Gene Expression in Bone Cells

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Thesis submitted for Doctor of Philosophy

Name: Michael S. Kim

Primary Supervisor: Dr. Nigel A. Morrison

Associate Supervisor: Dr. Stephen J. Ralph

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Abbreviations

BMP	Bone morphogenetic protein
bp	base pair
Cbfa1	Core-binding factor α1
CCL	CC chemokines ligand
CCR	CC chemokines receptor
cDNA	copy DNA
CT	Calcitonin
CTR	Calcitonin receptor
CsA	Cyclosporin A
CTSK	Cathepsin K
DNA	Deoxy-ribonucleic acid
dATP	deoxy adenosine triphosphate
dCTP	deoxy cytodine triphosphate
dGTP	deoxy guanosine triphosphate
dNTP	deoxy nucleotide triphosphate
dTTP	deoxy thymidine triphosphate
EDTA	Ethylene-diamine tetra acetic acid
FBP	Far upstream element (FUSE) binding protein
GABP	GA binding protein
GM-CSF	Granulocyte macrophage colony stimulating factor
IL	Interleukin
ILF	Interleukin binding enhancing factor
kb	Kilobase
MCP	Monocyte chemotactic protein

M-CSF	Macrophage colony stimulating factor
$MgCl_2$	Magnesium chloride
MIP	Macrophage inflammatory protein
MITF	Microphthalmia transcription factor
MMP	Metalloproteinases
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa B
OCIF	Osteoclastogenesis inhibitory factor
ODF	Osteoclast differentiation factor
OPG	Osteoprotegerin
OPGL	Osteoprotegerin ligand
Osf2	Osteoblast specific factor 2
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PTH	Parathyroid hormone
RANK	Receptor activator of NFκB
RANKL	Receptor activator of NFκB ligand
RANTES	Regulated upon activation, normally T-expressed, and presumably secreted
RNA	Ribonucleic acid
rRNA	ribosomal RNA
RT	Reverse transcriptase
RUNX2	Runt-related transcription factor 2
SCYA	Small inducible cytokine A family member

SDS	Sodium dodecyl sulfate
SSC	Sodium chloride, sodium citrate
Taq	*Thermus aquaticus*
TNF	Tumour necrosis factor
TRANCE	TNF-related activation-induced cytokine
TRAP	Tartrate resistant acid phosphatase
Tris	Tris hydroxymethyl amino methane
Tris-HCl	Tris-hydrogen chloride
V-ATPase	Vacuolar-type H+-ATPase

Summary

Osteoclast formation is a complex process that is yet to be clearly defined. Osteoclasts differentiate from monocytic precursors to large multinuclear cells via the actions of two crucial cytokines: macrophage colony stimulating factor (M-CSF) and receptor activator of NFκB ligand (RANKL). These two cytokines bind to the osteoclast precursor cells, activating various down stream signalling pathways, inducing genes required for differentiation and for activation of osteoclasts. Exposure of monocytic precursors to M-CSF alone leads to differentiation into macrophages.

Osteoclast differentiation was suppressed by granulocyte macrophage colony-stimulating factor (GM-CSF), resulting in mononuclear cells, lacking tartrate-resistant acid phosphatase (TRAP) and a bone resorptive phenotype. Further analysis determined GM-CSF dosage and temporal effects on osteoclast formation, where higher doses and earlier treatments of GM-CSF result in greater suppression of osteoclast formation. To understand the TRAP negative mononuclear cell phenotype, various osteoclast related markers and nuclear factors were tested using quantitative real-time PCR. GM-CSF suppressed the mRNA expression of osteoclast markers, including TRAP and cathepsin K (CTSK). CTSK is a cysteine protease, involved in osteoclast activity of bone resorption. Furthermore, GM-CSF down regulated the expression of critical osteoclast-related nuclear factors, including nuclear factor of activated T-cells, cytoplasmic (NFATc1), which has been identified as playing a critical role in osteoclast differentiation and function in mice and to some extent in humans. The suppression of crucial osteoclast markers and transcription factors by GM-CSF indicated an overriding of the RANKL signal and possible switching of the cellular phenotype away from osteoclasts.

To determine the cellular phenotype of GM-CSF driven cell differentiation, flow cytometry analysis was employed. As the cells visualised as dendritic cell like, CD1a, a dendritic cell surface marker, was selected for investigation. CD1a was highly expressed in GM-CSF, M-CSF and RANKL (GMR) treated cells and was absent in osteoclasts (M-CSF and RANKL treatment). The CD1a observations were indicative of GM-CSF overcoming the RANKL signal for osteoclastogenesis and directing differentiation to dendritic-like cells. To further understand the osteoclastogenesis suppressive effect of GM-CSF, a 19,000 gene cDNA microarray assay was examined. The microarray experiment showed that the CC chemokine, monocyte chemotactic protein 1 (MCP-1), was profoundly repressed by GM-CSF. CC chemokines are chemoattractants that are induced during inflammation and recruit monocytes to the site of inflammation. MCP-1 and other CC chemokines, RANTES (regulated on activation normal T cell expressed and secreted) and macrophage inflammatory protein 1 alpha (MIP1α) permitted formation of TRAP positive multinuclear cells in the absence of RANKL. However, these cells were negative for bone resorption. In the presence of RANKL, MCP-1 significantly increased the number of TRAP positive multinuclear bone resorbing osteoclasts ($p= 5.7\times10^{-6}$), while RANTES and MIP1α mildly increased the number of bone resorbing TRAP positive multinuclear cells. Furthermore, CC chemokines, MCP-1, RANTES and MIP1α are all induced when authentic bone resorbing human osteoclasts differentiate from monocyte precursors *in vitro* following M-CSF-RANKL treatment.

The addition of MCP-1, RANTES or MIP1α appeared to reverse GM-CSF suppression of osteoclast formation, resulting in TRAP positive multinuclear cells. However, only MCP-1 recovered the bone resorption phenotype, while other

chemokines, RANTES or MIP1α did not. The cognate receptors for MCP-1, in particular, CCR2b and CCR4, were potently induced by RANKL (12.6 and 49-fold, $p= 4.0\times10^{-7}$ and 4.0×10^{-8}, respectively), whereas the chemokine receptors for RANTES and MIP1α (CCR1 and CCR5) were not regulated by RANKL. Chemokine treatment in the absence of RANKL also induced MCP-1, RANTES and MIP1α. Unexpectedly, treatment with MCP-1 in the absence of RANKL resulted in 458-fold induction of CCR4 ($p= 1.0\times10^{-10}$), while RANTES treatment resulted in two fold repression ($p= 1.0\times10^{-4}$). Since CCR2b and CCR4 are cognate MCP-1 receptors, these data support the existence of an MCP-1 autocrine loop in human osteoclasts differentiated using RANKL.

All three chemokines in the absence of RANKL can induce TRAP positive multinuclear cells that are negative for bone resorption. However, as MCP-1 can significantly increase the number of osteoclast formation and recover the bone resorbing osteoclast phenotype from GM-CSF suppression, MCP-1 is the most potent chemokine involved in osteoclast formation. MCP-1 induced TRAP positive multinuclear cells were characterised and found to be positive for calcitonin receptor (CTR) and a number of other osteoclast markers, including NFATc1. As NFATc1 is associated with osteoclast maturity in mice and has even been referred to as a master regulator of osteoclast differentiation and function, a strong induction of NFATc1 should theoretically allow bone resorption of MCP-1 mediated TRAP positive multinuclear cells. Although great NFATc1 mRNA induction and activated nuclear NFAT were observed, MCP-1 did not result in the formation of bone resorbing osteoclasts in the absence of RANKL. Despite the similar visual phenotype and expression of mature osteoclast markers TRAP and CTR when compared to

osteoclasts, RANKL treatment was required for the MCP-1 induced TRAP positive, CTR positive, multinuclear cells to possess bone resorption activity. This suggested that MCP-1 mediated TRAP positive multinuclear cells were primed for RANKL signal, to further differentiate into authentic osteoclasts.

The lack of bone resorption was further correlated with a deficiency in expression of certain genes related to bone resorption, such as CTSK and matrix metalloproteinase 9 (MMP9) and integrin αV. Another observation with implications for absence of the bone resorptive activity in MCP-1 cell was the absence or disruption of the F-actin ring structure, correlating with the lack of integrin αV mRNA expression. It was hypothesised that as MCP-1 mediated TRAP positive multinuclear cells possessed a high induction of CTR, the addition of calcitonin would block multinucleation. Indeed, the exogenous calcitonin blocked the MCP-1 induced formation of TRAP positive, CTR positive, multinuclear cells as well as bone resorption activity in the osteoclast controls, indicating that calcitonin acts at two stages of osteoclast differentiation in the human PBMC model.

These data suggest that RANKL-induced chemokines are involved in osteoclast differentiation at the stage of multinucleation of osteoclast precursors and provides a rationale for increased osteoclast activity in inflammatory conditions where chemokines are abundant. Furthermore, MCP-1 induced TRAP positive, CTR positive multinuclear cells appear to represent an arrested stage in osteoclast differentiation, after NFATc1 induction and cellular fusion, but prior to the development of bone resorption activity and therefore, could be termed “pre-osteoclasts”.

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Chapter 1

Introduction

and

Literature Review

Bone.

Bone is classified as type of connective tissue composed of abundant mineralised matrix, incorporating different cell types (reviewed in Boskey, A.L., and Posner, A.S., 1984). Bone has many essential functions in the human body, including support and as framework for the body, points of muscle attachment, protection of vital internal organs, as site of blood cell production (hemopoiesis) and mineral homeostasis. Due to its various functions, bone is categorised into different types; flat bones, tubular bones and irregular bones. Flat bones are formed by intramembranous bone formation, while tubular bone elongate via endochondral ossification from cartilage growth plate (Woolf, A.D., and Dixon, A., 1998). Both classes of bone are composed of dense and compact outer surface with a porous middle section that provides maximum strength for the minimum weight (Woolf, A.D., and Dixon, A., 1998).

The matrix of the bone is primarily composed of hydroxyapatite, which is comprised of calcium and phosphate (reviewed in Becker, G.L., 1977). Type I collagen and non collagenous proteins such as osteopontin, osteocalcin and osteonectin (Woolf, A.D., and Dixon, A., 1998) provide an organic framework for the formation of hydroxyapatite. The combination of collagen fibres and hydroxyapatite provide resistance against the pulling forces and resistance against compression of bone, respectively (Woolf, A.D., and Dixon, A., 1998).

Bone is comprised of various cells; osteoblasts, osteoclasts, osteocytes and lining cells. Yet other cell types that are found in contact with these cells on the surfaces of bone including, bone progenitor cells, macrophages, mature and stem cells

from the hemopoietic series (Woolf, A.D., and Dixon, A., 1998). The osteoblasts are bone-forming cells that synthesise bone matrix and are involved in its subsequent mineralization, while osteoclasts are bone-resorbing cells (Woolf, A.D., and Dixon, A., 1998).

Osteoblasts

Osteoblasts are derived from pluripotent mesenchymal stem cells that are responsible for bone formation (osteogenesis) and replacement, through a process called ossification (reviewed in Mackie, E.J., 2003). Bone formation is a physiological process of critical importance, as it is involved in skeletal growth, bone remodelling and fracture repair (reviewed in Mackie, E.J., 2003). Osteoblasts form bone by synthesising a type I collagen-rich matrix that has the unique property of eventually becoming mineralised, by depositing hydroxyapatite (reviewed in Ducy, P. *et al.,* 2000). Osteoblasts are also believed to regulate the local concentrations of calcium and phosphate to promote hydroxyapatite formation (reviewed in Manolagas, S.C., 2000).

The molecular mechanism required for osteoblast differentiation is not clear, however, there are several hypotheses in the process of osteoblastogenesis. It is believed that osteoblast differentiation is initiated by bone morphogenetic proteins (BMPs), which have been implicated in skeletal development during embryonic life and the fracture healing (reviewed in Wan, M., and Cao, X., 2005). BMPs induce signal transduction pathways that ultimately activate core-binding factor α1 (Cbfa1) also known as osteoblast specific factor 2 (Osf2) or runt-related transcription factor 2

(RUNX2), a transcription factor described as the master regulator of osteoblast differentiation and function (reviewed in Ducy, P., and Karsenty, G.,1998).

Osteoclasts

Osteoclasts are highly motile, multinuclear cells that differentiate from a hematopoietic precursor of the monocyte/macrophage lineage in bone marrow through cell-cell interactions between osteoclast progenitors and stromal/osteoblastic cells (Udagawa, N., *et al.,* 1990; reviewed in Suda, T., *et al.,* 1992). Osteoclasts have several unique characteristics including, abundant mitochondria, free polysomes, coated transport vesicles, vacuoles and lysosomes and many stacks of golgi membranes (reviewed in Suda, T., *et al.,* 1992). The major function of osteoclasts is the resorption of vital bone or calcified cartilage, in contrast to the macrophage, whose bone resorbing activity is limited to dead bone (Woolf, A.D., and Dixon, A., 1998).

The osteoclasts lie on the bone surfaces or in the pits (Howship's lacunae), where osteoclasts resorb the calcified bone. The most characteristic feature of pits in the osteoclasts is the presence of the ruffled borders and clear zones (reviewed in Suda, T., *et al.,* 1992). The clear zone or sealing zone serves as the attachment area of osteoclasts to the bone surface. It is believed that integrin alpha v beta 3, a cell surface maker is responsible for the attachment of osteoclasts (reviewed in Stenbeck, G., 2002). When the osteoclasts are attached to the new site of resorptive area, ruffled border of the membrane is formed to begin bone resorption. The ruffled border is composed of deeply infolded finger-like plasma membranes adjacent to the bone surface (reviewed in Roodman, G.D., 1996a; Roodman, G.D., 1996b). The clear zone

and the ruffled border create a microenvironment favourable for the bone resorption (Woolf, A.D., and Dixon, A., 1998).

To degrade bone, osteoclasts use a proton pump of the vacuolar H^+ ATPase to release hydrogen ions across the ruffled border into the clear zone to create an acidic microenvironment (reviewed in Roodman, G.D., 1996a), which aids in demineralisation by solubilising hydroxyapatite (reviewed in Suda, T., *et al.,* 1992). The resorption of minerals and degradation of mineralised bone matrix (collagen and other matrix proteins) occurs beneath the ruffled border, due to release of acid proteolytic enzymes such as matrix metalloproteinases (MMPs), cathpesin K, B and L into the clear zone (reviewed in Roodman, G.D., 1996a; Manolagas, S.C., 2000). The resorbed calcium is then free to be used systemically to maintain extracellular calcium levels (reviewed in Blair, H.C., 1998).

Osteoclast activity is measured by the expression of tartrate resistant acid phosphatase (TRAP), calcitonin receptor (CTR) and cathepsin K (CTSK) (reviewed in Roodman, G.D., 1996a). Histologically, the presence of an osteoclast is detected through TRAP staining (reviewed in Roodman, G.D., 1996a). Mice deficient in TRAP exhibit a mild osteopetrotic phenotype and defective mineralisation of cartilage in developing bones (Hayman, A.R., *et al.,* 1996).

Osteoclastogenesis

Osteoclasts differentiate from precursor cells to large multinuclear cells through the process of osteoclastogenesis (reviewed in Takahashi, N., *et al.,* 1999). Osteoclast precursors are found in circulating blood as promonocytes, but the majority

of the osteoclast differentiation occurs within the bone marrow (Itonaga, I., *et al.,* 1999; reviewed in Manolagas, S.C., 2000). The process of osteoclast formation can be divided into two phases; a proliferation phase of early osteoclast precursors and a late differentiation phase. The osteoclast differentiation is a highly regulated process requiring cell to cell interaction between osteoblast/stromal cells and osteoclast progenitors (reviewed in Suda, T., *et al.,* 1992). Osteotropic factors such as 1α,25-dihydroxyvitamin D_3, parathyroid hormone (PTH) and interleukin(s)-1, -6, -11 (IL-1, IL-6, IL-11) act on osteoblast/stromal cells to release osteoclast differentiation factors, such as macrophage colony stimulating factor (M-CSF) and receptor activator of NFκB ligand (RANKL) (Nakagawa, N., *et al.,* 1998; reviewed in Boyce, B.F., *et al.,* 1999; Takahashi, N., *et al.,* 1999; Suda, T., *et al.,* 1999; Manolagas, S.C., 2000) (Fig. 1.1). Osteoprotegerin (OPG) released by osteoblasts act as a negative regulator of osteoclast differentiation (reviewed in Suda, T., *et al.,* 1999).

Initially, *in vitro* osteoclast cultures were dependent on the co-culturing of osteoclast precursors with osteoblast/stromal cells, with supplementation of M-CSF and osteotropic factors in the growth media (Tanaka, S., *et al.,* 1993; Suda, T., *et al.,* 1993). Currently, *in vitro* osteoclast formation can be performed using a wide range of cell models. Mouse osteoclast formation is studied using either primary bone marrow cells, (Takayanagi, H., *et al.,* 2000; Takayanagi, H., *et al.,* 2002a; Takayanagi, H., *et al.,* 2002b), spleen cells (Udagawa, N., *et al.,* 1989; Kurihara, N., *et al.,* 1989) or RAW-264.7, a mouse leukaemic monocyte/macropahge cell line (Ishida, N., *et al.,* 2002). Human osteoclast formation is studied using the adherent or CD14 positive fraction of peripheral blood mononuclear cells (PBMCs) (Matsuzaki, K., *et al.,* 1998; Nicholson, G.C., *et al.,* 2000) or using granulocyte/macrophage colony forming units

(CFU-GM) obtained from bone marrow (Kurihara, N., *et al.,* 1990) or umbilical cord blood (Hodge, J.M., *et al.,* 2004).

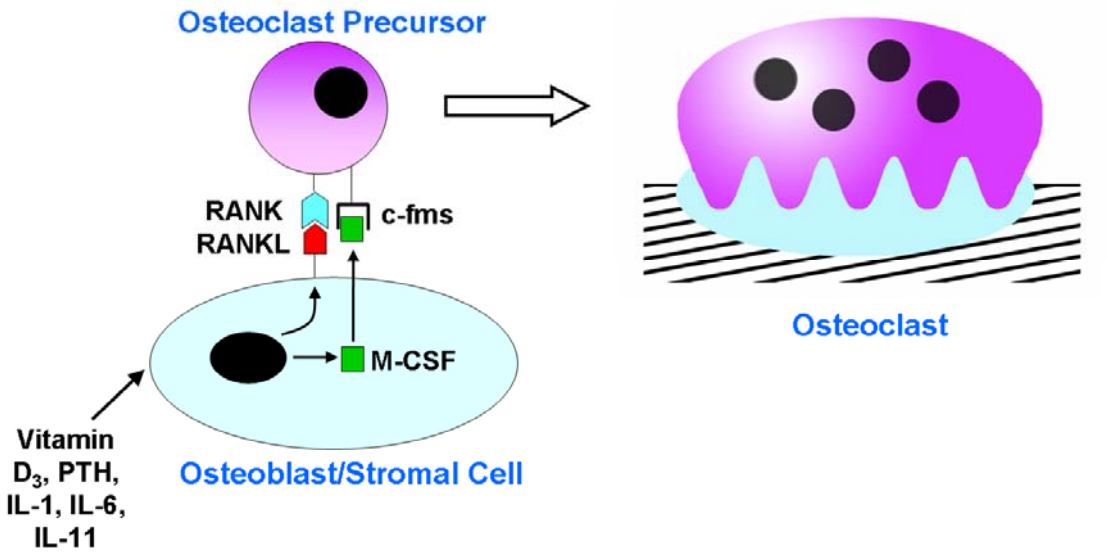

Fig. 1.1 Osteoclastogenesis (Adapted from Boyce, B.F., *et al.,* 1999). The figure depicts the osteoclast precursor interacting with an osteoblast/stromal cell, that is stimulated by osteotropic factors (1α,25-dihydroxyvitamin D3, PTH and IL-1,6 and 11). The stimulation of osteoblast/stromal cell by osteotropic factors, induces osteoclast differentiation factors (M-CSF and RANKL). Light blue colour underneath an osteoclast indicates bone resorbed area.

RANK and RANKL

RANKL is a member of tumour necrosis factor superfamily that is secreted by osteoblast/ stromal cells and is found to be a regulatory factor in differentiation of osteoclast, T-cells and dendritic cells (Anderson, D.M., *et al.,* 1997; reviewed in Takahashi N, *et al.,* 1999). RANKL is also referred as TNF-related activation-induced cytokine (TRANCE) (Wong, B.R., *et al.,* 1997a; Wong, B.R., *et al.,* 1997b), osteoprotegerin ligand (OPGL) (Lacey, D.L., *et al.,* 1998) and osteoclast differentiation factor (ODF) (Yasuda, H., *et al.,* 1998a (PNAS)). RANKL has many different functions, such as promoting the proliferation, differentiation, survival and

fusion of osteoclastic precursor cells. RANKL also activates osteoclastic precursor cells toward mature osteoclasts and induces inhibition of apoptosis of mature osteoclasts (reviewed in Hofbauer, L.C., and Heufelder, A.E., 2001a; Hofbauer, L.C., *et al.,* 2001b). RANKL acts in conjunction with M-CSF to induce osteoclast differentiation and also increase osteoclast function of bone resorption activity (Fuller, K., *et al.,* 1998; Yasuda, H., *et al.,* 1998a). In mature osteoclasts, RANKL treatment generated multiple cycles of bone resorption, by inducing actin ring formation and cytoskeletal rearrangement necessary for bone resorption (Burgess, T.L., *et al.,* 1999).

RANKL exerts its biological effects by binding to its receptor, receptor activator of NF-κB (RANK) on osteoclast lineage cells (Li, J., *et al.,* 2000). The RANK-RANKL interaction is essential in osteoclast differentiation and formation (Darnay, B.G., *et al.,* 1998; Galibert, L., *et al.,* 1998; Dougall, W.C., *et al.,* 1999). RANK-deficient mice were characterised to have osteopetrosis with blocked osteoclast differentiation (Dougall, W.C., *et al.,* 1999) and have defective peripheral and mesenteric lymph node organogenesis (Kong, Y.Y., *et al.,* 1999). The cytoplasmic terminus of RANK contains tumour necrosis factor receptor associated factor (TRAF) binding sites. TRAFs are responsible for induction of down-stream osteoclast differentiation signals when RANK signal is activated (Galibert, L., *et al.,* 1998). Of different TRAF molecules, TRAF6 activates several osteoclast signalling pathways, including translocation of transcription factor NFκB (Darnay, B.G., *et al.,* 1998; Galibert, L., *et al.,* 1998; Myers, D.E., *et al.,* 1999) and the activation of protein kinases, such as JNK and c-src (Galibert, L., *et al.,* 1998; Kobayashi, N., *et al.,* 2001). TRAF6-deficient mice exhibits severe osteopetrosis and are defective in osteoclast formation due to defective RANK-RANKL signalling (Naito, A., *et al.,* 1999).

Furthermore, Lomaga, M.A. *et al.*, (1999), have also demonstrated that TRAF-deficient mice are osteopetrotic with defects in bone remodelling and tooth eruption due to impaired osteoclast function (Lomaga, M.A., *et al.,* 1999).

Osteoprotegerin (OPG)

The osteoblasts/stromal cells produce osteoprotegerin (OPG) or osteoclastogenesis inhibitory factor (OCIF) (Yasuda, H., *et al.,* 1998b). OPG is identified as the only secreted member of the TNF receptor (TNFR) superfamily (Simonet, W.S., *et al.,* 1997; Yasuda, H., *et al.,* 1998b; reviewed in Hofbauer L.C., *et al.,* 2001b) and contains four cysteine-rich domains and two death domain homologous regions (Takahashi N, *et al.,* 1999). OPG acts as a decoy receptor of RANKL and competes with RANK receptor for RANKL binding (Yasuda, H., *et al.,* 1998a; Yasuda, H., *et al.,* 1998b). The binding of OPG to RANKL induces the inhibition of terminal stages of osteoclast differentiation, suppression of the activation of mature osteoclasts and induction of apoptosis (reviewed in Hofbauer, L.C., *et al.,* 2001b). Mice with targeted deletion of the OPG gene manifest severe osteoporosis (Bucay, N., *et al.,* 1998; Mizuno, A., *et al.,* 1998), whereas transgenic mice over-expressing OPG have severe osteopetrosis with a decrease in metaphyseal trabecular bone (Simonet, W.S., *et al.,* 1997). Bone remodelling is controlled by a balance between RANK-RANKL binding and OPG production. Therefore, the RANKL/OPG ratio determines overall osteoclast activity and bone turnover (Gori, F., *et al.,* 2000). Furthermore, OPG has been shown to act directly on mature osteoclasts and osteoclast precursors, inhibiting bone resorption activity (Hakeda, Y., *et al.,* 1998) by blocking TRAP and CTSK expression (Wittrant, Y., *et al.,* 2002).

Macrophage Colony Stimulating Factor (M-CSF)

M-CSF or Colony-Stimulating Factor-1 is a crucial soluble growth factor or cytokine responsible for the proliferation, differentiation, activation and survival of cells of the monocyte/macrophage lineage including the osteoclast precursors (Yoshida, H., *et al.,* 1990; Felix, R., *et al.,* 1990a; Felix, R., *et al.,* 1990b). M-CSF is released by osteoblasts to provide a signal for growth and maturation of osteoclast precursors (Felix, R., *et al.,* 1989).

It is believed that M-CSF is indispensable for the both proliferative phase and the differentiation phase of osteoclast development (reviewed in Suda, T., *et al.,* 1999). Even in the presence of RANKL, osteoclast precursors will not functionally differentiate into osteoclasts if M-CSF is absent *in vitro* (Matsuzaki *et al.,* 1998). Yoshida, H., *et al.,* (1990) found that in the osteopetrotic *op/op* mutant mouse, functional M-CSF is absent, causing inhibition of osteoclast proliferation and differentiation from progenitor cells. Furthermore, Amano, H., *et al.* (1998) demonstrated that M-CSF is required for the fusion of macrophages and mononuclear osteoclasts into multinuclear and active osteoclasts.

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a cytokine or growth factor that is produced by T cells following activation and by most myeloid lineage cells such as macrophages and granulocytes (reviewed in Burgess, A.W., and Metcalf, D., 1980; reviewed in Arai, K., *et al.,* 1986). GM-CSF is also produced at sites of activated inflammatory cells, such as endothelial and stromal cells (reviewed in Quesenberry, P., *et al.,* 1991; reviewed in Bab, I.A. and Einhorn, T.A., 1993).

GM-CSF has a number of biological functions and effects; maintaining the viability in haematopoietic progenitors cells and blood cells by suppressing apoptotic death and ensuring survival (reviewed in Wognum A.W., *et al.,* 1996; reviewed in Danova, M. and Aglietta, M., 1997). Moreover, GM-CSF has a role in the development and function of dendritic cells, granulocytes and macrophages (reviewed in Banyer, J.L., and Hapel, A.J., 1999).

There have been discrepancies over the exact actions of GM-CSF on osteoclast development. GM-CSF has been reported to be both a stimulator and inhibitor of osteoclast differentiation. GM-CSF has potential to induce differentiation into osteoclasts by proliferating the number of osteoclast precursors (Yamazaki, H., *et al.,* 2001). Recently, studies carried out by Cakouros, D., *et al.,* (2001), have demonstrated that transcription factor NFκB is required for correct transcription of a human GM-CSF transgene. In another study, GM-CSF increases AP-1 DNA binding in human alveolar macrophages (Flaherty, D.M., *et al.,* 2001). These recent results show that GM-CSF might stimulate osteoclast differentiation, as NFκB and AP-1 have been suggested to be important transcription factors in osteoclastogenesis (reviewed in Manolagas, S.C., 2000). Thus, GM-CSF might be a stimulator of osteoclast differentiation. In marked contrast, other studies suggested that GM-CSF is an inhibitor of osteoclastogenesis. Miyamoto, T., *et al.,* (2001) demonstrated that GM-CSF inhibits osteoclast formation by reducing the expression of c-fos, preventing AP-1 signalling. Furthermore, Hodge, J.M., *et al.,* (2004) showed that GM-CSF has biphasic effects on osteoclast formation, with persistent exposure of GM-CSF leads to formation of dendritic cells in human CFU-GMs.

Cathepsin K (CTSK)

Cathepsin K (CTSK) is a member of the papain superfamily of lysosomal cysteine proteases that includes Cathepsins B, L, H and S (reviewed in Turk, B., *et al.,* 1997). Cysteine proteases are believed to participate in a variety of physiological processes including lysosomal proteolysis (reviewed in Turk, B., *et al.,* 1997), cartilage proteoglycan breakdown (Buttle, D.J. and Saklatvala, J., 1992) matrix modification (Reddy, V.Y., *et al.,* 1995) and bone remodelling (Delaisse, J.M., *et al.,* 1984). CTSK is significantly expressed at higher levels in osteoclasts than other cathepsins (S, M, L, B or D) (Bossard, M.J., *et al.,* 1996; Drake, F.H., *et al.,* 1996; Mano H, *et al.,* 2000)

CTSK spans approximately 12.1kb of genomic DNA and has been mapped to chromosome 1q21. CTSK is composed of eight exons and seven introns with similar genomic organization to that of cathepsin S and L (Gelb, B.D., *et al.,* 1997; Rood, J.A., *et al.,* 1997). CTSK is synthesised as proenzyme (prepropeptide of 37kDa) and undergoes post-translational modification (Gelb BD, *et al.,* 1997). Pro-cathepsin K is subsequently transported to acidic lysosomal compartments, where the pro-peptide is cleaved to 27kDa and the enzyme is activated (Gelb BD, *et al.,* 1997). CTSK is demonstrated to be optimally active at pH of 6.1, but is functional at most pH (Bromme, D., *et al.,* 1996; Gelb, B.D., *et al.,* 1997).

CTSK is believed to be a key player in bone resorption, where osteoclasts synthesise and release CTSK into bone resorption lacunae. Mature and active CTSK is either directionally secreted into the resorption lacunae or undergoes proteolytic

degradation within the lysosome, which is then transported to the ruffled border (Rieman, D.J., *et al.,* 2001). The osteoclasts cultured with the CTSK antisense oligonucleotides (Ishikawa, T., *et al.,* 2001) and small inhibitory RNA to CTSK have shown significantly reduced bone resorption (Selinger, C.I., *et al.,* 2005). CTSK-deficient mice or knockout mice developed osteopetrosis and displayed the characteristics of pycnodysostosis (Saftig, P., *et al.,* 1998; Gowen, M., *et al.,* 1999), an uncommon human genetic disorder characterised by osteosclerosis of the skeleton, short stature, and bone fragility (reviewed in Fratzl-Zelman, N *et al.,* 2004). These results demonstrate that CTSK is critical in bone resorption by osteoclasts and is the major protease in osteoclast. Therefore, by inhibiting the function of CTSK, a possible treatment for osteoporosis can be designed (reviewed in Zaidi, M., *et al.,* 2001).

Tartrate-Resistant Acid Phosphatase (TRAP)

TRAP (type 5 acid phosphatase) is expressed during osteoclast differentiation and is also used as an osteoclast-specific marker (Hayman, A.R., *et al.,* 1996; reviewed in Roodman, G.D., 1996a and Manolagas, S.C., 2000). In cell culture, TRAP staining is adequate to identify osteoclasts from other monocyte-derived cells (Baroukh, B., and Saffar, J.L., 1991). TRAP is an acid phosphatase that contains a unique Fe^{2+}/Fe^{3+} metal centre that is able to catalyse hydroxyl radical formation (Hayman, A.R., *et al.,* 1996; reviewed in Kimura, E., 2000). TRAP is localized intracellularly in the lysosomal compartment (reviewed in Lamp, E.C. and Drexler, H.G., 2000), on the extracellular channels of the ruffled border and the space between the cells and bone (reviewed in Roodman, G.D., 1996a).

Hayman, A.R., *et al.,* (1996) have demonstrated that mice lacking TRAP have disrupted endochondral ossification and mild osteopetrosis, illustrating that TRAP is an enzyme necessary for osteoclast function. The function of TRAP in bone resorption is still unknown, but it is believed to have a role in bone resorption homeostasis (transport, metabolism) (reviewed in Lamp, E.C., and Drexler, H.G., 2000).

Calcitonin (CT) and calcitonin receptor (CTR)

Calcitonin (CT) is a 32 amino acid peptide hormone that is produced by the thyroid gland and is responsible for calcium homeostasis (reviewed in Pondel, M., 2000). Calcitonin regulates calcium homeostasis in conjunction with calcitonin receptor, by inhibiting osteoclast bone resorption and enhancing calcium excretion by the kidney (reviewed in Pondel, M., 2000). Calcitonin secretion is highly regulated by the plasma Ca^{2+} level. CT gene expression is conserved throughout many species and is regulated at the transcriptional level by transcriptional regulatory elements in its 5' flanking region (reviewed in Pondel, M., 2000). Calcitonin is found to be an effective inhibitor in the early stages of osteoclast development involving osteoclast recruitment and, at high concentration, is an effective inhibitor of late osteoclast development (Suzuki, H., *et al.,* 1996; Cornish, J., *et al.,* 2001).

Calcitonin receptor (CTR) is a member of the seven transmembrane domain G-coupled receptor superfamily that is expressed on a variety of cell types, but is not expressed on the macrophage polykaryons (reviewed in Pondel, M., 2000). CTR is a key marker of osteoclast formation and maturation (Hattersley, G., and Chambers, T.J., 1989; Lee, S.K., *et al.,* 1995). Exposure with calcitonin results in inhibition of

osteoclast mediated bone resorption and cessation of motility (Chambers, T.J., and Magnus, C.J., 1982). The calcitonin-CTR interaction causes a significant increase in the intracellular cAMP concentration (Nicholson, G.C., *et al.,* 1986) that leads to prolonged down-regulation of CTR by inhibiting the transcription of CTR gene expression (Inoue, D., *et al.,* 1999).

V-ATPase

Vacuolar-type H+-ATPase (V-ATPase) is a highly conserved multi-subunit enzyme that acidifies micro-environment by transporting protons across membranes (reviewed in Sun-Wada, G.H., *et al.,* 2003a; reviewed in Sun-Wada, G.H., *et al.,* 2003b; reviewed in Sun-Wada, G.H., *et al.,* 2004). The secretion of protons by osteoclasts is a critical mechanism that maintains the acidic environment required for proteolytic enzymes (Baron, R., *et al.,* 1985). The V-ATPases, the proton pumps required for acidification, are highly concentrated at the ruffled border of osteoclasts (Blair, H.C., *et al.,* 1989).

Suppression of V-ATPase by antisense RNA and small interfering RNA molecules inhibited bone resorption (Laitala, T., and Vaananen, H.K., 1994; Hu, Y., *et al.,* 2005). Furthermore, knock out of V-ATPase member I caused severe osteopetrosis, due to loss of osteoclast mediated extracellular acidification (Li, Y.P., *et al.,* 1999), demonstrating the importance of V-ATPase in the bone resorption activity of osteoclasts.

Integrins

Bone resorption activity of an osteoclast begins with the recognition of bone matrix components, followed by adhesion to bone via receptor-ligand interactions (Davies, J., *et al.,* 1989). The interaction between the osteoclasts and the bone matrix are mediated by members of integrin family of cell surface molecules and receptors (reviewed in Hynes, R.O., 1992). Integrins are family of heterodimeric transmembrane receptors composed of alpha and beta subunits, with 15 alpha subunits and 9 beta subunits. Integrins that mediate cell to cell and cell to extracellular matrix interactions (reviewed in Hynes, R.O., 1992). Integrins mediate cell adhesion by recognising specific peptide sequences of adhesion molecules (reviewed in Yamada, K.M., 1997). Extracellular matrix signalling is critical for the cell shape, polarity, gene expression, proliferation and cell survival (reviewed in Damsky, C.H., 1999). Furthermore, the integrin alphaV/beta3 complex is known to be involved in osteoclast formation, where the integrin alphaV/beta3 interacts with osteopontin in the matrix of bone (Reinholt, F.P. *et al.,* 1990). In addition, mice deficient in integrin beta3 have osteopetrotic phenotype (McHugh, K.P., *et al.,* 2000), indicating a crucial role of integrin in osteoclast activity. In osteoclasts, integrin alphaV/beta3 complex creates a unique cytoskeleton that prompts osteoclast to polarize its fibrillar actin into a circular structure known as the 'actin ring' or 'sealing zone'. The actin ring formation allows osteoclasts to create an acidified resorptive microenvironment (reviewed in Ross, F.P., and Teitelbaum, S.L., 2005). Furthermore, any structural abnormalities in the actin ring leads to arrested bone resorption and mice deficient in integrin alphaV/beta3 fails to form defined actin rings (McHugh, K.P., *et al.,* 2000), signifying the importance of the actin ring in osteoclast biology.

Chemokines

Chemokines are small proteins between 8 to 12 kD, that are classified into two main sub families, the CC (beta family) and the CXC (alpha family) chemokines according to the first two of the four cysteine residues (Gao, J.L., *et al.,* 1993; Gosling, J., *et al.,* 1999; reviewed in Rollins, B.J., 1997). The most CC chemokines genes are grouped at 17q11.2-12, while the most CXC chemokines are grouped at 4q12-13 (reviewed in Rollins, B.J., 1997). Both CC and CXC families of chemokines are involved in immune response, allergies and the development of several cell types (reviewed in Baggiolini, M., *et al.,* 1995; Horuk, R., 2001). There are over 20 known CC chemokine ligands (CCL) family that bind to 10 known CC chemokine motif receptors (CCR) and more than 15 CXC chemokine ligands and 6 CXC receptors (reviewed in Horuk, R., 2001; reviewed in New, D.C., and Wong, Y.H., 2003). All chemokine receptors are members of the 7-transmembrane spanning G-protein coupled receptor family. Chemokine receptor activation generally leads to inhibition of cAMP, regulating calcium flux, but receptors can activate other signalling pathways (reviewed in Rollins, B.J., 1997; reviewed in New, D.C., and Wong, Y.H., 2003).

Many chemokine ligands can bind multiple chemokine receptors, while several chemokine receptors can bind multiple chemokine ligands (reviewed in Rollins, B.J., 1997; reviewed in Horuk, R., 2001), creating confounding problems of understanding chemokine physiology. In relationship to bone disease, chemokines are discussed as candidates for involvement in bone loss in various inflammatory diseases through the recruitment of osteoclast precursors (Choi, S.J., *et al.,* 2000).

Furthermore, osteoblasts have also been proposed as a source of chemokines in bone (Yu, X., *et al.,* 2004).

Monocyte chemotactic protein 1 (MCP-1)

The most widely researched member of the CCL chemokine family is monocyte chemotactic protein 1 (MCP-1, CCL2 or SCYA2) (reviewed in Van Coillie, E., *et al.,* 1999). MCP-1 is a potent chemo-attractant for monocytes *in vitro* with an ED_{50} of 500ρmol/L. Monocyte chemotactic protein 1 (MCP-1) is commonly found at the site of tooth eruption, rheumatoid arthritic bone degradation and bacterially induced bone loss (reviewed in Wise, G.E., *et al.,* 2002a). MCP-1 is also expressed by a wide variety of cells including fibroblasts and endothelial cells (Yu, X., and Graves, D.T., 1995).

The cellular effect of MCP-1 is mediated by the CC chemokine receptor 2 (CCR2), a G-coupled receptor (reviewed in Rollins, B.J., *et al.,* 1997) and stimulates the phosphoinositide 3-kinase signalling pathways (Gerszten, R.E, *et al.,* 2001). At sites of tooth eruption MCP-1 is believed to recruit precursor cells to sites of tooth eruption where precursor cells fuse to form osteoclasts to degrade the area of bone (reviewed in Wise, G.E., *et al.,* 2002a), where molecules including, IL-1α (Wise, G.E., 1998; Que, B.G., *et al.,* 1999), M-CSF (Wise, G.E., *et al.,* 1998), MCP-1 (Que, B.G., and Wise, G.E., 1998; Wise, G.E., *et al.,* 1999), OPG (Wise, G.E., *et al.,* 2000; Wise, G.E., *et al.,* 2002b) and RANKL (Wise, G.E., *et al.,* 2000) regulate the fusion of mononuclear cells in the follicle. Recently, MCP-1 expression is regulated by NFκB

(Ueda, A., *et al.,* 1997) and microarray experiments found MCP-1 to be expressed in osteoclasts (Ishida, N., *et al.,* 2002).

RANTES

Another well documented CC chemokine is "regulated upon activation, normal T-cell expressed and presumably secreted" (RANTES) or small inducible cytokine A5 (SCYA5 or CCL5). RANTES is a small protein of 68 amino acids that binds CCR1, CCR3, and CCR5 (reviewed in Zlotnik, A., and Yoshie, O., 2000). RANTES is used by T-cells and monocytes to attract cells of the non-adaptable immune system and further T-cells to sites of infection or inflammation (Schall, T.J., *et al.,* 1990), but also acts on a range of other cells including basophils, eosinophils, natural killer cells, dendritic cells and mast cells (reviewed in Appay, V., and Rowland-Jones, S.L., 2001). RANTES is up-regulated during osteoclast formation (Cappellen, D., *et al.,* 2002; Ishida, N., *et al.,* 2002) and is assumed to have the role of attracting osteoclast precursor cells to sites of osteoclastogenesis to allow for fusion (Cappellen, D., *et al.,* 2002).

Macrophage inflammatory protein 1 alpha (MIP1α)

Macrophage inflammatory protein 1 alpha (MIP1α) or small inducible cytokine A3 (SCYA3 or CCL3) is a CC chemokine with 69 amino acids (Wolpe, S.D., and Cerami, A., 1989) that binds CCR1, and CCR5 (reviewed in Zlotnik, A., and Yoshie, O., 2000). MIP1α is exhibits a variety of proinflammatory activities *in vitro*, including leukocyte chemotaxis (reviewed in Cook, D.N., 1996), is a primary negative regulator of hematopoietic stem cell proliferation and protects hemopoietic stem cells

from damage during cytotoxic therapies for cancer (Graham, G.J., *et al.,* 1990; reviewed in Cook, D.N., 1996). MIP1α activates a number different cell types including, basophils, mast cells (Alam, R., *et al.,* 1992), and macrophages (Fahey, T.J. 3rd., *et al.,* 1992) and plays a role in the selective recruitment of macrophages in synovial inflammation associated with rheumatoid arthritis (Koch, A.E., *et al.,* 1994).

Recently, MIP1α is produced by myeloma cells and directly stimulates osteoclast progenitors and enhance osteoclast formation (Choi, S.J., *et al.,* 2000; Han, J.H., *et al.,* 2001; reviewed in Roodman, G.D., and Choi, S.J., 2004). Furthermore, antibodies to MIP1α inhibit the formation of TRAP positive multinuclear cells in favour of macrophage like cells (Scheven, B.A., *et al.,* 1999). In addition, MIP1α enhanced adhesive interactions between myeloma cells and marrow stromal cells increasing expression of PTHrP, RANKL, and 1α,25-dihydroxyvitamin D_3 (reviewed in Roodman, G.D., 2001; Roodman, G.D., and Choi, S.J., 2004). Recently, another CC chemokine has been linked to osteoclast differentiation. Macrophage inflammatory protein 1 gamma (MIP1γ) is the key CC chemokine expressed in mouse and rat osteoclast formation (Lean, J.M., *et al.,* 2002), but this chemokine is not found in human. Furthermore, this demonstrates that there is a difference in chemokine expression between human and mouse osteoclast differentiation.

Transcription factors involved in osteoclast formation.

PU.1

PU.1 is a member of the ETS binding transcription factor family, and is expressed only in the hematopoietic cells, with high levels detected in B lymphocytes, monocytes/macrophages, granulocytes and megakaryocytes (reviewed in Simon,

M.C., 1998) and osteoclast precursors (Tondravi, M.M., *et al.,* 1997, reviewed in Dahl, R., and Simon, M.C., 2003). PU.1 has been implicated in the expression of a large amount of genes needed by lymphoid and myeloid lineage cells including immunoglobulins, M-CSF receptor, GM-CSF receptor and the G-CSF receptor (reviewed in Simon, M.C., 1998). Mice lacking PU.1 gene exhibit osteopetrosis, with suppression of osteoclast formation at an early precursor stage (Tondravi, M.M., *et al.,* 1997). Furthermore, PU.1 expression was observed throughout the life cycle of the osteoclast, and PU.1 is crucial in osteoclast differentiation (Tondravi, M.M., *et al.,* 1997).

Nuclear Factor κB (NFκB)

The binding of RANKL to RANK causes translocation of NFκB (Wei, S., *et al.,* 2001), which is involved in activation of the osteoclasts rather than involvement in osteoclast survival (Wei, S., *et al.,* 2001). NFκB is a transcription factor; a heterodimer of members of Rel family of proteins, typically consisting of p50 and p65 monomeric proteins (reviewed in De Bosscher, K., *et al.,* 2003). The activation of NF-κB is regulated by TRAF6 mediated phosphorylation of IκB complex. IκB binds to NFκB and masks its nuclear localisation signal, thus retaining the transcription factor in the cytoplasm (reviewed in Ghosh, S., *et al.,* 1998). Once activated, NFκB binds to κB response elements on the target DNA sequences within the nucleus to initiate transcription of genes (reviewed in Ghosh, S., *et al.,* 1998). Genes expressed via activation of the NFκB are mainly involved in immune and inflammatory response (Rani, M.R., *et al.*., 2001), and is also involved in regulation of osteoclast activation for bone resorption (Miyazaki, T., *et al.,* 2000). The exact NFκB pathways involved in osteoclast differentiation are not known, however, the activation of NF-

κB leads to activation of downstream transcription factors, including NFAT (Schmidt, A., *et al.*., 1990); cytokines, including IL-1, IL-2, GM-CSF; and cell adhesion molecules, including VCAM-1 (reviewed in Ghosh, S., *et al.,* 1998).

c-Fos

The fos proto-oncogene (c-fos) is a cellular homolog of the oncogene that is carried by the Finkel-Biskis-Jinkins (FBK) murine sarcoma virus (reviewed in Grigoriadis, A.E., *et al.,* 1995). The c-fos protein, Fos is a nuclear phosphoprotein that is a major component of the Activator Protein-1 (AP-1) transcription factor (Chiu, R., *et al.*., 1988; Franza, B.R. Jr., *et al.*., 1988; Rauscher, F.J. 3rd, *et al.*., 1988a; Rauscher, F.J. 3rd, *et al.*., 1988b; reviewed in Curran, T., and Franza, B.R. Jr., 1988) and is a member of multi-gene family, including Fos-related proteins (*Fos B, Fra1, Fra2*) (reviewed in Grigoriadis, A.E., *et al.,* 1995). The function of Fos protein as a transcription factor is dependent on hetero-dimerisation with members of *Jun* family (Jun, Jun B, Jun D) (reviewed in Grigoriadis, A.E., *et al.,* 1995).

Fos knockout mice lack functional osteoclasts and are osteopetrotic (Johnson, R., *et al.,* 1992; Wang, Z.Q., *et al.,* 1992). The resulting cells from Fos knockout mice are also deficient in TRAP, CTSK and CTR expression (Matsuo, K., *et al.,* 2004). Conversely, over-expression of c-fos in osteoclast progenitors enhanced osteoclast differentiation (Miyauchi, A., *et al.,* 1994). It is also believed that Fos is critical in osteoclastogenesis, as Fos is an early mediator of other transcription factors required for osteoclast formation including NFAT (Matsuo, K., *et al.,* 2004).

c-Jun

The jun proto-oncogene (c-jun) is the cellular homolog of the oncogene that is carried by avian sarcoma virus 17 (reviewed in Bohmann, D., *et al.,* 1987). While the Fos proteins can only heterodimerise with members of the Jun family, Jun proteins can both homodimerise and heterodimerise to form transcriptionally active complexes (reviewed in Jochum, W., *et al.,* 2001). Jun protein is necessary for regulating the cell cycle and hence, cell development (reviewed in Shaulian, E. and Karin, M., 2001) and the inactivation of c-jun in chondrocytes developed impaired intervertebral discs and vertebral arches (reviewed in Jochum, W., *et al.,* 2001). Furthermore, c-jun have been shown to regulate osteoclast differentiation in co-operation with NFAT, mediating transcription of genes required for osteoclast formation (Ikeda, F., *et al.,* 2004)

Activator protein 1 (AP-1)

Activator protein 1 (AP1) is a ubiquitously expressed transcription factor formed from homo or hetero-dimers of the fos and jun families of proto-oncogenes (reviewed in Angel, P., and Karin, M., 1991). The action of AP-1 is controlled by interactions with other transcription factors and is further controlled by up-stream kinases such as c-Jun NH_2-terminal kinases (JNK) and mitogen activated protein kinases (MAPK) (reviewed in Dong, C., *et al.,* 2001). Furthermore, synthesis of AP-1 is further induced by the activation of Protein Kinase C (PKC) and Ras following T cell receptor engagement (reviewed in Isakov, N., and Altman, A., 2002). AP1 is important in organ development including cardiac and liver development and in the differentiation of several cell types including osteoclasts (reviewed in Jochum, W., *et al.,* 2001). In hematopoietic cell lineage, AP-1 plays multiple roles in the development of precursor cells into mature cells in most hematopoietic cell lineages,

including monocyte/macrophage, granulocyte, megakaryocyte, mastocyte (reviewed in Liebermann, D.A., *et al.,* 1998) and osteoclast lineages (reviewed in Wagner, E.F., 2002; reviewed in Wagner, E.F., and Matsuo, K., 2003).

Microphthalmia transcription factor (MITF)

Microphthalmia transcription factor (MITF) gene encodes a basic-helix-loop-helix zipper (bHLH-zip) transcription factor that has been mapped to chromosome 3p12.3-14.1 (Tachibana, M., *et al.,* 1994). MITF is believed to be required for terminal differentiation of osteoclasts (Mansky, K.C., *et al.,* 2002). MITF-deficient mice have osteopetrosis, demonstrating the importance of MITF in bone resorption and osteoclast function (Kawaguchi N, and Noda, M., 2000). MITF is also believed to regulate osteoclast target genes such as TRAP, CTSK and E-cadherin by binding to a 7 base pair conserved sequence TCANGTG found in the promoter region of these genes (Mansky, K.C., *et al.,* 2002). Therefore, studies have established that MITF is critical in regulation of osteoclast specific genes and MITF might be a direct target of a RANKL signalling pathway that is necessary for osteoclast differentiation and function (Mansky, K.C., *et al.,* 2002).

NFATc1.

Nuclear Factor of Activated T-cells (NFAT) is a multi-subunit transcription factor critical in regulating early gene transcription of several cytokines in response to T-cell receptor mediated signals in lymphocytes (Park, J., *et al.,* 1996; Ranger, A.M., *et al.,* 2000; reviewed in Horsley, V., and Pavlath, G.K., 2002). NFAT is not just implicated in immune cell function but also in muscle and myocardium development (reviewed in Horsley, V., and Pavlath, G.K., 2002). The NFATc family of proteins

has four members; NFATc1 (NFAT2), NFATc2 (NFAT1; NFATp), NFATc3 (NFAT4) and NFATc4 (NFAT3) (reviewed in Rao, A., *et al.,* 1997). The regulation of transcriptional activity of NFAT is dependent on Ca^{2+}/calmodulin dependent protein phosphatase, calcineurin (Northrop, J.P., *et al.,* 1994), a down stream target of increased intracellular calcium levels. NFAT protein is known to act synergistically with AP-1 to regulate the expression of diverse inducible genes including, IL-2 (Shaw, J.P., *et al.,* 1988), IL-3 (Cockerill, P.N., *et al.,* 1993), IL-4 (Chuvpilo, S., *et al.,* 1993), IL-5 (reviewed in De Boer, M.L., *et al.,* 1999) GM-CSF (Masuda, E.S., *et al.,* 1993), TNF-α (Goldfeld, A.E., *et al.,* 1993), IFN-γ (Sica, A., *et al.,* 1997) implicating its importance in gene regulation (reviewed in Rao, A., *et al.,* 1997).

In 2002, two separate groups demonstrated that NFATc1 is a key transcription factor in mouse osteoclast differentiation (Ishida, N., *et al.,* 2002; Takayanagi, H., *et al.,* 2002b). The importance of NFAT in osteoclast formation was re-iterated in human peripheral blood mononuclear cells (PBMC) osteoclast model (Day, C.J., *et al.,* 2004; Day, C.J., *et al.,* 2005). It is believed that NFAT is involved in multi-nucleation, the fusion and the function of osteoclasts (reviewed in Takayanagi, H., 2005a; reviewed in Takayanagi, H., 2005b). Addition of cyclosporin A (CsA), an inhibitor of calcineurin, in both mouse (Ishida, N., *et al.,* 2002; Hirotani, H., *et al.,* 2004) and human (Day, C.J., *et al.,* 2004) osteoclast differentiation models resulted in significant suppression of multinuclear cell formation.

Recently, NFATc1 has been reported as the master regulator of osteoclast formation (reviewed in Takayanagi, H., 2005a; reviewed in Takayanagi, H., 2005b). In mouse cells, transfected constitutive NFATc1 appears sufficient for induction of

bone resorbing osteoclasts (Takayanagi, H., *et al.,* 2002b; Hirotani, H., *et al.,* 2004). In mouse, NFATc1 regulates expression of major osteoclast markers including CTSK (Matsumoto, M., *et al.,* 2004) and TRAP (Matsuo, K., *et al.,* 2004). However, Day, C.J., *et al.,* (2004) has showed that suppression of NFATc1 using CsA does not influence the gene regulation of osteoclasts specific markers, including CTSK and TRAP (Day, C.J., *et al.,* 2004). Furthermore, suppression of NFATc1 suppressed the regulation of calcitonin receptor (CTR), another osteoclasts marker, demonstrating that CTR expression is linked to NFATc1 expression.

The purpose of this project is to understand the biology involved in osteoclast formation. The aim of this research was to understand and clarify the effects of cytokines involved in osteoclast formation. Furthermore, to understand the mechanism of action (autocrine or paracrine) undertaken by the cytokines in question. The investigating the effects of the cytokine using quantitative real-time PCR and microarray will enable understanding of down-stream genes affected by the cytokine. In addition, various doses and delayed additions would clarify the effects of the cytokine in osteoclast formation. Previously, GM-CSF receptor alpha was highly up regulated in RANKL mediated osteoclasts compared to M-CSF mediated macrophage-like cells (Day, C.J., *et al.,* 2005), thus it was hypothesised that exogenous GM-CSF would negatively regulate osteoclast formation. Furthermore, it was hypothesised that GM-CSF would inhibit expression of key osteoclast related genes and possibly other genes that may not have been linked previously to osteoclast formation. In addition, the fusion mechanism involved in osteoclast formation is a process that is yet to be established. Recently, a novel chemokine receptor, dendritic cell-specific transmembrane protein (DC-STAMP) has been linked to cell fusion of

osteoclast precursor cells to yield osteoclast formation (Yagi, M., *et al.*, 2005), indicating a possible role of chemokines in osteoclast formation. Furthermore, it was hypothesised that chemokines would regulate osteoclast formation by enhancing the cell to cell fusion mechanism.

Chapter 2

Materials and Methods.

Materials

0.2ml PCR strip tubes from Axygen.

1mM Dithiothreitol (DTT)

1.5mL microfuge tubes from Eppendorf

3.5ml transfer pipet from Becton Dickinson

5× First-strand buffer from Invitrogen

7.5% sodium barcabonate from Invitrogen

10× PCR buffer from Promega.

10ml serological pipet (with Plug) from Becton Dickinson

10mL centriguge tubes from Sarstedt

21 x 21cm glass plate (backing plate)

21 x 15.1cm glass plate (front plate)

21 x 1.4 x 0.4 teflon spacer

21 x 1.4 x 0.8 teflon spacer

25ml serological pipet (with Plug) from Becton Dickinson

50mL centrifuge tubes from BD Biosciences

75cm^2 tissue culture flask from Becton Dickinson

99.9% Acrylamide from Sigma-Aldrich

Ammonium persulfate from Amresco Inc.

Anti CD1a-FITC from Chemicon.

Anti human MCP-1 antibody from Pepro Tech.

Boric acid from Progen

Centrifuge 3K15 from Sigma.

Certified molecular grade agarose from Bio-Rad

Cesium chloride (CsCl) ultra pure grade from ICN Biomedical Inc.

Coolpix digital camera from Nikon.

Diethyl pyrocarbonate (DEPC) from Sigma-Aldrich.

FACS Calibur from BD Sciences

Fetal bovine serum (FBS) (heat inactivated) from Invitrogen

Ficoll-Paque solution from Amersham Pharmacia Biotech

Guanidine thiocyanate from Promega

Human GM-CSF (hGM-CSF) from Pepro Tech

Human MCP-1 (hMCP-1) from Pepro Tech

Human M-CSF (hM-CSF) from Pepro Tech

Human MIP1α (h MIP1α) from Pepro Tech

Human RANTES (hRANTES) from Pepro Tech

iCycler iQ Real-time detection system software version 3.0. from Bio-Rad

iCycler iQ System from Bio-Rad

iQ SYBR Green Supermix from Bio-Rad.

JVD-80 double-slab vertical gel system from Shelton Scientific

Leukocyte acid phosphatase staining kit from Sigma

Magnesium chloride ($MgCl_2$) from Promega

Microfuge-Centrifuge 5402 from Eppendorf

Minimum essential media (MEM) from Invitrogen

NFAT activation HitKit from Cellomics.

N,N'-Methylene-bis acrylamide from Sigma-Aldrich

N,N,N',N'-Tetramethyleneethylenediamine (TEMED) from Sigma-Aldrich.

Penicillin-streptomycin from Invitrogen

Phosphate buffered saline (PBS) from Amresco Inc.

Phalloidin (Rhodamine) from Invitrogen.

Polyallomer centrifuge tubes from Beckman Instruments.

Poly dA from Amersham Pharmacia Biotech

PowerPac 300 Power Supply from Bio-Rad

PowerPac 200 Power Supply from Bio-Rad

Primer sets from Invitrogen and Proligo.

RNasin Ribonuclease inhibitor from Promega

Set of 100mM dATP, dCTP, dGTP, dTTP (dNTP) from Promega

Soluble Human RANKL (sRANKL) from Pepro Tech

Sub-Cell GT electrophoresis system from Bio-Rad.

Superscript II Rnase H reverse transcriptase from Invitrogen

Taq polymerase from Promega.

TE-2000U inverted research-grade microscope from Nikon

Teflon well forming comb 18 x 6 x 0.4cm – 18 well spaces (0.7cm width)

Tris base from Amresco Inc.

Ultra centrifuge from Beckman

Table 2.1 Primers used for Q-PCR

Gene	Forward primer 5' - 3'	Reverse primer 5' - 3'
18S	CTTAGAGGGACAAGTGGCG	ACGCTGAGCCAGTCAGTGTA
c-Fos	CTGAGGTTGATGTTCTCGTC	CTAAGGCCACTGTCATCACA
c-Jun	CGTTAGCATGAGTTGGCAC	GCATGAGGAACCGCATCGC
Calmodulin 1	GGCATTCCGAGTCTTTGACAA	CCGTCTCCATCAATATCTGCT
Calcitonin Receptor	GCAGGAAGATGTATGCTTTGA	CTTTACAACAGCTAGGTCCTG
Cathepsin K	TGAGGCTTCTCTTGGTGTCCATAC	AAAGGGTGTCATTACTGCGGG
CCL1	GACACAGTTGGATGGGTTCA	CCTCTGTGACCTAGCAAAAG
CCR1	TTCCTGTTCACCCATGAGTG	AAGGGGAGCCATTTAACCAG
CCR2a	CATAGCTCTTGGCTGTAGGA	GTGAAGCCAGACGTGTGATT
CCR2b	AACAAACACGCCTTCCACTG	GTCAAAGTCTCTACCCACAG
CCR4	CTTATGGGGTCATCACCAGT	AGTAGGTATGGTTGCGCTCA
CCR5	ACCAAGCTATGCAGGTGACA	GAACAGCATTTGCAGAAGCG
CD14	CGTTCGACCCTGTCGGTG	AGTCCTCAACGTCCTGACG
CD44	TGGCACCCGCTATGTCCAG	GTAGCAGGGATTCTGTCTG
CSF1R	GGTACTGCTGTAATGAGCCAA	AGTTTGTGCTTCCTGCTTGGT
CSF2Rα	GGCACGAGGCGAGAGAAGA	ACGCAAACATCGCCGCTTCT
FBP	CATGGCGATGGACCGGGA	AGGTTTGTCAGCACCAGTGT
GABPα	AAAGAGCGCCGAGGATTTCAG	CCAAGAAATGCAGTCTCGAG
GABPβ	CCCAGAGAGTCCTGACACT	TCTGAAGAATTGGACAATGG
GPX1	TGGCTTCTTGGACAATTGCG	GATAAGTAGTACCTTGCCCC
ILF3	AGGCCTACGCTGCTCTTGCT	GCCGAAGCCAGGGTTATGTG
Integrin αV	CTGGTAGATCCTATTACACTTC	CCTTCAAGCCAACCTATACTA
KOX31	AAGATGTGACTGTGGGCTTCA	GATCACCTCTGGTTTGTGAAC
MCP-1	TCGCGAGCTATAGAAGAATCA	TGTTCAAGTCTTCGGAGTTTG
MIP-1α	CTATGGACTGGTTGTTGCCA	AGGGGAACTCTCAGAGCAAA
MK2	GAAGATGCATCCAACCCTCT	TGGACAGTTCTGTCTCTTCG
MKK7	CGATCAGAGTCGCTGTTCAT	AGACAAGGACAGAGAGGTAG
MMP9	GAGACGCCCATTTCGACGA	TCGAAGATGAAGGGGAAGTG
NFATc1	GCATCACAGGGAAGACCGTGTC	GAAGTTCAATGTCGGAGTTTCTGAG
RANK	CAGAACTAAGCTCAGTATGTGA	GAATGCCAAGCTGCAGCAAC
RANTES	GAGCTTCTGAGGCGCTGCT	TCTAGAGGCATGCTGACTTC
STAT1	GGCAAAGAGTGATCAGAAACAA	GTTCAGTGACATTCAGCAACTCT
STAT3	GCAGAAAGATACGACTGAGG	GCAGATCACCCACATTCACT
TBP2	CCTGAGTTCAAGTTCATGCCA	CACATGCTCACTGCACATTGT
TRAP	GACCACCTTGGCAATGTCTCTG	TGGCTGAGGAAGTCATCTGAGTTG
TXN	GTCAAATGCATGCCAACATTC	AGCTTTTCCTTATTGGCTCCA

Table 2.2 Standard Curves

Gene	R-squared	Gradient	Y-intercept	PCR efficiency
18S	0.997	-2.94	34.61	109.5
c-Fos	0.996	-3.35	39.85	98.5
c-Jun	0.998	-3.06	35.47	112.3
CCL1	0.989	-3.04	37.41	113.2
CCR1	0.999	-3.42	40.36	96.1
CCR2a	0.992	-3.34	41.41	99.3
CCR2b	0.984	-3.35	45.97	99.1
CCR5	0.996	-3.69	45.75	86.8
CD14	0.991	-2.95	32.34	118.1
CD44	0.992	-3.21	38.36	103.1
CSF1R	0.998	-3.40	39.01	97
CSF2RA	0.981	-3.56	43.87	91
CTR	0.999	-3.34	40.60	99.8
CTSK	0.989	-2.83	31.66	125.1
FBP	0.994	-3.01	34.78	114.9
GABPα	0.994	-2.82	34.49	121.6
GABPβ	0.979	-2.82	34.49	125.7
ILF3	0.996	-3.01	35.87	114.6
Integrin α V	0.995	-3.35	39.85	98.6
KOX31	0.996	-3.29	37.49	101.7
MCP-1	0.992	-2.86	34.20	123.7
MIP-1α	0.995	-2.77	35.81	129.5
MMP9	0.996	-2.63	33.96	139.2
NFATc1	0.992	-3.05	35.22	112.6
RANK	0.992	-3.25	39.80	100.5
RANTES	0.990	-2.95	33.94	118.6
TRAP	0.999	-3.15	34.55	107.9
Average	**0.993**	**-3.13**	**37.45**	**109.6**

Cell culture

Preparation and culture of human monocytes

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood obtained from healthy donors from lab member volunteers using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) as per Nicholson, G.C., *et al.,* 2000. Blood was diluted one to one with minimum essential medium and phoshate buffered saline (MEM-PBS) (1:4) and layered over Ficoll-Paque and centrifuged (at 1500 rpm for 30 minutes). The PBMC layer was removed using a sterile transfer pipette and was washed with MEM-PBS solution, which was isolated by centrifugation (at 1000g for 10 minutes). The isolated PBMCs were resuspended and cultured in MEM supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA) and 1% penicillin-streptomycin. PBMCs were plated at 10^6 cells per cm^2 in MEM and allowed to adhere for 1.5-hours at 37°C in a 5% CO_2 atmosphere. Non-adherent cells were removed from the flask by washing twice with MEM. The adherent PBMCs were supplemented with cytokines accordingly (shown below). The medium was replaced every 7-days for up to 21-days. PBMCs were cultured on plastic for staining and RNA isolation or sperm whale dentine slices to ascertain bone resorption.

The adherent PBMC differentiated into macrophage-like cells in the presence of 25ng/mL M-CSF alone. 40ng/mL RANKL and 25ng/mL M-CSF were supplemented to induce osteoclast formation. GM-CSF treatment was performed on standard M-CSF and RANKL treated cells at 25ng/ml to induce dendritic-like cells. Chemokines (MCP-1, MIP1α and RANTES) were added at 25ng/ml with standard M-CSF alone or M-CSF and RANKL treatment or GM-CSF, M-CSF and RANKL treated cells.

Addition of antagonists

Neutralizing antibody directed against MCP-1 was used at 4μg/ml according to the manufacturer's protocols. Control antibody of goat anti-rabbit IgG was used at 10μg/ml (Serotec). Dominant negative inhibitor of MCP-1 (7ND), a mutant MCP-1 lacking N-terminal amino acids 2 to 8 (obtained from Egashira, K.), was transfected into HEK293 cells using Fugene 6 (Roche). The final concentration of 5% of conditioned medium containing 7ND was added into the cultures. Calcitonin (CT) was used ranging from 10ng/ml to maximum of 50ng/ml. Antagonist to p38 MAPK pathway (SB203580) was used at 20μM. Antagonist to ERK1/2 MAPK pathway (U0126) was used at 10μM.

Bone resorption

Bone resorption is ascertained by both light microscopy and scanning electron microscopy (SEM). TRAP stained cells were removed from the bone by agitation in chloroform-methanol (2:1) solution. Dentine slices were painted with xylene free ink (Artline 70 permanent marker) and excess ink is rubbed clean on paper towel as described in Hodge, J., *et al.,* (2004) for visualisation by light microscopy. Pits are highlighted in black ink while un-resorbed areas remain unmarked. The painted dentine slices (with xylene free ink), were washed in chloroform-methanol (2:1) solution, before being prepared for SEM, by gold sputter coating using a Bio-Rad sputter coater. SEM was performed on dentine slices by FEI Quanta 200 SEM at the Analytical Electron Microscopy Facility (AEMF) at QUT with the aid of Mr Loc Duong and Dr Thor Bostrom.

Bone resorption assays using mature osteoclasts

PBMC were prepared as above using density separation and plated onto BioCoat collagen I coated 6-well plates or 75cm^2 flasks (BD Biosciences), and were grown for 14-days with medium replaced at 7-days. At 14-days, medium was washed twice with PBS and the cultures were treated with dissociation buffer (Invitrogen, Carlsbad, CA, USA) for 10 to 15 minutes at 37°C. Cells in dissociation buffer were transferred to 10mL tubes and remaining adherent cells were removed by scraping lightly. Cells were layered over 40% and 70% serum gradient and then were settled for 5 minutes at room temperature. The cells were then centrifuged through 40% and 70% serum gradient (at 100g for 5 minutes), to separate the large multinuclear cells from non-multinuclear cells (adapted from Collin-Osdoby, P., *et al.,* 2003). Cells were plated on dentine slices in 96 well plates at approximately 50% confluence.

Cell stains

Tartrate resistant acid phosphatase stain

TRAP stain was performed using the leukocyte acid phosphatase kit (Sigma). The TRAP stain solution consisting of 45 parts deionized water 1 part diazotized fast garnet GBC solution, 2 parts acetate solution, 1 part tartrate solution and 0.5 parts naphthol AS-Bl solution was made fresh prior use and warmed to 37ºC. The stain was effected on live or formaldehyde fixed cells for 1 hour at 37°C in 5% CO_2. Cells were then washed with distilled water prior to bright field microscopy.

F-actin ring stain

PBMC cultures were fixed in acetone, citrate and formaldehyde solution and were stained with rhodamine labelled Phalloidin (Invitrogen) for F-actin. The

phalloidin stock was diluted in 1% bovine serum albumin (BSA)-PBS solution (1:40). The stained cells were incubated at room temperature for 20 minutes before the staining solution was removed and washed with PBS for analysis.

Nuclear stain

PBMC cultures were fixed in acetone, citrate and formaldehyde solution before staining. Hoechst dye or 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) stain (Invitrogen) was used for nucleic acid stain. Hoechst dye was part of the NFAT activation HitKit (Cellomics, Pittsburgh, PA, USA). The DAPI stock solution was diluted in PBS (1:50,000). The stained cells were incubated at room temperature for 5 minutes, and then were washed twice with PBS for analysis.

Nuclear NFAT stain

NFAT activation HitKit HCS reagent kit (Cellomics, Pittsburgh, PA, USA) was used to visualize activation of NFAT transcription factor proteins. Cells were incubated with Thapsigargin solution at 37°C for 30 minutes, before fixing in acetone, citrate and formaldehyde solution. The fixed cells were treated with 0.2× permeabilisation solution for an hour, before washed twice in 1× blocking buffer. The NFAT antibody containing solution was added and incubated for an hour, and then was washed twice in 1× blocking buffer. The secondary antibody treated with Hoechst dye was added and incubated for an hour, and then was washed twice again in 1× blocking buffer. The stained cells were stored in PBS solution for analysis.

Flow cytometry

Cells cultured on BioCoat collagen I plates (BD Sciences, NJ, USA) for 21 days were dissociated using cell dissociation buffer (Invitrogen, Carlsbad, CA, USA), then incubated with FITC conjugated human CD1a antibody (Chemicon, Temecula, CA, USA) for 45 mins on ice and washed with PBS, prior to flow cytometry (FACS Calibur, BD Biosciences, NJ, USA). The unstained cells were gated out and data acquisition and analysis were done using CellQuest software (BD Biosciences, NJ, USA).

RNA isolation

Cesium chloride gradient ultracentrifugation of RNA.

The total RNA isolation process was initiated by lysing the cultured PBMC with Guanidine Thiocyanate. We used a modified version of RNA isolation described by Glisin V, et al, 1974. RNA isolation was prepared by adding 3.5ml of 5.7M CsCl-0.1M EDTA to Polyallomer Centrifuge tubes. 1ml of 10% sodium lauryl sacrcosinate (SLS) was added to the lysed cultures, allowing proteins to be denatured. The homogenates are layered onto CsCl cushion. The tubes are balanced with 4M guanidine thiocyanate and ultra centrifuged (27,000 rpm for 16-hours at 22°C). After centrifugation, CsCl solution was removed with transfer pipette, up to the DNA banding level. The tube was inverted, and all but the bottom 1cm was sheared off. The RNA in the clear pellet was rinsed with 100µl of 70% DEPC treated ethanol and the solution was discarded. The pellet was dissolved with 60µl TE-SDS (10mM Tris-Cl, 1mM EDTA pH 8.0 1% sodium dodecyl sulfate), washed with 40µl TE, rinsed with 250µl of ice-cold 100% ethanol and 10µl of sodium acetate and collected into 1.5ml microfuge tubes. Mix well, and store for at least 30 mins at 0°C. The precipitate of

RNA and supernatant was collected in separate microfuge tubes by centrifugation with Microfuge-Centrifuge 5402 at 14,000 rpm for 20 mins at 4°C. The precipitated pellet was washed with 100µl of 70% DEPC treated ethanol and re-centrifuged at 14,000 rpm for 5 mins at 4°C. The pellet of nucleic acid was then dried at room temperature.

Nucleospin column kit.

RNA isolation was also performed using a kit based method. The Macherey-Nagel nucleospin RNA II kit was employed to isolate RNA from small quantities of cells such as those from 24 well plates. RNA was isolated as per kit instructions.

Real-time quantitative PCR (Q-PCR).

Total RNA for Q-PCR was isolated as described above at three day, one week and three week time points and converted into cDNA using Superscript II reverse transcriptase (RT, Invitrogen, Carlsbad, CA, USA) and oligo dT primer. PCRs were performed using SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). PCR primers were designed using the assistance of the BLAT server (genome.ucsc.edu) to identify, where possible, reasonably short introns near the 3' end of the gene. PCR reactions were optimised on both genomic DNA and cDNA in order to verify the presence of the short intron in genomic DNAs. All PCR reactions were optimised for an annealing temperature of 55°C by varying magnesium or primer concentration. Primer concentrations were between 100 and 500nM in a final volume of 20µl. All primers used for Q-PCR assays are shown in Table 2.2. The reactions were then cycled as follows: step 1, 94°C for 2 minute; step 2, 94°C for 30 seconds, 55°C for 45 seconds, 72°C for 1 minute (45 cycles); step 3, melt curve analysis from 55 to 95°C in

0.5°C increments. Each Q-PCR reaction was set up using 50ng cDNA per reaction, along with a genomic DNA control (100ng per reaction), and a DNA-free water control. PCR were performed using the Bio-Rad iCycler iQ system. The cycle thresholds (Ct) of gene expression in different RNA samples acquired through the amplification curves were used to analyse the relative expression (fold regulation). To calculate the fold difference in gene expression, the difference Ct in each RNA sample was calculated using the equation: Fold = $2^{\Delta\Delta Ct}$ as per Livak, K.J., and Schmittgen, T.D. (2001). Correlation of Q-PCR results to array results was performed using SPSS statistical analysis software. Standard curves were constructed of all primer sets by analysing 10 to 10^9 copies of PCR product (Table 2.2). PCR products were run on 8-12% polyacrylamide gels as described above to verify product size.

Statistical analysis.

Analysis for cells counts was carried out using Excel for each treatment type. All data for cell counts are presented plus or minus the standard error of the mean. Statistical analysis of the Q-PCR was carried out using Excel and SPSS statistical analysis package. Significant difference was determined using paired samples T-tests and ANOVA of data obtained from three or more RNA samples from separate cell culture experiments. Each gene was analysed by Q-PCR across each treatment type simultaneously allowing for paired analysis. Analysis of the array data was determined by analysis of the 95% and 99% confidence interval of the mean of absolute fold change calculated as total radioactive counts from the probe derived from osteoclast mRNA (M-CSF and RANKL treatment) divided by the total counts

from the GM-CSF treatment probe (GM-CSF, M-CSF and RANKL treatment) sampled over all data from the array. Those genes whose ratios of relative gene expression were outside the 99% confidence interval were considered to represent a 1% false positive risk. That is, the extreme tails of the relative expression distribution represent the genes most likely to have demonstrated significant changes in expression; either induction or repression.

Results and Discussion:

Chapter 3

GM-CSF Suppression of Human Osteoclast Formation.

This Chapter includes data on GM-CSF inhibition of osteoclast formation published in:

1. Day, C.J., Kim, M.S., Stephens, S.J.R., Simcock, W.E., Aitken, C.J., Nicholson, G.C. and Morrison, N.A. (2004) *J Cell Biochem.*
2. Kim, M.S., Day, C.J. and Morrison, N.A. (2005) *J Biol Chem.*

RESULTS

Cellular phenotype of cells treated with GM-CSF in the presence of M-CSF and RANKL.

Previous studies have implicated GM-CSF as both a negative (Miyamoto, T., *et al.,* 2001; Hodge, J.M., *et al.,* 2004) and positive (Yamazaki, H., *et al.,* 2001) regulator of osteoclast formation. The effect of continuous exposure of human PBMC to GM-CSF on osteoclast differentiation mediated by M-CSF and RANKL treatment is shown below in Fig. 3.1. The appearance of TRAP positive multinuclear cells was suppressed dose dependently by GM-CSF, with osteoclast differentiation suppressed by as much as 97% when exposed to 25ng/mL GM-CSF (Fig. 3.1A). Delayed addition of GM-CSF at 25ng/mL had similar suppressive effects (Fig. 3.1B). While normal osteoclasts differentiated using M-CSF and RANKL treatment were TRAP positive and multinuclear with potent bone resorption (Fig. 3.1C), cultures treated with maximal doses of GM-CSF and M-CSF and RANKL treatment were TRAP negative, mononuclear and were completely negative for bone resorption (Fig. 3.1C).

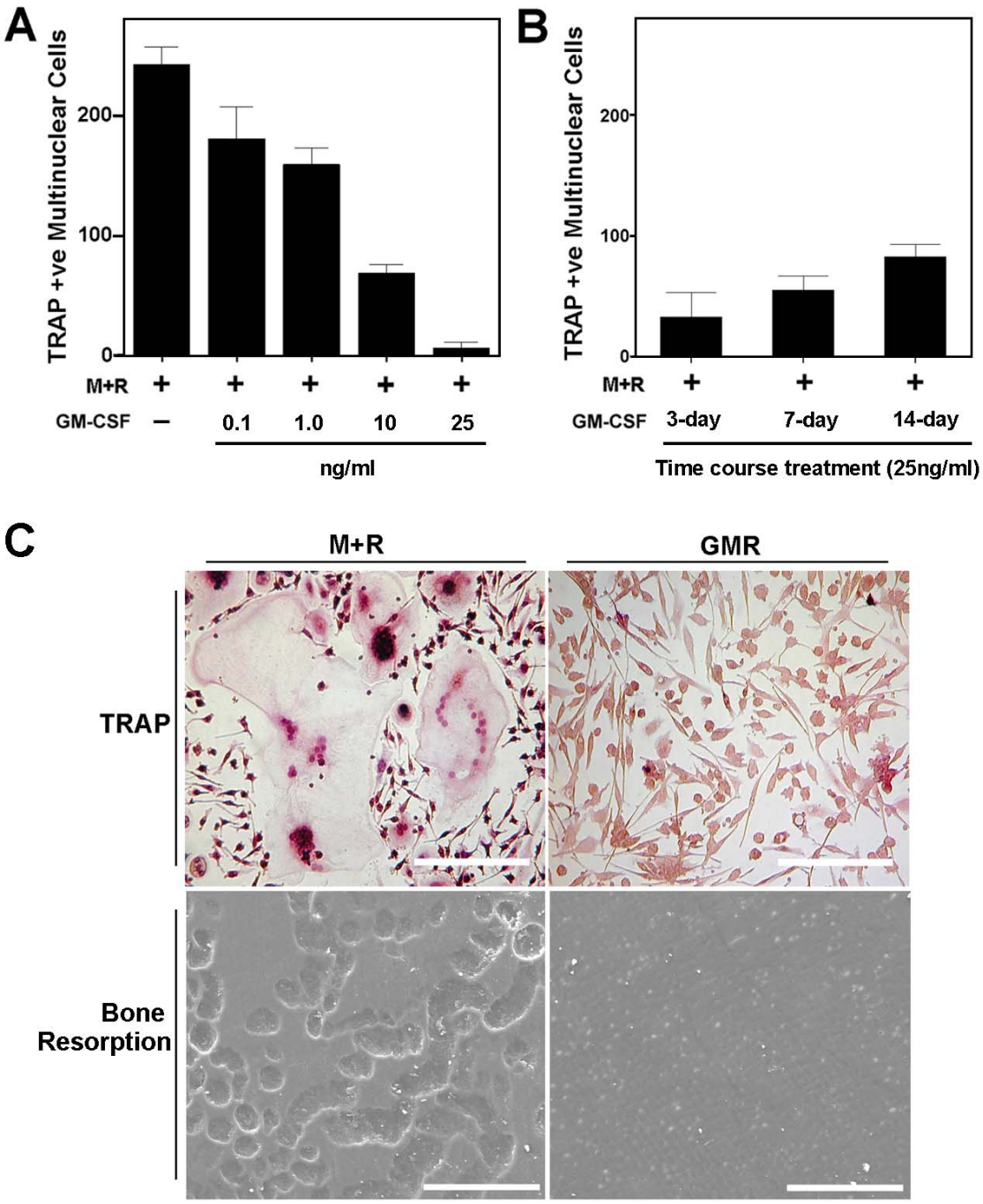

Fig. 3.1 GM-CSF suppresses osteoclast differentiation in PBMCs cultured with RANKL and M-CSF (M+R). (A) Cultures were treated continuously with GM-CSF at concentrations indicated (ng/ml) in the presence of RANKL and M-CSF (M+R). (B) Addition of GM-CSF (25ng/ml) at 3, 7 and 14-days suppresses osteoclast differentiation in cultures treated with RANKL and M-CSF. All the GM-CSF treated cultures had significantly lower numbers of TRAP positive multinuclear cells compared to M+R treated cultures (all p values <0.001). (C) Cellular and bone resorption phenotype after treatment with M+R or GMR (25ng/ml GM-CSF with M+R). TRAP: microscopy shows TRAP positive (purple colour) multinuclear giant cells in M+R treatment (upper left panel) and TRAP negative mononuclear cells in the GMR treatment (upper right panel). Bone resorption: scanning electron

microscopy of sperm whale dentine shows abundant resorption pits in M+R (lower left panel) and no bone resorption in GMR (lower right panel). Bar is 100 μm.

Characterisation of cells treated with GM-CSF in the presence of M-CSF and RANKL

To further characterize the phenotype of cells treated with GM-CSF in the presence of M-CSF and RANKL, the expression of a series of osteoclast related genes was examined (Figure 3.2A). Gene expression profiles were compared between osteoclasts (M+R treatment; 25ng/ml M-CSF and 20ng/ml RANKL), macrophage like cells (M-CSF alone treatment; 25ng/ml) and cells derived from continuous treatment with GM-CSF (25ng/ml), with M-CSF and RANKL (GMR treatment).

Osteoclast specific markers, including cysteine protease, cathepsin K (CTSK), G-coupled receptor, calcitonin receptor (CTR) and TRAP were analysed using quantitative real-time PCR. The expression of all three osteoclast specific markers was abolished in GMR treatment compared to M+R treated cells (Fig. 3.2), illustrating that GM-CSF treatment suppresses osteoclast formation and drives differentiation into different phenotype.

CSF2RA was induced in osteoclasts (M+R treatment) relative to macrophages and was further up regulated in GMR treatment (Fig. 3.2). The up regulation of GM-CSF receptor-α by RANKL provides an explanation why delayed addition of GM-CSF is still potent at suppressing osteoclast differentiation, since cells may be sensitised to inhibition by increased receptor content.

A series of nuclear factors that are regulated strongly by RANKL during osteoclast differentiation (Day, C.J., *et al.,* 2004), showed pronounced differential expression patterns in the three treatment groups (M-CSF alone, M+R and GMR treatments) (Fig. 3.2). In particular, the RANKL mediated induction of NFATc1 is suppressed by GM-CSF in GMR treated cells relative to osteoclasts. Since NFATc1 is considered necessary for induction of osteoclast genes (Takayanagi, H., *et al.*, 2002), inhibition of NFATc1 induction provides a rationale for the suppression of the osteoclast phenotype by GM-CSF. In contrast, the potent RANKL mediated induction of FUSE-binding protein (FBP) was essentially unaffected by GM-CSF in GMR treatment compared to M+R treatment. Other osteoclast nuclear factors, GABPα and ILF3, were potently down-regulated by GM-CSF, similar to the effect on NFATc1 (Fig. 3.2).

KOX31 is a zinc finger protein that is highly expressed in macrophage-like cells and repressed in osteoclasts (Day, C.J. *et al.,* 2004). GM-CSF does not reverse the RANKL mediated repression of KOX31: in fact, KOX31 is further repressed in GMR treated cells relative to M+R treatment (Fig. 3.2). The further repression of KOX31 by GMR eliminates any similarity between the M-CSF treated cells and the GMR treated cells, since potent up-regulation of KOX31 occurs in macrophage-like cells (Fig. 3.2).

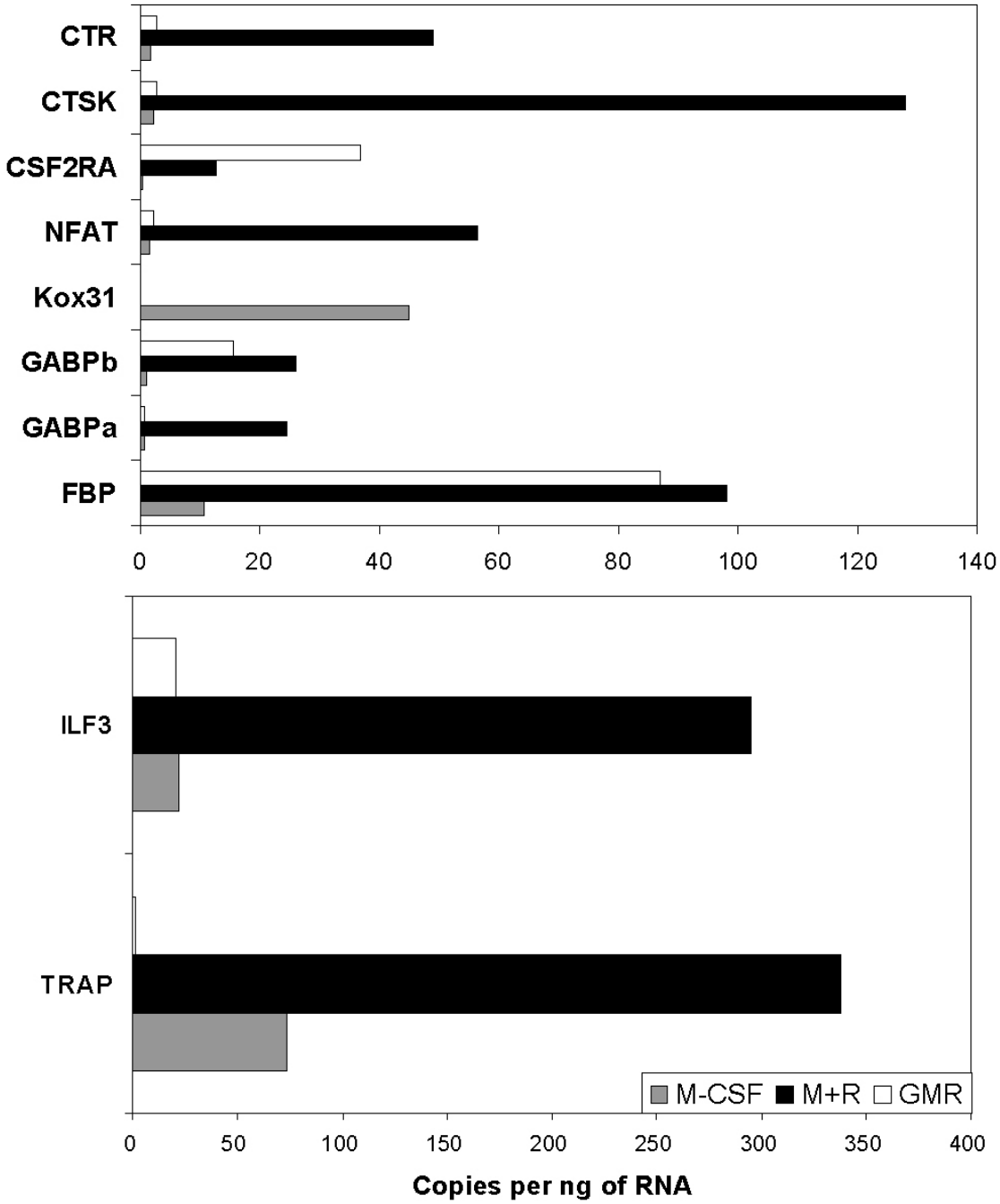

Fig. 3.2 Quantitative real time PCR analysis of gene expression of osteoclast-related genes. Graph shows calcitonin receptor (CTR), cathepsin K (CTSK), GM-CSF receptor-α (CSF2RA) TRAP and nuclear factors (NFATc1, KOX31, GABP and FBP, ILF3) in three treatment regimes; M-CSF, M+R, and GMR (shaded, black and white columns respectively).

Since GMR treatment suppressed NFATc1 and other osteoclast specific markers, it was hypothesised that GM-CSF in the presence of M-CSF and RANKL

inhibits the induction of key factors in osteoclast differentiation. Such factors would appear suppressed by GMR treatment compared to M+R treatment. Furthermore, a 19,000 gene cDNA microarray was performed to test gene regulation between M+R and GMR treatments (Kim, M.S., Honours Thesis, 2002). A large number of genes demonstrated significant differential expression in microarray analysis, although only a few are discussed. The expression of various genes was consistent with suppression of the osteoclast phenotype by GM-CSF (down-regulated by GM-CSF: GMR treatment vs M-CSF and RANKL treatment): cathepsin D, osteoclast specific vacuolar H+ ATPase proton pump (ATP6C) (-33.8-fold), and the osteoclast specific serine protease CTSK were repressed (-24.4-fold).

Table 3.1 Expression of osteoclast gene markers in GM-CSF treated microarray

Gene	Genbank	Fold
ATPase, H+ transporting, lysosomal, member D: ATP6D	R74157	-33.80
Cathepsin D: CTSD	R26954	-206
Cathepsin K: CTSK	R00859	-24.4
CD68 antigen: CD68	H77636	-148.00
Far upstream element (FUSE) binding protein 1: FBP1	W38369	-3.97
GA-binding protein transcription factor, α subunit: GABPA	W67265	-27.30
Interleukin enhancer binding factor 3: ILF3	H50941	-8.58
Nuclear factor of activated T-cells, cytoplasmic 1: NFATC1	H63794	-4.23
Nuclear factor of activated T-cells, cytoplasmic 4: NFATC4	H14072	-3.00
v-myc avian myelocytomatosis viral oncogene homolog: MYC	H43827	-3.95
v-jun avian sarcoma virus 17 oncogene homolog: JUN	N29139	-2.8
Vimentin: VIM	T40856	-43.6

Furthermore, nuclear factors that are found in osteoclasts (GABP, FBP, ILF3, c-Jun and c-myc and NFATc1) were also down-regulated by GM-CSF exposure, demonstrating the GM-CSF exposure does not lead to osteoclast differentiation, both by appearance and by gene expression profiles. CD68, a cell surface marker for osteoclast and macrophages was down regulated by GM-CSF (-148 fold) (Table 3.1).

The absence of CD68 expression in GM-CSF treated cells indicates that the phenotypes produced are divergent from osteoclasts.

The overall pattern of gene expression indicates that GMR treated cells are neither similar to M-CSF derived macrophage-like cells nor M-CSF and RANKL derived osteoclasts. From the cellular phenotype of GMR treated cells, it was hypothesised that GMR treated cells represent a dendritic cell phenotype. In doing so, we have examined a dendritic cell marker, CD1a (Kasinrerk, W., *et al.*, 1993) using FACS analysis (Fig. 3.3). FACS analysis using human CD1a antibody conjugated with fluorescein isothiocyanate (FITC) demonstrated that GMR treated cells had 82.5±1.0% of cells expressing CD1a, while M+R treated cells had 34.6±5.0% of cells expressing CD1a. These data shows that GMR treated cells express CD1a, a dendritic cell marker and have dendritic cell phenotype.

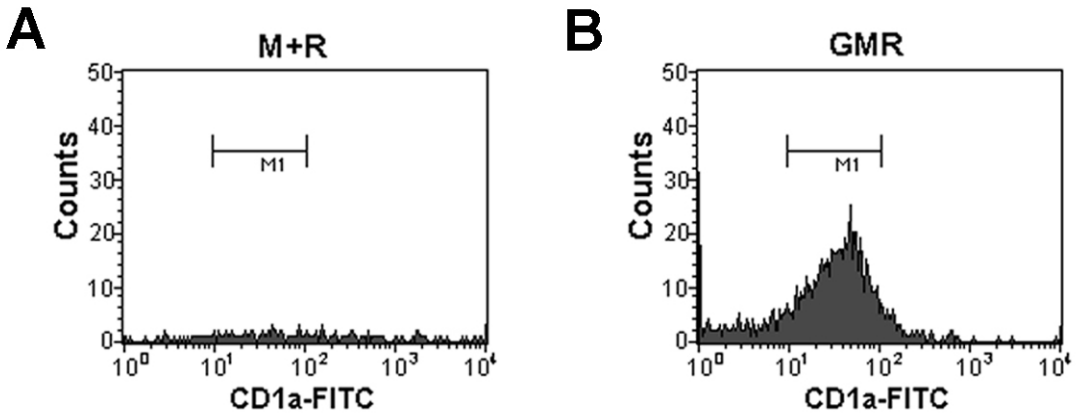

Fig. 3.3 FACS analysis of dendritic cell marker, CD1a. (A) Absence of dendritic cell marker CD1a in osteoclast cultures and (B) presence in GMR cultures as assessed with FACS analysis and FITC labelled antibody.

Of 19,000 genes assayed (Kim, M.S., Honours Thesis, 2002), the gene most repressed by GMR treatment in comparison to M+R treatment was monocyte chemotactic protein 1 (MCP-1), a CC chemokine not associated previously with osteoclast differentiation. In order to verify the regulation of MCP-1 by GM-CSF, new cultures were established on three separate occasions, differentiated over 21-days under comparable conditions with M-CSF alone, M+R and GMR treatment (Fig. 3.4). MCP-1 mRNA content, measured by quantitative real time PCR, was 17.1±0.1 (copies per ng total RNA) in M+R cultures compared to 0.24±0.06 in GMR treated cultures, resulting in a 72-fold decrease in MCP-1 mRNA ($p= 5\times10^{-9}$) with GM-CSF treatment. Furthermore, MCP-1 was induced by RANKL during osteoclast differentiation (15-fold) compared to macrophage cultures (M-CSF alone treatment) (Fig. 3.4). These data confirm that GM-CSF represses MCP-1 expression in the GMR treatment compared to M+R and also show that MCP-1 is induced in osteoclasts relative to macrophage like cells. Another CC chemokine, RANTES, is induced during human (Day, C.J., *et al.,* 2004) and mouse (Cappellen, D., *et al.,* 2002; Ishida, N., *et al.,* 2002) osteoclast differentiation. RANTES mRNA content was 5.3±0.4 in M+R cultures compared to 1.4±0.2 in GMR treated cultures, resulting in 4-fold decrease in RANTES mRNA ($p= 8.7\times10^{-4}$) (Fig. 3.4). MIP1α is also induced during osteoclast differentiation (Scheven, B.A., *et al.,* 1999; Choi, S.J., *et al.,* 2000). MIP1α mRNA content was 14.7±2.2 in M+R cultures compared to 3.3±1.5 in GMR treated cultures, resulting in 4.5-fold decrease in MIP1α mRNA ($p= 3.9\times10^{-2}$) (Fig. 3.4). The down-regulation of chemokines illustrate the magnitude of MCP-1, as GM-CSF suppresses MCP-1 by 72-fold, while GM-CSF only down-regulates RANTES or MIP1α by 4-fold.

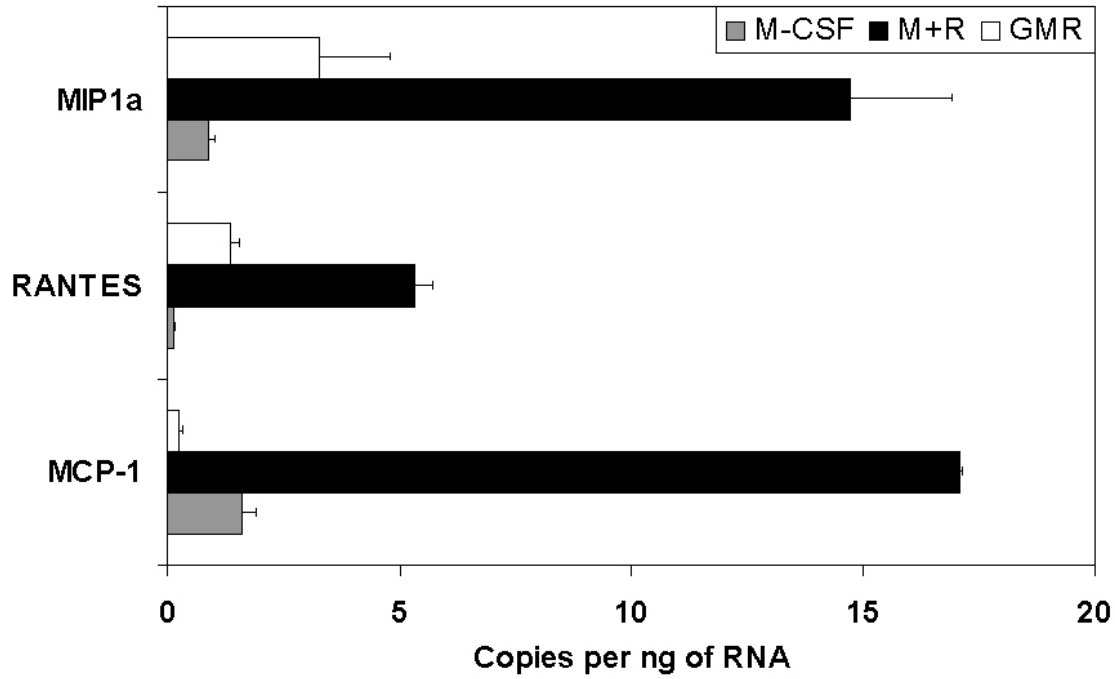

Fig. 3.4 Quantitative real time PCR analysis of MCP-1, RANTES and MIP1α gene expression in M-CSF alone, M+R, and GMR treated cultures. The data indicates induction of all three chemokines in M+R cultures relative to M-CSF alone treatment and repression in GMR compared to M+R treated cultures.

DISCUSSION

Cell fate is controlled by relative concentrations of cytokines, integrated over their respective signalling pathways. GM-CSF has been shown to be both stimulator (Fujikawa, Y., *et al.,* 2001; Yamazaki, H., *et al.,* 2001) and inhibitor (Shuto, T., *et al.,* 1994; Miyamoto, T., *et al.,* 2001; Day, C.J., *et al.,* 2004; Hodge, J.M., et al, 2004) of osteoclasts. Without RANKL, M-CSF treatment of monocytes results in macrophage-like cells; therefore, RANKL overrides the macrophage differentiation pathway to form osteoclasts in the presence of RANKL and M-CSF. Likewise, GM-CSF and RANKL represent two competing differentiation signals: RANKL to osteoclasts and GM-CSF to dendritic like cells. RANKL dependent up-regulation of GM-CSF receptor-α might provide a negative feedback signal (Day, C.J., *et al.,* 2004), sensitizing cells to the effect of GM-CSF found in the bone marrow milieu and thus,

aiding in regulation of osteoclast number. In the presence of RANKL and M-CSF, GM-CSF dominates cell fate and osteoclast differentiation is suppressed, with concomitant suppression of osteoclast-related genes.

The continuous exposure of GM-CSF has lead to morphological and phenotypic changes compared to the precursor cells treated with M+R. Continuous GM-CSF exposure has diverged morphology from osteoclast-like cells to TRAP-negative and dendritic-like cells, with suppression of bone resorbing capabilities of an osteoclast. The physiological GM-CSF level in normal human serum is around 8pg/ml, but it is believed that in bone marrow, the concentration is thought to be higher than in the serum. Furthermore, a patient with rheumatoid arthritis had GM-CSF level at around 0.1ng/ml (Ozaki, M., *et al.,* 2001). The dose response assay of GM-CSF (0.1ng/ml to 25ng/ml) showed a decrease in number of TRAP positive multinuclear cells, as the dosage increased. At 0.1ng/ml of GM-CSF, the osteoclast number was reduced by 25%. The 10ng/ml of GM-CSF dose reduced the osteoclast number by 75%, while 25ng/ml of GM-CSF reduced the number by 97%, indicating that 25ng/ml of GM-CSF had most potent suppression of osteoclast formation. The time course assay indicated that earlier GM-CSF exposure had increased suppressive effect, while the delayed exposure had minimal reduction, compatible with the biphasic effect of GM-CSF on osteoclast formation (Hodge, J.M., *et al.,* 2004).

The morphological changes and the high expression of CD1a (dendritic cell marker, Kasinrerk, W., *et al.,* 1993), indicate that GM-CSF mediates the differentiation of osteoclast precursors away from correct osteoclast formation rather, into a dendritic cell pathway. The microarray and quantitative real-time PCR

experiments suggest that GM-CSF exposure causes changes in gene expression, by down-regulating the key osteoclast specific markers, including vacuolar H^+ ATPase, CTSK and macrophage/osteoclast cell surface marker and osteoclast-related nuclear factors, including NFATc1. NFATc1 is a nuclear factor established as the master regulator of osteoclast formation (Takayanagi, H., *et al.,* 2002b; reviewed in Takayangi, H., *et al.,* 2005a; reviewed in Takayangi, H., *et al.,* 2005b) that is highly expressed in RANKL treated osteoclasts compared to M-CSF treated macrophage-like cells (Day, C.J., *et al.,* 2004). The down regulation of such an important nuclear factor by GM-CSF further indicate different signalling pathway undertaken by GM-CSF.

Furthermore, MCP-1, a member of CC chemokine superfamily was massively down regulated by GM-CSF. MCP-1 plays a critical role in the recruitment and activation of leukocytes during acute inflammation (Tangirala, R.K. *et al.,* 1997), and is also found at the site of tooth eruption, rheumatoid arthritic bone degradation and bacterially induced bone loss, a feature consistent with a role in osteoclast biology (reviewed in Wise, G.E., *et al.,* 2002a). Recently, MCP-1 is shown to participate in the formation of foreign body giant cells (Kyriakides, *et al.,* 2004). The potent down regulation of MCP-1 by GM-CSF is unsurpassed by either RANTES or MIP1α. Furthermore, MCP-1 expression in GM-CSF treated cells was even lower than the expression in M-CSF alone treated cells. Both RANTES and MIP1α were still down-regulated by GM-CSF compared to RANKL treated osteoclasts. However, this repression was still minor when compared to M-CSF alone treated cells, as GM-CSF treated cells had higher expression of RANTES and MIP1α compared to M-CSF alone treated cells.

Chapter 4

Chemokines and Osteoclasts.

This chapter includes data on chemokines in the formation of osteoclasts and osteoclast like polykaryons published in

1. Kim, M.S., Day, C.J. and Morrison, N.A. (2005) *J Biol Chem.*
2. Kim, M.S., Magno, C.L., Day, C.J. and Morrison, N.A. (2006) *J Cell Biochem.*
3. Kim, M.S., Day, C.J., Selinger, C.I., Magno, C.L., Stephens, S.R.J., and Morrison, N.A. (2006) *J Biol Chem.*

Chemokines are small cytokines known to be involved in immune response and in development of several cell types (reviewed in Horuk, R., 2001). Three most widely researched CC chemokines are monocyte chemotactic protein 1 (MCP-1), regulated upon activation, normally T-expressed, and presumably secreted (RANTES) and macrophage inflammatory protein 1-alpha (MIP1α). The effects of chemokines on osteoclast differentiation and formation are not clear, and require better understanding. Interestingly, in mouse, CCL9 or macrophage inflammatory protein 1 gamma (MIP1γ) is the major chemokine expressed by osteoclasts (Lean, J.M., *et al.,* 2002), but this chemokine is not found in human, illustrating that different chemokines are utilised between human and mouse.

RESULTS

Effects of chemokines on osteoclast differentiation.

Previous studies have shown that RANTES (Cappellen, D., *et al.,* 2002; Day, C.J., *et al.,* 2004) and MIP1α (Scheven, B.A., *et al.,* 1999; Choi, S.J., *et al.,* 2000) regulate osteoclast formation. Moreover, the suppression of MCP-1 expression by GM-CSF led to a hypothesis that exogenous MCP-1 would affect osteoclast differentiation.

Addition of exogenous MCP-1 to the standard M-CSF and RANKL (M+R) treatment protocol resulted in 34% more osteoclasts (324±21 n=17 versus 241±12 n=24, respectively; $p= 5.7\times10^{-6}$) (Fig. 4.1) that were TRAP positive and positive for bone resorption (Fig. 4.1D). Unexpectedly, MCP-1 treatment with M-CSF in the absence of exogenous RANKL, resulted in TRAP positive, multinuclear giant cells (Fig. 4.1C). Although these cells had the appearance of osteoclasts, they were unable to form resorption pits on dentine, suggesting that MCP-1 and M-CSF treatment results in an intermediate phenotype on the path to osteoclasts, permitting monocyte fusion but not further differentiation.

Unlike MCP-1, exogenous RANTES or MIP1α had minor or no significant effect on osteoclast number or bone resorption in the presence of RANKL, respectively (p= 0.05 and p= 0.43, respectively) (Fig. 4.1B and 4.1D). Interestingly, treatment with either exogenous RANTES or MIP1α in the absence of RANKL resulted in TRAP positive, multinuclear giant cells that are unable to resorb bone, consistent with the effects seen with MCP-1 (Fig. 4.1C). These data demonstrate that chemokines stimulate fusion of monocytes, into TRAP positive multinuclear cells, but do not permit further differentiation into bone resorbing osteoclasts.

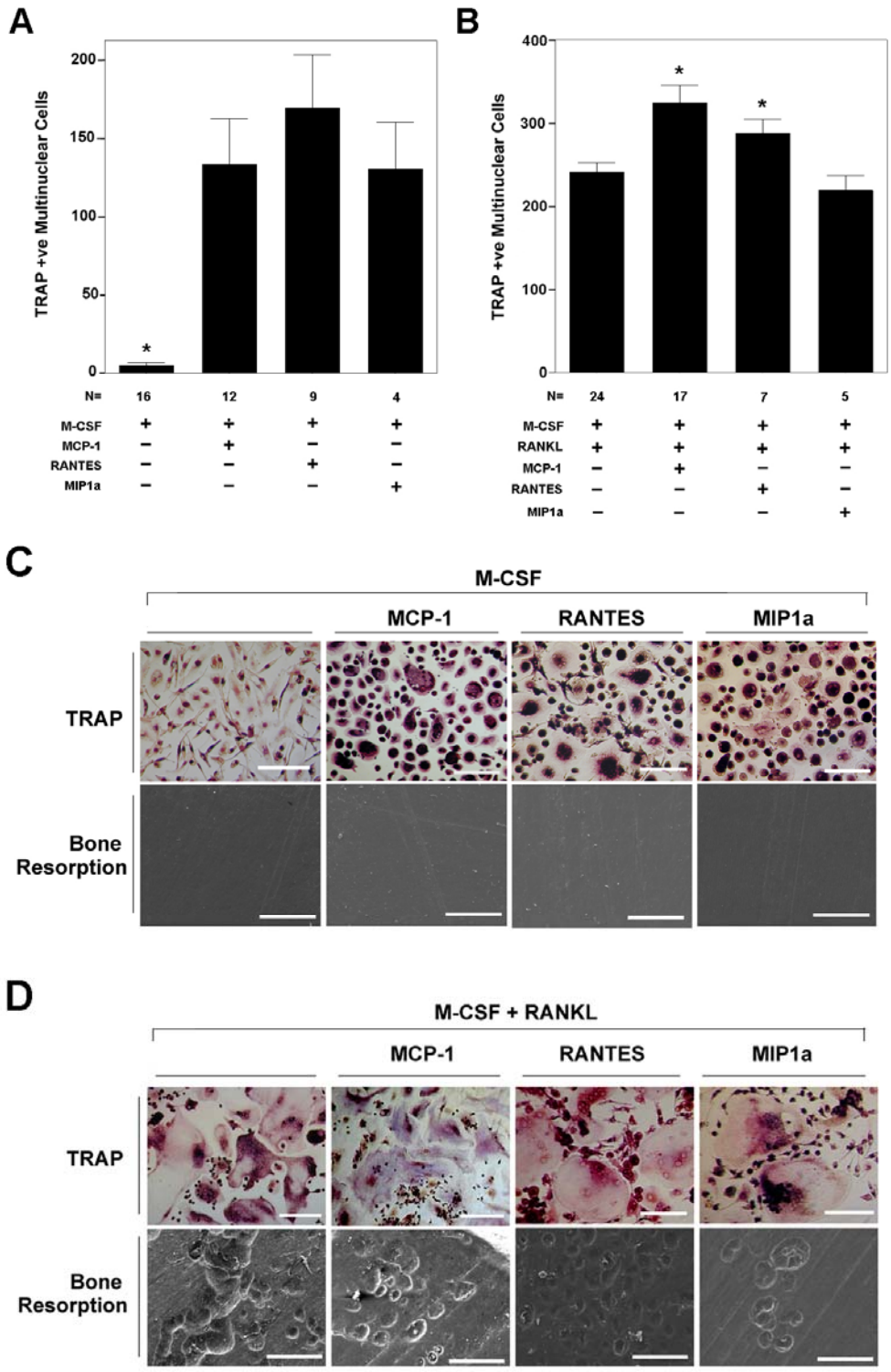

Fig. 4.1 Effect of chemokines MCP-1, RANTES and MIP1α on cellular phenotypes with and without RANKL. (A) In the presence of M-CSF and chemokines, TRAP positive multinuclear cells were formed. All three chemokines (MCP-1, RANTES and MIP1α) (at 25ng/ml each) significantly increased TRAP positive multinuclear cells compared to M-CSF alone treated cells. * significant difference compared to M-CSF and chemokine treated cells. (B) In the presence of M-CSF and RANKL,

continuous exposure to MCP-1 significantly increased TRAP positive multinuclear cells observed. In the presence of RANKL, RANTES had a mild effect on osteoclast number, while MIP1α had no significant effect on the osteoclast number. * significant difference compared to M+R treated PBMCs, where $p<0.05$. (C) In the presence of M-CSF, chemokines treatment resulted in the formation of TRAP positive multinuclear cells (upper panels) that were negative for bone resorption (lower panels). (D) In the presence of M-CSF and RANKL, continuous exposure to MCP-1, RANTES or MIP1α results in TRAP positive multinuclear osteoclasts (upper panels) that are positive for bone resorption (lower panels). Numbers of independent experiments are shown in (A and B). Bar is 100 μm.

Regulation of chemokine receptors by RANKL.

The exogenous CC chemokines have potent effect on osteoclast differentiation. Thus, CC chemokine receptors capable of interacting with MCP-1, RANTES and MIP1α were tested for RANKL regulation. In comparison to M-CSF treated cells, M-CSF and RANKL treatment resulted in the significant induction of chemokine receptors CCR2b and CCR4, (12.6 and 49-fold, $p= 4.0\times10^{-7}$ and 4.0×10^{-8}, respectively), that are capable of interaction with MCP-1. In contrast, the receptors for RANTES and MIP1α were not induced (CCR1 and CCR5; 1.0 and 1.4-fold, p= 0.96 and 0.22, respectively) (Fig. 4.2). The alpha isoform of CCR2, CCR2a was not induced by RANKL (p= 0.29) (Fig. 4.2). These data suggest that receptors for MCP-1, but not RANTES or MIP1α are induced by RANKL during osteoclast differentiation. Despite the lack of regulation by RANKL of the RANTES and MIP1α family receptors CCR1 and CCR5, reasonable amounts of the mRNA for CCR1 and CCR5 are present in osteoclasts, compared to macrophage-like cells, making it difficult to discount autocrine functions of RANTES and MIP1α. Moreover, RANKL regulates both MCP-1 and its possible cognate receptors (CCR2 and CCR4), providing evidence for an autocrine function of MCP-1 in osteoclasts.

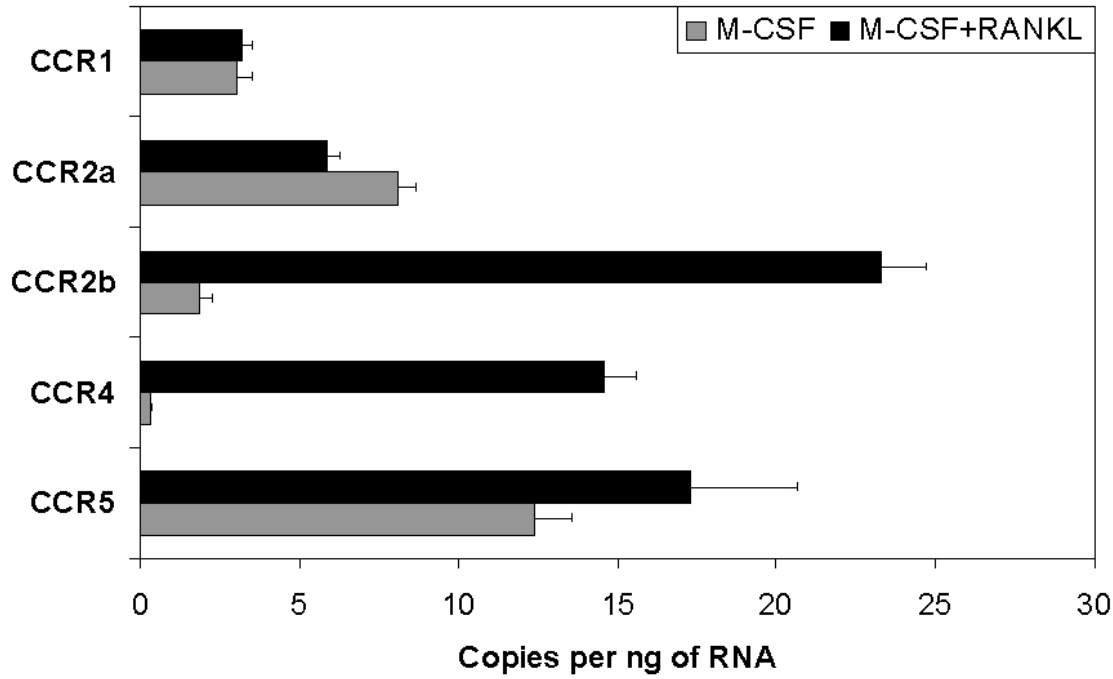

Fig. 4.2 Induction of CC chemokine receptors by RANKL. Graph shows the comparison in mRNA content measured by quantitative real time PCR of CCR1, CCR2a, CCR2b, CCR4 and CCR5 in macrophage-like cells (M-CSF alone treatment, grey columns) and osteoclasts (M-CSF+RANKL treatment, black columns). Error bars are SE from replicate experiments.

Regulation of chemokines and receptors by chemokines.

RANKL induced receptors for MCP-1, providing evidence for an autocrine cycle. It was further hypothesised that chemokines themselves may induce their own receptors to establish such autocrine loop. Cells were treated with either MCP-1 or RANTES in the presence of M-CSF and the expression of chemokines and their cognate receptors were examined at 21 days. MCP-1 treatment resulted in the dramatic induction of MIP1α and RANTES expression (45 and 90 fold with $p= 1.3\times10^{-10}$ and 8×10^{-12}, respectively) when compared to M-CSF alone treatment (Fig. 4.3A). MCP-1 was induced to a minor but significant extent (4.2 fold with $p= 2.4\times10^{-4}$) by MCP-1 treatment. In marked contrast, MCP-1 expression was induced 31 fold by RANTES treatment ($p= 5.0\times10^{-8}$). MIP1α and RANTES were significantly

induced by RANTES treatment (12.7 and 21.6 fold, p= 1.4×10^{-8} and 5.1×10^{-10}, respectively), but to a lesser extent than that observed with MCP-1 treatment. These data are consistent with feedback inhibition by the ligand through its receptor, limiting the production of the cognate ligand. In other words, RANTES family members (MIP1α and RANTES) are more potently induced by MCP-1 than by RANTES. Similarly, RANTES induces MCP-1 more potently than MCP-1 induces its own expression.

Chemokine receptor expression after treatment with chemokines MCP-1 and RANTES followed the same pattern as observed with RANKL treatment in that CCR1 and CCR5 were expressed but not significantly regulated, while CCR2 and CCR4 were profoundly regulated. CCR4 was induced 458 fold by MCP-1 treatment (p= 1.0×10^{-10}) and repressed two fold by RANTES (p= 1.0×10^{-4}). CCR2b was induced 26 fold by MCP-1 (p= 8.9×10^{-8}) and 8.0 fold by RANTES (p= $1.2\text{x}10^{-5}$). CCR2a was induced 3.5 fold by MCP-1 (p= 0.001) and repressed 10 fold by RANTES (p= 1.1×10^{-5}) (Fig. 4.3B).

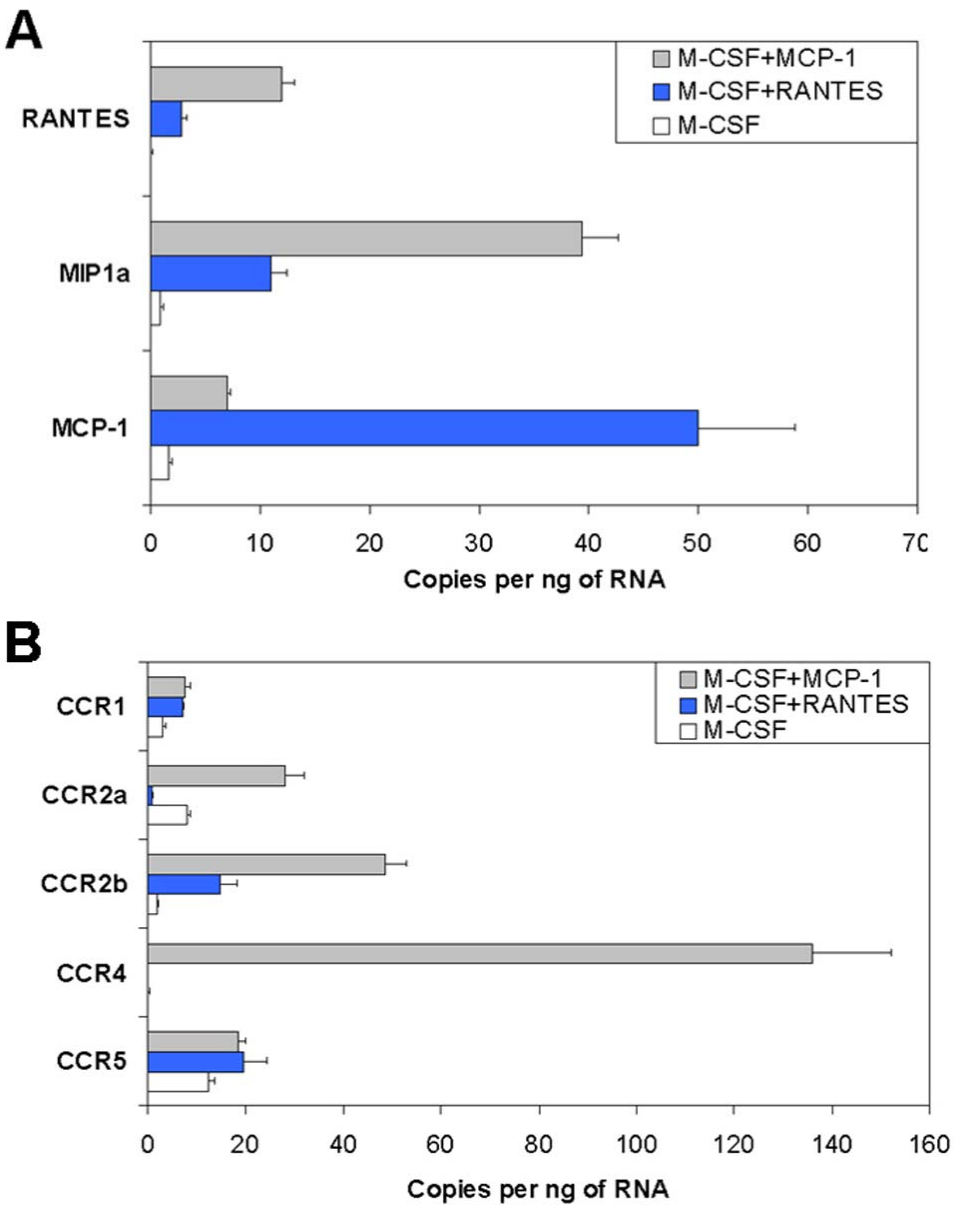

Fig. 4.3 Regulation of chemokines and their receptors by MCP-1 and RANTES treatment in the absence of RANKL. (A) Strong regulation of MCP-1, RANTES and MIP1α is observed after treatment with either MCP-1 (grey columns) or RANTES (blue columns) compared to M-CSF treatment alone (white columns). MCP-1 treatment in the absence of RANKL induces RANTES and MIP1α, whereas RANTES treatment potently induces MCP-1. (B) Receptors for MCP-1 (CCR2a, CCR2b and CCR4) are potently induced by MCP-1. In contrast, receptors for RANTES family members (CCR1 and CCR5) are not regulated.

MCP-1 reverses GM-CSF repression of osteoclast differentiation.

Fig. 3.1 showed that GM-CSF treatment led to TRAP negative, mononuclear dendritic-like cells that were also negative for bone resorption. MCP-1 acts as an enhancer of osteoclast differentiation and was strongly repressed by GM-CSF. It was hypothesised that the lack of MCP-1 may be influential in GM-CSF mediated suppression of osteoclast differentiation from PBMCs. This hypothesis was tested by addition of exogenous MCP-1, under conditions of maximal suppression of osteoclast differentiation by GM-CSF (25ng/ml). The addition of MCP-1 (at 25ng/ml) to the GMR treatment protocol dramatically increased the formation of TRAP positive multinuclear cells, compared to GMR treated cells (493±29, n=4, $p= 3.8\times10^{-8}$, Fig. 4.4A). Although the multinuclear cells derived from MCP-1 treated GMR cultures were generally smaller than osteoclasts derived from control M+R treated cultures (47±4 and 148±22 μm, respectively; average longest axis, $p= 2.2\times10^{-5}$) they were almost twice as abundant and were positive for bone resorption (Fig. 4.4B). Although RANTES overcame the block in multinucleation imposed by GM-CSF, resulting in a similar number of TRAP positive multinuclear cells as in M+R controls, the cells were negative for bone resorption (Fig. 4.4A and B).

It was hypothesised that RANTES and MIP1α would have similar effect as MCP-1, but less potent due to the lower regulation of these chemokines in the presence of GM-CSF, M-CSF and RANKL. In the presence of M-CSF and RANKL, MCP-1 enhanced osteoclast formation, whereas RANTES or MIP1α had mild or significant effect on osteoclast formation (Fig. 4.1B). RANTES and MIP1α treatment was able to overcome the potent suppression of human osteoclast formation by GM-CSF and resulted in a TRAP positive, multinuclear phenotype (Fig. 4.4A and B).

However, unlike MCP-1, neither RANTES nor MIP1α, were able to recover the formation of authentic osteoclasts capable of bone resorption activity from GM-CSF suppression (Fig. 4.4B).

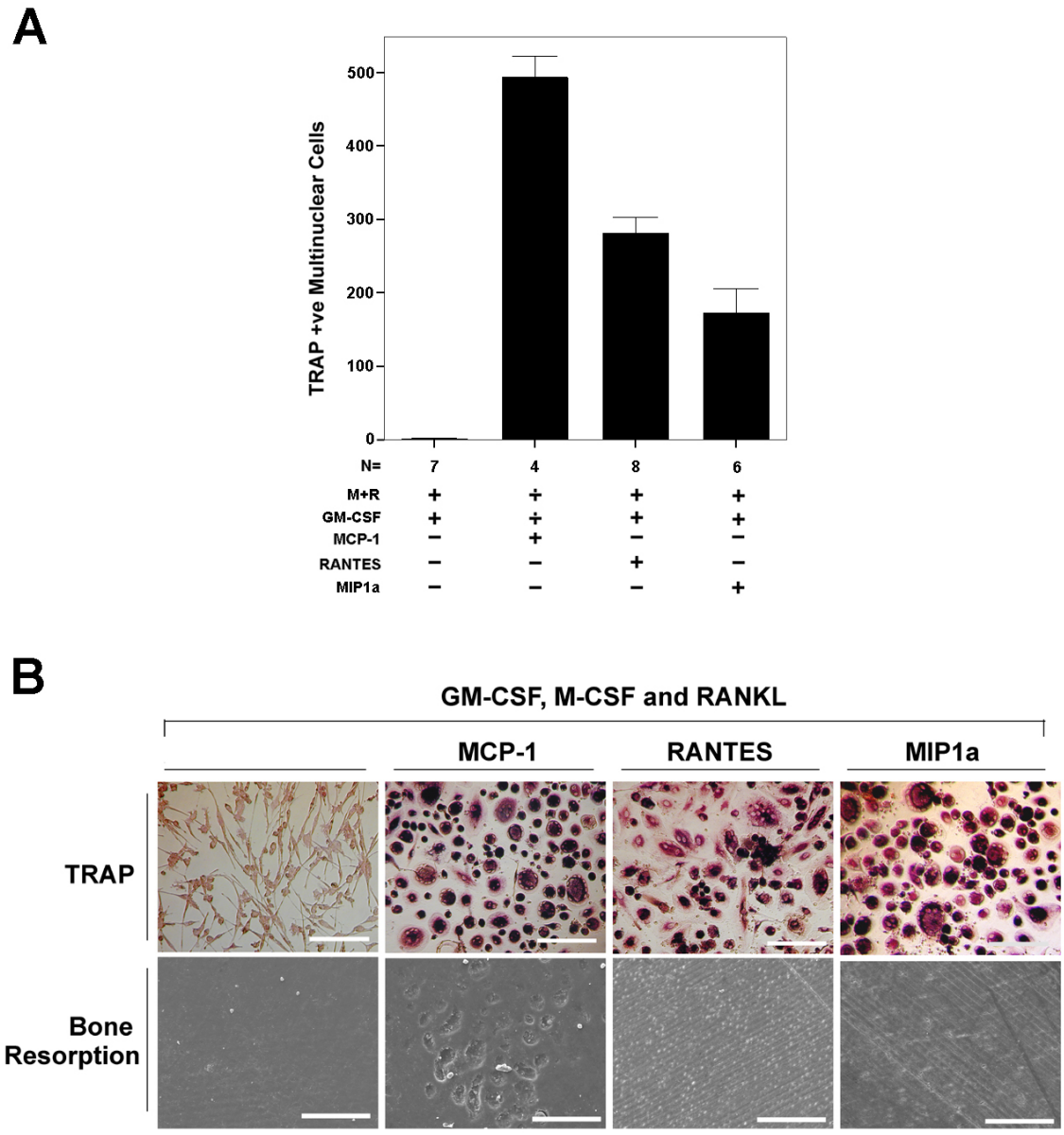

Fig. 4.4 Chemokines recover the multinuclear phenotype. (A) In the presence of M+R, TRAP positive multinuclear cells were suppressed by GM-CSF. All three chemokines (at 25ng/ml each) result in substantial recovery of TRAP positive multinuclear cells, indicating reversal of the suppressive effect of GM-CSF on osteoclast differentiation mediated by M-CSF and RANKL. (B) Microscopy of cellular phenotypes. Addition of MCP-1, RANTES or MIP1α resulted in TRAP positive multinuclear cells (upper panels). Furthermore, addition of MCP-1 recovered

the bone resorption activity. In contrast, addition of RANTES or MIP1α resulted in TRAP positive multinuclear cells that were negative for bone resorption (lower panels).

Neutralising the effects of MCP-1 on osteoclasts formation.

In the presence of RANKL, MCP-1 enhances osteoclast differentiation and formation, and in the absence of RANKL, MCP-1 promotes fusion of mononuclear cells into multinuclear cells. In doing so, it was hypothesised that neutralising the effect of MCP-1 would reverse the enhancement of osteoclast formation and the fusion events. The specific neutralising anti-MCP-1 antibody significantly reduced the number of osteoclasts in standard M-CSF and RANKL treated cultures (188±17, n=7, p= 0.01, Fig. 4.5A). The control antibody (IgG) had no effect on osteoclast number (p= 0.90, Fig. 4.5A and C). Furthermore, a construct for the mutant form of MCP-1 (7ND) was obtained from Dr. Kensuke Egashira. 7ND lacks the N-terminal amino acids 2 to 8 of MCP-1, and acts as a dominant-negative inhibitor of MCP-1 (Egashira, K., 2003). 7ND construct was transfected into HEK293 cells, then the conditioned media to final concentration of 5%, was added into the M-CSF and RANKL treated cultures. The 7ND significantly reduced the number of osteoclasts in M-CSF and RANKL treated cultures (21±1, n=4, $p= 4.3\times10^{-8}$, Fig. 4.5A) and blocked multinucleation, reverting back to mononuclear cell phenotype (Fig. 4.5C). The control media had no effect on osteoclast number (p= 0.95, Fig. 4.5A and C). The potent reductions in osteoclast numbers by MCP-1 inhibitors indicate that MCP-1 is crucial in osteoclast formation and differentiation.

Since, the neutralising anti-MCP-1 antibody significantly reduces osteoclast formation; the effects of the antibody were tested on other treatments. The addition of

neutralising anti MCP-1 antibody significantly reduced formation of M-CSF and MCP-1 mediated TRAP positive multinuclear cells (p= 0.003, Fig. 4.5B), and also reduced MCP-1 mediated enhancement of osteoclast formation (M-CSF, RANKL and MCP-1 treated cultures). The osteoclast number was reduced from 311±23 to 200±14, p= 0.008, reducing the counts to similar to the standard M-CSF and RANKL treated cultures (Fig. 4.5B) and the neutralising antibody also significantly reduced area of resorption pits (Fig. 4.5D and E). Furthermore, the addition of neutralising antibody also blocked the MCP-1 recovery of GM-CSF suppression (GM-CSF, M-CSF, RANKL and MCP-1 treated cultures). The number of osteoclast was reduced from 493±29 to 116±13, p= 1.3×10^{-8} (Fig. 4.5B), and also blocked resorption capabilities (Fig. 4.5 D and E).

Surprisingly, the neutralising anti-MCP-1 antibody had no effect on RANTES mediated TRAP positive multinuclear cell formation (p= 0.23, Fig. 4.5C). In contrast, the neutralising anti-MCP-1 antibody had mild effect on RANTES mediated osteoclast formation (M-CSF, RANKL and RANTES treated cultures), mildly reducing the number of osteoclasts from 288±16 to 243±5, p= 0.033 (Fig. 4.5B). This result illustrate that RANTES can moderately compensate for the reduction in the levels of MCP-1, but does not have the same potent abilities as MCP-1.

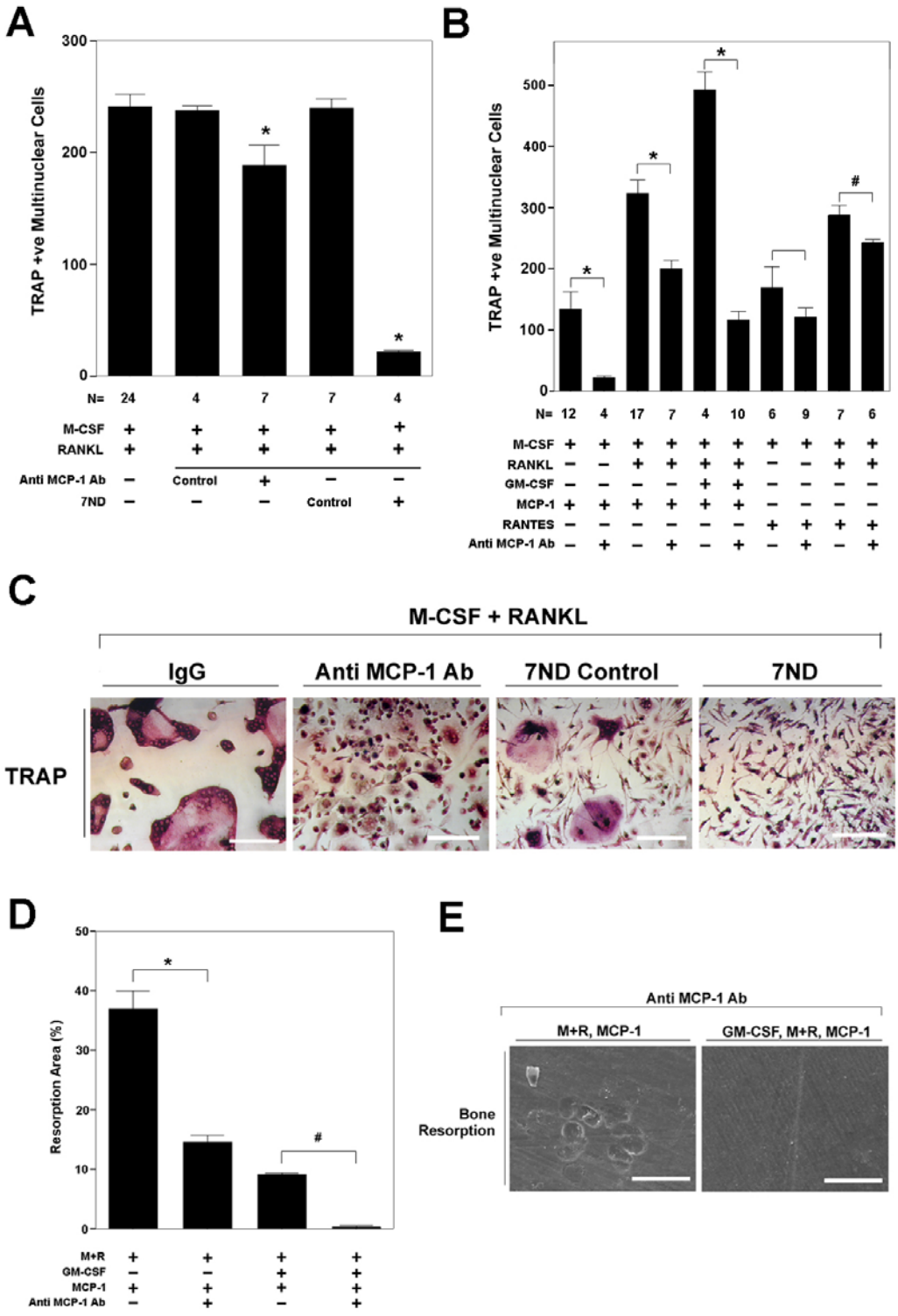

Fig. 4.5 Neutralising the effects of MCP-1. (A) In the presence of M-CSF and RANKL, specific neutralising anti-MCP-1 antibody significantly reduced the osteoclast numbers, compared to the control and standard osteoclast cultures. The mutant dominant negative MCP-1 (7ND) also suppressed osteoclast formation. * significant difference compared to standard M+R treated PBMCs, where $p<0.05$. (B) The neutralising antibody to MCP-1 severely reduced the effects of MCP-1 mediated

multinucleation (fusion), enhancement and the recovery of osteoclast formation from GM-CSF suppression. However, the neutralising antibody had no or mild effect on RANTES mediated multinucleation and enhancement of osteoclast formation, respectively. * significant difference, where $p<0.01$. # significant difference, where $p<0.05$. (C) Microscopy of cellular phenotypes. Addition of neutralising antibody to MCP-1, reduced the size of TRAP positive multinuclear cells. In marked contrast, the addition of dominant negative MCP-1 (7ND) blocked multinucleation and lingered as mononuclear phenotype. (D) The neutralising antibody to MCP-1 significantly reduced the percent resorption area of osteoclasts and the neutralising antibody also blocked the recovery of GM-CSF suppression. * significant difference, where $p<0.05$. # significant difference, where $p<0.01$. (E) The addition of neutralising antibody to MCP-1 reduced or suppressed the resorption area and the number of pits.

Discussion

MCP-1 is first of the four monocyte chemotactic proteins discovered at present (reviewed in Van Coillie, E., *et al.,* 1999) and is the most extensively researched human CC chemokine. MCPs are produced as precursor molecule containing 23 amino acids NH_2 terminal signal sequence. After cleavage of the signal peptide, a protein of 74 to 76 amino acids is secreted. MCP-2 and MCP-3 have a sequence homology with MCP-1 of 62 and 71%, respectively (reviewed in Van Coillie, E., *et al.,* 1999). Furthermore, MCP-2 and MCP-3 were initially characterised as protein co-produced with MCP-1 in osteosarcoma cells (Van Damme, J., *et al.,* 1992), indicating possible roles of MCP-2 and MCP-3 in osteoclast formation by potentially increasing multinucleation. Meanwhile, MCP-4 has 56 to 61% sequence homology with other MCPs (MCP-1 to 3) (reviewed in Van Coillie, E., *et al.,* 1999). To date, MCP-1 has not been implicated in osteoclast function, although data exists on its role in recruitment of monocytes during tooth eruption (reviewed in Wise, G.E., *et al.,* 2002a).

Effects of chemokines (MCP-1, RANTES and MIP1α) on osteoclast differentiation may explain why inflammatory diseases such as rheumatoid arthritis, which feature increased chemokine activity (Choi, S.J., *et al.,* 2000; reviewed in Horuk, R., 2001), are associated with increased osteoclast activity leading to bone degradation. Many of the ligands in the CC chemokine family have the capacity to interact with and share multiple chemokine receptors (reviewed in Mahalingam, S., and Karupiah, G., 1999; Horuk, R., 2001). Furthermore, CCR2 (Loetscher, P., *et al.,* 1996) and CCR4 (Power, C.A., *et al.,* 1995), the cognate receptors for MCP-1, are also induced by RANKL, providing evidence for an autocrine cycle involving MCP-1. CCR2 exists in two splicing variant forms, CCR2a and CCR2b, which differ in the carboxyl terminus (Sanders, S.K., *et al.,* 2000). CCR2b is reported to be the major cell surface form in monocytes (Tanaka, S., *et al.,* 2002) and, as monocytes differentiate into macrophages, total CCR2 content is reported to decrease (Wong, L.M., *et al.,* 1997). The data indicates that CCR2b is potently induced by both RANKL. CCR2a is less influenced by RANKL and MCP-1, suggesting that CCR2b and CCR4 may be of primary importance to MCP-1 signalling in osteoclasts. Moreover, CCR4 is substantially induced by both RANKL and MCP-1. CCR4 is reported as a MCP-1 receptor (Power, C.A., *et al.,* 1995), but it also responds strongly to CCL17 (Imai, T., *et al.,* 1997) and CCL22 (Imai, T., *et al.,* 1998). Consequently, the induction of MCP-1 receptors by MCP-1 is consistent with an autocrine loop and MCP-1 autocrine loop ramifies the effect of chemokines. In addition, chemokine receptors for RANTES and MIP1α (CCR1 and CCR5, reviewed in Horuk, R., 2001) are also highly expressed, but not highly regulated in osteoclasts compared to macrophage-like cells, indicating possible autocrine loop effect by RANTES and MIP1α.

All three chemokine treatments (MCP-1, RANTES and MIP1α) resulted in TRAP positive multinuclear cells in the absence of RANKL, suggesting that chemokines are sufficient for fusion events (Fig. 4.1). However, these TRAP positive multinuclear cells were unable for bone resorption, suggesting that differentiation is arrested prematurely. MCP-1 also enhanced osteoclast differentiation in the presence of RANKL, while RANTES or MIP1α had mild or no effect, indicating that MCP-1 has more potent role in osteoclast formation. Furthermore, MCP-1, unlike RANTES or MIP1α overcame GM-CSF mediated repression of osteoclast differentiation, by differentiating past multinucleation stage and to authentic bone resorbing osteoclasts. RANTES and MIP1α recovered the multinucleation phenotype of GM-CSF repression, but could not recover the bone resorbing capabilities of authentic osteoclasts.

The neutralising the effects of MCP-1 by adding specific neutralising antibody to MCP-1 or dominant negative mutant MCP-1 (7ND, Egashira, K., 2003) reduced the number of osteoclast formation and differentiation and also blocked recovery of osteoclast phenotype from GM-CSF suppression. Unlike competitive binding of MCP-1 to the neutralising MCP-1 antibody or MCP-1 receptors, the dominant negative MCP-1 binds to the cognate MCP-1 receptors, inhibiting the signalling effects of MCP-1, resulting in greater inhibitory effects. However, the neutralisation of MCP-1 had no effect on RANTES mediated TRAP positive multinuclear cell formation nor on RANTES mediate enhancement of osteoclast formation. This indicates that RANTES can mildly compensate for the offset of MCP-1, but is not optimal.

Taken together, these data suggest that RANKL induction of chemokines is an important component of osteoclast differentiation. Furthermore, from three chemokines analysed, MCP-1 is the most potent and most crucial chemokine involved in osteoclast differentiation and formation, by promoting fusion of mononuclear cells into multinuclear cells.

Chapter 5

Characterisation of Chemokine mediated Multinuclear Cells.

This chapter includes data on chemokines in the formation of osteoclasts and osteoclast like polykaryons published in

1. Kim, M.S., Day, C.J. and Morrison, N.A. (2005) *J Biol Chem.*
2. Kim, M.S., Magno, C.L., Day, C.J. and Morrison, N.A. (2006) *J Cell Biochem.*
3. Kim, M.S., Day, C.J., Selinger, C.I., Magno, C.L., Stephens, S.R.J., and Morrison, N.A. (2006) *J Biol Chem.*

Chemokine mediates TRAP positive multinuclear cells that lack bone resorption.

Treatment with M-CSF and chemokines resulted in multinuclear cells that had the appearance of osteoclasts but were negative for bone resorption (Fig. 4.1C). It was hypothesised that chemokines mediate cellular fusion of monocytes into multinuclear cells and these cells are pre-osteoclasts that have arrested differentiation at intermediate stage.

The levels of expression of two osteoclast related genes, TRAP and cathepsin K (CTSK), were compared by quantitative real-time PCR in cultures treated with M-CSF and either MCP-1, RANTES or MIP1α compared with standard M-CSF and RANKL treatment (Fig. 5.1). The mRNA content of TRAP was virtually identical in the multinuclear TRAP positive non-bone resorbing cells from M-CSF and chemokine treatments compared to authentic osteoclasts. In marked contrast, CTSK mRNA content was substantially lower in chemokine and M-CSF treated cells (Fig. 5.1). This may provide a rational for the inability of these osteoclast-like TRAP positive multinuclear cells to degrade bone: CTSK and possibly other essential osteoclast related genes are not appropriately induced in chemokine treated cells.

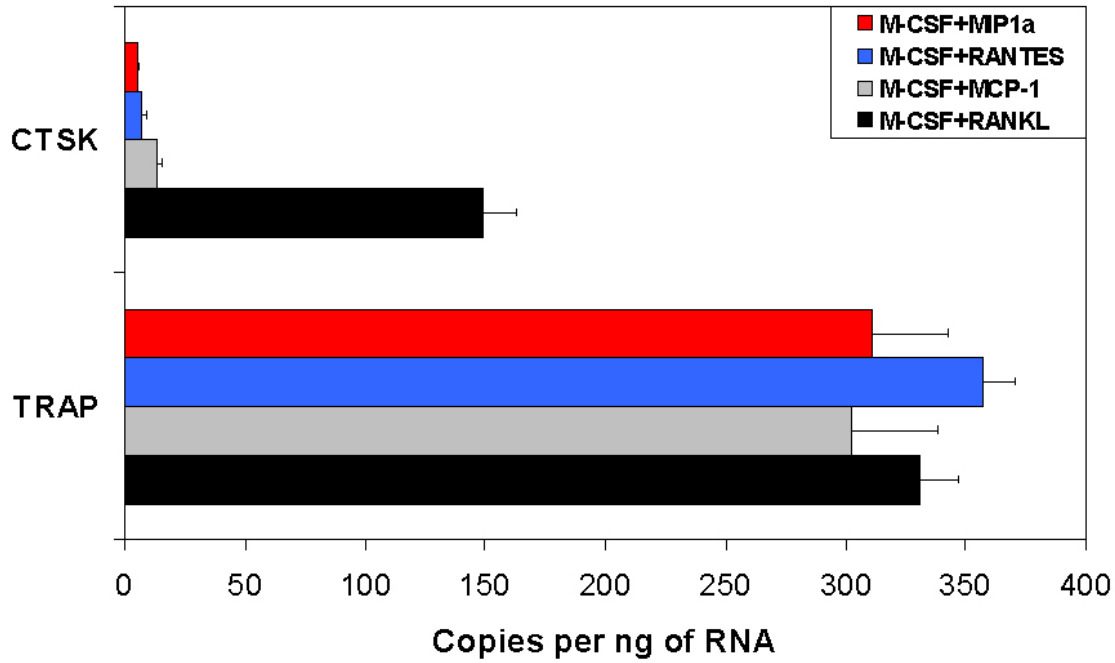

Fig 5.1 Quantitative real time PCR analysis of TRAP and cathepsin K (CTSK) expression in cultures treated with: M-CSF and RANKL (black columns); M-CSF and MCP-1 (gray columns); M-CSF and RANTES (blue columns); M-CSF and MIP1α (red columns).

Characterization of MCP-1 treated cells.

Of the three tested chemokines, all stimulated the formation of TRAP positive multinuclear cells in the absence of RANKL. Regardless of the chemokine used, such cells were negative for bone resorption. MCP-1 had two characteristics not shared by RANTES and MIP1a. These were the ability to rescue osteoclasts from GMR (continuous GM-CSF, M-CSF and RANKL) treatments the capacity to enhance the osteoclast number in the presence of M-CSF and RANKL. MCP-1 was therefore selected for further study. The phenotype of cells treated with MCP-1 in the presence of M-CSF, was compared to authentic osteoclasts differentiated with RANKL (Fig. 5.2). Confocal imagery using Hoechst stain confirmed multinuclearity of MCP-1 treated cells (Fig. 5.2B). In contrast to well defined F-actin rings in osteoclasts, TRAP positive multinuclear cells differentiated with MCP-1 did not show F-actin

rings. Both cell types demonstrated similar nuclear localization of the crucial osteoclast transcription factor NFAT (Fig. 5.2B).

The failure of MCP-1 treated cells to resorb bone may be due to a lack of expression of key osteoclast genes, despite the similarity in appearance of MCP-1 treated cells to osteoclasts. To further understand the phenotype of MCP-1 mediated TRAP positive multinuclear cells, the expression of genes related to osteoclast function was compared to that in authentic osteoclasts differentiated with RANKL (Fig. 5.2C-F). Matrix metalloproteinase 9 (MMP9) is a proteinase involved in bone resorption. MMP9 expression was similar in character to that of CTSK (Fig. 5.1), in that expression was lower in the non-bone resorbing TRAP positive multinuclear cells induced with MCP-1 (Fig. 5.2C). Integrin alpha-V (a subunit of the αV-β3 integrin) had a similar pattern of expression with lower levels in MCP-1 mediated TRAP positive multinuclear cells (Fig. 5.2C). These data provide further evidence that that MCP-1 mediated TRAP positive multinuclear cells lack essential genes required for the bone resorption function of an osteoclast (Fig. 5.2A).

Higher mRNA levels were observed for NFATc1 in MCP-1 treated cells compared to RANKL treated cells in all of six independent experiments with an average difference of 16-fold (n=6, $p= 4.8\times10^{-9}$), Similarly, CTR (6.3-fold, n=5, $p= 2.0\times10^{-5}$) and calmodulin 1 (Calm1, 3.4-fold, $p= 1.2\times10^{-8}$) were also higher in TRAP positive multinuclear cells induced by MCP-1 compared to osteoclasts. Surprisingly, the mRNA for these three genes were more abundant in the MCP-1 induced multinuclear cells than in osteoclasts differentiated with RANKL.

A series of transcription factors were examined (Fig. 5.2D): all were more abundant in MCP-1 treated cells than in RANKL treated cells. In particular, c-fos and c-jun were 26-fold ($p= 4.2\times10^{-4}$) and 2.7-fold ($p= 2.5\times10^{-3}$) respectively, more abundant in MCP-1 treated cells compared to RANKL treated cells. These data suggest that the failure of MCP-1 treated osteoclast-like cells to resorb bone cannot be attributed to insufficient NFATc1, c-fos or c-jun; transcription factors that are considered essential to osteoclast differentiation. Significantly greater levels of STAT1 ($p= 3.8\times10^{-6}$), STAT3 ($p= 4.4\times10^{-7}$), and FBP ($p= 1.5\times10^{-5}$), were observed in the MCP-1 treated cells compared to RANKL treatment. Kox31 is a transcription factor of the zinc finger family that was significantly repressed by RANKL during human osteoclast differentiation (Day, C.J., *et al.,* 2004). Kox31 mRNA is significantly higher in MCP-1 treated cells compared to authentic osteoclasts ($p= 0.01$).

A series of cell surface proteins were investigated: c-fms (CSF1R), RANK, CD44, CSF2RA and CD14. Of these proteins, c-fms, RANK, CD44 and CSF2RA are induced by RANKL and M-CSF treatment relative to macrophage-like cells treated with M-CSF alone (Day, C.J., *et al.,* 2004). The cell surface marker CD14 is higher in cells treated with M-CSF alone and is repressed by RANKL and M-CSF treatment (values are 265±33 versus 22.7±1.5 copies per ng total RNA, respectively with $p= 1.7\times10^{-5}$). MCP-1 represses CD14 in a manner similar to RANKL treatment relative to M-CSF alone ($p= 0.005$, Fig. 5.2E). In other respects, the TRAP positive multinuclear cells from MCP-1 treatment had significantly more mRNA for c-fms ($n=4$, $p= 2.3\times10^{-9}$), RANK ($n=4$, $p= 2.3\times10^{-6}$), CD44 ($p= 5.5\times10^{-6}$) and CSF2RA ($p= 0.009$) than in authentic osteoclasts differentiated with RANKL.

Finally, genes related to redox regulation were investigated (Fig. 5.2F). Thioredoxin (TXN), glutathione peroxidase-1 (GPX1) and thioredoxin binding protein-2 (TBP-2) were all significantly different in MCP-1 treated cells compared to authentic osteoclasts (Fig. 5.2F). Notably, it was previously shown that TBP-2 is down regulated in human osteoclast formation and that forced expression of TBP-2 prevents osteoclast formation (Aitken, C.J., *et al.,* 2004). TBP-2 levels in cells treated with MCP-1 and M-CSF are comparable with the levels in macrophage like cells differentiated with M-CSF alone. In other words, TBP-2 is not down regulated by MCP-1 as it is by RANKL. Likewise, increased TXN and GPX1 are features of osteoclasts. In TRAP positive multinuclear cells differentiated by MCP-1, these genes are not induced as in authentic osteoclasts. These redox regulators therefore fail to follow the pattern of gene expression found in osteoclasts.

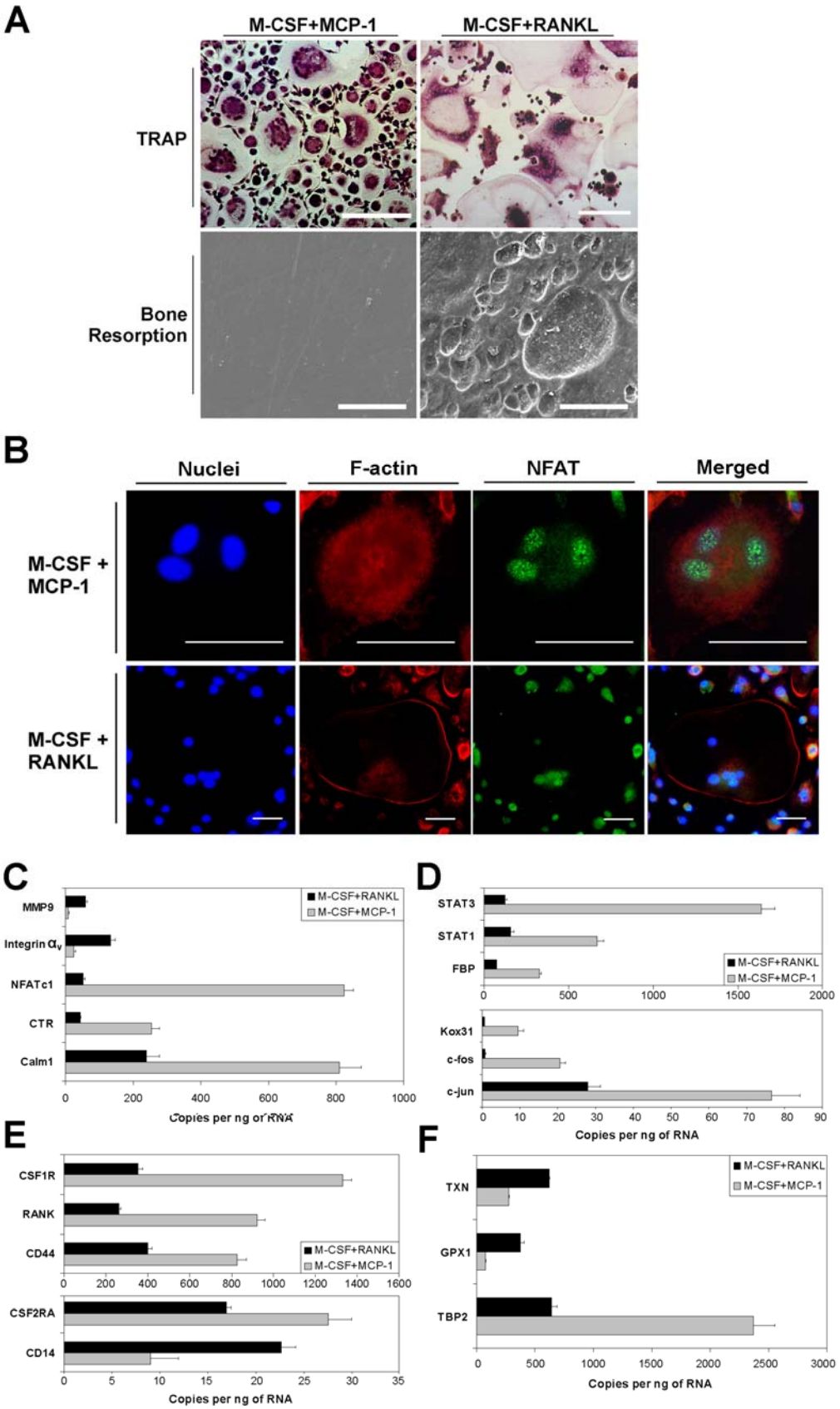

Fig. 5.2 Analysis of cellular and molecular phenotypes of multinuclear cells. (A) Light microscopy of cellular appearance and electron microscopy of bone resorption

phenotype of cells treated with either MCP-1 or RANKL. Both MCP-1 and RANKL treated cells are TRAP positive and multinuclear (upper panel), but MCP-1 treated cells are negative for bone resorption (lower panel). Bar represents 100 μm. (B) Confocal imagery of cellular phenotype after treatment with M-CSF and MCP-1 or M-CSF and RANKL. Hoechst stain for nuclei (blue): both treatment regimes show multiple nuclei in the cells. Rhodamine conjugated phalloidin stain for actin (red): cells from M-CSF and MCP-1 treatment are negative for the F-actin ring (upper panel), while M-CSF and RANKL treatment results in cells that are positive for the F-actin ring (lower panel). NFAT (green): both treatment regimes are positive for the presence of nuclear NFAT. Merged: M-CSF and MCP-1 treated cells are multinuclear cells that are positive for NFAT protein in the nuclei of the cells, but are negative for the F-actin ring (upper panel). M-CSF and RANKL treated cells are large multinuclear cells that are positive for NFAT protein within the nucleus and positive for the F-actin ring structure (lower panel). Bar is 50 μm. Quantitative real time PCR analysis of gene expression in cultures treated with M-CSF and RANKL (black columns) and M-CSF and MCP-1 (shaded columns). (C) Graph shows osteoclast markers; matrix metalloproteinase 9 (MMP9), integrin αV, NFATc1, calcitonin receptor (CTR) and calmodulin 1 (Calm1). (D) Graph shows nuclear factors; signal transducer and activator of transcription (STAT3), STAT1, far upstream element-binding protein (FBP), Kox31, c-fos and c-jun. (E) Graph shows cell surface markers and receptors; colony-stimulating factor 1 receptor (CSF1R), receptor activator of NF-κB (RANK), CD44, GM-CSF receptor-α (CSF2RA) and CD14. (F) Graph shows REDOX related genes; thioredoxin (TXN), glutathione peroxidase-1 (GPX1), thioredoxin binding protein-2 (TBP2). All data are mean copies per ng total RNA. Some axes have been changed to improve clarity.

Signalling pathway involved in MCP-1 mediated multinucleation.

Multinuclear cells derived from either MCP-1 treatment or RANKL treatment has substantial differences in gene expression profile and cellular characteristics. Yet despite these differences, these two diverse treatments (either MCP-1 or RANKL) both results in TRAP positive multinuclear cells. To understand the signalling pathway of multinucleation (MCP-1 mediated TRAP positive multinuclear cell formation) and activation of osteoclast (M-CSF and RANKL treated authentic osteoclasts), p38MAP kinase and MEK1/2 inhibitors (SB203580 and U0126, respectively), were utilised to segment the pathways involved in cell fusion. These inhibitors were tested in cultures treated with MCP-1, RANKL and MCP-1 plus RANKL combined treatment. Three parameters were measured to reflect the extent of

cell fusion in different cultures: the total number of TRAP+ cells with 3 or more nuclei, the number of nuclei per cell and the size of multinuclear cells measured on the longest axis.

Fig. 4.1 showed that treatment with M-CSF and MCP-1 resulted in TRAP positive multinuclear cells that were negative for bone resorption (134±29, n=12). The addition of p38 MAPK inhibitor, SB203580 had no effect on the number of TRAP+ multinuclear cells compared to MCP-1 treatment (126±3, p= 0.91, Fig. 5.3A), indicating that p38MAPK has no effect on cellular fusion into multinuclear cells. In contrast, the addition of SB203580 to RANKL treated cultures resulted in a 26% reduction in the number of TRAP+ multinuclear cells (from 217±3 to 160±7, p= 1.5×10^{-3}, Fig. 5.3A), a reduction in the number of nuclei per cell (from 7.2±0.7 to 3.2±0.6, p= 6.3×10^{-7}, Fig. 5.3B) and a reduction in the size of cells (from 136±6 to 49±5 μm, average longest axis, p= 9.2×10^{-11}, Fig. 5.3C), when compared to control RANKL treatment. SB203580 had a similar effect on cultures treated with MCP-1 plus RANKL. MCP-1 recovered SB203580 induced inhibition to a level of standard M-CSF and RANKL treatments (Fig. 5.3A), but was still reduced compared to the MCP-1 plus RANKL treated cultures (Fig. 5.3A), and SB203580 still reduced the number of nuclei (6.6±0.5 and 8.9±0.9, respectively, p= 0.03, Fig. 5.3B) and the size of the TRAP+ multinuclear cells compared (71±5 and 162±9 μm, respectively, average longest axis, p= 3.8×10^{-8}, Fig. 5.3C).

U0126 potently suppressed the number of TRAP+ multinuclear cells in all cultures, whether treated with either MCP-1, RANKL, or MCP-1 plus RANKL (p= 1.1×10^{-5}, 1.2×10^{-6}, and 8.9×10^{-5} respectively, Fig. 5.3A) and significantly reduced the

average of nuclei per cell (Fig. 5.3B). Moreover, the morphology of the cells changed from multinuclear cells to mononuclear cells (Fig. 5.3C), suggesting that U0126, inhibited multinucleation. Taken together, these data suggest that the effects of SB203580 and U0126 are consistent in the three treatment groups, with SB203580 treatment resulting in significant but quantitative effects on parameters of multinuclear cells while U0126 virtually eliminated multinuclear cells in all treatment regimes. Moreover, U0126 treated cells remained mononuclear (Fig. 5.3B and C), suggesting that U0126 inhibited multinucleation by blocking cell fusion.

High NFATc1 expression was present in both RANKL and MCP-1 induced TRAP+ multinuclear cells (Fig 5.2C). We measured the expression of NFATc1 in cultures exposed to SB203580 and U0126 (Fig. 5.3D). The potent RANKL mediated induction of NFATc1 was unaffected by SB203580, but was totally abolished by U0126 (Fig. 5.3D). Taken together, these data are consistent with the hypothesis that NFATc1 expression is correlated with multinuclear cell formation and that the induction of NFATc1 by RANKL flows through MEK1/2.

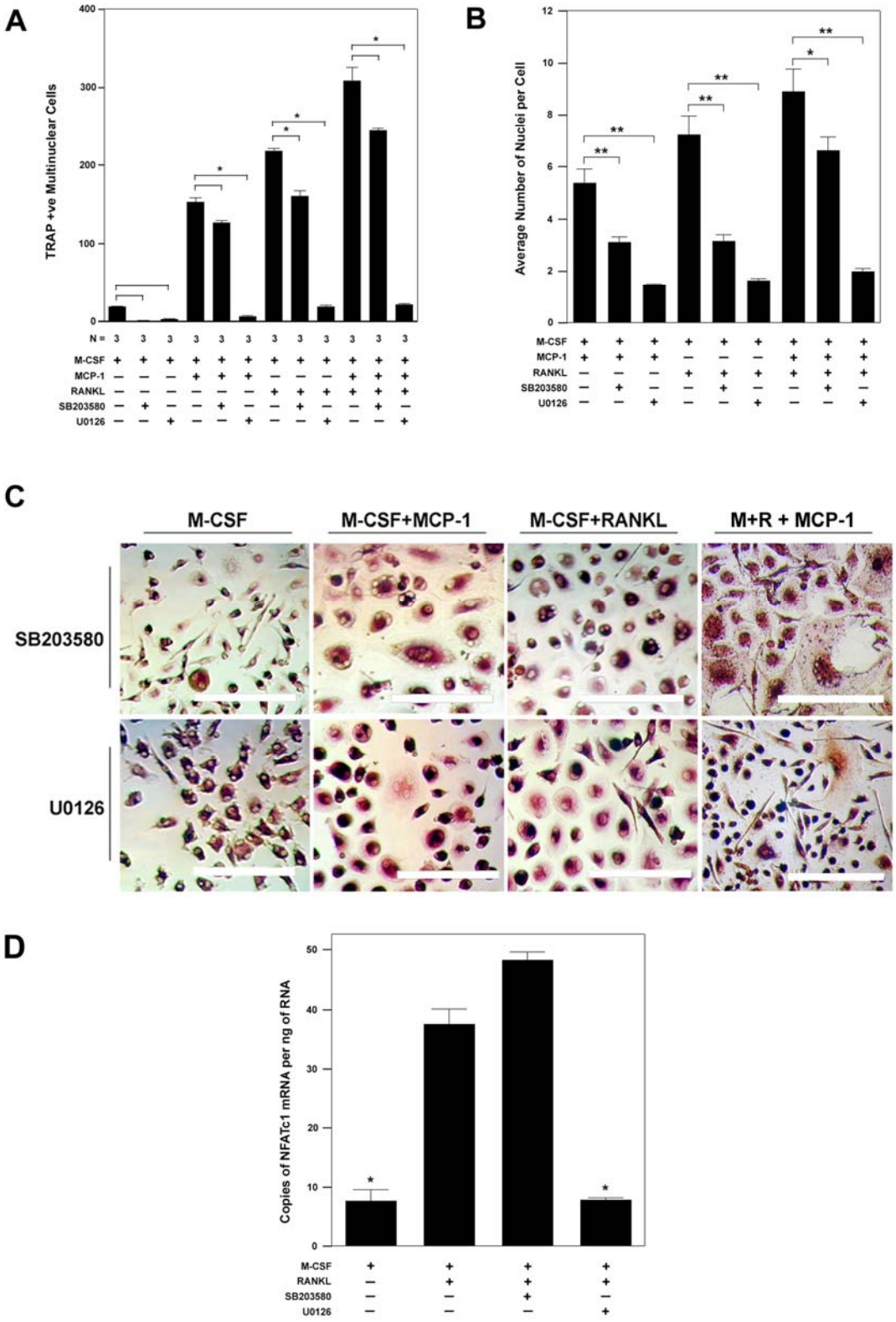

Fig. 5.3 Multinucleation is ERK1/2 dependent mechanism. (A) Cultures in four treatment regimes (M-CSF alone, M-CSF and MCP-1, M-CSF and RANKL, M-CSF, MCP-1, RANKL) were treated with either 20μM of p38 MAPK inhibitor, SB023580

or 10μM of ERK1/2 inhibitor, U0126. Addition of SB203580: There was no effect on TRAP+ multinuclear cells in M-CSF and MCP-1 treated cells, while significantly inhibited TRAP+ multinuclear cells in M-CSF and RANKL treatment. MCP-1 recovered SB203580 inhibition of M-CSF and RANKL mediated TRAP+ multinuclear cell formation. Addition of U0126: significantly suppressed TRAP+ multinuclear cells in all treatment regimes. *, significant differences, where $p<0.01$. (B) The average nuclei per cell were analyzed in three treatment regimes (M-CSF and MCP-1, M-CSF and RANKL, M-CSF, MCP-1 and RANKL) with either SB203580 or U0126. The adddition of either SB203580 or U0126, significantly reduces the average number of nuclei per cell in all three treatment regime. *, significant differences, where $p<0.05$. **, significant differences, where $p<0.01$. (C) Addition of SB203580 results in smaller TRAP+ multinuclear cells M-CSF and RANKL, M-CSF, MCP-1 and RANKL treatments (upper panels). Addition of U0126 results in TRAP+ mononuclear phenotype in all treatment regimes (lower panels). Bar is 100 μm. (D) Quantitative real time PCR analysis of NFATc1 in cultures treated with either SB203580 or U0126. Addition of SB203580 had no significant difference in the NFATc1 expression with control RANKL treated cells. However, U0126 significantly reduced expression of NFATc1 in RANKL treated cells. *, significant differences compared to M-CSF and RANKL or M-CSF, RANKL and SB203580 treatments, where $p<0.01$.

Addition of exogenous calcitonin inhibits fusion induced by MCP-1.

Calcitonin receptor (CTR) was highly up-regulated in MCP-1 treated cells compared to RANKL treated cells. This leads to the hypothesis that calcitonin may act to prevent fusion of multinuclear cells induced by MCP-1 as well as its recognized role in inhibiting fusion of osteoclasts differentiated with RANKL.

Exogenous calcitonin (up to 50ng/ml) was added to cultures treated with MCP-1 to generate TRAP positive multinuclear cells. Similarly, calcitonin was added to cells treated RANKL to produce authentic osteoclasts. Calcitonin resulted in a similar dose response of reduction in the number of multinuclear cells in both treatments (Fig. 5.4A and B), confirming the hypothesis that calcitonin receptor is active in MCP-1 generated TRAP positive multinuclear cells. Calcitonin (at 50ng/ml) significantly reduced the number of TRAP positive multinuclear cells in both MCP-1 treatment (133±29 versus 21±6, $p= 8\times10^{-3}$) and RANKL treated cells (240±11 versus

89±12, $p= 2\times10^{-4}$) (Fig. 5.4C). Calcitonin had no significant effect on cells treated with M-CSF alone (Fig. 5.4C). The visual appearance of RANKL and MCP-1 treated cultures was remarkably similar after treatment with calcitonin (Fig. 5.4D). Those TRAP positive multinuclear cells that remained after calcitonin treatment were generally of smaller size in both MCP-1 and RANKL treated cultures (Fig. 5.4D), indicating that calcitonin reduces the number and the size of multinuclear cells. Exogenous calcitonin reduced the size of RANKL treated cells by approximately three fold ($p= 2.2\times10^{-5}$). Calcitonin at 50ng/ml completely inhibited bone resorption by osteoclasts (Fig. 5.4D). Confocal imagery showed NFAT was still resident in the nuclei of the cells treated with calcitonin, regardless of whether they were from the MCP-1 or the RANKL treatments (Fig. 5.4E). Interestingly, the F-actin ring clearly evident in RANKL treated osteoclasts was absent after calcitonin treatment (Fig. 5.4E). These data suggest that calcitonin is able to prevent the MCP-1 mediated fusion of monocytes into TRAP positive multinuclear cells by paralysing the cell to cell fusion of mononuclear cells into multinuclear osteoclasts.

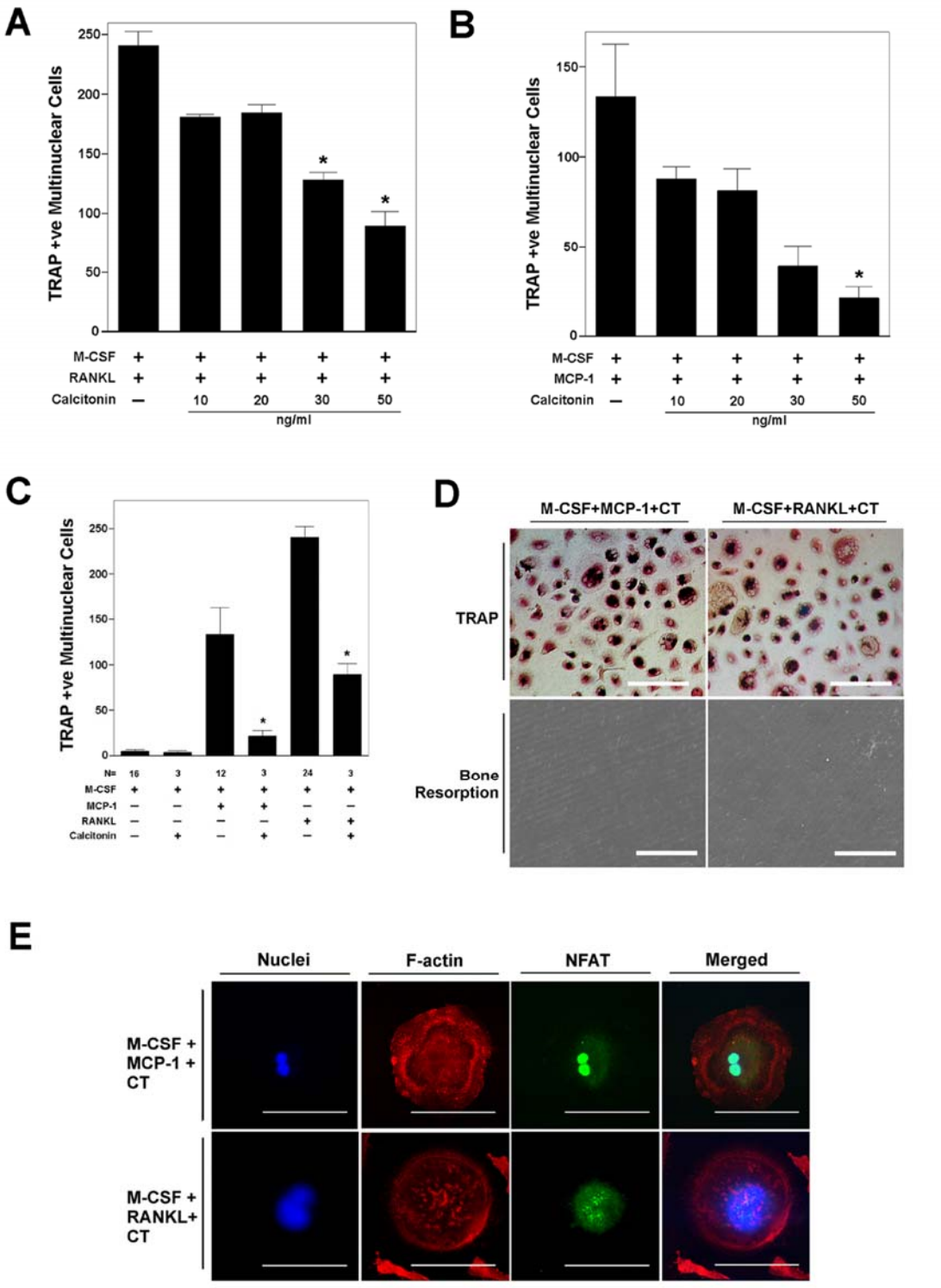

Fig. 5.4 Exogenous calcitonin inhibits the multinuclear phenotype. (A) Cultures were treated continuously with calcitonin at the concentration indicated (ng/ml) in the presence of M-CSF and RANKL. (B) Cultures were treated continuously with calcitonin at the concentration indicated (ng/ml) in the presence of M-CSF and MCP-1. (C) Addition of exogenous calcitonin (50ng/ml): TRAP positive multinuclear cells

were significantly suppressed in both M-CSF and MCP-1 treated cells and M-CSF and RANKL treated cells. *, significant differences compared with M-CSF and MCP-1 or M-CSF and RANKL treatment and exogenous calcitonin added cells, where $p<0.01$. (D) Simultaneous treatment with calcitonin and either MCP-1 (left panels) or RANKL (right panels) results in the same phenotype of smaller TRAP positive multinuclear cells that are negative for bone resorption (lower panels) Bar is 100 μm. (E) Confocal imagery of the effect of calcitonin. Hoechst stain for nuclei (blue): calcitonin (50ng/ml) inhibited multinucleation in both treatment regimes, reducing the number of nuclei present within cells. Phalloidin stain for actin (red): addition of calcitonin inhibited formation of the F-actin ring in RANKL treated cells. NFAT (green): calcitonin did not prevent the presence of nuclear NFAT in both treatment regimes. Bar is 50 μm.

MCP-1 treated cells are able to differentiate into osteoclasts and become positive for bone resorption activity after RANKL exposure.

The presence of RANK mRNA in TRAP positive multinuclear cells differentiated with MCP-1 suggests the hypothesis that such cells could become proficient for bone resorption if provided with RANKL. This hypothesis was tested by exposing TRAP positive multinuclear cells from MCP-1 treatment to RANKL on dentine slices.

Initially, the time course of bone resorption of RANKL treated human PBMC grown directly on dentine slices was established (Fig. 5.5A). PBMC grown on dentine slices and exposed to RANKL were negative for bone resorption activity at 7-days of culture, while at 14-days, bone resorption was evident on 7±2% of the surface of the dentine slice. By 21-days of continuous RANKL treatment, the amount of resorption significantly increased to 47±8% ($p= 3.4\times10^{-3}$). Thus, the percentage of

resorption increased exponentially, as more mature osteoclasts were formed (Fig. 5.5A). However, cultures exposed to continuous treatment with either M-CSF alone or M-CSF and MCP-1 were negative for bone resorption (Fig. 5.5B and D).

Multinuclear cells induced by MCP-1 were grown on collagen coated plates for 14-days then purified over serum step gradients and plated at appropriate densities onto dentine slices. Cells were then cultured on dentine for 7-days in the presence of RANKL. Controls were PBMC cultured on collagen coated plates for 14-days in the presence of RANKL, purified similarly over serum step gradients, plated onto dentine slices and then exposed to RANKL for 7-days. A further control was PBMC grown on collagen coated plates in the presence of M-CSF alone and then plated onto dentine slices and exposed to RANKL for 7-days.

Mononuclear cells treated with M-CSF for 14-days and then exposed to RANKL for 7-days showed a small amount of bone resorption (0.76±0.54%). This indicates that 14-days incubation with M-CSF does not eliminate the capacity to differentiate into an osteoclast (Fig. 5.5C). Incidentally, this represented more bone resorption than that observed in PBMC exposed for 7-days continuously to RANKL (Fig. 5.5A), perhaps suggesting that osteoclast progenitors are able to proliferate under the conditions of 14-days incubation with M-CSF.

Mature human osteoclasts from RANKL treated cells cultured on collagen coated plates survive serum gradient purification and accumulate many resorption pits and trails on dentine over the following 7-days of RANKL exposure. As expected, mature osteoclasts had significantly greater bone resorption activity (35±7.0% n=3,

$p = 8.4 \times 10^{-4}$), compared to M-CSF alone treated cells (Fig. 5.5C and E). Multinuclear cells purified from MCP-1 treated cultures and plated onto dentine in the presence of RANKL were positive for bone resorption activity, degrading 8.0±1.5% of the dentine surface after 7-days of exposure to RANKL (Fig. 5.5C and E). This amount of bone resorption was significantly greater than that compared to M-CSF alone treated cells ($p = 0.012$), but was significantly less than that from cells treated continuously with RANKL, whether replated from collagen or not. In order to put these data into perspective, the bone resorption activity of multinuclear cells produced by MCP-1 treatment, (purified, then exposed to dentine slices with RANKL), was compared to the time course of bone resorption from continuous exposure to RANKL (Fig. 5.5A). Cultures with prior MCP-1 treatment followed by RANKL treatment, had a similar amount of bone resorption activity after 7-days on dentine as did cultures from 14-days of continuous RANKL exposure on dentine (Pearson correlation, $R^2=1$). These data provide evidence that MCP-1 treated cells can differentiate efficiently into authentic bone resorbing osteoclasts and indicates that the induction of RANK by MCP-1 (Fig. 5.5E) may precondition such cells to differentiation in the presence of RANKL.

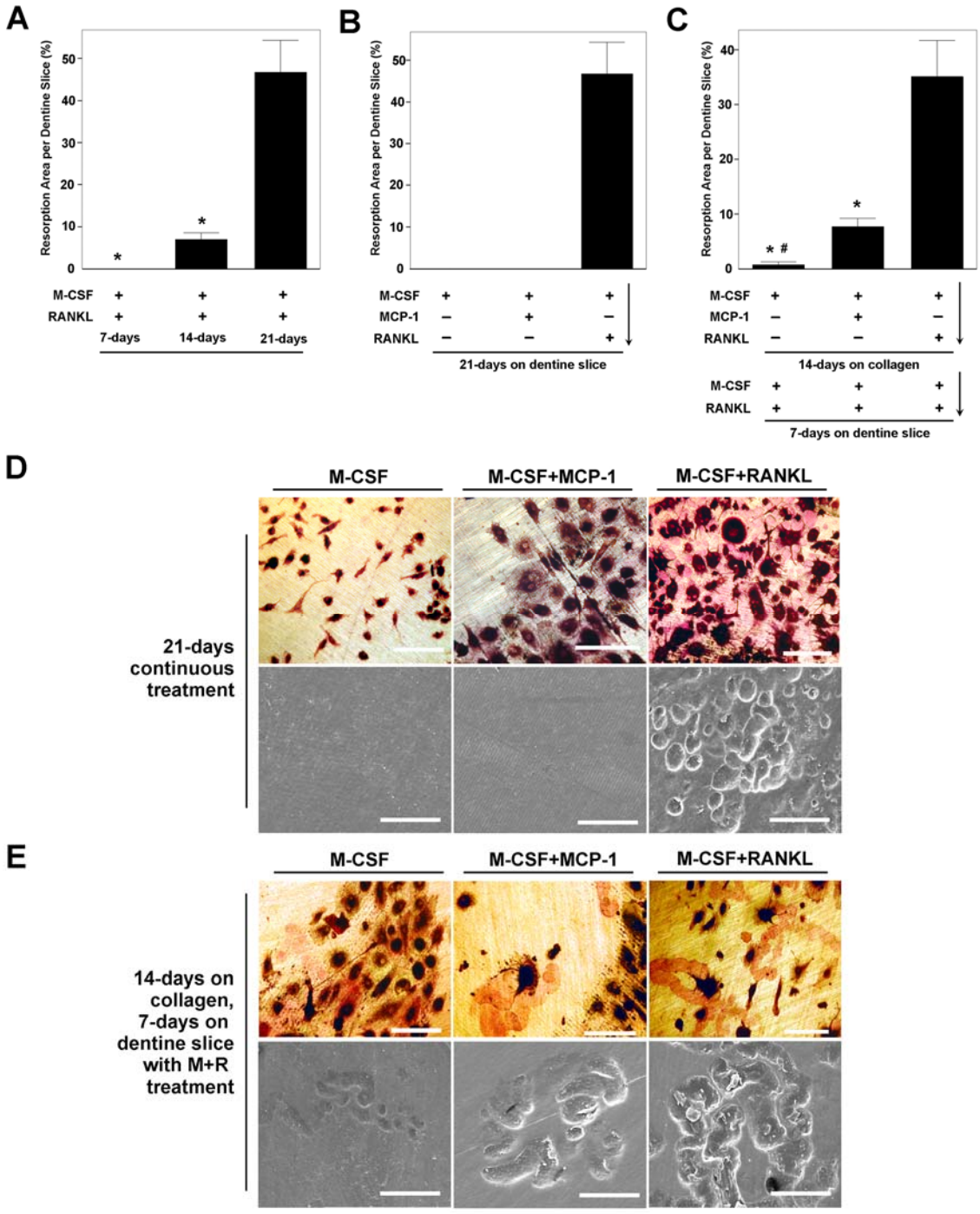

Fig. 5.5 MCP-1 induced TRAP positive multinuclear cells are positive for bone resorption after treatment with RANKL. (A) Graph shows percent of resorption area per dentine slice after continuous M-CSF and RANKL treatment for at 7, 14 and 21-days. Asterisk: *, significant difference compared with M-CSF plus RANKL treatment at 21-days, where $p<0.01$. (B) Graph shows percent of resorption area per dentine slice after 21-days continuous treatment with M-CSF alone, M-CSF plus MCP-1 or M-CSF plus RANKL. M-CSF and RANKL treated cells are positive for bone resorption, while others are negative for bone resorption. (C) Cells were grown on collagen coated plates for 14-days with M-CSF alone, M-CSF plus MCP-1 or M-CSF plus RANKL. All mature cells were then plated on dentine and treated with M-

CSF plus RANKL for 7-days. Graph shows percent of resorption area per dentine slice. M-CSF plus MCP-1 treated cells have significantly enhanced resorption activity compared to M-CSF treated cells. #, significant difference compared with M-CSF plus MCP-1 treatment, where $p<0.05$. *, significant difference compared with M-CSF plus RANKL treatment, where $p<0.05$. (D) Light and electron microscopy of cells and bone resorption on dentine slices. Cells were grown continuously on dentine slices as in graph B. Treatment with M-CSF alone (upper left panel) results in mononuclear macrophage-like cells that are negative for bone resorption (lower left panel). Treatment with MCP-1 (in the presence of M-CSF) results in TRAP positive multinuclear cells (upper center panel) that are negative for bone resorption (lower center panel). Treatment with RANKL (in the presence of M-CSF) results in TRAP positive osteoclasts (upper right panel) that have substantial bone resorption activity (lower right panel). Extensive resorption pits on dentine can be seen in both light and electron microscopy (upper and lower panels on right). (E). Cellular appearance and bone resorption activity of cells cultured on collagen coated plates for 14-days prior to exposure to RANKL for 7-days on dentine slices. Cells with prior M-CSF treatment (upper left panel) were able to produce a low number of osteoclasts, evidenced by bone resorption (lower left panel). TRAP positive multinuclear cells from MCP-1 treatment became positive for bone resorption after exposure to RANKL (upper and lower center panels), with visible osteoclast trails. Osteoclasts differentiated with RANKL for 14-days prior to plating on dentine showed substantial bone resorption with pits and trails (upper and lower right panel). Upper panels are all light microscopy with TRAP stain and lower panels are electron microscopy. Bar is 100 μm.

Discussion

MCP-1 is induced by RANKL in human osteoclast differentiation. MCP-1 stimulates the formation of osteoclasts in the presence of RANKL and, in the absence of RANKL, stimulates the formation of TRAP positive multinuclear cells that have the appearance of osteoclasts. To understand the difference in the signalling pathway between MCP-1 mediated multinucleation and RANKL induced osteoclast formation, MAP kinase inhibitors were utilised to test the hypothesis that there are separate pathways for multinucleation and activation of osteoclasts.

MAPK family members are classified into three groups: ERK, JNK and p38 MAPK groups. The phosphorylation of p38 MAPK by MAPK kinase (MKK) 3 or 6,

results in active p38 MAPK, which further activates the transcription factor, "activating transcription factor" (ATF-2), which then induces transcription of down stream target genes (reviewed in Cobb, M.H., and Goldsmith, E.J., 1995; Li, X., *et al.*, 2002). Using a specific p38 MAPK inhibitor (SB203580), the p38 MAPK signal was found to be involved in osteoclastic bone resorption induced by IL-1 in fetal rat long bones (Kumar, S., *et al.,* 2000), suggesting that p38 MAPK is involved in the regulation of osteoclast differentiation or function, or both (Li, X., *et al.,* 2002). Subsequently, p38 MAPK was shown to be crucial in osteoclast differentiation (Li, X., *et al.,* 2003) and essential in CTSK gene expression during osteoclast formation (Matsumoto, M., *et al.,* 2004). Taken together these observations confirm that p38 MAPK is involved in osteoclast formation. The absence of a reduction in MCP-1 mediated TRAP positive multinuclear cells by SB203580 indicates that p38 MAPK is not involved in the differentiation of precursors into multinuclear cells, but rather involved in the function of osteoclast, correlating with previous studies (Matsumoto, M., *et al.,* 2004).

Meanwhile, the ERK pathway is activated by Ras, a small GTP-binding protein that is ubiquitously expressed as inducer of various signalling pathway including proliferation and differentiation (reviewed in Bollag, G., and McCormick, F., 1991). Ras activates MAPK kinases (MEK1 and 2), which in turn activates ERK1 and 2 (Kyriakis, J.M., *et al.,* 1992), indicating that Ras/ERK pathway is important in cell signalling and survival. The MEK-ERK1/2 inhibitors, U0126 and PD98059 are specific inhibitors that bind common or overlapping MEK binding site. These compounds blocks activation of MEK substrates, but U0126 has approximately 100-fold higher affinity compare to PD98059 (Favata, M.F., *et al.,* 1998). Addition of

U0126 had completely suppressed both MCP-1 mediated TRAP positive multinuclear cell formation and RANKL induced osteoclast formation. These data correlated with previous study showing that inhibition of ERK activity by dominant negative Ras, rapidly induced apoptosis of osteoclasts (Miyazaki, T., *et al.,* 2000), indicating that ERK1/2 pathway is crucial in osteoclast differentiation and formation. However, human cells treated continuously with M-CSF, RANKL and U0126 do not appear apoptotic (Fig. 5.2C). Hence, the data illustrates that ERK1/2 pathway is required for differentiation of osteoclast precursors into giant multinuclear cells, and p38 MAPK might be required for the activation of osteoclasts.

MCP-1 induced TRAP positive multinuclear cells that are negative for bone resorption are indeed capable of differentiating into bone resorbing osteoclasts when provided with RANKL. Such TRAP positive multinuclear cells stimulated by MCP-1 may represent an intermediate stage of osteoclast differentiation. MCP-1 treated cells have many features in common with osteoclasts and are able to differentiate into authentic bone resorbing osteoclasts in the presence of RANKL. The further differentiation into osteoclasts may be attributed to the expression of RANK (receptor for RANKL), which is at a higher level in MCP-1 treated cells than that found in authentic osteoclasts. MCP-1 derived TRAP positive multinuclear cells had higher expression of osteoclast-related nuclear factors and cell surface receptors, showing a similar, but different phenotype to RANKL mediated osteoclasts. MCP-1 treated cells lacked expression of genes required for bone resorption activity, including proteases cathepsin K, MMP9 (Engsig, M.T., *et al.,* 2000; Kumta, S.M., *et al.,* 2003; Wittrant, Y., 2003; Dong, Z., *et al.,* 2005) and cell adhesion molecules, such as the αV subunit of integrin αVβ3, which is required for formation of F-actin ring

(Nakamura, I., *et al.,* 1996a; Nakamura, I., *et al.,* 1996b; reviewed in Duong, L.T., *et al.,* 2000; Akisaka, T., *et al.,* 2001). In keeping with the lack of expression of the αV subunit, MCP-1 meditated multinuclear cells lacked the F-actin ring, while RANKL mediated multinuclear cells clearly showed the F-actin ring structure (Fig. 5.2).

Interestingly, MCP-1 mediated TRAP positive multinuclear cells had abundant expression of NFATc1, calmodulin 1 and CTR. The presence of NFATc1 in MCP-1 treated cells was detected by quantitative PCR of mRNA and verified by nuclear staining for NFAT and confocal imagery. NFATc1 is induced by RANKL during mouse (Takayanagi, H., *et al.,* 2002b; Hirotani, H., *et al.,* 2004; Matsuo, K., *et al.,* 2004) and human (Day, C.J., *et al.,* 2004; Day, C.J., *et al.,* 2005) osteoclast differentiation and is claimed to be the master regulator of mouse osteoclast formation and function (reviewed in Takayanagi, H., 2005a; reviewed in Takayanagi, H., 2005b). However, the lack of bone resorption in the presence of high NFATc1 runs counter to the idea that NFATc1 alone is sufficient for osteoclast formation and function, as MCP-1 induced TRAP positive, CTR positive cells have abundant expression of NFATc1. In contrast, NFATc1 is required for the formation of multinuclear cells through cell fusion. Furthermore, the correlation of high NFATc1 with high CTR in MCP-1 treated cells suggests a direct regulatory function of NFATc1 to induce the CTR gene. Addition of exogenous calcitonin significantly reduced the number of MCP-1 mediated TRAP positive multinuclear cells, blocking the fusion process. Calcitonin significantly reduced the number of RANKL mediated TRAP positive multinuclear osteoclasts, and also suppressed the bone resorption capabilities, agreeing with previous observations (Suzuki, H., *et al.,* 1996). Calcitonin can inhibit chemokine stimulated cell fusion, presumably through the MCP-1 induced CTR.

These data suggest that CTR may be expressed under chemokine signalling and therefore may represent both the intermediate and late stages of osteoclast differentiation. In the presence of abundant calcitonin, one may expect the formation of osteoclasts through cell fusion to be inhibited. The existence of feedback regulation of osteoclast formation by physiological calcium levels through calcitonin seems a logical proposition. This idea also predicts that calcitonin may have benefits in inflammatory bone loss, where high chemokine production is observed, by inhibiting the fusion of pre-osteoclasts stimulated by chemokines in the absence of RANKL.

In a similar manner to the high levels of NFATc1 in MCP-1 treated cells, the other critical osteoclast related transcription factors, c-jun and c-fos, were also more abundant in MCP-1 treated cells than in RANKL treated cells. The c-fos is a nuclear phosphoprotein and is a major component of the activator protein-1 (AP-1) transcription factor (Candeliere, G.A., *et al*, 1995; Bakin, A.V., and Curran, T., 1999). The c-fos knockout mice studies have established that c-fos is involved in the differentiation and activity of progenitors of osteoclast lineage (Liebermann, D.A., *et al.,* 1998). c-fos was particularly more abundant (27-fold) in MCP-1 treated cells. This data is compatible with the suggestion that c-fos (as a component of AP1) is an activator of NFATc1 (Matsuo, K., *et al.,* 2004). Despite this possible relationship between c-fos and NFATc1, the lack of bone resorption activity in MCP-1 induced TRAP positive, CTR positive, multinuclear cells indicates that an unknown RANKL signalling pathway is required for activation of bone resorption in human osteoclasts even in the presence of nuclear NFAT.

CD44 is an integral cell membrane glycoprotein with a postulated role in matrix adhesion lymphocyte activation and lymph node homing (Aruffo, A., *et al.,* 1990). CD44 is shown to promote fusion in macrophages (Sterling, H., *et al.,* 1998; Cui, W., *et al.,* 2005). Interestingly, CD44 was up-regulated in MCP-1 mediated TRAP positive multinuclear cells compared to RANKL induced osteoclasts, indicating that MCP-1 mediates fusion of precursor cells into multinuclear cells. The cellular fusion process is a very complex mechanism that requires further understanding. Several proteins have been identified that play a role in macrophage/osteoclast fusion process, including signal regulatory protein alpha (SIRPα) or macrophage fusion receptor (MFR, Saginario, C., *et al.,* 1998), and its ligand CD47 (Han, X., *et al.,* 2000; reviewed in Vignery, A., 2005a; reviewed in Vignery, A., 2005a). Recently, a seven transmembrane protein, dendritic cell-specific transmembrane protein (DC-STAMP) has been reported to induce differentiation of osteoclasts (Kukita, T., *et al.,* 2004) and DC-STAMP is required for the cell to cell fusion of pre-osteoclastic event to yield formation of osteoclasts (Yagi, M., *et al.,* 2005; reviewed in Vignery, A., 2005b). Moreover, DC-STAMP is a chemokine receptor of unknown ligand, confirming at least a strong involvement of chemokines in the fusion process. MCP-1 might be the ligand for DC-STAMP, inducing fusion of osteoclast precursors into multinuclear cells, as MCP-1 participates in the formation of foreign body giant cell formation (Kyriakides, T.R., *et al.,* 2004; reviewed in Vignery, A., 2005b).

Thioredoxin and glutathione peroxidase-1 were lower in MCP-1 treated cells than in RANKL treated cells (2.3-fold and 4.9-fold, respectively). Thioredoxin binding protein-2 (TBP2) is repressed during osteoclast differentiation (Aitken, C.J.,

et al., 2004) but was up-regulated in MCP-1 treated cells (3.7-fold). TBP2 negatively regulates thioredoxin and other redox regulating molecules (Aitken, C.J., *et al.,* 2004), and is capable of blocking osteoclast differentiation when over expressed by transfection (Aitken, C.J., *et al.,* 2004). Thioredoxin mediates redox-regulated activation of AP1, NFκB and NFAT in osteoclasts (Aitken, C.J., *et al.,* 2004; Lean, J., *et al.,* 2004; Lean, J.M., *et al.,* 2005). NFATc1 and AP1 components (c-fos and c-jun) are more abundant in MCP-1 treated cells, compared to RANKL treated cells. Since MCP-1 treated TRAP positive multinuclear cells are capable of differentiating into osteoclasts on treatment with RANKL, the redox regulators such as TBP2 do not permanently block further differentiation and that a redox status permissive for osteoclast differentiation is acquired through RANKL. The functional consequences of redox regulation in controlling osteoclast differentiation and the phenotype of MCP-1 treated cells require further work.

In conclusion, MCP-1 mediated TRAP positive multinuclear cells express key osteoclast markers including CTR, TRAP and NFATc1, but lack genes required for bone resorption, including CTSK, MMP9 and integrin αV and the F-actin ring structure,. Furthermore, MCP-1 mediated TRAP positive multinuclear cells are able to differentiate into bone resorbing osteoclasts, once exposed to RANKL signal; hence these cells are "pre-osteoclasts" that have arrested differentiation at an intermediate stage.

Chapter 6

General Discussion

and

Conclusion.

Previous studies have shown that GM-CSF receptor alpha (CSF2RA) was highly up-regulated in M-CSF and RANKL treated TRAP positive multinuclear osteoclasts compared to M-CSF alone treated TRAP negative mononuclear macrophage-like cells (Day, C.J., *et al.*, 2004). This led to a hypothesis that exogenous GM-CSF would have an inhibitory effect on osteoclast formation. Additions of exogenous GM-CSF to the M-CSF and RANKL (GMR) treated cultures suppressed osteoclast formation, resulting in TRAP negative mononuclear cells with different cellular phenotype to macrophage-like cells. Quantitative real-time PCR and cDNA microarray experiments indicated that GM-CSF suppresses osteoclast markers and related genes, including cathepsin K (cysteine protease), vacuolar H^+ ATPase, TRAP and osteoclast related nuclear factors, including NFATc1, FUSE-binding protein 1, GA-binding protein and KOX31, a zinc finger protein. Furthermore, FACS analysis showed high amounts of CD1a in GM-CSF treated cells compared to authentic osteoclasts. CD1a is a cell surface marker for dendritic cells, indicating that GM-CSF diverts differentiation into a dendritic-like cell phenotype.

From the microarray experiment, the most down-regulated gene by GM-CSF treatment was MCP-1. MCP-1 is a member of the CC chemokine superfamily that recruits monocytes to the site of inflammation and is also involved in rheumatoid arthritis and tooth eruption. Addition of MCP-1 to M-CSF treated cultures, resulted in TRAP positive multinuclear cells that were negative for bone resorption. Furthermore, the addition of MCP-1 to M-CSF and RANKL treated cultures enhanced osteoclast formation by 33%. Moreover, neutralising antibody to MCP-1 inhibited the MCP-1 enhancement of osteoclast formation. Dominant negative MCP-1, which irreversibly binds to cognate MCP-1 receptors, abolished authentic osteoclast

formation. These data indicate that MCP-1 is involved in osteoclast formation and is crucial for multinucleation. Surprisingly, addition of MCP-1 to GMR cultures rescued the formation of functioning osteoclasts. Other CC chemokines, such as RANTES and MIP1α treatment with M-CSF also resulted in TRAP positive multinuclear cells that did not resorb bone. In addition, neither RANTES nor MIP1α had potent enhancement of osteoclast formation, as did MCP-1, when treated simultaneously with M-CSF and RANKL. Therefore, of the CC chemokines investigated, MCP-1 is the most potent chemokine in osteoclast formation.

MCP-1 treatment with M-CSF resulted in TRAP positive multinuclear cells that were negative for bone resorption. Through real-time PCR, the gene expression levels of osteoclast markers and related genes were examined. Surprisingly, MCP-1 mediated TRAP positive multinuclear cells induced higher expression of calcitonin receptor, c-fos and NFATc1 compared to authentic osteoclasts. However, these cells lacked expression of key genes involved in bone resorption, such as, cathepsin K, matrix metalloproteinase 9 and integrin αV, providing a rationale for inability for bone resorption. NFATc1, has recently been identified as the master regulator of osteoclast formation and function (reviewed in Takayanagi, H., 2005a and Takayanagi, H., 2005b). However, high expression of NFATc1 mRNA and nuclear NFAT in MCP-1 mediated TRAP positive multinuclear cells suggest that NFATc1 is involved in the cellular fusion of osteoclast precursors into multinuclear cells, but not in osteoclast function. Furthermore, exogenous calcitonin blocked formation of TRAP positive multinuclear cells, as well as bone resorption, demonstrating that calcitonin receptor is not a late marker of osteoclast maturity, rather a marker of an intermediate stage in osteoclast formation. In addition, treatment of the mature MCP-1 mediated

TRAP positive multinuclear cells with RANKL induced bone resorption. The data indicate that MCP-1 treated cells have an arrested osteoclast phenotype representing an intermediate stage of osteoclast differentiation.

The expression of osteoclast marker genes in cells treated with MCP-1 and M-CSF, suggests that some osteoclast characteristics can be acquired independently of RANKL. Since RANKL induces the MCP-1 receptor (CCR2), RANKL induction of MCP-1 sets up both autocrine (affecting the osteoclast producing MCP-1) and paracrine pathways (affecting cells destined to fuse with the RANKL stimulated osteoclast). A model is presented for chemokine action during osteoclast differentiation (Fig. 6.1). Osteoclasts form by fusion of RANK positive mononuclear precursors after contact with a cell expressing RANKL. Intimate cell-cell contact is necessary *in vivo* for RANKL signalling, a process mimicked *in vitro* with soluble recombinant RANKL. An osteoclast precursor in contact with a RANKL presenting cell will receive the RANKL signal and initiate a cascade of gene expression that includes the production of MCP-1, RANTES, MIP1α and possibly other chemokines. MCP-1, RANTES and MIP1α are chemotactic signals for monocytes, resulting in migration to the source of production of the chemokine. The data show that any of the three chemokines examined, can cause cell fusion in monocytes treated with M-CSF. It is proposed that cell fusion is a key event in the next step of osteoclast differentiation (Fig. 6.1), where monocyte-like cells, that have not yet seen RANKL, are attracted by chemokines to the site of coupling of the RANK positive precursor and the RANKL presenting cell. Chemokine mediated fusion increases the size of the osteoclast, and also transfers the RANKL signal to the additional nuclei that are now in the multinucleated cell. In the absence of RANKL, fusion can occur, but bone

resorption activity depends on RANKL. If the chemokine signal is strong enough, such monocyte cell fusion could occur prior to contact with the RANKL influenced osteoclast precursor. Such TRAP positive "pre-osteoclasts" would still require the RANKL signal to develop into an authentic osteoclast capable of bone resorption, since they have a deficit in genes required for bone resorption (i.e. CTSK, MMP9, integrin αV).

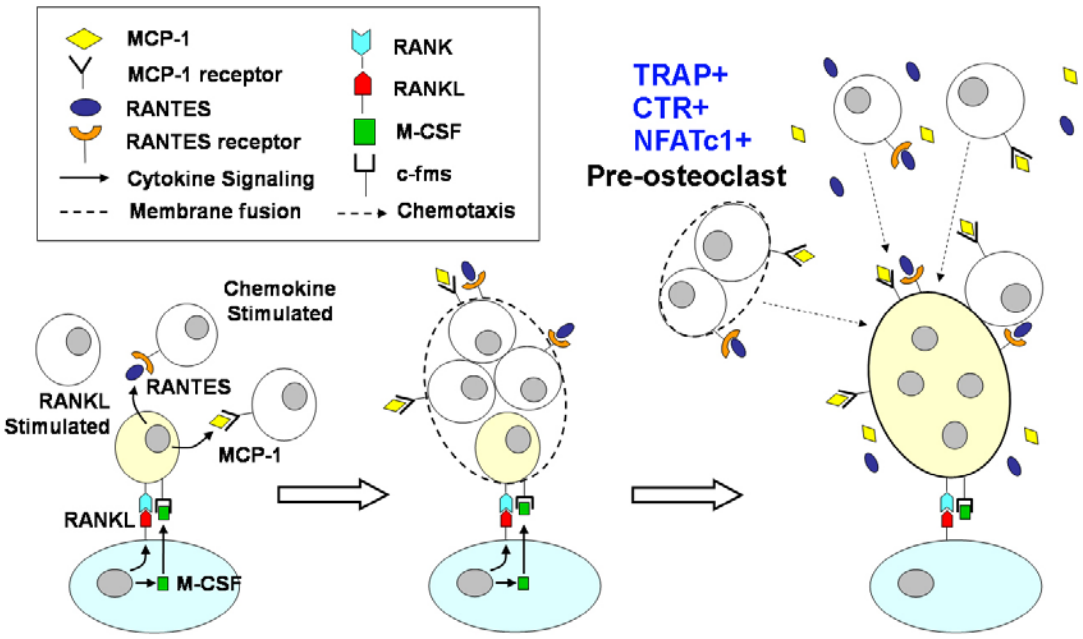

Fig. 6.1 A model for the role of chemokines MCP-1 and RANTES in cell differentiation from monocyte-like precursors to osteoclasts. In the initial stage (left side of the figure) a stromal cell (pale blue) presents membrane bound RANKL to a RANK+ monocyte (yellow). Once stimulated by RANKL, a signal cascade ensues, leading to increased expression of chemokines MCP-1 and RANTES. These chemokines act as chemical signals, attracting other monocytes to the site RANKL expression. Chemokines promote fusion of monocytes with the RANKL stimulated cell, illustrated in the intermediate stage, resulting in transference of the RANKL signal to the contiguous cytoplasm. Further chemokine production leads to the final stage, where sufficient chemokines are produced to stimulate an event "prefusion", that is, the fusion of monocyte precursors into multinucleate giant cells prior to fusion with the cell receiving the RANKL signal. Although multinuclear cells derived from prefusion may be TRAP positive, they are not competent for bone resorption, possibly due to a deficit in genes required for bone resorption and different redox status. This model explains a role for chemokines in RANKL signalling leading to osteoclast differentiation and also suggests that pathology associated with high levels of MCP-1 will result in both multinucleate giant cells and increased osteoclast differentiation.

To understand the signalling pathway involved in osteoclast differentiation and cellular fusion, p38 MAPK and MEK1/2 inhibitors (SB203580 and U0126, respectively) were utilised. SB203580 mildly inhibited RANKL mediated osteoclast formation, resulting in smaller TRAP positive multinuclear cells, compared to authentic osteoclasts. Interestingly, SB203580 had no effect on MCP-1 mediated TRAP positive multinuclear cells. Furthermore, exogenous MCP-1 overcame SB203580 inhibition of RANKL mediated osteoclast formation, suggesting that MCP-1 action is not dependent on the p38 MAPK pathway. U0126 abolished the both MCP-1 and RANKL mediated TRAP positive multinuclear cell formation, resulting in mononuclear cells that failed to fuse into multinuclear cells. Furthermore, NFATc1 mRNA expression was suppressed in U0126 treated cells compared to authentic osteoclasts. Sanna, B., et al., (2005) have shown that, in cardiomyocytes, calcineurin-dependent NFAT is linked to MEK-ERK1/2 signalling pathways (Sanna, B., et al., 2005). Furthermore, induction of AP-1 enhanced NFATc1 gene expression through MEK-ERK1/2 signalling (Sanna, B., et al., 2005, Fig. 6.2), consistent with the current data of inhibition of NFATc1 expression by U0126. Thus, it can be hypothesised that the process of multinucleation requires MEK dependent NFATc1 regulation (Fig. 6.2)

The data suggests that MCP-1 at high enough levels leads to cell fusion to form pre-osteoclasts, being NFATc1 positive, TRAP positive, CTR positive and multinuclear but lacking the capacity to degrade bone, possibly due to a lack of integrin αV, MMP9, cathepsin K and the F-actin ring. The MCP-1 induced osteoclast-like cells are able to differentiate into authentic osteoclasts when presented

with RANKL, suggesting that the MCP-1 induced TRAP positive, CTR positive, multinuclear cell is indeed a valid intermediate on the pathway to human osteoclast differentiation (Fig. 6.1). Calcitonin inhibited both bone resorption in osteoclasts and MCP-1 mediated cell fusion, suggesting that calcitonin can repress early cell fusion events as well as mature osteoclast function. Therefore, important calcitonin regulatory check-points exist at intermediate stage of osteoclast differentiation interacting with chemokines signalling.

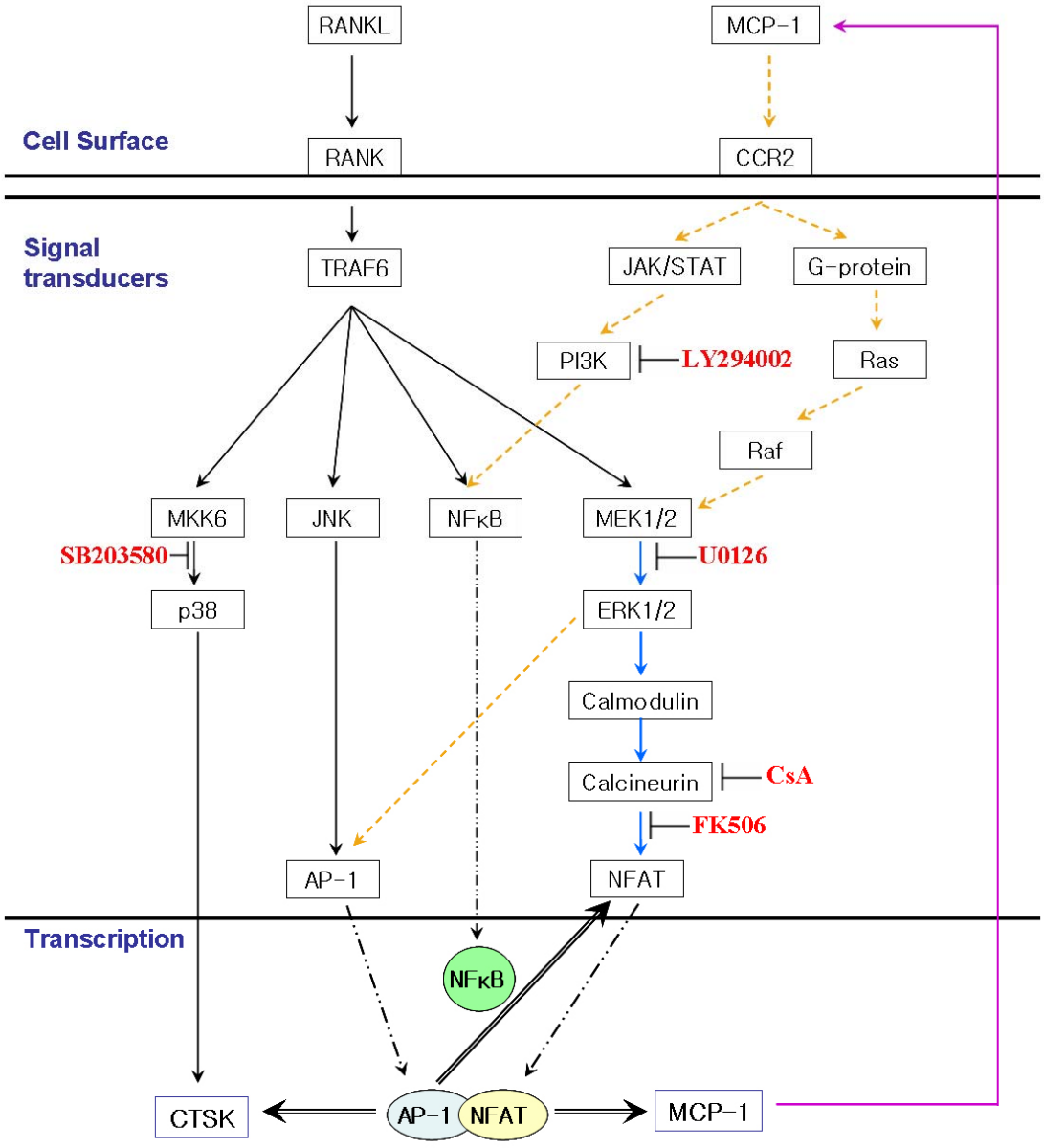

Fig. 6.2 RANKL and MCP-1 signalling network in osteoclast formation. RANK-RANKL interaction causes activation of TRAF6, which inturn induces signal transduction of different pathways. MKK6 activates, p38 MAPK, JNK is required for c-jun activation, MEK1/2 are required for activation of c-fos. c-jun and c-fos heterodimerise to form AP-1. Activation of MEK1/2 results in gene expression of NFAT. AP-1 and NFAT co-activates genes required for osteoclast formation, including cathepsin K and MCP-1. Expression of MCP-1 leads to secretion of MCP-1 to regulate its own cognate receptors, acting in autocrine manner. Binding of MCP-1 to its cognate receptor activates similar signal cascade of MEK (through Ras and Raf signalling) resulting in AP-1 and NFAT expression. Black arrows indicate RANKL signalling. Orange dotted arrows indicate MCP-1 signalling. Blue arrows indicate signal transduction through both RANKL and MCP-1 signals. Double lined arrows indicate gene expression. Purple arrow indicates autocrine effect of MCP-1. Black bars indicate target of known small molecule inhibitors.

In conclusion, MCP-1 is induced by RANKL during osteoclast differentiation, is repressed by GM-CSF. Supplementation of MCP-1 is able to overcome GM-CSF repression of osteoclast differentiation. Moreover, an osteoclast precursor that is exposed to RANKL produces MCP-1 and other chemokines that stimulate osteoclast differentiation through an autocrine loop and recruit other monocytes to the site of cell fusion, possibly through MEK1/2 pathway, rather than p38 MAPK that is required for bone resorption activity.

The present study only encompasses the *in vitro* analysis of MCP-1 on human osteoclasts. Thus, future direction is to obtain and analyse the *in vivo* effects of MCP-1 using MCP-1 and CCR2 knockout mice as well as creating and analysing MCP-1, CCR2 double knockout mice. Currently, there are no publications on the bone phenotype of MCP-1 knockout mice. In 2002, Graves, D.T., *et al.,* analysed the monocyte recruitment and bone resorption in MCP-1 deficient mice (Graves, D.T., *et al.,* 2002), but the authors had only analysed the osteoclasts in tooth eruption, which does not represent true nature of bone phenotypic effects. Furthermore, analysis of

polymorphism in MCP-1 and CCR2 gene would also provide new insight into the effects of chemokines in osteoporosis and other bone diseases.

Chapter 7

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Appendix

Publications from PhD.

Publications Accepted (published articles attached)

1. Day, C.J., Kim, M.S., Stephens, S.R., Simcock, W.E., Aitken, C.J., Nicholson, G.C., Morrison, N.A. (2004) Gene array identification of osteoclast genes: differential inhibition of osteoclastogenesis by cyclosporin A and granulocyte macrophage colony stimulating factor. *J Cell Biochem.* 91: 303-315.

2. Granfar, R.M., Day, C.J., Kim, M.S., Morrison, N.A. (2005) Optimised real-time quantitative PCR assays for RANKL regulated genes. *Mol Cell Probes.* 19: 119-126.

3. Kim, M.S., Day, C.J., Morrison, N.A. (2005) MCP-1 is induced by receptor activator of nuclear factor-{kappa}B ligand, promotes human osteoclast fusion, and rescues granulocyte macrophage colony-stimulating factor suppression of osteoclast formation. *J Biol Chem.* 280: 16163-16169.

4. Day, C.J., Kim, M.S., Lopez, C.M., Nicholson, G.C., Morrison, N.A. (2005) NFAT expression in human osteoclasts. *J Cell Biochem.* 95: 17-23.

5. Kim, M.S., Magno, C.L., Day, C.J., Morrison, N.A. (2006) Induction of chemokines and chemokine receptors CCR2b and CCR4 in authentic human osteoclasts differentiated with RANKL and osteoclast like cells differentiated by MCP-1 and RANTES. *J Cell Biochem.* 97: 512-518.

6. Kim, M.S., Day, C.J., Selinger, C.I., Magno, C.L., Stephens, S.R., Morrison, N.A. (2006) MCP-1-induced human osteoclast-like cells are tartrate-resistant acid phosphatase, NFATc1, and calcitonin receptor-positive but require receptor activator of NFkappaB ligand for bone resorption. *J Biol Chem.* 281: 1274-1285.