory cytokines, TNFα (210-TA, R&D Systems, MN, USA) and IL-1β (201-LB-005, R&D Systems, MN, USA). RNA from the HUVECs culture was collected at time points 0, 2, 6, 12, 24 and 48 hours post-treatment with cytokines.

For the BMEC cultures, the most suitable concentration of cytokines (TNFα and IL-1β) was tested at two different concentrations (5ng/mL and 20ng/mL) and the mRNA expression of OPG and OSCAR was determined. Based on the results obtained (see section Results 5.3.2), BMEC were left growing for up to 72 hours in duplicates either with or without 5ng/mL TNFα and IL-1β. The total RNA (for qRT-PCR, section 5.2.2 below) was collected at 0, 6, 12, 24, 48 and 72 hours (for BMEC only) post-treatment with those cytokines. The BMEC cell culture supernatant (section 5.2.3) was also collected and immediately kept at -20°C to be used for OSCAR ELISA.

5.2.2. Real Time qRT-PCR

In general, the procedure for performing qRT-PCR was as described in detail in Chapter 2 and 3 of this thesis (sections 2.2.3 and 3.2.5).

5.2.2.1. RNA Isolation and Spectrophotometry

The cell RNA was isolated following addition of 200µL TRIzol reagent per well as per manufacturers instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). Triplicates were pooled together, and at each time point RNA was harvested using routine procedure for RNA extraction from cell culture (section 2.2.3.1) and RNA concentration was measured using a spectrophotometer (section 2.2.3.1).

5.2.2.2. Real Time Reverse Transcription

cDNA was generated from 1µg RNA per reaction using Superscript III Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) (sections 2.2.3.1 and 3.2.5.1).

5.2.2.3. Polymerase Chain Reaction (PCR)

The PCR methods were similar to that described in earlier in this thesis (sections 2.2.3.3 and 3.2.5.3). The primer sequences for OSCAR gene were also the same as presented in the previous chapters. In contrast to the PCR work presented in Chapter 2, which used hARP as the reference gene, this study on mRNA expression in endothelial cell lines used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. GAPDH was chosen as reference gene since it was used routinely in other similar studies (Reid et al. 2009; Zannettino et al. 2005). The OPG and GAPDH primers used were the used in Atkins et al. (2009). All samples were in triplicates for the qPCR reactions. Unlike the earlier presented PCR data, in this chapter the data was calculated in form of fold induction based on the ΔΔCT method (Livak & Schmittgen 2001).

5.2.3. ELISA of OSCAR and OPG in Cell Culture Supernatant

ELISA was performed on supernatant of the cell culture to determine the levels of soluble OSCAR. Collected supernatants were left as triplicates for each treatment and time point and not pooled together. Similar ELISA kit (USCN Life Science Incorporation, Wuhan, China) and protocols as described in previous Chapter 4 (refer section 4.2.3 above) were used. 100µL

of undiluted supernatants was vortexed before being tested alongside with the appropriate standards. ELISA was only done for samples from the selected time points 0, 24, 48 and 72 hours post-treatment time points.

5.2.4. Immunofluoresence on Monolayer Cell Culture

The expression of OSCAR protein was examined more closely using immunofluoresence. Cells (BMEC) were seeded in chamber slides (Nalge Nunc International, Rochester, NY, USA) at concentration of 5 X 10^4 cells per well. Following 48 hours of cell culture with or without TNFα and IL-1β (at optimal 5ng/mL concentration determined in prior work), the cells were fixed with 1:1 acetone/methanol solution (300µL per well) for 5 minutes at room temperature. The acetone/methanol solution was replaced with filtered PBS, then cells were air dried in a fume hood before being stored at -20°C until ready for immunofluoresence.

Before incubating with the primary antibodies, cells were washed with PBS. The fixed cells were incubated with 200µL primary antibodies, either polyclonal anti-OSCAR (sc-34233 Santa Cruz Biotech., CA, USA) as described in page 77 at concentration of 8µg/mL or anti-OPG (MAB805 R&D Systems, MN, USA) at 10µg/mL concentration in PBS containing 1% BSA, per well for about 1 hour. Antibody isotype controls included normal goat serum (005-000-121, Jackson Immunoresearch, PA, USA)(for anti-OSCAR) and 1B5 (for the OPG antibody). Cells were then washed 3 times by 5 minutes incubation in PBS to remove the unbound primary antibodies. The specifically bound primary antibodies were later tagged with fluorophore-conjugated secondary antibodies, anti-goat IgG (whole-molecule)-FITC (green) produced in rabbit (F7367, ImL, Sigma Aldrich) diluted at 1:400 as recommended by the manufacturer and Cy3-conjugated (red) AffiniPure Fab Fragment Donkey anti-mouse IgG (H+L) (715-167-003, Jackson Immunoresearch, PA, USA) at concentration of 300ng/mL through incubation for 30 minutes in dark at room temperature. Similar to the previous step, the cells were washed with PBS 3 times before and after counter-staining. The cells were counter-stained through incubation at room temperature in dark for 10 minutes with 4', 6-Diamidino-2-phenylindole (DAPI) (Sigma Aldrich, MO) at 300µL per well at concentration of 100ng/mL (diluted in PBS). Finally, the chambers were removed to allow the coverslips to be mounted on the fixed cell culture using SlowFade Gold antifade reagent (S36937, Invitrogen, Mulgrave, Vic, Australia).

The immunofluoresence of the cells was viewed with an Olympus BX51 in Adelaide Microscopy, SA, Australia. Images were captured using Cell F software integrated into the computer attached to the machine. The images were captured at magnification of 200X.

5.2.5. Statistical Analysis

Statistical differences in levels of soluble OSCAR as detected by ELISA between treatments and time points were evaluated using Kruskal Wallis followed by Mann-Whitney test (GraphPad Software Inc. La Jolla, SA, USA). Differences in the mRNA level were evaluated using one-way ANOVA test (Graphpad Software, CA, USA). A $p<0.05$ was considered statistically significant.

5.3. Results

5.3.1. OSCAR and OPG mRNA Expression in HUVECs

The experiment used 3 different donors of HUVECs. The level of mRNA expression for OPG and OSCAR was assessed to see if there was any consistent pattern in the mRNA expression in response to treatment with and without TNFα and IL-1β cytokines. The OPG and OSCAR mRNA level in HUVECs at those investigated time points are presented in Figure 5.1.

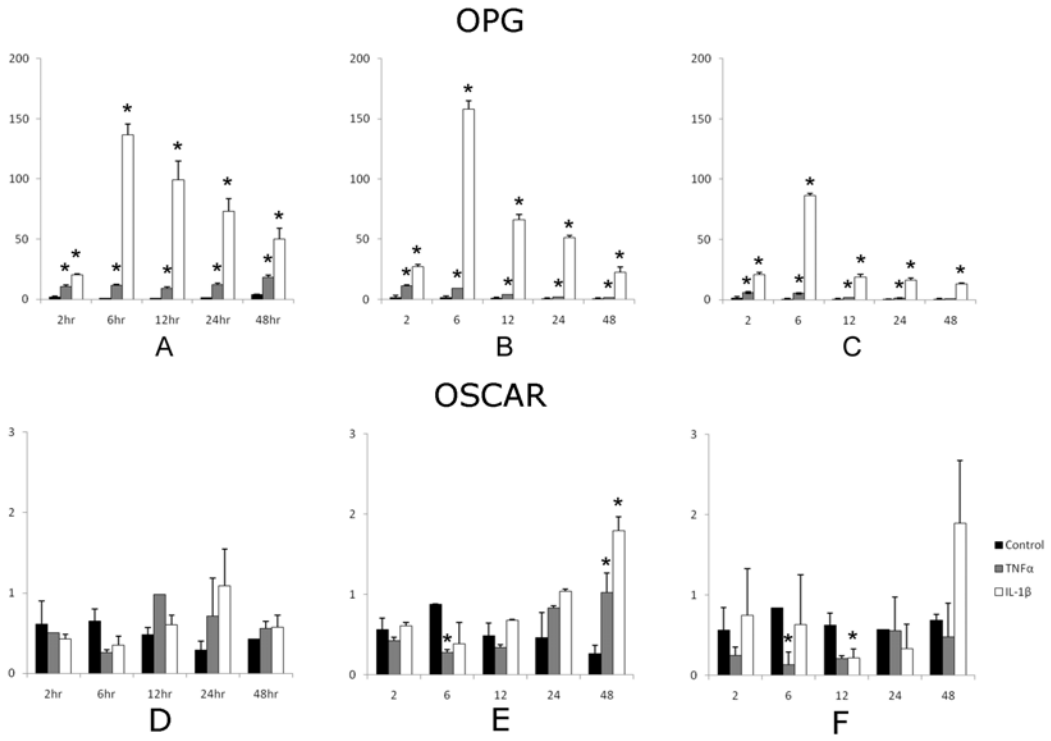

Figure 5.1: Fold change of OPG and OSCAR mRNA level in 3 donors of primary HUVECs over 48 hours period post-treatment with cytokines TNFα and IL-1β

This panel figure shows the fold change of OPG (top row) and OSCAR mRNA level (bottom row) in primary HUVECs from 3 donors (in panel column) at 2, 6, 12, 24 and 48 hours post-treatment with and without TNFα and IL-1β. The *y*-axis represents the fold change in the relative (to GAPDH) OPG mRNA level in comparison to the basal level, $2^{-\Delta\Delta CT}$, during the treatment with those cytokines. Significant difference from the controls is indicated by *, where $p<0.05$.

Based on the results obtained as shown in Figure 5.1 (upper panel row), IL-1β appeared to be a potent stimulator of OPG mRNA expression in all the HUVECs cultures as compared to the TNFα. There was also a consistent pattern between donors of largest stimulation on OPG mRNA expression seen 6 hours post-treatment with IL-1β before declining later. However, for TNFα, even though there was significantly higher OPG mRNA expression in the cytokine-treated samples, the stimulation appeared to be quite small compared to the IL-1β-induced levels. Consistent with the previous report from our group (Zannettino et al. 2005), it could be concluded that the data for OPG mRNA expression in primary HUVECs culture obtained from this study was reproducible and consistent with the previous report.

Unlike OPG, there was little change or induction of OSCAR mRNA in response to either TNFα or IL-1β (Figure 5.1 bottom row). In addition, there was no consistent trend in change of mRNA between donors that could be seen. In comparison to OPG, change in the OSCAR mRNA expression following treatment with TNFα and IL-1β in majority appeared to be very minimal (~ 1-fold change). Looking at the data on the level of OSCAR in relative to GAPDH, it could be concluded the level of OSCAR mRNA expression in those primary HUVECs was very low (Figure 5.2).

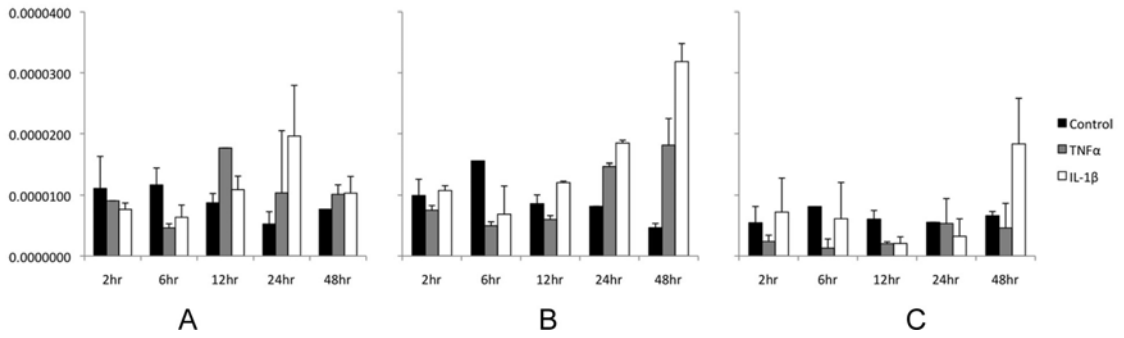

Figure 5.2: OSCAR mRNA level in relative to GAPDH in in 3 donors primary HUVECs over 48 hours period post-treatment in 3 treatment groups

This panel figure is consisted 3 graphs showing the relative OSCAR mRNA level (after normalised to GAPDH) in primary HUVECs from 3 donors at 2, 6, 12, 24 and 48 hours post-treatment with and without cytokines TNFα and IL-1β. The *y*-axis represents the relative (to GAPDH) OSCAR mRNA level, $2^{-\Delta CT}$, during the treatment with those cytokines. Significant difference from the control is indicated by *, where $p<0.05$.

5.3.2. Stimulation of OSCAR and OPG mRNA expression in BMEC by cytokines TNFα and IL-1ß

Since there was no significant change in the OSCAR mRNA expression seen in culture of primary HUVECs following treatments with pro-inflammatory cytokines TNFα and IL-1β, further investigations were carried out in BMEC.

Based on Figure 5.3 (top and bottom rows), there appeared to be no significant difference in both OSCAR and OPG mRNA expression between cytokine concentrations of 5 and 20ng/mL (except on OPG mRNA stimulation at 6 hours post-treatment). Therefore, it was concluded that higher concentration 20ng/mL of either TNFα and IL-1β did not result in significant change in the stimulation of mRNA expression. Hence, 5ng/mL of cytokine concentration was determined to be sufficient to induce an increase in mRNA expression in BMEC and used in the subsequent experiments on BMEC. Data from this preliminary experiment also gave an indication of the elevation of OPG and OSCAR mRNA levels following stimulation by either TNFα or IL-1β that be could seen particularly at the later time points.

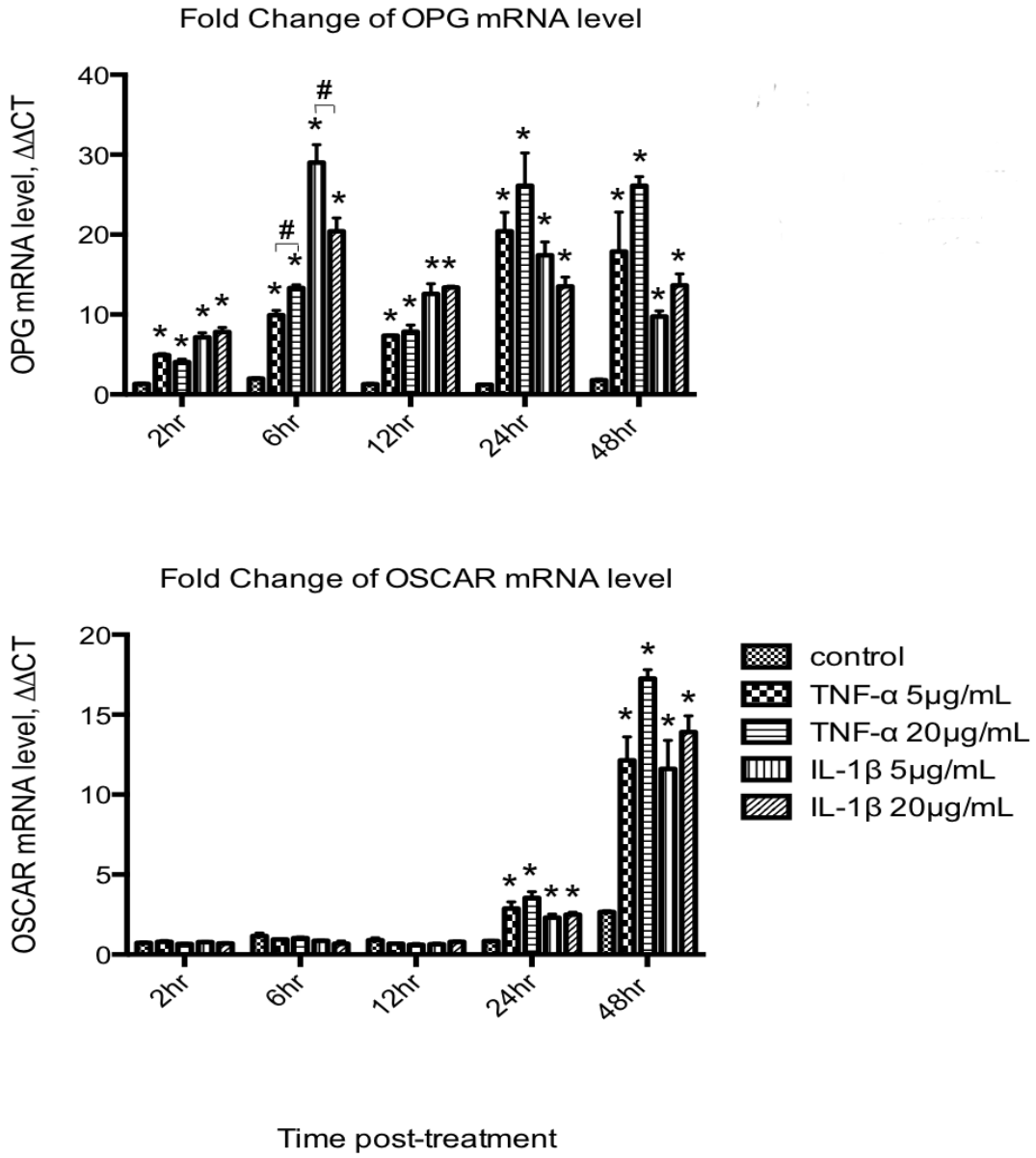

Figure 5.3: OPG and OSCAR mRNA expression in BMEC following treatment with either 5ng/mL or 20ng/mL of TNFα or IL-1β

This figure demonstrates the OPG (top graph) and OSCAR mRNA level (bottom graph) in BMEC at 2, 6, 12, 24 and 48 hours post-treatment with and without 5ng/mL or 20ng/mL cytokines TNFα and IL-1β. The *y*-axis represents the fold change in the relative (to GAPDH) OPG mRNA level in comparison to the basal level, ΔΔCT, during the treatment with those cytokines. Significant difference from the control is indicated by * and significant difference between cytokine concentrations is indicated by #, where $p<0.05$.

In the following experiment a 72 hour post-treatment time point was added in the setup since the finding from the earlier experiment demonstrated large change in mRNA expression modulated by TNFα and IL-1β took place at 24 and 48 hours post-treatment. As shown in Figure 5.4, there were large differences in the mRNA levels between treatment groups particularly at the later time points 24 to 72 hour time points.

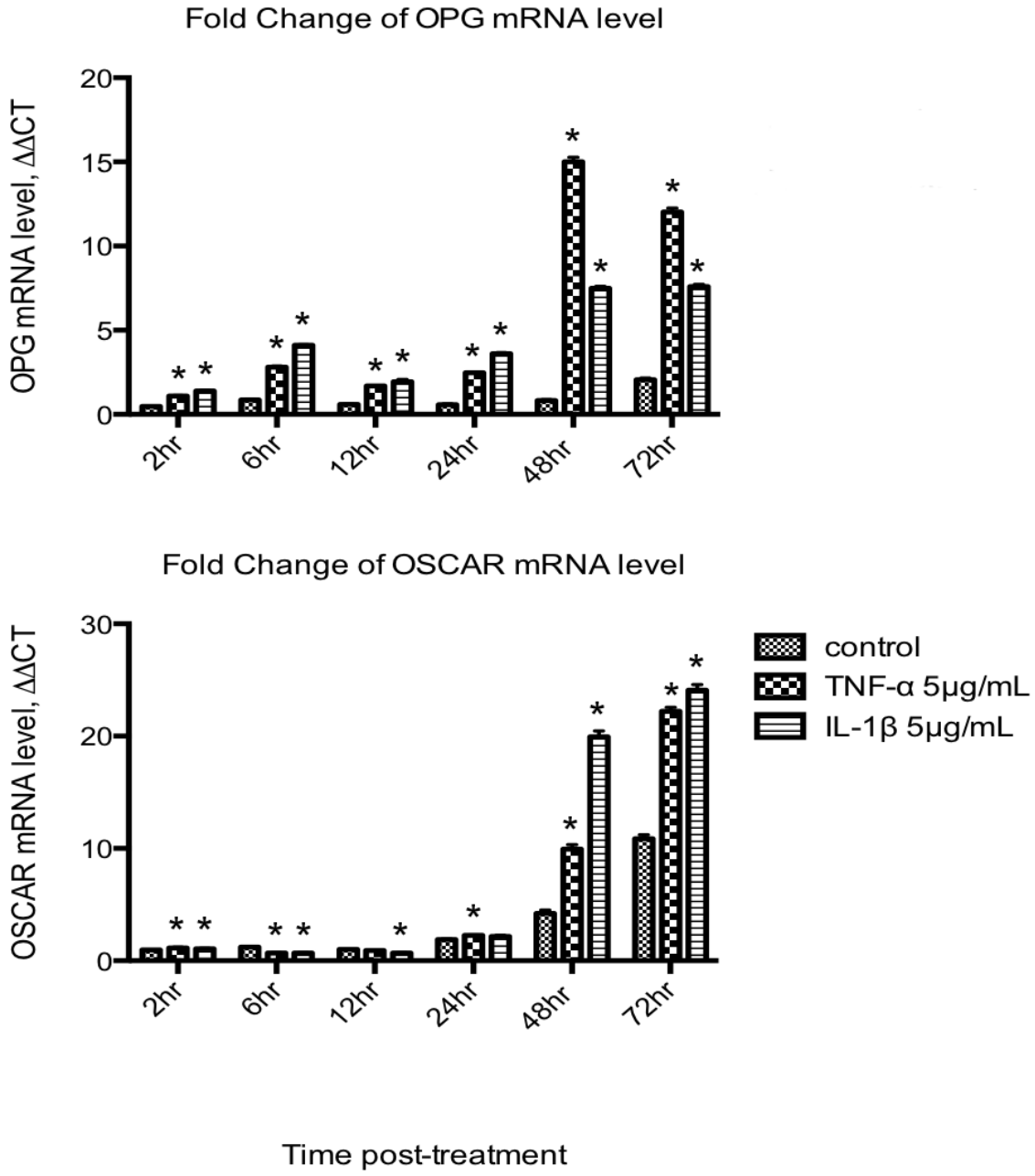

Figure 5.4: OPG and OSCAR mRNA expression in BMEC in response to 5ng/mL of TNFα or IL-1β up to 72 hours post-treatment

This figure demonstrates the OPG (top graph) and OSCAR mRNA level (bottom graph) in BMEC at 2, 6, 12, 24, 48 and 72 hours post-treatment with and without 5ng/mL cytokines TNFα and IL-1β. The *y*-axis represents the fold change in the relative (to GAPDH) OPG mRNA level in comparison to the basal level, ΔΔCT, during the treatment with those cytokines. Significant difference from the control is indicated by *, where $p<0.05$.

Another experiment incorporating samples in triplicates was carried out. However due to the large amount of samples the number of time points in the experiment was reduced to 24, 48 and 72 hours post-treatment. For the same reason, the PCR setup was also limited to duplicates instead of triplicates for each sample. Data from the corresponding experiment on BMEC culture is presented in the following Figure 5.5.

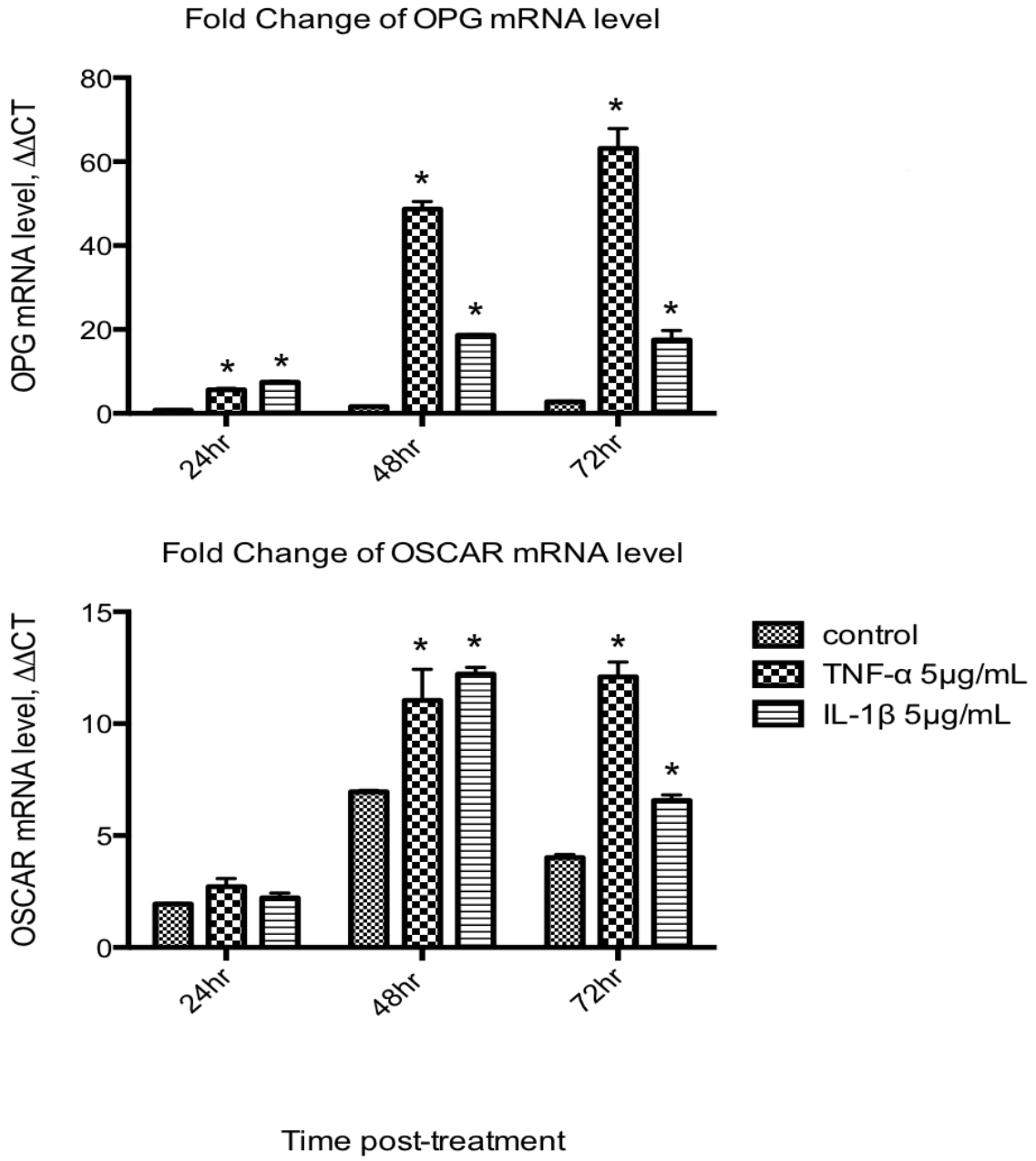

Figure 5.5: OPG and OSCAR mRNA expression in BMEC culture (in triplicates) in response to treatment with 5ng/mL TNFα or IL-1β

This figure demonstrates the OPG (top graph) and OSCAR mRNA level (bottom graph) in BMEC (in triplicates) culture at later time marks of 24, 48 and 72 hours post-treatment with and without 5ng/mL cytokines TNFα and IL-1β. The *y*-axis represents the fold change in the relative (to GAPDH) OPG mRNA level in comparison to the basal level, ΔΔCT, during the treatment with those cytokines. Significant difference from the control is indicated by *, where $p<0.05$.

Data from the experiment indicated that there were big variations in OPG and OSCAR mRNA level between replicates of BMEC. However, significant differences in OPG and OSCAR in response to TNFα and IL-1β treatment could still be seen particularly at the later time points.

Comparison of both OPG and OSCAR mRNA expression in BMEC culture during those series of three experiments could easily be seen by refering to Figure 5.6. Despite there were obvious differences in the mean magnitude of mRNA expression between experiments for the corresponding sample groups, it was noticed that data obtained from those experiment generally shared similar trend of mRNA regulation following TNFα and IL-1β treatment. Exception could be observed for IL-1β-induced OSCAR mRNA expression at 72 hours post-treatment that appeared to be lower than in the TNFα-treated samples. From Figure 5.6 (bottom row), the 48 hours post-treatment with cytokines TNFα and IL-1β could be regarded as the optimal time mark for stimulation of OSCAR mRNA expression in BMEC culture by TNFα and IL-1β.

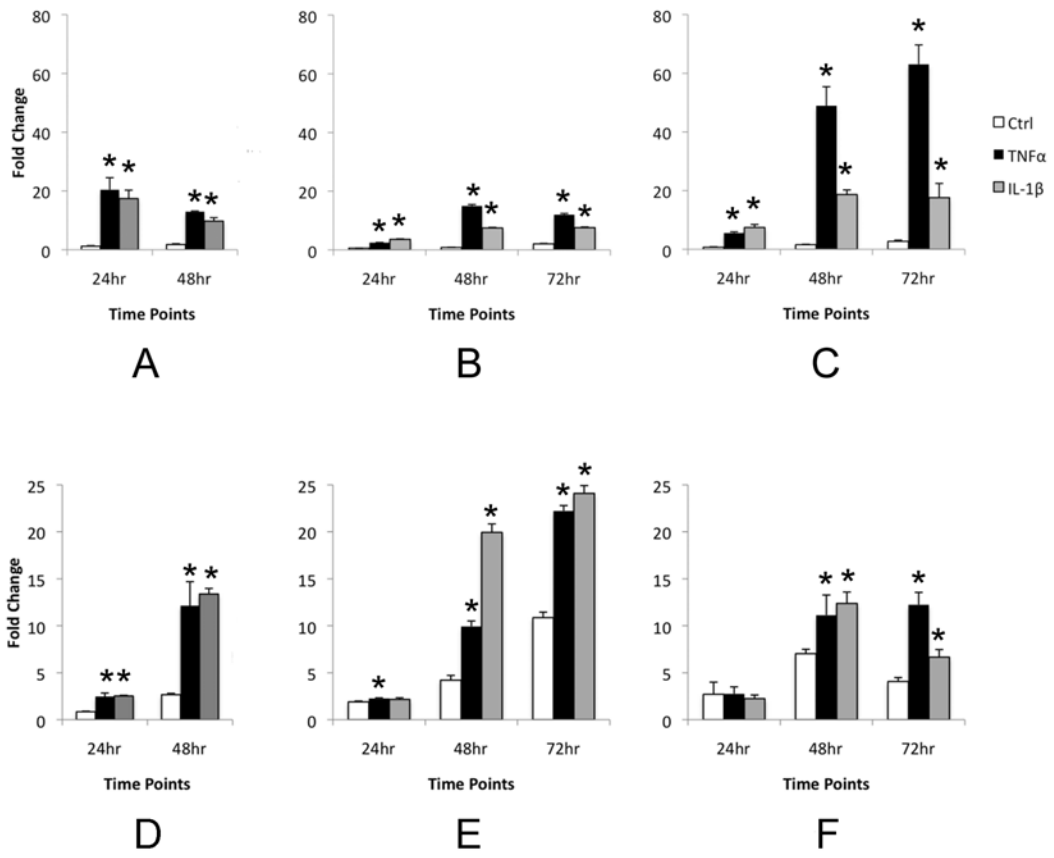

Figure 5.6: OPG (top) and OSCAR mRNA expression (bottom) in BMEC in response to treatment with 5ng/mL TNFα or IL-1β in 3 different experiments

This panel figure is consisted 6 graphs showing the OPG (top row) and OSCAR mRNA level (bottom row) in BMEC from 3 different experiments at 24, 48 and 72 hours post-treatment with and without 5ng/mL cytokines TNFα and IL-1β. The *y*-axis represents the fold change in the relative (to GAPDH) OPG mRNA level in comparison to the basal level, ΔΔCT, during the treatment with those cytokines. Significant difference from the control is indicated by *, where $p<0.05$.

5.3.3. Expression of soluble OSCAR following TNFα and IL-1β treatment

While many investigations on the regulation of OSCAR expression were carried out at the mRNA level, examination at protein expression was also done to complement the gene expression findings. Only BMEC cell culture supernatants from the time points 24, 48 and 72 hours post-treatment were subjected to the ELISA.

Data obtained from the OSCAR ELISA indicated that there was significantly higher level of soluble OSCAR being secreated by BMEC culture following stimulation by 5ng/mL of TNFα or IL-1β as compared to the controls at 24, 48 and 72 hour post-treatment. The pattern of soluble OSCAR expression in response to TNFα or IL-1β stimulation followed a similar pattern to the mRNA level (Figure 5.5 earlier). Both protein (Figure 5.7) and mRNA expression (Figure 5.5 bottom row and middle column) suggested that IL-1β was more potent in inducing OSCAR in BMEC than TNFα at 48 hours post-cytokine treatment.

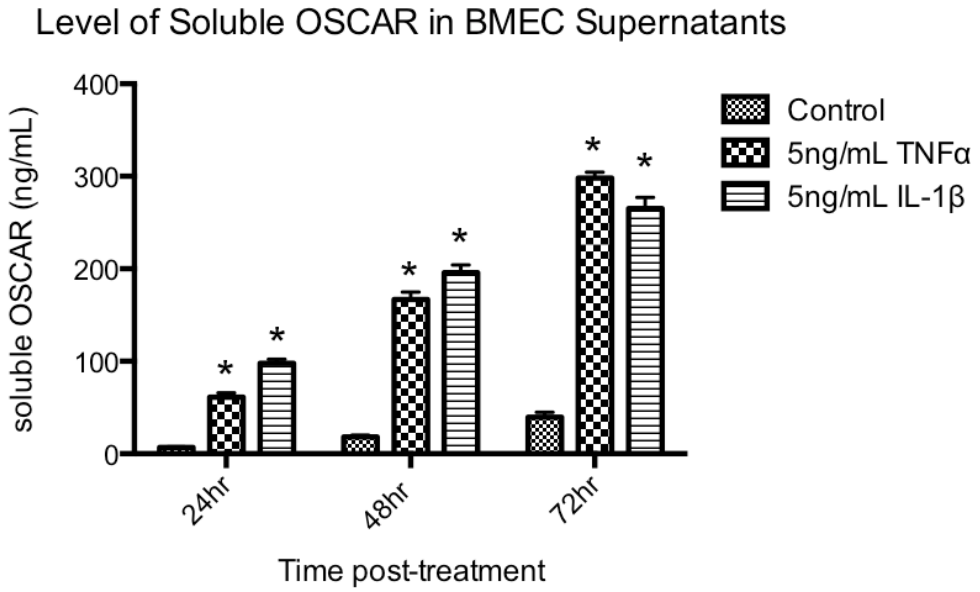

Figure 5.7: Expression of soluble OSCAR by BMEC culture following treatment with TNFα and IL-1β

This graph shows the level of soluble OSCAR secreated in BMEC media supernatant at 24, 48 and 72 hours treatment with and without 5ng/mL cytokines TNFα and IL-1β. The *y*-axis represents the fold change in the relative (to GAPDH) OPG mRNA level in comparison to the basal level, ΔΔCT, during the treatment with those cytokines. Error bars indicate the standard error of means obtained from 3 replicates within a sample group. Significant difference from the control is indicated by *, where $p<0.05$.

5.3.4. Detection of OSCAR expression in BMEC *in situ*

OSCAR protein expression following cytokines TNFα or IL-1β treatment was also investigated by immunofluoresence staining to detect membrane-bound OSCAR on the BMEC. Immunofluoresence was also carried out for OPG expression, as this has not been done in BMEC. Samples from 48 hours cytokines treatment only were included in the study as the PCR data demonstrated maximal stimulation of both OPG and OSCAR mRNA took place at this time point.

The immunofluorence for OPG demonstrated that the molecules could be detected in BMEC at 48 hours cytokine treatment particularly the one treated with 5ng/mL of TNFα and IL-1β (Figure 5.8B and C). It also appeared that there was more expression of OPG in BMEC treated with IL-1β as compared to TNFα. However, in the control, OPG was only minimally detected in the unstimulated BMEC suggesting that there was little expression of OPG taking place in the BMEC culture. In antibody isotype negative control fluorescence was also minimally detected (not shown).

As for the OSCAR expression, it appeared that there was induction of expression following stimulation by TNFα as compared to IL-1β (Figure 5.8E). There was minimal expression of OSCAR in non-cytokine treated BMEC (Figure 5.8D and F). This indicates that stimulation with TNFα resulted in increased production of OSCAR protein as detected by immunofluoresence.

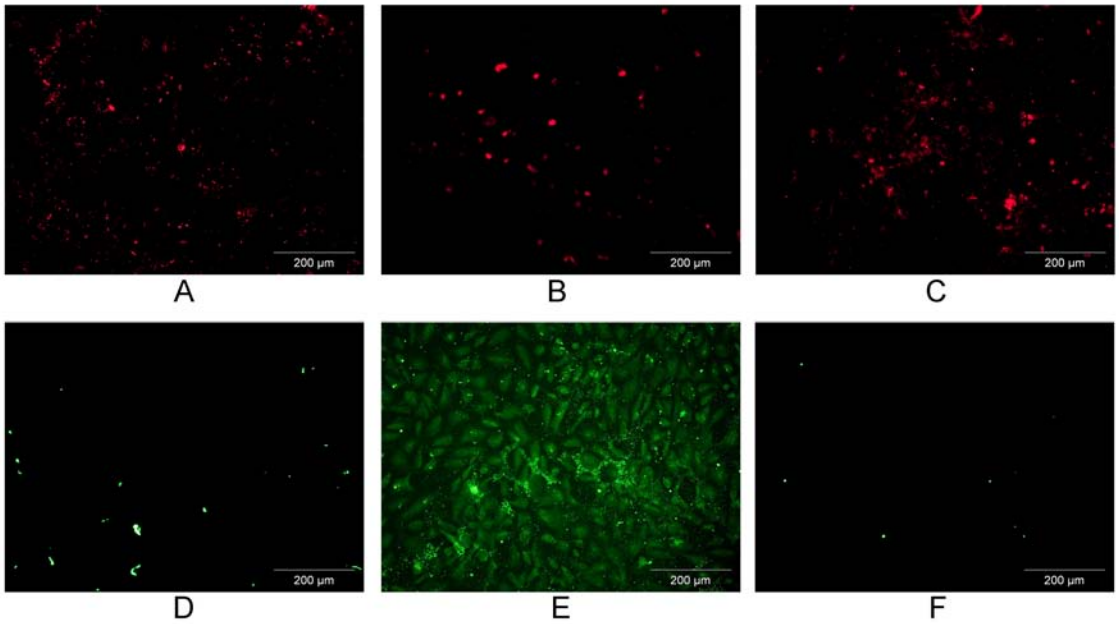

Figure 5.8: Detection of OPG and OSCAR expression by BMEC culture following 48 hours treatment with 5ng/mL of TNFα or IL-1β through immunofluoresence

This panel figure is consisted photos showing the detection of OPG (top row) and OSCAR (bottom row) proteins in non-cytokine treated control (**A** and **D**), as well as in the BMEC culture treated with 5ng/mL of TNFα (**B** and **E**) and IL-1β (**C** and **F**). Positive immunofluoresence for OPG and OSCAR is indicated by the red (Cy3-tagged) and green fluorescence (FITC-tagged) respectively. Photos were captured at original 200X magnification.

5.4. Discussions

The studies presented in this chapter were designed to complement work from the previous chapter. In Chapter 4, there was interesting finding that the vasculature was found to express osteoclast-associated ITAM immunoreceptors, TREM2 and OSCAR especially in RA tissues. OSCAR immunostaining of the vasculature was particularly interesting to be focused on as TREM2 was viewed more as having anti-inflammatory function. Chen and colleagues (2008) found treatment with TNFα and IL-1β upregulated protein expression of TREM1 and TREM3 detected by Western blotting, however, they also suppressed TREM2 in mice liver endothelial cells. This prompted further investigation on OSCAR expression *in vitro* using either primary endothelial cells or an endothelial cell line.

The observation of OSCAR immunostaining associated with vasculature structures, particularly in human RA synovial tissues, was similar to observations of OPG immunostaining reported about a decade ago (Crotti et al. 2004; Haynes et al. 2003). Subsequent studies on OPG expression in endothelial cell *in vitro* have reported abundant expression of OPG in HUVECs (Corallini et al. 2011; Stein et al. 2008; Zannettino et al. 2005) and the expression of OPG in endothelial cells was later found to affect various roles of endothelial cells. There were studies suggesting that the upregulated expression of OPG by proinflammatory cytokines could contribute towards the dysfunction of endothelial cells (Collin-Osdoby et al. 2001; Secchiero et al. 2006). Later studies found that expression in OPG in endothelial cells was associated with development and progression of artherosclerosis and CVD (Goettsch et al. 2011). It was found that higher levels of OPG could be secreted by inflammatory cells present in artherosclerotic plaques (Vik et al. 2007) and this was associated with the dysfunction of endothelial cells by inducing the expression of adhesion molecules like ICAM-1, VCAM-1 and E-selectin (Mangan et al. 2007; Zauli et al. 2007) that would promote attachment and accumulation of leukocytes. Therefore investigation of OPG expression was carried out to compare it to OSCAR expression and regulation *in vitro*. This was carried out with the HUVECs model used in previous publications (Corallini et al. 2011; Stein et al. 2008; Zannettino et al. 2005).

There has been a range of human endothelial cells used in *in vitro* experimental works done in past. Among them are HUVECs, BMEC, microvascular endothelial cells (Collin-Osdoby et al. 2001) and each cell type may behave differently. For instance, RANKL has been shown to be expressed in human microvascular endothelial cells (Collin-Osdoby et al. 2001). However there was no RANKL mRNA detected in HUVECs (Zannettino et al. 2005). In this particular work HUVECs was the first choice for the *in vitro* endothelial cell model before BMEC was also opted for the work following minimal changes in mRNA expression obtained from HUVECs culture.

As the name implies, primary HUVECs are isolated by flushing human umbilical cord with collagenase as reported in the literature (Gamble et al. 1985). Primary HUVECs were opted as the first choice of *in vitro* endothelial cell culture model for a couple of reasons. First HUVECs was a very commonly used *in vitro* endothelial cell model for studying OPG expression in many studies (Corallini et al. 2011; Stein et al. 2008; Zannettino et al. 2005). Following the discovery of positive OPG immunostaining on vasculature in tissues (Haynes et al. 2003), following-up study *in vitro* was conducted in HUVECs, in which induction of OPG expression following stimulation with either TNFα or IL-1β was observed (Zannettino et al. 2005). Considering the observation of OSCAR immunostaining on vasculature mainly in the human RA synovial tissues was quite similar to previous published finding of OPG on vessels (Haynes et al. 2003), the use of HUVECs to study OSCAR expression in endothelial cells *in vitro* following cytokine stimulation was very relevant. In addition, OSCAR expression has also been shown in HUVECs through immunofluoresence and RT-PCR (even though at low level) recently (Goettsch et al. 2011). Furthermore, HUVECs is a primary endothelial cell type that is closer to the endothelial cells in tissues and *in vivo*, not a cell line that more likely could undergo phenotype alternations following manipulation. All these together support the choice of using HUVECs in this study.

BMEC used in this study is a cell line that has been used for for studying human endothelial cells *in vitro*. BMEC was first isolated and characterised nearly 20 years ago (Almeida-Porada & Ascensao 1996; Masek & Sweetenham 1994; Schweitzer et al. 1995) and was recommended for studies investigating the role of endothelial cells for "homing" of hematopoietic progenitor cells to the bone marrow (Rood et al. 1999). Rood and colleagues (1999) found no difference or preferential adhesion of hematopoietic progenitor cells to human BMEC in comparison to HUVECs. However, an earlier study found otherwise and

showed there was better adherence of CD34-positive to TNFα-stimulated BMEC compared to similarly treated HUVECs (Schweitzer et al. 1997).

The expression of OSCAR and TREM2 in vasculature in rheumatoid arthritis was likely the result of inflammation in the tissues, presumably via the effect of pro-inflammatory cytokines like TNFα and IL-1β. These two cytokines are reported to be expressed in abundant in RA synovial membrane and may play important role in stimulating osteoclast formation in the tissues of RA (Chu et al. 1991). The regulation of OSCAR by either TNFα or IL-1β was first examined at the gene or mRNA level only (therefore based on the qRT-PCR data only) before proceeding to the investigation at the protein level. It should be noted that the qRT-PCR data was analysed using $2^{-\Delta\Delta CT}$ method (data in form of mean fold change) instead of $2^{-\Delta CT}$ (mRNA level in relative to the reference gene) (Livak & Schmittgen 2001) as applied for data presented in the previous chapters 2 and 3. This was justified by the presence of 0 hour time point (post-cytokine treatment) as the point for basal expression and data from the other evaluated time marks will be calculated as fold change over this reference point. GAPDH was used as the reference gene for normalisation as it is a common housekeeping gene used for endothelial cells reported in the literature, particularly for HUVECs (Mangan et al. 2007; Secchiero et al. 2008; Zhang et al. 2002).

In the experiment on HUVECs, cytokine concentration of 5ng/mL was chosen since this concentration has been reported to be able to activate HUVECs without causing induction of apoptosis (Molostvov et al. 2001). Zannettino and co-workers (2005) found that stimulation on HUVECs with either TNFα or IL-1β at 5ng/mL elevated OPG mRNA levels by 8 to 9-fold. This study saw the induction of OPG mRNA expression following the cytokine treatment was inconsistent between donors, even though all of them showed the increase. The present study also noted that 5ng/mL of IL-1β was far more potent in stimulating OPG mRNA expression (more than 50-fold induction) than TNFα of similar concentration (about 3 to 6-fold change) at 24 hours treatment. In another previous study, it was reported that OPG mRNA was also found to be expressed in primary HMVEC *in vitro* and could be upregulated by treatment with TNFα and IL-1α (with effective cytokine concentration ranging from 0.1 to 10nM) (Collin-Osdoby et al. 2001).

With regard to the OSCAR mRNA expression in HUVECs, there was variation in responses to the cytokines between donors. As a consequence it may be inappropriate to compare the

data obtained in this study to that previously reported (Goettsch et al. 2011), which did not use multiple donors of the primary cells. Due to the inconsistent change in OSCAR mRNA expression following TNFα or IL-1β treatment the experiment was not extended to investigation on protein expression in HUVECs. However, it was decided that the investigation was continued on BMEC instead of primary HUVECs. In addition, this would be novel as there has been no report on OSCAR in BMEC.

Unlike the studies in HUVECs in which 5ng/mL is regarded as an optimum cytokine concentration (Molostvov et al. 2001; Zannettino et al. 2005), there is not much literature reporting suitable concentration of TNFα and IL-1β cytokines to induce cell responses` in BMEC. Therefore in the first experiment with BMEC, the mRNA levels following induction by TNFα and IL-1β was compared between 5ng/mL and 20ng/mL. Study outcome there was little difference on the induction of OSCAR gene expression in BMEC at the 2 concentrations of TNFα and IL-1β (Figure 5.3 bottom row). Therefore, 5ng/mL was chosen as it was also used for HUVECs.

Overall in the 3 experiments with BMEC mRNA expression for both OPG and OSCAR following cytokine stimulation was consistent between experiments. Consistent with the findings here the induction of mRNA expression in endothelial cells has been reported as be able to peak as early as 1 hour post-stimulation (Hashimoto et al. 1994).

While it has been quite well established that OPG is detected in wide range of endothelial cell types such as in human HMVEC and HUVECs and could play significant role in endothelial cells (Collin-Osdoby et al. 2001; Secchiero et al. 2006; Secchiero et al. 2008; Stein et al. 2008; Zannettino et al. 2005; Zauli et al. 2007), to the author's knowledge, detection of OPG expression in human BMEC cell line (both at mRNA and protein level) has never been documented and hence could be considered as novel. Using the antibody MAB805, which has been thought to immunostain OPG in dimeric form expressed by endothelial cells (Haynes et al. 2003), this study demonstrated OPG expression at protein level in BMEC as detected by immunofluoresence.

This study provides support for the work by Goettsch and colleagues (2011) demonstrating endothelial cells as another cell type expressing OSCAR. This is also the first study to demonstrate the upregulation of OSCAR expression in endothelial cells by both TNFα and

IL-1β. Earlier induction of OSCAR has been shown in human CD14-positive monocytes (Herman et al. 2008) and in murine osteoclasts by TNFα and IL-1α respectively (Kim et al. 2009). A very recent study by Sinningen and colleagues (2013) found that treatment with 50ng/mL of TNFα resulted in stimulation of OSCAR mRNA expression in RAW264.7 cell line by 2-fold.

Significant induction of OSCAR mRNA expression following treatment with either TNFα or IL-1β from the first two experiments with BMEC was consistently noted at and after 24 hours post-cytokine treatment. Such finding may indicate that the induction in the OSCAR mRNA expression was a secondary response mediated by other pathway(s) and not directly stimulated by those TNFα and IL-1β cytokines. This sort of observation has helped in designing the experimental setup for the third experiment with BMEC by limiting the time points, hence allowing more replicates of samples to be included.

While the qRT-PCR data gave an indication of largest stimulation of OSCAR mRNA expression taking place at 48 hour post-treatment, it was quite surprising to note that only TNFα appeared to induce expression of OSCAR protein as detected by immunofluoresence, meanwhile data from the qPCR data indicated that IL-1β stimulated OSCAR mRNA expression more than TNFα did.

However, it was interesting to note that there was correlation in pattern after the cytokine treatment between the OSCAR mRNA level (refer Figure 5.6F) and the level (or concentration) of soluble OSCAR secreted into BMEC culture supernatant (Figure 5.7). ELISA was carried out on the supernatants of BMEC only (not HUVECs) since only the PCR data from the BMEC culture appeared to show induction of OSCAR mRNA expression. The high expression of soluble OSCAR in BMEC culture supernatant in response to TNFα and IL-1β stimulation may provide insights in explaining high level of soluble OSCAR in synovial fluids of RA patients (refer section 4.3.4). While the expression of membrane bound OSCAR in HUVECs would be detected by immunofluoresence (Goettsch et al. 2011), this study demonstrated protein expressed is most likely in soluble isoform of OSCAR, consistent with that previously described (Khan 2007; Kim et al. 2002).

While the association between OSCAR and its possible role in the development of artherosclerosis has been investigated (Goettsch et al. 2012; Goettsch et al. 2011), the present

study is the first in line to look into endothelial cell-expressed OSCAR in the context of chronic inflammation. It is consistent with the idea that OSCAR should have no longer seen as a molecule involved in bone homeostasis only, but also possibly plays other role in the progression of inflammation-mediated bone loss like RA.

Pro-inflammatory cytokines TNFα and IL-1β are likely to contribute to high OSCAR protein secreted in RA and OA synovial fluids described in previous Chapter 4 and in serum described by Herman and colleagues (2008) through induction on the expression of the molecule in the endothelial cells. Interestingly Herman et al. (2008) suggested that the level of soluble OSCAR in serum could be associated with protective function against bone erosion in RA, however this remains to be proven by further investigations.

It would be interesting to speculate on the role of OSCAR expressed by endothelial cells in the recruitment and infiltration of inflammatory cells into tissues. In dendritic cells activation of OSCAR in DC promotes expression of MCP-1 (Merck et al. 2006), which is the chemoatttractant recruiting leukocytes to site of inflammation. Literature has indicated that IL-1β and TNFα might be involved in promoting recruitment of osteoclast precursors (which is monocyte/macrophage-derived cells) (Issekutz et al. 1994) through endothelial cell activation (Kindle et al. 2006). Activated endothelial cells are believed to be important in supporting each phase of inflammatory process (Pober & Sessa 2007). Meanwhile a recent discovery by Goettsch and coworkers (2012) demonstrated induction in the expression of adhesion molecules (that mediating attachment of monocytes to the endothelium) following OSCAR overexpression by endothelial cells *in vitro*. Taken together, there is possibility that OSCAR may play role not just only in mediating osteoclastogenesis, but also in promoting the recruitment of inflammatory cells to the inflammation sites.

The findings of this chapter and the previous chapter open up new areas of research on OSCAR to be explored in the future. It would be interesting to investigate the regulation of the OSCAR expression in the presence of TNFα and IL-1β receptor antagonists in order to gain insight of the receptors involved in OSCAR regulation in the endothelial cells. It would also be interesting to investigate whether the stimulation of OSCAR expression in BMEC is mediated by NFATc1, which was observed in human coronary artery endothelial cells following stimulation by oxidized LDL (Goettsch et al. 2011).

OPG expression in endothelial cells (Haynes et al. 2003) has been associated with vascular homestasis and pathology (Corallini et al. 2008; Reid & Holen 2009; Secchiero et al. 2006). Similarly, cytokine regulation on OSCAR expression in endothelial cells as observed in this study alongside with the recent discovery of possible role in the inflammation and artherosclerosis (Goettsch et al. 2012; Goettsch et al. 2011) and the statistics of high mortalitiy in RA patients due to CVDs (Pham et al. 2006) could provide a meaningful insight on the role of endothelial cell-expressed OSCAR has in the context of RA.

5.5. Conclusion

This study is the first to report the regulation of OSCAR mRNA and protein expression in response to stimulation by pro-inflammatory cytokines TNFα or IL-1β in endothelial cells *in vitro*.

Despite the fact that there was induction of OPG mRNA expression observed following stimulation by either TNFα or IL-1β, in which OPG mRNA expression peaked at 6 hours post treatment and similar to the previous study in HUVECs (Zannettino et al. 2005), there was an inconsistency in the change in the OSCAR mRNA level between donors of primary HUVECs. Hence, BMEC was used in the *in vitro* model in the study. Data from these experiments consistently indicated that the largest increase in the OSCAR mRNA level took place at 48 and 72 hours post cytokine treatment.

The level of of soluble OSCAR in the supernatants of BMEC culture was consistent with the mRNA levels in response to the cytokines. Immunofluoresence for OSCAR on the BMEC culture at 48 hours post-treatment indicated that there was more OSCAR protein produced following stimulation by TNFα.

Together the data suggest that the regulation on OSCAR in RA tissues may have various roles in disease regulation. These include, adhesion and migration of cells from the vasculature, involvement endothelial cell damage (possibly associated with atherosclerosis) and the regulation of bone metabolism in disease. Further studies of OSCAR expression in endothelial cells open new gate for future scientific investigations.

6. SUMMARY AND CONCLUDING REMARKS

Bone remodelling is regulated by tight balance between bone resorption by osteoclasts and bone formation by osteoblasts. Imbalance between these two processes, either through reduced rate of bone formation or excessive osteoclast activity resulting net bone resorption could result in pathological bone loss. Peri-implant or peri-prosthetic osteolysis (PO) and rheumatoid arthritis (RA) are two examples of pathological bone loss condtions that have been associated with net bone resorption resulted from excessive osteoclast activity (Gravallese et al. 1998; Holding et al. 2006; Shen et al. 2006).

As a part of strategy for therapy against pathological bone loss as observed in RA and PO, the expression of molecules regulating of osteoclast differentiation and activity at the molecular level has long been studied. The OPG/RANK/RANKL triad has been found to be the main signalling pathway mediating osteoclast differentiation as well as activity and the expression of these molecules detected in RA and PO tissues has been demonstrated to have strong association with the feature of bone loss observed in those diseases (Crotti et al. 2002; Gehrke et al. 2003; Haynes et al. 2003; Haynes et al. 2001b; Haynes et al. 2004; Mandelin et al. 2003; Vanderborght et al. 2004). Studies have also indicated that regulation on these molecules has been found to be an effective approach in limiting bone loss in pathological conditions like RA (Pettit et al. 2001; Romas et al. 2002) and PO (Itonaga et al. 2000).

From accumulating evidence it has been strongly suggested that immunoreceptor tyrosine-based based activation motif (ITAM)-mediated pathway, which has been discovered in osteoclasts early in this century, plays role in mediating co-stimulatory signals for osteoclastogenesis (Humphrey et al. 2005; Koga et al. 2004). While two immunoreceptors, TREM2 and OSCAR together with their corresponding membrane adaptor molecules DNAX activation protein of 12kDa (DAP12) and Fc receptor gamma (FcRγ), have been identified as the molecules mediating the co-stimulatory ITAM-mediated signalling pathway in osteoclasts, there have been limited studies investigating on these molecules in the context of skeletal pathology. Hence the studies described here were designed to provide more holistic

investigation on osteoclast-associated ITAM-related molecules (TREM2, OSCAR, DAP12 and FcRγ), as well as the key transcriptional factor in osteoclasts nuclear factor of activated T-cells 1 (NFATc1), in the context of PO and RA pathology. Studies presented here investigate on the expression of those osteoclast-associated ITAM-related molecules in RA and PO tissues (in comparison to the control tissues) followed by *in vitro* work used to verify the observations on the tissues obtained earlier.

Presentation of the studies in this thesis commenced with investigation on both protein and mRNA expression of NFATc1, TREM2, DAP12, OSCAR and FcRγ in tissues adjacent to PO site in comparison to the osteoarthritis (OA) tissues as the controls. Generally there were more cells positive for immunostaining for all those ITAM-associated molecules, including multinucleated oscteoclast-like cells that were also positive for osteoclast cell markers tartrate acid phospotase (TRAP) and cathepsin K (based on serial immunolabeling comparison), in PO tissues as compared to the OA. Similar sort of outcome pattern was obtained for the examination on the mRNA levels, in which there was higher level of mRNA for all those ITAM-associated molecules in PO than in OA tissues.

Despite presence of more multinucleated osteoclast-like cells observed in PO tissues (supported by immunostaining for osteoclast cell markers TRAP and cathepsin K), study outcome found that there was not much positive immunostaining for NFATc1. Consistent with this, findings from the examination on mRNA level found that NFATc1 gene expression was not statistically higher in PO tissues in comparison to the control OA tissues.

More interestingly, polyethylene (PE) particles, a common type of wear debris causing osteolysis in PO (Campbell et al. 1995; Dowd et al. 2000; Hirakawa et al. 1996; Shanbhag et al. 1994b; Sochart 1999; Willert et al. 1990), were detected present in osteoclast-like cells positive for those osteoclast cell markers TRAP and cathepsin K. PE particles were also detected within and close to cells positive for TREM2, DAP12, OSCAR and FcRγ. All these study outcomes appeared to support one of the hypotheses presented in this thesis, in which ITAM-related molecules are expressed higher in human tissues of peri-implant osteolysis induced by PE and could be associated with the disease progression.

Detection of PE particles in some cells expressing osteoclast ITAM-related molecules, particularly in multinucleated cells, in PO tissues prompted further investigation on whether

those particles might affect the expression of those ITAM-related molecules in osteoclasts and hence could be associated with PE-induced osteoclastogenesis. This investigation was carried out in a novel *in vitro* model for PE-induced osteoclastogenesis assay that provided better representation of tissue microenvironment that may affect cellular interactions with the PE particles (Atkins et al. 2009). As part of assessment on the novel model of PBMC-derived osteoclast culture grown in 3D-collagen gel as an *in vitro* model for investigating PE-induced osteoclastogenesis, TRAP staining and dentine pit resorption assay were carried out to confirm increase in osteoclast formation and resorption activity in response to exposure to PE particles. Such outcomes were achieved following the assessment on the *in vitro* model. The study also detected presence of PE particles within the PBMC-derived osteoclasts in the culture. Together all these may suggest that PE particles could induce osteolysis in PO tissues through increase in the formation of osteoclasts and total resorption activity.

In conjunction to the intent of the study to further investigate the effect of PE particles on the expression of TREM2, DAP12, OSCAR and FcRγ in context of PE-induced osteoclastogenesis, the mRNA levels of those molecules were studied and compared between cells cultured with and without PE particles. The study outcome, which found that these factors were induced by PE particles during osteoclast formation *in vitro*, could suggest that the ITAM-associated receptor pathway is markedly activated by PE wear particles and may augment RANKL-mediated osteolysis in peri-implant tissues containing PE particles. The upregulation of osteoclast ITAM-related molecules *in vitro* as documented in Chapter 3, supported by higher expression of those molecules in peri-implant tissues as discussed earlier in Chapter 2, may indicate the significance those molecules possibly have in osteoclast formation and activity in the disease as hypothesized in the beginning of these studies.

Meanwhile in the context of RA, similar approach was used in which the expression of TREM2, DAP12, OSCAR and FcRγ alongside with NFATc1 and osteoclast cell marker cathepsin K was compared between in active and inactive RA tissues as well as OA and normal tissues following tissue immunostaining. Quite similar trend of study outcome (to PO tissues) was obtained, which demonstrated trends of more positive immunostaining of OSCAR, FcRγ, TREM2 and DAP12 in human RA tissues, particularly the active RA group, in comparison to the OA and normal tissue group. However, tissue immunostaining for the osteoclast cell marker cathepsin K indicated that those examined RA tissues were not heavily

populated with osteoclast-like cells. Instead, many of the cathepsin K-positive cells in the tissues examined were seen to have fibroblast-like morphology. Therefore, the finding of more expression of ITAM-related molecules in RA tissues could not be associated with an increase in osteoclast formation in RA that could be responsible for bone loss observed in the disease.

Despite the larger proportion of positively immunostained cells seen in the active RA tissues compared to the other tissue groups, unlike the other studied molecules, outcome from NFATc1 immunostaining found that most of the cells were in aggregations and appeared to be T-lymphocyte-like cells. Therefore based on the study outcome obtained, NFATc1 were mainly discussed in the context of T-lymphocytes in RA. There was no work on comparing the mRNA expression of these molecules in the RA and control tissues since there was technical limitation in finding appropriate tissue source for RNA extraction.

In addition to the findings that there was higher proportion of cells expressing ITAM-associated molecules in RA tissues, there was also another interesting observation noted, where positive immunostaining particularly for OSCAR was also obviously seen in vasculature structure especially in RA tissues. Positive immunostaining associated with vasculature, however, was not observed in PO tissues as described earlier. This sort of observation has become the basis for the work presented in the Chapter 5. Positive OSCAR immunostaining on vasculature has promoted further investigation on the existence of soluble form of OSCAR and the level of soluble OSCAR in RA patients. However since such study using patient sera has been reported in the literature this present work measured and compared the level of soluble OSCAR in the synovial fluids of patients with RA and OA to demonstrate novelty in the finding. Interesting data obtained from the work demonstrated quite high amount of soluble OSCAR measured in both groups of RA and OA synovial fluids.

In relation to the aim for further investigation following observation of OSCAR immunostaining on vasculature particularly in the RA tissues, experimental work was carried out on *in vitro* culture of endothelial cells, which were primary human umbilical vein endothelial cells (HUVECs) and bone marrow endothelial cell (BMEC) line. While there have been studies reporting on stimulation on OSCAR expression by oxidized low density lipoprotein (Goettsch et al. 2011) (more relevant to cardiovascular pathology) during the experimental work been carried out, this study provide novel finding of upregulation of

OSCAR expression in endothelial cells *in vitro* by pro-inflammatory cytokine TNFα and IL-1β, which is closer to the context of rheumatoid arthritis pathology. The expression of OSCAR in endothelial cells in the context of inflammation and rheumatoid arthritis also open a new dimension the current body of knowledge by taking OSCAR out of "monocyte-macrophage lineage and bone loss" scope only to broader context of inflammation. Like OPG, this study suggests bigger role and contribution OSCAR in a wider and more complex tissue microenvironment in inflammation-mediated bone loss pathology.

The findings presented in this thesis suggest that, in inflammation-mediated bone loss, there is alteration in the expression of osteoclast-associated ITAM-signalling molecules that may contribute towards the progression of PO and RA. Just like the revelation on the significance of OPG/RANK/RANKL axis in the pathology of those diseases (Crotti et al. 2004; Crotti et al. 2002) which has opened options for therapy approach wide, the discovery of significance on osteoclast ITAM-associated molecules in the context of inflammation-mediated bone loss as being proposed and documented here may provide new dimension for more targeted and specific treatment for those diseases. Further studies on ITAM-associated molecules in the future could identify that these molecules may potentially be developed as a promising therapy target.

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APPENDICES

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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RESEARCH ARTICLE Open Access

The immunoreceptor tyrosine-based activation motif (ITAM) -related factors are increased in synovial tissue and vasculature of rheumatoid arthritic joints

Tania N Crotti[1*], Anak ASSK Dharmapatni[1†], Ekram Alias[1,2†], Andrew CW Zannettino[3,4], Malcolm D Smith[5,6] and David R Haynes[1]

Abstract

Introduction: The immunoreceptor tyrosine-based activation motif (ITAM) pathway provides osteoclast co-stimulatory signals and regulates proliferation, survival and differentiation of effector immune cells. In the osteoclast, the receptors Triggering Receptor Expressed on Myeloid cells 2 (TREM2) and Osteoclast Associated Receptor (OSCAR) and their respective adaptor proteins, DAP12 and FcRγ mediate ITAM signals and induce calcium signaling and the crucial transcription factor, NFATc1. In rheumatoid arthritis (RA), OSCAR expression by monocytes is inversely correlated with disease activity. Additionally, serum levels of OSCAR are reduced in RA patients versus healthy controls suggesting that expression and secretion or cleavage of soluble (s) OSCAR is immune modulated. Recent data suggest that endothelial cells may also be a source of OSCAR.

Methods: ITAM receptors, their adaptor proteins, and NFATc1 and cathepsin K were detected in human synovial tissues by immunohistochemistry. Synovial tissues from patients with active RA were compared with tissue from patients in remission, osteoarthritis (OA) patients and healthy individuals. OSCAR was measured by immunoassay in synovial fluids recovered from active RA and OA patients. Endothelial cells were cultured with or without 5 ng/mL TNF-α or IL-1β over 72 hours. Temporal expression of OSCAR mRNA was assessed by qRT PCR and OSCAR protein in the supernatant was measured by ELISA.

Results: Significantly higher ($P < 0.05$) NFATc1-positive inflammatory cell aggregates were found in active RA tissues than in healthy synovial tissue. Similarly, the percentage of OSCAR, FcRγ, DAP12 and TREM2 positive cells was significantly higher in active RA tissues compared to the healthy synovial tissue. Notably, OSCAR was strongly expressed in the microvasculature of the active RA tissues (9/9), inactive RA (8/9) weakly in OA (4/9) but only in the lumen of healthy synovial tissue (0/8). OSCAR levels were detected in synovial fluids from both RA (47 to 152 ng/mL) and OA (112 to 145 ng/mL) patients. Moreover, OSCAR mRNA expression and soluble OSCAR release was stimulated by TNF-α and IL1-β in cultured endothelial cells.

Conclusions: Increased levels of ITAM related factors were present in synovial tissue from active RA joints compared to OA and healthy joints. OSCAR was strongly expressed by the vasculature of active RA patients and membrane bound and soluble OSCAR was stimulated by inflammatory mediators in endothelial cells *in vitro*.

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that involves dysregulated immune cell functions. It is characterized by joint damage and systemic bone loss associated with excessive osteoclast activity [1-4]. Human studies show that mature osteoclasts are in close proximity to the bone surface in affected joints of patients with RA [2]. Identifying factors that regulate the differentiation and activity of osteoclasts is crucial in identifying potential targets to abrogate this local and systemic bone loss in RA.

A key molecule known to stimulate osteoclast differentiation and activity is receptor activator NF kappa B ligand (RANKL). Elevated RANKL in active RA relative to its inhibitor osteoprotegerin (OPG) is associated with increased osteoclast differentiation and resorption [5]. In active RA synovial tissue we, and others, have demonstrated that RANKL expression is significantly increased in lymphocytes and fibroblasts [6,7]. RANKL binds to its receptor, RANK, on osteoclast precursors instigating the differentiation of monocytes into multinucleated mature osteoclasts via activation of key signaling cascades involving the transcription factor, nuclear factor of activated T cells (NFATc1) (reviewed by Asagiri *et al.* [8]).

NFATc1 is not only crucial in the regulation of terminal osteoclast formation, but also plays a role in the immune system [8,9]. NFATc1 also regulates T cell differentiation and activation [10], such as that seen in inflammatory diseases such as RA. In osteoclasts, NFATc1 directly induces early and late stage osteoclast specific gene expression in the absence of RANKL [9,11-14]. To our knowledge the distribution of NFATc1 expression in synovial tissue from active RA joints has not been previously characterized.

Recent studies suggest RANK-RANKL induced osteoclastogenesis is enhanced by co-stimulatory signals mediated by immunoreceptor tyrosine-based activation motif (ITAM) harboring adaptors [15-17]. ITAM signalling is also involved in the regulation of effector immune cells proliferation, survival and differentiation [16,17]. DNAX-activating protein 12kDa (DAP12) and Fc receptor common γ chain (FcRγ) are similar ITAM-containing adaptor proteins relevant to osteoclast formation in physiological bone turnover [17-19]. In pre-osteoclasts and osteoclasts the ITAM adaptor proteins DAP12 and FcRγ associate with innate immune receptors; in particular, Triggering Receptor Expressed on Myeloid cells 2 (TREM2) [15,20-22] and Osteoclast Associated Receptor (OSCAR) [17,23], respectively, to activate calcium, induce NFATc1 and convey ITAM signaling [17].

Mice deficient in both *DAP12* and *FcRγ* develop severe osteopetrosis [16] and exhibit an osteoclast multinucleation defect that is restored upon introduction of NFATc1 [17]. Evidence suggests DAP12 and TREM2 are required for differentiation and migration of osteoclasts as bone resorption is reduced in osteoclasts derived from mice with mutations in DAP12 and/or TREM2 *in vitro* [15,20,21]. In the context of RA, activation of macrophages by FcRγ is reported to induce cartilage destruction independent of inflammation [24]. It was thus suggested that interaction of FcRγ with immune complexes drives inflammation and induces bone loss indirectly [24]. We have recently demonstrated increased levels of OSCAR, FcRγ, TREM2 and DAP12 in periimplant tissues [25]. To our knowledge the expression of FcRγ, DAP12 and TREM2 has not yet been demonstrated in human RA.

A single-nucleotide polymorphism within the promoter of the OSCAR gene has been linked to increased risk of postmenopausal osteoporosis [26]. In RA, OSCAR has been related to disease activity [27] and the potential of cells to differentiate into osteoclasts [28]. These findings support the contention that OSCAR plays a role in human bone turn over [26,28,29]. In osteoclasts the crucial transcription factor, NFATc1, induces OSCAR gene expression [30]. Furthermore, ligand-activated OSCAR interacts with FcRγ to produce an increase in intracellular calcium [31] that further stimulates NFATc1 expression. This establishes a positive feedback loop that results in marked elevation of both OSCAR and NFATc1 expression in terminal stages of osteoclast formation [8]. Administration of an OSCAR-Ig fusion protein inhibits osteoclastogenes *in vitro* [30,32]. These findings establish OSCAR as not only an important immune modulator but also a major player in the regulation of osteoclastogenesis.

OSCAR is expressed by osteoclasts as well as dendritic cells in humans and is involved in antigen presentation and activation of dendritic cells [33,34]. OSCAR has also been reported to be associated with osteoclasts near sites of erosion and the mononuclear cells adjacent to the microvasculature in RA patients [28]. Interestingly, more recent studies have identified OSCAR expression by endothelial cells [35]. This is similar to our observation that OPG expression and release by endothelial cells is regulated by inflammatory cytokines [36]. However, production and possible release of soluble OSCAR by endothelial cells in response to inflammatory cytokines present in arthritides has not yet been reported.

High levels of OSCAR have recently been demonstrated in the synovial tissues and monocytes isolated from RA patients, with these cells having a greater propensity for differentiation into osteoclasts [28]. Tumor necrosis factor (TNF)-α was found to induce OSCAR expression in monocytes isolated from RA patients [28]. However, serum levels of OSCAR were lower in RA patients compared with normal controls. These findings further suggest cell associated and soluble OSCAR is regulated by inflammatory cytokines that play a significant role in the pathogenesis of RA.

We aimed to investigate the distribution of ITAM receptors (OSCAR and TREM2) and their adaptor proteins (FcRγ and DAP12) in synovial tissues from patients with active RA (as yet untreated with disease modifying anti-rheumatic drugs (DMARDs) and inactive RA (patients in remission following treatment) compared with tissues from OA and healthy joints. We also aimed to assess whether OSCAR is detectable in synovial fluid from active RA and OA as its close proximity to the joint might better reflect localized disease activity. In addition to this, we sought to determine whether OSCAR is expressed and released by endothelial cells *in vitro* and whether this expression is regulated by inflammatory cytokines.

Materials and methods

Patient samples

Synovial tissue samples were obtained from the rheumatology unit in the Repatriation General Hospital, Daw Park, South Australia. RA patients fulfilled the American College of Rheumatology criteria for RA [37]. Active RA patients were yet to undergo DMARD treatments and had active joint inflammation while inactive RA patients were in remission after successful DMARD treatment and undergoing follow-up. A small-bore arthroscopy (2.7 mm arthroscope, Dyonics, Andover, MA, USA) was performed under local anesthesia, as previously described [38]. Biopsies of synovial tissues from RA patients were obtained from all accessible regions of the knee joint, but mainly from the suprapatellar pouch. OA samples were obtained at the time of knee replacement surgery and fulfilled published criteria [39]. Healthy samples were from patients attending a sports medicine clinic with unexplained knee pain at the time of a diagnostic arthroscopy [40]. Details of the patients and medication at the time of surgery are summarized in Table 1. The study protocol was approved by the institutional Medical Ethics Committee. Written informed consent was obtained from patients with diseased (OA, active and inactive RA) and healthy joints that were included in the study.

Tissues were fixed in 10% buffered formalin and embedded in paraffin. Five-micrometer sections were mounted on 3-aminopropyltriethoxy-silane (APTS) (Sigma, St. Louis, MO, USA) coated glass slides for hematoxylin and eosin (H&E) staining and assessment of tissue histology.

Immunohistochemistry

Antibodies and reagents

Serial sections were stained with the following antibodies (Mab): mouse monoclonal IgG_1 anti-NFATc1 ((clone 7A6, sc-7294) Santa Cruz Biotechnology, Santa Cruz, CA, USA) (4 μg/mL), goat polyclonal anti-human OSCAR (sc-34233, Santa Cruz Biotechnology) (10 μg/mL), rabbit polyclonal anti-human FC€R1G (LS-B2169, Lifespan Biosciences, Inc., Seattle, WA, USA) (1.25 μg/mL), rabbit polyclonal anti-human TREM2 (HPA010917, Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia) and rabbit polyclonal anti-human DAP12 (sc-20783, Santa Cruz Biotechnology) (2 μg/mL). A mouse monoclonal IgG_1 anti-human cathepsin K (Cath K) (MAB3324 clone 182-12G5, Millipore (Billerica, MA, USA) (2 μg/mL) was used to detect the presence of any osteoclasts.

Secondary antibodies included 10 μg/mL polyclonal goat anti-mouse IgG (Dako Cytomation, Glostrup, Denmark), or 3 μg/mL goat anti-rabbit IgG (P0448, Dako, Glostrup, Denmark) or 7 μg/mL swine anti-goat IgG (ACI3404, Invitrogen Life Technology, CA, USA). Tertiary antibodies included 7 μg/mL swine anti-goat IgG (ACI3404, Invitrogen Life Technology) or 13 μg/mL rabbit anti-swine IgG (P0164, Dako, Glostrup, Denmark).

Immunohistochemistry (IHC)

Sections were dewaxed and pre-treated with either 10 mM sodium citrate buffer pH 6.0 or 10 mM Tris-ethylenediamine-tetraacetic acid (EDTA) buffer pH 9.0 at 90 to 95°C for 10 to 20 minutes for antigen retrieval. Sections were treated with phosphate buffered saline (PBS)/0.1% sodium azide and 0.3% v/v hydrogen peroxide to

Table 1 Details of the patients and medication at the time of surgery

Groups	Active RA	Inactive RA	OA	Normal
Age (years)	62.5 ± 19.28	72.33 ± 7.07	69.22 ± 7.98	36.3 ± 10.39
Gender (male/total)	2/10	6/9	6/9	6/10
CRP (IU/mL)	83.90 ± 83.78	9.78 ± 8.21	NA	NA
RF (mg/L)	19.40 ± 44.10	1.44 ± 0.53	<20	NA
Erosion Positivity per total samples	2/10	2/9	0/9	0/9
DMARDS	NSAIDs 9 Prednisolone 1	SSZ 1 Im Gold 5 MTX 2 Plaquenil 1	No NSAIDs 5 Panadeine 1 NSAIDs 3	None 9 Allopurinol 1

CRP, C-reactive protein; DMARD, disease modifying antirheumatic drug; Im Gold, intramuscular sodium aurothiomalate; MTX, methotrexate; NSAIDs, non-steroidal anti-inflammatory drugs; RF, rheumatoid factor; SSZ, sulphasalazine

inhibit endogenous peroxidase activity. A three-step peroxidase-based immunostaining technique, as previously described [41], with minor modifications, was employed. Sections were incubated with the primary antibodies (concentrations as described above) diluted in PBS and 1% bovine serum albumin (BSA) overnight at room temperature in a wet chamber. Sections were incubated with the appropriate HRP-conjugated secondary antibodies followed by incubation with the relevant HRP- conjugated tertiary swine anti-goat IgG or rabbit anti-swine IgG. HRP activity was detected using hydrogen peroxide as the substrate and 3-amino-9-ethylcarbazole (AEC) (K3469, Dako, **Carpinteria**, CA, USA) as the dye. Sections were counter-stained with Harris hematoxylin and lithium carbonate and mounted with GurrAquamount (British Drug House, Poole, UK). Negative controls included isotype-matched antibody controls (mouse IgG_{1kappa} for mouse IgG_1) and antibody-raised serum for polyclonal antibody (normal rabbit serum or goat serum) with equivalent concentration to the primary antibodies.

Semi quantitative scoring analysis (SQA) of IHC result

Sections were scanned at high resolution using a NanoZoomer (Hamatsu, Shizouka, Japan), Digital Pathology, to enable quantification and archival of the IHC results. Three areas of 2 mm^2 were randomly selected and sections were assessed in random order by two blinded observers. Semi-quantitative assessment (SQA) of OSCAR, FcRγ, DAP12 and TREM2 and Cath K staining was scored using a 5-scale (0 to 4) scoring system [25,42]. Assessment was according to the percentage of positive stained cells as follows; 0 represented 0 to 5%, 1 for 6 to 10%, 2 for 11 to 25%, 3 indicated between 26 and 50% and a score of 4 indicated more than 50% of positive cells (adapted from [42]) within the sublining of the synovial tissue. For NFATc1 immunostaining, the number of positive stained cell aggregations (defined as more than 25 cells) was used as a parameter for grading (adapted from [43]). A score of 0 represented no positive stained cell aggregation, a score of 1 indicated one to three positive cell aggregations, score of 2 indicated total positive cell aggregation between four and six, seven to nine positive cell aggregations gave a score of 3 and a score of 4 indicated more than nine positive cell aggregations were present. Observations were made as to the presence of positive cells in the vessels or lining.

Endothelial cell *in vitro* cultures

Bone marrow endothelial cells (BMEC) were cultured in triplicate in the presence and absence of 5 ng/mL TNF-α and IL1-β over a 72-hour time period. Expression of OSCAR and OPG mRNA levels by BMECs were assessed at 0, 6, 12, 24, 48 and 72 hours by quantitative reverse transcriptase polymerase chain reaction (QRT PCR). Only data from 0, 24, 48 and 72 are presented here. Supernatants were collected at 0, 24, 48 and 72 hours to assess soluble OSCAR and were stored at -20°C until use in ELISA assay. BMECs were also cultured on chamber slides for immunofluorescent detection of OSCAR and OPG.

ELISA analysis of soluble OSCAR

Synovial fluid was obtained from patients at the rheumatology unit in the Repatriation General Hospital, Daw Park, South Australia. The patient cohort consisted of synovial fluid from active RA (average age 67, CRP 67.4 IU/mL and RF 165 mg/L, 7 male/5 female, 4/12 with erosion) and OA joints (average age 70, 3 male/5 female). Levels of OSCAR were assessed by ELISA kit following manufacturer's instructions (USCN Life Science, Inc., Wuhan, China). Samples (synovial fluids and supernatants) were clarified by centrifugation at 13,000 rpm for five minutes at 4°C. The supernatant was transferred to 1.5 mL eppendorf tubes and diluted 1/5 for synovial fluid and 1/10 for supernatant in the sample diluent provided in the kit. One hundred microliters of pre-diluted samples were loaded into each well along with the protein standards provided. Assays were carried out in duplicate and the OSCAR protein concentration in each sample was determined based on the standard curve generated.

RNA extraction and cDNA synthesis

Total RNA was isolated from *in vitro* BMEC cultures following the addition of 500 μL TRIzol reagent per well, as per the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized from 1 μg RNA per reaction using Superscript III Reverse Transcriptase (Invitrogen Life Technologies), as previously described [44].

Quantitative real time reverse-transcription polymerase chain reaction (QRT-PCR)

Real-time PCR was performed using Platinum SYBR Green qPCRSupermix-UDG (Invitrogen Life Technologies), as per the manufacturer's recommendations. Amplification was carried out in a Rotor-Gene 3000. Reaction mixtures contained 1 μl of 1 in 5 pre-diluted cDNA, 7.5 μl Platinum SYBR Green qPCR Supermix-UDG, 300 nM each of forward and reverse primers and diethyl pyrocarbonate (DEPC)-treated water to a final volume of 15 μl. Primer3Plus freeware [45] was used to design oligonucleotide primers to human OSCAR Forward 'CCC AGC TTC ATA CCA CCC TA' and Reverse: 'GAA GAG AAG GGG AGC GAT CT' [46]. Primer sequences for OPG and the endogenous reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed as described previously [47]. All samples were investigated in triplicate

QRTPCR reactions. Fold induction was calculated as a measure of 2^{ddCT} [48].

Immunofluoresence of BMEC

BMECs were grown for 40 hours in the absence or presence of 5 ng/mL IL-1β or TNF-α. Cells were fixed with 1:1 methanol:acetone for five minutes and washed with PBS. Primary antibodies OPG MAB805 (10 μg/mL) or polyclonal goat anti-human OSCAR (8 μg/mL, sc-34233, Santa Cruz Biotechnology) were diluted in PBS with 1% BSA and incubated overnight at room temperature. To control for non-specific staining of the OSCAR antibody, wells were incubated with normal goat serum in the absence of the antibody to OSCAR. Following washing with PBS, OSCAR and OPG antibodies were detected with secondary antibodies to goat conjugated with FITC (green) and mouse conjugated to cy3 (red) (both from Southern Biotechnology Associates, Inc., Birmingham, AL, USA), respectively, diluted in PBS plus 1% BSA for 30 minutes then washed three times with PBS.

Statistical analysis

Power calculations demonstrate sufficient power to detect differences between the healthy subjects and joints with active RA for each of the molecules detected (92.9 to 100%). To assess the SQAs assigned by analysis of the IHC staining statistical analysis was performed using SPSS version 11.5 **(SPSS Inc**, Chicago, IL, USA). A non-parametric Kruskal-Wallis analysis was used to compare the mean of the SQA score between the groups. A Mann-Whitney-U test was used to examine the significant difference between two groups, with a P-value <0.05 accepted as statistically significant.

Differences in the soluble OSCAR levels in synovial fluids between the two groups were analyzed by Student's t-test and $P < 0.05$ was considered significant. Statistical significance between treatments and time points was determined using Kruskal-Wallis followed by the Mann-Whitney test using GraphPad Prism version 5.0d (GraphPad Software Inc. La Jolla, CA, USA).

Differences in mRNA levels between the groups at each time point were analyzed using a Two-way Anova test.

Results

Expression of ITAM modulatory factors in active RA

While NFATc1 expression was localized in isolated cells throughout the tissue, NFATc1 was mainly expressed by cells within lymphocyte aggregates in tissues from untreated active RA joints (Figure 1). The SQA grading for NFATc1 immunostaining was, therefore, based on the number of cell aggregates expressing NFATc1 (described in Methods). The number of NFATc1-positive cell aggregations was found to be significantly more in the active RA group compared to all other groups ($P < 0.05$) (Table 2).

Cathepsin K (Cath K) is routinely used as a marker of osteoclast-like cells as it is expressed at high levels in osteoclasts [49,50] and is the main bone-matrix degrading enzyme [51,52]. Immunostaining for Cath K was found to be predominantly in the sublining of the synovium and expressed by synovial fibroblasts (images not shown). The proportion of Cath K positive cells was not significantly different between groups.

OSCAR has been reported to be increased in RA compared to that in OA tissues [28]. OSCAR expression was associated with macrophage-like cells in the sublining of the synovium of inactive and active RA patients as well as in the lining of OA patients (Figure 1). Strongly positive OSCAR staining was associated with the microvasculature of the synovium in patients with active RA, nine of nine (Figure 2A) and with inactive RA (eight of nine) (Figure 2B). OSCAR was weakly expressed in four out of eight of the tissue samples from OA patients (Figure 2C) and was absent in the microvasculature of the healthy tissues (zero of nine) (Figure 2D). Interestingly, OSCAR appeared in the lumen of the microvasculature of the synovium from healthy patients that was suggestive of cleaved or soluble protein in the serum.

TREM2 was highly expressed throughout tissue from active RA patients (Figure 1). Many types of cells appeared to express TREM2, including mononuclear cells in lymphoid aggregates and fibroblasts with expression significantly greater than all other groups (Table 2). TREM2 expression was also associated with the microvasculature of active and inactive RA patients.

FcRγ was also highly expressed in active RA tissues and OA patient tissues (Figure 1). This was significantly higher in comparison to the inactive RA and the control tissues ($P < 0.05$) (Table 2). FcRγ was strongly expressed in macrophage-like synovial lining cells particularly in the OA patients. In active RA, FcRγ appeared to be expressed by macrophage and fibroblast-like cells throughout the tissue but was absent in the lymphoid aggregates and not associated with the microvasculature.

DAP12 expression was significantly higher in active RA compared with all other groups ($P < 0.05$) (Table 2). DAP12 appeared predominantly associated with macrophage-like cells in the sublining of the synovial tissue and the macrophage-like lining cells of the OA group (Figure 1). Of note, the microvasculature was negative in all groups.

Soluble OSCAR in OA and RA synovial fluid

Soluble OSCAR was detected in serum from RA patients and normal individuals by Herman [28], suggesting a cleaved or released form of OSCAR. To more

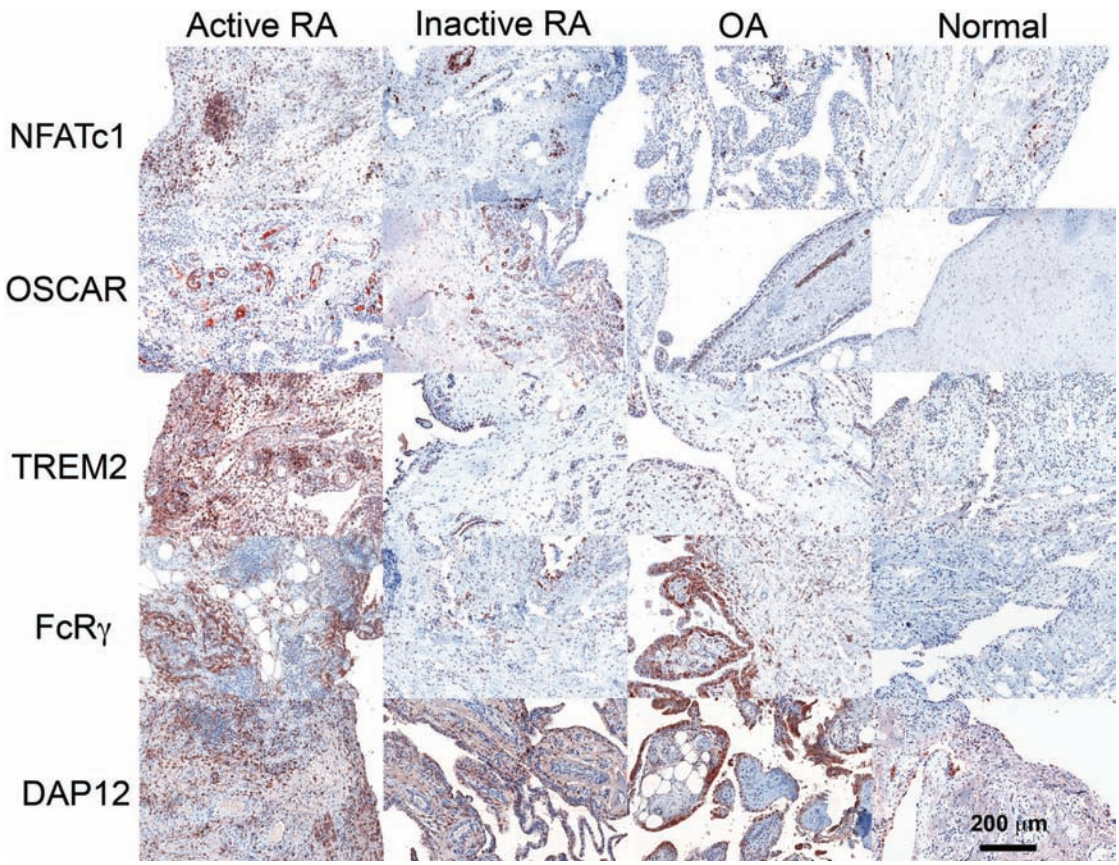

Figure 1 Expression of NFATc1 and ITAM associated molecules in human synovial tissues. Detection of factors was visualised with AEC (red) and counterstained with heamatoxilyn (blue) 100X mag.

closely assess OSCAR in relation to disease activity near the joint, we measured soluble OSCAR in the synovial fluid from OA (average age 70, 3 male/5 female) and RA patients (average age 67, 7 male/5 female). We detected soluble OSCAR in OA, (112 to 145 ng/mL) with more variable levels in active RA groups (47 to 152 ng/mL) and no significant difference between the groups (Figure 3).

Table 2 Semi-quantitative analysis of staining for NFATc1 and ITAM factors within tissue

	Act RA	Inact RA	OA	Norm
NFATc1	2.50 ± 0.21 ♦‡⊠ (n = 10)	0.75 ± 0.23 (n = 8)	0.77 ± 0.24 (n = 9)	0.28 ± 0.21 (n = 7)
Cath K	0.6 ± 0.22 (n = 10)	1.12 ± 0.35 (n = 8)	1.33 ± 0.41 (n = 9)	1.89 ± 0.35 (n = 9)
OSCAR	1.44 ± 0.24 (n = 9)	0.89 ± 0.39 (n = 9)	0.89 ± 0.26 (n = 9)	0 ± 0 (n = 8)
TREM2	3.44 ± 0.17♦‡⊠ (n = 9)	2.55 ± 0.34⊠ (n = 9)	2.44 ± 0.24⊠ (n = 9)	1.77 ± 0.22 (n = 9)
FcRγ	2.50 ± 0.31 ♦⊠ (n = 10)	0.88 ± 0.40 (n = 8)	2.22 ± 0.40 ♦⊠ (n = 9)	0.70 ± 0.26 (n = 10)
DAP12	1.70 ± 0.21♦‡⊠ (n = 10)	0.67 ± 0.29 (n = 9)	0.89 ± 0.11⊠ (n = 9)	0.40 ± 0.22 (n = 10)

Mean Score ± SEM; ♦ $P < 0.05$ significantly different from Inactive RA, ‡ $P < 0.05$ significantly different from OA, ⊠ $P < 0.05$ significantly different from Normal.

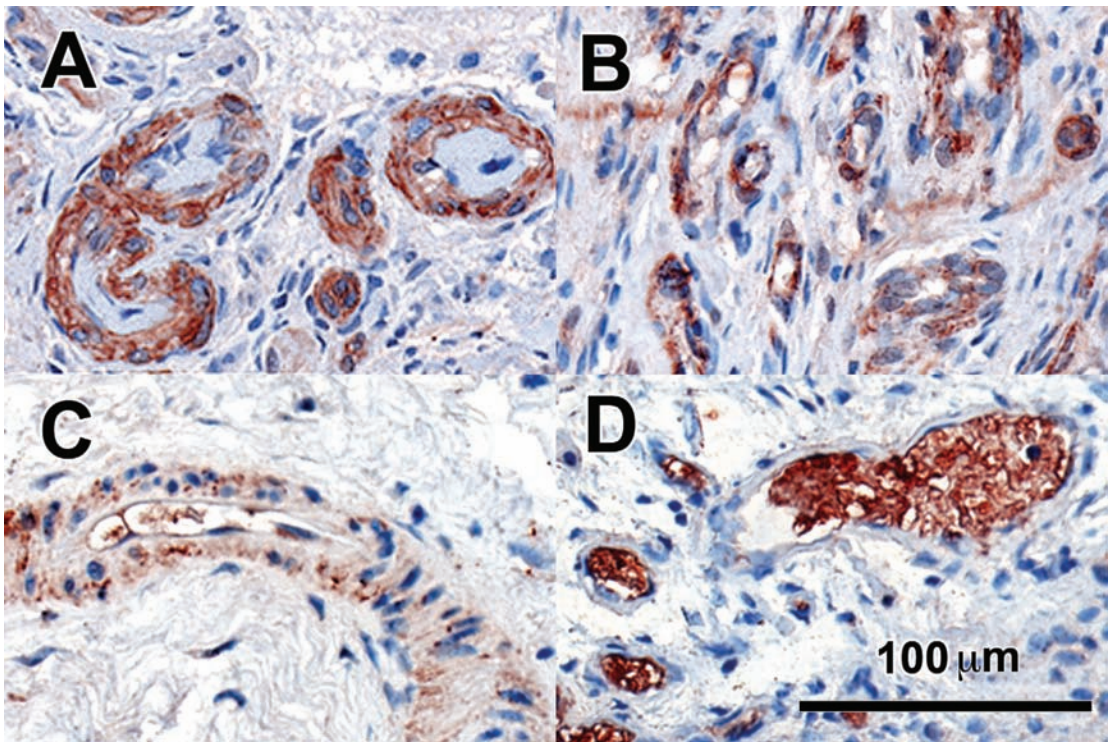

Figure 2 OSCAR staining in associated with the vasculature in active (A) and inactive RA (B) synovial tissues as well as OA (C) and normal synovial tissues (D) (red stain) at 200X mag.

OSCAR expression in BMECs

OSCAR expression by human umbilical vein endothelial cells (HUVEC) has been recently reported [35]. In our IHC analysis, we observed high levels of OSCAR associated with the microvasculature in the sublining region of synovial tissue from active and inactive RA synovial tissue joints (Figure 2A, B), with low levels associated with OA microvasculature (Figure 2C). It was interesting to observe that OSCAR was present in the lumen of the microvasculature in the synovium of normal patients (Figure 2D). These findings suggest soluble OSCAR may be present that is likely mediated by inflammatory cytokines present in RA tissue [53]. In view of this, we assessed whether OSCAR mRNA expression and release of soluble OSCAR by endothelial cells was modulated by inflammatory mediators associated with RA. OSCAR mRNA was significantly increased in cultured endothelial cells by IL-1β at 48 and 72 hours ($P < 0.05$ and 0.001 respectively) and TNF-α at 48 and 72 hours (P 0.001) compared with untreated cells at these time points (Figure 4A). Similarly, TNF-α and IL-1β significantly increased OPG expression by BMECs both at 48 and 72 hours ($P < 0.001$), consistent with our previous report using HUVEC cells [36] (Figure 5B). Based on these findings, supernatants from the cytokine stimulated BMEC cultures were assessed for OSCAR protein after 24, 48 and 72 hours of treatments (Figure 4C). We found increasing levels of soluble OSCAR over a 72-hour time period in response to both TNF-α and IL-1β with $P < 0.001$ at all time points compared with untreated BMECs.

Confocal detection of OSCAR in BMECs

To ascertain whether cell bound OSCAR is regulated by inflammatory cytokines, OSCAR was detected by immunofluorescence in cytokine stimulated BMECs. This was compared to OPG in the light of our previous findings that showed OPG is up-regulated by TNF-α in endothelial cells [36]. Following IL-1β treatment, little OSCAR but higher levels of OPG were detected (Figure 5B, E, respectively). TNF-α induced strong protein expression of OSCAR and little OPG expression. OSCAR appeared to be present in vesicles as there was strong punctate staining as well as diffuse cellular staining (Figure 5C). In all the treatments, the pattern of OSCAR expression was distinctly different from that of OPG.

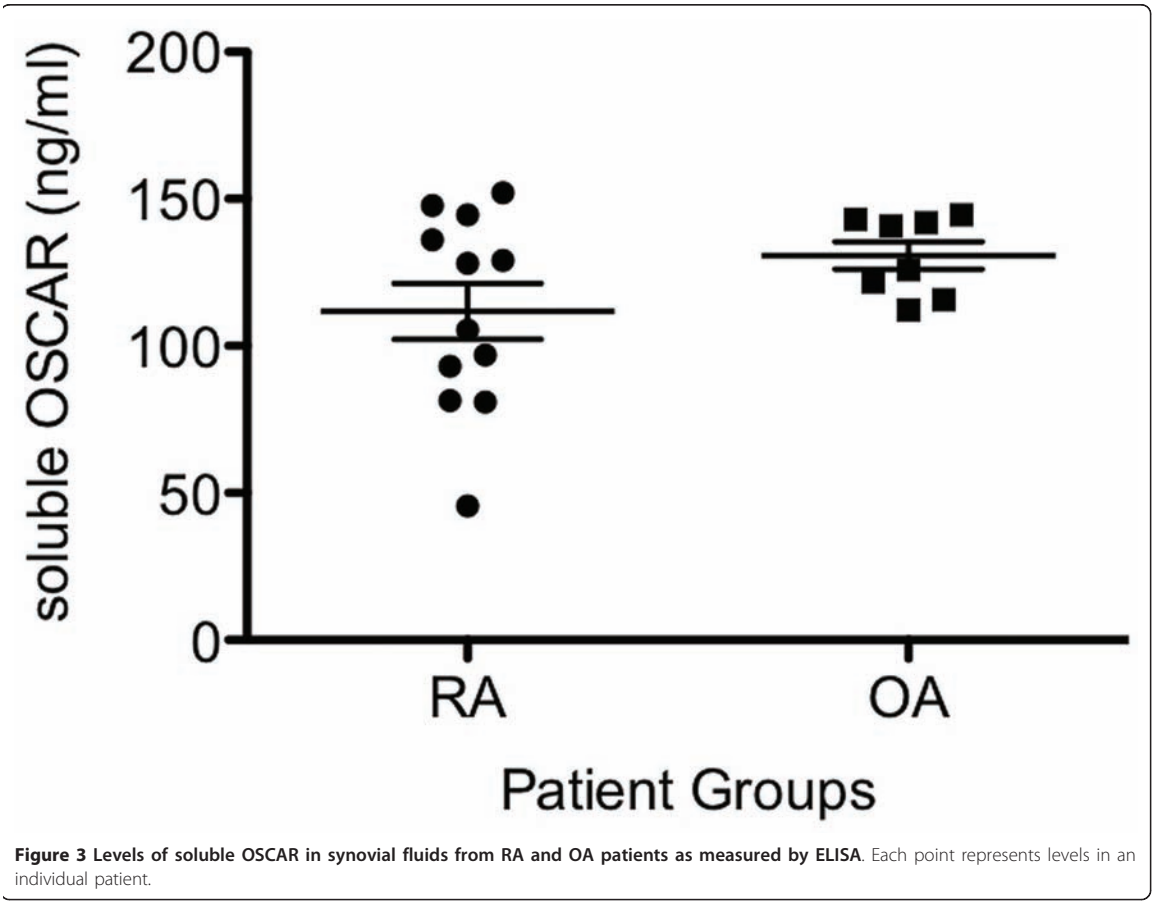

Figure 3 Levels of soluble OSCAR in synovial fluids from RA and OA patients as measured by ELISA. Each point represents levels in an individual patient.

Discussion

ITAM-related molecules are likely to be important mediators of RA joint destruction through their regulation of immune-mediated inflammation and bone erosion by osteoclasts [29]. In osteoclasts, the ITAM co-stimulatory pathways activate calcium and induce NFATc1 in pre-osteoclasts [17] to further enhance osteoclast differentiation and activation [9]. NFATc1 in activated T-cells regulates their differentiation and activation [10] and may further stimulate osteoclastogenesis via stimulating RANKL expression [54]. We observed significantly increased aggregates of NFATc1 positive cells in active RA synovial tissue. These are likely to represent populations of activated T-cells in the rheumatoid tissues. While low numbers of NFATc1 positive cells with the morphology of multinucleated pre-osteoclasts were observed in the current study, this may be due to the tissue sections not including juxtaposing bone where later stage active osteoclasts would be present [55]. Furthermore, our samples consisted of synovial tissue rather than pannus where osteoclast precursors are higher in number [2]. For similar reasons we did not note a significant increase in osteoclast-like cells expressing Cath K in active RA synovial tissue. Additionally, we observed Cath K associated with fibroblasts, consistent with published reports describing Cath K expression in skin fibroblasts [56,57]

Consistent with the findings reported here, TREM2 has been reported as expressed by macrophages, osteoclasts and, more recently, endothelial cells [15,22,58]. The ITAM-containing adapter molecule, DAP12, associates with TREM2 receptors in a number of cell types, including osteoclasts [15,20,22,59]. DAP12/TREM2 signaling has been shown to regulate inflammatory responses [59], play an important role in antigen presentation by dendritic cells [20] and has been implicated in T cell regulatory activity [60]. We observed markedly higher levels of DAP12 and TREM2 in active RA patients. Multiple cell types expressed TREM2, including mononuclear cells in lymphoid aggregates and fibroblasts. TREM2 expression was also associated with the microvasculature of active and inactive RA patients. Interestingly, DAP12 appeared

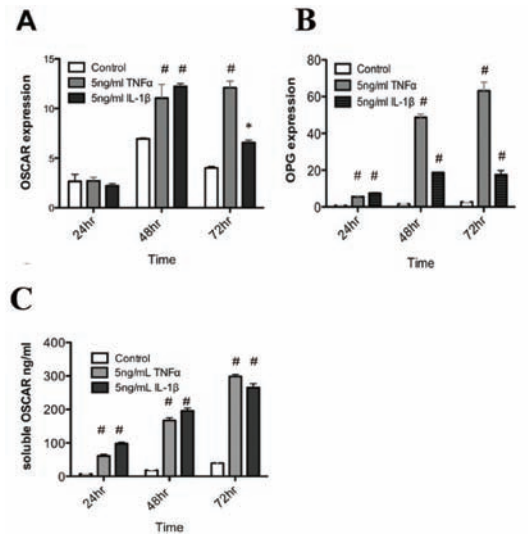

Figure 4 Expression of OSCAR by endothelial cells in response to inflammatory cytokines. BMECs were cultured in triplicate in the presence or absence of recombinant TNF-α and IL1-β. **A**. OSCAR gene expression was assessed at 0, 24, 48 and 72 hours by QRT PCR. GAPDH was used as the housekeeping gene. Fold induction was calculated as a measure of 2^{ddCT}. **B**. Induction of OPG gene expression at 0, 24, 48 and 72 hours. **C**. Release of soluble OSCAR was assessed in duplicate, by ELISA, as per the manufacturer's instructions. The mean values were calculated. Significance of $P < 0.05$ (*) and $P < 0.001$ (#) are indicated.

predominantly associated with macrophage-like cells in the sublining of the synovial tissue, particularly in the macrophage-like cells in the lining of the OA group.

OSCAR and FcRγ form an ITAM receptor/signaling pair that is expressed in osteoclasts and transmits ITAM signaling to induce osteoclast differentiation [16,23]. We observed high levels of FcRγ in association with fibroblasts and monocytes of the synovial sublining while lymphoid aggregates and the vasculature did not express FcRγ. Of note, similar to DAP12, FcRγ was associated with macrophage-like synoviocytes in the synovial lining with some scattered monocytes in the sublining of the OA tissue. The increased DAP12 and FcRγ expression may indicate a role in the pathogenesis of OA but this is yet to be determined.

OSCAR is a functional receptor on monocytes and neutrophils involved in the induction of the primary pro-inflammatory cascade and the initiation of downstream immune responses [31]. Ligation of human OSCAR on monocytes and neutrophils also results in the induction of a pro-inflammatory cascade and downstream immune responses [31]. High levels of OSCAR have recently been demonstrated in the tissues of RA patients and the serum of healthy individuals [28]. This is consistent with our findings that OSCAR protein was increased on monocytes from RA patients compared with healthy individuals with expression correlating with inflammatory disease activity [28].

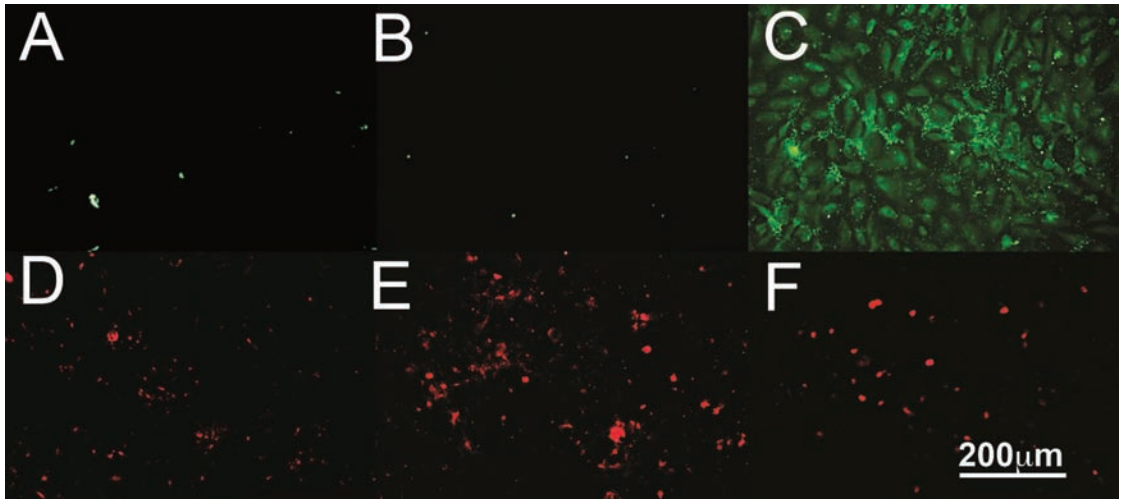

Figure 5 OSCAR and OPG expression by BMECs. BMECs were cultured in the absence (**A** and **D**) or presence of 5 ng/mL IL-1β, (**B**, **E**) and 5 ng/mL TNF-α (**C**, **F**) for 40 hours and fixed for visualization by con-focal microscopy. OSCAR is seen as green (A, B, C) and OPG as red (D, E, F). Original magnification was 200X.

Interestingly, OSCAR expressing monocytes were seen adjacent to microvasculature, consistent with observations by Herman *et al.* [28]. In a previous study, the expression of OSCAR was shown in multinucleated osteoclast-like cells attached to the bone [28]. We could not confirm this as the synovial tissue specimens from the RA patients used in this study did not include bone [55,61]. However, we did detect positive macrophage-like cells expressing OSCAR in the lining of the OA specimens similar to that previously reported [28]. Herman and colleagues have suggested that OSCAR expression might be regulated by pro-inflammatory cytokines [28]. Therefore, increased expression of OSCAR in active RA synovia compared to other groups is likely to be due to elevated inflammatory cytokines present in RA synovial tissues.

A possible limitation of the study is that both groups were not perfectly age- and sex-matched. It is very difficult to age- and sex-match the RA, OA and normal tissues due to the nature of the joint diseases affecting predominantly different age groups and sexes. In addition, patients presenting with active RA can take several years of treatment before the disease is in remission and classified as inactive. While to our knowledge there is are no reports that OSCAR expression changes with age, it is possible the differences in age may influence the results of our study.

It is possible that soluble OSCAR may act as a decoy receptor and suppress ITAM signaling. Of particular interest is the observation that RA patients had reduced levels of soluble OSCAR in serum compared to healthy individuals [28]. However, we noted high levels of OSCAR in the synovial fluid of our active RA patients and OA patients. A possible reason for this is that in the Herman *et al.* study patients with RA were being treated with anti-TNF therapy, unlike our active RA patients. It is also possible that levels in the joint locally are not necessarily reflected systemically in the circulation. The lack of significant differences between soluble OSCAR in OA and RA patients may be due to the low sample number or may reflect the increased release of OSCAR in response to inflammatory cytokines present in both of these pathologies [38,53].

High levels of OSCAR were associated with the microvasculature of the synovium of active RA specimens as well as tissues taken from our inactive RA patient group, who have had successful DMARD treatments. In the synovial tissue of healthy joints, positive OSCAR staining was present only in the lumen of the vasculature, suggesting the presence of soluble OSCAR in the blood. These observations were consistent with the recent finding that human primary endothelial cells express OSCAR [35]. Contrary to our findings, Herman *et al.* did not observe OSCAR staining of the microvasculature of his RA patients. This again may be due to the fact that, unlike our active RA patients, the patients in the Herman study were being treated with anti-TNF therapy.

The increased expression of OSCAR associated with the microvasculature in RA compared with normal tissue suggests that OSCAR expression is modulated by immune mediators or cytokines. Our previous studies have shown a reverse pattern of OPG expression in the vasculature, with low OPG levels associated with RA, compared with high levels associated with normal tissue [41]. Additionally, our *in vitro* studies showed that OPG expression in HUVEC is regulated by cytokines [36]. Our *in vitro* studies extend the findings by Goettsch *et al.* [35] and demonstrate that the expression of OSCAR by endothelial cells is stimulated by the inflammatory cytokines, TNF-α and IL-β, and is consistent with our *in vivo* findings showing OSCAR expression was elevated in the untreated RA synovial vasculature. Importantly, endothelial cells are a likely source of OSCAR and not just binding soluble OSCAR present in the serum, as we demonstrated that the inflammatory cytokines stimulated both mRNA and protein *in vitro*. TNF-α also induces OSCAR expression in monocytes *in vitro* [28]. This indicates that soluble OSCAR in the serum [28] and synovial fluid in RA is released by a variety of cells and is stimulated by inflammatory cytokines.

As recombinant human OSCAR-Fc is able to act as a decoy receptor for cell bound OSCAR [28], soluble OSCAR might provide a protective mechanism against bone erosion *in vivo* by competing with a ligand for cellular OSCAR and reducing signaling for inflammatory cells and osteoclasts. We propose that successful treatment of RA results in increased cleavage of OSCAR resulting in increased soluble OSCAR levels [27,28]. This is consistent with the observation that patients in remission have higher levels of soluble OSCAR and suggests a role in modulating osteoclastic bone resorption.

While we were able to demonstrate OSCAR expression in RA and OA synovial fluids for the first time, our data on the relative levels suggest that it is not a good discriminator between RA and OA. The data of Herman [28] and Zhao [27], who measured blood levels, suggest blood levels are lower in active RA compared to inactive disease. However, induction of OSCAR release *in vitro* and the levels of expression in the tissue indicate its expression, at least locally, may be related to disease activity. Further studies following patients during treatment may help resolve whether it is a marker for assessing disease activity and joint erosion in RA.

Conclusions

Here we present evidence that NFATc1 and the ITAM-related molecules FcRγ, TREM2, DAP12 and NFATc1 are up-regulated in active RA synovia in comparison to healthy control or inactive RA and OA tissues. In addition

to this, we find higher proportions of OSCAR positive vessels in both active RA patients and inactive RA patients compared with healthy controls. Our *in vitro* studies also confirm OSCAR expression by endothelial cells and demonstrate regulation of production and release of soluble protein by inflammatory cytokines, IL-1β and TNF-α. Of interest, we were able to demonstrate OSCAR expression in RA and OA synovial fluids for the first time.

Abbreviations

AEC: 3-amino-9-ethylcarbazole; APTS: 3-aminopropyltriethoxy-silane; BMEC: bone marrow endothelial cells; BSA: bovine serum albumin; Cath K: cathepsin K; DAP12: DNAX-activating protein 12kDa; DEPC: diethyl pyrocarbonate; DMARDs: disease-modifying antirheumatic drugs; EDTA: ethylenediamine-tetraacetic acid; FcRγ: Fc receptor common γ chain; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; H&E: hematoxylin and eosin; HRP: horse-radish peroxidase; HUVEC: human umbilical vein endothelial cells; IHC: immunohistochemistry; ITAM: immunoreceptor tyrosine-based activation motif; NFATc1: nuclear factor of activated T cells; OA: osteoarthritis; OPG: osteoprotegerin; OSCAR: osteoclast associated receptor; PBS: phosphate-buffered saline; RA: rheumatoid arthritis; RANKL: receptor activator NFkappa B ligand; SQA: semi-quantitative assessment; TREM2: triggering receptor expressed on myeloid cells 2.

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Authors' contributions

TC, EA and AD made substantial contributions to conception of the study, the design of experiments, the acquisition of data, data analysis and interpretation of data. TC, EA, AD, AZ MS and DH made substantial contributions to conception of the study, the interpretation of data and were involved in drafting the manuscript. All authors have read and approved the manuscript for publication.

Competing interests

The authors declare that they have no competing interests.

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