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

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

**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-1β, 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-1β, 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-κB 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-1β 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-1β, 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
Introduction

Patients with Crohn’s disease (CD) may develop secondary osteoporosis with fractures in the course of the disease.\cite{1,2,3,4} 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.\cite{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.\cite{6,7,8} These cytokines are key regulators of bone turnover, and are involved in postmenopausal osteoporosis, as well as osteoporosis in rheumatic diseases.\cite{9,10,11,12} 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 of NF-κB ligand (RANKL) and osteoprotegerin (OPG), factors synthesized by osteoblasts and osteocytes, but acting only on osteoclasts.\cite{13}

Inflammatory cytokines play a major role in shifting the RANKL/OPG balance toward excessive RANKL, which induces osteoclast activity and greater bone degradation.\cite{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.\cite{15} Additionally, in patients with CD, inflammatory cytokines are correlated with markers of osteoclast activity.\cite{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.\cite{17} These findings were surprising, because IL-6 is an osteoclast-activating factor in other inflammatory diseases such as rheumatic disorders.\cite{18} In addition, it has been suggested that other cytokines, especially TNF-α, are the most relevant cytokines in CD.\cite{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.\cite{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.\cite{21}

Cytokine production

The serum levels of the cytokines were determined using commercially available enzyme-linked immunosor- bent 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, USA; 0.155–5.00 pg/mL, minimal detectable level 0.051–0.161 pg/mL), and TNF-α (R&D systems, Minneapolis, USA; 0.016–0.110 pg/mL), which are associated with increased bone turnover, and are involved in postmenopausal osteoporosis, inflammation from CD in children affects osteoblasts and bone formation, but not osteoclasts. 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
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<td>β-actin</td>
<td>Fwd: 5′-tcgggaagctgtaggagctac-3′ Rev: 5′-agaattcagccttacatgcc-3′</td>
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<tr>
<td>OPG</td>
<td>Fwd: 5′-ctcggactctggagcttcc-3′ Rev: 5′-gacagctggtaaagagga-3′</td>
</tr>
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<tr>
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</tr>
<tr>
<td>VNTR</td>
<td>Fwd: 5′-actaagcagaaaggagcgcaat-3′ Rev: 5′-agatctcgcggcgtataccg-3′</td>
</tr>
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</table>

OPG – osteoprotegerin; RANK – 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. This SCP-1 cell line can function as a progenitor cell but differentiates into an osteoblast phenotype when appropriately stimulated. 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 (PPARγ; 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 × 10⁴ 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-1β, 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-1β, 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 µM 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-1β (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)](image-url)
Furthermore, experiments were also performed with or without dexamethasone (10 µM) 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...
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-1β, 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 x 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 µL, 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-1β, 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, asP) 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 µM 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 (Ct) values (2-ΔΔCt method). Statistical analysis

The data was analyzed using Prism GraphPad 4 software (San Diego, USA). All the data is presented as means ±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-1β (~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-κB 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-1β, 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-1β 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-1β 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-1β 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-1β; 5 pg/mL TNF-α (Table 1).

After 48 h, the cytokine combination showed no influence on the expression of IL-1β, 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 proceeded 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>
<thead>
<tr>
<th>Parameter</th>
<th>CD-patients (n = 7)</th>
<th>Healthy controls (n = 16)</th>
<th>p-value (Mann Whitney U)</th>
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<tr>
<td>IL-6 (&lt;0.447–996 pg/mL)</td>
<td>11.28 ±11.66</td>
<td>0.89 ±0.59</td>
<td>0.012</td>
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<tr>
<td>IL-1β (&lt;1.996 pg/mL)</td>
<td>0.62 ±0.99</td>
<td>0.09 ±0.08</td>
<td>0.012</td>
</tr>
<tr>
<td>TNF-α (&lt; 8.1 pg/mL)</td>
<td>2.12 ±2.02</td>
<td>0.2781 ±1.07</td>
<td>0.0055</td>
</tr>
</tbody>
</table>

CD – Crohn’s disease; IL-1β – 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-1β, 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-1β, 5 pg/mL TNF-α). Dexamethasone (10 µM) 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).
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-1β, 5 pg/mL TNF-α. β-actin was used as the housekeeping gene. P-values were calculated via unpaired t-tests.

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...
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-1β 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-κB (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, 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-6, IL-1β, and TNF-α were measured. Based on studies of pediatric patients with CD, all bone markers had returned to normal 1 month after the withdrawal of glucocorticoid treatment.

As expected, we detected increased levels of IL-6, IL-1β, 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.

To replicate the conditions occurring during the acute phase of CD, the osteoblast cell models were subjected to cytokines IL-6, IL-1β, 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. 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-1β, 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-1β 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-1β or TNF-α at higher concentrations has already been described, although that was in murine cells via the Stat3 pathway. 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. 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.

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. 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 further, possibly mimicking their expression when applied individually, and partly with cytokines applied in the concentrations found in adult CD patients. Assuming a decrease in osteoclastic activity, 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. Nevertheless, it has been shown that TNF-α as well as IL-1β can have inhibitory effects on osteoclastic function. 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. 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 osteoclasts resorption. The appropriate control of the conditioned cytokine medium clearly induced less osteoclast activity, suggesting an effect independent of “incubated” cytokines.

In a recent study, we demonstrated that the combination of the 3 cytokines IL-6, IL-1β, 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-1β, 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.

References
Methods using real-time quantitative PCR and the 2(-Delta Delta C(T)) Methodologies of primary human mesenchymal progenitor cells.

J Cell Biochem and adipogenic differentiation markers in selected subpopulations of mouse bone marrow cells with active Crohn's disease. Osteology as an osteoporosis risk factor in patients with active Crohn's disease.

Disease Study.


Weitzmann MN. The role of inflammatory cytokines, the RANKL/OPG axis, and the immunoskeletal interface in physiological bone turnover and osteoporosis. Scientifica (Cairo). 2013;2013:125705.