IL-6, *IL-1 β* , and *TNF- α* only in combination influence the osteoporotic phenotype in Crohn's patients via bone formation and bone resorption

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Abstract

Background. Crohn's disease (CD) is associated with a higher prevalence of osteoporosis. The pathogenesis of bone affliction remains controversial, especially if inflammatory cytokines or glucocorticoid therapy are the main contributors. In postmenopausal osteoporosis, bone resorption is induced by *IL-6*, *IL-1 β* and *TNF- α* . In contrast, in children with CD, *IL-6* exclusively decreased bone formation without affecting bone resorption.

Objectives. The objective of this study was to further clarify the pathophysiology of bone affliction in adult patients with CD with the use of an osteoblast and osteoclast cell model.

Material and methods. Inflammatory cytokines *IL-6*, *IL-1 β* , and *TNF- α* were measured in adult CD patients' serum. Mean values of these cytokines were applied with or without dexamethasone to the human cell line SCP-1 (osteoblastic cell model). Also, the effect of cytokines on primary human osteoclast differentiation and activity was determined.

Results. The combined cytokine application increased the receptor activator of NF- κ B ligand/osteoprotegerin (*RANKL/OPG*) ratio 2-fold after 2 and 14 days. Additional application of dexamethasone to SCP-1 cells further increased the *RANKL/OPG* ratio 3-fold, but decreased *IL-6* and *IL-1 β* expression to 10% and 50%, respectively. *TNF- α* expression was maximally suppressed to 16% by dexamethasone in the presence of cytokines. In osteoclasts, the combined cytokine treatment decreased expression of characteristic genes to approx. 30%, while increasing osteoclast resorption activity to 148%. In addition, a cytokine stimulated osteoblast cell culture-generated supernatant stimulated osteoclast resorption activity by 170%.

Conclusions. Our results suggest that *IL-6*, *IL-1 β* , and *TNF- α* only in combination induced osteoclast-stimulating activity represented by the *RANKL/OPG* ratio in osteoblasts. Dexamethasone further increased this effect in osteoblasts, while decreasing cytokine expression. The results in osteoclasts support a direct and osteoblast-mediated effect on bone resorption. Our in vitro results differentiate for the first time the effect of cytokines on bone turnover as measured in adult CD patients from the additional dexamethasone effect on osteoblasts as part of the pathophysiology of osteoporosis.

Key words: cytokines, osteoprotegerin, Crohn's disease, bone remodeling, *RANK ligand*

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Introduction

Patients with Crohn's disease (CD) may develop secondary osteoporosis with fractures in the course of the disease.^{1–4} The pathophysiology of osteoporosis in CD has not been clearly established, but, aside from steroid therapy, the disease itself appears to be a key factor.⁵ Cytokines, such as interleukin 1 beta (*IL-1β*), interleukin-6 (*IL-6*), and tumor necrosis factor alpha (*TNF-α*), have been implicated in the pathogenesis of CD.^{6–8} These cytokines are key regulators of bone turnover, and are involved in postmenopausal osteoporosis, as well as osteoporosis in rheumatic diseases.^{9–12} Bone turnover is characterized by balanced bone formation by osteoblasts and the degradation of bone by osteoclasts. The most important factor for bone turnover is the ratio of the receptor activator for NF-κB ligand (*RANKL*) and osteoprotegerin (*OPG*), factors synthesized by osteoblasts and osteocytes, but acting only on osteoclasts.¹³

Inflammatory cytokines play a major role in shifting the *RANKL/OPG* balance toward excessive *RANKL*, which induces osteoclast activity and greater bone degradation.¹⁴ We know from studies of postmenopausal osteoporosis that *IL1β*, *IL-6*, and *TNF-α* are associated with increased bone resorption in in vivo models.¹⁵ Additionally, in patients with CD, inflammatory cytokines are correlated with markers of osteoclast activity.¹⁶ However, in an in vitro organ culture model of bone formation, serum from children with CD exclusively influenced bone formation without inducing changes in bone resorption, suggesting that the inflammation from CD in children affects osteoblasts and bone formation, but not osteoclasts.¹⁷ These findings were surprising, because *IL-6* is an osteoclast-activating factor in other inflammatory diseases such as rheumatic disorders.¹⁸ In addition, it has been suggested that other cytokines, especially *TNF-α*, are the most relevant cytokines in CD.¹⁹

We now hypothesize that multiple factors in serum, but not *IL-6* or *TNF-α* alone, are primarily responsible for bone disease in adult CD patients, and that these factors are more highly expressed in patients with an acute phase of CD. In addition, we determined the effects of these cytokines with and without glucocorticoid on bone formation and/or bone resorption by monitoring the *RANKL/OPG* ratio using an osteoblast cell culture model.

We have also investigated the direct influence of the combined cytokine treatment on bone resorption using primary human osteoclasts.

Material and methods

Serum sampling in patients with acute CD

Patients with acute CD were recruited either at the emergency clinic or at our Clinic of Gastroenterology and Gastrointestinal Oncology (UMG, Germany) for chronic

inflammatory bowel diseases, and the diagnosis of CD was based on endoscopic, histological, or radiological findings. "Acute" disease was identified using the Crohn's disease activity index (CDAI), in which a score greater than 150 is defined as active disease.²⁰ Patients were excluded if they had been treated with any steroid or immunosuppressive compound during the previous 3 months. The use of calcium and vitamin D supplements, or estrogen prior to the study was allowed. Upon inclusion in the study, routine blood analysis was performed. An additional 50 mL of blood was drawn, placed directly on ice, and centrifuged within minutes, and the resulting serum was batched and stored at -70°C . Sera obtained from healthy, age- and gender-matched controls were treated under identical conditions. This study was approved by the Ethics Committee of Göttingen University Medical Center, and informed consent was signed by all subjects. The characteristics of these patients as well as detailed inclusion and exclusion criteria had been published previously.²¹

Cytokine production

The serum levels of the cytokines were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's specifications (Table 1). *IL-6* (R&D Systems, Minneapolis, USA; 0.447–9.96 pg/mL, minimal detectable level 0.016–0.110 pg/mL), *IL-1β* (R&D systems, Minneapolis,

Table 1. Primer sequences used in the present study for real-time polymerase chain reaction (PCR)

Gene	Primer sequence
<i>β-actin</i>	Fwd:5'-ctggaacggtgaaggtgacg-3' Rev:5'-agtctctcgccacattgtga-3'
<i>OPG</i>	Fwd:5'-ctcaggcacttgaggcttctc-3' Rev:5'-tgcaagcagtaataaggga-3'
<i>RANKL</i>	Fwd:5'-agctcagcctttgtctcatc-3' Rev:5'-tggtgctctctcttcac-3'
<i>IL-6</i>	Fwd:5'-tggctgaaaagatggatgct-3' Rev:5'-aactccaaagaccagtgatgatt-3'
<i>IL-1β</i>	Fwd:5'-aatttgagtctgccagttccc-3' Rev:5'-agtcagttatctcggccgccc-3'
<i>TNF-α</i>	Fwd:5'-cccaggcagtcagatcatctc-3' Rev:5'-agctgccctcagctga-3'
<i>Cathepsin K</i>	Fwd:5'-tgaggcttctctgtgtccatc-3' Rev:5'-aaagggtgtcattactgcggg-3'
<i>RANK</i>	Fwd:5'-ggtgcagccttaactcctg-3' Rev:5'-ttgagaccaggctgggaac-3'
<i>TRAP</i>	Fwd:5'-gaccaccttggaatgtctctg-3' Rev:5'-tggctgaggaagtcattcagttg-3'
<i>VNTR</i>	Fwd:5'-actcaagaaaaggagcaat-3' Rev:5'-agattcatccgcagatcacgc-3'

OPG – osteoprotegerin; *RANKL* – receptor activator of NF-κB ligand; *IL-1β* – interleukin-1 beta; *IL-6* – interleukin-6; *TNF-α* – tumor necrosis factor alpha; *RANK* – receptor activator of NF-κB; *TRAP* – tartrate-resistant acid phosphatase; *VNTR* – vitronectin receptor.

USA; <1.996 pg/mL, minimal detectable level <0.1 pg/mL), and *TNF- α* (DPC Biermann GmbH, Bad Nauheim, Germany) reference values from the manufacturer were <8.1 pg/mL. Intra-assay precision was 2.6–3.6%, and inter-assay precision was 4–6.5%. The minimal detectable level was 0.1 pg/mL.

Experimental procedures

Cell culture

Disposable cell culture products were purchased from Nunc (Roskilde, Denmark). Fetal calf serum (FCS) was obtained from Lonza (Köln, Germany), cell culture medium and the medium supplements (antibiotics and glutamine) were obtained from GIBCO-BRL (Eggenstein, Germany). All reagents were purchased from Sigma Chemical Co. (Munich, Germany), unless otherwise stated.

Treatment of SCP-1 cells with cytokines

To simulate the conditions occurring during the acute phase of CD, a combination of the cytokines, rather than the single cytokines alone at the detected levels, was applied to an osteoblast cell model. To avoid the variability of primary human cell culture, an immortalized mesenchymal cell line (SCP-1; a single-cell-picked clone of hTERT immortalized human mesenchymal stem cells) was used as an osteoblast model.²² This SCP-1 cell line can function as a progenitor cell but differentiates into an osteoblast phenotype when appropriately stimulated.²² Osteogenic stimulation resulted in increased expression of alkaline phosphatase (*ALPL1*; 3.5-fold) and osteocalcin (*OC*; 6.6-fold). Adipogenic stimulation increased the expression of adipocyte protein 2 (*aP2*; 82-fold) and peroxisome proliferator activated receptor gamma (*PPAR γ* ; 8.5-fold) (data not shown). The cells were split 1:5 weekly and cultured under standard conditions (10% FCS in MEM alpha). For these experiments, the cells were trypsinized and plated on 6-well dishes at a density of 5×10^4 cells/mL.

For short-duration stimulation in bovine serum albumin (BSA) (dose response experiments), 10%

FCS medium was changed to 0.1% BSA after reaching confluence, and the cytokines were applied as individual agents at various concentrations (*IL-1 β* , 0.01–100 ng/mL; *TNF- α* , 0.01–100 ng/mL; or *IL-6*, 0.01–100 ng/mL) or as a combination (10 pg/mL *IL-6*, 1 pg/mL *IL-1 β* , and 5 pg/mL *TNF- α*) to the cell medium without osteogenic differentiation. After incubation for 2 days, total RNA was isolated using a Qiagen RNeasy Mini Kit, and the RNA purity was confirmed based on the 260/280 ratio.

For SCP-1 cell experiments using 1% human serum from a healthy blood donor (Department of Transfusion Medicine), 10% FCS was exchanged for 1% human serum after 4 days of culture. After reaching confluence, the cells were supplemented with 10 mM β -glycerophosphate (bGP) and 10 μ M ascorbic acid 2-phosphate (ascP) (osteogenic differentiation) and stimulated with the cytokines either individually or in combination (see above) to evaluate the effect of the cytokines on the osteoblasts.

Fig.1. Gene expression of *RANKL* (A) and *OPG* (B) in SCP-1 cells after 48 h in 0.1% bovine serum albumin (BSA) and in the presence of various cytokine concentrations: *IL-6* (0.01–100 ng/mL), *IL-1 β* (0.01–100 ng/mL), or *TNF- α* (0.01–100 ng/mL). β -actin was used as the housekeeping gene (n = 4). Significance is indicated as follows: ***p < 0.001; **p < 0.01; *p < 0.05 (Dunnett's test, treatments vs BSA)

Furthermore, experiments were also performed with or without dexamethasone (10 μ M) to assess the dexamethasone effect in the presence of cytokines. The *RANKL/OPG* ratio and the self-induction of cytokines in osteoblasts were investigated to imitate glucocorticoid effects during the acute phase in CD. Based on the experimental design, treatment with cytokines, bGP, asCP, and dexamethasone was performed every 3–4 days.

Treatment of osteoclasts with cytokines

For osteoclast culture, monocytes obtained from healthy individuals were isolated using the magnetic-activated cell sorting (MACS) cell separation method (CD14) and cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, glutamine, and penicillin/streptomycin. Differentiation into mature osteoclasts

Fig. 2. Gene expression of *IL-1 β* (A), *TNF- α* (B), and *IL-6* (C) in SCP-1 cells after 48 h in 0.1% bovine serum albumin (BSA) in the presence of various cytokine concentrations: *IL-6* (0.01–100 ng/mL), *IL-1 β* (0.01–100 ng/mL), or *TNF- α* (0.01–100 ng/mL). D – Normalized gene expression of *IL-1 β* , *TNF- α* , and *IL-6* in the absence or presence of the combined cytokines (10 pg/mL *IL-6*; 1 pg/mL *IL-1 β* ; 5 pg/mL *TNF- α*) from 3 independent experiments. β -actin was used as the housekeeping gene. P-values were calculated using Dunnett's test (A, B, and C) ($n = 4$) or unpaired t-test (D) ($n = 12$). Significance is indicated as follows: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ (treatments vs BSA)

was achieved via the addition of *RANKL* (100 ng/mL) and macrophage colony-stimulating factor (M-CSF) (25 ng/mL) to the cells in 6-well plates for 17 days, and differentiation was monitored using toluidine blue (Sigma-Aldrich, St. Louis, USA) and tartrate-resistant acid phosphatase (TRAP) staining (Sigma-Aldrich). On days 9 and 13, the cells were treated with the combined cytokines (*IL-1 β* , *IL-6*, and *TNF- α*) for 48 h. After 17 days, the total RNA from the osteoclasts was isolated. In 3 independent experiments, the expression of the markers was determined by real-time polymerase chain reaction (PCR), and β -actin (*ACTB*) was used as the housekeeping gene. The osteoclasts expressed a characteristic protein profile and resorbed osteoblast-derived matrix in a bone resorption assay.²³

Bone resorption activity was determined as follows. Briefly, isolated monocytes (1×10^4 cells/mL) were plated onto a 24-well plate coated with a dense layer of SAOS-2-derived extracellular matrix (provided from Dr. Hempel) in the presence of 100 ng/mL *RANKL* and 25 ng/mL M-CSF. After 9 and 13 days, osteoclasts were stimulated for 48 h with either the control medium, the cytokine combination, an SCP-1 cell culture supernatant, or an “incubated” cytokine

combination (incubated without cells for 48 h at 37°C in SCP-1 medium to be used as supernatant control). SCP-1 cells were treated with the cytokine combination for 48 h for the culture supernatant (Fig. 2d). To determine the pit area (resorptive activity), the osteoclasts were removed after 17 days by treating the coated plate with 5% sodium hypochlorite for 5 min and washing with water. The pits were photographed and analyzed using image analyzing software (Image J). Experiments were performed with $n = 4$.

RNA isolation and complementary DNA (cDNA) synthesis

Total RNA was isolated after treatment with cytokines and/or dexamethasone, depending on the individual experiment, using a Qiagen RNeasy Mini Kit, and the RNA purity was confirmed based on the 260/280 ratio. For each sample, 400 ng of RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase, as previously described.²⁴ The real-time-PCR (RT-PCR) reactions were performed using a Peq-Lab Primus 96 thermal cycler in a total volume of 40 μ L, as previously described.²⁵

Fig. 3. Normalized gene expression of *RANKL* and *OPG* and the *RANKL/OPG* ratio in SCP-1 cells after 48 h in 0.1% bovine serum albumin (BSA) (A) or after 14 days in 1% human serum (B) with osteogenic stimulation (bGP, ascP) in the absence or presence of the combined cytokines (10 pg/mL *IL-6*; 1 pg/mL *IL-1 β* ; 5 pg/mL *TNF- α*). The data were obtained from 3 (A; $n = 12$) or 2 (B; $n = 8$) independent experiments. β -actin was used as the housekeeping gene. Significance is indicated as follows: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ (unpaired t-test)

Fig. 4. Influence of dexamethasone on the gene expression of RANKL and OPG and the RANKL/OPG ratio in SCP-1 cells after 48 h in 0.1% bovine serum albumin (BSA) (A) or after 14 days in 1% human serum (B) and with osteogenic stimulation (bGP, ascP) in the absence (clear) or presence (gray) of the combined cytokines (10 pg/mL *IL-6*; 1 pg/mL *IL-1β*; 5 pg/mL *TNF-α*). Dexamethasone was added at a concentration of 10 μ M at the beginning of the experiment and every time the medium was changed (4 times in 14 days). β -actin was used as the housekeeping gene. Significance is indicated as follows: for the samples with or without dexamethasone, ***p < 0.001; **p < 0.01; *p < 0.05; and for the samples in the presence or absence of cytokines, +, p < 0.05 (unpaired t-test)

RT-PCR analysis of cDNA was performed at 60–95°C for 40 cycles using the ABI Prism StepOnePlus sequence detection system (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. The SYBR Green Reaction Master Mix (ABI Prism; Applied Biosystems) and the primers listed in Table 1 were used. All primers were synthesized by Invitrogen. Based on our experience from previous experiments, β -actin (*ACTB*) mRNA was used as an internal control in each RNA sample, because the expression of this gene is constant under different experimental conditions.

The results were normalized to *ACTB*, and the fold change in expression was calculated based on the threshold cycle (C_t) values ($2^{-\Delta C_t}$ method).²⁶

Statistical analysis

The data was analyzed using Prism GraphPad 4 software (San Diego, USA). All the data is presented as means $\pm$ SEM (standard error of the mean). Statistical significance was calculated using the Mann-Whitney U test for clinical patient data, unpaired t-test (cytokine combination) or one-way analysis of variance ANOVA, followed by Dunnett's test (dose response experiments). The significance was set at $p < 0.05$.

Ethical considerations

All the patients provided informed consent, and the study was approved by the Ethics Committee of Göttingen University Medical Center (11/5/00). The study was conducted in accordance with good clinical practice and the Declaration of Helsinki.

Results

Serum parameters

The cytokines were significantly increased in sera from CD patients compared with those from healthy, age- and gender-matched controls (Table 2), i.e., *IL-6* (~100-fold), *IL-1 β* (~10-fold), and *TNF- α* (~10-fold). Due to the small number of carefully chosen CD patients ($n = 7$), results must be judged carefully.

Treatment of SCP-1 cells with cytokines

To determine whether single cytokines influence bone turnover in SCP-1 cells, we applied cytokines for 2 days (dissolved in 0.1% BSA) and analyzed receptor activator of NF- κ B ligand (*RANKL*) and osteoprotegerin (*OPG*), as shown in Fig. 1. During the short incubation, the cells remained vital and healthy. The expression of *RANKL* and *OPG* was not influenced by treatment with the lowest

concentrations of *IL-6*, *IL-1 β* , or *TNF- α* (0.01 ng/mL) comparable to acute phase in CD patients. Higher concentrations of *TNF- α* increased *RANKL* expression (2.4-fold, 10 and 100 ng/mL) and *OPG* expression (3–12.5-fold; 10 and 100 ng/mL). The expression of *OPG* was also induced by higher concentrations of *IL-1 β* beginning at 0.1 ng/mL (4.4-fold) (Fig. 1).

The potential self-induction of exogenous applied cytokines in SCP-1 cells was examined by stimulation with each of the individual cytokines at increasing concentrations (Fig. 2a, 2b, 2c), or with the combined cytokines (Fig. 2d). As shown in Fig. 2a, 2b, and 2c, none of the cytokines was able to self-induce its own expression or the expression of the other cytokines in the lowest concentration adapted to the concentration found in CD patients.

Regarding higher concentrations, *IL-6* had no influence on the expression of itself or the other cytokines, even at the highest concentrations. *IL-1 β* did induce its own expression and expression of *IL-6* and *TNF- α* beginning at 0.1 ng/mL. This influence accumulated with increasing *IL-1 β* concentration (up to 100-fold). *TNF- α* induced its own expression and the expression of the remaining 2 cytokines only at the second highest and the highest concentrations (up to 50-fold).

In Fig. 2d the results of 3 independent experiments for the application of a combination of cytokines is presented. The concentration of cytokines in the combined treatment was chosen based on the measured cytokine concentrations in the sera of CD patients: 10 pg/mL *IL-6*; 1 pg/mL *IL-1 β* ; 5 pg/mL *TNF- α* (Table 1).

After 48 h, the cytokine combination showed no influence on the expression of *IL-1 β* , a tendency towards increased *IL-6* expression (to 120%), and a significant increase in *TNF- α* expression to 140% compared with the control.

Based on the fact that the single cytokines at relevant concentrations had no effect on SCP-1 cells, we preceded with the experiments on SCP-1 cells with the cytokine combination.

To determine whether *RANKL* and/or *OPG* expression were affected, we treated undifferentiated SCP-1 cells with the combined cytokines for 2 days in 0.1% BSA solution

Table 2. Cytokine levels in CD patients during active disease and in healthy controls

Parameter	CD-patients ($n = 7$)	Healthy controls ($n = 16$)	p-value (Mann Whitney U)
<i>IL-6</i> (0.447–9.96 pg/mL)	11.28 $\pm$ 11.66	0.89 $\pm$ 0.59	0.012
<i>IL-1β</i> (<1.996 pg/mL)	0.62 $\pm$ 0.99	0.09 $\pm$ 0.08	0.012
<i>TNF-α</i> (< 8.1 pg/mL)	2.12 $\pm$ 2.02	0.2781 $\pm$ 1.07	0.0055

CD – Crohn's disease; *IL-1 β* – interleukin-1 beta; *IL-6* – interleukin-6; *TNF- α* – tumor necrosis factor alpha.

Fig. 5. Influence of dexamethasone on the gene expression of IL-6, IL-1 β , and TNF- α in SCP-1 cells after 48 h in 0.1% bovine serum albumin (BSA) (A) or after 14 days in 1% human serum (B), and with osteogenic stimulation (bGP, ascP) in the absence (clear) or presence (gray) of the combined cytokines (10 pg/mL IL-6; 1 pg/mL IL-1 β ; 5 pg/mL TNF- α). Dexamethasone (10 μ M) was added at the beginning of the experiment and every time the medium was changed (4 times in 14 days). β -actin was used as the housekeeping gene. Significance is indicated as follows: for samples with or without dexamethasone, ***p < 0.001; **p < 0.01; *p < 0.05; and, for the samples in the presence or absence of cytokines, + p < 0.05 (unpaired t-test)

(identical to that used for the treatment of the individual cytokines in Fig. 1), or for 14 days in 1% human serum obtained from a healthy donor under osteogenic stimulation.^{6,27} Fig. 3 shows that the combined cytokine treatment increased *RANKL* expression by 2-fold after both the short-duration incubation in BSA ($p < 0.001$) (Fig. 3a) and the 14-day incubation in 1% human serum ($p = 0.1$) (Fig. 3b). *OPG* expression was not significantly affected by the combined cytokine treatment under these experimental conditions (Fig. 3b).

These findings indicate a clear influence of the combined cytokine treatment on the expression of *RANKL*, while *OPG* appeared to be unaffected. Therefore, the combined cytokine treatment shifted the *RANKL/OPG* ratio toward *RANKL* (Fig. 3).

Influence of dexamethasone on SCP-1 cells in the presence or absence of cytokines

CD patients are regularly treated with glucocorticoids during the acute phase of the disease. Therefore, we applied

a dose-adapted concentration of dexamethasone to SCP-1 cells in either 0.1% BSA for 48 h (Fig. 4a) or in 1% human serum for 14 days (Fig. 4b) in the absence (white bars) or presence (black bars) of the combined cytokines. The data is shown in Fig. 4. The induction of the *RANKL/OPG* ratio by the cytokine combination was detectable at both time points. The ratio further increased by addition of dexamethasone for 48 h (right panel); this effect was independent of the cytokine combination ($p = 0.026$; 48 h in the presence of dexamethasone, absence of cytokines). The strongest induction (7-fold) occurred with dexamethasone and cytokines in combination ($p = 0.07$). The effect of dexamethasone on the *RANKL/OPG* ratio was caused by both an increase in *RANKL* expression and a decrease in *OPG* expression after 48 h.

After 14 days, the effect on *OPG* reversed, showing an increase in the presence of dexamethasone and, therefore, attenuated the effect on the *RANKL/OPG* ratio.

The effect of dexamethasone on cytokine expression is shown in Fig. 5. Dexamethasone significantly suppressed

Fig. 6. Gene expression of *cathepsin K*, *RANK*, *TRAP*, and *VNTR* in osteoclast primary cultures after 17 days with or without (48 h bovine serum albumin (BSA)) the addition of the combined cytokines (at days 9 and 13 for 48 h) at the concentrations: 10 pg/mL *IL-6*; 1 pg/mL *IL-1 β* ; 5 pg/mL *TNF- α*). β -actin was used as the housekeeping gene. P-values were calculated via unpaired t-tests

IL-6 expression to less than 10% at both time points, regardless of the presence of the cytokine combination (Fig. 5a and 5b, left panel). *IL-1 β* expression was not influenced by dexamethasone after 48 h, but decreased to approx. 50% after 14 days (Fig. 5b, middle panel). *TNF- α* expression was not affected by dexamethasone after 48 h, but decreased to 20% under those conditions after 14 days. Notably, *TNF- α* was the only cytokine in which an additional effect evoked by the cytokine combination in the presence of dexamethasone was observed ($p = 0.02$) (Fig. 5b, right panel). This resulted in a decreased *TNF- α* expression to 16% in the presence of dexamethasone and cytokines of basal expression ($p = 0.10$).

Effect of cytokine combination on osteoclasts

Osteoclasts were incubated with the cytokine combination from day 6 to day 17 in the culture. The gene expression levels of the osteoclast markers *cathepsin K*, receptor activator of NF- κ B (*RANK*), tartrate-resistant acid phosphatase (*TRAP*), and vitronectin receptor (*VNTR*) are presented in Fig. 6. The expression of these osteoclastic marker genes was significantly reduced (*cathepsin K* to 70%; *RANK* to 80%; *TRAP* to 64%, and *VNTR* to 53%, respectively) by treatment with the combined cytokines (Fig. 6), indicating a reduction in osteoclast function.

Osteoclast resorption assay

Osteoclast function was further analyzed by a bone resorption assay (Fig. 7). When comparing the freshly applied cytokine combination, bone resorption increased in relation to the control medium ($p = 0.12$; ns). In addition,

Fig. 7. Osteoclast resorption assay analyzing the pit area, indicating osteoclast activity after 17 days in culture. Osteoclasts were differentially stimulated at days 9 and 13 for 48 h with either control medium, cytokine combination (10 pg/mL *IL-6*; 1 pg/mL *IL-1 β* ; 5 pg/mL *TNF- α*), 48 h SCP-1 cell supernatant (*), cytokines incubated for 48 h in medium without cells. Every approach was performed 4-fold ($n = 4$). * SCP-1 cells were treated with cytokine combination for 48 h (Fig. 2d)

we applied supernatant from the SCP-1 experiments for 48 h as conditioned medium, showing the same effect as the freshly applied cytokine combination.

As a direct control for the supernatant approach, cytokines were incubated in control medium without SCP-1 cells for 48 h and supplied to the osteoclasts. Under these conditions, a significantly lower osteoclast activity was observed ($p = 0.017$) compared with the stimulation with the 48-h supernatant. These results indicate an effect on the conditioned SCP-1 supernatant that is independent of the cytokines.

Discussion

The pathogenesis of osteoporosis in CD patients is multifactorial, but both the disease itself and the administration of glucocorticoids are thought to be among the primary contributors. We, therefore, analyzed sera from patients with active CD that had been glucocorticoid-free (steroid-free) for at least 1 month prior to analysis. Concentrations of cytokines *IL-1 β* , *IL-6*, and *TNF- α* were measured.²¹ Based on studies of pediatric patients with CD, all bone markers had returned to normal 1 month after the withdrawal of glucocorticoid treatment.^{28–30}

As expected, we detected increased levels of *IL-6*, *IL-1 β* , and *TNF- α* in sera from CD patients in the acute phase of the disease compared with age- and gender-matched controls. The data has to be interpreted carefully due to the small number of patients, but similar concentrations were measured in adult and pediatric CD patients.^{6,16} Therefore, these cytokines possibly represent major pathogenic factors that influence the bone metabolism.³¹

To replicate the conditions occurring during the acute phase of CD, the osteoblast cell models were subjected to cytokines *IL-6*, *IL-1 β* , and *TNF- α* , individually and in combination, at the detected levels.

In the following experiments of bone formation, the individual treatment of SCP-1 cells with *IL-6* did not influence the expression of *RANKL* or *OPG*. It has been suggested that *IL-6* is the main effector of bone resorption in children with CD.⁶ In our system, none of the individually applied cytokines affected *RANKL* or *OPG* expression at the concentrations measured in CD patients. However, when applied in combination, the cytokines did influence the *RANKL/OPG* ratio toward bone resorption.

To verify the potential of self-induction, we investigated cytokine expression induced by the application of *IL-1 β* , *IL-6*, and *TNF- α* , and produced dose-response curves. These experiments, applying the individual cytokines, showed a clear increase in *IL-6* expression via the single application of either *IL-1 β* or *TNF- α* , but only at higher concentrations than those measured in the CD sera. An increase in *IL-6* after the application of *IL-1 β* or *TNF- α* at higher concentrations has already been described, although that was in murine cells via the Stat3 pathway.¹⁸ The cytokine levels as measured in CD patients

had no effect on *IL-6* expression when applied individually, and no self-induction of *IL-6* was observed. Therefore, our results using adult CD serum and SCP-1 cells are clearly different from those obtained by the organ culture model using serum from children with CD.⁶ The slight increase in *TNF- α* and *IL-6* expression following the combined cytokine treatment gave no clear indication for a feedforward signaling cascade, because *IL-6* did not influence the expression of the other cytokines, and the effect of *TNF- α* on the expression of the other cytokines was only observed at considerable concentrations.

Therefore, treatment of osteoblasts with the combined cytokines applied in the concentrations found in adult CD patients produced results that contrasted absolutely with those observed using single cytokines, and partly with those observed in children with CD. The effects of cytokines on murine and human mesenchymal cells appear to be similar at high concentrations.¹⁸

To imitate the effects of glucocorticoid in the acute phase of CD, we performed experiments in the presence of dexamethasone. The effect of the combined cytokine treatment on the *RANKL/OPG* ratio was further increased when dexamethasone was added, regardless of the presence or absence of the cytokines. Dexamethasone reduced *OPG* transcript expression and protein secretion, and slightly increased *RANKL* gene expression in ST2 osteoblasts.³² Our findings might be interpreted as being parallel to the clinical state in CD patients, indicating that an increased level of all 3 cytokines in combination shifts the *RANKL/OPG* ratio toward bone resorption, and that the additional application of dexamethasone further increases the *RANKL/OPG* ratio.

Dexamethasone strongly decreased *IL-6* expression, and the detectable original cytokine effect was small relative to the substantial dexamethasone effect. Therefore, in our model, *IL-6* stimulation in the presence of dexamethasone may have only minor effects on the bone. Other researchers have reported a similar inhibitory effect of glucocorticoids on the expression of *IL-6*.^{33–35} Thus, the application of glucocorticoids to cultured cells exceeds the cytokine effect on the *RANKL/OPG* ratio, possibly mimicking their administration as a medication to CD patients.

Dexamethasone also reduced the expression of *IL-1 β* independently of the presence of cytokines; this decrease was not as dominant as the *IL-6* decrease, but it was significant. *TNF- α* expression was decreased by the addition of dexamethasone, but, in contrast to the other 2 cytokines, the effect was further modified by the combined cytokine treatment. This supports the special role of *TNF- α* in CD.^{19,36,37}

The expression levels of *IL-6*, *IL1 β* , and *TNF- α* were strongly reduced by dexamethasone. Moreover, dexamethasone shifted the *RANKL/OPG* ratio toward *RANKL*, independently of the presence or absence of cytokines. These findings demonstrate that the effect of dexamethasone on SCP-1 cells is not mediated by the reduced expression of cytokines in osteoblasts, but by the direct effect of dexamethasone on the expression of *RANKL* and *OPG*.

In addition to the treatment of osteoblasts with cytokines, we treated primary osteoclasts with the combined cytokines *IL-6*, *IL-1 β* , and *TNF- α* . This treatment decreased all the examined markers of osteoclast function in cultures from 3 different donors. Assuming a decrease in osteoclastic function, this would pathophysiologically be in contrast to the effect observed in osteoblasts, where cytokines increase the *RANKL/OPG* ratio and, therefore, induce bone resorption via osteoclasts. The investigation of the effect of cytokines on osteoclasts as described in the literature mainly focuses on the differentiation between monocytes and mature osteoclasts, and not on the effects on mature osteoclasts, as in our experiments.³⁸ Nevertheless, it has been shown that *TNF- α* as well as *IL-1 β* can have inhibitory effects on osteoclastic function.^{39–41} However, we would have expected an increase in osteoclast number or function, because the development of monocytes or pre-osteoclasts into osteoclasts is strongly regulated by cytokines via *IL-6* and *RANKL*.^{18,42} We, therefore, investigated osteoclast activity, using a bone resorption assay. The cytokine combination induced bone resorption activity. The conditioned cytokine medium from SCP-1 cells also increased osteoclast resorption, suggesting that factors produced by the osteoblasts and secreted into the medium stimulate increased osteoclast resorption. The appropriate control of the conditioned cytokine medium clearly induced less osteoclast activity, suggesting an effect independent of “incubated” cytokines.

In the present study, we demonstrated that the combination of the 3 cytokines *IL-6*, *IL-1 β* , and *TNF- α* increased the *RANKL/OPG* ratio in an osteoblast cell model, suggesting an indirect effect of cytokines on bone turnover via *RANKL/OPG*. The addition of dexamethasone to the osteoblast system caused a marked decrease in cytokine expression, especially that of *IL-6*, as well as a shift in favor of the *RANKL/OPG* ratio. In osteoclasts, treatment with the combined cytokines resulted in a decrease in osteoclast characteristic gene expression; however, bone resorption activity increased with the cytokine combination. In addition, a similar increase of osteoclast resorption activity by the conditioned medium suggested an additional influence of the SCP-1 supernatant independently of a direct *IL-6*, *IL-1 β* , or *TNF- α* effect.

In conclusion, our in vitro results explain why in CD patients the increased cytokine levels together with glucocorticoid therapy will be deleterious to bone over an extended period, and may result in osteoporosis. Our results are the next step toward understanding osteoporosis in adult CD patients.

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