Redundant modulatory effects of proinflammatory cytokines on human osteoblastic cells *in vitro*

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Abstract

Objective

The aim of our study was to investigate possible interaction of IL-17, TRAIL, and TNF-α in the modulation of osteoblast homeostasis in vitro, using human differentiated osteoblastic Saos-2 cells as in vitro model.

Methods

The effects of these cytokines on osteoblastic cell viability were assessed, by MTT assay, alone or in combination, at different times and concentrations. The effects of IL-17 and TNF-α on the regulatory system of osteoclast activity RANK / RANKL / OPG were evaluated by Western blot and ELISA techniques in cell culture media. Quantitative expression of RANKL, OPG and pro-inflammatory factors were analysed at the mRNA level by quantitative real time RT-PCR.

Results

Effects of IL-17, TNF-α and TRAIL on osteoblastic cell viability indicated that IL-17 alone, or in combination with TNF-α did not alter Saos-2 cell viability. On the other hand, TRAIL, as expected, exhibited time- and concentration-dependent cytotoxicity. The expression both RANKL and OPG were increased at the mRNA level and protein release by IL-17 and TNF-α, either alone or in combination. The analysis of IL-17 and TNF-α on pro-inflammatory molecules mRNA expression, such as CXC family chemokines CXCL-1 and CXCL-5, COX-2 and IL-6 demonstrated an increase in these pro-inflammatory cytokines with cooperative effects of the combination.

Conclusion

Overall, these results suggest that IL-17, TRAIL and TNF-α sustain bone tissue inflammation associated with decrease of calcified component. To do so, they act redundantly each other, to amplify the inflammatory response in the bone. In conclusion, unravelling novel molecular targets within the bone-cytokine network represents a platform for innovative treatment of bone diseases due to immunological diseases such as psoriatic arthritis.

Key words

osteoblasts, IL17, IL6, TRAIL, RANKL

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Introduction

Rheumatic diseases include a series of pathological conditions, due to several independent pathogenetic factors, such as, for example alterations in cytokine release. Indeed, cytokines promote the inflammatory/immune response in several organs, including the bone, a tissue often affected by pathological processes associated with an impaired peripheral immune response (1).

Psoriasis, is a chronic, autoimmune inflammatory skin disease, which is characterised by an abnormal differentiation and hyperproliferation of epidermal keratinocytes (2), a condition affecting two to four percent of the world population (3, 4). Psoriasis displays various systemic effects, and has been associated with various comorbidities, including psoriatic arthritis. In fact, approximately 20–30% of patients develop psoriatic arthritis as a long-term consequence of psoriasis, namely a form of spondyloarthritis (5, 6) characterised by synovial hyperplasia, immune cell infiltrates and substantial and rapidly occurring bone erosion, essentially due to accelerated resorption processes (7, 8).

Indeed, a healthy calcified tissue implies an equilibrium of underlying remodelling processes, and results from a proper balance between both bone formation and resorption. These two aspects of bone physiology are sustained by two effector cell types, respectively, osteoblasts, mesenchymal derived cells, responsible for new bone formation, and osteoclasts, monocyte-macrophage-derived cells, underlying mineralised bone resorbtion. The two processes are tightly linked; osteoblasts, indeed, promote osteoclast differentiation through cell-to-cell interactions and two major cytokines – receptor activator of NF-κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) (9, 10), that, in turn, induce bone formation via osteoblastic mediators released during bone degradation. RANKL interaction with its receptor, RANK, expressed by the osteoclast is inhibited by the decoy receptor OPG (11, 12) and, thus, the RANKL/OPG ratio exerts relevant influence on bone resorption processes.

The activity of osteoblasts and osteoclasts may be influences by an array of factors including, several hormones, such as parathyroid hormone, 1,25-dihydroxyvitamin D3, sex hormones and calcitonin (13, 14), as well as mechanical factors (15), nitric oxide, prostaglandins, growth factors and cytokines (16-18) and others. Among the mediators of immune response, the pro-inflammatory cytokines tumour necrosis factor-α (TNF-α), interleukin1β (IL-1β), and Interleukin 17 (IL-17) appear crucial for the regulation of bone resorption.

In fact, IL-1 released in response to TNF-α, a proinflammatory/proapoptotic macrophagic cytokine (19, 20), induces, in turn, the expression of RANKL in the bone tissue, leading to increased osteoclastogenesis and consistent bone resorption (21, 22). IL-17, a member of the pro-inflammatory family of cytokines produced by a subset of T helper cells (23-25), is abundantly expressed in the synovial membrane of rheumatoid arthritis patients, where it induces the synthesis of matrix-degrading enzymes, such as matrix metalloproteinases (MMPs), which contribute to destruction of cartilage and bone tissue during inflammatory processes (26-30). Moreover, a number of recent studies enlighten the role of IL-17 in the pathogenesis of psoriasis and psoriasis arthritis (31). Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), a proapoptotic cytokine belonging to the TNF superfamily, is known to induce apoptosis of tumour and normal cells (32-34). It binds to death domain receptors which mediate its potent apoptotic effect, DR4 and DR5, as well as to a soluble decoy receptor, osteoprotegerin, involved in regulation of bone tissue formation (35-38).

Considered involvement of pro-inflammatory cytokines in bone resorption associated with psoriasis (24, 27, 39-45) and the relative lack of knowledge on their role in bone formation. the reciprocal role of TNF-α, IL-17 and TRAIL in mechanisms leading to psoriatic arthritis, it appears of interest to study the effects of these molecules in modulating bone formation. With the aim to identify and characterise potential molecular targets for neutralisation of detrimental effects exerted by these

proinflammatory cytokines upon osteoblasts, their effects and relative underlying mechanisms have been studied in a human osteoblastic cell line *in vitro*.

Materials and methods

Reagents

All buffers, media, and reagents of tissue culture were purchased from EuroClone S.p.A. (Milan, Italy). Recombinant human IL-17A, IL-17F, and TNF-α were from PeproTech (Rocky Hill, NJ, USA). Recombinant human TRAIL was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). ELISA kits were from Diaclone SAS (Besançon, France) for IL-6 and Biomedica Medizinprodukte GmbH& KG (Vienna, Austria) for RANKL and OPG.

Cell culture and IL-17, TNF-α and TRAIL treatments

Human SAOS-2 osteoblastic cells (OBS) were obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 10% fetal bovine serum (FBS), and kept at 37°C in humidified 5% CO_2/95% O_2 atmosphere. For cytokine treatments with IL-17, TNF-α and TRAIL OBS were seeded onto 10 cm dishes and grown to confluence in DMEM/10% FBS. Following attachment, cells were washed twice in phosphate-buffered saline (PBS), incubated in DMEM/0.3% FBS overnight, and stimulated with the different cytokines and combinations for 72 h and/or TNF-α (2 ng/ml) for 2, 24 or 48 h.

Cell viability analysis

Cell viability was determined by 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. Briefly, OBS were plated on 96-well cultured plates at a density of $5x10^3$ cells per well and incubated for 72 h in DMEM 0.3% FBS with IL-17A, IL-17F, or TRAIL at 50, 100 and 200 ng/ml for dose-response studies and with IL-17A (200 ng/ml), IL-17F (200 ng/ml), TNF-α (2 ng/ml) and/or TRAIL (100 ng/ml) for combination studies. At the end of the treatment period, cell viability was measured by the reduction of 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution (0.5 mg/mL) (Sigma, St. Louis, MO, USA). After additional 3 h incubation at 37°C, this solution was removed, and produced blue formazan crystals were solubilised in dimethyl sulfoxide. The optical density of the formed blue formazan was measured at 545 nm (ref. 630 nm). All tests were performed in triplicate, at least twice.

RNA isolation, RT-PCR and quantitative real-time PCR

Total RNA was isolated using the RNeasy kit (Qiagen) with on-column DNase digestion to eliminate DNA contamination according to the manufacturer's instructions. Concentration was determined by spectrophotometry while purity and integrity of total RNA were monitored by electrophoretic analysis on denaturing agarose gel. Single-stranded cDNA was synthesised using RETROscript kit (Ambion, Life Technologies) and random hexamer primers following the manufacturer's recommendations.

Basal expression of the different receptors for IL-17 and TRAIL was asserted by RT-PCR and products visualised by electrophoresis on agarose gel. Sequences of primers, reported in Table I, were designed to span introns so that signal from genomic DNA could be distinguished from cDNA. Amplification conditions were determined for each pair of primers.

Quantitative real-time PCR was performed in the Rotor- Gene Q (Qiagen) using QuantiNova SYBR green PCR kit (Qiagen) according to the manufacturer's protocol. PCR reactions consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 30 s and 72°C for 60s and a final elongation step at 72°C for 10 min. Sequences of primers were designed to span introns and are given in Table II. All measurements were performed in triplicate and the comparative Ct method was used to calculate relative mRNA expression. Ct values of both the calibrator and the samples of interest were normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The comparative Ct method is also known as the $2^{\Delta\Delta Ct}$ method, where, $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$. ΔCT, sample is the Ct value for the samples normalised to the endogenous housekeeping gene, and $\Delta Ct_{reference}$ is the Ct value for the calibrator, also normalised to the endogenous housekeeping gene.

Protein extraction

Cells were harvested in a lysis buffer containing 150 mM NaCl, 50 mM Tris–HCl [pH 7.5], 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na_3VO_4, 30 mM Na pyrophosphate, 50 mM NaF, 1 mM acid phenyl-methyl-sulfonyl-fluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 10% glycerol and 0.2% Triton x-100. The homogenates were then centrifuged at 14,000 rpm for 10 min at 4°C. For analysis of secreted proteins the culture medium samples were

Table I. Primers used in RT-PCR experiments.

Gene	GenBank Accession number	Forward sequence	Reverse sequence	Amplicon length
IL-17	NM_ 002190	AACGCTGATGGGAACGTGGA	GCAGCCCACGGACACCAGTA	132 bp
IL-17RA	NM_014339	CGCGGAAAAGAAAGCCTCAG	GGCAGGTACTATTCTTGACCGT	250 bp
IL-17RC	NM_153461	ACCAGAACCTCTGGCAAGC	GAGCTGTTCACCTGAACACA	231 bp
DR4	NM_003844.3	CTACCTCCATGGGACAGCAC	ATGAGATCCTGGTGGACACAAC	245 bp
DR5	NM_003842.4	ACTCCTGCCTCTCCCTGTTC	CAGGTCGTTGTGAGCTTCTGT	187 bp
DcR1	NM_003841.3	GACAAGTGACCACGCCTTTTC	GTCCCCAGAGTTCCCTAACG	190 bp
DcR2	NM_003840.4	GCACAAACTACGGGGACGA	GGGATGGTGGCAGAGTCAAC	241 bp
TRAIL	NM_003810.3	TCCCCTAGTGAGAGAAAGAGGT	GCCTTTTCATTCTTGGAGTTTGGA	105 bp

Table II. Primers used in real-time PCR experiments.

Gene	Accession number	Forward sequence	Reverse sequence
ALP	NM_000478.4	GGTCCAGGGATAAAGCAGGT	AGTGTCTCTTGCGCTTGGTCT
Col	NM_000088.3	AGGGCTCCAACGAGATCGAGATCCG	TCAGGAAGCAGACAGGGCCAACGTCG
COX2	NM_000963	CCCATGTCAAAACCGAGGTG	CCGGTGTTGAGCAGTTTTCTC
CXCL1	NM_001511.3	AGGCAGGGGAATGTATGTGC	AAGCCCCTTTGTTCTAAGCCA
GADPH	NM_002046	GAAAGCCTGCCGGTGACTAA	GCCCAATACGACCAAATCAGAGA
IL-6	NM_000600.3	TTCGGTACATCCTCGACGGC	TCTGCCAGTGCCTCTTTGCT
MMP13	NM_002427.3	CAAGATGCGGGGTTCCTGAT	TCGCCATGCTCCTTAATTCCA
OPG	NM_002546.3	GCTCACAAGAACAGACTTTCCAG	CTGTTTTCACAGAGGTCAATATCTT
RANKL	NM_003701.3	TCAGCCTTTTGCTCATCTCACTAT	CCACCCCCGATCATGGT

transferred to Amicon Ultra-15 centrifugal filter devices- 10,000NMWL (Millipore) and centrifuged at 4,000g for 10 min at room temperature. The protein concentration was determined by the Bradford method (46).

Western blot analysis

Cellular protein (30 μg) was mixed with an equal volume of sodium dodecyl sulfate (SDS) loading buffer [20% glycerol, 100 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol, 4% SDS, and 0.1% bromophenol blue], boiled for 5 min, and separated by electrophoresis in either 10% or 12% SDS-polyacrylamide gels (Bolt® Gels and Mini Gel Tank; Life Technologies, Monza, Italy), and the separated proteins were transferred to nitrocellulose (iBlot® Gel Transfer Device; Life Technologies). The membranes were blocked 60 min at room temperature with 5% nonfat dry milk (EuroClone) in phosphate buffered saline Tween-20 and then incubated at 4°C overnight with the following primary antibodies: rabbit polyclonal anti-Alkaline Phosphatase (Abcam, Cambridge, UK), rabbit polyclonal anti-DcR1 (ProSci Inc., Poway, CA, USA), rabbit polyclonal anti-DcR2 (Abcam), rabbit polyclonal anti-DR4 (ProSci Inc.), rabbit polyclonal anti-DR5 (Abcam), rabbit polyclonal anti-IL-1β (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-IL-6 (Abcam), rabbit polyclonal anti-IL-17A (PeproTech), mouse monoclonal anti-IL-17RA (Novus Biologicals, Cambridge, UK), rabbit polyclonal anti-IL-17RC (Novus Biologicals), rabbit polyclonal anti-osteoprotegerin (Abcam), mouse monoclonal anti-RANKL (Novus Biologicals), rabbit polyclonal anti-TRAIL (Abcam). A mouse monoclonal anti-α-Tubulin antibody (Novus Biologicals) was used as an internal control to validate the right amount of protein loaded in the gels. The membranes were following incubated for 60 min at room temperature with the corresponding secondary antibody: horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Detection was performed by means of chemiluminescence assay (Merck Millipore, Darmstadt, Germany).

ELISA

IL-6, RANKL and OPG levels in the culture supernatant were evaluated by using specific enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions.

Statistical analysis

Experiments were carried out in triplicate. Data were analysed by the one-way analysis of variance (ANOVA), and then the least significance Duncan's test was applied. Vertical bars are means ± SEM of at least three different experiments; significance was set at a p-value <0.05.

Results

Presence of IL-17, TRAIL and their receptors in Saos-2 cells

First experiments were performed to verify whether Saos-2 cells expressed IL-17 and TRAIL, as well as their respective receptors. RT-PCR analysis showed that IL-17A, IL-17RA and IL-17RC mRNAs are expressed in Saos-2 osteoblastic cells, as well as TRAIL and decoy receptors DcR1 and DcR2, and its death receptors DR4 and DR5 (Fig. 1). Moreover, expression of corresponding proteins was evaluated by Western blot analysis. The expression of the respective proteins was analysed in cells cultured for 3 and 7 days confirming the presence of the specific protein, which was not modified by the cellular density of the culture (Fig. 2A-B).

Effects of IL-17 and/or cytokines on viability of Saos-2 cells

On the basis of published studies on cooperation between IL-17 and low concentration of TNF-α (47, 48), in all studies treatments of Saos-2 cells were performed with the following concentrations, 200 ng/ml for IL-17A or IL-17F and 2 ng/ml TNF-α.To assess potential effects of IL-17 and/or TNF-α on Saos-2 cell viability, the cells were incubated for 72 h with IL-17A, IL-17F and/or TNF-α in DMEM 0.3% FBS. MTT cell viability assay showed that IL-17 alone or in combination with TNF-α had no effect on cell viability, nor it induced cell death (Fig. 3A).

In addition, in order to evaluate the effects of IL-17 in combination with TRAIL, another important cytokine belonging to the TNF superfamily, we incubated Saos-2 cells for 72 h with IL-17A, IL-17F and/or TRAIL in DMEM 0.3% FBS. MTT cell viability assay revealed that TRAIL has a toxic effect on Saos-2 cells as it reduces their viability, but the association with IL-17 has no additive toxic effect (Fig. 3B). In addition, treatment with different concentrations of the single substances confirmed that only TRAIL had a toxic effect on Saos-2 cell viability detectable already at 50 ng/ml while IL17-A and F had no effect (Fig. 3C).

Effects of IL-17 and/or TNF-α on IL-17RA, IL-17RC, DR4 and DR5 receptors expression

Since level of receptors are critical for

cytokines activity regulation, we tested whether IL-17 and /or TNF-α treatment affected the levels of IL-17RA, IL-17RC, DR4 and DR5 expression. Densitometry data of Western blot analysis indicated that when Saos-2 cells were treated for 48 h with IL-17A and/or TNF-α the protein expression of IL-17RA did not change compared with untreated control (Fig. 4A), while level of IL-17RC slightly decreased upon TNF-α treatment alone or in combination. On the other hand, the same treatments resulted in an increased protein expression of TRAIL death receptors, DR4 and DR5 respect to untreated cells (Fig. 4B).

IL-17 synergises with TNF-α to induce IL-6 secretion

As observed in other cell types (30, 49-51), we found that IL-17 induced the production of IL-6 in Saos-2 cells in a time-dependent manner following 2 and 24 h stimulation (Fig. 5). However, in the presence of low serum levels (0.3% FBS), IL-6 secretion was low, while in contrast, IL-17 triggered higher levels of IL-6 when cells were incubated with high concentration (10%) of FBS (data not shown). Thus, IL-17 alone is a poor inducer of IL-6 secretion, although it is likely that IL-17 can function cooperatively with an unknown factor in serum to stimulate IL-6 expression. Accordingly, all subsequent experiments were performed following an overnight pre-incubation in low serum containing medium (0.3% FBS) to eliminate the effects of confounding factors in serum.

Although relatively high concentration of IL-17 (200 ng/ml) was insufficient to induce significant IL-6 secretion under low serum conditions, results showed that this concentration of IL-17 could potently synergise with a suboptimal dose of TNF-α (2 ng/ml) as demonstrated by real-time PCR (Fig. 5A), ELISA (Fig. 5B) and western blot (Fig. 5C). Moreover, experiments were performed to evaluate whether treatment with IL17 (200 ng/ml) and/or TNF-α (2 ng/ml) could induce production of IL-1β from osteoblastic cell. Evaluation by bot Elisa and western blot on either culture medium and cell lysate after 24h and 48h did not demonstrate any production of IL-1β (data not shown).

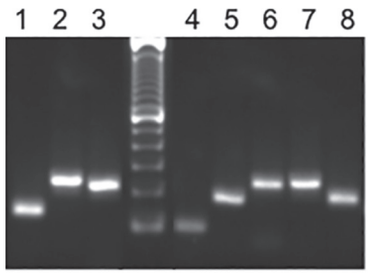

Fig. 1. mRNA expression of IL-17, TRAIL and their respective receptors in Saos-2 cells analysed by RT-PCR. Amplification products for IL-17, IL-17RA, IL-17RC, TRAIL, DcR1, DcR2, DR4 and DR5, respectively lane 1 to 8, were analysed on 2% agarose gel electrophoresis.

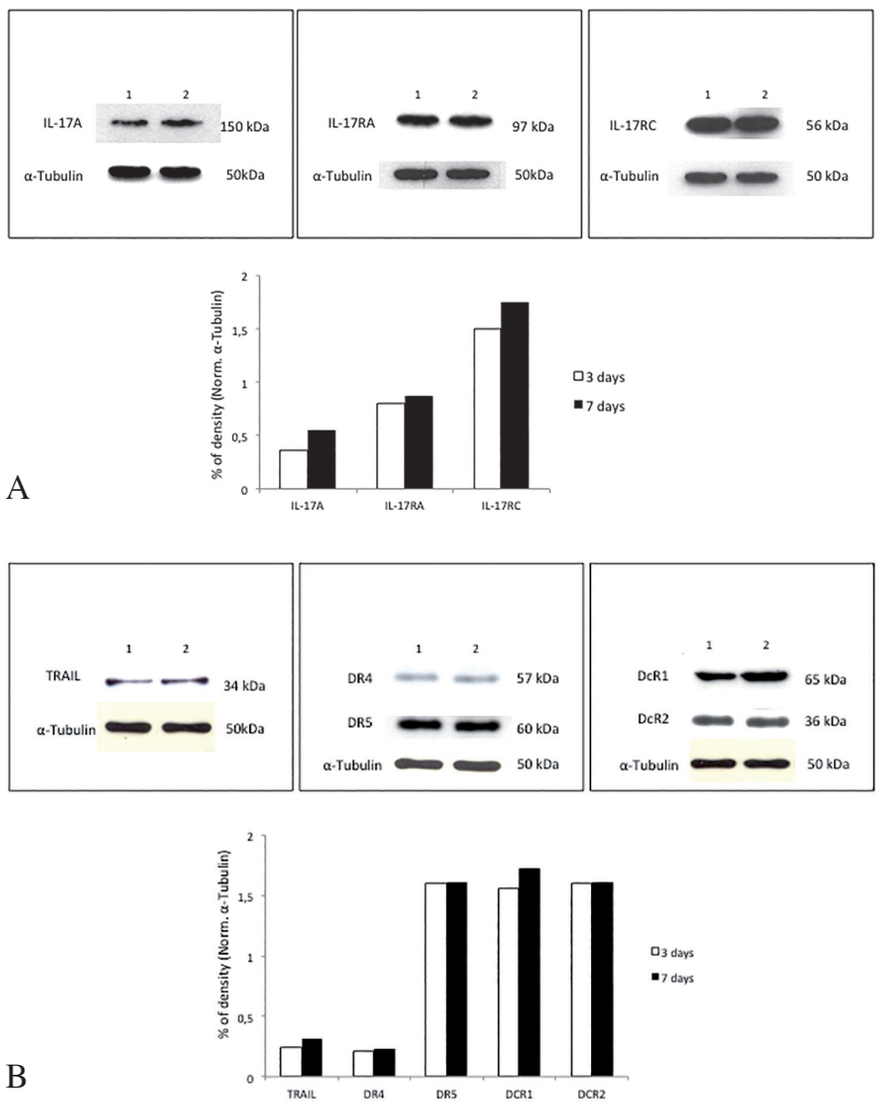

Fig. 2. Basal protein expression of IL-17, TRAIL and their receptors in Saos-2 cells.
A: Western blot analysis of IL-17 and its receptors IL-17RA and IL-17RC in Saos-2 cells was examined at days 3 and 7 of culture, lanes 1 and 2, respectively.
B: Western blot analysis of TRAIL and its death receptors DR4 and DR5, and decoy receptors DcR1 and DcR2 in Saos-2 cells was examined at days 3 and 7 of culture, lanes 1 and 2, respectively.

Effects of IL-17 and/or TNF-α on bone remodelling factors

To evaluate the effect of cytokines on bone remodelling factors, we analysed RANKL and OPG protein expression. Saos-2 cells were treated with IL-17A and/or TNF-α, and after 48 h proteins secreted in the culture medium were analysed by Western blot. Data showed that IL-17 and TNF-α alone are able to increase RANKL and OPG secretion, and their association resulted in an ad-

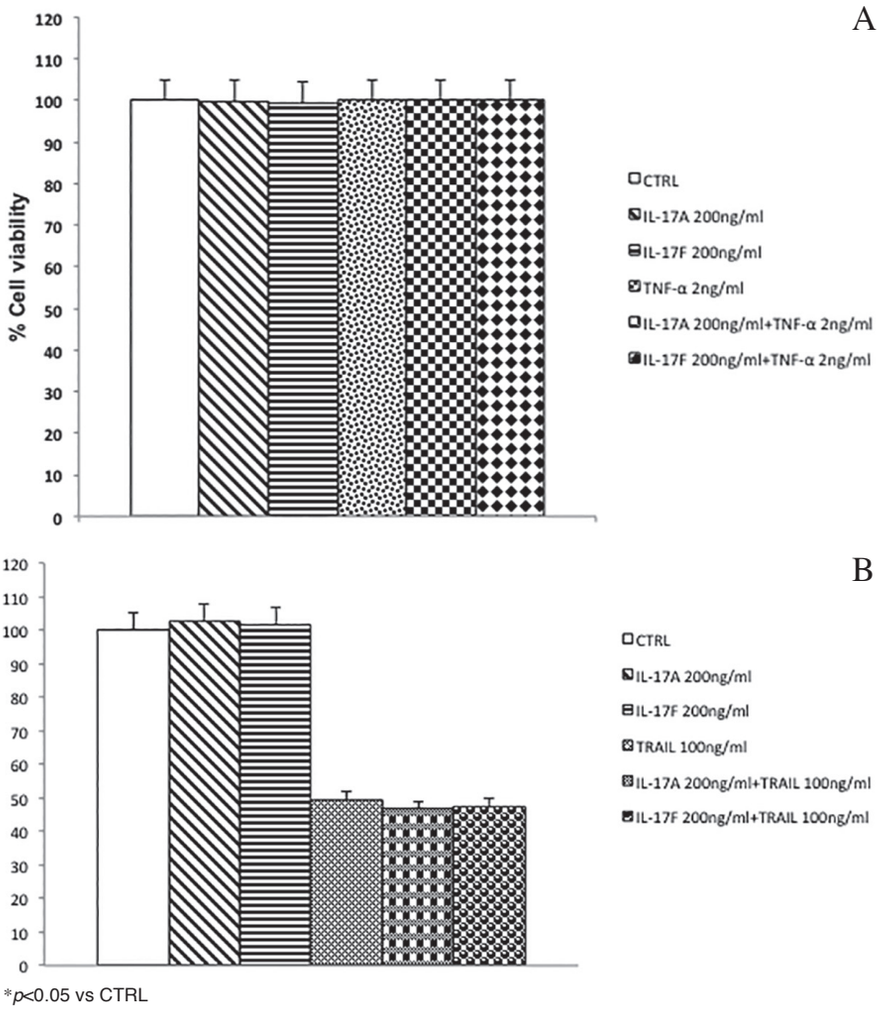

Fig. 3. Effects of IL-17, TNF-α and TRAIL on Saos-2 cell viability.
A: Saos-2 cells after 72h treatment with IL-17A (200 ng/ml), IL-17F (200 ng/ml) and/or TNF-α (2 ng/ml) in DMEM 0.3% FBS.
B: Saos-2 cells after 72h treatment with IL-17A (200 ng/ml), IL-17F (200 ng/ml) and/or TRAIL (100 ng/ml) in DMEM 0.3% FBS.
C: Saos-2 cells after 72h treatment with IL-17A, IL-17F or TRAIL at 50, 100 or 200 ng/ml in DMEM 0.3% FBS Results were analysed by the one-way ANOVA, followed by the Fisher's Least Significant Difference test. Significance was set at a $p<0.05$. Vertical bars are means ± SEM.

ditive effect on the release of osteoclast markers (Fig. 6A). Accordingly, we found that IL-17 and TNF-α increased intracellular levels of RANKL, and the association augmented their effects (Fig. 6B). To evaluate the results observed by Western blot analysis on secreted RANKL and OPG we performed enzyme-linked immunosorbent assay (ELISA). Saos-2 cells were treated with IL-17A and/or TNF-α, and after 24 and 48 h we measured the total amount of RANKL (Fig. 6C, (a)) and OPG (Fig. 6C, (b)) secreted into the culture medium. Results reported an enhanced secretion of RANKL and OPG after 48 h treatment.

The relative mRNA expression level of bone remodelling markers ALP, RANKL and OPG were analysed by real-time PCR (Fig. 7) after incubation of the cells with IL-17A and/or TNF-α for 2, 24 and 48 h. IL-17 treatment alone did not induce change in the ratio of ALP mRNA expression at any time point, whereas TNF-α treatment resulted in a progressive decrease of the level of mRNA observed after 24 and 48 h of treatment (Fig. 7A). The elevated threshold cycle (Ct), value inversely related to the initial amount of target in sample, observed for RANKL in untreated cells (data not shown) indicated a limited basal mRNA expression of RANKL. The association of IL-17 to TNF-α did not modify the effect induced by TNF-α alone. After 2 h treatment with TNF-α and TNF-α plus IL-17 the mRNA level of RANKL was lightly increased, respectively 1.3- and 1.5-fold the untreated level; however after 24 and 48 h of treatment the ratio of expression decreased from 0.8 to 0.5 for TNF-α alone, and from 0.9 to 0.4 for IL-17 plus TNF-α. The limited increase in relative amount of RANKL mRNA expression respect to untreated level is detectable from 2 to 48 h after IL-17 treatment (Fig. 7B) while it is decreasing for TNF-α and combination. After 2 h treatment the relative level of OPG mRNA expression remained unchanged while after 24 h treatments with TNF-α alone or combined with IL-17 showed an increased relative amount (Fig. 7C).

Effects of IL-17 and/or TNF-α on pro-inflammatory mediators

To assess the effect of IL-17 and/or TNF-α on pro-inflammatory factors we analysed the relative expression of selected mRNA markers by means of quantitative RT-PCR. Saos-2 cells were treated with IL-17A (200 ng/ml) and/or TNF-α (2 ng/ml), and mRNAs for CXCL-1, CXCL-5, and cyclooxygenase-2 (COX2) were analysed after 2 and 24 h (Fig. 8). Analysis of CXCL1 mRNA indicated that TNF-α treatment strongly enhanced the relative expression rate, 8-fold the basal level, while IL-17 presented a limited effect the combination of the cytokines synergistically potentiated the individual effect, with an increased rate of 25-fold after 2 h treatment. The same effects, slightly attenuated, were observed after 24 h treatment (Fig. 8A). The results of the analysis of CXCL5 mRNA showed a more marked effect for TNF-α treatment and combined treatment with IL-17, respectively 20- and 50-fold increase as compared to basal expression level at 2 h treatment. The stimulatory effect was observed in a lesser extend after 24 h treatment (Fig. 8B). The level of expression of COX2 mRNA appeared modulated by IL-17 treatment after 2 h, the effect was enhanced by the presence of TNF-α that alone showed

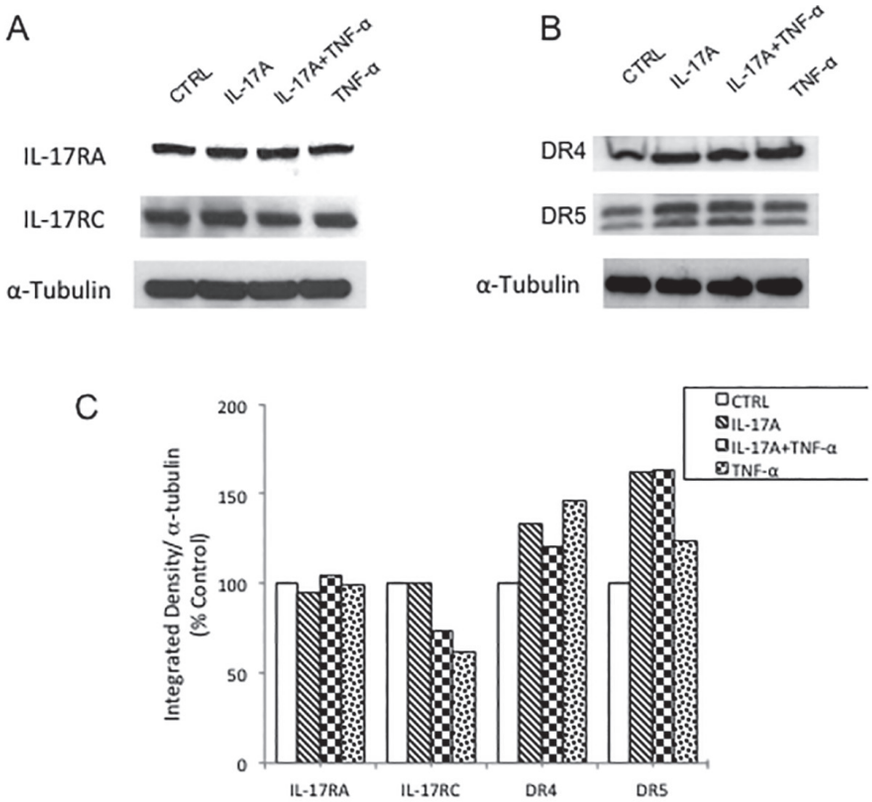

Fig. 4. Expression of IL-17 and TRAIL respective receptors in Saos-2 cells after treatment with IL-17 and /or TNF-α.
A: Western blot analysis of IL-17RA (upper blot) and IL-17RC (middle blot) protein expression in Saos-2 cells after 48h treatment with IL-17A (200 ng/ml) and/or TNF-α (2 ng/ml); α-Tubulin (lower blot) was used as control.
B: Western blot analysis of TRAIL death receptors DR4 (upper blot) and DR5 (middle blot) in Saos-2 cells after 48h treatment with IL-17A (200 ng/ml) and/or TNF-α (2 ng/ml); α-Tubulin (lower blot) was used as control.
C: Densitometric analysis of western blot.
Results are expressed as percent variation *vs*. control values.

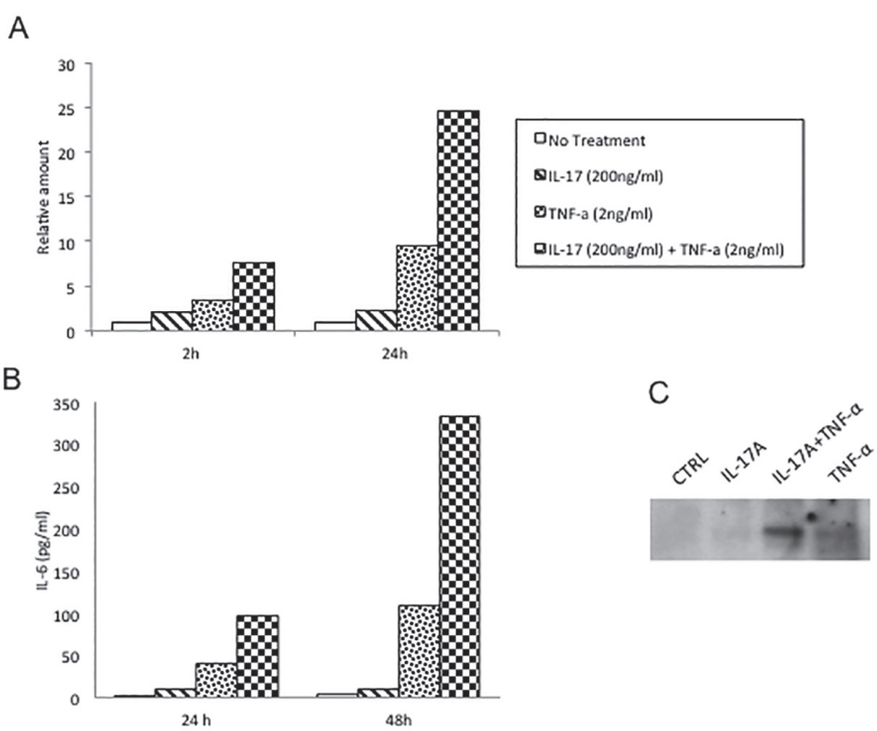

Fig. 5. Effects of IL-17 and/or TNF-α on IL-6 expression.
A: Real-time PCR of IL-6 gene expression in Saos-2 cells after 2h and 24h of treatment with IL-17 (200 ng/ml) and/or TNF-α (2 ng/ml). Results are expressed as mRNA relative amounts of untreated cells.
B: ELISA of IL-6 protein amounts measured in the culture medium of Saos-2 cells challenged with IL-17 (200 ng/ml) and/or TNF-α (2 ng/ml) after 24h and 48h. Vertical bars are means ± SEM; **p*<0.05 *vs*. no treatment (one-way ANOVA followed by the least significance Duncan's test).
C: Western blot analysis of IL-6 protein expression in culture media of Saos-2 cells challenged with IL-17 (200 ng/ml) and/or TNF-α (2 ng/ml) after 48h.

no stimulatory effect. After 24 h treatment IL-17 effect was no more detected and only treatment with the combined cytokines still showed a slightly increased level of expression of COX2 (Fig. 8C).

Discussion

The results presented herein demonstrate that the pro-inflammatory cytokines IL-17 and TNF-α can modulate the homeostasis of osteoblasts by altering specific gene expression, the observed changes concerted to a potential consecutive stimulation of the osteoclast activity and bone resorption. It is well known that a coordinated activation of the immune systems is essential for the efficient suppression

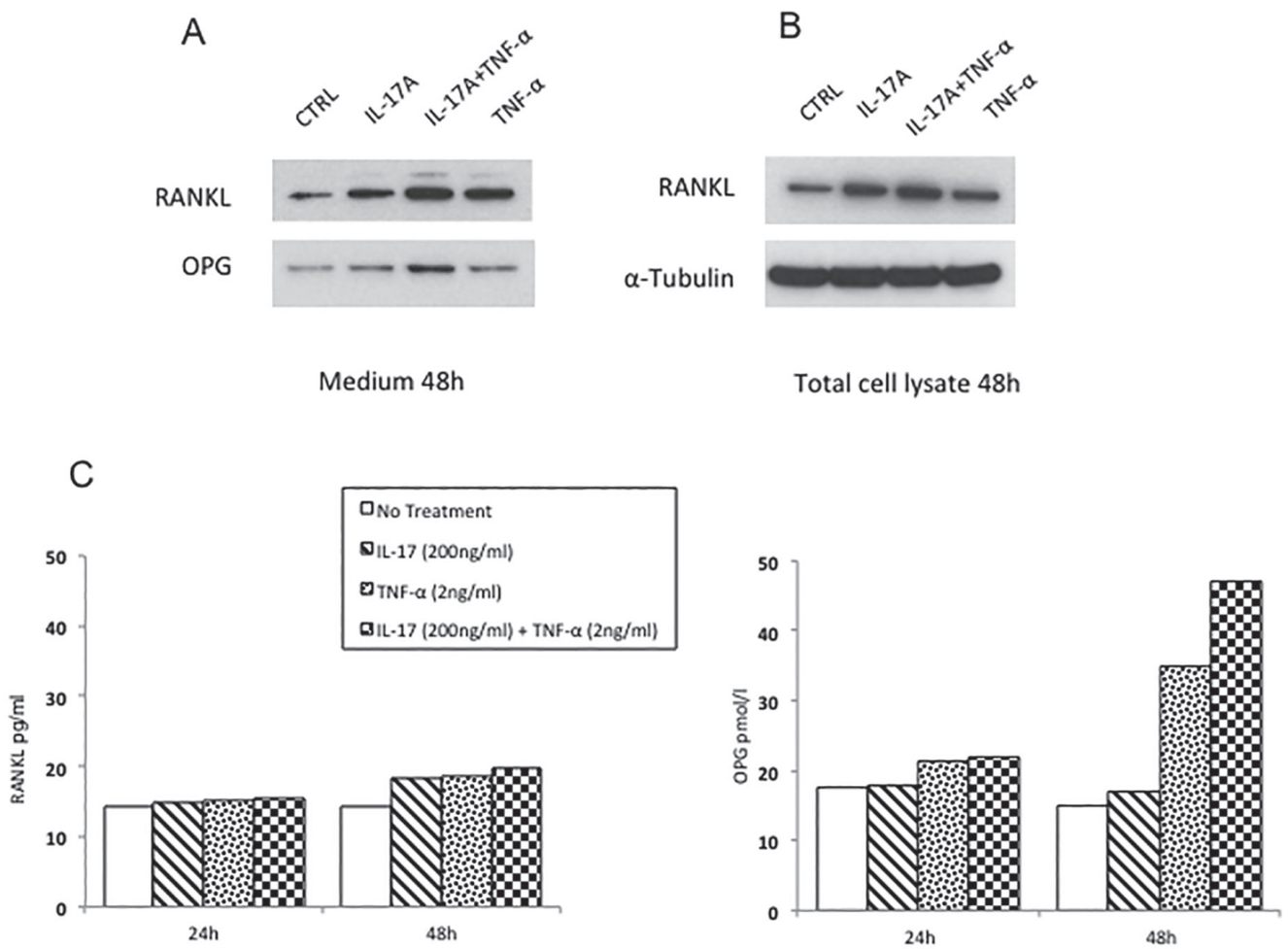

Fig. 6. Effects of IL-17 and/or TNF-α on osteoclastic regulatory molecules.
A: Western blot analysis of RANKL (upper blot) and OPG (lower blot) protein amounts in Saos-2 culture medium after 48h treatment with IL-17 (200 ng/ml) and/or TNF-α (2 ng/ml).
B: Western blot analysis of RANKL protein expression in Saos-2 total cell lysate after 48h treatment with IL-17 (200 ng/ml) and/or TNF-α (2 ng/ml).
C: ELISA of RANKL (a) and OPG (b) protein secretion in the culture medium of Saos-2 cells challenged with IL-17 (200 ng/ml) and/or TNF-α (2 ng/ml) after 24h and 48h.
Vertical bars are means ± SEM; **p*<0.05 *vs*. no treatment (one-way ANOVA followed by the least significance Duncan's test).

of pathogens. However, abnormal or protracted immune activation under specific pathological conditions, such as autoimmune inflammation, leads to tissue damage through activation of effector cells. Indeed in the autoimmune diseases psoriatic arthritis and rheumatoid arthritis, an increased number of osteoclasts have been observed in synovia together with a substantial infiltration of different cell types of the innate and adaptive immune response (52, 53) raising the challenging question of how the abnormality of the immune system induces the skeletal damage.

Pro-inflammatory cytokines modulate bone remodelling by increasing differentiation, maturation and bone resorption activity of osteoclasts and several studies provided evidence of their role in the skeletal alterations of rheumatic diseases such rheumatoid arthritis and psoriasis (30). In particular, growing evidences are supporting the prominent involvement of IL-17 in the highly intricate cytokine context associated to these autoimmune diseases. Furthermore, it has been well documented that IL-17 acts cooperatively, in an additive and often synergistic mode, with other cytokines such as TNF-α, IL-1β, IFN-γ and IL-4. In inflammation conditions, where TNF-α, IL-1β, IFN-γ and IL-4 are commonly expressed, IL-17, which secretion is limited by the presence of infiltrating Th17 lymphocytes, may represent a regulatory cytokine responsible for a fine tuning of the inflammatory response. In fact, IL-17 alone is not a potent inducer of inflammatory pathways and the mechanisms responsible of synergy are not fully understood. Interestingly, IL-17 and TNF-α are known to interplay and drive common molecular pathways, indeed, their cooperation has been described in multiple cells types, synoviocytes, chondrocytes, osteoblasts and myoblasts (28, 54-56). On the other hand, considering the regulatory effect of the pro-inflammatory cytokine TRAIL on bone metabolism through its interaction with OPG, its relationship with TNF-α and its well-recognis ed important role in the regulation of immune responses in various autoimmune-mediated pathologies included psoriasis (39-40),

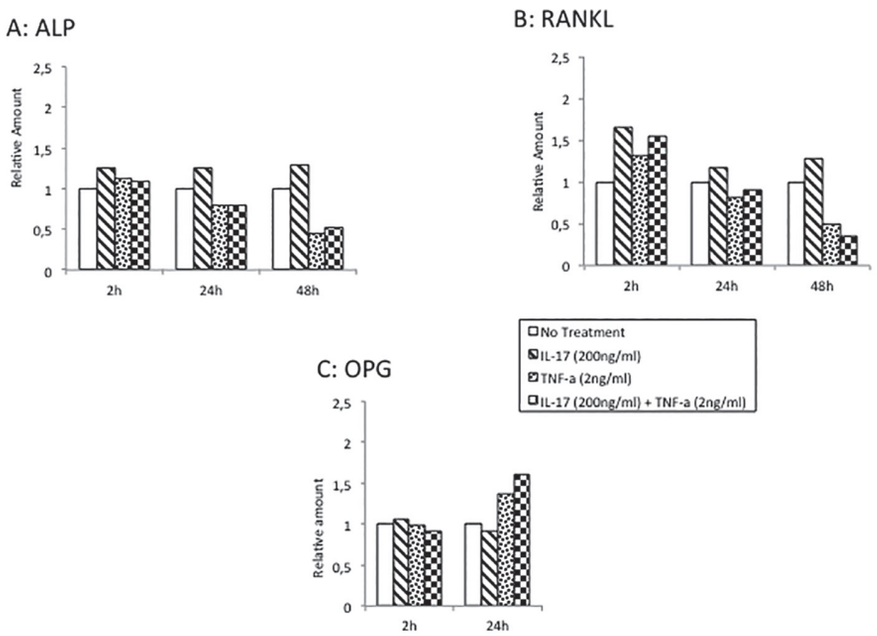

Fig. 7. Effects of IL-17 and/or TNF-α on bone remodelling markers. Real-time PCR analysis of ALP (**A**), RANKL (**B**) and OPG (**C**), mRNAs in Saos-2 cells treated for the indicated times with IL-17 (200 ng/ml) and/or TNF-α (2 ng/ml). Results are expressed as mRNA relative amounts of untreated cells.

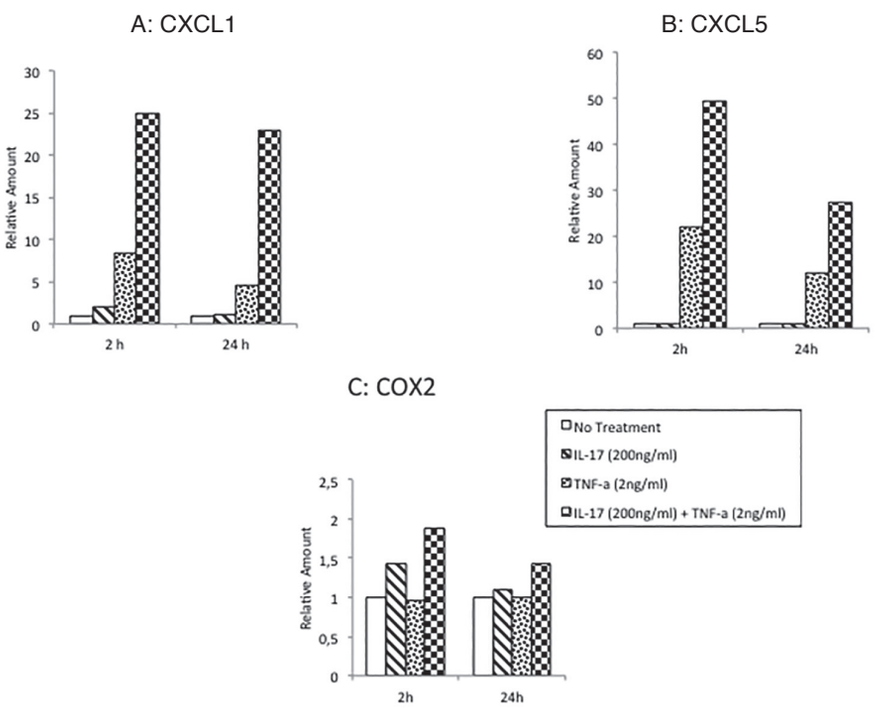

Fig. 8. Effects of IL-17 and/or TNF-α on pro-inflammatory factors. Real-time PCR analysis of CXCL-1, CXCL-5, and COX2 mRNAs, in Saos-2 cells treated for the indicated times with IL-17 (200 ng/ml) and/or TNF-α (2 ng/ml). Results are expressed as mRNA relative amounts of untreated cells.

it appeared interesting to access the effect of combination between IL-17 and TRAIL.

In addition to the well-acknowledged pivotal role of IL-17 in pathogenesis of bone-destructive diseases, it has been suggested that IL-17 may be involved as well in the regulation of bone formation. This dual role has been demonstrated in studies on human mesenchymal stem cells showing that, in addition to support osteoclastogenesis, IL-17 stimulates the proliferation and the osteoblastic differentiation (57, 58). In addition, osteoblast maturation induced by the isoform IL-17 F has been observed in mice during early phase of bone repair (59). Contrasting results were however observed in studies on osteogenesis in rats both *in vitro* and *in vivo* suggesting a species or cell type-specific role of IL-17 in bone formation (60). On the basis of these, still limited, observations on osteoblastogenesis, we were interested in studying the effect of IL-17 on the human osteosarcoma cell line Saos-2, presenting osteoblastic cell characteristics, to access direct effect on osteoblastic cells and potential indirect effect on osteoclastogenesis.

We showed that both IL-17A and IL-17F treatment did not induce proliferation or apoptosis of Saos-2 cells and did not influence the effect of associated cytokine (TNF-α or TRAIL) indicating no direct effect on osteoblast cell viability. Analysis of the effect of IL-17 and/or TNF-α treatment on the expression of receptors showed a limited effect on the expression of IL-17 RC receptor and TRAIL death receptors likely not reflecting a physiological change.

The RANK/RANKL/OPG system plays a pivotal regulatory role for bone turnover. Osteoblasts express RANKL on the cell surface, which binds its counter-receptor RANK on osteoclast precursors triggering their maturation. On the other hand, OPG is a soluble decoy receptor, which prevents signalling from RANK, thus antagonising the biological effects. The balance between OPG and RANKL regulates the degree of osteoclastogenesis and, thus, bone destruction. In relation to bone alteration situation, several experimental and clinical reports have implicated IL-17 in the regulation of OPG and RANKL expression from various cell types, such as human periodontal ligament cells (61), in osteoblasts (62), neutrophils (63) and synovia circumstantial cells (64). Our results demonstrate that in our model of osteoblastic cells, IL-17 and TNF-α only slightly modulate the expression of the main factors, ALP, RANKL and OPG, involved in osteoclastogenesis and osteoclast activity, with an eventually additive effect. The increase in RANKL expression corre-

spond to a rapid reaction to treatment while the OPG response seems to be a secondary event. Nevertheless, IL-17 and/or TNF-α efficiently stimulated the secretion of the pro-inflammatory cytokine IL-6 and TNF-α at sub-optimal concentration exerted a synergistic effect in Saos-2 cells.

In this study we also showed that IL-17 induces the expression of COX2, an immediate-early response gene mainly induced at sites of inflammation in response to inflammatory stimuli (65). Interestingly COX2 is an inducible enzyme, involved in the production of prostaglandin E2 (PGE2), and previous reports have suggested that PGE2 might be involved in bone pathogenesis via autocrine mechanism on murine osteoblastic MC3T3-E1cells (66, 67) and on differentiation of osteoclast precursors cells RAW264.7 (68).

Finally our data showed that IL-17 and TNF-α synergistically up-regulate mRNA expression of two chemokines, CXCL1 (or keratinocyte-derived chemokine) and CXCL5 (or LPS-induced CXC chemokine). These two chemokines have been demonstrated to be very potent neutrophil attractant (69) and inappropriate or excessive activation of neutrophils results in host tissue damage. For example, neutrophils contribute to lung tissue destruction in different pathologies such as acute asthma, chronic pulmonary disease, and during chronic infection with Mycobacterium tuberculosis. It is interesting that previous studies have shown that IL-17 cooperates with innate cytokines such as TNF-α to induce the synthesis of factors involved in neutrophil chemotaxis (24). The synergistic effect IL-17A and TNF-α on astrocytes resulted in enhanced secretion of CXCL-1 leading to the inflammation tissue damage in cerebral ischemia (70). Finally, Ruddy (71) demonstrated that IL-17 and TNF-α cooperatively induce CXCL5 in the pre-osteoblast cell line MC3T3. Our results corroborate the previous observations and suggest that synergistic expression of the two chemokines CXCL1 and CXCL5 triggered by IL-17 and TNF-α in osteoblasts may represent a mechanism for neutrophil recruitment in inflammatory tissue contributing to the deleterious effects observed in bone disease.

Further investigation on the mechanism by which IL-17 provides a redundant inflammatory response, particularly for neutrophils recruitment, may highlight new molecular targets for innovative treatment of bone pathologies associated with immunological diseases such as psoriatic arthritis.

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