Effects of Interleukin-4 and Interleukin-13 on Bone

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Abstract

Cytokines play important roles in bone metabolism, participating in the complex interplay necessary for normal bone formation and turnover. The aim of the present thesis was to investigate the effects of two anti-inflammatory cytokines, interleukin-4 (IL-4) and interleukin-13 (IL-13) on bone.

Influence of pro- and anti-inflammatory cytokines on interleukin-6 (IL-6) formation in cultured human osteoblasts (hOBs) was investigated. IL-4 and IL-13 as well as interleukin-1 (IL-1) and tumour necrosis factor alpha and beta (TNF-α/β) stimulated IL-6 secretion in hOBs. Also, IL-4 and IL-13 synergistically potentiated the effect of IL-1 and TNFs on IL-6 secretion.

Effects of IL-4 and IL-13 on markers of osteoblastic activity in hOBs were investigated. IL-4 and IL-13 induced a dose-dependent increase in the formation of alkaline phosphatase (ALP) and pro-collagen type I carboxy-peptide (PICP) together with enhanced mineralization rate in hOBs. Formation of osteocalcin (OC) was unaffected.

The mechanism behind inhibited proliferation by IL-4 and IL-13 in hOBs was investigated. IL-4 and IL-13 caused a dose-dependent increase in DNA-fragmentation together with escalating Caspase-3 activity in hOBs, reflecting induced apoptosis. Osteoblast apoptosis was also confirmed by TNF-α, dexamethasone and by serum starvation.

The skeletal phenotype of IL-13−/−, IL-4−/−IL-13−/− and WT mice was compared. An altered cortical bone mass was detected in adult male IL-4−/−IL-13−/− mice. They displayed a reduction in cortical bone mineral content (BMC) secondary to reduced cortical thickness. Mechanical strength of the cortical bone was reduced in level with the reduction detected in BMC. Trabecular bone mineral density (tvBMD) was unaffected.

Callus formation in IL-4−/−IL-13−/− and WT male mice was compared. No differences were found concerning radiological healing, biomechanical properties, callus parameters or histology. Heterotopic bone formation in IL-4−/−IL-13−/− and WT mice was compared using DXBM implants. No differences were found concerning mineralization of implants. Immuno-histology showed inhibition of autonomic nerves and lack of implant vascularization in IL-4−/−IL-13−/− mice.

In summery, the two anti-inflammatory cytokines IL-4 and IL-13 influence osteoblast activity and apoptosis in vitro. They also selectively influence cortical bone formation in vivo. These findings suggest a role for IL-4 and IL-13 in osteoblast differentiation, in bone metabolism and in bone formation.

Keywords: Bone, Osteoblasts, Interleukin-4, Interleukin-13, Knockout

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I Silfverswärd C-J, Frost A, Brändström H, Nilsson O and Ljunggren Ö. Interleukin-4 and Interleukin-13 potentiate Interleukin-1 induced secretion of Interleukin-6 in Human Osteoblast-like Cells.


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III Silfverswärd C-J, Winqvist O, Nilsson O and Ljunggren Ö. Induction of Apoptosis in Human Osteoblasts by Interleukin-4 and Interleukin-13

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Front page: Representative diaphyseal femoral cortical cross-sectional pQCT scan of a WT (left) and an IL-4−/−IL-13−/− (right) mouse. Cross-section level is defined at 36% of the total femoral length proximal to the distal epiphyseal plate
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Abbreviations

ALP  Alkaline phosphatase
α-MEM  Alpha modification of Eagle’s medium
BMC  Bone mineral content
BMD  Bone mineral density
BMP  Bone morphogenetic protein
BMU  Basic multicellular unit
CGRP  Calcitonin gene-related peptide
ddH₂O  Double destilled water
DNA  Deoxy ribonuclear acid
DXBM  Demineralized xenogenic bone matrix
EC₅₀  Concentration giving 50% of maximal effect
Elisa  Enzyme linked immunosorbent assay
EtOH  Ethanol
FCS  Foetal calf serum
FGF  Fibroblast growth factor
GAP-43  Growth associated protein-43
GH  Growth hormone
H&E  Haematoxylin and Eosin
hOBs  Human osteoblast-like cells
IL  Interleukin
IL-(x)R  Interleukin-receptor
IGF-1  Insulin growth factor-1
JAK  Janus family of tyrosine kinases
KO  Knock-out
LIF  Leukemia inhibitory factor
MCS-F  Macrophage colony stimulating factor
MgCl  Magnesium chloride
NPY  Neuropeptide-Y
OC  Osteocalcin
OI  Osteoinductive (medium)
OP  Osteopontin
OPG  Osteoprotegrin
OVX  Ovariectomised
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PECAM-1  Platelet endothelial cell adhesion molecule-1
PGE  Prostaglandin E
PGP 9.5  Protein gene product 9.5
PICP  Procollagen type I carboxyterminal peptide
pQCT  Peripheral quantitative computerized tomography
PTH  Parathyroid hormone
PTHrP  Parathyroid hormone related protein
RANK  Receptor activator of nuclear factor-κB
RANK-L  Receptor activator of nuclear factor-κB ligand
RIA  Radioimmuno assay
RT-PCR  Reversed transcriptase polymerase chain reaction
STAT  Signal transducers and activators of transcription
TGF-β  Transforming growth factor beta
Th2  T-helper 2 lymphocytes
TNF-(α/β)  Tumour necrosis factor (alpha, beta)
Vit-D  Vitamin-D
WT  Wild-type (genetically intact)
Introduction

Bone tissue

Bone is a living, biologically active, tissue nourished by blood supply and to some extent innervated. Microscopically bone is comprised of an organic part called the matrix, containing matrix-proteins and cells, and an inorganic crystal-like part containing calcium-rich hydroxyapatite, Ca_{10}(PO_{4})_{6}(OH)_{2} (1). The main protein in bone matrix is collagen type I forming a fibrillar network to which other proteins attach. Three cell types are represented in bone, osteoblasts, osteoclasts and osteocytes. (Fig. 1)

Osteoblasts are differentiated cells originating from pluripotent mesenchymal stem cells. Once primed for the osteoblastic lineage they follow a set-up path from precursor cells through different preosteoblastic stages to fully differentiated osteoblasts. (Fig. 2) Multiple factors influence this process containing proliferation and differentiation steps and thereby govern the development, function and destiny of osteoblasts (2-5). During this development osteoblasts express different markers of activity e.g. alkaline phosphatase (ALP), osteopontin (OP), osteocalcin (OC) and collagen. Expression of these markers is tightly controlled genetically. Through mapping of the variance in expression of osteoblastic markers one finds that they follow a pattern correlating to different developmental stages (6, 7). The main function of the mature osteoblast is to create bone tissue. Therefore most osteoblasts end up as working cells building new bone by synthesis of bone matrix that is concomitantly mineralized. After a normal life-cycle of approximately three months the osteoblast dies through apoptosis (8).

Some osteoblasts, ending up inside the mineralized bone, differentiate further to osteocytes. These cells develop dendrite-like extensions connecting to other osteocytes in a network through the canaliculi inside bone. They probably function as sensor-cells detecting mechanostatic loads (9, 10).

The third cell-type, osteoclasts, originates from a haematopoetic background. They are formed from mononuclear precursors that merge to multinuclear, macrophage-like cells. With further differentiation osteoclasts become specialised in bone resorption degrading bone tissue by acidification and proteolysis (11, 12).
Figure 1. Histological section in HE-staining showing osteoblast (blue arrow), osteoclasts (green arrows and osteocytes (yellow arrows. Yellow dashed line outlines resorption lacunae.

Figure 2. Developmental stages for the osteoblast from mesenchymal stem-cell to fully differentiated bone forming osteoblasts that finally become lining-cells, osteocytes or undergo programmed cell death (apoptosis).

Differentiation of bone cells, both osteoblasts and osteoclasts, is governed genetically and is carefully controlled by intricate multifactorial systems (13-15). Also important, bone tissue is situated in direct proximity to bone marrow containing cells of haematological origin and is therefore exposed to immunological influences in its immediate environment.

Macrosopically one distinguishes between two types of bone tissue in the mature skeleton. Trabecular bone situated in the metaphyseal regions of long bones or inside vertebral bodies function as important calcium reservoirs but also building a framework of supportive tissue adjacent to joints. The other type, cortical bone, is the cover of flat bone structures like the skull, pelvis or ribs, structures that also works as a protection of internal organs. Cortical bone is most abundant in long bone diaphysis where it has a main function of skeletal support.
Bone modelling and remodelling

Bone is built up, modelled, during foetal life and childhood either from a cartilage template, enchondral bone synthesis, or directly from fibrous tissue, intramembranous bone synthesis. After final completion of skeletogenesis the skeleton is continuously rebuilt, remodelled, in a process referred to as bone turnover.

During normal bone remodelling osteoclasts and osteoblasts work closely together under the influence of different signalling mechanisms to fulfil the bone regeneration process (16). Microscopically this process is carried out in basic multicellular units, BMUs, containing osteoclasts and osteoblasts. Osteoclasts attach to bone, forming a seal towards the bone surface. Inside this sealing zone lysosomal enzymes are secreted through foldings in the plasma membrane, called “ruffled border”. These enzymes resorb bone through demineralization by acidic phosphatases and protein denaturation by collagenases. The microscopical pits formed, also called resorption lacunae, are sequentially filled out with new bone by osteoblasts synthesising fresh bone matrix, also called osteoid, which concomitantly becomes mineralized (17). (Fig. 3) In trabecular bone this process continuously takes place on the surfaces of the trabeculi. In cortical bone regeneration appears endostally, periostally or inside the bone corresponding to the Haversian systems. The co-working situation between osteoblasts and osteoclasts is referred to as the coupling mechanism. It is dependent on a sophisticated communication system between participating cells where cytokines and growth factors regulate the process at a local level in a paracrine or autocrine manner (18-20).

Normal bone turnover is crucial for the very sensitive calcium homeostasis. It is also important to optimise bone structure and vitality with regards to skeletal function e.g. movement, strength and weight bearing. Because of its implications in calcium balance trabecular bone has a fast turnover (appr. 25%/year) while cortical bone regenerates at a much slower rate (appr. 3%/year) (21). In total the skeleton is renewed every 10 years.
Regulation of bone modelling and remodelling

During embryogenesis the development of bone is strictly regulated. Limb formation starts with a mesenchymal limb-bud covered by epithelium and interaction between the cells in these two tissues determines the position and fate of the cells. The mesenchymal tissue condenses, and cells differentiate further to chondrocytes with the ability to form a cartilage template. The cartilage is then gradually resorbed and replaced by bone synthesised from invading osteoprogenitor cells. In this process factors from the TNF-β superfamily (BMPs) have been found to be of fundamental importance (22).

Mature bone is also influenced by numerous factors, either systemic or in the micro-environment. Systemically hormones play important roles in bone homeostasis, e.g. Vitamin-D, PTH, glucocorticoids, and sex-steroid hormones (23). Systemic influence also comes from innervation to some extent, although this parameter of bone influence is as yet not so well understood (24, 25). In the micro-environment numerous local factors affect bone metabolism such as growth-factors, cytokines, acute phase reactants and matrix proteins (19, 26). Factors like FGFs and BMPs are important regulators of
osteoblastic differentiation and activity. BMPs together with TGF-β regulate bone synthesis by influence on bone matrix production. MCS-F, and also BMPs are crucial for osteoclast differentiation and activation (27). Recent research has elucidated the powerful RANK/RANK-L pathway pointing out the importance of communication between bone cells in the regulation of bone turnover. RANK-L expressed on the surface of osteoblasts and stromal cells interact with membrane-bound RANK on the surface of osteoclast precursor cells. This leads to the differentiation and maturation of osteoclasts. Osteoblasts and stromal cells also express OPG, a protein working as a decoy receptor, that inhibit RANK/RANK-L interaction by competitively binding to RANK-L (28-30).

Many cytokines, e.g. TNFs and interleukins also affect bone turnover pointing at the intricate co-operation between bone metabolism and the immunological system (31). Under normal conditions these systemic and local factors act in concert to keep calcium homeostasis at an optimum and at the same time regulate bone renewal for the benefit of skeletal function. Taken together bone metabolism is governed by a complicated multi-factorial system of influences that has to be carefully regulated. (Fig. 4) Imbalance in this regulation leads to disturbances in the bone turnover process represented in different types of bone pathologies.

![Figure 4](image.png)

*Figure 4. Numerous factors regulate bone metabolism. The two cytokines of interest for the present thesis highlighted.*
Apoptosis and Osteoblasts

Apoptosis is characterized as a physiological form of cell death or programmed cell death differing from necrotic cell death by the lack of inflammatory response. It comprises a number of morphological changes, initially defining the term, including nuclear chromatin condensation, DNA-degradation, shrinkage of the cytoplasm together with membrane blebbing and finally cell disintegration by formation of apoptotic bodies that ultimately get phagocytized by neighbouring cells (32, 33).

Later research has outlined the mechanisms and biochemical background of apoptosis. Genetic studies on the nematode Caenorhabditis Elegans defined a number of death and survival genes, encoding proteins important for apoptosis and also represented by homologous proteins, caspases, in mammals (34, 35). In recent research two principle pathways of apoptosis-activation have been elucidated. The mitochondrial pathway activated by cellular stress or cytotoxic stimuli leading to mitochondrial membrane instability and release of cytochrome c into cytosol. Cytochrome c interacts with apoptotic proteases activating initiator caspases, e.g. caspase-9, starting a chain-reaction with further activation of downstream execution caspases. The receptor-activated pathway is triggered by ligand-binding to cell-surface death receptors belonging to the TNF receptor superfamily (Fas/TNF-R). Given a critical density of receptors, ligand-binding can enable clustering of associated intracellular death-domain adaptor proteins forming complexes that activate initiator caspases, e.g. caspase-8, -10 and -2. Also this pathway is run by a chain-reaction leading to the activation of downstream execution caspases. Caspase-3 represents the final, and probably non-reversible, step of the caspase chain in both pathways and initiates non-caspase effectors ultimately leading to the DNA fragmentation typical for apoptosis. (36, 37).

DNA is degraded by enzymes, endonucleases, cleaving DNA that is still bound to its core proteins (histones), resulting in specific histone-bound DNA complexes. (Fig. 5)

The size of the osteoblastic cell population is determined by the rate of cell proliferation contra cell death or progression to the osteocyte phenotype (8). Osteoblast cell death by apoptosis plays an important role in normal bone tissue development (38) as well as in pathological conditions like fracture healing (39, 40). It is also implicated in diseases like osteoporosis related to age as well as sex-hormone deprivation or glucocorticoids (8, 41). Previous in vitro findings has indicated that osteoblast apoptosis is regulated by cytokines and growth factors (42). There is also evidence for regulation of osteoblast apoptosis by extracellular matrix components released during bone resorption (43). Interestingly, induction of osteoblast apoptosis, mediated via activation of caspase-8 and caspase-3, is also seen as a result of compressive force (44).
Figure 5. Schematic figure showing the two principle pathways leading to apoptosis. The extrinsic pathway is receptor mediated and activated by ligands such as TNF or FAS-L. The intrinsic pathway is activated by cell-stress/cytotoxic stimuli, e.g. cytotoxic drugs, irradiation etc.

Pathological events in bone

Many pathological events in bone are related to disturbances in the bone turnover process. Osteoporosis arises as a consequence of imbalance between bone resorption and bone formation (18, 45). In this condition age-related, post-menopausal or pharmacological, bone resorption exceeds bone formation (8, 46) leading to a net loss of bone. Post-menopausal osteoporosis is hormone related, connected to the withdrawal of female sex-steroids important for normal bone metabolism. Estrogen has been shown to influence bone at several levels in co-operation with different cytokines (23). While post-menopausal osteoporosis is primarily associated with excessive osteoclast activity, age-related osteoporosis results from a decline in the supply of osteoblasts in proportion to the ongoing formation of new BMUs (47). Pharmacological osteoporosis usually provoked by glucocorticoids, another steroid hormone, affects both osteoblasts and osteoclasts resulting in negative bone turnover (48, 49). Nutrition defects, e.g. vitamin-D deficiency, results in defective bone formation causing rickets. Inborn errors of bone
metabolism e.g. osteogenesis imperfecta, are caused by a genetic defect in collagen expression, also resulting in defective bone formation. Certain diseases like malignancies (myeloma, metastasis etc) (50, 51) or inflammatory joint diseases (rheumatoid arthritis) (4, 52, 53) are associated with deteriorated bone metabolism as well, either generalised or locally in the skeleton. Furthermore, pathological bone reactions sometimes occur when the system’s condition to deal with injury fails such as in periprosthetic osteolysis after hip replacement (54), defective fracture healing (pseudarthrosis) or aberrant soft tissue healing (heterotopic bone formation) (55). In many of these pathological conditions cytokines have been implicated as important regulators.

Fracture healing
Injury affects the immunological system with alterations in T-cell responses and cytokine production (56). Suppression of T-cell proliferation is seen in the early post-traumatic stages. Concerning Th2-type cytokines earlier studies are inconclusive. However some data indicates a possible shift towards Th2 cytokine production following trauma (57-59).

Fracture healing is regulated by a number of important events aiming to restore the strength and integrity of bone (60). A key event is the formation of new blood vessels, angiogenesis, which in fact is the hallmark of all types of wound healing. Callus is characterized by rapidly developing new vessels that are necessary to provide nutrients to the new growing bone tissue (61, 62). Recruitment of pleuripotent cells, their proliferation and differentiation into bone forming cells in response to trauma, or bone loss, is also crucial. Bone progenitor cells are recruited from the periosteum and endosteum by chemotaxis. They differentiate to become osteoblasts producing extracellular matrix, most importantly type I collagen. The matrix is mineralized by hydroxyapatite crystals deposited around collagen fibrils (5, 63). This process is orchestrated by a variety of factors in the micro-environment. Growth factors and cytokines originating from the immunological response play important roles in regulating chemotaxis, proliferation, differentiation and communication throughout the healing of bone (64-66).

Heterotopic bone formation
Bone formation may occur heterotopically, i.e. outside normal bone tissue borders. It is seen in some hereditary disorders (67), as a complication to endoprosthetic surgery (68) or soft tissue trauma e.g. myositis ossificans traumatica (69) and also secondary to neurological injury or neoplasms (55). Heterotopic bone formation is characterised by a number of key events
where formation of new blood vessels, just like in fracture healing, is a pre-
requisite. Also here, angiogenesis is followed by chemotaxis, proliferation
and migration of primitive mesenchymal cells that become differentiated to
highly specialised bone forming cells. This is made possible by the complex
process of bone induction where undifferentiated mesenchymal cells become
primed to differentiation along the chondroblastic and osteoblastic pathways
for bone formation (70).

Osteoinduction is dependent on cytokine influences in the micro-
environment, with BMPs as important regulators (71-74). Especially BMP-2,
4 and 7 have shown the ability to stimulate the entire process of stem cell
differentiation into mature osteoblast cells in vitro. BMPs have also been
tested in preclinical and clinical studies showing their definite potential in
osteoinduction (75). Apart from BMPs other cytokines and growth factors
and also hormones modulate osteoinduction, systemically or locally (76).
Pro-inflammatory cytokines such as IL-1 and TNF-α has attracted most in-
terest in bone research (19, 40, 77) whereas anti-inflammatory cytokines
have been less extensively investigated.

Cytokines
Pro-inflammatory cytokines
Interleukin-1 (IL-1), Interleukin-6 (IL-6) and Tumour Necrosis Factor (TNF)
are multifunctional cytokines involved in the regulation of the immune re-
sponse, hematopoiesis and inflammation. These cytokines are mainly de-
derived from the monocyte lineage but they are also produced by other cell-
types including bone marrow stromal cells (78).

Interleukin-6 (IL-6), a member of the LIF/IL-6 cytokine family, was dis-
covered and characterised in the late 1980’s. It was originally identified as a
B-cell differentiating factor (78). Apart from the main source monocytes, IL-
6 is produced by a variety of tissue cells and also by many tumours (79, 80).
Osteoblasts produce IL-6 (81, 82), and also express IL-6 receptors indicating
a possible autocrine function (83-85). Osteotropic factors, systemic like PTH
and 1,25-(OH)2-Vit-D3 or local like TNF and IL-1, stimulate the production
of IL-6 in osteoblasts (86, 87). IL-6 activity is reported to be dependent on
either IL-6 receptors on osteoblasts or a complex of IL-6 and a soluble IL-6
receptor (88). However, there is also evidence for IL-6 receptors expressed
by osteoclasts (89). The role of IL-6 in bone is predominantly osteoclasto-
genic and initial in vitro studies pointed out IL-6 as a key effector in osteo-
clast development and activation (83). Later research, especially completed
with in vivo data showing IL-6 not to be essential for normal bone resorption
and homeostasis, has toned down the central role of IL-6. Nevertheless, this
pro-inflammatory cytokine seems to play an important role in bone metabo-
lism also in vivo as depletion of IL-6 results in protection against joint destruction secondary to inflammation in induced arthritis. Oestrogen loss result in IL-6 mediated stimulation of osteoclastogenesis and bone resorption due to oestrogen depletion has also been reported to decrease in IL-6−/− mice (90, 91). The importance of IL-6 in bone pathologies is further pointed out by its role in diseases like multiple myeloma (92), Paget’s disease (83) and rheumatoid arthritis (93).

IL-1 together with TNF are two of the most potent pro-inflammatory cytokines, both mainly derived from activated macrophages (20). However, osteoblasts also release IL-1 and TNF and evidence shows that these two cytokines can act as endogenous mitogenic factors for human osteoblastic cells (94). Also, enhanced osteoblastic proliferation is seen as a result of exogenous stimulation by TNF and IL-1 (95). TNF and IL-1 are both powerful stimulators of bone resorption. In vitro TNF stimulate osteoclastic bone resorption via induction of osteoblasts (96). IL-1, on the other hand, has the capacity to stimulate bone resorption either by direct stimulation of osteoclasts or by acting via osteoblasts stimulating their expression of RANKL and IL-6 (53).

Both cytokines are markedly active in different inflammatory diseases concerning bone, typically rheumatoid arthritis (RA) (53, 97). IL-1, TNF and also IL-6 are detected in the synovial fluid of patients with RA and dexamethasone together with other glucocorticoids suppress the production of all three cytokines (78). In inflammatory conditions the action of pro-inflammatory cytokines are counteracted by anti-inflammatory cytokines such as IL-4 and IL-13 (98).

Anti-inflammatory cytokines IL-4 & IL-13

The two cytokines interleukin-4 and interleukin-13 are mainly derived from Th2-cells, and they are encoded from the same region on human chromosome no. 5 (99). Like other cytokines IL-4 and IL-13 are relatively small glycoproteins with a molecular weight of approximately 14 kD. Apart from T-lymphocytes, both cytokines are also constitutively expressed by mast cells, eosinophils and basophils and stimulation of these cells is required for cytokine production and secretion (100). It has previously been demonstrated that IL-4 and IL-13 act in concert to initiate Th2-like responses, and that their combined disruption can either abolish such responses or significantly delay their onsets, resulting in an inappropriate Th1 response (101). Although IL-4 and IL-13 share many immunoregulatory effects they also show some discrepancies concerning biological actions (102-105). IL-4 and IL-13 also influence other tissue cells e.g. endothelial cells, microglial cells and keratinocytes (106-108). These two interleukins belong to the family of anti-inflammatory cytokines because of their ability to suppress pro-inflammatory cytokines. This is seen for example in conditions like rheuma-
toid arthritis where both cytokines suppress the production of IL-1, TNF and IL-6 (109).

Receptors for IL-4 and IL-13 have been investigated in various cell systems, and at least four different subunits have been elucidated which in different constellations are believed to activate the intracellular JAK/STAT signalling pathway (110-113). Murata and colleagues have presented an interesting receptor subunit model involving subunits IL-4R, IL-13R, IL-13Rα and IL-2Rγc with evidence that IL-4 and IL-13 share these receptor subunits (114). Also, alternative splicing of the IL-4R gene can result in the production of a soluble form of IL-4R (sIL-4R) which has no signalling abilities and therefore probably works as a decoy and an antagonist to its ligand (115, 116). A combined mechanism of action of IL-4 and IL-13 on Th2 cell responses is supported by that IL-4 and IL-13 share the α-chain of the IL-4 receptor and consequently signal through related pathways including STAT6 (117). In human osteoblasts, expression of IL-4R, IL-13R and IL-13Rα, but not IL-2γc, has been demonstrated (82).

Previous in vitro studies have indicated that IL-4 and IL-13 may regulate bone metabolism. There is data showing that IL-4 and IL-13 act as chemo-tactic factors for human osteoblasts (118). ALP activity in osteoblasts is enhanced by stimulation with IL-4 shown in the osteoblastic cell line MG-63 (119) and in human osteoblast cells (120). IL-4 stimulates collagen-1 expression (119) and also induces mineralization in human osteoblast-like cells (121). Further studies have demonstrated that both IL-4 and IL-13 inhibit osteoblast proliferation in human cell cultures (119, 122). Contrary to these findings Ura and colleagues reported enhanced proliferation and inhibited differentiation by IL-4 and IL-13 in murine osteoblastic cell-line MC3T3-E1 (123). IL-4 and IL-13 up-regulate IL-6 transcription and secretion in cultured human osteoblasts, an effect shown to be transmitted via the receptor unit IL-4R (82). This trigger-effect on IL-6 could possibly result in osteoblast mediated activation of osteoclasts since IL-6 is known to promote osteoclastogenesis (81, 83).

Studies on osteoclasts have shown that IL-4 inhibits their development and activation (124-128). In fact both IL-4 and IL-13 have been shown to influence osteoclast formation, though with bi-directional effects depending on the micro-milieu and/or state of cell activation (129). There is also evidence that IL-4 and IL-13 inhibit bone resorption induced by pro-inflammatory cytokines, PTH, PTHrP, Vit.-D and PGE2 (130, 131). Recently inhibition of hormone and cytokine stimulated osteoclastogenesis and bone resorption by IL-4 and IL-13 in rat calvaria was reported. This effect was associated with increased OPG expression together with a decrease in RANK/RANKL expression showing that IL-4 and IL-13 also has the capacity to interfere with crucial osteoclastogenic pathways (132). In vivo, over-production of IL-4 in transgenic mice results in a low-turnover osteoporosis with both a trabecular and cortical bone loss (133).
In summery divergent results regarding the role of IL-4 and IL-13 in the regulation of bone metabolism have been presented with most data pointing towards a negative effect on bone mass. As with other cytokines the action of IL-4 and IL-13 in bone is not isolated or restricted to only one cell-type, mechanism or pathway. They rather seem to exert different actions depending on the target and the situation in a complex multifactorial system.
Aims

*General aim:* To investigate the effects of two anti-inflammatory cytokines, Interleukin-4 and Interleukin-13 in bone.

- To study potential interactions between anti-inflammatory (IL-4 & IL-13) and pro-inflammatory cytokines concerning IL-6 formation in human osteoblasts.
- To investigate if and how IL-4 and IL-13 affect the activity of human osteoblasts in culture.
- To study the mechanism by which IL-4 and IL-13 inhibit proliferation of human osteoblasts in culture.
- To elucidate effects on bone tissue *in vivo* by genetic depletion of IL-4 and IL-13.
- To study potential effects *in vivo* on different types of bone formation in the absence of IL-4 and IL-13.
Methods

Cell cultures (Papers I - III)
Primary cultures of human osteoblasts (hOBs) were isolated from cancellous human bone, obtained from the proximal femur in patients undergoing total hip replacement. Bone specimens were cut into small fragments, 1-2 mm in diameter, thoroughly rinsed with PBS, and cultured in 75 cm² tissue culture flasks containing α-MEM supplemented with PEST (100 U/mL of penicillin, 100 μg/mL of streptomycin), L-glutamine (2 mmol/L) and 10% FCS. Culture conditions in paper I and II also contained amphotericin B (0.5 μg/mL). In differentiation-experiments (paper II) ascorbic acid (50 μM) was added to the culture media. After 4-5 weeks, the culture dishes were confluent with cells that had migrated from the trabecular bone. The cells were detached with trypsin-EDTA, reconstituted in 10 ml of α-MEM and counted in a hemocytometer (Bürkerchamber). Only first passage cells were used.

Interleukin-6 assay (Paper I)
Cells were seeded in 24-well culture plates at a density of 30,000 cells/well and left to adhere in α-MEM supplemented with 10% FCS and antibiotics for 72 h. The cells were washed in PBS and the medium was changed to α-MEM with 0.1% FCS. Cytokines were added, except in control wells, in quadrupule series for each sample. After 72 h of incubation the culture media were harvested and IL-6 levels analysed by ELISA.

Test for endotoxin (Paper I)
Checking of the test media for lipopolysaccharide (LPS) contamination was performed using the Limulus Amebocyte Lysate assay "E-Toxate" with a sensitivity of 0.03 EU/ml according to the manufacturer. No traces of LPS were detected in our test media.
Pro-Collagen type I Carboxy-Peptide (PICP) assay
(Paper II)

Cells were seeded in a 24-well culture plate at a density of 1 x 10^5 cells/well. They were left to adhere in α-MEM supplemented with antibiotics, ascorbic acid (50 μM) and 10% FCS. After 72 hours cells were washed twice in PBS and stimulation was started. 4 wells received the same medium mentioned above, although serum free (control), and 4 wells received the same serum free medium supplemented with 100 nM 1,25(OH)2D3 (control). The other wells also received the same serum free medium supplemented with 100 nM 1,25(OH)2D3 and IL-4 or IL-13 (1-100 pM) and quadruple series for each sample. The plates were incubated for 72 hours. Supernatants were collected and immediately frozen to -70°C. Prior to the analysis the samples were lyophilized and dissolved in 120 μl assay buffer. The PICP propeptides were measured in the supernatants according to the instruction of the RIA manufacturer.

Osteocalcin assay (Paper II)

100 μl of the above mentioned supernatants in each sample was used for measurement of osteocalcin-secretion by ELISA and the assay was performed according to the instructions provided by the manufacturer.

Alkaline phosphatase assay (Paper II)

ALP activity in the cell layers in the above-mentioned 24-well plates was measured by the specific conversion of p-nitrophenyl phosphate (p-NPP) into nitrophenol and quantified in a multititer spectrophotometer. The cell layers were washed with PBS and lysed in 1% Triton X-100 in PBS. The lysates were frozen and stored in –70°C. To further release the enzyme activity the lysates were freeze-thawed twice. The enzyme reaction was set up by mixing 50 μl of lysate with 50 μl of substrate buffer containing 0.5 M 2-amino 2-methyle-1-propanol, 2 mM MgCl and 10 mM p-NPP at a pH of 10.3. A microtiter plate containing the enzyme reaction was incubated in a heated (37°C) multititer spectrophotometer and absorbency at 405 nm was determined for one hour.
Alkaline phosphatase histochemistry (Paper II)

Chamber slides were seeded with 2 x 10^4 cells/well and cultured in α-MEM with 10% FCS for seven days. Then the medium was changed to ones containing IL-4 or IL-13 in the concentrations 1 or 100 pM. α-MEM with 10% FCS and no additives was used as negative control and osteoinductive (OI) medium containing 10 mM β-glycerophosphate, 100 nM dexamethasone and L-ascorbic acid (50 μg/ml) was used as positive control. The media were changed after 1 week and after two weeks the wells were rinsed with PBS, fixed for 30 seconds in 60% citrate buffered acetone and incubated with 0.25 mg/ml fast Blue RR salt in 0.1 % naphtol AS-MX phosphate at pH 8.6 for 30 minutes. Slides were rinsed with tap water and covered with a glass slip before they were photographed.

von Kossa mineralization assay (Paper II)

Cells were seeded in 6-well plates at a density of 1 x 10^5 cells/well and cultured in α-MEM supplemented with PEST (100 U/ml of penicillin, 100 μg/ml of streptomycin), amphotericin-B (0.5 μg/ml), L-glutamine (2 mM) and 10% FCS. After one week the medium was changed and IL-4 or IL-13 was added in the concentrations 1 or 100 pM. OI medium was used as control and medium without additives as negative control. Medias were changed weekly. After two and four weeks respectively the experiment was interrupted. The cell layers were washed with PBS, fixed in neutral formalin and then incubated in a 5% (w/v) silver nitrate solution for 60 minutes under a UV light board. The wells were then rinsed in water and neutralized with 5% sodium thiosulfate. The culture plates were then photographed.

Alizarin-Red mineralization assay (Paper II)

Cells were seeded, handled and stimulated in the exact same manner as mentioned for v. Kossa above. After two and four weeks respectively the experiment was interrupted. The cell layers were washed with PBS and then fixed in ice-cool EtOH 70% for 1 h. After another washing-procedure in ddH2O cell-layers were incubated in Alizarin-Red colour-media, 40 mM AR-S pH 4.2, for 10 minutes in room temperature. Following repeated washing in ddH2O and once in PBS for 15 minutes in room temperature the culture plates were photographed. For colour quantification each cell layer was dissolved in 10% CPC for 15 minutes in room temperature, samples were pipetted to a micro-titre plate and absorbance was read at 540 nm.
Apoptotic DNA-fragmentation (*Paper III*)

Cells were seeded in 24-well culture plates and left to adhere in α-MEM supplemented with 10% FCS and antibiotics for 72 h. During initial experiments culture conditions were optimised concerning cell-number, serum-concentration and timing of DNA-histone complex measurements. Stimulation of 100.000 cells/well at a serum concentration of 0.7% FCS for 8h was considered optimal. Cells were subsequently seeded in 24-well culture plates at a density of 100.000 cells/well and left to adhere in α-MEM supplemented with 10% FCS and antibiotics for 72 h. The cells were then washed twice in PBS and the medium was changed to α-MEM with 0.7% FCS. Cytokines were added in triplicate series for each sample of either IL-4 or IL-13. After 8 h of incubation at 37°C stimulation was interrupted, cells were washed twice in PBS and then exposed to lysis. Samples were placed in streptavidin-coated microtitre plates and absorbance measurements (A405 - A490 nm) of DNA-histone complex binding from cell-lysates were carried out in a sandwich-Elisa-setting according to instructions from the manufacturer.

Measurement of Caspase-3 activity (*Paper III*)

Cells were seeded in 6-well culture plates at a density of 500.000 cells/well and left to adhere in α-MEM supplemented with 10% FCS and antibiotics for 48 h. The cells were washed twice in PBS and the medium was changed to 0.7% FCS. Time-course experiments revealed an optimum time-point of Caspase-3 measurement at 120 min post-stimulation determined by various stimuli-factors (Dexamethasone, TNF-α, IL-4 and IL-13) and by serum-starvation. Subsequent experiments, performed in medium supplemented with 0.7% FCS and antibiotics, contained cytokines (IL-4 1 nM and IL-13 1 nM) with double wells for each sample and in triplicate series. Dexamethasone (10 μM) and TNF-α (10 nM) in 0.7% FCS and also serum-starvation (0% FCS) were used as positive controls. After 120 min of incubation at 37°C stimulation was interrupted and supernatants were collected. Cells were detached by trypsin-EDTA and collected together with their respective supernatant. The double wells for each sample were pooled so that each sample contained a total of 1 million cells that were pelleted and frozen to –70°C. For analysis pellets were thawed, cells were dissolved in cell-lysis buffer and exposed to freeze-thawing. Following cell-lysis cell debris was pelleted and 50 μl of supernatant for each sample was used for Caspase-3 detection by fluorometric analysis according to the instructions provided by the manufacturer.
Animals (*Paper IV*)

IL-13\(^{-/-}\), IL-4\(^{-/-}\)IL-13\(^{-/-}\) and wild type (WT) mice were maintained on a more than 10 times backcrossed BALB/c background. Mice homozygous for the disrupted IL-13 or IL-4 and IL-13 genes were obtained by interbreeding the heterozygotes. Genotyping of tail DNA was performed at the age of 3 weeks using PCR as previously described (101). (Fig. 6) For the single knock-out (IL-13\(^{-/-}\)) four primers with the following sequences were used (5’ GGG TGA CTG CAG TCC TGG CT 3’), (5’ GTT GCT CAG CTC CTC AAT AAG C 3’), (5’ CTT GGG TGG AGA GGC TAT TC 3’), (5’ AGG TGA GAT GAT GAC AGG AGA TC 3’). Program conditions were 94\(^{o}\)C for 2 minutes (94\(^{o}\)C for 30 sec., 64\(^{o}\)C for 30 sec., 72\(^{o}\)C for 1 minute) for 30 cycles followed by 72\(^{o}\)C for 10 minutes. For the double knock-out (IL-4\(^{-/-}\)IL-13\(^{-/-}\)) three primers with the following sequences were used (5’ CCT GGA TTC CCT GAC CAA CAT C 3’), (5’ GGC CTT GCG GTT ACA GAG GCC 3’), (5’ ACC ACA CTG CTC GAC ATT GGG TG 3’). Program conditions were 94\(^{o}\)C for 2 minutes (94\(^{o}\)C for 30 sec., 60\(^{o}\)C for 30 sec., 72\(^{o}\)C for 1 minute) for 30 cycles followed by 72\(^{o}\)C for 10 minutes. Animals had free access to fresh water and food pellets consisting of cereal products (76.9% barley, wheat feed, wheat and maize germ), vegetable proteins (14.0% hipro soy) and vegetable oil (0.8% soy oil). The mice were anaesthetized and examined by pQCT at 6 (juvenile) and 20 (adult) weeks of age. The animals were sacrificed at 20 weeks of age, serum samples taken, and dissected with excision of internal organs and bones. Excised bones were then analysed by three-point bending. Each group contained seven animals.

In an additional experiment, female IL-4\(^{-/-}\)IL-13\(^{-/-}\) and WT mice were either sham operated (n= 8+8) or ovariectomized (ovx) (n=12+12), at 12 weeks of age and then analysed by pQCT at 20 weeks of age.
Animals (Paper V)

IL-4^{-/-}IL-13^{-/-} and wild type (WT) mice were maintained on a more than 10 times backcrossed BALB/c background. In the fracture-investigation mice homozygous for the disrupted IL-4 and IL-13 genes were obtained by interbreeding the heterozygotes. In the DXBM investigation homozygous mice of each type (WT and IL-4^{-/-}IL-13^{-/-}) were obtained by interbreeding homozygots (WT + WT and IL-4^{-/-}IL-13^{-/-}) respectively. Genotyping of tail DNA was performed at 3 weeks of age using PCR with primers as described above for the double knock-out (IL-4^{-/-}IL-13^{-/-}) mice. Animals had free access to fresh water and food pellets consisting of wheat, barley, soy, wheat-germ, wheat meal flour, wheat-bran, potato protein, minerals, vegetable fat, vitamins and trace elements.

Fracture model (Paper V)

A modified version of a mouse fracture model, earlier described by Skoglund et.al. (134) was used. Adult male mice, IL-4^{-/-}IL-13^{-/-} and WT, were anaesthetized by intraperitoneal injection 0.2 ml of a mixture of Dormitor® Vet. (1 mg/ml) 1 ml and Ketalar® (50 mg/ml) 1.5 ml suspended in 2.5 ml of NaCl. Under sterile conditions the distal part of the left femur was exposed by a lateral incision. The patella was dislocated medially to expose the femur condyles. A cannulated needle (diameter 0.6 mm) was drilled into the bone marrow cavity to the level of the major trochanter. The needle was backed 1 mm, cut off and reinserted to minimize damage to the knee. A standardized diaphysial fracture of the femur was then created by specially constructed tongs with semi lunar cutting edges sparing precisely the intramedularly placed needle. (Fig.7) After repositioning of the patella muscles covered the fracture and the skin was closed by sutures. Post-operatively the mice showing slow recovery were given a wake-up dose of Antisedan® Vet. (5 mg/ml) 0.1 ml suspended in NaCl in a 1:4 ratio. They were initially left to recover in separate heated cages. The mice started to use their operated leg within 1-3 days post-operatively where-after, apart from initial limping, they showed normal behaviour. Five weeks post-operatively the animals were sacrificed by CO2 inhalation, examined by faxitrone and dissected with removal of the femora. Before further analysis femurs were fixed by immersion in Zamboni’s solution for 24 hours and the specimens were then incubated for two days in 20% sucrose with Sörensen phosphate buffer, containing 0.01% sodium acid and 0.02% Bacitracin and there after kept in the same solution until further analysis.
Heterotopic bone model (Paper V)

In a parallel investigation adult male and female mice (IL-4\(^-/-\)IL-13\(^-/-\) and WT) were operated with implantation of demineralized, xenogenic bone matrix (DXBM) prepared from long bones of Sprague-Dawley rats (135). The mice were anaesthetized as described above. Under sterile conditions a small incision was made bilaterally on the lateral proximal thigh and a pouch was created in the rectus femoris muscle by blunt dissection. One DXBM-implant (2 mg) was placed in each muscle-pouch where after the fascia and skin was closed by sutures. (Fig. 8) The above-mentioned post-operative regime was applied and most of the animals started to use their operated leg within 1-2 days. Five weeks post-operatively the animals were sacrificed by CO\(_2\)-inhalation and examined by faxitrone. The implants were dissected free and removed for further analysis.
Peripheral quantitative computerized tomography
(Paper IV)

Computerized tomography (CT) analyses was performed *in vivo* at 6 and 20 weeks of age using the Stratec peripheral quantitative computerized tomography (pQCT) XCT Research M (software version 5.4B) operating at a resolution of 70 μm as previously described (136). Cortical bone parameters were determined with a mid-diaphyseal pQCT scan of the tibiae and femora. Trabecular volumetric BMD (tvBMD) was determined with a metaphyseal pQCT scan of the proximal tibiae and distal femora and defined as the inner 45% of the total area (137).

Peripheral quantitative computerized tomography
(Paper V)

Dissected fracture-bones, IL-4−/−IL-13−/− and WT (n=8+8), were analysed by Computerized tomography (CT) as described above. The callus was measured by performing five consecutive pQCT sections, starting from the centre of the callus, each with a thickness of five μm. Analysis of these five sections was used to determine the average cross-sectional area, calculation of callus volume and for determination of Bone Mineral Density (BMD) and Bone Mineral Content (BMC) in each fracture.
Mechanical testing (*Paper IV*)

The excised right femora were kept frozen until a few hours before mechanical testing. All specimens were tested to failure in three-point bending at room temperature on an electromechanical testing machine at a rate of 1 mm/second. The distance between the end supports was 8 mm and the load was applied in the middle between the two supports. The specimens were placed with the posterior part of the femoral condyles resting against one of the two end supports in such a way that the load was applied at 6.5 mm from the distal joint surface with an antero-posterior direction. An axial load cell with the range 0-100 N was used. Values for load and displacement were collected 50 times per second until failure, using software provided with the testing machine (Testware II). The collected data were stored as data files including the variables time, displacement and load. Based on the collected data load at failure, displacement at failure, stiffness and energy to failure were calculated. The bending stiffness was calculated by using the load and displacement data to define a slope after which the tangent of the maximal slope for each sample was used. Energy to failure was defined by the area under the load-displacement curve. (Fig. 9)

![Figure 9](image)

*Figure 9.* Representative load–displacement curve resulting from biomechanical test using three point bending with loading until fracture (failure). Y-axis represents breaking force (bone strength or load at failure) and X-axis is the displacement until failure. Blue bar defines the tangent of the maximal slope representing the stiffness. The area under the curve (yellow) represents the energy required to break the bone.
Mechanical testing *(Paper V)*
Fracture specimens, IL-4⁻/⁻IL-13⁻/⁻ (n=6) and WT (n=10), were tested to failure by three-point bending as described above. The distance between the end supports was 8 mm. The specimens were placed in such a way that the load was applied in an anterio-posterior direction with the centre of the callus in the middle between the two supports. An axial load cell with the range 0-25 lb was used. Values for load and displacement were collected 50 times per second until failure, using software provided with the testing machine (Testware II). The collected data were stored and analysed as mentioned above.

Serum Parameters *(Paper IV)*
Serum osteocalcin levels were measured by ELISA using a monoclonal antibody raised against human osteocalcin. The sensitivity of the osteocalcin assay was 21.1 ng/ml and intra- and inter-assay coefficients of variation (CVs) were less than 10%. Serum IGF-I levels were measured by double antibody IGF binding protein-blocked radioimmunoassay (RIA) (138).

Radiology by Faxitron *(Paper V)*
X-ray pictures of animals, in both investigations, were taken immediately after sacrifice and prior to dissection using faxitron. Voltage and duration was optimised, for animals included in the fracture experiment to 40 V and 40 sec. respectively, and for animals in the DXBM experiment to 46 V and 40 sec. respectively.

Measurement of mineralization by ash-weight *(Paper V)*
Retrieved (left-side) DXBM implants from IL-4⁻/⁻IL-13⁻/⁻ and WT male (8+10) and female (n=11+12) mice, were ashed in a muffle furnace at 600 °C for 24 hours and weighed. Mean values of ash-weights in the two groups (WT and IL-4⁻/⁻IL-13⁻/⁻) were compared for male and female animals respectively.
Histological analysis (*Paper V*)

**Fracture model.**

In the fracture experiment histological analysis was performed in mechanically tested callus specimens from male, IL-4⁻/⁻IL-13⁻/⁻ (n=6) and WT (n=5), animals. Also, 4 intact calluses, IL-4⁻/⁻IL-13⁻/⁻ (n=2) and WT (n=2) were examined. For histochemistry the specimens were demineralized in EDTA in 4°C for 4 weeks before sectioned longitudinally on a Leica cryostat at a thickness of 14 μm and then kept in minus 70°C. Frozen sections were consecutively chosen for haematoxylin-eosin (H&E) staining for morphological analysis and indirect immunofluorescence technique. Antibodies to protein gene product 9.5 (PGP 9.5) and growth-associated protein-43 (GAP-43) were used as markers of mature and newly formed immature nerve fibres respectively. Antibodies to calcitonin gene-related peptide (CGRP) and neuropeptide-Y (NPY) were used as markers of sensory and autonomic nerves. For identification of newly formed vessels, platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) was used. The sections were initially incubated with 10% normal serum, followed by incubation with the primary antibody, all five mentioned above in dilution 1:200, for 12 hours in 4°C. The secondary antibody, fluorescein isothiocyanate (FITC)-conjugated diluted 1:20 was applied for half an hour in 37°C. As antifader Vectashield™ was used. The sections were analysed by 2 independent observers in a Nikon and a Leica epifluorescens-microscope regarding morphology and occurrence of positive immunoreactive staining for nerve fibres and vessels. A finding was regarded as positive when confirmed by both observers in at least 1/3 of the sections, and in the majority of the implants. A positive finding was defined as a positive immunostaining for the specific antibody in at least five femora out of eight (KO) or four out of seven (WT).

**Heterotopic bone model.**

Retrieved (right-side) DXBM implants from IL-4⁻/⁻IL-13⁻/⁻ and WT male (10+8) and female (n=11+12) mice, were harvested five weeks following implantation. Twenty-seven specimens of the total were handled and processed for histochemistry, H&E and indirect immunofluorescence staining in accordance with the technique given for the fractures previously mentioned except that no demineralization was needed. After fixation and rinsing the sections were kept in -70°C before staining. Specimens were prepared for histological and immune-histological analysis of nerve ingrowth and vascularization as specified above. The DXBM implants were analysed separately for DXBM, bone marrow formed within the implant, capsule around the implant, and surrounding muscle. A finding was regarded as positive when
confirmed by both observers in at least 1/3 of the sections, and in the majority of the implants. Thus, a positive finding demanded a positive immunostaining for the specific antibody in four specimens out of seven in male and female IL-4<sup>−/−</sup>IL-13<sup>−/−</sup> mice and male WT mice and in four specimens out of six in female WT mice.

Statistics (Papers I, IV & V)

(Paper I) Two-tailed Student’s t-test for independent groups was used for statistical analysis.

(Paper IV) Based on power-calculations, knowledge of the precision of measurement of the outcome variables, and on the hypothesis that we should be able to determine differences in bone phenotypes of more than 5 to 10%, we determined the number of animals to be 7-8 in each group. This number was also regarded as ethically justified by the Ethical councils for animal research. Due to favourable breeding outcomes some of the groups in the ovx-study contained 12 mice. For statistical evaluation two-tailed Student’s t-test and Mann-Whitney U test was applied.

(Paper V) For statistical evaluation of ash-weight and pQCT-data (Callus Volume, BMD, BMC) two-tailed Student’s t-test was used. Biomechanical data was statistically evaluated by Mann-Whitney U-test.
RESULTS & DISCUSSION

Effects of pro- and anti-inflammatory cytokines on IL-6 formation in human osteoblasts (Paper I)

Communication between osteoblasts and osteoclasts is of crucial importance for the regulation of normal bone turnover. In this communication system IL-6 has been a prime candidate where the role of IL-6 in bone is predominantly believed to be osteoclastogenic (83).

In this study we have focused on the action of, as well as the interaction between, pro- and anti-inflammatory cytokines affecting IL-6 secretion in osteoblasts. We found that both pro-inflammatory cytokines IL-1 and TNF-(α/β) and anti-inflammatory cytokines IL-4 and IL-13 stimulate the secretion of IL-6 in cultured human osteoblasts dose-dependently and with a rank order of potency IL-1 > TNFs > IL-4 > IL-13. To investigate possible interactions we performed co-stimulation experiments between the pro- and anti-inflammatory cytokines at EC$_{50}$-doses. No additive or synergistic effect was detected on IL-6 secretion in hOB cells co-stimulated with the two anti-inflammatory cytokines IL-4 and IL-13. As IL-4 and IL-13 share receptor subunits this lack of potentiated effect might be explained by competition between the two anti-inflammatory cytokines on the receptor level.

On the other hand co-stimulation with IL-1 together with IL-4 resulted in a significant synergistic effect on IL-6 secretion. Co-stimulation with IL-1 together with IL-13 also resulted in a synergistic effect on IL-6 secretion although not as pronounced as that exerted by IL-1 + IL-4. From earlier experiments we have learned that there is a difference in potency where the effect of IL-4 is approximately 10 times that of IL-13. Again, referring to the shared activation mechanisms through the same receptor subunits of IL-4 and IL-13, this difference in potency might be explained on the receptor level in terms of affinity. To investigate the specificity of the synergistic effect by IL-4/13 and pro-inflammatory cytokines we further analysed the interaction in co-stimulatory experiments with TNF α and β. Also together with these two pro-inflammatory cytokines respectively IL-4 or IL-13 potentiated IL-6 secretion in hOBs.

Bone remodelling, like many other homeostatic systems, is dependent on a variety of factors. Previous studies on IL-6 has pointed out this pro-inflammatory cytokine as an important osteoclast activating factor in different conditions with pathological osteoclastic bone resorption, e.g. arthritis.
and bone malignancies (4, 79, 139, 140). The activity of IL-6 in bone seems to be dependent on either IL-6 receptors on osteoblasts or a complex of IL-6 and a soluble IL-6 receptor (88), but there is also evidence for IL-6 receptors expressed by osteoclasts (89). Recently the powerful axis of RANK/RANKL/OPG in bone metabolism has been elucidated (141). There is now also data indicating interactions between IL-6 and the RANK/RANKL pathway enhancing bone resorption in conditions like Paget’s disease and possibly bone metastases or myeloma (142). Hence, osteoclast activity may be regulated by IL-6 via different pathways where osteoblasts play a crucial role as IL-6 producers and mediators of IL-6 effects. Apart from IL-6 production (81, 82) osteoblasts also express IL-6 receptors (85) indicating yet another possible, autocrine way of osteoclast regulation.

It might be tempting to look at pro- and anti-inflammatory cytokines as two entities always counteracting each other. Interestingly, the results in this study show that the anti-inflammatory interleukins IL-4 and IL-13, otherwise known to suppress pro-inflammatory cytokines, here work synergistically with IL-1 and TNF, promoting IL-6 secretion in human osteoblasts. These data suggest a possibility for IL-4 and IL-13 to affect osteoclast activity not merely by promoting IL-6 secretion directly, but maybe more importantly by facilitating IL-6 secretion from human osteoblasts stimulated with other cytokines promoting bone resorption such as IL-1 and TNF.

Influence on markers of osteoblastic differentiation and mineralization by IL-4 & IL-13. (Paper II)

Historically distinct markers of osteoblastic proliferation and differentiation stages are still lacking, although a picture of the kinetics in osteoblastic development is beginning to appear (7). In this study we investigated the effects of anti-inflammatory cytokines IL-4 and IL-13 on the expression of activity markers in cultured human osteoblasts. Treatment with IL-4 and IL-13 dose-dependently increased the secretion of PICP from cultured hOBs, reflecting increased collagen synthesis and indicating a positive role for IL-4 and IL-13 in bone matrix synthesis. This result strengthens earlier data showing IL-4 induced collagen-1 expression in human osteoblast-like cells (120). In our study IL-4 and IL-13 also stimulated the secretion of ALP in a dose-dependent manner evaluated quantitatively and histochemically. Apart from one previous study showing inhibited ALP-activity in murine osteoblast cell-line MC3T3-E1 (123) the present data is in agreement with earlier research concerning effects of IL-4 on ALP activity in osteoblast cell-line MG63 (119) and in human osteoblasts (120). Furthermore it is interesting to note that IL-13 exerts similar effects on both ALP and collagen-1 ex-
pression as the related cytokine IL-4. This effect of IL-13 on osteoblast activity markers has, to our knowledge, never been shown before. Concerning the formation rate of osteocalcin, which is a relatively late marker of bone activity maximally expressed during mineralization (6), no effect was seen by IL-4 or IL-13 stimulation. Most interestingly we found that mineralization, the final part in the cycle of bone regeneration, was dose-dependently stimulated by both IL-4 and IL-13. Similar results in human osteoblast-like cells have been presented previously, although restricted to IL-4 induced mineralization by periosteal cells (121). Hence, the present study not only strengthens this earlier evidence of IL-4 induced mineralization in human osteoblasts, but it also points to the same effect from the related anti-inflammatory cytokine IL-13. In addition it shows that not only periosteal, but also endosteal osteoblasts react on anti-inflammatory cytokines with induced mineralization. The total rate of bone synthesis is related to the number of active osteoblasts and to the grade of activity in each osteoblastic cell. From earlier research we know that IL-4 and the related cytokine IL-13 inhibit cell proliferation in isolated human osteoblasts (82). Therefore, including the results from the present study, the net effect of IL-4 and IL-13 indicates a role of maintaining the osteoblast in a differentiated phase or potentially even to provoke a shift of the osteoblast from a proliferative to a more mature, productive stage.

Apoptosis in human osteoblasts, induced by IL-4 and IL-13 (Paper III)

IL-4 and IL-13 influence osteoblast proliferation, and both cytokines have presented a dose-dependent negative effect shown by inhibited thymidine incorporation in osteoblast cell line MG-63 (119) and also in hOBs (82). Since the reason for this inhibition of proliferation is unknown we hypothesized that apoptosis may be the underlying mechanism. By the use of Elisa, detecting specifically cleaved histone-bound DNA fragmentation representing the final step in apoptosis, we investigated apoptosis in cultured human osteoblasts. Setting up the system, we cultured hOBs for different time intervals, with increasing cell density and increasing serum concentrations. As expected osteoblasts exposed to serum starvation were more vulnerable to apoptosis, especially at higher cell densities. Osteoblasts cultured in 10% FCS were relatively insensitive to the induction of apoptosis even at higher cell densities. Comparing different levels of serum starvation at equivalent cell density, we found a dose-dependent increase in DNA-fragmentation with declining serum concentrations. Also this finding is expected and well in agreement with earlier data showing declining levels of activity and induction of apoptosis in osteoblasts exposed to serum-
starvation (143). The concentration limit for survival was found to be between 1 and 3% of FCS and EC_{50} approximately 0.7% FCS. Investigating the timing for measurement of DNA-fragmentation we found a time dependent increase in DNA fragmentation escalating up to 8 h post stimuli. Thus, we conclude that primary human osteoblasts are sensitive to serum deprivation causing apoptosis as identified by dose and time dependent increase in DNA fragmentation.

To test the postulated apoptotic effect by IL-4 and IL-13 we cultured primary human osteoblasts at the lowest possible amount of FCS (0.7%) in order to avoid serum factors rescuing from apoptosis. We found a dose dependent increase in DNA fragmentation by stimulation with IL-4. The response of the DNA fragmentation was two-phased which may suggest that IL-4 signals through a high and a low affinity receptor respectively. Also by stimulation with IL-13 a dose dependent increase in DNA fragmentation was observed.

To confirm these results we analysed caspase-3 activity, the final step of caspase activation during apoptosis, preceding the onset of DNA fragmentation. Caspase-3 activation was stimulated by serum starvation as well as by dexamethasone or TNF-α at high doses with a maximum effect at 120 minutes post-stimuli. Osteoblasts cultured in decreasing amounts of FCS responded dose-dependently with increased caspase-3 enzymatic activity verifying that osteoblasts are sensitive to FCS deprivation. Stimulation by IL-4 and IL-13 (1 nM) also caused an increase in caspase-3 activity confirming that IL-4/IL-13 induce apoptosis in primary human osteoblasts. We also noted a two-phased dose-response pattern in caspase-3 activity following IL-4 and IL-13 stimulation. The increase in caspase-3 activity by IL-4 and IL-13 found in our study was well in level with the effect of high-dose stimulation by TNF-α or dexamethasone.

Expression of death-receptors (Fas) and apoptosis mediated by Fas-L or TNF-α has previously been shown in human osteoblasts (144-146) and in murine osteoblasts (147, 148). According to these studies TNF-α exerts its apoptotic action, either by upregulation of Fas-expression facilitating activation of apoptosis by Fas-L binding, or as a direct effect of TNF-α without the need for co-activation with Fas-L. Our results concerning TNF-α suggest a direct effect as no Fas-L was added. However we used a relatively high dose of TNF-α and perhaps the question of Fas-L dependency is also a matter of TNF-α dose. Glucocorticoids increase osteoblast apoptosis in vitro and in vivo (41) and there is some evidence of activation via the mitochondrial pathway by induction of cytochrome c release. Suppression of survival factors may also be a mechanism explaining glucocorticoid induced apoptosis (37).

Concerning IL-4 and IL-13 the present data show induction of apoptosis via activation of caspase-3 in hOBs. Thereby it also seems reasonable to
believe that the inhibited proliferation seen by IL-4 and IL-13 in hOBS can be caused by induced apoptosis.

Bone loss in IL-4 and IL-13 inactivated mice (Paper IV)

In this investigation we describe in vivo effects in bone by the two anti-inflammatory cytokines IL-4 and IL-13 using gene-depleted mice. As both cytokines share receptors (110-112) we chose gene depleted mice with either a single gene-knockout (IL-13⁻/⁻) or knockout of both genes (IL-4⁻/⁻IL-13⁻/⁻) in comparison to WT-mice. The skeletal phenotype was analysed at 6 weeks of age (before sexual maturation) and at 20 weeks of age (adult mice) in IL-13⁻/⁻, IL-4⁻/⁻IL-13⁻/⁻ and WT mice of both genders.

Adult male IL-4⁻/⁻IL-13⁻/⁻ mice displayed a significant reduction of cortical BMC as measured by pQCT compared to WT mice. The reduction in cortical BMC was due to a decreased cross-sectional area correlating to reduced cortical thickness while cortical volumetric bone mineral density (cvBMD) was unaffected. Thus, the amount but not the density of cortical bone was reduced. From pQCT values it is also possible to theoretically estimate mechanical strength by calculation of the area moment of inertia. As predicted the area moment of inertia was also decreased in adult male IL-4⁻/⁻IL-13⁻/⁻ mice. When the quality of the bone is unchanged, the area moment of inertia is normally proportional to the mechanical strength of the bone determined by three-point bending (149). This was confirmed by biomechanical testing, where we found significant reduction in displacement, maximal load and total energy to failure in adult male IL-4⁻/⁻IL-13⁻/⁻ mice. Also, the reduction of maximal strength was not decreased more than suggested by the changes in the area moment of inertia, supporting the notion that the amount but not the quality of cortical bone is affected in adult male mice with depletion of IL-4 and IL-13.

As the reduction in cortical bone formation was seen in the double knock-out mice alone and not in the IL-13 single knock-out mice the effect is either a result of the combined gene-disruption or a solitary effect of IL-4 depletion. Unfortunately we did not have access to IL-4 single KO mice to draw a more precise conclusion. The altered skeletal phenotype of adult male IL-4⁻/⁻IL-13⁻/⁻ mice was not seen in juvenile mice. Also in adult female IL-4⁻/⁻IL-13⁻/⁻ mice no altered skeletal phenotype was found indicating a protective role of female sex steroids. However, ovariectomy did not provoke the same cortical phenotype seen in adult male IL-4⁻/⁻IL-13⁻/⁻ mice. Neither was ovarietomy-induced trabecular bone loss dependent on disruption of IL-4 and IL-13.

Weight of total body, kidney, liver and heart was unaffected in gene-depleted mice of both genders indicating that the altered phenotype is restricted to bone tissue. The effect was also restricted within bone tissue af-
fecting only cortical bone. No influence was detected in trabecular bone, on bone length or on osteocalcin levels. All together this isolated influence on cortical bone suggests a specific effect on periosteal bone formation rather than a metabolic effect in bone. In spite of the rather small number of mice in each group (n=7) there were no clear tendencies in the outcome variables indicating that larger samples might have yielded significant differences, other than those for cortical bone in adult male mice.

Results from previous studies show that the two anti-inflammatory Th2 cytokines IL-4 and IL-13 are involved in the regulation of bone mass. A majority of these studies also points towards a metabolic role of IL-4 and IL-13 potentially yielding a negative effect on bone turnover (82, 119, 122, 124, 133). The present study support earlier evidence of a role for IL-4 and IL-13 in the regulation of bone mass. However, while overproduction of IL-4 and IL-13 yield metabolic bone effects, inactivation of these cytokines seems to be more specific affecting cortical bone modelling and macro-structure. The present results raises questions concerning potential effects of IL-4 and IL-13 on other modelling processes like fracture healing and bone induction.

**Bone formation in IL-4 and IL-13 inactivated mice**

*(Paper V)*

Based on our previous findings showing decreased cortical bone formation in IL-4−/−IL-13−/− mice (Paper IV) we wanted to further elucidate the role of IL-4 and IL-13 in different situations of bone formation in vivo. For this purpose we used two experimental models reflecting physiological processes, i.e. fracture healing and heterotopic bone formation respectively.

Fracture healing has been studied earlier in different rodent models (40, 150, 151). Also, treatment studies related to growth factors have been presented, e.g. with positive effects on fracture healing by GH, IGF-1 and TGF-β1. (72, 152, 153). We compared adult male WT mice to IL-4−/−IL-13−/− mice in which we previously detected a selective cortical bone phenotype. In both groups animals displayed radiological healing with mineralized callus formation of the fractures at 5 weeks but no visual differences were detected concerning callus development between the two groups. A slight, though non-significant, decrease in callus volume and bone mineral content (BMC) was detected in IL-4−/−IL-13−/− male mice compared to WT male mice as determined by pQCT. Bone mineral density (BMD) was unaffected comparing the two groups. Comparison of mechanical strength by three-point bending revealed no differences concerning the parameters load, displacement, stiffness or energy to failure between IL-4−/−IL-13−/− and WT mice. Histological evaluation by staining with H&E showed sparse fracture callus in all femora. In both groups vascular endothelial staining, by PECAM-1, was positive.
only in the Volkmann’s canals. Positive immunoreactivity to all four neuronal antibodies (GAP-43, PGP 9.5, CGRP or NPY) in use was found in the periosteum and surrounding muscular tissue but not in the callus tissue or adjacent cortical bone. Also in this aspect no differences between the groups were detected. Although autonomous (NPY) and sensory (CGRP) nerve fibres could be detected in the marrow cavities of the fractured bones of IL-4−/−IL-13−/− mice as opposed to WT mice this difference was not associated with any alteration in fracture healing or gross morphology of the calluses. In summery, depletion of the two Th2 derived cytokines IL-4 and IL-13 was not reflected by any alterations in fracture healing in adult male mice.

In the second part of this study we investigated the capacity to form heterotopic bone by induction of DXBM intramuscularly implanted in IL-4−/−IL-13−/− adult mice compared to WT mice of both genders. Bone formation by demineralized bone matrix has been studied in various animal models with implantation subcutaneously, intramuscularly or in bone defects (154, 155).

Radiological evaluation at 5 weeks showed mineralization of implants in all groups. Analysis of net bone formation by ash-weight measurements revealed no differences in either gender comparing IL-4−/−IL-13−/− to WT mice, although a tendency towards less bone in the IL-4/13-deficient mice was noted. Evaluating bone formation histologically by H&E staining an ossicle was found with a surrounding periosteum-like capsule of connective tissue in all implants, WT as well as IL-4−/−IL-13−/−. Immuno-histological staining by (PECAM-1) revealed an endothelial network in the capsule around the implant, in the surrounding muscular tissue and also in the newly formed marrow. This too, was seen in all specimens. However, inside the implant-matrix positive PECAM-1 staining was found only in WT animals. Inhibited vascularization indicate one possible mechanism for inhibition of bone formation since vascularization of the inductive implant-matrix is a prerequisite for the induction of cartilage and bone (71).

Immunoreactivity to all types of nerve fibres was found in the surrounding muscle while none of the nerve fibres stained for was found in the DXBM matrix or marrow in any mouse type. In the capsule immature (GAP-43) and mature (PGP 9.5) nerve fibres were present in all mice. Autonomic nerve fibres (NPY) were found only in the capsule in WT male and female mice. The absence of autonomous nerves in the capsule of IL-4−/−IL-13−/− mice is interesting since osteoblasts have been shown to express receptors for NPY indicating a role for this neuropeptide in bone formation (24). In addition, NPY has been shown to inhibit the effects of PTH on osteoblastic cells (156). Thus, the lack of expression of NPY in IL-4/13-deficient mice indicates another possible mechanism by which the reduced cortical bone in these mice may have been produced.

In summery an altered pattern of nerve expression is detected in connection to the fracture callus and around the DXBM implants respectively. Also, reduced angiogenesis within the implants of IL-4/13-deficient mice is seen.
However, these changes were not connected to any major effects on fracture healing or heterotopic bone formation.
CONCLUSIONS

- IL-4 and IL-13 stimulate IL-6 secretion in cultured human osteoblasts and also enhance IL-6 secretion in cultured human osteoblasts stimulated by pro-inflammatory cytokines.

- IL-4 and IL-13 dose-dependently stimulate collagen I secretion and ALP expression in human osteoblast cell cultures.

- IL-4 and IL-13 enhance mineralization in human osteoblast cell cultures.

- IL-4 and IL-13 induce apoptosis through activation of caspase-3 in cultured human osteoblasts.

- Depletion of IL-4 and IL-13 cause reduction of cortical bone thickness and strength without affecting cortical bone density or trabecular bone synthesis in mice. This effect is gender-specific, detected only in male mice and not influenced by female gonad hormones.

- Depletion of IL-4 and IL-13 cause minor alterations in neuro- and angiogenesis during bone formation in mice; however no net effect on new bone formation is detected.
Cytokines are important actors in many aspects of bone metabolism. Their actions are implicated in normal bone development and regeneration, and also in different pathological conditions causing imbalance in the process of bone turnover. Even though many cell types have the capacity to produce cytokines, they primarily originate from the immunological system where activated T-cells constitute their primary source. Given the close proximity between bone tissue and the haematopoietic tissue in bone marrow it is of great interest to investigate potential interactions between the immunological system and bone. Also, immunological reactions are mandatory in conditions like infectious bone diseases or fracture healing where the reaction aims to heal and restore the bone. Sometimes the reaction becomes unbalanced, which is seen in rheumatoid arthritis (RA) and bone malignancies or metastasis, where cytokines are involved. Cytokines also play a role in defective bone healing (pseudarthrosis) and in aberrant (heterotopic) bone formation. Most pathological conditions result in loss of bone (osteolysis) which is seen in bone infections, RA, hypotrophic pseudarthrosis and in most malignancies involving bone tissue. However, sometimes generation of bone excess is seen e.g. in osteoarthritis (osteophytes), in heterotopic bone formation, in osteopetrosis and in hypertrophic callus development. Cytokines are also involved in osteoporosis, one of the fastest growing diseases in modern society, leading to increased morbidity and fracture risk. Unlike the pro-inflammatory cytokines, the anti-inflammatory cytokines have played a quite anonymous role in bone biology.

In the present thesis we have focused on the action of the two anti-inflammatory cytokines IL-4 and IL-13 investigating their effects in vitro in cultured human osteoblasts and in vivo using gene-depleted mice. We consider our in vitro culturing system to be very reliable since more than 600 bone samples from patients undergoing total hip replacement has been isolated and investigated in different aspects including repeated checking of the osteoblast phenotype. These cells represent the common denominator in the first three papers. Although osteoblasts act primarily as bone producers they also play an important role in bone resorption as regulators of osteoclastogenesis and osteoclast activity. Therefore, our finding of enhanced IL-6 formation by pro- and anti-inflammatory cytokine stimulation in hOBs (Paper I) is interesting. The isolated effect of each cytokine, is confirmatory of earlier data. However, the combined and synergistic effect on IL-6 formation by
pro- and anti-inflammatory cytokines is a novel discovery. This shows that pro- and anti-inflammatory cytokines do not always work to antagonize each other but on the contrary sometimes enhance each other’s effects.

In papers II and III we show that IL-4 and IL-13 enhance the expression of osteoblastic markers of activity and also that they can induce apoptosis in cultured human osteoblasts. ALP expression, and especially the ability to produce collagen type I together with induction of mineralization are typical for the differentiated osteoblast phenotype. Both IL-4 and IL-13 were shown to increase ALP formation and PICP production and also to induce mineralization in hOBs (Paper II). Considering the similarity between IL-4 and IL-13 with respect to other biological activities, it is not surprising to find similar actions also in this aspect. The effects of IL-13 presented are, to our knowledge, shown for the first time, while the same effects by IL-4 are mostly confirmatory. As IL-4 and IL-13 are known to inhibit osteoblast proliferation, the increased expression of late osteoblastic markers cannot be explained by a larger number of cells. We believe the explanation to be rather that IL-4 and IL-13 enhance the differentiation of human osteoblasts so that more cells reach a late differentiation step earlier.

Investigating the mechanism behind inhibited cell proliferation by IL-4 and IL-13 we found that both cytokines are able to induce apoptosis in hOBs (Paper III). However, if apoptosis explains the whole inhibitory effect remains to be proven since for example cytokine induced cell cycle arrest could constitute an additional effect. The exact mechanism behind the apoptotic effect by IL-4 and IL-13 is not elucidated in this study and can only be speculated upon. Either, IL-4 and IL-13 induce apoptosis directly in hOBs via the extrinsic or intrinsic apoptotic pathways, or, considering the previous results (Paper II), IL-4 and IL-13 enhance the differentiation of hOBs so that more cells reach maturation earlier and thereby approaches their final destination and the naturally occurring apoptosis faster.

In vitro culturing of primary human osteoblasts gives us the opportunity to study isolated events like the interaction between cytokines and the osteoblast. Human osteoblasts are also advantageous, compared to rodent bone cells or immortalized osteoblastic cell-lines, with respect to possible conclusions for the human species. At the same time one has to be aware that these in vitro investigations study isolated events with the risk of yielding findings of limited importance to the in vivo situation.

Papers IV and V are in vivo studies investigating the phenotype and capacity for bone formation in gene depleted mice. In the phenotype-study (Paper IV) mice with depletion of either IL-13 or IL-4/13 were examined. The finding of reduced cortical bone mass in only adult male IL-4/13 KO mice was somewhat intriguing in many aspects, raising questions about cytokine specificity, gender specificity, bone specificity (only cortical) and age-relation. These questions are also discussed above (Results and Discussion Paper IV). Access to single IL-4 KO mice and extended experiments by
orchidectomy in male IL-4/13 KO mice might have made it possible to address the gender and cytokine issue more specifically. As this is an in vivo situation the exact mechanism behind the sole effect on cortical bone is difficult to determine, and can only be speculated upon at this point. The cytokines might provoke different actions in osteoblasts and/or osteoclasts depending on location, trabecular vs. cortical bone. In this aspect the effect might be dependent on different pattern of receptor expression. The access to other local factors interacting with IL-4 and IL-13 might also be different in cortical bone compared to trabecular bone. Furthermore, it is possible that IL-4 and IL-13 work through actions on other tissues in close proximity to bone. Influences on vascularization, innervation or on the periosteum covering only cortical bone are all possible pathways.

Expanding on the results from the phenotype study we investigated the effects of IL-4 and IL-13 on two physiological types of bone formation, again using gene-depleted mice (Paper V). In the fracture part of the study no differences were found concerning callus development or net bone formation between IL-4/13 KO and WT mice. In the DXBM part of the study minor effects were shown where vascularization of the implants and autonomous nerve ingrowth in the surrounding capsule were inhibited in IL-4/13 KO mice. However, no effect was seen in net bone synthesis.

The reason for the lack of effect on bone regeneration seen in these two investigations can only be speculated upon. Experimental conditions with a strong signal for new bone formation or a possible alteration in immunological response secondary to the operative trauma might have influenced the results. A possible weakness in our animal studies is the number of animals included. Power calculations indicated seven to eight animals in each group to be adequate with respect to catching any differences in the variables measured. Animal groups were bred within ethical guidelines with a small surplus; however, due to fatal drop-out, the groups in the biomechanical part (Paper V) became uneven. A possible weakness in the histological parts of the last study is the nature of the method, relying on the subjective judgement of the observer. In this aspect we have tried to compensate for bias as far as possible by using predetermined criteria for histological evaluations and by the use of two independent observers.

The results presented in this thesis clearly show that IL-4 and IL-13 affect human osteoblasts in different respects. Interpreting the in vitro data we find no uniform explanation as to whether the net effect is negative or positive for the osteoblast or possibly for bone formation. In vivo data point towards a positive effect on cortical bone formation as withdrawal of IL-4 and IL-13 leads to reduction of cortical bone mass. However, this effect is limited and also specific concerning age, gender and bone type. Compared to other powerful cytokines it seems that IL-4 and IL-13 play a more discrete role with diverging actions depending on the situation in bone formation and metabolism.
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