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TNFα inhibits the development of osteoclasts through osteoblast-derived GM-CSF

Elvis Atanga [a], Silvia Dolder [a], Tina Dauwalder [b], Antoinette Wetterwald [a], Willy Hofstetter [a,*]

[a] Group for Bone Biology and Orthopaedic Research, Department Clinical Research, University of Bern, Murtenstrasse 35, CH-3010 Bern, Switzerland
[b] CSL Behring Biotherapies for Life™, Wankdorfstrasse 10, CH-3022 Bern, Switzerland

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ABSTRACT

Inflammatory cytokines such as tumor necrosis factor-alpha (TNFα) are potent stimulators of osteoclast formation and bone resorption and are frequently associated with pathologic bone metabolism. The cytokine exerts specific effects on its target cells and constitutes a part of the cellular microenvironment. Previously, TNFα was demonstrated to inhibit the development of osteoclasts in vitro via an osteoblast-mediated pathway. In the present study, the molecular mechanisms of the inhibition of osteoclastogenesis were investigated in co-cultures of osteoblasts and bone marrow cells (BMC) and in cultures of macrophage-colony stimulating factor (M-CSF) dependent, non-adherent osteoclast progenitor cells (OPC) grown with M-CSF and receptor activator of NF-κB ligand (RANKL). Granulocyte-macrophage colony stimulating factor (GM-CSF), a known inhibitor of osteoclastogenesis was found to be induced in osteoblasts treated with TNFα and the secreted protein accumulated in the supernatant. Dexamethasone (Dex), an anti-inflammatory steroid, caused a decrease in GM-CSF expression, leading to partial recovery of osteoclast formation. Flow cytometry analysis revealed that in cultures of OPC, supplemented with 10% conditioned medium (CM) from osteoblasts treated with TNFα/1,25$(OH)_2D_3$, expression of RANK and CD11c was suppressed. The decrease in RANK expression may be explained by the finding, that GM-CSF and the CM from *wt* osteoblasts were found to suppress the expression of *c-Fos*, *Fra-1*, and *Nfatc-1*. The failure of OPC to develop into $CD11c^+$ dendritic cells suggests that cell development is not deviated to an alternative differentiation pathway, but rather, that the monocytes are maintained in an undifferentiated, $F4/80^+$, state. The data further implies possible interactions among inflammatory cytokines. GM-CSF induced by TNFα acts on early hematopoietic precursors, inhibiting osteoclastogenesis while acting as the growth factor for M-CSF independent inflammatory macrophages. These in turn may condition a microenvironment enhancing osteoclast differentiation and bone resorption upon migration of the OPC from circulation to the bone/bone marrow compartment.

Introduction

Differentiation and activation of bone cells is regulated by their respective microenvironment, which is composed of cells, cytokines and growth factors, and of the extracellular matrix. A shift in this fine-tuned equilibrium may lead to qualitative and quantitative changes that constitute the base for the onset of pathological conditions [1–3]. The close functional and anatomical relationship between bone and the cells of the immune system was demonstrated in the recent past, when increasing evidence has been gathered supporting a role for immune cells in defining the bone microenvironment through a network of regulatory molecules. Furthermore, immune cells develop within the bone marrow, and as a consequence, bone and bone cells form part of their specific microenvironment [4].

The inflammatory response is a vital component of the immune system's defense against infectious agents. An abnormally prolonged activation may be followed by skeletal pathologies, including focal bone erosions and cartilage degeneration. Rheumatoid arthritis (RA) is an example of a disease in which activation of the immune system leads to an increase in bone resorption [4–6]. Elevated levels of interleukins (IL) such as IL-1, IL-6, IL-15, IL-17 and of TNFα secreted by activated T-lymphocytes and monocyte/macrophage lineage cells have been associated with bone loss in RA through their stimulatory effects on osteoclast development and activation [7–9]. The efficiency of therapeutic protocols [10,11], employing antibodies against IL-17, IL-1AR, IL-6R [12,13] and TNFα [10,14–16], in preventing bone erosions, confirms the interdependence of the cell lineages of bone and of the immune system.

Osteoclasts are multinucleated cells of hematopoietic origin that degrade bone [17]. Two essential growth factors required for the development of osteoclast lineage cells are M-CSF, belonging to a group of lineage-specific hematopoietic growth factors [17–19], and RANKL, a member of the TNF-superfamily of proteins [20–22]. RANKL is expressed, apart from osteoblasts, by many cell types, including

* Corresponding author. Fax: +41 31 632 32 97.
E-mail addresses: elvis.atanga@dkf.unibe.ch (E. Atanga), silvia.dolder@dkf.unibe.ch (S. Dolder), tina.dauwalder@cslbehring.com (T. Dauwalder), antoinette.wetterwald@dkf.unibe.ch (A. Wetterwald), hofstetter@dkf.unibe.ch (W. Hofstetter).

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monocytes, neutrophils [23], fibroblasts [6,24,25], and by B- [26–28] and T-lymphocytes. M-CSF is widely expressed and inflammatory cytokines increase its synthesis by mesenchymal and endothelial cells. By this mechanism, cells of the immune system can actively modulate the differentiation and activation of osteoclasts, and, as a consequence, of bone resorption. Furthermore, additional cytokines originating in the immune system, such as IL-3, and IL-15, sustain osteoclast differentiation and mediate bone loss either by increasing the pool of osteoclast precursors, as does IL-3, or by inducing the expression of RANKL by NK cells, which is stimulated by IL-15 [29]. IL-4, IL-5, IL-12, GM-CSF and interferon-gamma (IFN-γ), on the other hand, are known inhibitors of osteoclastogenesis [30].

TNFα is released during inflammation by monocyte/macrophage lineage cells and is a potent, multifunctional modulator of inflammatory and osteolytic processes. The cytokine has been reported to support differentiation and activation of osteoclasts [31–34] while inhibiting the activity of osteoblast lineage cells, thus causing bone loss [35]. While not essential for osteoclastogenesis, TNFα potently modulates bone resorption by acting in synergy with RANKL [33]. The cytokine enhances osteoclastogenesis through direct and indirect mechanisms, by stimulating accessory cells to produce an osteoclastogenic environment, by mobilizing osteoclast precursors from bone marrow and by prolonging the survival of mature osteoclasts. Exposure to TNFα was found to result in increased levels of RANKL in several cell types, including osteoblasts, synoviocytes, stromal cells, and T- and B-cells. The long established concept that TNFα is solely an activator of osteoclastogenesis is changing and studies have shown that, while the cytokine enhances osteoclastogenesis by acting directly on osteoclast progenitors, it can inhibit the same process indirectly by inducing the expression of inhibitors by different cell types such as fibroblasts and osteoblasts. Expression levels of osteoprotegerin (OPG) a decoy receptor for RANKL were upregulated in human synovial fibroblasts treated with TNFα [36,37]. Along a similar line, previous studies in our group have shown that TNFα in vitro does exert an inhibitory effect on the development of osteoclasts through osteoblasts, which, however, seemed not to depend on OPG [38].

In the present study, we investigated the molecular mechanisms that mediate the inhibitory activity of TNFα on osteoclastogenesis in vitro. The data demonstrates that the inhibition of osteoclastogenesis by TNFα is partially mediated by GM-CSF, which is released by osteoblast lineage cells in response to TNFα. Furthermore, it is demonstrated that the mechanism of inhibition of osteoclastogenesis includes the downregulation of RANK, probably as a consequence of a downregulation of *c-Fos* and *Fra-1*, on osteoclast progenitors during the early phase of osteoclast differentiation.

Methods

Animals

Animals used in this study include C57Bl/6J *wt*, *GM-CSF*$^{-/-}$ and *p55TNFR*$^{-/-}$/*p75TNFR*$^{-/-}$ mice. Animals were bred in the Central Animal Facility of the Medical Faculty of the University of Bern, complying with the Swiss Guidelines for care and use of experimental animals. The experiments of the study were approved by the State Committee for the Control of Animal Experimentation (permit number 13/07 to WH).

Isolation of osteoblasts and production of conditioned media

Primary murine osteoblasts were isolated by sequential collagenase digestion of calvariae from 1 and 2 day old mice as described previously [38]. Briefly, 25 calvariae were digested for 5×20 min in Hank's balanced salt solution (Sigma, Buchs, CH) containing 3 mg/ml collagenase II (Worthington, NJ, USA). Fractions 3 to 5 were pooled, 10^6 cells were seeded into 75 cm^2 culture flasks and grown in culture medium [αMEM containing 10% FBS (Inotech AG, Dottikon, CH) and pen/strep (100 U/ml and 100 μg/ml; respectively; GIBCO BRL Life Technologies, Basle, CH)]. After 4 days in culture, the cells were aliquoted and stored in liquid nitrogen. Before the experiments, the cells were thawed, grown in culture medium for 4 days and subsequently used according to the experimental protocols.

To investigate the inhibitory activity of osteoblasts on osteoclast formation, 5×10^4 osteoblasts were seeded in 6-well plates in 2.5 ml of cell culture medium supplemented with 10^{-8} M $1,25(OH)_2D_3$ and TNFα (1 and 5 ng/ml) for 72 h. The resulting conditioned media (CM) were centrifuged and stored at 4 °C for later use.

Quantification of GM-CSF

GM-CSF protein levels were quantified in CM of primary osteoblasts from C57Bl/6J *wt*, *GM-CSF*$^{-/-}$ and *p55TNFR*$^{-/-}$/*p75TNFR*$^{-/-}$ mice treated with TNFα and $1,25(OH)_2D_3$ (specific conditions are indicated in the Results section) using a sandwich ELISA Kit as recommended by the manufacturer (BD OptEIA™ Set Mouse GM-CSF ELISA kit, BD Bioscience, San Diego, CA, USA).

PCR analysis

Levels of transcripts were assessed by quantitative real-time PCR. For this purpose, cells were cultured according to specified protocols and total RNA was isolated using the RNeasy Mini Kit (Qiagen, Basle, CH) according to the recommendation of the manufacturer. After reverse transcription with MMuLV RT (Roche Diagnostics, Rotkreuz, CH), PCR was performed using an ABI PRISM 7500 System (Applied Biosystems, Rotkreuz, CH). The C_T values were normalized against 18S rRNA. The reactions were performed using TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Rotkreuz, CH), the respective Assays-on-Demand (ABI, Rotkreuz, CH) and 5 ng to 10 ng cDNA. The reaction mixes were preincubated for 2 min at 50 °C, followed by 10 min at 95 °C. Thereafter, 45 cycles of 5 sec at 95 °C and 15 sec at 60 °C each were performed. The reactions were analyzed using the ABI PRISM sequence detection software 7500 Fast System v1.4.0. For quantitative PCR the following Assays-on-Demand were used: GM-CSF Mm00434162_m1; RANKL/Tnfsf11 Mm00441908_m1; RANK/TNFRSF-11A Mm00437135_m1; c-Fms Mm01266652_m1: IL-6 Mm00446190_m1; IL-11 Mm00434162_m1; *c-Fos* Mm00487425_m1; *Fra-1* Mm00487429_m1; Nfatc-1 Mm00479445_m1; NAH-2 Mm00724785_m1.

Alkaline phosphatase assay

Osteoblasts (4×10^3 cells per well) were grown in 96-well tissue culture plates. At the end of the culture, the cells were washed with PBS and lysed in 20 μl of 0.1% Triton-X-100 in water by freezing and thawing three times. After incubation with 0.1 ml of 3 mM *p*-nitrophenylphosphate in 1 M ethanolamine, pH 9.5, for 1 h, the reaction was stopped with 50 μl of 0.1 M EDTA, pH 8.0 and the absorption was measured at 405 nm with a multi-well spectrophotometer (Tecan Infinite 200, Maennedorf, CH).

Cell viability (XTT) assay

Osteoblasts (4×10^3 per well) were grown in 96-well tissue culture plates. Cell proliferation was determined using the Cell Proliferation Kit II (XTT) (Roche Diagnostics, Rotkreuz, CH), on days 3 and 6 of the culture according to the recommendation of the manufacturer. Briefly, at the end of the culture period, the medium was discarded, and cells were incubated (37 °C and 5% CO_2) with 100 μl of freshly prepared culture medium and 50 μl of the XTT labeling mixture (5 ml XTT labeling reagent and 0.1 ml of electron-coupling reagent) for 3 h. At the end of the incubation period, the absorption was measured at 472 nm with a multi-well spectrophotometer (Tecan Infinite 200, Maennedorf, CH).

Cultures of osteoclast precursor cells

To assess the development of osteoclasts in vitro, M-CSF-dependent non-adherent osteoclast precursor cells (OPC) were grown in the presence of M-CSF and RANKL and, depending on the experimental protocol, with additional growth factors and CM. For the preparation of OPC, bone marrow cells (BMC) were flushed in αMEM/4 mM $NaHCO_3$ from femora of 6 week old *C57/Bl6J* mice and 6×10^7 cells were grown in 15 ml of culture medium containing M-CSF (30 ng/ml) for 24 h. Thereafter, the non-adherent cells were used for further experiments in cultures supplemented with M-CSF (Cetus Corporation, CA, USA), RANKL (5 and 20 ng/ml) (Preprotech, Lucerne, CH) and CM. The culture period lasted 5 days with a renewal of media after 3 days.

Osteoclast development was also investigated in co-cultures of total BMC and primary osteoblasts. 6×10^4 BMC and 4×10^3 osteoblasts were seeded into 48-well plates in 0.2 ml of cell culture medium supplemented with 10^{-8} M $1,25(OH)_2D_3$ and TNFα (0, 0.3, 1, 3, 10, and 30 ng/ml). Cultures were kept for up to 5 days and media were replaced after 3 days.

Tartrate resistant acid phosphatase (TRAP) staining

In vitro osteoclast formation was assayed by staining the cultures for the marker enzyme TRAP. Briefly, the cells were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) in PBS for 10 min at room temperature. After washing with deionized water, the cells were stained by adding TRAP solution [0.3 mg/ml diazotized fast garnet GBC, 2.5 M acetate solution, 0.67 M tartrate and 12.5 mg/ml naphthol; (Leukocyte acid phosphatase kit, Sigma Aldrich, Buchs, CH)] for 5 min. After washing to remove the staining solution, TRAP positive cells with 3 and more nuclei were counted as multinucleated osteoclast-like cells.

Flow cytometry

Osteoclast lineage cells were analyzed for the expression of cell surface markers after 4 days in culture. The culture media were supplemented with cytokines (M-CSF, RANKL, rGM-CSF (recombinant murine GM-CSF), and IL-4) or with 10% CM from C57Bl/6J *wt* and *GM-CSF*$^{-/-}$ osteoblasts treated with TNFα (5 ng/ml)/±$1,25(OH)_2D_3$ (10^{-8} M) as described above. The cells were harvested using a cell scraper (Greiner Bio-One GmbH, Frickhausen, D) and washed twice with FACS buffer (PBS, 0.5% FBS and 0.2% sodium azide). Cell suspensions (1×10^5 cells) were incubated with a cocktail of antibodies, containing FITC-conjugated anti-mouse CD11c, APC-conjugated anti-mouse CD115, APC-Cy7 conjugated anti-mouse F4/80, PB-conjugated anti-mouse *c-Kit* and PE-conjugated anti-mouse RANK (Biolegend, San Diego, CA, USA) on ice for 20 min. Isotype-matched monoclonal antibodies were used as negative controls (Biolegend, San Diego, CA, USA). After the final wash, a total of 2×10^4 cells was analyzed using a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA) and FLOWJO analysis software version 7.6.1 (FLOWJO, Ashland, OR, USA).

Statistical analysis

Differences in osteoclast numbers, release of GM-CSF, and cell viability were evaluated by one-way ANOVA, with Bonferroni's post tests using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Primary murine osteoblasts release GM-CSF upon treatment with TNFα and 1,25(OH)$_2$D$_3$

In separate experiments analyzing the cellular transcriptome, transcripts encoding GM-CSF, a known inhibitor of osteoclastogenesis, were found to be upregulated within 24 h in primary osteoblasts upon treatment with TNFα (1 ng/ml) and $1,25(OH)_2D_3$ (10^{-8} M). This was confirmed by real-time PCR, treating the cells for 24 h, 48 h, and 72 h with TNFα/$1,25(OH)_2D_3$. Levels of transcripts encoding GM-CSF were found to be increased within 24 h, reaching a maximum around 48 h, and decreasing thereafter (Fig. 1A). GM-CSF protein, as determined by ELISA, accumulated and increased during the whole culture period (Fig. 1B). No GM-CSF was detected in CM in the absence of TNFα. Osteoblasts from *GM-CSF*$^{-/-}$ and *p55TNFR*$^{-/-}$/*p75TNFR*$^{-/-}$ animals did not release GM-CSF at detectable levels (not shown).

Osteoblasts from GM-CSF$^{-/-}$ *mice are less efficient in the inhibition of osteoclast development as compared to cells from wt animals*

To further confirm the role of GM-CSF in the TNFα/$1,25(OH)_2D_3$-mediated inhibition of osteoclastogenesis, co-cultures with osteoblasts from C57Bl/6J *wt* and *GM-CSF*$^{-/-}$ mice and BMC from *wt* and *p55TNFR*$^{-/-}$/*p75TNFR*$^{-/-}$ animals, respectively, were performed (Fig. 2). In co-cultures with *wt* osteoblasts, TNFα blocked the development of osteoclasts in a dose-dependent manner, with a complete inhibition of osteoclast formation at a concentration of ≥3 ng/ml (Figs. 2A, B). In co-cultures of *GM-CSF*$^{-/-}$ osteoblasts and *wt* BMC, grown in media supplemented with $1,25(OH)_2D_3$, the number of osteoclasts was reduced by max. 60% with TNFα concentrations of up to 30 ng/ml (Figs. 2C, D). In co-cultures of *GM-CSF*$^{-/-}$ osteoblasts and *p55TNFR*$^{-/-}$/*p75TNFR*$^{-/-}$ BMC, TNFα at 10 ng/ml reduced the number of osteoclasts by ca. 90%. In cultures grown without $1,25(OH)_2D_3$, no osteoclasts developed at low concentrations of TNFα, independently of

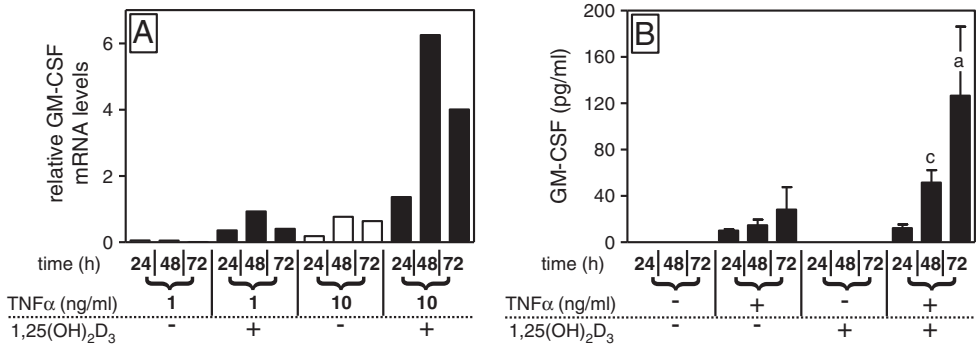

Fig. 1. Osteoblasts release GM-CSF upon treatment with TNFα and $1,25(OH)_2D_3$. Primary osteoblasts from murine calvariae were treated with TNFα (1 and 10 ng/ml) and 1,25 $(OH)_2D_3$ (10 nM). After 24 h, 48 h and 72 h, transcripts encoding GM-CSF were determined by quantitative real-time PCR (A) and protein levels were quantified in the culture supernatants by ELISA (B). Levels of transcript increased in dependence of the dose of TNFα, reaching a maximum after 48 h, while the protein accumulated during the culture period. Induction of GM-CSF was higher in cultures treated with TNFα in combination with $1,25(OH)_2D_3$, reaching a concentration of 126 pg/ml after 72 h, as compared to TNFα alone. Bars represent the average ± SD of 4 wells from one representative experiment. (a) $p<0.0001$ and (c) $p<0.01$; significant difference to respective controls.

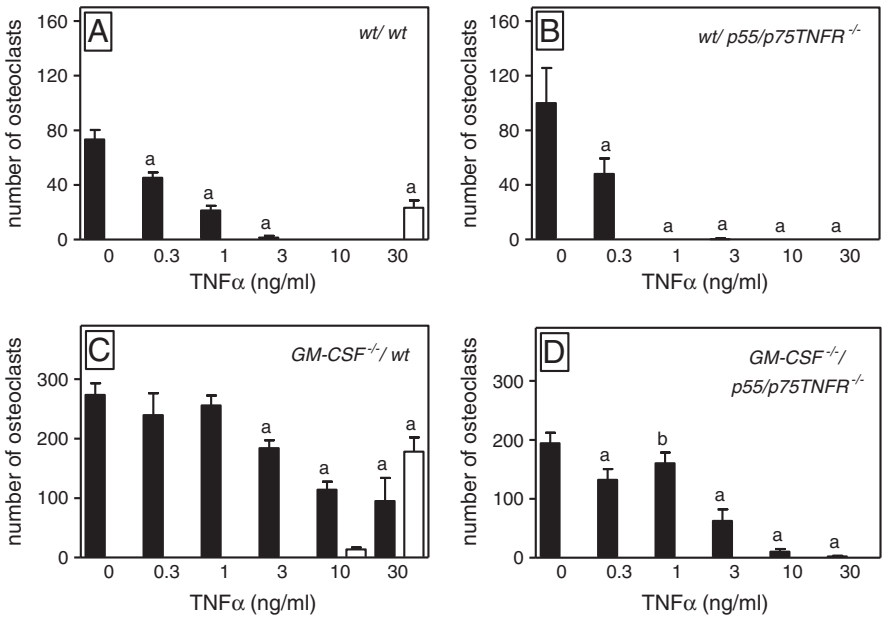

Fig. 2. Osteoblasts from *GM-CSF*$^{-/-}$ mice are less efficient than *wt* cells in inhibiting osteoclastogenesis. Co-cultures of C57Bl/6J *wt* (A, B) and *GM-CSF*$^{-/-}$ (C, D) osteoblasts with *wt* (A, C) or *p55TNFR*$^{-/-}$/*p75TNFR*$^{-/-}$ (B, D) BMC were grown with TNFα only (white bars) and TNFα/1,25(OH)$_2$D$_3$ (black bars). In co-cultures of *wt* osteoblasts and *wt* BMC (A), and *wt* osteoblasts and *p55TNFR*$^{-/-}$/*p75TNFR*$^{-/-}$ BMC (B), treatment with TNFα caused a dose-dependent inhibition of osteoclast formation. In co-cultures of *GM-CSF*$^{-/-}$ osteoblasts and *wt* BMC (C), or *GM-CSF*$^{-/-}$ osteoblasts and *p55TNFR*$^{-/-}$/*p75TNFR*$^{-/-}$ BMC (D), the efficiency of the inhibition was decreased. Higher levels of TNFα (≥10 ng/ml) induced partial restoration of osteoclast formation in cultures with *wt* BMC in the absence of 1,25(OH)$_2$D$_3$. Bars represent the average ± SD of 4 wells from one representative experiment. (a) $p<0.0001$ and (b) $p<0.001$; significant difference to respective controls without TNFα.

the origin of osteoblasts and BMC. At 30 ng/ml TNFα, osteoclastogenesis was restored to ca. 30% in cultures with *wt* osteoblasts and to ca. 65% in cultures with osteoblasts derived from *GM-CSF*$^{-/-}$ mice.

CM from *wt* and from *GM-CSF*$^{-/-}$ osteoblasts both mediated an inhibition of osteoclastogenesis in *wt* OPC cultured with M-CSF (30 ng/ml) and RANKL (5 ng/ml). As shown in Fig. 3, 10% of CM, obtained from osteoblasts after a 72 h culture period in medium supplemented with 1,25(OH)$_2$D$_3$ (10^{-8} M) and TNFα (5 ng/ml), inhibited osteoclast development by 100% in the case of *wt* osteoblasts, and by approx. 50% in the case of *GM-CSF*$^{-/-}$ osteoblasts. No inhibition in the number of osteoclasts was found in CM from control cells or from cells treated with either 1,25(OH)$_2$D$_3$ or TNFα alone.

Dexamethasone reversed the TNFα/1,25(OH)$_2$D$_3$-dependent induction of GM-CSF and the inhibition of osteoclastogenesis

Previously it was observed that Dex restored osteoclastogenesis in co-cultures treated with TNFα/1,25(OH)$_2$D$_3$ [38]. Therefore, the expression levels of GM-CSF transcripts and protein were assessed in primary osteoblasts grown in medium supplemented with 1,25 (OH)$_2$D$_3$ (10^{-8} M) and TNFα (3 ng/ml) in the presence or absence of Dex (10^{-8} M). At this concentration, Dex was found to greatly reduce (Fig. 4) levels of transcripts encoding GM-CSF (>80% after 72 h) as well as of GM-CSF protein (>90% after 72 h). The inhibitory effects of CM on the development of osteoclasts mirrored these changes in

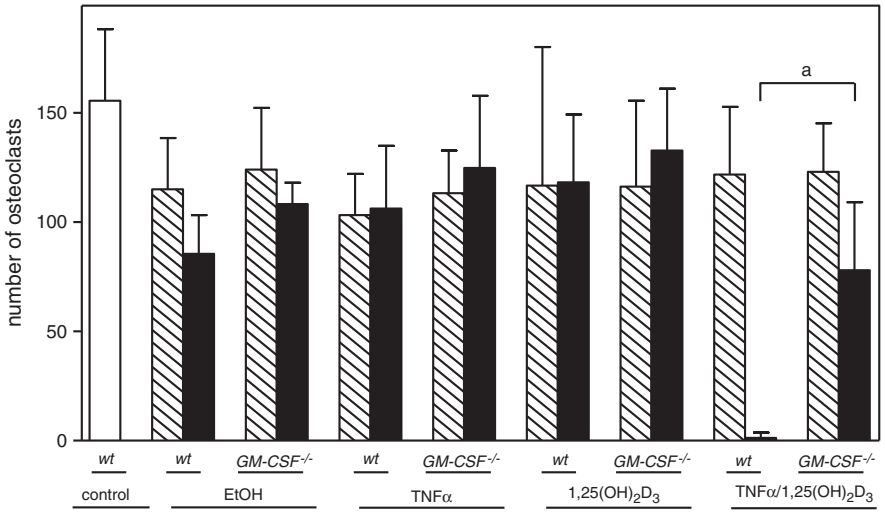

Fig. 3. GM-CSF partially mediates the TNFα-dependent inhibition on osteoclast formation by osteoblasts. CM was collected from *wt* and *GM-CSF*$^{-/-}$ osteoblasts grown in the presence of TNFα (5 ng/ml) and 1,25(OH)$_2$D$_3$ (10 nM) and tested in cultures of *wt* OPC containing M-CSF (30 ng/ml) and RANKL (5 ng/ml). CM was added at concentrations of 0% (white bar), 1% (hatched bars) and 10% (black bars). Addition of CM from *wt* osteoblasts treated with TNFα/1,25(OH)$_2$D$_3$ completely blocked osteoclast formation, while CM from *GM-CSF*$^{-/-}$ osteoblasts caused a partial reduction (15–30%) in osteoclast number only. Bars represent the average ± SD of 4 wells from one representative experiment. (a) $p<0.0001$; significant difference between *wt* and *GM-CSF*$^{-/-}$ CM from osteoblasts treated with TNFα/1,25(OH)$_2$D$_3$.

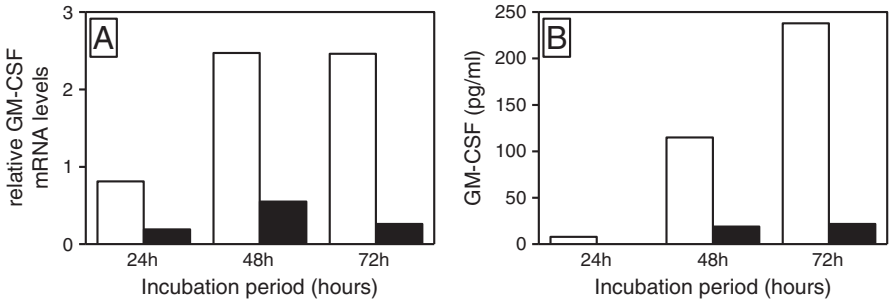

Fig. 4. Dexamethasone downregulates the expression of *GM-CSF*. To verify the effect of Dex on GM-CSF expression, CM from *wt* osteoblasts were prepared from cultures containing TNFα (5 ng/ml), 1,25$(OH)_2D_3$ (10 nM) and Dex (10 nM) (black bars) or no Dex (white bars). GM-CSF encoding transcripts were measured by quantitative real-time PCR (A), GM-CSF protein levels were quantified by ELISA (B). GM-CSF transcript and protein levels were found to be decreased by more than 80% after growing the cells for 72 h with Dex.

levels of GM-CSF, osteoclastogenesis being partially restored in media supplemented with Dex (Supplementary Fig. 1).

TNFα enhances the expression of stimulators of bone resorption, but not of RANKL, by osteoblasts

Given the osteoblast-mediated inhibition of osteoclastogenesis by TNFα, the expression of cytokines known to support the development of osteoclasts was investigated (Fig. 5). For this purpose, primary osteoblasts were cultured for 48 h with 1,25$(OH)_2D_3$ (10^{-8} M) and TNFα (1 ng/ml and 10 ng/ml). Total RNA was prepared and levels of transcripts encoding GM-CSF (Fig. 5A), RANKL (Fig. 5B), IL-6 (Fig. 5C), and IL-11 (Fig. 5D) were determined. GM-CSF mRNA levels were highly increased by TNFα in C57Bl/6J *wt* osteoblasts. No transcripts encoding GM-CSF were detected in total RNA from *p55TNFR*$^{-/-}$/*p75TNFR*$^{-/-}$ and *GM-CSF*$^{-/-}$ osteoblasts, respectively. Levels of transcripts encoding RANKL were induced upon treatment with 1,25$(OH)_2D_3$, no further stimulation by TNFα was attained. To the contrary, at 10 ng/ml, TNFα caused a decrease in RANKL mRNA (Fig. 5B). Levels of transcripts encoding IL-6 (Fig. 5C) and IL-11 (Fig. 5D) were highly dependent on the presence of TNFα and 1,25$(OH)_2D_3$, which act synergistically. 1,25$(OH)_2D_3$ alone did not cause any increase in transcript levels, while the effects of TNFα alone were modest only.

GM-CSF suppresses RANK expression in OPC

To characterize the effects of GM-CSF on OPC, levels of transcripts encoding RANK were quantified. OPC grown with CM from TNFα/1,25 $(OH)_2D_3$ treated C57Bl/6J *wt* osteoblasts showed a decrease in RANK mRNA levels during the 6 day culture period (Fig. 6A). When OPC

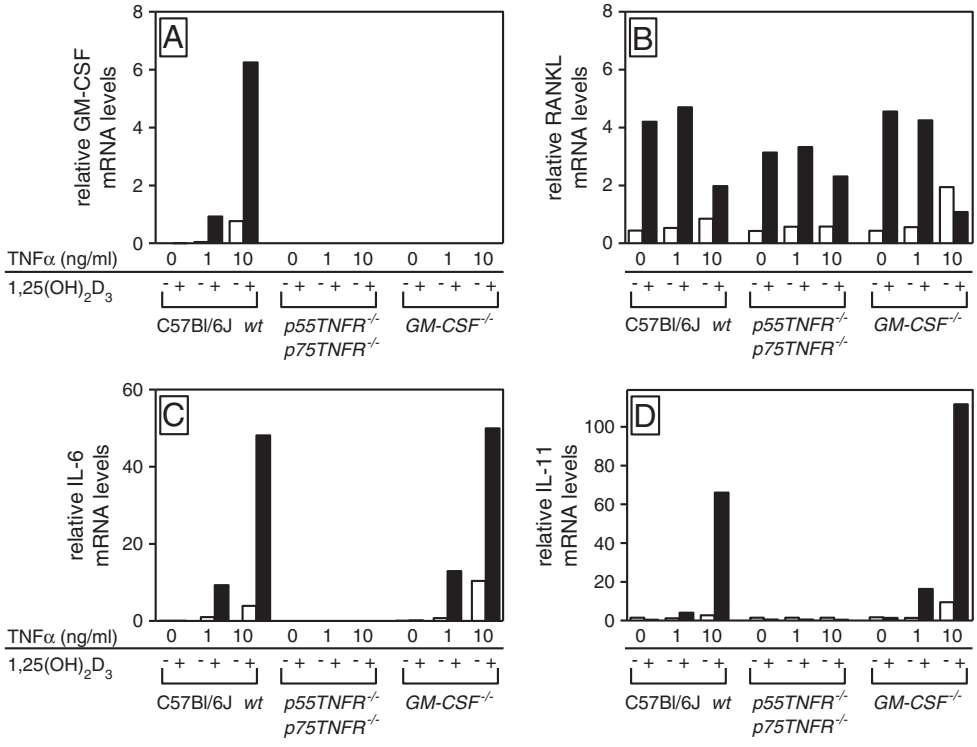

Fig. 5. The expression of osteoclastogenic factors is enhanced by TNFα. To assess whether the expression levels of osteoclastogenic growth factors was affected by TNFα, in *wt*, *p55TNFR*$^{-/-}$/*p75TNFR*$^{-/-}$ and *GM-CSF*$^{-/-}$ osteoblasts, the cells were treated with increasing doses of TNFα (0, 1 and 10 ng/ml) with (black bars) or without (white bars) 1,25 $(OH)_2D_3$ (10 nM) for 48 h. Transcripts encoding GM-CSF (A), RANKL (B), IL-6 (C) and IL-11 (D) were quantified by real-time PCR. Levels of transcripts encoding GM-CSF, IL-6 and IL-11 were increased with increasing doses of TNFα in cultures with TNFα/1,25$(OH)_2D_3$ (A, C and D). RANKL expression was stimulated by 1,25$(OH)_2D_3$ and these levels were no further enhanced by TNFα at low concentration of TNFα (0, 1 ng/ml) but were decreased at 10 ng/ml (B).

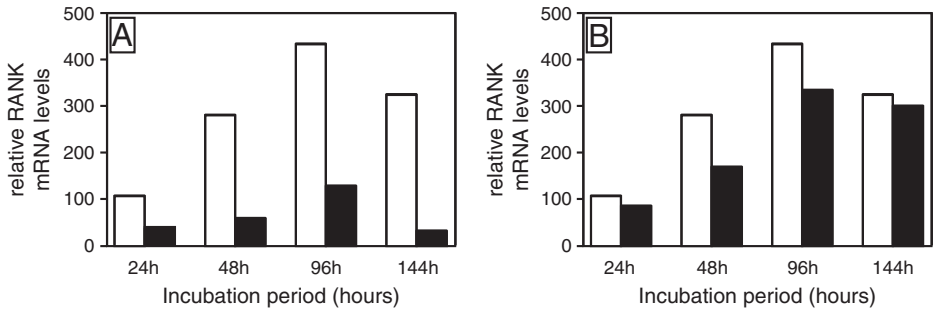

Fig. 6. Levels of transcripts encoding RANK are suppressed in OPC cultures with *wt CM*. To verify the effect of CM on the expression of RANK mRNA in OPC, CM from *wt* and *GM-CSF*$^{-/-}$ osteoblasts treated with TNFα (5 ng/ml) and 1,25$(OH)_2D_3$ (10 nM) was added to cultures of *wt* OPC supplemented with RANKL (5 ng/ml) and M-CSF (30 ng/ml). RANK mRNA levels were determined in cultures with *wt* CM (A) and *GM-CSF*$^{-/-}$ CM (B) by real-time PCR. White bars represent culture with control CM and black bars cultures with CM from TNFα/1,25$(OH)_2D_3$ treated osteoblasts. RANK mRNA levels were decreased by 60%, 75%, 65% and 90% in cultures with *wt* CM (A) and by 10%, 35% and 20% in cultures with CM from *GM-CSF*$^{-/-}$ cells (B) after 24 h, 48 h and 96 h, respectively. After 6 days, no differences between treated and control were observed in cultures of OPC supplemented with CM from *GM-CSF*$^{-/-}$ cells (B).

were grown with CM from TNFα/1,25$(OH)_2D_3$ treated *GM-CSF*$^{-/-}$ osteoblasts, the detected decrease in RANK mRNA levels was reduced as compared to *wt* CM (Fig. 6B). Using these experimental protocols, levels of transcripts encoding *c-Fms* remained unchanged (Supplementary Fig. 2).

To characterize the expression of RANK protein, OPC were analyzed by flow cytometry after 4 days of culture (Fig. 7). RANK was expressed by OPC grown with M-CSF/RANKL (Fig. 7B), and, marginally in OPC grown in cultures supplemented with M-CSF/RANKL/rGM-CSF (Fig. 7C), No expression of RANK by OPC was detected in cultures with M-CSF (Fig. 7A) only and with M-CSF/RANKL/rGM-CSF/IL-4 (Fig. 7D). In cultures supplemented with CM, RANK was induced when control CM from *wt* and *GM-CSF*$^{-/-}$ osteoblasts were added to OPC (Figs. 7E, G). However, while OPC grown in medium with CM from *GM-CSF*$^{-/-}$ osteoblasts treated with TNFα/1,25$(OH)_2D_3$ did express surface RANK (Fig. 7H), receptor expression was completely blocked with CM from treated *wt* osteoblasts (Fig. 7F).

CM from TNFα treated osteoblasts support maintenance of a macrophage/monocyte phenotype

To characterize the differentiation of hematopoietic progenitors in the presence of CM, expression of CD11c (Figs. 8C, D, G, H), *c-Kit* (not shown), *c-Fms* (Supplementary Fig. 3) and *F4/80* (Figs. 8A, B, E, F) by OPC were analyzed by flow cytometry after 4 days of culture. In these cultures, no cells positive for *c-Kit* could be observed, the expression of the antigen being lost within 24 h of the culture period (not shown). Expression of *c-Fms* and F4/80 were very similar, a major portion of the cells staining positive for these markers, independently, whether the media were supplemented with growth factors

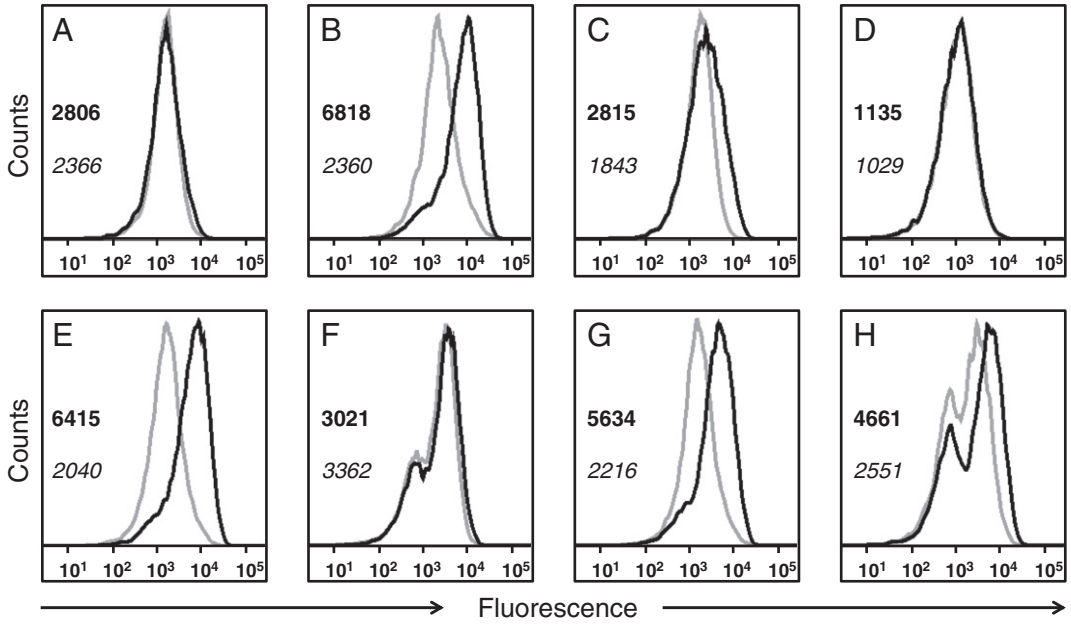

Fig. 7. RANK protein expression by OPC is suppressed in the presence of CM from *wt* osteoblasts. To assess the effects of CM on the expression of RANK, OPC were grown with CM [from *wt* and *GM-CSF*$^{-/-}$ osteoblasts treated with TNFα (5 ng/ml) and 1,25$(OH)_2D_3$ (10 nM) for 72 h], in media supplemented with M-CSF (30 ng/ml)/RANKL (20 ng/ml), or were grown with M-CSF (30 ng/ml)/RANKL (20 ng/ml)/rGM-CSF (50 pg/ml)/IL-4 (500 pg/ml). Culture conditions were: M-CSF alone (A), M-CSF/RANKL (B), M-CSF/RANKL/GM-CSF (C), M-CSF/RANKL/GM-CSF/IL-4 (D), CM from non-treated (control) *wt* osteoblasts CM (E), CM from treated *wt* osteoblasts (F), and CM from control (G) and treated (H) *GM-CSF*$^{-/-}$ osteoblasts. The values of the mean fluorescence intensity (MFI) are given in each graph, bold numbers for anti-RANK antibodies and *italics* for the isotype controls. RANK was induced in cultures with M-CSF/RANKL (B). In cultures with CM, expression of RANK was induced in cultures supplemented with control CM (E and G), and with CM from *GM-CSF*$^{-/-}$ osteoblasts (H). Expression of RANK was not detected in OPC cultures grown in media supplemented with CM from treated *wt* osteoblasts (F).

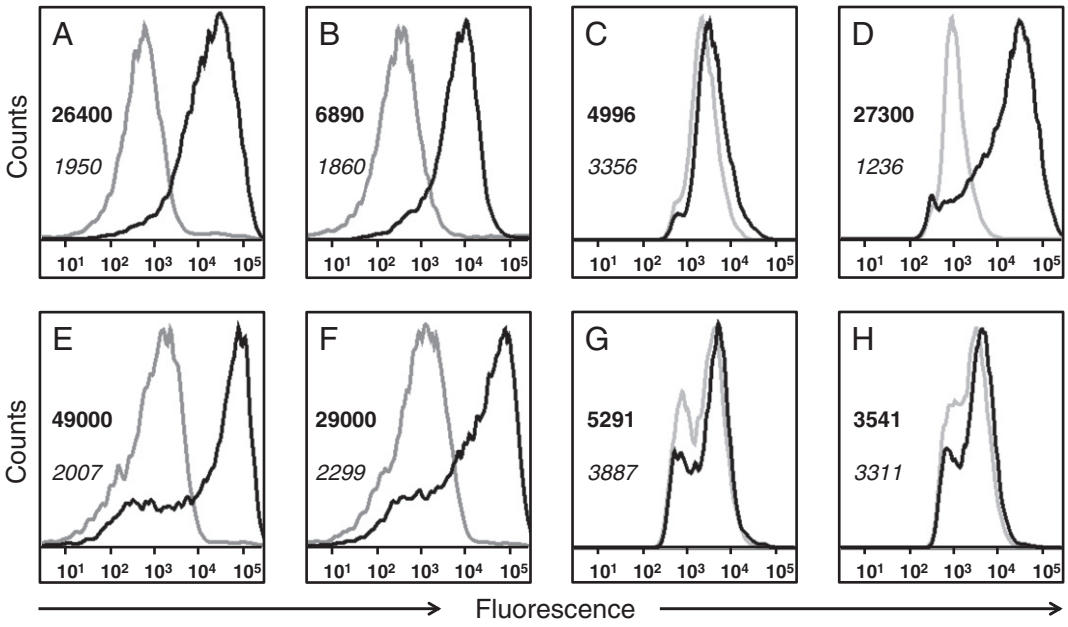

Fig. 8. OPC do not differentiate to dendritic cells in the presence of *CM*. To assess the fate of the OPC grown with CM from osteoblasts treated with TNFα/1,25$(OH)_2D_3$, the expression of F4/80 (A, B, E, F), as a pan-monocyte marker, and of CD11c (C, D, G, H) as a marker for dendritic cells were determined by flow cytometry. OPC were grown with M-CSF/RANKL (A, C), M-CSF/RANKL/GM-CSF/IL-4 (B, D), and with CM from *wt* (E, G) and *GM-CSF*$^{-/-}$ (F, H) osteoblasts exposed to TNFα/1,25$(OH)_2D_3$. Expression of F4/80 was high in each of the culture conditions. In cultures of OPC supplemented with M-CSF/RANKL/GM-CSF/IL-4, expression of CD11c was detected (D), a process that was not observed with CM either from *wt* (G) or from *GM-CSF*$^{-/-}$ (H) osteoblasts. The values of the mean fluorescence intensity (MFI) are given in each graph, bold numbers for the specific antibodies and *italics* for the isotype controls.

(Figs. 8A, B) or with CM from *wt* (Fig. 8E) or *GM-CSF*$^{-/-}$ (Fig. 8F) osteoblasts. The dendritic cell surface marker CD11c was only induced in cultures treated with M-CSF/RANKL/rGM-CSF/IL-4 (Fig. 8D). No induction was observed with M-CSF, M-CSF/RANKL (Fig. 8C) or in cultures supplemented with CM from *wt* (Fig. 8G) or *GM-CSF*$^{-/-}$ (Fig. 8H) osteoblasts treated with TNFα/1,25$(OH)_2D_3$.

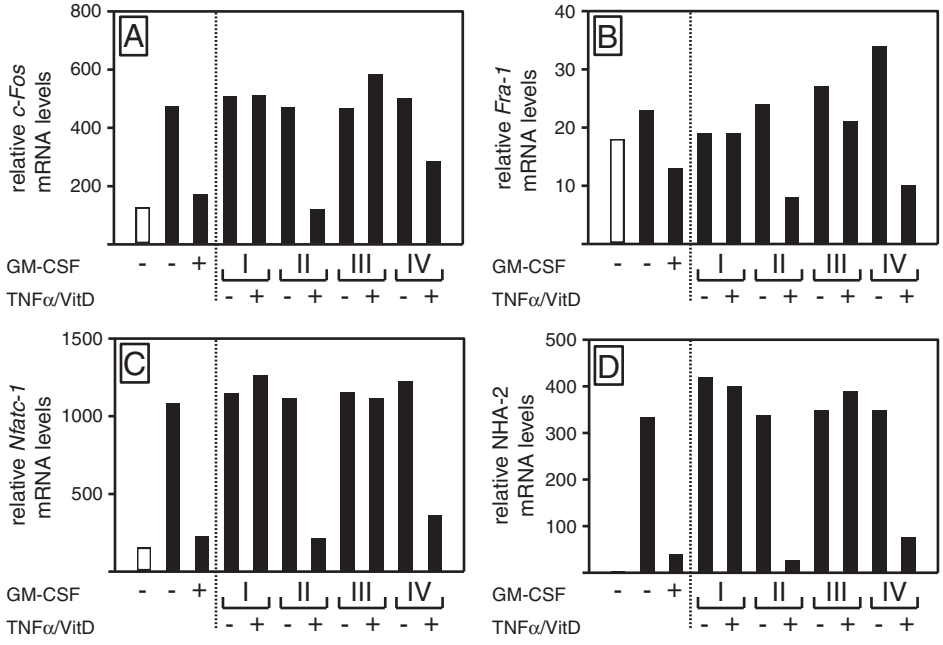

Fig. 9. Suppression of *c-Fos* and *Fra-1* is involved in the inhibition of osteoclastogenesis. To assess the mechanisms by which CM from *wt* osteoblasts treated with TNFα/1,25$(OH)_2D_3$ mediate the inhibition of OPC differentiation, levels of transcripts encoding *c-Fos* (A), *Fra1* (B), *NFATc1* (C), and NHA2 (D) were quantified after growing the cells for 48 h with M-CSF (30 ng/ml)/RANKL (5 ng/ml) [white bar: M-CSF only] and GM-CSF (10 pg/ml) or with CM from primary osteoblasts ± TNFα/1,25$(OH)_2D_3$ [(I) no cells; (II) C57Bl/6 J *wt* osteoblasts (the concentration of GM-CSF in the CM was determined beforehand and CM was added to a final concentration of GM-CSF of 10 pg/ml. The same concentration of CM was used for control CM and those from the other osteoblast populations); (III) *p55TNFR*$^{-/-}$/*p75TNFR*$^{-/-}$ osteoblasts; (IV) *GM-CSF*$^{-/-}$ osteoblasts]. *c-Fos* transcript levels were found to be decreased by GM-CSF, *wt* CM and to a lesser extent *GM-CSF*$^{-/-}$ CM (A), while levels of transcripts encoding *Fra1* were not affected by GM-CSF and no difference was observed for CM from *wt* and *GM-CSF*$^{-/-}$ cells, respectively (B). *NFATc1* mRNA was downregulated by GM-CSF and by CM from *wt* and *GM-CSF*$^{-/-}$ osteoblasts (C), and the detection of mRNA encoding the osteoclast product NHA2 was dependent on the development of osteoclasts. CM from *p55TNFR*$^{-/-}$/*p75TNFR*$^{-/-}$ did not affect the expression of any of these transcripts.

GM-CSF and CM from osteoblasts suppress c-Fos and Fra-1 transcripts in OPC

Previously, the inhibitory effects of GM-CSF on the development of osteoclasts in vitro were attributed to the suppression of *c-Fos* and *Fra-1* in OPC upon exposure to the growth factor [39]. Levels of transcripts encoding *c-Fos*, *Fra-1*, *Nfatc-1*, and a sodium/hydrogen exchanger highly expressed in mature osteoclasts, NHA-2, were quantified in OPC after a culture period of 2 days (Fig. 9). Levels of transcripts encoding *c-Fos* were increased by the treatment of OPC with M-CSF/RANKL as compared to M-CSF alone, and further addition of GM-CSF prevented this increase in transcript levels. CM from *wt* osteoblasts treated with TNFα/1,25$(OH)_2D_3$ caused a decrease in *c-Fos* transcripts by ca. 75%, and a smaller decrease was found in OPC exposed to CM from treated *GM-CSF*$^{-/-}$ cells (Fig. 9A). Medium alone or CM from control cells or *p55TNR*$^{-/-}$/*p75TNFR*$^{-/-}$ osteoblasts did not affect transcript levels. Similar results were obtained for *Fra-1* (Fig. 9B) with the exception that transcript levels were not dependent on the presence of RANKL and suppression by GM-CSF was less pronounced. Culture of the cells in media supplemented with CM from treated *wt* and *GM-CSF*$^{-/-}$ osteoblasts, however, caused a similar decrease of approx. 60%. *Nfatc-1* mRNA levels paralleled the mRNA levels of *c-Fos*, the RANKL induced increase was prevented by GM-CSF and the effects of CM from treated *wt* osteoblasts were more pronounced than were those of CM from *GM-CSF*$^{-/-}$ cells (Fig. 9C). Lastly, levels of transcripts encoding the osteoclast product NHA-2 again followed the levels of transcripts encoding *Nfatc-1*, with an efficient decrease by GM-CSF and CM from *wt* osteoblasts and CM from *GM-CSF*$^{-/-}$ osteoblasts allowing for a partial recovery (Fig. 9D).

TNFα affects osteoblast viability and release of GM-CSF by osteoblasts

Primary murine osteoblasts were cultured in the presence of TNFα and 1,25$(OH)_2D_3$ for up to 6 days. Viable cells were determined using an XTT assay and the release of GM-CSF was quantified by ELISA. In the absence of 1,25$(OH)_2D_3$, the number of viable cells after 3 days was not affected by TNFα concentrations up to 10 ng/ml and decreased at 30 ng/ml (Fig. 10A), while at TNFα concentrations of 10 ng/ml and 30 ng/ml a decrease of more than 50% was observed in the presence of 1,25$(OH)_2D_3$. The release of GM-CSF was found to be stimulated by increasing doses of TNFα with or without 1,25$(OH)_2D_3$ (Fig. 10B). After a culture time of 6 days, increasing doses of TNFα in the presence of 1,25$(OH)_2D_3$ caused a decrease in cell viability (Fig. 10C), but little effects only were found in the absence of 1,25$(OH)_2D_3$. GM-CSF release in the presence of 1,25$(OH)_2D_3$ reached a maximum at TNFα concentrations of 3 ng/ml, and declined with higher doses (Fig. 10D) whereas in the absence of 1,25$(OH)_2D_3$, the release of GM-CSF increased with increasing doses of TNFα.

Discussion

In the present study, we report that osteoblast-derived GM-CSF functions as a mediator in the previously described [38] TNFα-dependent inhibition of osteoclast formation. The data indicates that the GM-CSF suppressed upregulation of *c-Fos* and *Nfatc-1* in OPC in response to RANKL leads to a decrease in RANK expression, which may be the mechanism causing the block in osteoclastogenesis. The failure of OPC to express specific cell lineage markers characteristic for dendritic cells and osteoclasts suggests that they are maintained as monocytes in a multipotential precursor state rather than being directed towards an alternative differentiation pathway. In the previous study, experiments using inactivating antibodies, it was concluded that GM-CSF might not play a major role in the TNFα/1,25$(OH)_2D_3$-mediated inhibition of osteoclastogenesis. The experiments presented in this study, employing primary osteoblasts from *GM-CSF*$^{-/-}$ mice, however, clearly demonstrate an involvement of GM-CSF in this effect.

Osteoclastogenesis is a multistep process, guided by numerous growth factors and cytokines. Several lines of evidence support a malregulation of osteoclast development and activity as the underlying cause for the occurrence of bone erosions in inflammatory diseases such as rheumatoid arthritis or inflammatory bowel diseases, and in animal models of arthritis. In these pathologies, the inflammatory

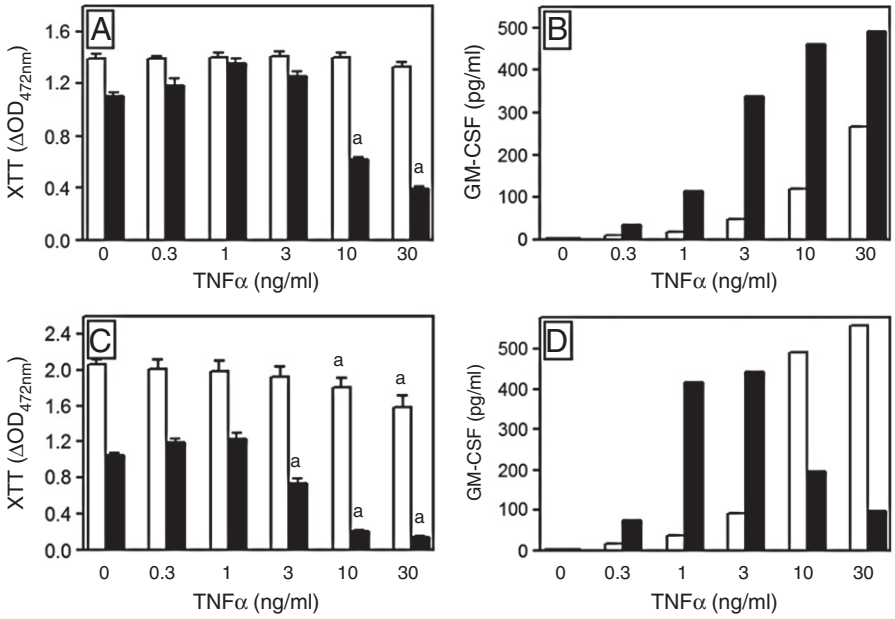

Fig. 10. Effects of TNFα treatment on primary osteoblasts. To assess the effects of TNFα on osteoblasts, the cells were exposed to increasing concentrations of TNFα (0, 0.3, 1, 10, and 30 ng/ml) with (black bars) or without (white bars) 1,25$(OH)_2D_3$. After 3 (A, B) and 6 (C, D) days, cell viability (A, C) and the release of GM-CSF (B, D) were determined. After 3 days, at high concentrations of TNFα (≥10 ng/ml) and in the presence of 1,25$(OH)_2D_3$, the number of viable cells was decreased by more than 50% (A) while GM-CSF protein increased with increasing concentrations of TNFα (B). After 6 days, cell proliferation was decreased by 40% at low concentrations of TNFα (≤1 ng/ml) and by more than 80% at TNFα levels ≥10 ng/ml (C). GM-CSF levels increased in dependence of the dose of TNFα in the absence of 1,25$(OH)_2D_3$, while in the presence of the hormone, a decrease in GM-CSF levels was observed at TNFα≥10 ng/ml (D). Bars represent the average ± SD of 6 wells from one representative experiment. (a) $p<0.0001$; significant decrease compared to respective controls without TNFα.

environments are characterized by increased levels of growth factors that stimulate osteoclast formation, activity and/or survival [40–45]. These factors, including M-CSF, TNFα, IL-1, and RANKL, act either directly on osteoclast precursor cells, or on accessory cells, as do IL-1, TNFα, IL-11, IL-15, IL-17, PTHrP, which in turn increase the expression of RANKL.

TNFα acts as a link between inflammatory processes and osteoclastogenesis. Its pro-osteoclastic effects were found to be mediated mainly by two mechanisms, namely by an indirect mechanism in which TNFα induces stromal cells to express RANKL, M-CSF and IL-1 and by a direct mechanism, in which TNFα synergizes with RANKL to enhance osteoclast formation from progenitor cells, both mechanisms contributing to the formation of bone erosions [46]. In the past, the induction of inhibitors of osteoclast formation by TNFα, such as OPG and GM-CSF [47] has been described. The hematopoietic growth factor GM-CSF was initially characterized for its ability to generate colonies of granulocytes and macrophages in vitro [48]. Endogenous levels of GM-CSF under physiological conditions are undetectable but higher levels have been registered at sites of inflammation in inflammatory arthritis and in lung tissue of mice following administration of lipopolysaccharide (LPS).

The role of TNFα as an inducer of GM-CSF had been investigated in detail in cells and tissues of human origin. Recombinant human TNFα was found to induce GM-CSF expression in normal lung fibroblasts and vascular endothelial cells [47,49,50]. Feldmann et al. demonstrated GM-CSF to be present in cultures of synovial cells from RA and OA patients. Furthermore, the release of GM-CSF was suppressed by the treatment of these cell cultures with neutralizing anti-TNFα antibodies [51,52]. Although human osteosarcoma cell lines MG-63 and SaOS-2 were found to release GM-CSF constitutively [53,54], little had been reported on the expression of GM-CSF by primary osteoblasts and stromal cells. Here it is shown that GM-CSF is induced in cultures of primary murine osteoblasts treated with TNFα in vitro and that this induction was increased by $1,25(OH)_2D_3$. It is also shown that TNFα, at concentrations up to 30 ng/ml, did not affect osteoblast viability and activity in the absence of $1,25(OH)_2D_3$, while in the presence of the hormone, increasing doses of TNFα caused a decrease in the viability and activity of osteoblasts, and in their ability to support osteoclastogenesis.

NF-κB binding sites [55,56] and vitamin D responsive elements (VDRE) [57] have been identified in the GM-CSF promoter region. The downstream activation of NF-κB by TNFα and its subsequent binding to the GM-CSF promoter has been shown to be important in the expression of the protein in activated T-lymphocytes. The downregulation of GM-CSF by Dex, which has been reported to inhibit NF-κB signaling, illustrates the importance of this transcription factor in GM-CSF expression. VDREs on the GM-CSF promoter were reported to have a suppressive effect on GM-CSF expression in T-lymphocytes and peripheral blood mononuclear cells (PBMC). However, in the present study, $1,25(OH)_2D_3$ was found to synergize with TNFα in the upregulation of the release of GM-CSF by osteoblasts. The partial recovery of osteoclastogenesis in cultures of OPC grown with CM from *GM-CSF*$^{-/-}$ osteoblasts, as compared to CM from *wt* cells, demonstrates the contribution of GM-CSF in the inhibition to osteoclast formation. Similar results were obtained in co-culture experiments and at low concentrations of TNFα (<10 ng/ml). At TNFα concentrations exceeding 10 ng/ml, osteoclastogenesis was governed by two processes. Firstly, the inhibitory effects of TNFα on osteoblast function, leading not only to a decrease in GM-CSF levels but also in the ability of the osteoblasts to support osteoclast formation through the synthesis of RANKL and M-CSF and, secondly, the direct stimulation of the osteoclast precursors by TNFα, which was not observed in cultures with OPC deficient in TNF receptors. Nevertheless, although it is shown herein that GM-CSF contributes to the inhibition of osteoclastogenesis, the data indicates that the growth factor did not account for the full inhibition. Furthermore, given the low levels of TNFα present in physiological condition, GM-CSF may not be induced and its inhibitory effect on osteoclast development may not be relevant in normal bone physiology. At inflammatory sites and in circulation, with elevated levels of TNFα, however, the released of GM-CSF may cause an increase in the pool of osteoclast progenitor cells and thus affect the balance between bone formation and bone resorption.

Mechanisms inhibiting osteoclast are numerous and they mainly affect RANKL–RANK signaling and to a lesser extent M-CSF signaling. RANKL was found to be a major regulator of osteoclast development and activity and the biological efficacy of the factor is modulated by the ratio between RANKL and its decoy receptor OPG [18]. Deficiency in either RANKL or RANK leads to severe osteopetrosis [58] due to a failure to form osteoclasts. Pro-inflammatory and pro-osteolytic factors known to shift the ratio of RANKL:OPG, including IL-1α, IL-6 and IL-11, have been investigated in this study. These factors were found to be induced by TNFα in osteoblasts. Furthermore, levels of RANKL were found to be increased in the presence of $1,25(OH)_2D_3$ and were decreased only at high TNFα concentrations. Collectively, no evidence pointing to a deregulation or limitation of RANKL levels, or a decrease in other pro-osteoclastogenic growth factors, was found to be associated with the observed inhibition. However, levels of transcripts encoding RANK were decreased in cultures of OPC grown with CM from *wt* osteoblasts during the entire culture period. This downregulation was reduced in cultures supplemented with CM from *GM-CSF*$^{-/-}$ osteoblasts, suggesting the decrease in RANK expression as one of the mechanisms by which osteoclast formation is inhibited. In agreement with the observed downregulation of RANK, osteoclastogenesis was inhibited only after the addition of *wt* CM during the early phase (0–72 h) of the cultures. Addition of *wt* CM after 72 h did no longer affect osteoclast formation. Expression of *c-Fms* both at the transcript and the protein levels was not affected by *wt* CM and increased continuously during the culture, irrespective of the treatment protocols. To further elucidate the molecular mechanisms of the TNFα/$1,25(OH)_2D_3$-dependent osteoblast-mediated inhibition of osteoclastogenesis, the regulation of transcripts encoding proteins that were previously shown to be critical for the differentiation of OPC, and were demonstrated to be strongly suppressed by GM-CSF [39], were investigated. Deficiency in *c-Fos* [59] was previously demonstrated to lead to osteopetrosis. The phenotype could be overcome in knock-in mice, expressing *Fra-1* in place of *c-Fos* [60]. Another transcription factor, whose expression is modulated by RANK/RANKL signaling [61] and whose deficiency leads to osteopetrosis is *Nfatc-1* [62]. In the present study, levels of transcripts encoding *c-Fos* were greatly enhanced by the exposure of OPC to RANKL, an upregulation that was fully blunted by GM-CSF and CM from TNFα/$1,25(OH)_2D_3$-treated *wt* osteoblasts. If cultured with CM from *GM-CSF*$^{-/-}$ osteoblasts, the reduction in *c-Fos* mRNA was less pronounced, which may result in levels of AP-1 permissive for osteoclastogenesis. *Fra-1* transcript levels on the other hand were found not to be dependent on the growth of OPC in the presence of RANKL and only to a small extent on GM-CSF, and as a consequence, no difference was detected in transcript levels of OPC cultured with CM from *wt* and *GM-CSF*$^{-/-}$ cells. The residual levels of *c-Fos* may allow for a partial recovery of the expression of RANK, which is paralleled by the partial restoration of the mRNA levels encoding *Nfatc-1* and the osteoclastic sodium/hydrogen transporter NHA2 [63]. Thus, the CM from *GM-CSF*$^{-/-}$ may be less efficient in the inhibition of osteoclast development due to its reduced inhibitory potential on the expression of *c-Fos*, while the GM-CSF-independent inhibition of *Fra-1* is still maintained, preventing a full recovery of osteoclastogenesis.

Monocyte/macrophage lineage cells are still multipotential with respect to their differentiation and serve as precursors for osteoclasts, macrophages and dendritic cells, terminal differentiation being regulated by the repertoire of factors and cytokines in the local environment. The differentiation pathway can be characterized by the expression of cell lineage specific surface markers. In the present study the expression of marker molecules, including *c-Kit* (hematopoietic stem cells, early

precursors), CD11c (dendritic cells), *F4/80* (monocytes), *c-Fms* (monocytes/macrophages), and RANK (macrophages/osteoclasts), was analyzed in cultures of OPC treated with CM and rGM-CSF by flow cytometry. *c-Kit* was lost in the early phase, within 1 day, of the culture, demonstrating that cell differentiation was not blocked at the level of early hematopoietic precursors. While monocyte markers including F4/80 and *c-Fms* expression increased and remained high during the whole culture period for all culture conditions, expression of the dendritic and osteoclast markers CD11c and RANK was not induced in control cultures with M-CSF only. CD11c was induced in cultures with GM-CSF and the failure of CM containing equivalent amount of GM-CSF to induce the expression of CD11c demonstrates the capacity of CM to block GM-CSF driven dendritic cell differentiation. The increase in RANK protein expression, albeit reduced when compared to M-CSF/RANKL cultures, in the presence of CM from *GM-CSF*$^{-/-}$ osteoblasts treated with TNFα/1,25 $(OH)_2D_3$, again suggests a major role for the growth factor in the prevention of RANK expression.

Levels of GM-CSF were reported to be elevated in synovial fluid under inflammatory conditions such as rheumatoid arthritis and yet, osteoclastic activity is severely enhanced, suggesting a contradiction with the data presented herein. During inflammation, however, numerous pro-osteoclastic cytokines, including TNFα, are highly expressed and the total effects may drive osteoclast formation and activity and as a consequence bone destruction. Based on the present results, we postulate a model in which GM-CSF acts on osteoclast lineage cells in separate physiological compartments. In circulation, the growth factor may act as a proliferation factor for monocyte lineage cells, increasing the pool of potential osteoclast progenitors. Upon migration to the bone/bone marrow compartment [64], which will be the focus of future studies, the progenitors are exposed to a local osteoclastogenic environment, giving rise to osteoclasts leading subsequently to an increase in bone resorption and the observed osteolytic lesions frequently associated with inflammatory diseases.

Conclusion

In summary, the present data demonstrates that GM-CSF is playing a part in the TNFα-dependent inhibition of osteoclastogenesis, which is mediated by osteoblast lineage cells. The repression of RANK expression demonstrates that the prevention of RANK–RANKL signaling is responsible for the inhibition of osteoclastogenesis by TNFα in vitro. The failure in the development of GM-CSF-dependent $CD11c^+$ dendritic cell with *wt* CM suggest that these osteoclast precursors are not deviated to an alternative differentiation pathway but rather are maintained in a multipotential monocytic state.

Supplementary materials related to this article can be found online at doi:10.1016/j.bone.2011.08.003.

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References

[1] Boyce BF, Li P, Yao Z, Zhang Q, Badell IR, Schwarz EM, et al. TNF-alpha and pathologic bone resorption. Keio J Med 2005;54:127–31.
[2] Lee SH, Kim TS, Choi Y, Lorenzo J. Osteoimmunology: cytokines and the skeletal system. BMB Rep 2008;41:495–510.
[3] Wakkach A, Mansour A, Dacquin R, Coste E, Jurdic P, Carle GF, et al. Bone marrow microenvironment controls the in vivo differentiation of murine dendritic cells into osteoclasts. Blood 2008;112:5074–83.
[4] Ochi S, Shinohara M, Sato K, Gober HJ, Koga T, Kodama T, et al. Pathological role of osteoclast costimulation in arthritis-induced bone loss. Proc Natl Acad Sci U S A 2007;104:11394–9.
[5] Schett G. Osteoimmunology in rheumatic diseases. Arthritis Res Ther 2009;11:210.
[6] Schett G, Teitelbaum SL. Osteoclasts and arthritis. J Bone Miner Res 2009;24:1142–6.
[7] Li X, Yuan FL, Lu WG, Zhao YQ, Li CW, Li JP, et al. The role of interleukin-17 in mediating joint destruction in rheumatoid arthritis. Biochem Biophys Res Commun 2010;397:131–5.
[8] Yamashita A, Nishikawa S, Rancourt DE. Microenvironment modulates osteogenic cell lineage commitment in differentiated embryonic stem cells. PLoS One 2010;5:e9663.
[9] Yuan FL, Li X, Lu WG, Xu RS, Zhao YQ, Li CW, et al. Regulatory T cells as a potent target for controlling bone loss. Biochem Biophys Res Commun 2010;402:173–6.
[10] Feldmann M, Maini RN, Bondeson J, Taylor P, Foxwell BM, Brennan FM. Cytokine blockade in rheumatoid arthritis. Adv Exp Med Biol 2001;490:119–27.
[11] Moreland LW. Cytokines as targets for anti-inflammatory agents. Ann N Y Acad Sci 2009;1182:88–96.
[12] Plushner SL. Tocilizumab: an interleukin-6 receptor inhibitor for the treatment of rheumatoid arthritis. Ann Pharmacother 2008;42:1660–8.
[13] Woodrick R, Ruderman EM. Anti-interleukin-6 therapy in rheumatoid arthritis. Bull NYU Hosp Jt Dis 2010;68:211–7.
[14] Feldmann M, Maini RN. Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? Annu Rev Immunol 2001;19:163–96.
[15] Maini RN. Anti-TNF therapy from the bench to the clinic: a paradigm of translational research. Clin Med 2010;10:161–2.
[16] McCluggage LK, Scholtz JM. Golimumab: a tumor necrosis factor alpha inhibitor for the treatment of rheumatoid arthritis. Ann Pharmacother 2010;44:135–44.
[17] Shinohara M, Takayanagi H. Novel osteoclast signaling mechanisms. Curr Osteoporos Rep 2007;5:67–72.
[18] Aeschlimann D, Evans BA. The vital osteoclast: how is it regulated? Cell Death Differ 2004;11(Suppl 1):S5–7.
[19] Teitelbaum SL. Osteoclasts: what do they do and how do they do it? Am J Pathol 2007;170:427–35.
[20] Boyce BF, Yamashita T, Yao Z, Zhang Q, Li F, Xing L. Roles for NF-kappaB and c-Fos in osteoclasts. J Bone Miner Metab 2005;23(Suppl):11–5.
[21] Xing L, Schwarz EM, Boyce BF. Osteoclast precursors, RANKL/RANK, and immunology. Immunol Rev 2005;208:19–29.
[22] Wada T, Nakashima T, Hiroshi N, Penninger JM. RANKL–RANK signaling in osteoclastogenesis and bone disease. Trends Mol Med 2006;12:17–25.
[23] Chakravarti A, Raquil MA, Tessier P, Poubelle PE. Surface RANKL of Toll-like receptor 4-stimulated human neutrophils activates osteoclastic bone resorption. Blood 2009;114:1633–44.
[24] Goldring SR. Bone loss in chronic inflammatory conditions. J Musculoskelet Neuronal Interact 2003;3:287–9 [discussion 292-4].
[25] Goldring SR. Pathogenesis of bone and cartilage destruction in rheumatoid arthritis. Rheumatology (Oxford) 2003;42(Suppl 2):ii11–6.
[26] Choi Y, Woo KM, Ko SH, Lee YJ, Park SJ, Kim HM, et al. Osteoclastogenesis is enhanced by activated B cells but suppressed by activated CD8(+) T cells. Eur J Immunol 2001;31:2179–88.
[27] Manabe N, Kawaguchi H, Chikuda H, Miyaura C, Inada M, Nagai R, et al. Connection between B lymphocyte and osteoclast differentiation pathways. J Immunol 2001;167:2625–31.
[28] Kawai T, Matsuyama T, Hosokawa Y, Makihira S, Seki M, Karimbux NY, et al. B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease. Am J Pathol 2006;169:987–98.
[29] Soderstrom K, Stein E, Colmenero P, Purath U, Muller-Ladner U, de Matos CT, et al. Natural killer cells trigger osteoclastogenesis and bone destruction in arthritis. Proc Natl Acad Sci U S A 2010;107:13028–33.
[30] Quinn JM, Gillespie MT. Modulation of osteoclast formation. Biochem Biophys Res Commun 2005;328:739–45.
[31] Inoue M, Ross FP, Erdmann JM, Abu-Amer Y, Wei S, Teitelbaum SL. Tumor necrosis factor alpha regulates alpha(v)beta5 integrin expression by osteoclast precursors in vitro and in vivo. Endocrinology 2000;141:284–90.
[32] Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, et al. Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL–RANK interaction. J Exp Med 2000;191:275–86.
[33] Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL. TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. J Clin Invest 2000;106:1481–8.
[34] Zhang Q, Badell IR, Schwarz EM, Boulukos KE, Yao Z, Boyce BF, et al. Tumor necrosis factor prevents alendronate-induced osteoclast apoptosis in vivo by stimulating Bcl-xL expression through Ets-2. Arthritis Rheum 2005;52:2708–18.
[35] Benayahu D, Gurevitch O, Zipori D, Wientroub S. Bone formation by marrow osteogenic cells (MBA-15) is not accompanied by osteoclastogenesis and generation of hematopoietic supportive microenvironment. J Bone Miner Res 1994;9:1107–14.
[36] Brandstrom H, Jonsson KB, Vidal O, Ljunghall S, Ohlsson C, Ljunggren O. Tumor necrosis factor-alpha and -beta upregulate the levels of osteoprotegerin mRNA in human osteosarcoma MG-63 cells. Biochem Biophys Res Commun 1998;248:454–7.
[37] Kubota A, Hasegawa K, Suguro T, Koshihara Y. Tumor necrosis factor-alpha promotes the expression of osteoprotegerin in rheumatoid synovial fibroblasts. J Rheumatol 2004;31:426–35.
[38] Balga R, Wetterwald A, Portenier J, Dolder S, Mueller C, Hofstetter W. Tumor necrosis factor-alpha: alternative role as an inhibitor of osteoclast formation in vitro. Bone 2006;39:325–35.
[39] Miyamoto T, Ohneda O, Arai F, Iwamoto K, Okada S, Takagi K, et al. Bifurcation of osteoclasts and dendritic cells from common progenitors. Blood 2001;98:2544–54.

[40] Bluml S, Binder NB, Niederreiter B, Polzer K, Hayer S, Tauber S, et al. Antiinflammatory effects of tumor necrosis factor on hematopoietic cells in a murine model of erosive arthritis. Arthritis Rheum 2010;62:1608–19.
[41] Polzer K, Joosten L, Gasser J, Distler JH, Ruiz G, Baum W, et al. Interleukin-1 is essential for systemic inflammatory bone loss. Ann Rheum Dis 2010;69:284–90.
[42] Herman S, Muller RB, Kronke G, Zwerina J, Redlich K, Hueber AJ, et al. Induction of osteoclast-associated receptor, a key osteoclast costimulation molecule, in rheumatoid arthritis. Arthritis Rheum 2008;58:3041–50.
[43] Zwerina J, Redlich K, Polzer K, Joosten L, Kronke G, Distler J, et al. TNF-induced structural joint damage is mediated by IL-1. Proc Natl Acad Sci U S A 2007;104:11742–7.
[44] Redlich K, Hayer S, Ricci R, David JP, Tohidast-Akrad M, Kollias G, et al. Osteoclasts are essential for TNF-alpha-mediated joint destruction. J Clin Invest 2002;110:1419–27.
[45] Redlich K, Hayer S, Maier A, Dunstan CR, Tohidast-Akrad M, Lang S, et al. Tumor necrosis factor alpha-mediated joint destruction is inhibited by targeting osteoclasts with osteoprotegerin. Arthritis Rheum 2002;46:785–92.
[46] Lacativa PG, Farias ML. Osteoporosis and inflammation. Arq Bras Endocrinol Metabol 2010;54:123–32.
[47] Koeffler HP, Gasson J, Ranyard J, Souza L, Shepard M, Munker R. Recombinant human TNF alpha stimulates production of granulocyte colony-stimulating factor. Blood 1987;70:55–9.
[48] Burgess AW, Metcalf D. The nature and action of granulocyte-macrophage colony stimulating factors. Blood 1980;56:947–58.
[49] Munker R, DiPersio J, Koeffler HP. Tumor necrosis factor: receptors on hematopoietic cells. Blood 1987;70:1730–4.
[50] Munker R, Gasson J, Ogawa M, Koeffler HP. Recombinant human TNF induces production of granulocyte–monocyte colony-stimulating factor. Nature 1986;323:79–82.
[51] Haworth C, Brennan FM, Chantry D, Turner M, Maini RN, Feldmann M. Expression of granulocyte-macrophage colony-stimulating factor in rheumatoid arthritis: regulation by tumor necrosis factor-alpha. Eur J Immunol 1991;21:2575–9.
[52] Alsalameh S, Firestein GS, Oez S, Kurrle R, Kalden JR, Burmester GR. Regulation of granulocyte macrophage colony stimulating factor production by human articular chondrocytes. Induction by both tumor necrosis factor-alpha and interleukin 1, downregulation by transforming growth factor beta and upregulation by fibroblast growth factor. J Rheumatol 1994;21:993–1002.
[53] Taichman RS, Emerson SG. Human osteosarcoma cell lines MG-63 and SaOS-2 produce G-CSF and GM-CSF: identification and partial characterization of cell-associated isoforms. Exp Hematol 1996;24:509–17.
[54] Taichman RS, Emerson SG. Human osteoblasts support hematopoiesis through the production of granulocyte colony-stimulating factor. J Exp Med 1994;179:1677–82.
[55] Schreck R, Baeuerle PA. NF-kappa B as inducible transcriptional activator of the granulocyte-macrophage colony-stimulating factor gene. Mol Cell Biol 1990;10:1281–6.
[56] Brettingham-Moore KH, Rao S, Juelich T, Shannon MF, Holloway AF. GM-CSF promoter chromatin remodelling and gene transcription display distinct signal and transcription factor requirements. Nucleic Acids Res 2005;33:225–34.
[57] Towers TL, Freedman LP. Granulocyte-macrophage colony-stimulating factor gene transcription is directly repressed by the vitamin D3 receptor. Implications for allosteric influences on nuclear receptor structure and function by a DNA element. J Biol Chem 1998;273:10338–48.
[58] Li J, Sarosi I, Yan XQ, Morony S, Capparelli C, Tan HL, et al. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. Proc Natl Acad Sci U S A 2000;97:1566–71.
[59] Grigoriadis AE, Wang ZQ, Cecchini MG, Hofstetter W, Felix R, Fleisch HA, et al. c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. Science 1994;266:443–8.
[60] Fleischmann A, Hafezi F, Elliott C, Reme CE, Ruther U, Wagner EF. Fra-1 replaces c-Fos-dependent functions in mice. Genes Dev 2000;14:2695–700.
[61] Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, et al. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cell 2002;3:889–901.
[62] Asagiri M, Sato K, Usami T, Ochi S, Nishina H, Yoshida H, et al. Autoamplification of NFATc1 expression determines its essential role in bone homeostasis. J Exp Med 2005;202:1261–9.
[63] Hofstetter W, Siegrist M, Simonin A, Bonny O, Fuster DG. Sodium/hydrogen exchanger NHA2 in osteoclasts: subcellular localization and role in vitro and in vivo. Bone 2010;47:331–40.
[64] Ishii T, Shimazu Y, Nishiyama I, Kikuta J, Ishii M. The role of sphingosine 1-phosphate in migration of osteoclast precursors; an application of intravital two-photon microscopy. Mol Cells 2011;31:399–403.