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**Osteotropic Cytokines:  
Expression in Human Gingival Fibroblasts and Effects on Bone**

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Cover Picture: Primary human gingival fibroblasts visualized by light microscopy.

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## ABSTRACT

### **Osteotropic Cytokines: Expression in Human Gingival Fibroblasts and Effects on Bone.**

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Bone metabolism is regulated by endocrine and paracrine signalling molecules influencing bone cells in the continuously remodelling bone tissue. These molecules include a variety of osteotropic stimulatory and inhibitory cytokines. Degradation of alveolar bone in periodontal disease is believed to be a result of local release of such osteotropic cytokines, although the relative importance of particular cytokines and their cellular origin is currently unknown.

The aim of the present project was to study if, and how, pro-inflammatory cytokines in the interleukin-6 (IL-6) family of cytokines, and anti-inflammatory IL-4 and IL-13 type of cytokines, can affect osteoclast differentiation and bone resorption. Additionally, the objective was to study if gingival fibroblasts may influence alveolar bone resorption through secretion of IL-6 type cytokine release and if the secretion is regulated by pro-inflammatory as well as anti-inflammatory mediators such as IL-4 and IL-13.

IL-6 in combination with its soluble receptor (sIL-6R) was found to stimulate mouse calvarial bone resorption. Similarly, two other IL-6 family members, leukemia inhibitory factor (LIF) and oncostatin M (OSM) were found to stimulate bone resorption. The stimulatory effect on bone resorption induced by the three cytokines was associated with increased expression of receptor activator of NF- $\kappa$ B ligand (RANKL), a cytokine which is essential in osteoclast formation and activation through binding to receptor activator of NF- $\kappa$ B (RANK) on osteoclastic cells. The interaction between RANKL and RANK can be inhibited by binding of the decoy receptor osteoprotegerin (OPG) to RANKL, and the expression of OPG was also regulated by IL-6, LIF and OSM (Paper I). The two related cytokines IL-4 and IL-13 were found to inhibit osteoclastogenesis and mouse calvarial bone resorption by mechanisms involving a decreased RANKL/OPG ratio in osteoblasts and decreased RANK expression in osteoclastic cells. The results further demonstrated that IL-4 and IL-13 exert their effects on both osteoblasts and osteoclasts by a mechanism involving the transcription factor signal transducer and activator of transcription 6 (STAT6) (Paper II). Constitutional expression of IL-6, LIF and another member of the IL-6 family of cytokines, IL-11, was demonstrated in human gingival fibroblasts. IL-6 type cytokine expression levels were found to be enhanced by IL-1 $\beta$  and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Paper III), whereas IL-4 and IL-13 inhibited IL-11 and LIF release from gingival fibroblasts (Paper IV).

In conclusion, IL-6 type cytokines were found to be stimulators and IL-4 and IL-13 inhibitors of bone resorption *in vitro* via mechanisms involving RANK/RANKL/OPG interactions. Additionally, gingival fibroblasts were able to secrete several cytokines in the IL-6 family. Secretion was further enhanced by pro-inflammatory mediators and inhibited by IL-4 and IL-13. These findings support the view that resident cells may influence the pathogenesis of periodontal disease through osteotropic cytokine production.

*Key words: osteoblasts, osteoclasts, fibroblasts, cytokines, IL-6, LIF, OSM, IL-4, IL-13, bone resorption*

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## ABBREVIATIONS

$\alpha(1)$ - coll. I	$\alpha(1)$ - collagen I
ALP	alkaline phosphatase
$\alpha$ -MEM	$\alpha$ -modification of Minimum Essential Medium
BCA	bicinchoninic acid
BSA	bovine serum albumin
BMM	bone marrow macrophage
BMP-2	bone morphogenetic protein-2
BSP	bone sialoprotein
cDNA	complementary deoxyribonucleic acid
CNTF	ciliary neurotrophic factor
CT-1	cardiotrophin-1
CTR	calcitonin receptor
D3	1,25(OH) <sub>2</sub> vitamin D <sub>3</sub>
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated protein kinase
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
I $\kappa$ B	inhibitor of NF- $\kappa$ B
IKK	I $\kappa$ B kinase
IL	interleukin
IL-1R1	interleukin-1 receptor 1
IL-1R2	interleukin-1 receptor 2
IL-1 $\alpha$	interleukin-1 $\alpha$
IL-1 $\beta$	interleukin-1 $\beta$
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LIF	leukemia inhibitory factor
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NFAT	nuclear factor of activated T-cells
NF- $\kappa$ B	nuclear factor $\kappa$ B
NNT-1/BSF-3	novel neutrophin-1/B-cell stimulatory factor-3
OC	osteocalcin
OPG	osteoprotegerin
OSM	oncostatin M
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGE <sub>2</sub>	prostaglandin E <sub>2</sub>
PTH	parathyroid hormone
RANK	receptor activator of nuclear factor $\kappa$ B
RANKL	RANK ligand
RIA	radioimmunoassay
RPL13A	ribosomal protein L13A
sIL-6R	soluble IL-6 receptor
STAT	signal transducer and activator of transcription
TGF- $\beta$	transforming growth factor- $\beta$
TNF	tumor necrosis factor
TNFR I	tumor necrosis factor receptor I
TNFR II	tumor necrosis factor receptor II
TRAF	TNF receptor-associated factor
TRAP	tartrate-resistant acid phosphatase

## PREFACE

The thesis is based on the following papers, which will be referred to by their Roman numerals:

- I. Palmqvist P\*, Persson E\*, Conaway HH and Lerner UH.  
Interleukin-6, Leukemia Inhibitory Factor and Oncostatin M Stimulate Bone Resorption and Regulate the Expression of Receptor Activator of NF-kappa B Ligand, Osteoprotegerin and Receptor Activator of NF-kappa B in Mouse Calvariae.  
*J Immunol* 2002, 169: 3353-3362 \* contributed equally to the study
- II. Palmqvist P, Lundberg P, Persson E, Johansson A, Lundgren I, Lie A, Conaway HH and Lerner UH.  
Inhibition of Hormone and Cytokine Stimulated Osteoclastogenesis and Bone Resorption by Interleukin-4 and Interleukin-13 is Associated with Increased OPG and Decreased RANKL and RANK in a STAT6 Dependent Pathway.  
*J Biol Chem* 2006, 281: 2414-2429
- III. Palmqvist P, Lundberg P, Hånström L, Lundgren I and Lerner UH.  
IL-1 $\beta$  and TNF- $\alpha$  Regulate IL-6 Type Cytokines in Gingival Fibroblasts.  
*Submitted.*
- IV. Palmqvist P, Lundberg P, Lundgren I, Hånström L, Conaway HH and Lerner UH.  
Regulation of IL-6 Type Cytokines in Human Gingival Fibroblasts by Interleukin-4 and Interleukin-13.  
*Manuscript.*

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# INTRODUCTION

## Periodontium

### *General introduction*

The supportive tissues surrounding the teeth are collectively termed the periodontium and comprise the root cementum, the periodontal ligament, the gingival tissue and the alveolar bone. Together, the components of the periodontium provide attachment and stability to the teeth as well as mobility and mechanical force adaptation. Moreover, it offers nutrition and regenerative capacities. The anatomical vicinity of the major components of the periodontium, the connective tissue and the alveolar bone, makes cellular and molecular cross-talk possible. The objective of this thesis was to further explore the putative interactions between molecules expressed by master cells of the gingival connective tissue, the fibroblasts, and inflammatory cells and bone.

### *Gingival connective tissue*

The gingival tissue is composed of a network of fibrous connective tissue covered by gingival epithelium. The underlining connective tissue occupies the area between the tooth, the alveolar bone and the basal membrane and attaches to the tooth and the alveolar bone through fibrous attachments, thus providing tooth attachment to bone as well as epithelial support. The connective tissue offers tissue architecture and maintenance of the extracellular matrix (ECM). Mast cells, macrophages and occasional lymphocytes are present in healthy gingival connective tissue, although fibroblasts are the most abundant cells, making up 10% of the tissue volume.

### *Gingival fibroblasts*

Fibroblasts are stellate or spindle-shaped cells with an oval-shaped nucleus whose main function is remodelling of the ECM, which is undertaken through production and degradation of ECM components. Fibroblasts, similar to cementoblasts and osteoblasts, produce ECM, including fibers such as collagen, reticulin, oxytalan and elastic fibers, of which collagen is predominant in gingival tissue. ECM also contains proteoglycans, including glycosaminoglycans, and glycoproteins. Glycosaminoglycans are large and negatively charged molecules which serve as a filter, thus, smaller molecules such as water and electrolytes are incorporated into the ECM whereas larger molecules are prevented from entering the tissue. Other components of ECM are minerals, lipids, water and tissue-bound growth factors (Bartold *et al.*, 2000). Maintenance requires degradation of ECM components, and fibroblasts also produce proteolytic enzymes, a group of zinc- and calcium-dependent endopeptidases, so called matrix metalloproteases (MMPs) and their controlling inhibitors, tissue inhibitors of matrix metalloproteases (TIMPs) (Birkedal-Hansen, 1993). The fibroblasts also rid the tissue from degraded collagen through phagocytosis (Deporter and ten Cate, 1980; Everts *et al.*, 1996). Fibroblasts secrete and are responsive to growth factors and inflammatory mediators such as cytokines, thus interacting with other cells such as leukocytes. Bacterial components and products also evoke responses in fibroblasts, reflecting the meshwork of reactions needed to maintain normal tissue homeostasis

and offer protection of host cells to infectious or injurious agents. The tissue remains healthy as long as formation and degradation of connective tissue are in balance (Lindhe *et al.*, 2003).

### *Alveolar bone*

The alveolar bone refers to the maxillar and mandibular bone parts surrounding and supporting the teeth. The remodelling rate of the alveolar bone is high due to the adaptive demands made by e.g. tooth eruption and mastication (Sodek and McKee, 2000). The alveolar bone is structurally comparable to other bone tissues in the body, which will be described below.

## **Bone tissue**

### *Function*

The skeleton is a large organ composed of bone tissue and cartilage. The strong and rigid skeleton supports the soft parts of the body making an upright posture possible. Skeletal bones also provide sites for muscle attachment, thus enabling movement. Moreover, the skeleton protects inner organs, encapsules bone marrow and serves as a mineral supply which is essential for maintenance of the mineral homeostasis in the body.

### *Composition*

Bone tissue is a mineralized form of connective tissue in which mineral salts are incorporated into an organic extracellular matrix mainly made up of a network of collagen type I fibers. Approximately 25% of the bone tissue is made up of organic matrix, of which 90-95% is collagen type I. The collagen type I fibers provide architecture, strength and elasticity to the bone tissue, as it does in other types of connective tissues in the body. The remaining five percent of the organic matrix contains proteoglycans (e.g. decorin, osteoadherin and biglycan), glycoproteins (e.g. osteopontin, osteonectin, vitronectin and bone sialoprotein), GLA-containing proteins, such as osteocalcin, as well as a vast number of non-collagenous proteins (e.g. insulin growth factor I and II, transforming growth factor- $\beta$  and bone morphogenetic proteins) and bone cells. Five percent of the bone tissue is water and the major part, 70%, is inorganic and mainly composed of calcium and phosphate in the form of hydroxyapatite crystals  $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ . These are dispersed along the collagen fibers and contribute to the compressive strength of bone as well as serving as a mineral supply. All non-resorbing bone surfaces are covered by a layer of unmineralized bone called osteoid and bone tissue is nutriated via blood vessels in Haversian and Volkmanns channels and via canaliculi (Buckwalter *et al.*, 1996; Robey *et al.*, 1993; Weiner and Traub, 1992).

### *Structure*

Differences in morphology divide bone into two distinct forms, i.e. the cortical and the trabecular bone. Although the composition is similar, the two types of bone differ in both structure and function. Cortical bone has a protective (bone marrow) and mechanical role whereas the role of trabecular bone is mainly metabolic.

Cortical bone makes up 80% of the skeleton and is formed by dense apposition of layers forming lamellae, which contributes to the organized and compact structure of this bone type. The cortical bone forms the outer bone layer which surrounds the bones in the body. Its high density offers high resistance to compressive strength whereas, in contrast, trabecular bone forms a network of bone trabeculae surrounded by bone marrow. This structure offers a greater surface area per unit of bone (Buckwalter *et al.*, 1996) and bone remodelling rate and metabolic activity is greater in trabecular than in cortical bone, since bone remodelling is a surface dependent mechanism. Outer surfaces of the cortical bone are covered by the periosteum, separating the bone from the surrounding tissues. The periosteum is made up of two layers of which the outer collagen-rich layer contains fibroblasts, nerves and blood vessels and the inner cellular layer includes bone cells, fibroblasts and nerve cells (Allen *et al.*, 2004). The inner surfaces of the cortical bone as well as the trabecular bone surfaces are covered by the endosteum, which separates the bone from the bone marrow.

### *Bone cells*

Similar to other tissues in the body, bone tissue is maintained through continuous formation and degradation. Regulators of these processes are the bone forming osteoblasts, the bone lining cells and the bone resorbing osteoclasts, all of which are present on bone surfaces, and the osteocytes, which are located in the bone matrix.

### *Osteoblasts*

Active osteoblasts are the cuboidal, polarized bone forming cells which are aligned on bone surfaces undergoing bone formation. Similar to fibroblasts, myoblasts, chondrocytes and adipocytes, these cells originate from mesenchymal stem cells located in the bone marrow, endosteum or periosteum. During differentiation of multipotent mesenchymal cells into different lineages, the progenitors of the different lineages acquire specific phenotypes under the control of regulatory factors of the restricted lineages. Two transcription factors have been identified as essential in osteoblast differentiation, the runt-related transcription factor 2 (Runx2) and Osterix (Osx). Although necessary, Runx2 is not solely sufficient for obl differentiation. Osx is a downstream target for Runx2 and is also essential for osteoblast differentiation (Nakashima *et al.*, 2002). The expression of Runx2 is regulated by many factors, including bone morphogenetic proteins and wnt signalling (Ryoo *et al.*, 2006; Canalis *et al.*, 2005; Ducy *et al.*, 2000; Leboy, 2006; Westendorf *et al.*, 2004). Mesenchymal cells can differentiate into either osteoblasts, as is the case in intramembranous ossification, or into chondrocytes, as is the case in the indirect endochondral ossification, both resulting in formation of bone. In similarity to fibroblasts, the osteoblasts produce extracellular matrix. However, the two different cell types differ in the way that matrix secretion is undertaken. Whereas the fibroblastic secretion is pericellular, the osteoblastic secretion is usually polarized towards the bone surface.

Initially, the organic extracellular matrix of collagen type I and various noncollagenous proteins, called the osteoid, is secreted by the osteoblasts, and in a second event of action the osteoblasts mineralize the matrix (Aubin, 2001; Bilezikian *et al.*, 2002). Osteoblasts are also responsible for degradation of the osteoid, and many of the agents known to regulate osteoclast formation and activity act via osteoblasts, thus regulating the osteoclasts. Consequently, receptors for the calcium-regulating hormones parathyroid hormone (PTH) and 1,25 (OH)<sub>2</sub> vitamin D<sub>3</sub> (D3) as well as receptors for various osteotropic cytokines are expressed by osteoblasts. The expression of a number of bone-related extracellular matrix proteins, high activity of the mineralization-associated enzyme alkaline phosphatase as well as responses to osteotropic hormones and cytokines are regarded as characteristics of osteoblasts (Katagiri and Takahashi, 2002; Mundlos and Olsen, 1997; Rodan and Martin, 1981).

Bone lining cells are inactive, flat and elongated osteoblastic cells covering bone surfaces which are not being remodelled. Little is known about the function of these cells, but they have been suggested to be precursors for osteoblasts and are the cells which have been proposed to be responsible for guidance of osteoclast precursor cells to the bone surface (Bilezikian *et al.*, 2002).

### *Osteocytes*

The osteocytes are the most abundant bone cells, representing 90% of the cells in bone. In contrast to osteoblasts, bone lining cells and osteoclasts, which are located on bone surfaces, osteocytes are located in the bone matrix. Osteocytes are osteoblasts which have become imbedded in the bone matrix during bone formation. As mentioned previously, osteoblastic matrix secretion is normally polarized, but pericellular secretion sometimes occurs. The latter event encapsules the matrix secreting osteoblasts, which become osteocytes eventually making up the next osteocyte layer. Osteocytes establish cytoplasmic connections to other cells before the matrix is mineralized. Osteocytes are small, stellate and terminally differentiated osteoblasts (Franz-Odenaal *et al.*, 2006) whose cell bodies reside in lacunae and whose cytoplasmic processes extend through canaliculi in the bone matrix towards other osteocytes, osteoblasts at the cell surface as well as blood vessels. The cytoplasmic processes enable communication with other cells through gap junctions, and as diffusion through the bone matrix is limited the canaliculi provide the osteocytes with nutrients. The functions of the osteocytes remain partly unclear but they have been suggested to serve as mechanosensors responding to mechanical stimuli, such as load, thus regulating bone metabolism together with osteoblasts and osteoclasts (Buckwalter *et al.*, 1996; Ducey *et al.*, 2000; Klein-Nulend *et al.*, 2003; Knothe Tate *et al.*, 2004).

### *Osteoclasts*

Osteoclasts, like leukocytes, arise from hematopoietic stem cells of the monocyte/macrophage lineage, and sequential expression of different sets of genes at different stages of differentiation determines lineage commitment. The RANK-RANKL-OPG pathway plays a crucial role in osteoclast differentiation and the process is also influenced by several cytokines and growth factors. In hematopoietic tissues, such as the bone marrow, osteoclast progenitor cells proliferate and differentiate into

mononuclear osteoclast precursor cells. Aspects of time and place as well as regulating mechanisms for fusion of mononuclear preosteoclasts into multinuclear osteoclasts are not known. Similarly, the actions regulating late stages of differentiation are not fully understood. In the bone marrow, however, fusion takes place in the vicinity of bone surfaces of resorptive sites. Osteoclast precursors are recruited to peripheral bone surfaces of forthcoming resorption via the circulation and are guided by a so called homing process, of which the mechanisms are currently unknown. The identity of the cell type responsible for osteoclast precursor recruitment is not known. At the bone surfaces of the marrow cavities, fusion of mononuclear preosteoclasts leads to formation of multinucleated osteoclasts. Active osteoclasts are giant, multinucleated cells located on bone surfaces, and it is generally accepted that the osteoclasts are the only cells capable of resorption of mineralized bone, dentin, cementum and mineralized cartilage. Osteoclasts share many similarities with macrophages, but unlike the intracellular mechanism of phagocytosis utilized by macrophages, the osteoclasts degrade tissue components extracellularly. Multinucleation, tartrate resistant acid phosphatase (TRAP)-activity, calcitonin receptor (CTR) and cathepsin K expression as well as bone resorbing activity are considered as characteristics for osteoclasts (Boyle *et al.*, 2003; Lerner, 2000). Further details of osteoclastogenesis are discussed below.

### *Bone metabolism*

Embryonic development of the skeleton is followed by two postnatal decades of modelling, where bone formation exceeds bone resorption, i.e. the two processes are uncoupled. During this period the bones reach their final length and shape and peak bone mass is reached. The first two decades are followed by three decades where formation equals resorption, i.e. the processes are coupled, and localized resorption is followed by equal deposition of bone at the same site. Hence, bone is remodelled and through this process maintained and renewed. Anabolic and catabolic processes in the skeleton are coupled in order to maintain homeostasis. If the equilibrium is altered, as is the case in diseases such as osteoporosis, osteopetrosis, rheumatoid arthritis or periodontitis, general or local loss of bone or altered bone composition may be the result.

### *The remodelling cycle*

Continuous formation and resorption of bone is a prerequisite for normal skeletal development, growth and maintenance, and bone is remodelled in so-called bone multi-cellular units (BMU). These units are temporary structures of osteoblasts and osteoclasts, whose role it is to maintain the skeleton through degradation of old bone and formation of new bone. The number and activity of these cell units are regulated by systemic hormones such as PTH and D3 as well as growth hormone, sex hormones, steroids and local factors such as growth factors, cytokines and prostaglandins. Mechanical load also regulates the BMUs. (Bilezikian *et al.*, 2002; Harada and Rodan, 2003).

Osteoblastic cells are covering all bone surfaces in the body, and when bone remodelling is not taking place these cells remain in a quiescent state. Hence,

activation of quiescent osteoblasts initiates the remodelling cycle, and this event is regulated by a vast number of factors such as systemic hormones and local agents. By the use of proteolytic enzymes, the osteoblasts degrade the osteoid (Vaes, 1988) which in turn exposes the bone surface to the bone resorbing osteoclasts. Osteoclast progenitor cells are recruited to the resorptive site by a homing process, a mechanism of which the regulation is still unknown. In the field of osteoclastogenesis, however, much progress in the understanding of regulating mechanisms has been made in the past decades. Initially, stromal cells were shown to be essentially required for induction of osteoclastogenesis in hematopoietic cell cultures (Takahashi *et al.*, 1988) and the same group later revealed that the reason for their previous findings was the necessity of cell-to-cell contact between stromal cells and osteoclastic cells for osteoclast differentiation (Udagawa *et al.*, 1989). The ground for this observation was later demonstrated to be two proteins produced by osteoblasts/stromal cells. The proteins which were shown to be crucial factors for osteoclastogenesis were macrophage colony-stimulating factor (M-CSF) (Felix *et al.*, 1994; Tanaka *et al.*, 1993) and receptor activator of nuclear factor  $\kappa$ B ligand (RANKL) (Kong *et al.*, 1999; Lacey *et al.*, 1998). M-CSF is secreted from osteoblasts/stromal cells and binds to its receptor c-Fms on osteoclast progenitor cells, thus stimulating proliferation and survival of the osteoclast progenitor cells as well as contributing to their differentiation (Teitelbaum, 2000; Teitelbaum and Ross, 2003). The second protein discovered, RANKL, is expressed on the surface of osteoblasts/stromal cells and binds to its cognate receptor termed receptor activator of nuclear factor  $\kappa$ B (RANK) (Hsu *et al.*, 1999) on osteoclast precursors and multinuclear osteoclasts. RANKL binding to RANK stimulates differentiation of mononuclear osteoclast progenitor cells, fusion of preosteoclasts and activation of multinucleated osteoclasts, resulting in bone-resorbing mature osteoclasts. The interaction between RANKL and RANK can be blocked by the soluble glycoprotein osteoprotegerin (OPG), which is secreted by osteoblasts/stromal cells (Simonet *et al.*, 1997; Yasuda *et al.*, 1998a). OPG is a decoy receptor acting as an inhibitor of osteoclastogenesis and bone resorption. *In vivo* data in mice have demonstrated the importance of these factors since *rankl*<sup>-/-</sup> and *rank*<sup>-/-</sup> mice lack osteoclasts and exhibit an osteopetrotic phenotype, whereas *opg*<sup>-/-</sup> mice show increased numbers of osteoclasts resulting in osteoporosis (Lerner, 2000; Suda *et al.*, 1999; Boyle *et al.*, 2003; Hofbauer *et al.*, 2000; Teitelbaum, 2000).

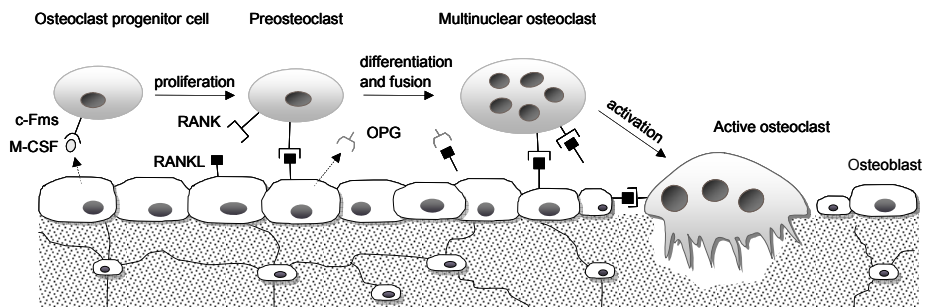


Fig. 1. Osteoclast proliferation, differentiation, fusion and activation.

In addition, activation of RANK has been shown to induce osteoclastogenesis in cooperation with immunoreceptor tyrosine-based activation motif (ITAM) signalling, and the co-stimulatory immunoglobulin-like receptors/Fc receptor common  $\gamma$  subunit (FcR $\gamma$ )/DNAX-activating protein 12 (DAP12) pathway has been shown to be essential in osteoclastogenesis (Asagiri and Takayanagi, 2006; Koga *et al.*, 2004).

Moreover, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) has been suggested to stimulate osteoclastogenesis by mechanisms independent of the RANK/RANKL interaction.

However, recent reports suggest that the effect of TNF- $\alpha$  is partly dependent on permissive RANKL levels to induce osteoclastogenesis (Lam *et al.*, 2000).

RANKL is not only expressed by osteoblasts/stromal cells, but also by T-lymphocytes (Takayanagi *et al.*, 2000b), synovial fibroblasts (Takayanagi *et al.*, 2000a) and, interestingly, periodontal ligament (PDL) cells (Belibasakis *et al.*, 2005a). RANKL expression by T-lymphocytes and synovial fibroblasts has been demonstrated to stimulate osteoclastogenesis and has been suggested to influence bone turnover in inflammation induced bone resorption (Takayanagi *et al.*, 2000a; Takayanagi *et al.*, 2000b; Teng *et al.*, 2000).

How resorption sites are determined is currently unknown, but the first sign is retraction of the bone-lining cells which exposes the osteoid, allowing for degradation of osteoid by adjacent osteoblasts. These actions uncover the mineralized bone surface to the osteoclasts, making it possible for these cells to attach. Resorbing osteoclasts express adhesion receptors on their cell membranes, so called integrins, including the  $\alpha_v\beta_3$  integrin (vitronectin receptor). These adhesion receptors bind to several different extracellular matrix proteins in bone which serve as adhesion proteins, including vitronectin, collagen, osteopontin and bone sialoprotein, which offers attachment to bone (Nesbitt *et al.*, 1993). The active osteoclast is polarized through reorganization of cytoskeletal components such as microtubules and actin filaments as well as lysosomal vesicles, and the polarized cell exhibits different plasma membrane domains, i.e. the sealing zone, the ruffled border and the basolateral domain, including the functional secretory domain. In the periphery of the osteoclast cell membrane, the firm attachment to bone creates a tight sealing zone which functions as a barrier, isolating the resorption pit area from the surrounding tissues. Osteoclast attachment initiates signalling, which results in transport of vesicles containing vacuolar type electrogenic proton pumps, H<sup>+</sup> ATPases, to the resorption site, referred to as Howship's lacuna. Fusion of the proton pump containing acidic intracellular vesicles with the cell membrane facing the lacuna creates a second specialized membrane domain of the osteoclast, termed the ruffled border. This domain shares many common proteins with endosomal or lysosomal membranes, including the vacuolar proton pump (Zhao 2000). The enzyme carbonic anhydrase II transforms hydrogen carbonate into H atoms which are released by the H<sup>+</sup> ATPase proton pumps into the lacuna via exocytosis. To maintain electroneutral balance in the osteoclast, chloride anions are transported via chloride channels in the ruffled border into the lacuna. Hence, hydrochloric acid is formed in the lacuna resulting in a local decrease of pH to approximately 4.5. At this pH, hydroxyapatite crystals are dissolved. Furthermore, various proteolytic enzymes are released via exocytosis, including matrix metalloproteinases (MMPs) and cysteine proteinases such as cathepsin K, thus degrading the organic components of bone

including collagen fibers, extracellular matrix components and bone cells. Although several mechanisms have been suggested, including a role in degradation of bone matrix components, the function of the TRAP enzyme in osteoclasts is not completely understood. TRAP deficient mice, however, exhibit disrupted ossification and mild osteopetrosis whereas transgenic mice overexpressing TRAP show decreased trabecular density and mild osteoporosis (Angel *et al.*, 2000; Hayman *et al.*, 1996). The resorption lacuna is cleared from degraded components by the osteoclast which, through endocytosis, engulfs the components which are transported through the osteoclast in transcytotic vesicles and released at the opposite side of the cell in the functional secretory domain of the basolateral membrane. Osteoclasts undergo several resorption cycles during their life span and after a number of weeks, when the resorption process is terminated, the osteoclasts detach from the bone surface. Non-collagenous proteins, including osteoblastic growth factors such as insulin-like growth factor I (IGF-1), IGF II and cytokines of the transforming growth factor  $\beta$  superfamily such TGF- $\beta$  and BMPs, are produced by osteoblasts and incorporated in the bone matrix during bone formation. These factors are released in the resorptive process and are believed to activate osteoblasts in the vicinity of the resorbed area, thus recruiting osteoblasts to the resorption site where new bone formation is initiated. This process is called coupling. In contrast to bone resorption, bone formation takes several months (Bilezikian *et al.*, 2002; Lerner, 2000; Rousselle and Heymann, 2002; Teitelbaum, 2000; Vaananen *et al.*, 2000).

## **Regulators of osteoclastogenesis**

The network of factors required to regulate bone formation and bone resorption include systemical and local factors, and local regulation of bone cells by paracrine factors is performed by bone cells. However, other cell types such as leukocytes can also influence bone cells. Locally produced osteotropic agents include growth factors and prostaglandins, but also pro-inflammatory cytokines such as interleukin-1 (IL-1), TNF- $\alpha$  and IL-6 type cytokines as well as anti-inflammatory cytokines such as IL-4 and IL-13. Below, osteotropic agents of particular interest for this thesis will be introduced.

### *M-CSF*

Macrophage colony stimulating factor (M-CSF), or colony stimulating factor -1 (CSF-1), is a protein which is secreted by osteoblasts/stromal cells but also by several other cell types such as fibroblasts, endothelial cells, granulocytes and monocytes. M-CSF exists in several forms, both secreted and membrane bound, due to differential splicing (Stanley *et al.*, 1997), and it stimulates survival, proliferation and differentiation of monocytes and macrophages in addition to its crucial role for osteoclast progenitor survival and proliferation. Observations in the *op/op* mouse, exhibiting a natural genetic mutation causing defect M-CSF protein (Yoshida *et al.*, 1990), demonstrated the essential role of M-CSF in osteoclastogenesis, as the mice lacked osteoclasts and were osteopetrotic (Wiktor-Jedrzejczak *et al.*, 1990). M-CSF was later shown to play a role in both proliferation and differentiation of osteoclasts

(Felix *et al.*, 1994; Tanaka *et al.*, 1993). Recent studies have indicated that secreted forms of M-CSF are necessary for stimulation of osteoclastogenesis (Dai *et al.*, 2004).

In osteoclast progenitor cells, the interaction between M-CSF and its membrane bound receptor c-Fms on osteoclast progenitor cells induces intracellular signalling cascades including phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) cascades (Bourette and Rohrschneider, 2000) resulting in promotion of cell survival and proliferation. c-Fms has, similar to M-CSF, been demonstrated to be necessary for osteoclastogenesis, as c-Fms deficient mice develop osteopetrosis (Dai *et al.*, 2002).

### *RANK*

Receptor activator of NF- $\kappa$ B (RANK) is a transmembrane protein which is expressed by osteoclast precursors (Li *et al.*, 2000). RANK was detected on osteoclasts as the receptor for RANKL (Hsu *et al.*, 1999), and it is the sole signalling receptor for RANKL in stimulation of differentiation and activation of osteoclasts. RANK contains an N-terminal extracellular portion including 4 cysteine-rich domains, similar to OPG and other members of the TNF receptor superfamily. The extracellular ligand-binding portion is connected via a transmembrane domain to a cytoplasmic portion of the receptor which mediates signals through interaction with several signalling pathways. RANK signalling, similar to other TNF receptor family members, involves adaptor proteins, so called TNF receptor-associated factors (TRAFs). An important adaptor protein in RANK signalling is TRAF6, which binds to the cytoplasmic tail, closely to the membrane (Darnay and Aggarwal, 1999; Darnay *et al.*, 1999; Galibert *et al.*, 1998). Activation of TRAF6 mediates intracellular signalling by several pathways, including nuclear factor  $\kappa$ B (NF- $\kappa$ B), c-Src, kinases including p38, extracellular signal-regulated protein kinases (ERKs) and c-Jun-N-terminal kinase (JNK) (Lerner, 2004). Eventually, signalling by the various pathways leads to translocation of a number of transcription factors to the nucleus, among them NF- $\kappa$ B, activator protein-1 (AP-1) and nuclear factor of activated t-cells 2 (NFAT2). These transcription factors regulate transcription of genes which are necessary for osteoclastogenesis. Genetically modified mice have revealed the essential roles of both RANK and TRAF6 in osteoclastogenesis and bone resorption. RANK deficient mice demonstrated total absence of multinucleated osteoclasts and developed severe osteopetrosis (Dougall *et al.*, 1999; Li *et al.*, 2000) and similar to the *rank*<sup>-/-</sup> mice the *traf6*<sup>-/-</sup> mice developed osteopetrosis (Lomaga *et al.*, 1999; Naito *et al.*, 1999).

### *RANKL*

Receptor activator of NF- $\kappa$ B ligand (RANKL) is a protein which is a member of the TNF ligand superfamily. The protein is present in a transmembrane form, but also in a soluble form produced by proteolytic cleavage of the extracellular domain of RANKL by the enzyme metalloprotease-disintegrin TNF- $\alpha$  convertase (TACE) (Lum *et al.*, 1999). Both forms are metabolically active but the membrane bound form has been suggested to more efficiently induce osteoclastogenesis *in vitro* (Nakashima *et al.*, 2000). RANKL, like OPG, was simultaneously discovered by different groups. In studies of T cells, a TNF-related protein which bound to TNF receptors was found and

termed TNF-related activation-induced cytokine (TRANCE) (Wong *et al.*, 1997), while another group reported a protein which was found to stimulate T cell growth and named it RANKL (Anderson *et al.*, 1997). Yet two additional groups reported the discovery of a protein which stimulated osteoclast differentiation and named it osteoprotegerin ligand (OPGL) (Lacey *et al.*, 1998) and osteoclast differentiation factor (ODF) (Yasuda *et al.*, 1998b). RANKL is expressed by osteoblasts/stromal cells in bone but is also expressed by lymphocytes (Takayanagi *et al.*, 2000b), PDL cells (Belibasakis *et al.*, 2005a), synovial fibroblasts (Takayanagi *et al.*, 2000a) and several other cell types (Kartsogiannis *et al.*, 1999) and has an important role in the development and function of T cells, dendritic cells and osteoclasts. RANKL binds to the extracellular domain of RANK in form of a homotrimer, thus activating RANK signalling (Ito *et al.*, 2002; Lam *et al.*, 2001). Gene deletion studies in mice have shown the importance of RANKL in osteoclastogenesis and *rankl*<sup>-/-</sup> mice have been demonstrated to, similar to the *rank*<sup>-/-</sup> mice, be osteopetrotic due to absence of osteoclasts (Kong *et al.*, 1999). Mice overexpressing soluble RANKL, however, showed an osteoporotic phenotype with increased number of osteoclasts and decreased bone mineral density (Mizuno *et al.*, 2002). Additionally, many osteotropic factors (PTH, D3, PGE<sub>2</sub>) stimulate RANKL expression in mouse osteoblasts and OPG expression is simultaneously decreased by these factors (O'Brien *et al.*, 1999).

### OPG

Osteoprotegerin (OPG) is a soluble glycoprotein without transmembrane and cytoplasmic domains serving as a soluble receptor. Similar to RANK, it belongs to the TNF receptor superfamily and like the other members it has four cysteine-rich domains in the N-terminal which are essential for inhibition of osteoclast differentiation and function. OPG was observed as a protein which was released from human fibroblasts and inhibited bone resorption, and it was termed osteoclastogenesis inhibitory factor (OCIF) (Tsuda *et al.*, 1997), while at the same time another group reported a protein, termed OPG, with actions similar to those of OCIF (Simonet *et al.*, 1997). A third group showed a protein which inhibited osteoclast formation and was termed TNF receptor-like molecule (TR1) (Tan *et al.*, 1997). Later, OCIF, TR 1 and OPG were found to be the same protein (Kwon *et al.*, 1998; Yasuda *et al.*, 1998a). Moreover, a TNF-related receptor in lymphoid cells named follicular dendritic cell-derived receptor-1 (FDCR-1) was also found to be identical to OPG (Yun *et al.*, 1998). OPG is produced by osteoblasts/stromal cells as well as fibroblasts and a vast number of different cell types, and is thus widely expressed in the body (Lerner, 2004). Gene-modified mice have been used to map out the functions of OPG in bone tissue. *opg*<sup>-/-</sup> mice exhibit severe osteoporosis due to strongly enhanced osteoclast formation (Bucay *et al.*, 1998; Mizuno *et al.*, 1998) and overexpression of OPG in mice results in osteopetrotic animals with increased bone mineral density and a decreased number of osteoclasts (Simonet *et al.*, 1997), together showing that OPG produced by osteoblasts/stromal cells is an important suppressing regulator of osteoclast differentiation and activation.

## **Osteopathological conditions**

At physiological conditions, the processes of bone formation by osteoblasts and bone resorption by osteoclasts are in balance, i.e. the processes are coupled. In pathological conditions, however, these two processes are uncoupled, which normally means that bone resorption exceeds bone formation. This results in bone loss, as is the case in hyperparathyroidism or post-menopausal osteoporosis. Bone formation sometimes exceeds bone resorption, however, resulting in sclerosis. Inflammatory processes in bone or in the vicinity of bone tissue usually cause bone loss, as is the case in rheumatoid arthritis (RA), marginal periodontitis, apical periodontitis, osteomyelitis and most malignant tumors that metastasize to bone. However, inflammatory diseases or malignant tumours in the vicinity of bone tissue may also induce increased bone formation compared to resorption resulting in local sclerosis, which can be observed in apical periodontitis and adjacent to certain malignant tumour metastases in bone. Inflammatory conditions resulting in either loss of bone or gain of bone both display increased bone formation as well as bone resorption. It is the relative proportions of the two processes which determine the outcome. The central role of the osteoblast as a regulator of both bone formation and bone resorption may be a possible explanation for this situation.

### *Periodontal inflammation*

Microbial colonization of the dental surfaces in a few days leads to signs of inflammation of the gingival tissues, i.e. gingivitis. Microorganisms contain and release compounds (e.g. LPS, bacterial toxins) which induce signs of inflammation such as increased vascularisation and increased amounts of leukocytes, in particular neutrophils, which are recruited to the area for the purpose of antigen clearance and tissue healing. Pro-inflammatory cytokines, e.g. IL-1 and TNF- $\alpha$ , enhance the expression of adhesion molecules on endothelial cells (including intercellular adhesion molecule-1 (ICAM-1), endothelial leukocyte adhesion molecule-1 (ELAM-1) and P selectin) which promotes leukocyte migration through venule walls. Leukocytes migrate along a chemoattractant gradient towards the dental crevice. Numerous neutrophils migrate through the junctional epithelium and into the oral cavity but lymphocytes are retained in the connective tissue where B- and T-cells maintain the local cell-mediated and humoral immune response. Only a few plasma cells are present at an early stage of inflammation but as the lesion becomes more advanced the plasma cells become increasingly dominant. Local macrophages which are activated by the invading leukocytes release IL-1, TNF- $\alpha$  and IL-6 which mediate many local responses. Components of microorganisms, such as LPS, also interact with epithelial cells, leukocytes and fibroblasts thus inducing cytokine production, e.g. IL-1 $\beta$ , TNF- $\alpha$  and IL-6. These cytokines act on fibroblasts and endothelial cells and induce coagulation, increased vascular permeability and production of MMPs, among many functions. The MMP production of fibroblasts, and other gingival cell types, cause degradation of the ECM components to permit leukocyte infiltration, and fibroblasts degenerate, probably by apoptosis. Although host-defence is essential for elimination of infectious agents it may lead to excessive tissue destruction. MMP production may lead to loss of connective tissue and tooth attachment, and as many of the locally

released cytokines have osteotropic effects, e.g. IL-1, TNF- $\alpha$  and IL-6, degradation of alveolar bone may occur (Kinane, 2001; Kinane and Lappin, 2001). The gingival inflammation may remain in an unaltered state but if the persistence of antigen is prolonged or if the net effect of the host response is proinflammatory enough in intensity or duration the production of lytic enzymes and osteotropic mediators may lead to tissue destruction (Graves and Cochran, 2003). A chronic inflammation may follow as a consequence of persistent antigen exposure, which is the case in chronic infectious diseases such as periodontitis or in autoimmune diseases such as rheumatoid arthritis (RA).

Periodontitis is a common infectious disease among adults and 10-15% of the population develop the disease. It is a multifactorial disease which is influenced by microorganisms present in the subgingival lesion, systemic diseases, which have negative effects on the efficacy of the hosts' resistance to infection, as well as environmental factors such as smoking. Certain genetic disorders are also related to periodontitis in certain populations. Increasing evidence suggests that certain individuals are susceptible to periodontitis, exhibiting an imbalance between microbial load and host response. Moreover, an overreactive immune response has been suggested to be a contributing factor and antibodies produced by plasma cells of successfully compared to unsuccessfully treated periodontitis patients have been suggested to differ in efficacy (Gemmell and Seymour, 2004; Kumar *et al.*, 2005; Lindhe *et al.*, 2003; Madianos *et al.*, 2005; Mooney *et al.*, 1995).

### **Osteotropic cytokines**

A number of cytokines which were initially discovered as regulators of immune and hematopoietic cells have later been shown to be produced in bone and to be of osteotropic importance. Cytokines are small (molecular weight < 30 kDa), soluble messenger or regulatory proteins or glycoproteins secreted by various cell types, mainly by Th cells and macrophages but also by other cell types such as osteoblasts, fibroblasts and epithelial cells. They serve as messenger molecules and the local signals are mainly autocrine or paracrine. Binding of cytokines to specific receptors on target cells induces certain biological responses. Cytokines have numerous functions in different cell types and tissues, including their functions in initiation and maintenance of immune and inflammatory responses and hematopoiesis as well as regulation of cell growth and differentiation. Cytokines also regulate the production of other cytokines. The number of cytokines is vast and many of them have overlapping functions (Okada and Murakami, 1998). *In vivo*, cytokines are expressed together with other cytokines leading to synergistic and antagonistic effects and cascades of cytokine release. The non-specific character of cytokines requires a careful regulation of expression of cytokine receptors, and cell-to-cell contact ensures that effective concentration of cytokine is achieved. Furthermore, the half-life of cytokines in circulation is very short in order to ensure limitation of cytokine activities.

### *Interleukins*

One group of cytokines is the interleukins and a major function of these messenger molecules, as their name implies, is communication between leukocytes. However, interleukins are also messengers between numerous other cell types, e.g. fibroblasts, osteoblasts, epithelial and endothelial cells. Interleukins are released in small amounts and induce responses in cells expressing the specific interleukin receptors. A network of cytokines is controlling the release and actions of cytokines, and modulation is also governed by cytokine receptors and their inhibitors. Cytokines such as IL-1, TNF- $\alpha$  and IL-6 are often expressed together and induce the expression of each other, thus being important cytokines promoting the inflammatory response. Such cytokines are referred to as pro-inflammatory cytokines. Furthermore, cytokines need to be carefully regulated and negative regulators of cytokines include IL-4, IL-13 and IL-10. These cytokines are referred to as anti-inflammatory cytokines (Graves and Cochran, 2003; Madianos *et al.*, 2005).

### *Interleukin receptors*

Interleukin receptors are expressed by various cell types. These receptors are generally di- or trimeric molecules consisting of subunits where one subunit is generally cytokine-specific whereas other subunits are required for high affinity binding of cytokine and/or signal transduction. Interleukins bind to the extracellular ligand binding domains of transmembrane cytokine-specific ligand binding receptors. These contain an N-terminal extracellular domain including four cysteine residues and a site which participates in ligand binding. The extracellular part of the receptor is connected by a transmembrane domain to a cytoplasmic domain exhibiting sites participating in intracellular signalling. The cytoplasmic tails of many cytokine receptors are associated to intracellular protein kinases which induce tyrosine phosphorylation and subsequent propagation of intracellular signalling by intracellular signalling cascades. The signal transduction cascade is thus initiated by binding of the cytokine to its receptor and finally results in production of DNA-binding proteins which affect transcription of various genes through binding to promoter and enhancer sequences in genes.

### *IL-6 family of cytokines*

The members of the IL-6 family of cytokines have pleiotropic functions and exert widespread effects in various tissues in the body. They are known to have pro-inflammatory properties and to be regulators of bone metabolism (Bilezikian *et al.*, 2002; Martin *et al.*, 1998). Within the frame of this project IL-6, IL-11, leukemia inhibitory factor (LIF) and oncostatin M (OSM) have been studied.

The IL-6 family of cytokines has 7 members: IL-6, IL-11, LIF, OSM, cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF) and novel neutrophin-1/B cell stimulating factor-3 (NNT-1/BSF-3) (Heinrich *et al.*, 2003; Sehgal *et al.*, 1995; Senaldi *et al.*, 1999). The members have overlapping biological functions, among others modulation of inflammatory and immune responses as well as regulation of the acute phase response. Functional similarities are partly related to sequence homology and binding to common receptors, but also to a common transmembrane receptor subunit, the glycoprotein 130 (gp130), which is used by all members for signal transduction

(Kishimoto *et al.*, 1995b; Taga *et al.*, 1992). The IL-6 type cytokines bind to their ligand-binding receptor subunits which homo dimerize (LIF, OSM) or heterodimerize (IL-6, IL-11) with gp130, which in turn initiates intracellular signalling cascades involving Janus kinases (JAKs), a family of tyrosine kinases binding close to the membrane of many cytokine receptor complexes. A chain of events subsequently leads to dimerization of signal transducers and activators of transcription (STATs) which translocate to the nucleus where they regulate gene expression (Levy and Darnell, 2002). Several other intracellular signalling pathways have been suggested to be involved in IL-6 family signalling, including the Ras pathway (a MAP kinase signalling cascade) and the PI3K (Heinrich *et al.*, 2003; Muller-Newen, 2003).

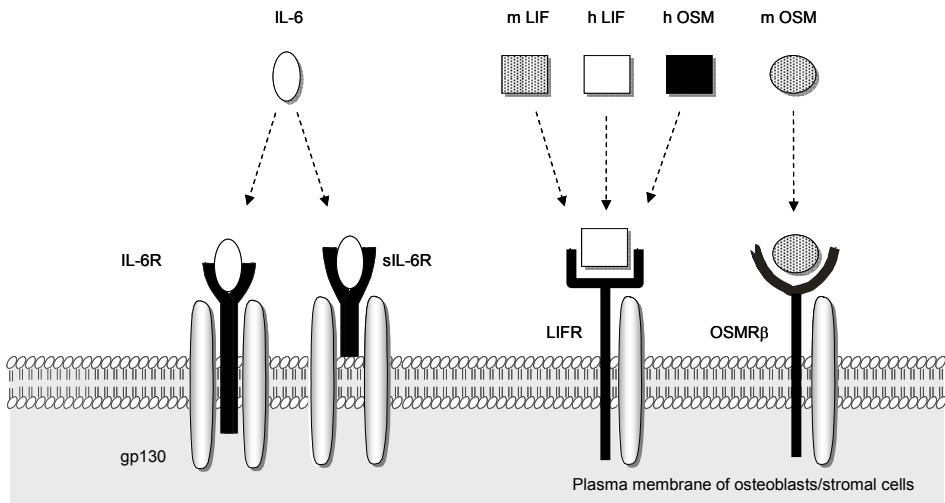


Fig. 2. Ligand-receptor interactions of IL-6, mouse (m) and human (h) LIF and m and h OSM and their receptors in mouse osteoblasts/stromal cells.

### *IL-6 family of cytokines and bone*

IL-6 type cytokines, including IL-6, IL-11, LIF and OSM, are produced by bone cells, and osteoblasts are the primary source of these cytokines in bone. Osteoblasts also express receptors for IL-6, IL-11, LIF and OSM (Bellido *et al.*, 1996; Heymann and Rousselle, 2000).

### *IL-6*

IL-6 is a pleiotropic cytokine produced by osteoblasts/stromal cells but also by other cell types including monocytes, macrophages, Th2 cells and fibroblasts. IL-6 is an important regulator of bone metabolism as well as a regulator of immune and hematopoietic cells. It is also an acute phase mediator. The IL-6 receptor exists in two forms, a membrane bound form, IL-6 receptor (IL-6R, gp80), and a soluble form, the soluble IL-6 receptor (sIL-6R) (Kishimoto *et al.*, 1992; Kishimoto *et al.*, 1995a) which are both active and binding to IL-6. The soluble form is produced through proteolytic cleavage of the IL-6R or through differential splicing (Jones *et al.*, 2001). Since synthesis of the membrane bound IL-6R is relatively sparse the presence of sIL-6R is necessary to evoke a response in many cells. The latter receptor induces biological responses in target cells as long as gp130 is present, and gp130 has been shown to be more widely expressed than IL-6R (Saito *et al.*, 1992). Ligand-binding to IL-6R or sIL-6R results in homodimerization of gp130 which initiates signalling via activation of members of the Janus kinase (JAKs) family and subsequent activation of STATs, including STAT3 (Heinrich *et al.*, 1998; O'Brien *et al.*, 1999; Takeda *et al.*, 1998). Other intracellular signalling pathways are also activated, such as MAP kinase (MAPK) cascades.

### *IL-6 and bone*

The reports on the effects of IL-6 in bone were initially diverging. IL-6 was reported to be a stimulator of osteoclastogenesis in human bone marrow cultures as well as being a stimulator of bone resorption in mouse calvariae (Ishimi *et al.*, 1990; Kurihara *et al.*, 1990). In contrast to these results, however, were reports demonstrating no effects of IL-6 on bone resorption in mouse calvariae (al-Humidan *et al.*, 1991; Holt *et al.*, 1994). IL-6 was suggested to mediate the stimulatory effects of PTH on bone resorption *in vivo* (Grey *et al.*, 1999) and moreover, osteoclast formation in mouse bone marrow cultures stimulated by IL-6 + sIL-6R was abolished by the cyclooxygenase inhibitor indomethacin, suggesting that the effects by IL-6 + sIL-6R were prostaglandin-dependent (Tai *et al.*, 1997). Results showed that in some systems the presence of sIL-6R was required for IL-6 to exert its effects, as neither IL-6 nor sIL-6R alone induced osteoclastogenesis in mouse bone marrow cultures, but when combined, osteoclast formation was noted (Tamura *et al.*, 1993). It was later demonstrated that the effects of IL-6 on osteoclast differentiation and bone resorption were indirect, by IL-6 binding to receptors on osteoblasts (Udagawa *et al.*, 1995). These findings were later confirmed as both expression of membrane bound and sIL-6R were found in human osteoblasts (Vermes *et al.*, 2002).

Surprisingly, *gp130*<sup>-/-</sup> fetal mice exhibited increased number of osteoclasts (Kawasaki *et al.*, 1997) and, moreover, the role of IL-6 in bone metabolism has been suggested to

be more evident in conditions of increased bone turnover than in physiological conditions (Kotake *et al.*, 1996; Manolagas, 1998), which is supported by the not obviously pathological bone phenotype of *il-6*<sup>-/-</sup> mice (Kopf *et al.*, 1994).

IL-6 has been suggested to be a mediator of bone resorption in pathological systemic conditions such as osteoporosis, hyperparathyroidism and Paget's disease (Grey *et al.*, 1996; Manolagas and Jilka, 1995; Roodman and Windle, 2005). Moreover, IL-6 serves as an important mediator of bone loss in postmenopausal osteoporosis. The decreased estrogen levels in this condition cause bone loss since estrogen limits the synthesis of IL-6 in osteoblasts/stromal cells (Girasole *et al.*, 1992), and ovariectomy induced bone loss in mice has been shown to be prevented by infusion of neutralising IL-6 antibodies (Jilka *et al.*, 1992). Further supporting these findings were results from ovariectomized *il-6*<sup>-/-</sup> mice where bone loss was decreased (Poli *et al.*, 1994).

In local inflammation-induced bone loss it is plausible that local bone cells, infiltrating immune cells as well as local cells such as fibroblasts participate in regulation of bone metabolism through IL-6 production. IL-6 has been suggested to be a mediator of bone resorption in inflammatory conditions such as periodontitis (Loos *et al.*, 2000; Okada and Murakami, 1998) and rheumatoid arthritis (Kotake *et al.*, 1996). Different arthritis models have shown the importance of IL-6 in bone and joint degeneration in rheumatoid arthritis (Alonzi *et al.*, 1998; Ohshima *et al.*, 1998) and studies on the *il-6*<sup>-/-</sup> mice have shown delayed onset and reduced severity of arthritis in these mice (Sasai *et al.*, 1999). The convincing data have lead to the production of an anti-IL-6R antibody which is given to arthritis patients and currently tested in clinical trials (Maini *et al.*, 2006; Naka *et al.*, 2002).

### LIF

LIF is a pleiotropic cytokine which, among other cell types, is produced by stromal cells/osteoblasts and fibroblasts. Although LIF is widely expressed by many cell types the constitutive expression levels are usually low (Brown *et al.*, 1994). Similar to IL-6, LIF has various physiologic functions in the body including being a mediator in the acute phase response, stem cell proliferation and hematopoiesis (Auernhammer and Melmed, 2000). The LIF receptor (LIFR, gp190) has a ligand-binding extra cellular domain, a transmembrane domain and a cytoplasmic domain, similar in structure to gp130, and is involved in signal transduction. Differential splicing results in two forms of LIFR, a membrane bound (LIFR) and a soluble form (sLIFR) (Owczarek *et al.*, 1996; Zhang *et al.*, 1998). sLIFR is detected in serum and has been suggested to serve as an inhibitor of LIF (Gearing *et al.*, 1993; Layton *et al.*, 1992). When LIF binds to the membrane bound LIFR the receptor heterodimerizes with gp130. Similar to IL-6, activation of JAKs is initiated by ligand-binding to the receptor complex, and subsequent activation of transcription factors including STATs (Baumann *et al.*, 1994; Stahl and Yancopoulos, 1994), of which STAT3 in the JAK/STAT pathway is involved, similar to IL-6. MAP kinase (MAPK) cascades are also activated (Heinrich *et al.*, 2003; Lowe *et al.*, 1995; O'Brien *et al.*, 1999; Schiemann and Nathanson, 1998).

### *LIF and bone*

Observations in mice have indicated that LIF is involved in regulation of bone turnover and bone cell functions (Auernhammer and Melmed, 2000; Heymann and Rousselle, 2000) and like IL-6, LIF is produced by osteoblasts in response to PTH (Greenfield *et al.*, 1993; Pollock *et al.*, 1996). LIF has demonstrated variable effects on bone resorption *in vitro* depending on model system used. LIF stimulated osteoclastogenesis and bone resorption in mouse calvariae both *in vitro* and *in vivo* (Cornish *et al.*, 1993; Reid *et al.*, 1990) and increased osteoclast formation in mouse bone marrow cultures was noted upon LIF stimulation (O'Brien *et al.*, 2000; Richards *et al.*, 2000). In line with these findings were reports where increased osteoclastic bone resorption was noted in mice engrafted with cells overexpressing LIF (Metcalf and Gearing, 1989). In contrast, bone resorption in fetal rat and mouse long bones (Lorenzo *et al.*, 1990) and in fetal mouse metacarpals (Van Beek *et al.*, 1993) was inhibited by LIF *in vitro*, and in addition *lifr*<sup>-/-</sup> knockout mice show increased number of osteoclasts resulting in decreased bone mass (Ware *et al.*, 1995).

### *OSM*

OSM, similar to other IL-6 family members, exerts biological functions involving roles in the acute phase response, inflammation and hematopoiesis. Although produced by many different cell types, it is mainly produced by monocytes, macrophages and T-cells (Tanaka and Miyajima, 2003). OSM is structurally similar to LIF, and the two cytokines have overlapping functions. One reason for this is the common use of the receptor subunit gp130 for signalling. In murine cells, human OSM does not bind to the mouse OSMR but to the mouse LIFR, which also binds murine and human LIF, whereas murine OSM uniquely binds to the murine OSM receptor  $\beta$  (OSMR $\beta$ ), an additional OSM receptor, and not to the LIFR (Ichihara *et al.*, 1997; Lindberg *et al.*, 1998; Tanaka *et al.*, 1999). OSMR $\beta$  only binds OSM, not LIF (Mosley *et al.*, 1996). Intracellular signalling pathways initiated by OSM binding to OSMR $\beta$  are similar to those mediated through the LIFR, thus evoking similar biological responses. OSM signalling, similar to LIF, involves heterodimerization of gp130 and activation of JAKs and STATs, including STAT3 (Levy *et al.*, 1996; O'Brien *et al.*, 1999).

### *OSM and bone*

OSM has, similar to LIF, been suggested to be involved in regulation of bone turnover and bone cell functions (Auernhammer and Melmed, 2000; Heymann and Rousselle, 2000), although it is unclear whether OSM is expressed in bone. OSM has been suggested to be a potent stimulator of IL-6 (Jay *et al.*, 1996) and similar to LIF, OSM has been shown to stimulate osteoclast formation in mouse bone marrow cultures (Richards *et al.*, 2000) but in contrast to these findings, and similar to LIF, was a report demonstrating inhibition of bone resorption in fetal mouse long bones treated with OSM (Jay *et al.*, 1996). The latter report is supported by observations in mice overexpressing OSM which exhibit an osteopetrotic phenotype (Malik *et al.*, 1995). Interestingly, a neutralizing OSM-antibody showed inhibition of disease progression in a mouse model of experimental arthritis (Plater-Zyberk *et al.*, 2001).

### *IL-6 type cytokines and gingival fibroblasts*

Cytokines play important roles not only in tissue homeostasis but also in the pathogenesis of many infectious diseases. Excessive and/or continuous production of cytokines in inflamed periodontal tissues has been suggested to partly account for the progress of periodontitis and periodontal tissue destruction. Inflammatory cytokines such as IL-1, TNF- $\alpha$  and IL-6 are present in the diseased periodontal tissues, and enhanced production of these cytokines seems to influence chronic leukocyte recruitment and tissue destruction (Okada and Murakami, 1998). Bacterial components (e.g. LPS) and cytokines released from mononuclear leukocytes has been shown to evoke responses and induce phenotypical changes in resident gingival fibroblasts (Murakami *et al.*, 1999) and in addition to cytokines produced by leukocytes, resident cells such as epithelial cells, fibroblasts and periodontal ligament cells also express various cytokines.

Osteoclasts are stimulated by cytokines produced by infiltrating immune cells (Graves and Cochran, 2003; Lerner, 2006) and, moreover, direct interactions between gingival fibroblasts and osteoclast progenitor cells have been shown in co-cultures of human cells (de Vries *et al.*, 2006). Thus, additional cytokine release from resident cells may contribute to the stimulation of osteoclasts, or exert modulatory actions. In analogy, resident synovial fibroblasts have been suggested to play central roles in tissue remodelling in rheumatoid arthritis, not only by production of MMPs but also by possible osteotropic effects (Ritchlin, 2000). Inflammatory leukocytes and resident cells may interact in a similar way resulting in osteotropic actions by cytokines released from inflammatory gingiva.

IL-6 type cytokines stimulate osteoclastogenesis and bone resorption, as described previously, and IL-6 has been suggested to play a regulatory role in periodontal disease (Okada and Murakami, 1998). Elevated IL-6 expression levels in human gingival fibroblasts and gingival crevicular fluid have been associated with periodontitis (Graves and Cochran, 2003; Kent *et al.*, 1999; Lin *et al.*, 2005; Mogi *et al.*, 1999; Wang *et al.*, 2003). IL-6 has been reported to be expressed in human gingival fibroblasts (Belibasakis *et al.*, 2005b; Kent *et al.*, 1998; Modeer *et al.*, 1998) and, interestingly, enhanced IL-6 mRNA expression levels were demonstrated in fibroblasts from inflammatory gingiva compared to cells from healthy tissue (Kent *et al.*, 1999; Wang *et al.*, 2003). Single nucleotide polymorphisms in the human IL-6 promoter have also been shown to be associated with periodontal disease (Holla *et al.*, 2004; Trevisatto *et al.*, 2003).

IL-11, similar to other IL-6 type cytokines, has been reported to stimulate osteoclastogenesis and bone resorption in mice (Ahlen *et al.*, 2002; Girasole *et al.*, 1994; Morinaga *et al.*, 1998), and IL-11 mRNA and protein expression in gingival fibroblasts of healthy patients has been reported (Yashiro *et al.*, 2006). However, no reports have demonstrated LIF or OSM expression in human gingival fibroblasts, although elevated levels of LIF and OSM have been noted in GCFs of diseased periodontal pockets (Lin *et al.*, 2005; Sakai *et al.*, 2006).

### *IL-4 and IL-13*

IL-4 and IL-13 are cytokines which are secreted by activated Th2 cells and mast cells (Howard and Paul, 1982). The major functions of these cytokines include regulatory actions on Th-cells and B-cells as well as inhibition of macrophage function (Hart *et al.*, 1989; Stuart *et al.*, 1988), thus being important regulators of inflammatory responses. The two cytokines are referred to as inhibitory cytokines as these two proteins are capable of modulating the effects of pro-inflammatory cytokines by regulating the synthesis of these cytokines or their antagonists as well as interfering with binding of these cytokines to their soluble receptors (Burger and Dayer, 1995). Although sharing only 25% homology, the two proteins have many overlapping functions and they use closely related receptors.

IL-4 signalling can occur in two alternative ways. One alternative is binding of IL-4 to IL-4 receptor  $\alpha$  (IL-4R $\alpha$ ), which is a receptor providing both ligand-binding specificity and signal transduction (Idzerda *et al.*, 1990). The IL-4R $\alpha$  heterodimerizes with the  $\gamma$  common chain ( $\gamma$ c) (which is also a receptor component of receptors for IL-2, IL-7, IL-9, IL-15 and IL-21), together making up the complete receptor assembly of the type 1 IL-4 receptor (Russell *et al.*, 1993). The IL-4R $\alpha$  can also heterodimerize with IL-13 receptor  $\alpha$ 1 (IL-13R $\alpha$ 1), making up the type 2 IL-4 receptor, and thus induce signalling (Kelly-Welch *et al.*, 2003). IL-13 signalling is achieved by binding of IL-13 to IL-13R $\alpha$ 1, which heterodimerizes with IL-4R $\alpha$  (Hershey, 2003). Binding of either IL-4 to IL-4R $\alpha$  in type 1 or type 2 receptors or binding of IL-13 to IL-13R $\alpha$ 1 initiates binding of JAK-1 in the Janus kinase (JAK) family to the cytoplasmic tail of IL-4R $\alpha$  where tyrosine residues are phosphorylated. Tyrosine residues in IL-4R $\alpha$  act as docking sites binding the transcription factor STAT6, which after tyrosine phosphorylation detaches from the docking sites, homodimerizes and translocates to the nucleus. Ligand binding to IL-4 and IL-13 receptors also initiates binding of Tyk2, which is another member of the JAK family, to the cytoplasmic tail of IL-13R $\alpha$ . Moreover, activation of IL-4R $\alpha$  induces phosphorylation of another tyrosine residue of the IL-4R $\alpha$  tail, which serves as a docking site for insulin receptor substrate 1 (IRS-1) and IRS-2, which subsequently become phosphorylated leading to activation of the Ras/mitogen-activated protein kinase and phosphatidylinositol 3 kinase pathways. IL-13 signalling, however, is less well known than IL-4 signalling, and although IL-13 is known to bind to IL-13R $\alpha$ 2 with high affinity, the function of this receptor is poorly understood. It has been suggested to function as a decoy receptor (Hershey, 2003; Kelly-Welch *et al.*, 2003).

### *IL-4 and IL-13 and bone*

IL-4 has been shown to inhibit *in vitro* bone resorption in mouse long bones induced by several stimulators, e.g. PTH, D3, IL-1 and PGE<sub>2</sub>, (Watanabe *et al.*, 1990) and in neonatal mouse calvarial bones stimulated by IL-11 (Ahlen *et al.*, 2002). The inhibitory effect of IL-4 on bone resorption *in vitro* is thought to involve decreased osteoclastogenesis. IL-4 inhibited osteoclastogenesis in bone marrow cultures (Kasono *et al.*, 1993; Shioi *et al.*, 1991) as well as osteoclastogenesis induced by PTH, PTHrP, D3, IL-1 and PGE<sub>2</sub> in co-cultures of bone marrow macrophages and stromal cells (Lacey *et al.*, 1995) and osteoclast formation in co-cultures of mouse

spleen cells and ST-2 stromal cells stimulated by D3 (Riancho *et al.*, 1993). Similar effects of IL-4 noted in bone marrow macrophage (BMM) cultures suggested that osteoclast precursors were the target cells for IL-4. However, as osteoblasts have been shown to express IL-4 receptors (Riancho *et al.*, 1993; Ueno *et al.*, 1992) and IL-4 induces M-CSF release in the murine osteoblast-like cell line MCT3T3-E1 as well as in primary osteoblasts (Lacey *et al.*, 1994), both osteoblasts and osteoclasts appear to be target cells for IL-4.

*In vivo*, PTHrP and IL-1 secreting transplanted tumors in mice stimulated bone resorption which was inhibited by infusions of IL-4 (Nakano *et al.*, 1994), and hypercalcemia induced by parathyroid hormone-related peptide (PTHrP) in mice was inhibited by IL-4 administration *in vivo* (Nagasaki *et al.*, 1991). Bone loss caused by ovariectomy was similarly inhibited by IL-4 (Okada *et al.*, 1998).

In an *in vivo* model of collagen induced arthritis, an adenovirus vector producing IL-4 decreased RANKL production and decreased bone resorption (Lubberts *et al.*, 2000). Similar results were observed following intraperitoneal injection of IL-4 (Joosten *et al.*, 1999) and bone resorption in adjuvant-induced arthritis in rats can be decreased by injection of an adenovirus producing IL-4 (Woods *et al.*, 2001). Moreover, an *ex vivo* model of bone resorption in rheumatoid arthritis showed that IL-4 inhibited the appearance of osteoclasts (Miossec *et al.*, 1994). IL-4 expression has been shown in arthritic joints, although expression levels were suggested to be too low to prevent actions of proinflammatory cytokines in progressive RA (Isomaki and Punnonen, 1997). Part of the inhibitory effects of IL-4 and IL-13 has been suggested to be related to an IL-4 induced decrease in prostaglandin production by the bone cells (Kawaguchi *et al.*, 1996; Onoe *et al.*, 1996) and in line are organ culture studies where inhibition exerted by IL-4 of the IL-1 induced stimulation of bone resorption was suggested to be due to inhibition of COX-2 expression (Kawaguchi *et al.*, 1996; Onoe *et al.*, 1996).

Additionally, one study has demonstrated that IL-4 can inhibit osteoclast formation not uniquely by direct actions on cells of the myelomonocytic lineage but also by indirect actions on T-cells, which induce the expression of an unknown membrane-associated inhibitor (Miroslavljec *et al.*, 2003).

IL-4 transgenic mice overexpressing IL-4, surprisingly, showed cortical thinning in long bones and vertebrae (Lewis *et al.*, 1993). These mice had normal osteoclast numbers but the osteoclasts had altered function, as measured by a decrease in TRAP activity. A marked decrease in bone formation was also noted in these mice, as well as decreased number and function of cells lining the bone surface, possibly explaining the phenotype.

Several molecular mechanisms have been suggested for the inhibition of osteoclast formation by IL-4, including inhibition of phosphorylation of inhibitor of  $\kappa$ B (I $\kappa$ B) and decreased NF- $\kappa$ B nuclear translocation (Abu-Amer, 2001), decreased RANK stimulation of osteoclast progenitor cells by activation of peroxisome proliferator-activated receptor  $\gamma$ 1 (PPAR  $\gamma$ 1) (Bendixen *et al.*, 2001), and inhibitory effects on

signalling through c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK) (Wei *et al.*, 2002).

#### *IL-4 and IL-13 and gingival fibroblasts*

IL-4 and IL-13 have anti-inflammatory properties as well as being inhibitors of osteoclastogenesis and bone resorption, as previously described. There is a possibility that tissue remodelling, anti-inflammatory cytokines may be expressed by resident cells, and not only by leukocytes. Reports have demonstrated the presence of Th2 lymphocytes expressing IL-4 in gingival biopsies from patients with periodontitis (Berglundh *et al.*, 2002; Ito *et al.*, 2005). One study demonstrated a higher number of samples positive for IL-4 protein in healthy gingiva compared to samples from periodontitis patients (Gorska *et al.*, 2003) and, similarly, IL-4 protein levels have been reported to be lower in crevicular fluid of periodontitis patients compared to healthy controls (Bozkurt *et al.*, 2006). However, another study reported no difference in IL-4 mRNA expression between the gingiva of patients with gingivitis compared to patients with periodontitis (Honda *et al.*, 2006) and an additional study reported that IL-4 protein levels were more elevated in the gingiva of periodontal sites in the vicinity of deep periodontal pockets compared to those from healthy gingiva (Johnson and Serio, 2005). Furthermore, gingival fibroblasts have been shown to express receptors for IL-4 (Kunii *et al.*, 2005).

Although reports on IL-4 levels in gingiva in correlation to periodontal disease are diverging, IL-4 and IL-13 may be of importance in regulation of inflammation induced bone remodelling. This notion is supported by studies in several models of experimental arthritis where bone loss has been shown to be prevented by IL-4 (Joosten *et al.*, 1999; Lubberts *et al.*, 2000). Interestingly, in a similar study, IL-4 reduced both pro-inflammatory cytokine levels and bone resorption, further supporting the possible potential of the inhibitory effects of IL-4 and IL-13 (Woods *et al.*, 2001).

## AIMS

The general aim of the thesis was to study the effects of IL-6 type cytokines as well as IL-4 and IL-13 on osteoclastogenesis and bone resorption. Furthermore, the objective was to determine if human gingival fibroblasts expressed these cytokines and, in addition, if IL-6 type cytokine expression by gingival fibroblasts was modulated by inflammatory mediators as well as by IL-4 and IL-13, mechanisms of possible importance for alveolar bone resorption in periodontal disease. The specific purposes of the individual papers were:

### Paper I

To investigate if the IL-6 type cytokines IL-6, LIF and OSM were stimulators of bone resorption in neonatal mouse calvarial bones *in vitro* and to determine the possible involvement of RANK, RANKL and OPG interactions in the putative calvarial effects of these cytokines.

### Paper II

To study if IL-4 and IL-13 were inhibitors of osteoclastogenesis and bone resorption and if the two cytokines induced effects in both stromal cells/osteoblasts and osteoclast progenitor cells through mechanisms involving RANK, RANKL and OPG interactions, and, furthermore, to determine whether possible effects exerted by IL-4 and IL-13 were STAT6 dependent.

### Paper III

To determine if IL-6 type cytokines were expressed in human gingival fibroblasts and if their expression was regulated by IL-1 $\beta$  and TNF- $\alpha$ .

### Paper IV

To study if IL-4 and IL-13 regulated the expression of IL-6 type cytokines in human gingival fibroblasts stimulated by IL-1 $\beta$  and TNF- $\alpha$ .

## METHODS

### Humans

Gingival fibroblasts were isolated from gingival papillar explants/biopsies obtained from clinically and systemically healthy voluntary donors.

### Mice

CsA mice from our own inbred colony were used in most experiments. Additionally, Balb/c mice homozygous for the STAT4<sup>tm1Gru</sup> mutation (C.129S2-Stat4<sup>tm1Gru</sup>/J; stock no. 002826), mice homozygous for the STAT6<sup>tm1Gru</sup> mutation (C.129S2-Stat6<sup>tm1Gru</sup>/J; stock no. 002828) and their corresponding wild type mice (stock no. 000651) were used. These mice were purchased from JAX<sup>®</sup>MICE, The Jackson Laboratory and bred in our animal facility unit. Calvarial bones and spleen cells from STAT6 deficient mice were confirmed not to express STAT6 mRNA as assessed by reverse transcriptase polymerase chain reaction (RT-PCR) analyses.

### Ethical evaluation

Humans: Donors' rights were protected by the Swedish Ethical Committee for Human Research (99 03 29) and informed consent was granted.

Mice: Animal care and experiments were approved and conducted in accordance with accepted standards of humane animal care and use as deemed appropriate by the Animal Care and Use Committee of Umeå University, Umeå, Sweden.

### Isolation and culture of primary human fibroblast cultures

Explants were dissected into 0.5 cm<sup>2</sup> pieces and placed at the bottom of 25 cm<sup>2</sup> culture flasks containing  $\alpha$ -MEM ( $\alpha$  modification of Minimum Essential Medium) supplemented with 10% foetal calf serum (FCS), L-glutamine and antibiotics (bensylpenicillin, gentamycin sulphate, streptomycin) (basic medium) followed by incubation at 37°C in humidified air containing 5% CO<sub>2</sub>. The flasks were left untouched for 7-10 days until outgrowth of fibroblasts from the explants was observed. Medium was changed 3 times per week. Fibroblasts were seeded at a density of 3.5 x 10<sup>4</sup> cells/cm<sup>2</sup> in basic medium and cultured until cells were 80-90% confluent. Media were changed and cells were incubated in the absence (controls) or presence of test substances for 24 h (mRNA) and 48 h (protein). Fibroblasts from passages 4-7 were used in the experiments. For mRNA analyses, cells were lysed in TRIzol LS Reagent (Applied Biosystems, Warrington, UK) or the lysis buffer supplied with the RNAqueous 4-PCR kit (Ambion Inc., Austin, TX, USA), after which samples were stored at -80 °C until RNA extraction and PCR analyses were performed. For protein analyses, culture media were collected and cells were washed thoroughly in serum-free medium followed by lysis in 0.2% Triton X-100 for 24 h. The cell cultures used in the experiments were confirmed to be free of mycoplasma-infection by DAPI fluorescent staining.

### **Isolation and culture of primary mouse calvarial osteoblasts**

Primary mouse osteoblasts were isolated from calvariae of 2-3 day-old CsA mice. Calvariae were dissected in phosphate-buffered saline (PBS) substituted with 10% fetal calf serum (FCS) using a modified time sequential enzyme-digestion technique described by Boonecamp *et al.* in 1984. Cells from populations 6 to 10 were used in the experiments. The isolated cells demonstrated an osteoblastic phenotype as assessed by their cyclic AMP-responsiveness to PTH, expression of alkaline phosphatase (ALP), osteocalcin (OC) and bone sialoprotein (BSP) as well as their capacity to form mineralized bone noduli. The cells were seeded in culture flasks containing  $\alpha$ -MEM supplemented with 10% FCS, L-glutamine and antibiotics (basic medium) at 37°C in humidified air containing 5% CO<sub>2</sub>. After four days in flasks, the cells were seeded at a density of 10<sup>4</sup> cells/cm<sup>2</sup> in culture dishes containing basic medium. After attachment overnight, medium was changed and cells were cultured in basic medium in the absence or presence of test substances. Each group contained four dishes and cells were cultured for 8 h (mRNA) and 48 h (mRNA, protein). Cells were collected and, following RNA extraction, used for subsequent PCR analyses. For protein analyses, cells were treated with Triton X-100 for 24 h and lysates were subsequently analyzed by ELISA.

### **Mouse spleen cell cultures**

Spleen cells were isolated from 5-7 week-old mice (CsA, *stat6*<sup>-/-</sup>, *stat4*<sup>-/-</sup>, Balb/cJ) and seeded in basic medium on cover slips in 24 well plates at a density of 1 x 10<sup>6</sup> cells/cm<sup>2</sup>. After attachment overnight, the medium was changed and the cells cultured in the absence and presence of M-CSF (25 ng/ml) and RANKL (100 ng/ml), with and without test substances, for 4-5 days and medium was changed after three days. At the end of the experiments, the cells were stained for tartrate resistant acid phosphatase (TRAP) using the Sigma Diagnostics Acid Phosphatase kit by following the manufacturer's instructions. Cells which were positive for TRAP and exhibiting three or more nuclei were considered osteoclasts, and the number of multinucleated osteoclasts was counted. Osteoclasts formed in the spleen cell cultures stimulated by M-CSF and RANKL were able to form pits when cultured on slices of bovine bone. Osteoclast formation was associated with increased mRNA expression of calcitonin receptor (CTR), TRAP and cathepsin K. No osteoclasts were formed when cells were treated with M-CSF or RANKL alone. Neither were osteoclasts formed in the presence of PTH or D3, demonstrating the lack of stromal cells in the spleen cell cultures. Osteoclast formation caused by M-CSF and RANKL was abolished by OPG. For semi-quantitative RT-PCR and quantitative real-time RT-PCR analyses, spleen cells were seeded at a density of 1 x 10<sup>6</sup> cells/cm<sup>2</sup> in culture dishes. After attachment overnight, cells were incubated in basic medium in the absence and presence of M-CSF (25 ng/ml) and RANKL (100 ng/ml), with or without test substances, for 4-5 days after which RNA was extracted and used for PCR analyses. 4 wells per group were used in the experiments.

### **Bone marrow macrophage cultures (BMM)**

The femurs and tibiae from 5-7 week-old male CsA mice were dissected and cleaned from adhering tissues. The cartilage ends were cut off and the cells in the marrow cavity were flushed out using  $\alpha$ -MEM in a syringe with a sterile needle. The marrow cells were collected in basic medium and the erythrocytes lysed in red blood cell lysis buffer (0.16 M  $\text{NH}_4\text{Cl}$ , 0.17 M Tris, pH 7.65). The remaining bone marrow cells were washed and suspended in basic medium containing M-CSF (100 ng/ml), in order to stimulate proliferation of cells of the macrophage lineage. The cells were seeded at a density of  $8 \times 10^4$  cells/cm<sup>2</sup> in culture dishes, to which stromal cells and lymphoid cells cannot adhere. After 3 days, the cultures were vigorously washed with PBS and cells attached to the bottom were detached using 0.002% EDTA in PBS. These cells were resuspended in basic medium containing M-CSF (100 ng/ml) and seeded at a density of  $0.5 \times 10^4$  cells/cm<sup>2</sup> in culture dishes. After another 3 days, the cells were washed and detached as described above after which cells were used as bone marrow macrophages (BMM). These cells did not express alkaline phosphatase, RANKL, OPG or CTR mRNA, but mRNA for RANK, c-Fms, cathepsin K and TRAP, as assessed by quantitative real-time PCR. Using flow cytometry, cell surface expression of the typical macrophage markers F4/80 and c-Fms, but not the lymphoid cell markers CD3 and B 220, was demonstrated (Granholm *et al.*, submitted).

For osteoclastogenesis experiments, BMM were seeded on 0.8 cm<sup>2</sup> glass chamber slides at a density of  $10^4$  cells/cm<sup>2</sup> in basic medium containing either M-CSF (100 ng/ml, controls) or of M-CSF (100 ng/ml) and RANKL (50 ng/ml), with and without test substances. After 4 days, with a change of medium after 3 days, the cultures were harvested and the cells fixed with acetone in citrate buffer. Cells stained positively for TRAP and having three or more nuclei were considered osteoclasts, and the number of multinucleated osteoclasts were counted. Osteoclasts formed in these cultures stimulated by M-CSF and RANKL were able to form pits when cultured on slices of bovine bone, and osteoclast formation was associated with increased mRNA expression of CTR, TRAP and cathepsin K. Osteoclasts were not formed in the presence of M-CSF and either PTH or D3, indicating the lack of stromal cells in the cultures. Osteoclast formation caused by M-CSF and RANKL was abolished by OPG.

For quantitative real-time PCR analyses, BMM were seeded at a density of  $10^4$  cells/cm<sup>2</sup> in culture dishes and incubated in basic medium in the presence of M-CSF (100 ng/ml; control), M-CSF (100 ng/ml) and RANKL (50 ng/ml), with or without test substances, for three days after which RNA was extracted and used for subsequent PCR analyses. 4 wells per group were used in the experiments.

### **Raw 264.7 cell cultures**

For PCR analysis, the murine macrophage cell line RAW 264.7 was seeded at a density of  $10^3$  cells/cm<sup>2</sup> in culture dishes containing basic medium. After attachment overnight, cells were incubated in basic medium containing RANKL (100 ng/ml) and with or without test substances. In addition, cells were cultured in basic medium without test substances as a control group. After 2, 4 and 6 days, RNA was extracted and used for semi-quantitative RT-PCR analyses.

For FACS analysis, RAW 264.7 cells were seeded in culture dishes at a density of  $2 \times 10^5$  cells/dish and preincubated in basic medium for 24 h, followed by incubation for 48 h in basic medium with or without test substance (50 ng/ml IL-4). After the incubation cells were used for FACS analysis. 4 wells per group were used in the experiments.

### **Mouse bone organ culture**

Calvarial bones were dissected from 6-7 day-old mice (CsA, *stat4*<sup>-/-</sup>, *stat6*<sup>-/-</sup>, Balb/cJ) which were killed by cervical dislocation. The bones were divided into halves (PCR, ELISA) or quarters (<sup>45</sup>Ca) and preincubated for 18-24 h in  $\alpha$ -MEM supplemented with 0.1% bovine serum albumin (BSA), 1  $\mu$ g/mL Fe(NO<sub>3</sub>)<sub>3</sub>, 0.1 mg/ml ascorbic acid, L-glutamine and antibiotics (basic calvarial medium) and 1  $\mu$ M indomethacin. Preincubation was performed in order to prevent initial bone resorption due to PGE<sub>2</sub> release induced by dissection trauma. Following preincubation, the bones were extensively washed and subsequently cultured for 24-96 h in multiwell culture dishes containing indomethacin free basic calvarial medium in the absence or presence of test substances. The bones were incubated in the presence of 5% CO<sub>2</sub> in humidified air at 37°C (Lerner, 1987).

For bone mineral release analysis, a total of 6 prelabelled calvarial bone quarters per group were preincubated, followed by individual incubation in 24 well plates in the absence or presence of test substances for 24-96 h. At the end of culture, culture media and bones were collected and used for subsequent <sup>45</sup>Ca analysis.

For bone matrix degradation analysis, a total of 6 prelabelled calvarial bone quarters per group were preincubated, followed by individual incubation in 24 well plates in the absence or presence of test substances for 96 h. At the end of culture, culture media and bones were collected and used for subsequent <sup>3</sup>H analysis.

For mRNA analysis, a total of 5 calvarial bone halves per group were preincubated, followed by individual incubation in the absence or presence of test substances in 24 well plates in the absence or presence of test substances for 24 h prior to RNA extraction. For semi-quantitative RT-PCR, bones were homogenized and RNA was extracted from bones and pooled for subsequent analyses, whereas for quantitative real-time RT-PCR, bones were homogenized and RNA was extracted from individual bones and used for PCR analyses.

For protein analysis, a total of 8 calvarial halves per group were preincubated, followed by individual incubation in 24 well plates in the absence or presence of test substances for 48 h. Following treatment with 0.2% Triton-X 100 for 24 h, extracted bone samples were analyzed by ELISA.

For PGE<sub>2</sub> analysis, bone culture media were collected at the end of culture and used for RIA analysis.

### **RNA extraction and cDNA synthesis**

Total RNA was extracted from mouse calvarial bones, mouse calvarial osteoblasts, mouse RAW 264.7 cells, mouse spleen cells, mouse BMM and human gingival fibroblasts by TRIzol LS reagent or by the RNAqueous-4PCR kit by following the manufacturer's protocols. Extracted total RNA was treated with DNase I to eliminate genomic DNA according to the instructions supplied by the manufacturer. The RNA was quantified spectrophotometrically and the integrity of the RNA preparations was examined by agarose gel electrophoresis. Only RNA preparations showing intact species were used for subsequent cDNA synthesis.

One microgram of total RNA was reverse transcribed into single-stranded cDNA with a 1<sup>st</sup> Strand cDNA Synthesis Kit using AMV reverse transcriptase and oligo-p(dT)<sub>15</sub> primers. After incubation at 25°C for 10 min and at 42°C for 60 min, the AMV reverse transcriptase was denatured at 99°C for 5 min, followed by cooling to +4°C for 5 min. The cDNA was kept at -20°C until used for PCR analysis.

### **Semi-quantitative Reverse Transcription Polymerase Chain Reactions (RT-PCR)**

First-strand cDNA mixture was amplified by RT-PCR using a PCR Core Kit, a PC-960G Gradient Thermal Cycler (Corbett Research, Australia) or a Mastercycler Gradient (Eppendorf), oligonucleotide primers and Taq DNA polymerase or HotStar Taq DNA polymerase. The PCR analyses were performed using a PCR Core Kit standard protocol using 1 µl template, 0.2 µM of each primer, 2.5 U Taq DNA polymerase or Hot Star Taq DNA polymerase, 1x PCR buffer, 0.2 mM dNTPs and 1.5 mM MgCl<sub>2</sub> (100 µl total volume). For some primers, MgCl<sub>2</sub> concentration was altered to 1 or 2 mM and the PCR reactions were initiated using either Taq DNA polymerase or HotStar Taq Polymerase. The conditions for PCR were denaturing at 94°C for 2 min (Taq DNA polymerase) or 15 min (Hot Star Taq DNA polymerase), annealing for 35 s at temperatures optimized for the individual primer pairs, followed by elongation at 72°C for 45 s; in subsequent cycles denaturing was performed at 94°C for 35 s. Some PCRs were performed using a step down technique in which, after the initial 10 cycles with the annealing temperature set to 65°C, the primer annealing temperature was decreased stepwise by 5°C every 5 cycles down to 45°C. The specific PCR conditions, sequences of primers, GenBank accession numbers, positions for the 5' and 3' ends of the nucleotides for the predicted PCR products and the estimated size of the PCR products are given in the individual papers. mRNA expressions of target genes were compared at the logarithmic phase of the PCR reaction and normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal standard. No amplification was detected in samples in which the RT reaction had been omitted. The PCR products were electrophoretically size fractionated in 1.5% agarose gel and visualized using ethidium bromide. The identities of the PCR products were confirmed using a QIAquick purification kit and a Thermo Sequenase-TM II DYEnamic ET terminator cycle sequencing kit with sequences analysed on an ABI 377 XL DNA Sequencer. Densities of electrophoretic gel bands were calculated using the 1D Image Analysis software from Kodak.

### **Quantitative real-time RT-PCR**

Quantitative real-time RT-PCR analyses were performed using Taqman kinetics with fluorescence labeled probes (reporter fluorescent dye VIC at the 5' end and quencher fluorescent dye TAMRA at the 3' end). Oligonucleotide primers and TaqMan probes for most genes were designed using Primer Express (Applied Biosystems), based on sequences from the GenBank database (<http://www.ncbi.nlm.nih.gov>). For some genes Assay on Demand kits or TaqMan Inventoried Gene Expression assays (Applied Biosystems) were used. The sequences of primers and probes are listed in the individual papers. In each reaction, cDNA was diluted 20-fold with nuclease-free water and amplified using the TaqMan Universal PCR Master Mix, 300 nM of each primer and 100 nM of probe. The reaction conditions were an initial step of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The amplifications were performed using the ABI PRISM 7900 HT Sequence Detections System and software (Applied Biosystems). No amplifications were detected in samples where the RT reaction had been omitted. To control for variability in amplification due to differences in starting mRNA concentrations,  $\beta$ -actin (mouse) or ribosomal protein L13A (RPL13A) (human) were used as internal standards. The relative expressions of target mRNAs were computed from the target Ct values and  $\beta$ -actin or RPL13A Ct values using the standard curve method. Ct values for  $\beta$ -actin and RPL13A were confirmed not to vary over time or due to treatment. In some experiments, the data were recalculated and the results expressed as percent of control, which was set at 100%, allowing for accumulation of data from several experiments.

### **Protein analysis by ELISA**

Protein synthesis in cultures of mouse calvarial osteoblasts, calvarial bones and gingival fibroblasts was assessed using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Protein release from cells was analyzed in culture media and intracellular protein content in cells or calvariae was analyzed in cell or calvarial lysates according to the manufacturer's protocols for the ELISAs. In gingival fibroblasts, cytokine protein expression was normalized to total protein concentration in cell lysates, determined by the bicinchoninic acid (BCA) method. The sensitivities of the RANKL and OPG immunoassays were 5 pg/ml, respectively, those for IL-11 and LIF were both 8.0 pg/ml and the sensitivity for the IL-6 immunoassay was 0.7 pg/ml. In some experiments, the data were recalculated and the results expressed as percent of control, which was set at 100%, allowing for accumulation of data from several experiments.

### **FACS analysis**

The expression of RANK protein was assessed by analyzing cell surface expression of RANK in RAW 264.7 cells by the use of fluorescein isothiocyanate (FITC)-labelled anti-RANK antibodies. After incubation, the adherent RAW 264.7 cells were treated with 0.2% EDTA and detached with a rubber cell scraper, followed by fixation with 2% paraformaldehyde in PBS, pH 7.2, for 10 min at room temperature. The cells were subsequently washed once with PBS containing 1% bovine serum albumin (PBS-BSA) and harvested by centrifugation at 300 x g for 5 min. Cell pellets were re-suspended in

PBS-BSA containing goat RANK specific IgG (5 µg/ml) and incubated at room temperature for 1 h under gentle agitation. Thereafter, the cells were washed once in PBS-BSA and re-suspended in PBS-BSA containing FITC-labelled rabbit antibodies to goat IgG (2 µg/ml). Following incubation in the dark, the cells were washed once in PBS-BSA and suspended in PBS to a final concentration of  $10^6$  cells/ml before being analyzed using a fluorescence-activated cell sorter (Facstar) equipped with LYSIS II software (Becton Dickinson Immunocytometry Systems). The effect of IL-4 on the expression of RANK was calculated in relation to the control cells that had not been exposed to IL-4. The mean fluorescence of each cell population was compared and expressed in relation to fluorescence of control cultures.

### **Bone mineral release assay**

Bone resorption was determined by analyzing mineral release from cultured mouse calvarial bones, as assessed by the release of  $^{45}\text{Ca}$  from bones from mice prelabelled *in vivo*. 2-3 day-old mice (CsA, *stat6*<sup>-/-</sup>, *stat4*<sup>-/-</sup>, Balb/cJ) were injected with 1.5 µCi  $^{45}\text{Ca}$  3 days prior to dissection. Following culture, the amounts of  $^{45}\text{Ca}$  in bones and culture media were analyzed by liquid scintillation. For time course experiments, mice were injected with 12.5 µCi  $^{45}\text{Ca}$  and radioactivity was analyzed at different time points by withdrawal of small amounts of culture media at the stated time points. Release of isotope was expressed as the percentage release of the initial amount of isotope, calculated as the sum of radioactivity in medium and bone after culture (Lerner, 1987). In some experiments, the data were recalculated and the results expressed as percent of control, which was set at 100%, allowing for accumulation of data from several experiments.

### **Bone matrix degradation assay**

Bone matrix degradation was assessed by analyzing the release of  $^3\text{H}$  from bones dissected from CsA mice prelabelled *in vivo* with 10 µCi of [ $^3\text{H}$ ]-proline four days prior to dissection. Following culture, bones were hydrolyzed and the radioactivity ( $^3\text{H}$ ) in the hydrolysates and culture media was analysed by liquid scintillation. The release of  $^3\text{H}$  ([ $^3\text{H}$ ]-proline + [ $^3\text{H}$ ]-hydroxyproline) parallels the release of [ $^3\text{H}$ ]-hydroxyproline and thus is a reliable indicator of collagen breakdown (Brand and Raisz, 1972).

### **Measurements of prostaglandin synthesis**

Prostaglandin formation in calvarial bones was assessed by analyzing the  $\text{PGE}_2$  concentration in culture media at the end of the cultures.  $\text{PGE}_2$  was determined using a commercially available radioimmunoassay (RIA) according to the instructions supplied by the manufacturer.

### **Statistical analysis**

Statistical analysis was performed using one-way analysis of variance (ANOVA) with Levene's homogeneity test and subsequent Bonferroni's, Dunnett's 2-sided or Dunnett's T3 post hoc test. In addition, the non-parametric Kruskal-Wallis and Mann-Whitney tests were used.

## RESULTS AND DISCUSSION

### Effects of IL-6 type cytokines on mouse calvarial bone resorption and RANK, RANKL and OPG interactions

The first task of the project was to elucidate the effects of IL-6 on bone resorption, since previous reports had demonstrated conflicting results where IL-6, LIF and OSM had been reported to exert both inhibitory and stimulatory effects on osteoclastogenesis and bone resorption. Initially, the effect of IL-6 on neonatal mouse calvarial bone resorption was studied. However, neither human nor mouse IL-6 induced bone resorption in this system, as assessed by the release of  $^{45}\text{Ca}$ , reflecting mineral release, or  $^3\text{H}$ , reflecting matrix degradation, from prelabelled bones. The results were in line with previous reports (al-Humidan *et al.*, 1991; Holt *et al.*, 1994). When IL-6 was combined with its soluble receptor (sIL-6R), however, both  $^{45}\text{Ca}$  and  $^3\text{H}$  release from calvarial bones were stimulated. The enhanced mineral release from bones was concentration-dependent with respect to concentration of both IL-6 and sIL-6R, and increased over time up to 72 h. Previous reports have similarly shown that IL-6 stimulated osteoclast formation in bone marrow cultures only when combined with sIL-6R (Tamura *et al.*, 1993). RT-PCR-analysis revealed low IL-6R mRNA expression levels in both calvarial bones and calvarial osteoblasts, which probably explains the absent effects on bone resorption by IL-6 alone (Hofbauer *et al.*, 1999; Naruishi *et al.*, 1999).

The enhanced  $^{45}\text{Ca}$  release induced by IL-6 + sIL-6R was abolished by three mechanistically different inhibitors of osteoclast activity (calcitonin, acetazolamide and AHPBP) and mRNA expression of the osteoclastic marker TRAP was induced in calvarial bones stimulated by IL-6 + IL-6R. Taken together these results indicate that the bone resorptive effects of IL-6 + sIL-6R were mediated by osteoclasts.

Since IL-6 has been suggested to be a mediator of excessive bone resorption in hyperparathyroidism (Grey *et al.*, 1996) and both IL-1 $\beta$  and PTH have been shown to stimulate IL-6 release in mouse calvarial bones (own unpublished observations) (Lacey *et al.*, 1993) there was a possibility that the effects of these agents were mediated by IL-6. However, the effects of IL-1  $\beta$  and PTH on  $^{45}\text{Ca}$  release were unaffected by the presence of sIL-6R and similarly unaffected by the presence of a neutralizing gp130 antibody, indicating that the effects of IL-1  $\beta$  and PTH on  $^{45}\text{Ca}$  release in this model were not mediated by IL-6 release in bones.

The fact that an antibody neutralizing gp130 inhibited  $^{45}\text{Ca}$ -release induced by IL-6 + sIL-6R and has also been shown to inhibit  $^{45}\text{Ca}$ -release stimulated by IL-11 (Ahlen *et al.*, 2002), whereas no effect on  $^{45}\text{Ca}$ -release induced by human OSM, mouse OSM or human LIF was noted, may be explained by interference of the antibody with homodimerization in IL-6 and IL-11 signalling compared to heterodimerization in LIF and OSM signalling.

Moreover, IL-6 + sIL-6R treatment of mouse calvariae resulted in significantly, 4-fold, increased PGE<sub>2</sub> formation, and two different inhibitors of cycloxygenase activity, indomethacin and flurbiprofen, inhibited <sup>45</sup>Ca release by 40%, suggesting that part of the bone resorptive effect induced by IL-6 + sIL-6R was prostaglandin independent.

We further evaluated if the stimulatory effects of IL-6 + sIL-6R on bone resorption act via RANK, RANKL, OPG interactions. IL-6 + sIL-6R increased both RANKL and OPG mRNA and protein expressions in calvarial bones whereas RANK mRNA expression was decreased. The fact that the induced effects on both RANKL mRNA and protein expression were more efficient than those on OPG probably explains the demonstrated stimulatory effect on bone resorption induced by IL-6 + sIL-6R in this system. Moreover, the dual stimulatory effects of IL-6 + sIL-6R on both RANKL and OPG most likely explain why IL-6 + sIL-6R less efficiently stimulate bone resorption compared to PTH or D3 since the latter two increase RANKL whereas OPG is decreased. OPG concentration-dependently inhibited <sup>45</sup>Ca release induced by IL-6 + sIL-6R which brings further support to the view that RANK, RANKL, OPG interactions are involved in IL-6 + sIL-6R stimulated bone resorption. These observations are supported by a study demonstrating enhanced RANKL expression in osteoblasts/stromal cells stimulated by IL-6 type cytokines (O'Brien *et al.*, 1999).

Similar to IL-6 + sIL-6R, the two other IL-6 family members studied, leukemia inhibitory factor (LIF) and oncostatin M (OSM), were found to concentration-dependently stimulate calvarial bone resorption, effects which were inhibited by OPG. Moreover, in similarity to the effects induced by IL-6 + sIL-6R, enhanced mRNA expressions of RANKL and OPG were noted in mouse calvarial bones following treatment with human OSM, mouse OSM and human LIF. Mouse OSM was also shown to stimulate RANKL and OPG protein synthesis in calvarial bones. In contrast to the inhibitory effects induced by IL-6 + sIL-6R, none of the latter three cytokines altered RANK mRNA expression.

Human OSM and human LIF showed similar efficiency in inducing <sup>45</sup>Ca-release and mRNA expression of RANKL and OPG in calvariae while the efficiency in inducing <sup>45</sup>Ca-release exerted by mouse OSM appeared to be slightly higher. The explanation may be the fact that human OSM and mouse OSM activate different receptors in mice. Thus, murine and human LIF, as well as human OSM, bind to the mouse LIF receptor in mice, whereas mouse OSM binds to the mouse OSM receptor (OSMR $\beta$ ). The observation that mouse OSM more efficiently stimulated <sup>45</sup>Ca-release, compared to the effect by human OSM, may be explained by the more abundant mRNA expression of OSMR than LIFR in both calvariae and osteoblasts isolated from calvarial bones. Later studies in our group have further explored the different effects of m LIF and m OSM, and m OSM was confirmed to be a more efficient stimulator of <sup>45</sup>Ca-release than m LIF. One explanation for the more efficient signalling through the OSMR $\beta$  appears to be OSMR $\beta$  autostimulation by m OSM in osteoblasts, and another may be the use of different intracellular signalling pathways, as m OSM more prominently induced STAT3 activation compared to LIF and in addition activated ERK1/2, in contrast to m LIF.

To conclude the first paper, IL-6, in combination with sIL-6R, LIF and OSM were all stimulators of bone resorption in mouse calvarial bones *in vitro*. The stimulatory effects on bone resorption involved regulation of RANKL and OPG in osteoblasts.

### **Effects of IL-4 and IL-13 on osteoclastogenesis and mouse calvarial bone resorption - regulation of RANK, RANKL, OPG and STAT6 dependency**

The anti-inflammatory cytokine IL-4 has previously been shown to inhibit bone resorption in neonatal mouse calvariae stimulated by IL-11 (Ahlen *et al.*, 2002) and in mouse long bones stimulated by PTH, D3, IL-1 and PGE<sub>2</sub> (Watanabe *et al.*, 1990), and initially the effects of these two cytokines on bone resorption, as assessed by <sup>45</sup>Ca-release from cultured neonatal mouse calvarial bones, were examined. Results showed that bone resorption stimulated by several cytokines, peptides and steroid hormones, i.e. PTH, D3, IL-1 $\beta$ , TNF- $\alpha$ , IL-6 + sIL-6R, OSM and RANKL, was inhibited by both IL-4 and IL-13. IL-4 and IL-13 were more efficient inhibitors of cytokine-stimulated bone resorption than resorption stimulated by peptide and steroid hormones. Furthermore, the inhibitory effects of IL-4 and IL-13 were concentration-dependent and the two cytokines were equipotent in D3 as well as RANKL stimulated bones.

In organ culture studies, the inhibitory effect of IL-4 on bone resorption has been suggested to be due to inhibition of cyclo oxygenase-2 expression in osteoblasts, thus decreasing prostaglandin synthesis in these cells (Kawaguchi *et al.*, 1996; Onoe *et al.*, 1996). Our results revealed that bone resorption stimulated by IL-1 $\beta$ , IL-6 + sIL-6R and OSM was partly affected by the cyclo oxygenase inhibitor indomethacin. In contrast, bone resorption stimulated by PTH, D3, TNF- $\alpha$  and RANKL was unaffected by the cyclooxygenase inhibitor indomethacin, whereas inhibition was noted by IL-4 and IL-13. Our observations suggest that the inhibitory effects of IL-4 and IL-13 on bone resorption are both dependent and independent of cyclo oxygenase-2 inhibition.

The inhibitory effects of IL-4 on bone resorption *in vitro* are believed to involve decreased osteoclastogenesis and IL-4 has been shown to inhibit osteoclast formation in mouse bone marrow cultures as well as co-cultures of mouse spleen cells and ST-2 stromal cells (Kasano *et al.*, 1993; Riancho *et al.*, 1993; Shioi *et al.*, 1991). One study has demonstrated inhibition of mature osteoclast activity in cell cultures by IL-4, but not by IL-13 (Mangashetti *et al.*, 2005). In our study, the inhibitory effect of calcitonin (CT) on bone resorption was lost over time, whereas the inhibitory effect of IL-4 persisted in long term cultures up to 120 hours. Unlike CT, IL-4 failed to inhibit bone resorption in bones where resorption was prestimulated by D3, suggesting that IL-4 does not affect the activity of mature osteoclasts but inhibits differentiation of mononuclear osteoclast progenitor cells. The reasons for the different findings in our study and the study of Mangashetti *et al.* are not clear, although one possible explanation may be differential receptor expression in the different systems. In support of the notion that IL-4 inhibits differentiation of mononuclear osteoclast progenitor cells were results demonstrating decreased mRNA expression levels of TRAP and CTR in unstimulated and D3 stimulated calvariae treated with IL-4 and IL-13. Similar inhibitions were observed in RANKL-stimulated monocytic/macrophage RAW 264.7

cells and M-CSF/RANKL stimulated spleen cells. In addition, mRNA expression levels of the osteoclast marker cathepsin K in spleen cell cultures and BMM were decreased by IL-4 and IL-13, and the increased formation of TRAP<sup>+</sup> OCL in M-CSF/RANKL stimulated mouse spleen cells and BMM was inhibited by IL-4 and IL-13.

The fact that IL-4 and IL-13 inhibited bone resorption induced by many different stimulators, together with data indicating inhibitory effects by IL-4 and IL-13 on osteoclast progenitor cell differentiation suggested that the two cytokines could be interfering with a commonly used, essential mechanism in osteoclast differentiation and bone resorption. RANK, RANKL, OPG interactions were plausible targets, which made us study the effects of IL-4 and IL-13 on the expression levels of RANK, RANKL and OPG in calvarial bones.

RANK mRNA expression in both unstimulated calvarial bones and bones stimulated by D3 was inhibited by IL-4 and IL-13, and similar inhibitory effects were noted in mouse spleen cells, BMM and RAW 264.7 cells. In RAW 264.7 cells, inhibition of RANK mRNA by IL-4 was shown to be concentration-dependent, and in addition IL-4 was shown to inhibit cell surface expression of RANK protein. Together these results indicate that IL-4 and IL-13 receptors are present on osteoclast progenitor cells and that activation of these decrease RANK expression.

In support of this view, IL-4 was reported to irreversibly inhibit osteoclast formation in mouse marrow cells, human CD14<sup>+</sup> monocytes and RAW 264.7 cells by a mechanism involving decreased RANK mRNA expression. Interestingly, the study showed that IL-4 was unable to inhibit osteoclast formation in RAW 264.7 cells overexpressing RANK, controlled by a cytomegalovirus promoter (Moreno *et al.*, 2003). The authors proposed that the mechanism by which IL-4 inhibits RANK may involve STAT6 binding to the promoter of RANK.

The inhibitory effects by IL-4 on RANK expression in osteoclast progenitor cells may be cell specific, since stimulatory effects by IL-4 on RANK expression in activated human peripheral blood T-lymphocytes has been reported (Anderson *et al.*, 1997).

Moreover, the effects on osteoclastogenesis exerted by IL-4 have been suggested to be indirect and mediated by T-cells (Miroslavljevic *et al.*, 2003). However, our data show direct effects on RAW cells and the BMM cells used were shown to be free from T-cells, which together suggest that IL-4 acted directly on osteoprogenitor cells.

The stimulated osteoclastogenesis in spleen cell cultures and BMM was also associated with increased mRNA levels of the master transcription factor for osteoclastogenesis, nuclear factor of activated T-cells 2 (NFAT2), which was decreased by IL-4 and IL-13. These observations suggest that the ITAM-harboursing adaptor molecules FcR $\gamma$  and DAP 12 may also be inhibited by the two cytokines, as these proteins are upstream regulators of ITAM mediated regulation of calcium signalling, which regulates NFAT2. In agreement with these findings, preliminary data

from our laboratory (Persson *et al.*) have revealed that osteoclast-associated receptor (OSCAR) and DAP12 mRNA in mouse spleen cells and BMM is inhibited by IL-4 and IL-13, results indicating that IL-4 and IL-13 inhibit osteoclast differentiation not only by actions on RANK but also on the co-stimulatory immunoglobulin-like receptors/FcR $\gamma$ /DAP12 pathway. Moreover, the latter mechanism was shown to be STAT6 dependent.

Osteoclastogenesis and bone resorption are dependent on RANK expression by osteoclast progenitor cells but also on RANKL and OPG expression by stromal cells/osteoblasts (Lerner, 2000). In D3 stimulated calvarial bones the increased mRNA and protein expressions of RANKL were inhibited by IL-4 and IL-13 whereas the decreased mRNA and protein expressions of OPG were counteracted by IL-4 and IL-13. Enhanced OPG protein levels were also noted in unstimulated bones treated with IL-4 and IL-13. In agreement with the calvarial data, results in D3 stimulated mouse calvarial osteoblasts showed inhibitory effects by IL-4 and IL-13 on the increased RANKL mRNA levels whereas the decreased OPG mRNA and protein expressions in the osteoblasts were reversed by IL-4 and IL-13. However, the protein levels of RANKL in both unstimulated and stimulated osteoblasts were beneath the detection limit of the ELISA method used.

The observations suggest that IL-4 and IL-13 receptors are present on osteoblasts and that the two cytokines activate the receptors, thus decreasing the ability of osteoblasts/stromal cells to induce osteoclastogenesis. In line with these observations, IL-4 has been shown to decrease RANKL and increase OPG expression in human fibroblast-like synoviocytes in rheumatoid arthritis and to inhibit osteoclast formation in co-cultures of synoviocytes and human peripheral blood mononuclear cells (Lee *et al.*, 2004). Interestingly, IL-4 and IL-13 also stimulate OPG mRNA expression in human gingival fibroblasts (own unpublished observations).

The effects of IL-4 and IL-13 on mRNA expression of RANKL, RANK and OPG suggested that IL-4 and IL-13 receptors were present in both osteoblasts and osteoclast progenitor cells. In agreement with this notion, constitutional mRNA expression of the four components of the IL-4 and IL-13 receptor subtypes, i.e.  $\gamma c$ , IL-4R $\alpha$ , IL-13R $\alpha 1$  and IL-13R $\alpha 2$ , was demonstrated in mouse calvarial osteoblasts, mouse spleen cells and in mouse BMM cells.

IL-4 and IL-13 receptors have been demonstrated to regulate gene expression by mechanisms dependent on homodimerization of the transcription factor STAT6 (Kelly-Welch *et al.*, 2003). To explore the involvement of the transcription factor STAT6 in the effects of IL-4 and IL-13 on bone resorption, osteoclast formation and mRNA expression of RANKL and RANK, we compared the effects of IL-4 and IL-13 in *stat6*<sup>-/-</sup> mice, *stat4*<sup>-/-</sup> mice, which were used as controls, and *wt* mice. Results showed that no inhibition of bone resorption by IL-4 and IL-13 occurred in D3 or IL-1 $\beta$  stimulated calvariae from *stat6*<sup>-/-</sup> mice, in contrast to the inhibition noted in *stat4*<sup>-/-</sup> and *wt* mice. Similarly, IL-4 and IL-13 failed to decrease D3 stimulated RANKL and RANK mRNA expression levels in calvariae from *stat6*<sup>-/-</sup> mice in

contrast to *wt* mice. In M-CSF/RANKL stimulated spleen cell cultures, IL-4 and IL-13 failed to inhibit osteoclast formation, mRNA expression levels of the osteoclastic markers CTR, TRAP (own unpublished observations) and cathepsin K as well as mRNA expression of RANK in *stat6*<sup>-/-</sup> mice, in contrast to the other two genotypes. The results suggest that STAT6 is an essential mediator of the inhibitory effects of IL-4 and IL-13 on osteoclast formation and bone resorption.

Taken together, the results of the second paper indicate that IL-4 and IL-13 can inhibit osteoclast differentiation and bone resorption both by activating receptors on osteoclastic cells, leading to decreased RANK formation in osteoclast progenitor cells, and by activating receptors on osteoblasts, causing decreased RANKL formation and increased OPG formation. Both mechanisms are dependent on STAT6 activation by the two cytokines.

### **Expression and regulation of IL-6 type cytokines in human gingival fibroblasts**

Since IL-6 type cytokines are stimulators of bone resorption and are expressed not only by mononuclear leukocytes but also by resident cells we hypothesized that gingival fibroblasts, being the most abundant cells in healthy gingival tissue, may influence the pathogenesis of periodontal disease by IL-6 type cytokine expression. Human gingival fibroblasts from non-inflammatory gingival explants from nine individuals demonstrated mRNA expression of  $\alpha$ (1)-collagen I, alkaline phosphatase and bone morphogenetic protein-2, whereas no expression of osteocalcin or bone sialoprotein was noted. In contrast, these genes were all expressed in primary human osteoblasts, indicating that the cells used expressed a fibroblastic rather than a periodontal ligament cell or osteoblastic phenotype. Results also revealed constitutional mRNA expression of IL-6, LIF and another member of the IL-6 family of cytokines, IL-11, whereas no OSM expression was noted. Gingival fibroblasts stimulated by the pro-inflammatory cytokines IL-1 $\beta$  or TNF- $\alpha$  showed increased mRNA expression of IL-6 and LIF in isolates from five out of five individuals whereas increased IL-11 mRNA was noted in five out of five isolates treated with IL-1 $\beta$ . Furthermore, IL-1 $\beta$  and TNF- $\alpha$  were shown to concentration-dependently stimulate mRNA and protein expressions of IL-6 and LIF, whereas IL-1 $\beta$ , but not TNF- $\alpha$ , concentration-dependently stimulated IL-11 mRNA and protein expression. The observations that IL-6 and LIF were enhanced by IL-1 $\beta$  and TNF- $\alpha$  whereas IL-11 was uniquely enhanced by IL-1 $\beta$  suggest that IL-1 $\beta$  and TNF- $\alpha$  regulate IL-6 type cytokine expression by different molecular mechanisms. The results of this study are in line with previous reports demonstrating enhanced IL-6 expression levels in human gingival fibroblasts stimulated by IL-1 $\beta$  and TNF- $\alpha$  (Kent *et al.*, 1998; Modeer *et al.*, 1998). Moreover, mRNA expressions of IL-1R1, TNFRI and TNFRII were shown in the fibroblasts, which is in line with the cellular responses to the two cytokines. Expression of IL-11 in human gingival fibroblasts has been demonstrated previously (Yashiro *et al.*, 2006) and these reports were confirmed by the present study. In addition, IL-11 mRNA and protein expression was shown to be stimulated by IL-1 $\beta$ . LIF or OSM expression in human gingival fibroblasts has not been reported previously, although detected in gingival crevicular fluids (GCFs) from diseased

periodontal pockets of periodontitis patients (Lin *et al.*, 2005; Sakai *et al.*, 2006). Our observations suggest that LIF production by gingival fibroblasts may be one source of LIF detected in GCFs.

In RA, resident cells such as fibroblast-like synovial cells have been suggested to influence bone destruction through their production of osteolytic factors (Walsh and Gravallese, 2004), and the noted expressions of IL-6, IL-11 and LIF, but not OSM, in the human gingival fibroblasts in this study are in line with similar observations in fibroblast cell lines derived from human synovium of RA patients (Okamoto *et al.*, 1997).

Treatment strategies targeting locally produced inflammatory cytokines have been shown to successfully retard tissue degeneration in rheumatoid arthritis (Walsh and Gravallese, 2004) and anti-cytokine therapies targeting IL-6 have been proposed to be useful in treatment of inflammatory osteolytic conditions (Ancey *et al.*, 2003).

Interestingly, blocking of IL-6 signalling has been shown to prevent tissue erosion in animal models of arthritis (Gabay, 2006) and a randomized clinical trial of an IL-6 receptor antagonist has shown successful results decreasing tissue destruction in rheumatoid arthritis (Maini *et al.*, 2006).

Taken together, the findings demonstrate osteotropic IL-6 type cytokine production by resident human gingival fibroblasts, a production which is further enhanced by pro-inflammatory cytokines. These observations support the view that resident cells may influence tissue remodelling in periodontal disease.

### **Regulation of IL-6 type cytokines in human gingival fibroblasts by IL-4 and IL-13**

IL-4 and IL-13 are known to have anti-inflammatory properties and to be inhibitors of osteoclastogenesis and bone resorption. We studied the effects of IL-4 and IL-13 on osteotropic IL-6 type cytokine expression in human gingival fibroblasts, in order to study possible modulatory actions of these cytokines in periodontal tissues.

In our study, no constitutional mRNA expression of IL-4 and IL-13 was noted in fibroblasts isolated from seven patients and none was observed in five fibroblast isolates stimulated by IL-1 $\beta$  and TNF- $\alpha$ .

As shown in the previous study, mRNA and protein levels of IL-6 were induced by IL-1 $\beta$  and TNF- $\alpha$ . However, the stimulated IL-6 mRNA and protein expressions remained unaffected by IL-4 and IL-13. In contrast, the LIF mRNA and protein expression levels stimulated by IL-1 $\beta$  and TNF- $\alpha$  were inhibited by IL-4 and IL-13. Similar results were demonstrated in MG-63 cells, a human osteoblastic osteosarcoma cell line (own unpublished observations). The increased expression of IL-11 mRNA and protein induced by IL-1 $\beta$  was reversed by IL-4 and IL-13. Results showed that the gingival fibroblasts were responsive to IL-4 and IL-13. Thus IL-11 and LIF secretion by human gingival fibroblasts is negatively regulated by IL-4 and IL-13, observations suggesting a modulatory role for IL-4 and IL-13 on osteotropic cytokine production by resident mesenchymal cells. In line with these results is a report demonstrating

increased production of intracellular IL-1 $\alpha$  in human gingival fibroblasts stimulated with IL-1 $\beta$  or TNF- $\alpha$ , levels which are decreased by IL-4 (Kobayashi *et al.*, 1999). Furthermore, IL-4 and IL-13 have been reported to inhibit IL-1 $\beta$ , TNF- $\alpha$  and IL-6 production by synovial cells, and in addition IL-1 receptor antagonist was enhanced by IL-4 and IL-13 in this study (Morita *et al.*, 2001). Interestingly, IL-4 and IL-13 have been shown to stimulate OPG mRNA expression in human gingival fibroblasts (own unpublished observations) and moreover, when studying the effects of IL-4 on extracellular matrix metabolism, IL-4 was reported to stimulate the production of proteoglycans (Hashimoto-Uoshima *et al.*, 2002). The potential roles of IL-4 and IL-13 in periodontal disease are not known but, as shown previously, IL-4 and IL-13 are inhibitors of osteoclastogenesis and bone resorption stimulated by several different mediators. Moreover, IL-4 inhibits the expression of matrix metalloproteinase-3 in human gingival fibroblasts (Kunii *et al.*, 2005). Together these observations imply that IL-4 and IL-13 may function as inhibitors of degradation of both ECM of soft tissues and mineralized bone. In support of this notion, IL-4 expression in gingival tissue has been shown to decrease with severity of inflammation and collagen type I degradation (Ejeil *et al.*, 2003), suggesting that progression of periodontal inflammation may be due to a lack of, or inappropriate response of, anti-inflammatory cytokines.

The data presented in this study indicate that IL-4 and IL-13 may inhibit bone resorption not only by mechanisms involving direct effects on osteoblasts and osteoclastic cells affecting the RANK, RANKL, OPG system, as demonstrated in paper II, but also indirectly, by inhibition of osteotropic cytokines released from resident mesenchymal cells.

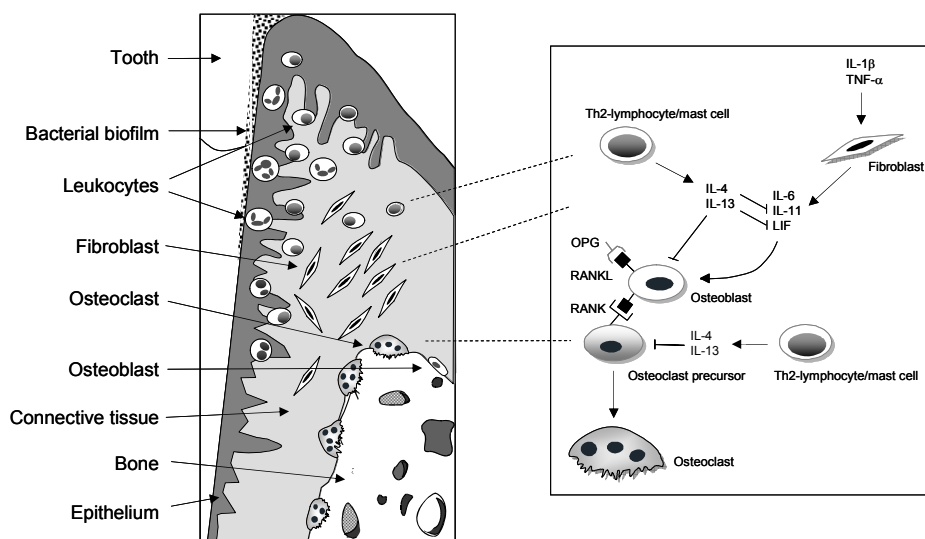


Fig. 3. IL-6 type cytokine release by gingival fibroblasts and regulation by IL-4 and IL-13.

## CONCLUSIONS

The observations demonstrated in the present thesis show that IL-6, LIF and OSM, which are all members of the IL-6 family of cytokines, exert resorptive effects in calvarial bones primarily by effects on osteoblasts altering RANKL and OPG expression.

Moreover, IL-4 and IL-13 inhibit bone resorption via receptors on both osteoblasts and osteoclast progenitor cells in turn regulating RANK, RANKL and OPG interactions. Both effects in osteoblasts and effects in osteoclast progenitor cells are mediated by STAT6.

Furthermore, IL-6, IL-11 and LIF are expressed in gingival fibroblasts, and expression of these cytokines is further enhanced by the pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  and inhibited by IL-4 and IL-13. These findings provide further support to the notion that resident cells may influence the pathogenesis of periodontal disease through cytokine production.

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## REFERENCES

- Abu-Amer Y (2001). IL-4 abrogates osteoclastogenesis through STAT6-dependent inhibition of NF-kappaB. *J Clin Invest* 107(11):1375-85.
- Ahlen J, Andersson S, Mukohyama H, Roth C, Backman A, Conaway HH, *et al.* (2002). Characterization of the bone-resorptive effect of interleukin-11 in cultured mouse calvarial bones. *Bone* 31(1):242-51.
- al-Humidan A, Ralston SH, Hughes DE, Chapman K, Aarden L, Russell RG, *et al.* (1991). Interleukin-6 does not stimulate bone resorption in neonatal mouse calvariae. *J Bone Miner Res* 6(1):3-8.
- Allen MR, Hock JM, Burr DB (2004). Periosteum: biology, regulation, and response to osteoporosis therapies. *Bone* 35(5):1003-12.
- Alonzi T, Fattori E, Lazzaro D, Costa P, Probert L, Kollias G, *et al.* (1998). Interleukin 6 is required for the development of collagen-induced arthritis. *J Exp Med* 187(4):461-8.
- Ancey C, Kuster A, Haan S, Herrmann A, Heinrich PC, Muller-Newen G (2003). A fusion protein of the gp130 and interleukin-6Ralpha ligand-binding domains acts as a potent interleukin-6 inhibitor. *J Biol Chem* 278(19):16968-72.
- Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, *et al.* (1997). A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* 390(6656):175-9.
- Angel NZ, Walsh N, Forwood MR, Ostrowski MC, Cassady AI, Hume DA (2000). Transgenic mice overexpressing tartrate-resistant acid phosphatase exhibit an increased rate of bone turnover. *J Bone Miner Res* 15(1):103-10.
- Asagiri M, Takayanagi H (2006). The molecular understanding of osteoclast differentiation. *Bone*.
- Aubin JE (2001). Regulation of osteoblast formation and function. *Rev Endocr Metab Disord* 2(1):81-94.
- Auernhammer CJ, Melmed S (2000). Leukemia-inhibitory factor-neuroimmune modulator of endocrine function. *Endocr Rev* 21(3):313-45.
- Bartold PM, Walsh LJ, Narayanan AS (2000). Molecular and cell biology of the gingiva. *Periodontol* 2000 24(28-55).
- Baumann H, Symes AJ, Comeau MR, Morella KK, Wang Y, Friend D, *et al.* (1994). Multiple regions within the cytoplasmic domains of the leukemia inhibitory factor receptor and gp130 cooperate in signal transduction in hepatic and neuronal cells. *Mol Cell Biol* 14(1):138-46.

Belibasakis GN, Johansson A, Wang Y, Chen C, Kalfas S, Lerner UH (2005a). The cytolethal distending toxin induces receptor activator of NF-kappaB ligand expression in human gingival fibroblasts and periodontal ligament cells. *Infect Immun* 73(1):342-51.

Belibasakis GN, Johansson A, Wang Y, Chen C, Lagergard T, Kalfas S, *et al.* (2005b). Cytokine responses of human gingival fibroblasts to *Actinobacillus actinomycetemcomitans* cytolethal distending toxin. *Cytokine* 30(2):56-63.

Bellido T, Stahl N, Farruggella TJ, Borba V, Yancopoulos GD, Manolagas SC (1996). Detection of receptors for interleukin-6, interleukin-11, leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor in bone marrow stromal/osteoblastic cells. *J Clin Invest* 97(2):431-7.

Bendixen AC, Shevde NK, Dienger KM, Willson TM, Funk CD, Pike JW (2001). IL-4 inhibits osteoclast formation through a direct action on osteoclast precursors via peroxisome proliferator-activated receptor gamma 1. *Proc Natl Acad Sci U S A* 98(5):2443-8.

Berglundh T, Liljenberg B, Lindhe J (2002). Some cytokine profiles of T-helper cells in lesions of advanced periodontitis. *J Clin Periodontol* 29(8):705-9.

Bilezikian JP, Raisz LG, Rodan GA, editors (2002). Principles of Bone Biology. San Diego, USA: Academic Press.

Birkedal-Hansen H (1993). Role of matrix metalloproteinases in human periodontal diseases. *J Periodontol* 64(5 Suppl):474-84.

Bourette RP, Rohrschneider LR (2000). Early events in M-CSF receptor signaling. *Growth Factors* 17(3):155-66.

Boyle WJ, Simonet WS, Lacey DL (2003). Osteoclast differentiation and activation. *Nature* 423(6937):337-42.

Bozkurt FY, Yetkin Ay Z, Berker E, Tepe E, Akkus S (2006). Anti-inflammatory cytokines in gingival crevicular fluid in patients with periodontitis and rheumatoid arthritis: A preliminary report. *Cytokine* 35(3-4):180-5.

Brand JS, Raisz LG (1972). Effects of thyrocalcitonin and phosphate ion on the parathyroid hormone stimulated resorption of bone. *Endocrinology* 90(2):479-87.

Brown MA, Metcalf D, Gough NM (1994). Leukaemia inhibitory factor and interleukin 6 are expressed at very low levels in the normal adult mouse and are induced by inflammation. *Cytokine* 6(3):300-9.

Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, *et al.* (1998). osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 12(9):1260-8.

Buckwalter JA, Glimcher MJ, Cooper RR, Recker R (1996). Bone biology. I: Structure, blood supply, cells, matrix, and mineralization. *Instr Course Lect* 45(371-86).

- Burger D, Dayer JM (1995). Inhibitory cytokines and cytokine inhibitors. *Neurology* 45(6 Suppl 6):S39-43.
- Canalis E, Derogowski V, Pereira RC, Gaggero E (2005). Signals that determine the fate of osteoblastic cells. *J Endocrinol Invest* 28(8 Suppl):3-7.
- Cornish J, Callon K, King A, Edgar S, Reid IR (1993). The effect of leukemia inhibitory factor on bone in vivo. *Endocrinology* 132(3):1359-66.
- Dai XM, Ryan GR, Hapel AJ, Dominguez MG, Russell RG, Kapp S, *et al.* (2002). Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood* 99(1):111-20.
- Dai XM, Zong XH, Sylvestre V, Stanley ER (2004). Incomplete restoration of colony-stimulating factor 1 (CSF-1) function in CSF-1-deficient Csf1op/Csf1op mice by transgenic expression of cell surface CSF-1. *Blood* 103(3):1114-23.
- Darnay BG, Aggarwal BB (1999). Signal transduction by tumour necrosis factor and tumour necrosis factor related ligands and their receptors. *Ann Rheum Dis* 58 Suppl 1(I2-I13).
- Darnay BG, Ni J, Moore PA, Aggarwal BB (1999). Activation of NF-kappaB by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF-kappaB-inducing kinase. Identification of a novel TRAF6 interaction motif. *J Biol Chem* 274(12):7724-31.
- de Vries TJ, Schoenmaker T, Wattanaroonwong N, van den Hoonaard M, Nieuwenhuijse A, Beertsen W, *et al.* (2006). Gingival fibroblasts are better at inhibiting osteoclast formation than periodontal ligament fibroblasts. *J Cell Biochem* 98(2):370-82.
- Deporter DA, ten Cate AR (1980). Collagen resorption by periodontal ligament fibroblasts at the hard tissue-ligament interfaces of the mouse periodontium. *J Periodontol* 51(8):429-32.
- Dougall WC, Glaccum M, Charrier K, Rohrbach K, Brasel K, De Smedt T, *et al.* (1999). RANK is essential for osteoclast and lymph node development. *Genes Dev* 13(18):2412-24.
- Ducy P, Schinke T, Karsenty G (2000). The osteoblast: a sophisticated fibroblast under central surveillance. *Science* 289(5484):1501-4.
- Ejeil AL, Gaultier F, Igondjo-Tchen S, Senni K, Pellat B, Godeau G, *et al.* (2003). Are cytokines linked to collagen breakdown during periodontal disease progression? *J Periodontol* 74(2):196-201.
- Everts V, van der Zee E, Creemers L, Beertsen W (1996). Phagocytosis and intracellular digestion of collagen, its role in turnover and remodelling. *Histochem J* 28(4):229-45.
- Felix R, Hofstetter W, Wetterwald A, Cecchini MG, Fleisch H (1994). Role of colony-stimulating factor-1 in bone metabolism. *J Cell Biochem* 55(3):340-9.
- Franz-Odenaal TA, Hall BK, Witten PE (2006). Buried alive: how osteoblasts become osteocytes. *Dev Dyn* 235(1):176-90.

Gabay C (2006). Interleukin-6 and chronic inflammation. *Arthritis Res Ther* 8 Suppl 2(S3).

Galibert L, Tometsko ME, Anderson DM, Cosman D, Dougall WC (1998). The involvement of multiple tumor necrosis factor receptor (TNFR)-associated factors in the signaling mechanisms of receptor activator of NF-kappaB, a member of the TNFR superfamily. *J Biol Chem* 273(51):34120-7.

Gearing DP, Druck T, Huebner K, Overhauser J, Gilbert DJ, Copeland NG, *et al.* (1993). The leukemia inhibitory factor receptor (LIFR) gene is located within a cluster of cytokine receptor loci on mouse chromosome 15 and human chromosome 5p12-p13. *Genomics* 18(1):148-50.

Gemmell E, Seymour GJ (2004). Immunoregulatory control of Th1/Th2 cytokine profiles in periodontal disease. *Periodontol* 2000 35(21-41).

Girasole G, Jilka RL, Passeri G, Boswell S, Boder G, Williams DC, *et al.* (1992). 17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens. *J Clin Invest* 89(3):883-91.

Girasole G, Passeri G, Jilka RL, Manolagas SC (1994). Interleukin-11: a new cytokine critical for osteoclast development. *J Clin Invest* 93(4):1516-24.

Gorska R, Gregorek H, Kowalski J, Laskus-Perendyk A, Syczewska M, Madalinski K (2003). Relationship between clinical parameters and cytokine profiles in inflamed gingival tissue and serum samples from patients with chronic periodontitis. *J Clin Periodontol* 30(12):1046-52.

Graves DT, Cochran D (2003). The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. *J Periodontol* 74(3):391-401.

Greenfield EM, Gornik SA, Horowitz MC, Donahue HJ, Shaw SM (1993). Regulation of cytokine expression in osteoblasts by parathyroid hormone: rapid stimulation of interleukin-6 and leukemia inhibitory factor mRNA. *J Bone Miner Res* 8(10):1163-71.

Grey A, Mitnick MA, Shapses S, Ellison A, Gundberg C, Insogna K (1996). Circulating levels of interleukin-6 and tumor necrosis factor-alpha are elevated in primary hyperparathyroidism and correlate with markers of bone resorption--a clinical research center study. *J Clin Endocrinol Metab* 81(10):3450-4.

Grey A, Mitnick MA, Masiukiewicz U, Sun BH, Rudikoff S, Jilka RL, *et al.* (1999). A role for interleukin-6 in parathyroid hormone-induced bone resorption in vivo. *Endocrinology* 140(10):4683-90.

Harada S, Rodan GA (2003). Control of osteoblast function and regulation of bone mass. *Nature* 423(6937):349-55.

Hart PH, Vitti GF, Burgess DR, Whitty GA, Piccoli DS, Hamilton JA (1989). Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor alpha, interleukin 1, and prostaglandin E2. *Proc Natl Acad Sci U S A* 86(10):3803-7.

Hashimoto-Uoshima M, Noguchi K, Suzuki M, Murata A, Yanagishita M, Ishikawa I (2002). Effects of interleukin-4 on proteoglycan accumulation in human gingival fibroblasts. *J Periodontal Res* 37(1):42-9.

Hayman AR, Jones SJ, Boyde A, Foster D, Colledge WH, Carlton MB, *et al.* (1996). Mice lacking tartrate-resistant acid phosphatase (Acp 5) have disrupted endochondral ossification and mild osteopetrosis. *Development* 122(10):3151-62.

Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Graeve L (1998). Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J* 334 ( Pt 2)(297-314.

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F (2003). Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J* 374(Pt 1):1-20.

Hershey GK (2003). IL-13 receptors and signaling pathways: an evolving web. *J Allergy Clin Immunol* 111(4):677-90; quiz 691.

Heymann D, Rousselle AV (2000). gp130 Cytokine family and bone cells. *Cytokine* 12(10):1455-68.

Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S (1999). Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. *Bone* 25(3):255-9.

Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL (2000). The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. *J Bone Miner Res* 15(1):2-12.

Holla LI, Fassmann A, Stejskalova A, Znojil V, Vanek J, Vacha J (2004). Analysis of the interleukin-6 gene promoter polymorphisms in Czech patients with chronic periodontitis. *J Periodontol* 75(1):30-6.

Holt I, Davie MW, Braidman IP, Marshall MJ (1994). Interleukin-6 does not mediate the stimulation by prostaglandin E2, parathyroid hormone, or 1,25 dihydroxyvitamin D3 of osteoclast differentiation and bone resorption in neonatal mouse parietal bones. *Calcif Tissue Int* 55(2):114-9.

Honda T, Domon H, Okui T, Kajita K, Amanuma R, Yamazaki K (2006). Balance of inflammatory response in stable gingivitis and progressive periodontitis lesions. *Clin Exp Immunol* 144(1):35-40.

Howard M, Paul WE (1982). Interleukins for B lymphocytes. *Lymphokine Res* 1(1):1-4.

Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, *et al.* (1999). Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci U S A* 96(7):3540-5.

Ichihara M, Hara T, Kim H, Murate T, Miyajima A (1997). Oncostatin M and leukemia inhibitory factor do not use the same functional receptor in mice. *Blood* 90(1):165-73.

- Idzerda RL, March CJ, Mosley B, Lyman SD, Vanden Bos T, Gimpel SD, *et al.* (1990). Human interleukin 4 receptor confers biological responsiveness and defines a novel receptor superfamily. *J Exp Med* 171(3):861-73.
- Ishimi Y, Miyaura C, Jin CH, Akatsu T, Abe E, Nakamura Y, *et al.* (1990). IL-6 is produced by osteoblasts and induces bone resorption. *J Immunol* 145(10):3297-303.
- Isomaki P, Punnonen J (1997). Pro- and anti-inflammatory cytokines in rheumatoid arthritis. *Ann Med* 29(6):499-507.
- Ito H, Honda T, Domon H, Oda T, Okui T, Amanuma R, *et al.* (2005). Gene expression analysis of the CD4+ T-cell clones derived from gingival tissues of periodontitis patients. *Oral Microbiol Immunol* 20(6):382-6.
- Ito S, Wakabayashi K, Ubukata O, Hayashi S, Okada F, Hata T (2002). Crystal structure of the extracellular domain of mouse RANK ligand at 2.2-Å resolution. *J Biol Chem* 277(8):6631-6.
- Jay PR, Centrella M, Lorenzo J, Bruce AG, Horowitz MC (1996). Oncostatin-M: a new bone active cytokine that activates osteoblasts and inhibits bone resorption. *Endocrinology* 137(4):1151-8.
- Jilka RL, Hangoc G, Girasole G, Passeri G, Williams DC, Abrams JS, *et al.* (1992). Increased osteoclast development after estrogen loss: mediation by interleukin-6. *Science* 257(5066):88-91.
- Johnson RB, Serio FG (2005). Interleukin-18 concentrations and the pathogenesis of periodontal disease. *J Periodontol* 76(5):785-90.
- Jones SA, Horiuchi S, Topley N, Yamamoto N, Fuller GM (2001). The soluble interleukin 6 receptor: mechanisms of production and implications in disease. *Faseb J* 15(1):43-58.
- Joosten LA, Lubberts E, Helsen MM, Saxne T, Coenen-de Roo CJ, Heinegard D, *et al.* (1999). Protection against cartilage and bone destruction by systemic interleukin-4 treatment in established murine type II collagen-induced arthritis. *Arthritis Res* 1(1):81-91.
- Kartsogiannis V, Zhou H, Horwood NJ, Thomas RJ, Hards DK, Quinn JM, *et al.* (1999). Localization of RANKL (receptor activator of NF kappa B ligand) mRNA and protein in skeletal and extraskeletal tissues. *Bone* 25(5):525-34.
- Kasono K, Sato K, Sato Y, Tsushima T, Shizume K, Demura H (1993). Inhibitory effect of interleukin-4 on osteoclast-like cell formation in mouse bone marrow culture. *Bone Miner* 21(3):179-88.
- Katagiri T, Takahashi N (2002). Regulatory mechanisms of osteoblast and osteoclast differentiation. *Oral Dis* 8(3):147-59.
- Kawaguchi H, Nemoto K, Raisz LG, Harrison JR, Voznesensky OS, Alander CB, *et al.* (1996). Interleukin-4 inhibits prostaglandin G/H synthase-2 and cytosolic phospholipase A2 induction in neonatal mouse parietal bone cultures. *J Bone Miner Res* 11(3):358-66.

- Kawasaki K, Gao YH, Yokose S, Kaji Y, Nakamura T, Suda T, *et al.* (1997). Osteoclasts are present in gp130-deficient mice. *Endocrinology* 138(11):4959-65.
- Kelly-Welch AE, Hanson EM, Boothby MR, Keegan AD (2003). Interleukin-4 and interleukin-13 signaling connections maps. *Science* 300(5625):1527-8.
- Kent LW, Rahemtulla F, Hockett RD, Jr., Gilleland RC, Michalek SM (1998). Effect of lipopolysaccharide and inflammatory cytokines on interleukin-6 production by healthy human gingival fibroblasts. *Infect Immun* 66(2):608-14.
- Kent LW, Rahemtulla F, Michalek SM (1999). Interleukin (IL)-1 and Porphyromonas gingivalis lipopolysaccharide stimulation of IL-6 production by fibroblasts derived from healthy or periodontally diseased human gingival tissue. *J Periodontol* 70(3):274-82.
- Kinane DF (2001). Causation and pathogenesis of periodontal disease. *Periodontol* 2000 25(8-20).
- Kinane DF, Lappin DF (2001). Clinical, pathological and immunological aspects of periodontal disease. *Acta Odontol Scand* 59(3):154-60.
- Kishimoto T, Akira S, Taga T (1992). Interleukin-6 and its receptor: a paradigm for cytokines. *Science* 258(5082):593-7.
- Kishimoto T, Akira S, Narazaki M, Taga T (1995a). Interleukin-6 family of cytokines and gp130. *Blood* 86(4):1243-54.
- Kishimoto T, Tanaka T, Yoshida K, Akira S, Taga T (1995b). Cytokine signal transduction through a homo- or heterodimer of gp130. *Ann N Y Acad Sci* 766(224-34).
- Klein-Nulend J, Nijweide PJ, Burger EH (2003). Osteocyte and bone structure. *Curr Osteoporos Rep* 1(1):5-10.
- Knothe Tate ML, Adamson JR, Tami AE, Bauer TW (2004). The osteocyte. *Int J Biochem Cell Biol* 36(1):1-8.
- Kobayashi M, Okada N, Okamatsu Y, Mugikura K, Nishihara T, Hanazawa S, *et al.* (1999). Intracellular interleukin-1 alpha production in human gingival fibroblasts is differentially regulated by various cytokines. *J Dent Res* 78(4):840-9.
- Koga T, Inui M, Inoue K, Kim S, Suematsu A, Kobayashi E, *et al.* (2004). Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. *Nature* 428(6984):758-63.
- Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, *et al.* (1999). OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 397(6717):315-23.
- Kopf M, Baumann H, Freer G, Freudenberg M, Lamers M, Kishimoto T, *et al.* (1994). Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature* 368(6469):339-42.

Kotake S, Sato K, Kim KJ, Takahashi N, Udagawa N, Nakamura I, *et al.* (1996). Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation. *J Bone Miner Res* 11(1):88-95.

Kumar V, Abbas AK, Fausto N, editors (2005). Robbins and Cotran Pathologic Basis of Disease

Kunii R, Nemoto E, Kanaya S, Tsubahara T, Shimauchi H (2005). Expression of CD13/aminopeptidase N on human gingival fibroblasts and up-regulation upon stimulation with interleukin-4 and interleukin-13. *J Periodontal Res* 40(2):138-46.

Kurihara N, Bertolini D, Suda T, Akiyama Y, Roodman GD (1990). IL-6 stimulates osteoclast-like multinucleated cell formation in long term human marrow cultures by inducing IL-1 release. *J Immunol* 144(11):4226-30.

Kwon BS, Wang S, Udagawa N, Haridas V, Lee ZH, Kim KK, *et al.* (1998). TR1, a new member of the tumor necrosis factor receptor superfamily, induces fibroblast proliferation and inhibits osteoclastogenesis and bone resorption. *Faseb J* 12(10):845-54.

Lacey DL, Grosso LE, Moser SA, Erdmann J, Tan HL, Pacifici R, *et al.* (1993). IL-1-induced murine osteoblast IL-6 production is mediated by the type 1 IL-1 receptor and is increased by 1,25 dihydroxyvitamin D3. *J Clin Invest* 91(4):1731-42.

Lacey DL, Erdmann JM, Shima M, Kling S, Matayoshi A, Ohara J, *et al.* (1994). Interleukin 4 enhances osteoblast macrophage colony-stimulating factor, but not interleukin 6, production. *Calcif Tissue Int* 55(1):21-8.

Lacey DL, Erdmann JM, Teitelbaum SL, Tan HL, Ohara J, Shioi A (1995). Interleukin 4, interferon-gamma, and prostaglandin E impact the osteoclastic cell-forming potential of murine bone marrow macrophages. *Endocrinology* 136(6):2367-76.

Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, *et al.* (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93(2):165-76.

Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL (2000). TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *J Clin Invest* 106(12):1481-8.

Lam J, Nelson CA, Ross FP, Teitelbaum SL, Fremont DH (2001). Crystal structure of the TRANCE/RANKL cytokine reveals determinants of receptor-ligand specificity. *J Clin Invest* 108(7):971-9.

Layton MJ, Cross BA, Metcalf D, Ward LD, Simpson RJ, Nicola NA (1992). A major binding protein for leukemia inhibitory factor in normal mouse serum: identification as a soluble form of the cellular receptor. *Proc Natl Acad Sci U S A* 89(18):8616-20.

Leboy PS (2006). Regulating bone growth and development with bone morphogenetic proteins. *Ann N Y Acad Sci* 1068(14-8).

- Lee CK, Lee EY, Chung SM, Mun SH, Yoo B, Moon HB (2004). Effects of disease-modifying antirheumatic drugs and antiinflammatory cytokines on human osteoclastogenesis through interaction with receptor activator of nuclear factor kappaB, osteoprotegerin, and receptor activator of nuclear factor kappaB ligand. *Arthritis Rheum* 50(12):3831-43.
- Lerner UH (1987). Modifications of the mouse calvarial technique improve the responsiveness to stimulators of bone resorption. *J Bone Miner Res* 2(5):375-83.
- Lerner UH (2000). Osteoclast formation and resorption. *Matrix Biol* 19(2):107-20.
- Lerner UH (2004). New Molecules in the Tumor Necrosis Factor Ligand and Receptor Superfamilies with Importance for Physiological and Pathological Bone Resorption. *Crit Rev Oral Biol Med* 15(2):64-81.
- Lerner UH (2006). Inflammation-induced Bone Remodeling in Periodontal Disease and the Influence of Post-menopausal Osteoporosis. *J Dent Res* 85(7):596-607.
- Lewis DB, Liggitt HD, Effmann EL, Motley ST, Teitelbaum SL, Jepsen KJ, *et al.* (1993). Osteoporosis induced in mice by overproduction of interleukin 4. *Proc Natl Acad Sci U S A* 90(24):11618-22.
- Levy DE, Darnell JE, Jr. (2002). Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 3(9):651-62.
- Levy JB, Schindler C, Raz R, Levy DE, Baron R, Horowitz MC (1996). Activation of the JAK-STAT signal transduction pathway by oncostatin-M cultured human and mouse osteoblastic cells. *Endocrinology* 137(4):1159-65.
- Li J, Sarosi I, Yan XQ, Morony S, Capparelli C, Tan HL, *et al.* (2000). RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. *Proc Natl Acad Sci U S A* 97(4):1566-71.
- Lin SJ, Chen YL, Kuo MY, Li CL, Lu HK (2005). Measurement of gp130 cytokines oncostatin M and IL-6 in gingival crevicular fluid of patients with chronic periodontitis. *Cytokine* 30(4):160-7.
- Lindberg RA, Juan TS, Welcher AA, Sun Y, Cupples R, Guthrie B, *et al.* (1998). Cloning and characterization of a specific receptor for mouse oncostatin M. *Mol Cell Biol* 18(6):3357-67.
- Lindhe J, Karring T, Lang MP, editors (2003). *Clinical Periodontology and Implant Dentistry*.
- Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A, *et al.* (1999). TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev* 13(8):1015-24.
- Loos BG, Craandijk J, Hoek FJ, Wertheim-van Dillen PM, van der Velden U (2000). Elevation of systemic markers related to cardiovascular diseases in the peripheral blood of periodontitis patients. *J Periodontol* 71(10):1528-34.

- Lorenzo JA, Sousa SL, Leahy CL (1990). Leukemia inhibitory factor (LIF) inhibits basal bone resorption in fetal rat long bone cultures. *Cytokine* 2(4):266-71.
- Lowe C, Gillespie GA, Pike JW (1995). Leukemia inhibitory factor as a mediator of JAK/STAT activation in murine osteoblasts. *J Bone Miner Res* 10(11):1644-50.
- Lubberts E, Joosten LA, Chabaud M, van Den Bersselaar L, Oppers B, Coenen-De Roo CJ, *et al.* (2000). IL-4 gene therapy for collagen arthritis suppresses synovial IL-17 and osteoprotegerin ligand and prevents bone erosion. *J Clin Invest* 105(12):1697-710.
- Lum L, Wong BR, Josien R, Becherer JD, Erdjument-Bromage H, Schlondorff J, *et al.* (1999). Evidence for a role of a tumor necrosis factor-alpha (TNF-alpha)-converting enzyme-like protease in shedding of TRANCE, a TNF family member involved in osteoclastogenesis and dendritic cell survival. *J Biol Chem* 274(19):13613-8.
- Madianos PN, Bobetsis YA, Kinane DF (2005). Generation of inflammatory stimuli: how bacteria set up inflammatory responses in the gingiva. *J Clin Periodontol* 32 Suppl 6(57-71).
- Maini RN, Taylor PC, Szechinski J, Pavelka K, Broll J, Balint G, *et al.* (2006). Double-blind randomized controlled clinical trial of the interleukin-6 receptor antagonist, tocilizumab, in European patients with rheumatoid arthritis who had an incomplete response to methotrexate. *Arthritis Rheum* 54(9):2817-29.
- Malik N, Haugen HS, Modrell B, Shoyab M, Clegg CH (1995). Developmental abnormalities in mice transgenic for bovine oncostatin M. *Mol Cell Biol* 15(5):2349-58.
- Mangashetti LS, Khapli SM, Wani MR (2005). IL-4 inhibits bone-resorbing activity of mature osteoclasts by affecting NF-kappa B and Ca<sup>2+</sup> signaling. *J Immunol* 175(2):917-25.
- Manolagas SC, Jilka RL (1995). Bone marrow, cytokines, and bone remodeling. Emerging insights into the pathophysiology of osteoporosis. *N Engl J Med* 332(5):305-11.
- Manolagas SC (1998). The role of IL-6 type cytokines and their receptors in bone. *Ann N Y Acad Sci* 840(194-204).
- Martin TJ, Romas E, Gillespie MT (1998). Interleukins in the control of osteoclast differentiation. *Crit Rev Eukaryot Gene Expr* 8(2):107-23.
- Metcalf D, Gearing DP (1989). Fatal syndrome in mice engrafted with cells producing high levels of the leukemia inhibitory factor. *Proc Natl Acad Sci U S A* 86(15):5948-52.
- Miossec P, Chomarat P, Dechanet J, Moreau JF, Roux JP, Delmas P, *et al.* (1994). Interleukin-4 inhibits bone resorption through an effect on osteoclasts and proinflammatory cytokines in an ex vivo model of bone resorption in rheumatoid arthritis. *Arthritis Rheum* 37(12):1715-22.
- Mirosavljevic D, Quinn JM, Elliott J, Horwood NJ, Martin TJ, Gillespie MT (2003). T-cells mediate an inhibitory effect of interleukin-4 on osteoclastogenesis. *J Bone Miner Res* 18(6):984-93.

- Mizuno A, Amizuka N, Irie K, Murakami A, Fujise N, Kanno T, *et al.* (1998). Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. *Biochem Biophys Res Commun* 247(3):610-5.
- Mizuno A, Kanno T, Hoshi M, Shibata O, Yano K, Fujise N, *et al.* (2002). Transgenic mice overexpressing soluble osteoclast differentiation factor (sODF) exhibit severe osteoporosis. *J Bone Miner Metab* 20(6):337-44.
- Modeer T, Anduren I, Yucel-Lindberg T (1998). Bradykinin synergistically stimulates interleukin 6 production in human gingival fibroblasts challenged with interleukin 1 or tumour necrosis factor alpha. *Cytokine* 10(1):26-31.
- Mogi M, Ootogoto J, Ota N, Inagaki H, Minami M, Kojima K (1999). Interleukin 1 beta, interleukin 6, beta 2-microglobulin, and transforming growth factor-alpha in gingival crevicular fluid from human periodontal disease. *Arch Oral Biol* 44(6):535-9.
- Mooney J, Adonogianaki E, Riggio MP, Takahashi K, Haerian A, Kinane DF (1995). Initial serum antibody titer to Porphyromonas gingivalis influences development of antibody avidity and success of therapy for chronic periodontitis. *Infect Immun* 63(9):3411-6.
- Moreno JL, Kaczmarek M, Keegan AD, Tondravi M (2003). IL-4 suppresses osteoclast development and mature osteoclast function by a STAT6-dependent mechanism: irreversible inhibition of the differentiation program activated by RANKL. *Blood* 102(3):1078-86.
- Morinaga Y, Fujita N, Ohishi K, Zhang Y, Tsuruo T (1998). Suppression of interleukin-11-mediated bone resorption by cyclooxygenase inhibitors. *J Cell Physiol* 175(3):247-54.
- Morita Y, Yamamura M, Kawashima M, Aita T, Harada S, Okamoto H, *et al.* (2001). Differential in vitro effects of IL-4, IL-10, and IL-13 on proinflammatory cytokine production and fibroblast proliferation in rheumatoid synovium. *Rheumatol Int* 20(2):49-54.
- Mosley B, De Imus C, Friend D, Boiani N, Thoma B, Park LS, *et al.* (1996). Dual oncostatin M (OSM) receptors. Cloning and characterization of an alternative signaling subunit conferring OSM-specific receptor activation. *J Biol Chem* 271(51):32635-43.
- Muller-Newen G (2003). The cytokine receptor gp130: faithfully promiscuous. *Sci STKE* 2003(201):PE40.
- Mundlos S, Olsen BR (1997). Heritable diseases of the skeleton. Part I: Molecular insights into skeletal development-transcription factors and signaling pathways. *Faseb J* 11(2):125-32.
- Murakami S, Hino E, Shimabukuro Y, Nozaki T, Kusumoto Y, Saho T, *et al.* (1999). Direct interaction between gingival fibroblasts and lymphoid cells induces inflammatory cytokine mRNA expression in gingival fibroblasts. *J Dent Res* 78(1):69-76.
- Nagasaki K, Yamaguchi K, Watanabe K, Eto S, Abe K (1991). Interleukin-4 blocks parathyroid hormone-related protein-induced hypercalcemia in vivo. *Biochem Biophys Res Commun* 178(2):694-8.

- Naito A, Azuma S, Tanaka S, Miyazaki T, Takaki S, Takatsu K, *et al.* (1999). Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes Cells* 4(6):353-62.
- Naka T, Nishimoto N, Kishimoto T (2002). The paradigm of IL-6: from basic science to medicine. *Arthritis Res* 4 Suppl 3(S233-42).
- Nakano Y, Watanabe K, Morimoto I, Okada Y, Ura K, Sato K, *et al.* (1994). Interleukin-4 inhibits spontaneous and parathyroid hormone-related protein-stimulated osteoclast formation in mice. *J Bone Miner Res* 9(10):1533-9.
- Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, *et al.* (2002). The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 108(1):17-29.
- Nakashima T, Kobayashi Y, Yamasaki S, Kawakami A, Eguchi K, Sasaki H, *et al.* (2000). Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines. *Biochem Biophys Res Commun* 275(3):768-75.
- Naruishi K, Takashiba S, Chou HH, Arai H, Nishimura F, Murayama Y (1999). Role of soluble interleukin-6 receptor in inflamed gingiva for binding of interleukin-6 to gingival fibroblasts. *J Periodontal Res* 34(6):296-300.
- Nesbitt S, Nesbit A, Helfrich M, Horton M (1993). Biochemical characterization of human osteoclast integrins. Osteoclasts express alpha v beta 3, alpha 2 beta 1, and alpha v beta 1 integrins. *J Biol Chem* 268(22):16737-45.
- O'Brien CA, Gubrij I, Lin SC, Saylors RL, Manolagas SC (1999). STAT3 activation in stromal/osteoblastic cells is required for induction of the receptor activator of NF-kappaB ligand and stimulation of osteoclastogenesis by gp130-utilizing cytokines or interleukin-1 but not 1,25-dihydroxyvitamin D3 or parathyroid hormone. *J Biol Chem* 274(27):19301-8.
- O'Brien CA, Lin SC, Bellido T, Manolagas SC (2000). Expression levels of gp130 in bone marrow stromal cells determine the magnitude of osteoclastogenic signals generated by IL-6-type cytokines. *J Cell Biochem* 79(4):532-41.
- Ohshima S, Saeki Y, Mima T, Sasai M, Nishioka K, Nomura S, *et al.* (1998). Interleukin 6 plays a key role in the development of antigen-induced arthritis. *Proc Natl Acad Sci U S A* 95(14):8222-6.
- Okada H, Murakami S (1998). Cytokine expression in periodontal health and disease. *Crit Rev Oral Biol Med* 9(3):248-66.
- Okada Y, Morimoto I, Ura K, Nakano Y, Tanaka Y, Nishida S, *et al.* (1998). Short-term treatment of recombinant murine interleukin-4 rapidly inhibits bone formation in normal and ovariectomized mice. *Bone* 22(4):361-5.

- Okamoto H, Yamamura M, Morita Y, Harada S, Makino H, Ota Z (1997). The synovial expression and serum levels of interleukin-6, interleukin-11, leukemia inhibitory factor, and oncostatin M in rheumatoid arthritis. *Arthritis Rheum* 40(6):1096-105.
- Onoe Y, Miyaura C, Kaminakayashiki T, Nagai Y, Noguchi K, Chen QR, *et al.* (1996). IL-13 and IL-4 inhibit bone resorption by suppressing cyclooxygenase-2-dependent prostaglandin synthesis in osteoblasts. *J Immunol* 156(2):758-64.
- Owczarek CM, Layton MJ, Robb LG, Nicola NA, Begley CG (1996). Molecular basis of the soluble and membrane-bound forms of the murine leukemia inhibitory factor receptor alpha-chain. Expression in normal, gestating, and leukemia inhibitory factor nullizygous mice. *J Biol Chem* 271(10):5495-504.
- Plater-Zyberk C, Buckton J, Thompson S, Spaul J, Zanders E, Papworth J, *et al.* (2001). Amelioration of arthritis in two murine models using antibodies to oncostatin M. *Arthritis Rheum* 44(11):2697-702.
- Poli V, Balena R, Fattori E, Markatos A, Yamamoto M, Tanaka H, *et al.* (1994). Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion. *Embo J* 13(5):1189-96.
- Pollock JH, Blaha MJ, Lavish SA, Stevenson S, Greenfield EM (1996). In vivo demonstration that parathyroid hormone and parathyroid hormone-related protein stimulate expression by osteoblasts of interleukin-6 and leukemia inhibitory factor. *J Bone Miner Res* 11(6):754-9.
- Reid LR, Lowe C, Cornish J, Skinner SJ, Hilton DJ, Willson TA, *et al.* (1990). Leukemia inhibitory factor: a novel bone-active cytokine. *Endocrinology* 126(3):1416-20.
- Riancho JA, Zarrabeitia MT, Mundy GR, Yoneda T, Gonzalez-Macias J (1993). Effects of interleukin-4 on the formation of macrophages and osteoclast-like cells. *J Bone Miner Res* 8(11):1337-44.
- Richards CD, Langdon C, Deschamps P, Pennica D, Shaughnessy SG (2000). Stimulation of osteoclast differentiation in vitro by mouse oncostatin M, leukaemia inhibitory factor, cardiotrophin-1 and interleukin 6: synergy with dexamethasone. *Cytokine* 12(6):613-21.
- Ritchlin C (2000). Fibroblast biology. Effector signals released by the synovial fibroblast in arthritis. *Arthritis Res* 2(5):356-60.
- Robey PG, Fedarko NS, Hefferan TE, Bianco P, Vetter UK, Grzesik W, *et al.* (1993). Structure and molecular regulation of bone matrix proteins. *J Bone Miner Res* 8 Suppl 2(S483-7).
- Rodan GA, Martin TJ (1981). Role of osteoblasts in hormonal control of bone resorption--a hypothesis. *Calcif Tissue Int* 33(4):349-51.
- Roodman GD, Windle JJ (2005). Paget disease of bone. *J Clin Invest* 115(2):200-8.
- Rousselle AV, Heymann D (2002). Osteoclastic acidification pathways during bone resorption. *Bone* 30(4):533-40.

- Russell SM, Keegan AD, Harada N, Nakamura Y, Noguchi M, Leland P, *et al.* (1993). Interleukin-2 receptor gamma chain: a functional component of the interleukin-4 receptor. *Science* 262(5141):1880-3.
- Ryoo HM, Lee MH, Kim YJ (2006). Critical molecular switches involved in BMP-2-induced osteogenic differentiation of mesenchymal cells. *Gene* 366(1):51-7.
- Saito M, Yoshida K, Hibi M, Taga T, Kishimoto T (1992). Molecular cloning of a murine IL-6 receptor-associated signal transducer, gp130, and its regulated expression in vivo. *J Immunol* 148(12):4066-71.
- Sakai A, Ohshima M, Sugano N, Otsuka K, Ito K (2006). Profiling the cytokines in gingival crevicular fluid using a cytokine antibody array. *J Periodontol* 77(5):856-64.
- Sasai M, Saeki Y, Ohshima S, Nishioka K, Mima T, Tanaka T, *et al.* (1999). Delayed onset and reduced severity of collagen-induced arthritis in interleukin-6-deficient mice. *Arthritis Rheum* 42(8):1635-43.
- Schiemann WP, Nathanson NM (1998). Raf-1 independent stimulation of mitogen-activated protein kinase by leukemia inhibitory factor in 3T3-L1 cells. *Oncogene* 16(20):2671-9.
- Sehgal PB, Wang L, Rayanade R, Pan H, Margulies L (1995). Interleukin-6-type cytokines. *Ann N Y Acad Sci* 762(1-14).
- Senaldi G, Varnum BC, Sarmiento U, Starnes C, Lile J, Scully S, *et al.* (1999). Novel neurotrophin-1/B cell-stimulating factor-3: a cytokine of the IL-6 family. *Proc Natl Acad Sci U S A* 96(20):11458-63.
- Shioi A, Teitelbaum SL, Ross FP, Welgus HG, Suzuki H, Ohara J, *et al.* (1991). Interleukin 4 inhibits murine osteoclast formation in vitro. *J Cell Biochem* 47(3):272-7.
- Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, *et al.* (1997). Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 89(2):309-19.
- Sims NA, Jenkins BJ, Quinn JM, Nakamura A, Glatt M, Gillespie MT, *et al.* (2004). Glycoprotein 130 regulates bone turnover and bone size by distinct downstream signaling pathways. *J Clin Invest* 113(3):379-89.
- Sodek J, McKee MD (2000). Molecular and cellular biology of alveolar bone. *Periodontol* 2000 24(99-126).
- Stahl N, Yancopoulos GD (1994). The tripartite CNTF receptor complex: activation and signaling involves components shared with other cytokines. *J Neurobiol* 25(11):1454-66.
- Stanley ER, Berg KL, Einstein DB, Lee PS, Pixley FJ, Wang Y, *et al.* (1997). Biology and action of colony--stimulating factor-1. *Mol Reprod Dev* 46(1):4-10.

Stuart PM, Zlotnik A, Woodward JG (1988). Induction of class I and class II MHC antigen expression on murine bone marrow-derived macrophages by IL-4 (B cell stimulatory factor 1). *J Immunol* 140(5):1542-7.

Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ (1999). Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr Rev* 20(3):345-57.

Taga T, Hibi M, Murakami M, Saito M, Yawata H, Narazaki M, *et al.* (1992). Interleukin-6 receptor and signals. *Chem Immunol* 51(181-204.

Tai H, Miyaura C, Pilbeam CC, Tamura T, Ohsugi Y, Koishihara Y, *et al.* (1997). Transcriptional induction of cyclooxygenase-2 in osteoblasts is involved in interleukin-6-induced osteoclast formation. *Endocrinology* 138(6):2372-9.

Takahashi N, Akatsu T, Udagawa N, Sasaki T, Yamaguchi A, Moseley JM, *et al.* (1988). Osteoblastic cells are involved in osteoclast formation. *Endocrinology* 123(5):2600-2.

Takayanagi H, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, *et al.* (2000a). Involvement of receptor activator of nuclear factor kappaB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. *Arthritis Rheum* 43(2):259-69.

Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, *et al.* (2000b). T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. *Nature* 408(6812):600-5.

Takeda K, Kaisho T, Yoshida N, Takeda J, Kishimoto T, Akira S (1998). Stat3 activation is responsible for IL-6-dependent T cell proliferation through preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice. *J Immunol* 161(9):4652-60.

Tamura T, Udagawa N, Takahashi N, Miyaura C, Tanaka S, Yamada Y, *et al.* (1993). Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6. *Proc Natl Acad Sci U S A* 90(24):11924-8.

Tan KB, Harrop J, Reddy M, Young P, Terrett J, Emery J, *et al.* (1997). Characterization of a novel TNF-like ligand and recently described TNF ligand and TNF receptor superfamily genes and their constitutive and inducible expression in hematopoietic and non-hematopoietic cells. *Gene* 204(1-2):35-46.

Tanaka M, Hara T, Copeland NG, Gilbert DJ, Jenkins NA, Miyajima A (1999). Reconstitution of the functional mouse oncostatin M (OSM) receptor: molecular cloning of the mouse OSM receptor beta subunit. *Blood* 93(3):804-15.

Tanaka M, Miyajima A (2003). Oncostatin M, a multifunctional cytokine. *Rev Physiol Biochem Pharmacol* 149(39-52.

Tanaka S, Takahashi N, Udagawa N, Tamura T, Akatsu T, Stanley ER, *et al.* (1993). Macrophage colony-stimulating factor is indispensable for both proliferation and differentiation of osteoclast progenitors. *J Clin Invest* 91(1):257-63.

- Teitelbaum SL (2000). Bone resorption by osteoclasts. *Science* 289(5484):1504-8.
- Teitelbaum SL, Ross FP (2003). Genetic regulation of osteoclast development and function. *Nat Rev Genet* 4(8):638-49.
- Teng YT, Nguyen H, Gao X, Kong YY, Gorczynski RM, Singh B, *et al.* (2000). Functional human T-cell immunity and osteoprotegerin ligand control alveolar bone destruction in periodontal infection. *J Clin Invest* 106(6):R59-67.
- Trevilatto PC, Scarel-Caminaga RM, de Brito RB, Jr., de Souza AP, Line SR (2003). Polymorphism at position -174 of IL-6 gene is associated with susceptibility to chronic periodontitis in a Caucasian Brazilian population. *J Clin Periodontol* 30(5):438-42.
- Tsuda E, Goto M, Mochizuki S, Yano K, Kobayashi F, Morinaga T, *et al.* (1997). Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochem Biophys Res Commun* 234(1):137-42.
- Udagawa N, Takahashi N, Akatsu T, Sasaki T, Yamaguchi A, Kodama H, *et al.* (1989). The bone marrow-derived stromal cell lines MC3T3-G2/PA6 and ST2 support osteoclast-like cell differentiation in cocultures with mouse spleen cells. *Endocrinology* 125(4):1805-13.
- Udagawa N, Takahashi N, Katagiri T, Tamura T, Wada S, Findlay DM, *et al.* (1995). Interleukin (IL)-6 induction of osteoclast differentiation depends on IL-6 receptors expressed on osteoblastic cells but not on osteoclast progenitors. *J Exp Med* 182(5):1461-8.
- Ueno K, Katayama T, Miyamoto T, Koshihara Y (1992). Interleukin-4 enhances in vitro mineralization in human osteoblast-like cells. *Biochem Biophys Res Commun* 189(3):1521-6.
- Vaananen HK, Zhao H, Mulari M, Halleen JM (2000). The cell biology of osteoclast function. *J Cell Sci* 113 ( Pt 3):377-81.
- Vaes G (1988). Cellular biology and biochemical mechanism of bone resorption. A review of recent developments on the formation, activation, and mode of action of osteoclasts. *Clin Orthop Relat Res* 231:239-71.
- Walsh NC, Gravallesse EM (2004). Bone loss in inflammatory arthritis: mechanisms and treatment strategies. *Curr Opin Rheumatol* 16(4):419-27.
- Van Beek E, Van der Wee-Pals L, van de Ruit M, Nijweide P, Papapoulos S, Lowik C (1993). Leukemia inhibitory factor inhibits osteoclastic resorption, growth, mineralization, and alkaline phosphatase activity in fetal mouse metacarpal bones in culture. *J Bone Miner Res* 8(2):191-8.
- Wang PL, Ohura K, Fujii T, Oido-Mori M, Kowashi Y, Kikuchi M, *et al.* (2003). DNA microarray analysis of human gingival fibroblasts from healthy and inflammatory gingival tissues. *Biochem Biophys Res Commun* 305(4):970-3.

Ware CB, Horowitz MC, Renshaw BR, Hunt JS, Liggitt D, Koblar SA, *et al.* (1995). Targeted disruption of the low-affinity leukemia inhibitory factor receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death. *Development* 121(5):1283-99.

Watanabe K, Tanaka Y, Morimoto I, Yahata K, Zeki K, Fujihira T, *et al.* (1990). Interleukin-4 as a potent inhibitor of bone resorption. *Biochem Biophys Res Commun* 172(3):1035-41.

Wei S, Wang MW, Teitelbaum SL, Ross FP (2002). Interleukin-4 reversibly inhibits osteoclastogenesis via inhibition of NF-kappa B and mitogen-activated protein kinase signaling. *J Biol Chem* 277(8):6622-30.

Weiner S, Traub W (1992). Bone structure: from angstroms to microns. *Faseb J* 6(3):879-85.

Vermes C, Jacobs JJ, Zhang J, Firneisz G, Roebuck KA, Glant TT (2002). Shedding of the interleukin-6 (IL-6) receptor (gp80) determines the ability of IL-6 to induce gp130 phosphorylation in human osteoblasts. *J Biol Chem* 277(19):16879-87.

Westendorf JJ, Kahler RA, Schroeder TM (2004). Wnt signaling in osteoblasts and bone diseases. *Gene* 341(19-39).

Wiktor-Jedrzejczak W, Bartocci A, Ferrante AW, Jr., Ahmed-Ansari A, Sell KW, Pollard JW, *et al.* (1990). Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. *Proc Natl Acad Sci U S A* 87(12):4828-32.

Wong BR, Rho J, Arron J, Robinson E, Orlinick J, Chao M, *et al.* (1997). TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J Biol Chem* 272(40):25190-4.

Woods JM, Katschke KJ, Volin MV, Ruth JH, Woodruff DC, Amin MA, *et al.* (2001). IL-4 adenoviral gene therapy reduces inflammation, proinflammatory cytokines, vascularization, and bony destruction in rat adjuvant-induced arthritis. *J Immunol* 166(2):1214-22.

Yashiro R, Nagasawa T, Kiji M, Hormdee D, Kobayashi H, Koshy G, *et al.* (2006). Transforming growth factor-beta stimulates interleukin-11 production by human periodontal ligament and gingival fibroblasts. *J Clin Periodontol* 33(3):165-71.

Yasuda H, Shima N, Nakagawa N, Mochizuki SI, Yano K, Fujise N, *et al.* (1998a). Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. *Endocrinology* 139(3):1329-37.

Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, *et al.* (1998b). Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A* 95(7):3597-602.

Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, *et al.* (1990). The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* 345(6274):442-4.

Yun TJ, Chaudhary PM, Shu GL, Frazer JK, Ewings MK, Schwartz SM, *et al.* (1998). OPG/FDCR-1, a TNF receptor family member, is expressed in lymphoid cells and is up-regulated by ligating CD40. *J Immunol* 161(11):6113-21.

Zhang JG, Zhang Y, Owczarek CM, Ward LD, Moritz RL, Simpson RJ, *et al.* (1998). Identification and characterization of two distinct truncated forms of gp130 and a soluble form of leukemia inhibitory factor receptor alpha-chain in normal human urine and plasma. *J Biol Chem* 273(17):10798-805.