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Estrogen deficiency increases osteoclastogenesis upregulating T cells activity: a key mechanism in osteoporosis

Running title: T cells promote osteoclastogenesis

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ABSTRACT

Compelling evidences suggest that increased production of osteoclastogenic cytokines by activated T cells plays a relevant role in the bone loss induced by estrogen deficiency in the mouse. However, little information is available on the role of T cells in postmenopausal bone loss in humans. To investigate this issue we have assessed the production of cytokines involved in osteoclastogenesis (RANKL, TNFα and OPG), *in vitro* osteoclast (OC) formation in pre and postmenopausal women, the latter with or without osteoporosis. We evaluated also OC precursors in peripheral blood and the ability of peripheral blood mononuclear cells to produce TNF α in both basal and stimulated condition by flow cytometry in these subjects.

Our data demonstrate that estrogen deficiency enhances the production of the pro-osteoclastogenetic cytokines TNFα and RANKL and increases the number of circulating OCs precursors. Furthermore, we show that T cells and monocytes from women with osteoporosis exhibit a higher production of TNFα than those from the other two groups.

Our findings suggest that estrogen deficiency stimulates OCs formation both by increasing the production of TNFα and RANKL and increasing the number of OCs precursors. Women with postmenopausal osteoporosis have a higher T cells activity than healthy postmenopausal subjects; T cells thus contribute to the bone loss induced by estrogen deficiency in humans as they do in the mouse.

Introduction

Postmenopausal osteoporosis is a systemic skeletal disorder characterised by reduced bone mineral density (BMD), micro architectural deterioration of bone tissue resulting in fragility and susceptibility to fractures [1] and uncoupling of osteoblast-mediated bone formation and osteoclast (OC)-mediated bone resorption.

Postmenopausal osteoporosis stems from the cessation of ovarian function at menopause and from genetic and non genetic factors which heighten and prolong the rapid phase of bone loss characteristic of the early postmenopausal period. The anti resorptive activity of estrogen is a result of multiple genomic and non genomic effects on bone marrow and bone cells, which leads to decreased OC formation, increased OC apoptosis and decreased capacity of mature OCs to reabsorb bone. Although it is now recognized that stimulation of bone resorption in response to estrogen deficiency is mainly due to cytokine-driven increases in OC formation, the responsible factors are not completely understood. OC formation occurs when monocytes are co-stimulated by the essential osteoclastogenic factors RANKL and M-CSF [2-4], but additional inflammatory cytokines are responsible for the up-regulation of OC formation observed in estrogen deficiency. One of the cytokines responsible for the augmented osteoclastogenesis in states of estrogen deficiency is TNFα, a factor which enhances OC formation by up-regulating stromal cells production of RANKL and M-CSF, and by augmenting the responsiveness of OCs precursors to RANKL [5, 6]. Furthermore, TNF directly induces marrow precursors differentiation into OCs in the absence of RANKL, although according to some studies, TNF is not osteoclastogenic in cells not previously primed by RANKL [7]. However, the source of this cytokine, which is relevant to postmenopausal bone loss

in humans, remains to be determined. Studies in mice suggest that activated T cells are the most relevant source of TNF in conditions of estrogen deficiency [8, 9]. In support of this hypothesis there are reports demonstrating that T cell deficient nude mice are protected against post-ovariectomy bone loss [10, 11]. Furthermore, data show that adoptive transfer of wild type T cells restores the capacity of ovariectomy to induce bone loss, while transfer of T cells from TNF null mice does not [8-10, 12]. Other studies argued against a pivotal role of T cells in bone loss induced by ovariectomy in mice models [13-15]. In particular, Lee et al [13] suggested that nude mice lose trabecular bone as well as wild type after ovariectomy and that T cells may have important effects on the cortical rather than on trabecular compartment.

Little information is available regarding the role of T cells in human bone loss. Studies show a key role of T cell produced TNF in rheumatoid arthritis [16], multiple myeloma [17, 18] and bone metastasis [5, 19]. Other reports show that estrogen deficiency increases the production of TNF and RANKL by bone marrow cells, including T cells, and that their increase correlate with indices of bone resorption [20-22].

This cross-sectional investigation was designed to determine how estrogen deficiency affects the production of osteoclastogenic cytokines by peripheral blood mononuclear cells (PBMC), OC formation and bone resorption.

We report that estrogen deficiency increases the ability of PBMC to form OCs *in vitro* and that this ability is due both to increased output of OCs precursors from bone marrow and to an increased production of TNFα. Furthermore, we demonstrate that T cells are more active in women affected by postmenopausal osteoporosis as respect to postmenopausal healthy women and to pre-menopausal healthy controls. We suggest T cells cytokines production as primary driver of osteoclastogenesis in

postmenopausal osteoporosis.

Materials and Methods

Experimental subjects and markers of bone turnover.

The study was approved by the human study review board of the Azienda Sanitaria Ospedaliera San Giovanni Battista in Torino. The study population included twenty-five postmenopausal women with osteoporosis, 23 healthy postmenopausal and 10 healthy pre-menopausal women. All study subjects had levels of 25-OH vitamin D, bone alkaline phosphatase (BAP) activity and routine blood tests within normal limits. Subjects with secondary forms of osteoporosis or taking drugs active on bone turnover such as calcium and vitamin D, thyroid hormones, corticosteroids, estrogen, bisphosphonates and raloxifene were excluded. The patients and the postmenopausal controls were matched for age, years since menopause and body mass index. Patients had a bone mineral density (BMD) T score of -2.5 S.D. or less according to the WHO criteria as measured by double-emission X-ray absorptiometry with a Hologic QDR 4500. BMD was measured at both lumbar spine and femoral neck; none of the patients had sustained recent fragility fractures. Postmenopausal controls had a T score >-1 SD in both the lumbar spine and the femoral neck. BMD was not measured in pre-menopausal controls.

Serum osteocalcin and urinary CTX were measured by RIA using commercially available kits (DiaSorin for osteocalcin and α-Cross Laps ® RIA from Osteometer BioteTech A/S CTX, respectively) in patients and postmenopausal controls. All biochemical measurements were performed on a single blood sample at a single time point per subject.

Cell isolation and cultures

PBMCs were obtained in all the subjects with the Ficoll-Paque method from 40 ml peripheral blood in lithium heparin as previously described [22]. All cultures were

performed in triplicate in 16-well plates BD BioCoat™ Osteologic™ Bone Cell Culture System (Becton Dickinson & Co. 4 x 10^5 cell/well) [17, 23] using alpha minimal essential medium (α-MEM: Gibco) supplemented with 10% fetal bovine serum (FBS), benzyl penicillin (100 IU/ml) and streptomycin (100 µg/ml) (called "complete medium"), or complete medium plus M-CSF [25 ng/ml] and RANKL [30 ng/ml]. RPMI (Gibco) was used for cell isolation. All cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. To validate resorption of the hydroxyapatite matrix, cells from 5 patients, 5 postmenopausal and 5 pre-menopausal controls randomly selected were also double-plated on dentin slices [2x10^6 cells/slice]. The capacity of OCs to resorb bone *in vitro* was equally detected by measuring resorption pits on dentin slices and the resorption of hydroxyapatite-coated wells (data not shown) as also demonstrated by other studies [17, 18, 24]. In this study bone resorption was quantified by measuring the resorption of hydroxyapatite in all the subjects.

Osteoclast formation and activity

PBMCs were fed every 3 days. On the 21st day, they were fixed and stained for Tartrate Resistant Acid Phosphatase (TRAP) (Acid Phosphatase, Leukocyte staining kit, Sigma Diagnostics) and stained with an immuno technique to express the vitronectin receptor (VNR) (Becton Dickinson & Co). The supernatants of cultures without M-CSF and RANKL (unstimulated conditions) after 3 and 6 days of culture were harvested and frozen at -80°C until the measurement were done. OCs were recognized as TRAP+ and VNR+ multinucleated cells (> 3 nuclei) according to the literature [22, 25] and were counted in each well. In order to avoid possible bias the count was blind to subject status and OCs were always identified by the same operator as previously described [22, 23], the mean per subject for 3 wells with and without M-CSF and RANKL was calculated. In order to evaluate OCs activity, cells

were removed from the wells with 14% sodium hypochlorite after the count and a Nikon Coolpix digital camera attached to an inverted research microscope was used to photograph the entire surface of each well. Lacunar resorption was determined by examining each micrograph with a dedicated computer image analysis system developed in our lab (patent n Italian Patent Application n TO2006A00565), and was expressed as the reabsorbed and total examined surface percentage ratio [23].

Cytokine measurement

ELISA kits were used to measure TNFα (Quantikine; R&D System), OPG (Biomedica; Biomedica Medizinprodukte GmbH & Co KGA) and free s-RANKL (Biomedica; Biomedica Medizinprodukte GmbH & Co KGA) levels in 3 and 6 day unstimulated culture supernatants from all the subjects.

To evaluate the *in vivo* cytokines production we measured the same cytokines also in the serum. We measured the levels of total s-RANKL (Apotech; Apotech Corporation & Immunodiagnostik) in the serum to exclude possible variation in free s-RANKL due to OPG level as previously suggested [26]. Each measurement was performed in duplicate for each patient.

Western blot for RANKL

In order to ensure the specificity of the antibody used for the RANKL measurement, we performed a Western blot analyses on PBMC cultures supernatants. Western blot analysis was performed following standard protocols using the following equipment: Biorad electrophoresis/ western blot system, 10% SDS mini gels, broad range molecular weight standards, and ECL chemiluminescence detection reagents (Amersham). For detection of RANKL, Western blot analysis was performed on protein extracted from PBMC cultures supernatants from three osteoporotic women whose RANKL levels correspond to the mean of the analysed patients. Purified

RANKL 0.25, 0.5 and 1 pg were used as positive control. Immunoprecipitation of RANKL was performed using the ELISA antibody or a mouse anti-human s-RANKL Ab (Abcam). Samples were loaded in growing doses onto 10% SDS page minigel along with broad range standards. The Western blot was run at 120 V for 90 min, and then gel was transferred to PVDF membrane. Blots were incubated in HRP-conjugated secondary antibody or in HRP-streptavidin for the ELISA antibody, followed by chemiluminescence substrate, developed, and exposed to X-ray film for 20 s.

Real-time RT-PCR.

Considering the low amount of RANKL detected, we performed real-time RT-PCR experiments to further confirm the results. Real time RT-PCR was performed on PBMC from 5 osteoporotic women, 5 postmenopausal and 5 pre-menopausal controls randomly selected at baseline or after stimulus with phytoemoagglutinin (PHA: Sigma Aldrich s.r.l.), a mitogen that predominantly stimulates T cells [10μg/ml], for 24 h. In order to evaluate the role of T cells in RANKL production we obtained monocytes and T cells by negative separation with monocyte and Pan T cell isolation kits (Miltenyi Biotech) in 3 osteoporotic women and 3 postmenopausal controls randomly selected and performed the real-time RT-PCR experiments on each population. 3 x 10^6 cells were resuspended in 500 µl sol A of RNA extraction Kit, (Nurex) and total RNA was purified according to the manufacturer's instructions and resuspended in 30 µL of elution solution. cDNA was prepared adding to 30 µL of extracted RNA 48 µL reaction mixture containing 300 ng oligo-dT and 600 U M-MLV reverse transcriptase (both Invitrogen). Oligonucleotide sequences (RANKL: Fwd 5'-3' ATC ACA GCA CAT CAG AGC AGA G, Rev GGA CAG ACT CAC TTT ATG GGA ACC; S14: Fwd AGG TGC AAG GAG CTG GGT AT, Rev TCC AGG GGT CTT GGT

CCT ATT T) were identified using Beacon Designer Software from PREMIER Biosoft International and designed to be intron spanning allowing the differentiation between cDNA and DNA-derived PCR products. Real-time quantitative RT-PCR was performed on the iCycler instrument (Bio-Rad). PCR amplification was carried out in 25 µl of reaction mixture. 5 µl of cDNA and primers 300 nM for RANKL and 200 nM for S14 were added to the amplification mixture iQ SYBR Green Supermix (Bio-Rad). DNA polymerase was pre-activated for 2 min at 94°C, and the amplification was performed by a 50-cycle PCR (94°C, 30s, 60°C, 30s and 72°C, 30 s). A standard curve, with four dilutions of PBMC treated with PHA [10µg/ml] for 24 h cDNA, was included in each respective PCR run to quantify the number of RANKL producing cells in clinical samples. S14 was used as reference gene to normalize cDNA across samples [27]. All samples were analyzed in triplicate. The specificity of the PCR results was confirmed by melt curve analysis.

Flow cytometry.

OC precursors were detected in all the subjects by staining the fresh blood samples with FITC-conjugated anti-VNR, PE-conjugated anti CD14 and APC-conjugated anti-CD11b mAb, or with the corresponding isotype control followed by incubation at 4°C for 30 min. We treated the triple-positive (CD14+/CD11b+/VNR+) cells as OC precursors according to previous literature [19, 23, 28-32].

To assess TNFα production by PBMC in conditions similar to those *in vivo,* free from the possibility of alteration due to residence in culture, and to detect the cells involved, we compared PBMC at baseline and after stimulation with PHA. Freshly isolated PBMC [$1x10^6$ PBMC/well] were analyzed for the basal production or TNF α or plated in triplicate in 96-well plates using RPMI (supplied by GIBCO) supplemented with 10% fetal bovine serum (FBS), benzyl penicillin (100 IU/ml) and

streptomycin (100 μg/ml), plus PHA [10 μg/ml] and analyzed after 24 hrs of culture for the production of TNF after stimulus in all the subjects. For intra-cytoplasmic detection of TNF α in these subsets, 1 x 10^6 PBMC were stained with PerCP-conjugated anti-CD3 and APC-conjugated anti-CD14 or with the corresponding isotype control and then incubated for 30 min at 4°C, with a sample stained with the isotypes as the negative control. Then the cells were fixed with 1 ml cold 0.4% paraformaldehyde in PBS and incubated for 15 min at 4°C. Fixed cells were washed twice with PBS supplemented with 0.5% BSA, 0.1% sodium azide and 500 μl of FACS permeabilizing solution (Becton Dickinson & Co.) was added to the pellet. After 10 min incubation at room temperature, cells were washed with PBS supplemented with 0.5% BSA, 0,1% sodium azide and incubated with PE-conjugated anti-TNF alpha mAb (according to the manufacturer's protocols) or with the corresponding isotype control for 30 min at 4°C, as previously described [33]. Flow cytometry was performed on a FACSCalibur flow cytometer (Becton Dickinson & Co.). Membrane antigens expression was analyzed through CellQuest (Becton Dickinson & Co.) software and displayed as bivariate dot plots or histograms. Each plot represents the results from 10,000 events of viable cells gated by size and granularity. All the antibodies were purchased from Becton Dickinson & Co.

Cell co-cultures

To clarify the role of lymphocytes in OCs differentiation and activation in vitro, we cultured monocytes alone and together with either T or B cells obtained by negative separation with monocyte, Pan T cell and Pan B cell isolation kits (Miltenyi Biotech). CD14+ cells were cultured alone (200,000/well), with CD3+ cells (420,000/well) and with CD19+ cells (360,000/well) on biocoated plates with and without growth factors (25 ng/ml M-CSF, 30 ng/ml RANKL). Flow cytometry of the separated fractions

showed a 90% separation purity for the CD14+ monocytes, 94.9% for the CD3+ T cells and 96.2% for the CD19+ B cells. The cells for these experiments were obtained from 5 osteoporotic women randomly selected.

Cytokine inhibition experiment

To evaluate the role of endogenous TNFα and RANKL production in supporting osteoclastogenesis, we carried out a double cytokine inhibition experiment on three osteoporotic patients whose RANKL and TNF levels correspond to the mean of the analysed patients. Increasing doses of anti-TNF mAb (Peproteck) and anti-RANKL mAb (RANKL-Fc, R&D System) or a neutral isotype were added during a 21-day culture, after which the cells were fixed, stained and counted under a light microscope as described above.

The FACS analyses, the cultures and the cytokines measurement experiments were performed for all the enrolled subjects.

Statistics

The data were processed with the SPSS 14.0 for Windows software package, with $p<0.05$ as the significance cut-off. One-way ANOVA was used to compare patients and controls for menopausal period, BMI, bone metabolism markers, TNFα, RANKL and OPG levels in the supernatants, number of OCs per well, percentage of resorbed hydroxyapatite and TNFα production measured with FACS. The post hoc Bonferroni test was used to point out differences between groups. To analyze the differences among patients and controls for serum levels of TNF, RANKL and OPG we used a Kruskal-Wallis test as these variables were not distributed according to a Gaussian curve. To determine which of the significantly different variables were predictors of the number of OCs, we used a linear regression model with stepwise analysis.

Results

Osteoclasts formation is higher in women affected by postmenopausal osteoporosis. Unstimulated PBMC cultures from osteoporotic women produced more OCs than those from controls, whereas this difference disappeared when the cultures were stimulated with M-CSF and RANKL. Furthermore, the OCs formed in patients are more active than those formed in controls in unstimulated condition as demonstrated by the higher percentage of hydroxyapatite resorbed (Fig.1). PBMC from pre-menopausal controls do not form OCs nor reabsorb hydroxyapatite in unstimulated condition by contrast with postmenopausal controls. This finding confirms that estrogen deficiency induces osteoclastogenesis. The addition of osteoclastogenic cytokines in culture annuls the difference between the three groups, suggesting that an adequate stimulation is able to induce the formation of mature and active OCs even in PBMC from subjects in pre-menopausal period. The markers of bone metabolism confirm the experimental data on the higher OCs activity: in fact the bone resorption marker (CTX) is higher in patients, as respect to postmenopausal controls (8.02±1.88 versus 4.17±2.56 μg/L, p=0.04) and correlated to the number of OCs formed in culture (r= 0.7, p=0.03).

To assess the effects of menopause and osteoporosis on the process of OC differentiation we next measured the number of OC precursors in PBMC harvested from the 3 study groups. The percentage of OC precursors (CD14+/CD11b+/VNR+ cells) was found higher in the patients than in the two control groups. The mean fluorescence intensity (MFI) of CD11b and VNR was higher in patients than in samples from the control groups, while the MFI of CD14 was higher in the pre-menopausal controls and inversely correlated with age (R=-0.5, p=0.002) (Fig.2). This finding suggests that OC precursors in patients were more committed toward

osteoclastic lineage as compared to controls.

Patients produce more TNF and RANKL as respect to both postmenopausal and pre-menopausal controls. The production of RANKL and TNF by unstimulated PBMC was assessed by measuring the levels of these two cytokines in 3 and 6 day culture media by ELISA. TNFα levels were significantly higher in the patients than in both control groups at each point studied. Furthermore, postmenopausal had higher levels of TNF than pre-menopausal controls. These findings suggest that estrogen loss induce a greater production of TNFα by PBMC and that osteoporosis further increases this production. At day 3 and 6 RANKL levels were significantly higher in the patients than in controls (Tab. 1). The Western blot technique used to verify the specificity of the antibody at low RANKL concentration confirms the reliability of our results: serial dilutions of PBMC supernatants protein revealed dominant bands of RANKL at 49 and 29 kDa with intensities that were linear over the range from 100-600 µg protein. Thus, the antibody recognized RANKL in a linear fashion over the protein range studied. The lower RANKL dose (0.25 pg) was also recognized (Fig.3). The real-time RT-PCR data confirm that PBMC from patients produce more RANKL than those from controls (Fig. 4).

In contrast, OPG levels were similar in all three groups at each point studied (Tab.1). The ratio between RANKL and OPG measured at day 6 was significantly higher in patients (0.7±0.2) as respect to postmenopausal and pre-menopausal controls (0.5±0.4 and 0, respectively).

In order to evaluate the *in vivo* cytokines, production we measured the serum levels of TNFα, OPG and total RANKL (free RANKL + OPG-bound RANKL). We choose to evaluate total RANKL in serum to avoid possible interference due to changes in OPG levels in osteoporotic subjects [26, 34]. Our data show that both TNF α and OPG are

higher in patients as compared to the controls, while RANKL is not statistically different even if higher in patients (Tab.2). The ratio between serum RANKL and OPG was not significantly different between patients and controls.

T cells are the major source of TNF and RANKL in osteoporosis. To determine whether the increased levels of TNFα *in vitro* were due to a greater production of the cytokine per cell, or to an increase in their number, we measured the number of TNF producing cell by FACS in PBMC and in PBMC after stimulus with PHA for 24 hours. We found that TNF was produced by monocytes and T cells both with and without PHA. As expected, PHA increased TNF production by T cells, but not by monocytes. T cells from pre-menopausal and postmenopausal controls also displayed a greater response to PHA than those from patients (80, 30 and 1 fold respectively) (Fig.5). These data suggest that T cells from postmenopausal women are less prone to immune stimulation as respect to pre-menopausal healthy women. However in patients TNF production was significantly higher at baseline and after PHA stimulation as respect to controls. Monocytes from pre-menopausal produced more TNFα than those from postmenopausal controls also (Fig.5), and this points to a greater reactivity of immune system in youth.

To investigate the role of T cells in the RANKL production, we performed a real-time RT-PCR experiment on T cells and monocytes separated from 3 osteoporotic and 3 postmenopausal healthy controls to check for RANKL production; our data demonstrated that T cells produced a greater amount of RANKL as respect to monocytes and that T cells from osteoporotic subjects produced more RANKL than healthy controls (Fig.4), furthermore stimulus with PHA increases the amount of RANKL in T cells both in patients and in controls, as expected ($p=0.016$). The percentage of T cells in PBMC range between 58 and 63% without significant

differences between patients and controls.

T cells are pivotal for OCs formation. To further investigate the effects of T cells on OCs formation *in vitro*, we examined the capacity of cultures of monocytes to form OCs in the presence and in the absence of T cells. The number of OCs formed in the monocyte plus T cell co-cultures was the same as in the un-fractionated PBMCs, while the absence of T cells suppressed OCs formation in cultures without the addition of exogenous growth factors. The addition of permissive amount of M-CSF and RANKL in the cultures of monocytes and of monocytes plus B cells restored osteoclastogenesis (Fig.6). These findings suggest that T cells are pivotal in OCs formation through the production of osteoclastogenic cytokines. This datum is also supported by dose response studies conducted by adding increasing amounts of neutralizing antibodies directed against TNFα and RANKL, these experiments revealed that neutralization of both TNFα and RANKL reduces OCs formation in a dose dependent fashion (Fig.7).

Analysis by linear regression showed that the number of OCs formed in the absence of exogenous growth factors was 100% (R square=1) predicted by the circulating precursor percentage, the precursors' degree of maturation and the RANKL level. BMD levels were 94% (R square=0.94) predicted by the RANKL production (Tab. 3).

Discussion

Although estrogen deficiency is known to induce bone loss through various mechanisms, to our knowledge, this report is the first to demonstrate the contribution of T cells to cytokine driven osteoclastogenesis in postmenopausal osteoporosis. We found menopause to increase the number of OCs precursors in the PBMC and the ability of T cells to produce proosteoclastogenic cytokines. Estrogen deficiency leads to an increase in OCs formation more relevant in patients affected by osteoporosis. Menopause stimulates osteoclastogenesis through increased production of RANKL and TNF by monocytes and T cells and this phenomenon is more relevant in osteoporotic women. Our findings confirm the result of an earlier report from our laboratory [22] and demonstrate that T cells play a pivotal role in the stimulation of osteoclastogenesis induced by postmenopausal osteoporosis in humans. Thus, this study validates in humans earlier findings in ovariectomized mice.

A relevant finding of our study is that PBMC from healthy postmenopausal women produce fewer OCs than those from osteoporotic subjects, but only in the absence of exogenous cytokines. In fact, the addition of permissive doses of M-CSF and RANKL induces the formation of a similar number of OCs in cultures from all groups, suggesting that estrogen deficiency does not increase the capacity of OCs precursors to differentiate into mature OCs, but rather increases the levels of osteoclastogenic cytokines in the bone microenvironment. Here we show that mature and active osteoclasts can be differentiated from PBMC cultures and that their activity is significantly greater in patients with postmenopausal osteoporosis compared with postmenopausal and premenopausal healthy controls, and that enhanced osteoclast formation is largely dependent on the greater ability of PBMC from such patients to produce pro-osteoclastogenic cytokines (TNF alpha and

RANKL). It has been demonstrated that circulating osteoclast precursors exist primarily within the monocytic fraction of peripheral blood, [19, 28-32] and their presence in the circulation serves both as a reservoir for replenishing pre-osteoclast populations in the bone marrow as needed and as a potentially abundant source of pre-osteoclasts that can be recruited into bone or joint tissue in response to reparative or pathological signals. In contrast to the beneficial nature of pre-osteoclast recruitment during normal bone remodelling or fracture repair, excessive pre-osteoclast recruitment in pathological conditions causes significant bone loss in many skeletal disorders characterized by increased osteoclast formation and activity [35-37]. Several studies have identified the surface markers that define the phenotype of monocytes with osteoclastogenic potentials [19, 28, 29, 31, 38]. Analysis of the MFI of these markers by flow cytometry can be used as an indirect indicator of greater precursor differentiation in the osteoporotic patients. Here we demonstrate that there are an increased number of mature circulating OC precursors in osteoporotic patients as respect to controls.

Furthermore, we found that pre-menopausal controls had a larger expression of CD14 on cellular surface, as compared to menopausal controls and to patients, and that CD14 MFI was inversely correlated with age. This points to greater reactivity of the immune system in youth, as suggested by the greater TNFα expression induced by PHA stimulation of young women CD3+ cells, whereas it had little effect in healthy postmenopausal women.

Our findings suggest the hypothesis that estrogen deficiency reduces the ability of T cells to answer to immune stimulation both in healthy than in osteoporotic subject, whilst in patients T cells are already activated to a greater degree at baseline. Greater spontaneous and PHA stimulated TNFα and RANKL production by

monocytes, and even more so by T cells in samples from patients suggest that estrogen deficiency stimulates OCs formation through TNF and RANKL. T cells are a key source of these cytokines not only because a significant fraction of TNF and RANKL producing cells are T cells, but also because in vitro OCs formation was impeded by the absence of T cells and restored by the addition of exogenous cytokines. It has been suggested that T cells survive in PBMC cultures and are able to prolong osteoclast viability in cultures until 35 days in myeloma patients [17].

Our data on the role of T cells and of the production of TNFα in culture confirm recent data obtained both in humans, in vivo and in vitro [5, 16-19], and in the animal model [12]. The present study demonstrates an increase in the production of two potent pro-osteoclastogenetic cytokines, TNFα and RANKL, by PBMC cultures from patients as compared to controls. These findings may provide an explanation of the increased osteoclastogenesis characteristic of women with osteoporosis. In postmenopausal women we observed a greater production of pro-osteoclastogenetic cytokines as compared to pre-menopausal subjects, in confirmation of the previous data in the literature which established a relationship between estrogen deficiency and the greater production of pro-inflammatory cytokines [39-41]. These findings are consistent with the result of the antibody neutralization experiments which show that silencing of TNF and RANKL blocks OCs formation and with the linear regression model that suggests the MFI of the VNR, the percentage of osteoclast precursors and the RANKL levels in the supernatant are able to predict the formation of osteoclast *in vitro*.

The level of TNF is greater in patients than in controls also in the serum, this datum confirms that there is a higher production of this cytokine also *in vivo*. Total s-RANKL was not significantly different in patients and controls even if higher in patients, the

lack of statistic significance could be due to the small cohort studied and to the high individual variability. Serum OPG were higher in patients than in controls: this datum confirms previous studies by Yano et al [26] in postmenopausal osteoporosis and by Oh et al in males [34].

In conclusion, the present study suggests that estrogen deficiency up-regulate osteoclastogenesis through the production of the pro-osteoclastogenic cytokines TNF alpha and RANKL. It confirms previous reports demonstrating that osteoclastogenesis is greater after the menopause in women suffering from postmenopausal osteoporosis compared with controls matched for menopausal period. It also suggests that the factors predictive of osteoclastogenesis are to be sought in both the greater marrow output of precursors committed in the osteoclast direction and in the cytokine production, and in particular of RANKL and TNF. Furthermore, it suggests a fundamental role for T lymphocytes in postmenopausal bone loss and in the induction of osteoclastogenesis in humans.

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Table 1. PBMCs from osteoporotic produce more TNF and RANKL as respect to both postmenopausal and pre-menopausal controls. Supernatant TNFα, OPG and free s-RANKL values (pg/ml: means ± SE) at 3 and 6 days of culture of PBMC cultures without addition of M-CSF and RANKL from the patients and the two control groups. The p values were obtained with the one way ANOVA corrected with the Bonferroni post hoc test. The values in parenthesis correspond to the number of analysed subjects.

	PATIENTS	CONTROLS		P values			
	OSTEOPOROTIC (25)	POST-MENOPAUSAL (23)	PRE-MENOPAUSAL (10)	Across groups	Patients vs postmenopausal	Patients vs pre-menopausal	Postemenopausal vs pre-menopausal
TNF α day 3	40±3.8	30.4±2.7	0.4±0.05	0.001	0.04	0.001	0.037
TNF α day 6	178.2±2.5	53.7±7.2	38.2±7.7	0.029	0.024	0.03	0.029
RANKL day 3	0.29±0.09	0.05±0.03	0	0.024	0.024	0.000	0.04
RANKL day 6	0.32±0.08	0.036±0.01	0	0.002	0.003	0.002	0.04
OPG day 3	11.39±23.66	10.57±2.88	12.38±16.62	NS	NS	NS	NS
OPG day 6	14.45±24.61	29.31±26.63	19.31±2.39	NS	NS	NS	NS

Table 2. *In vivo* cytokines production. Serum TNFα, OPG and total s-RANKL values (pg/ml: means ± SE) in patients and the two control groups. The p values were obtained with the Kruskal-Wallis test. The values in parenthesis correspond to the number of analysed subjects.

	PATIENTS	**CONTROLS**		**P values**			
	OSTEOPOROTIC (25)	POST-MENOPAUSAL (23)	PRE-MENOPAUSAL (10)	Across groups	Patients vs postmenopausal	Patients vs pre-menopausal	Postemenopausal vs pre-menopausal
TNF α	163.04 ± 107	25.14±2.65	23.23±1.78	0.028	0.021	0.003	NS
RANKL	31.07±28.96	0.1±0.05	0.7±0.5	NS	NS	NS	NS
OPG	1863.9±373.1	1191±141.65	812.55±111.23	0.014	0.018	0.0003	NS

Table 3. Factors predictive of osteoclast formation and of bone density. Stepwise linear regression models for osteoclast (OC) formation without added M-CSF and RANKL and bone mineral density (BMD) as dependent variables.

OCs without factors	**Standardized B**	**T**	**P**
MIF VNR	0.95	5.5	0.012
OC precursors %	0.98	227.57	0.000
6th culture day RANKL level	0.022	70.21	0.000
BMD	**B**	**T**	**P**
6th culture day RANKL level	-0.461	-6.9	0.006

FIGURE LEGENDS.

Figure 1. Osteoclast formation and activity in patients, postmenopausal and pre-menopausal controls. (A) Upper panels: TRAP staining (10 x) of cultures (unstimulated) of PBMC from an osteoporotic patient, a postmenopausal and a premenopausal control. The OCs are indicated with arrows. Lower panels: micrographs of the hydroxyapatite matrix resorbed by the OCs from these three subjects.

(B) Histograms showing the number of OCs (a) and the percentage of hydroxyapatite matrix resorbed (b), with (stimulated) or without (unstimulated) M-CSF [25 ng/ml] and RANKL [30 ng/ml] , in PBMC cultures from patients (25), postmenopausal (23) and pre-menopausal (10) controls. The bars show the mean and SD obtained for all the experiments. P values were calculated with ANOVA: * p = 0.000, **p= 0.030

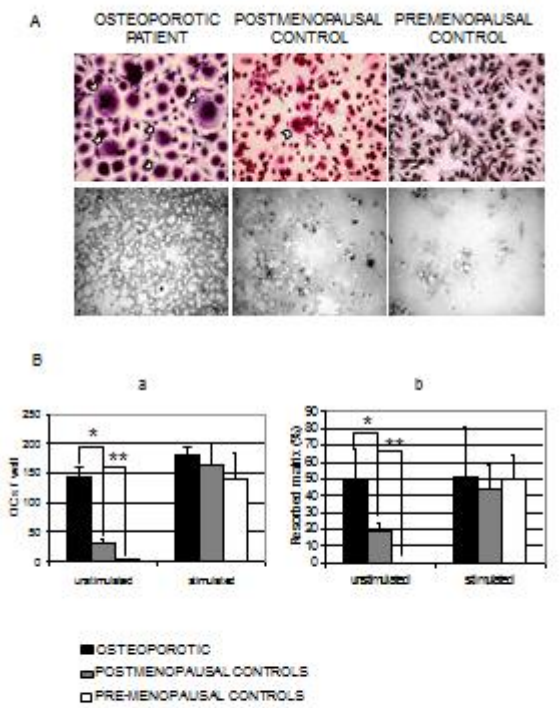

Figure 2. Osteoclasts precursors. FACS analysis of circulating OC precursors from PBMC of an osteoporotic woman, a postmenopausal control and a pre-menopausal control labelled with FITC-conjugated anti-VNR, PE-conjugated anti-CD14 and APC-conjugated anti-CD11b mAbs. Dot plots with CD14+ cells gated (upper panels), middle panels displays of VNR+ and CD11b+ cells gated on CD14+ cell. The graphs show MFI and percentages of positive cells, the bars represent the mean and SD obtained for all the experiments performed in 25 osteoporotic, 23 postmenopausal and 10 pre-menopausal controls. The quadrant gates were set against isotype control. P values were calculated with ANOVA: °p=0.02, * p=0.04, **p=0.04.

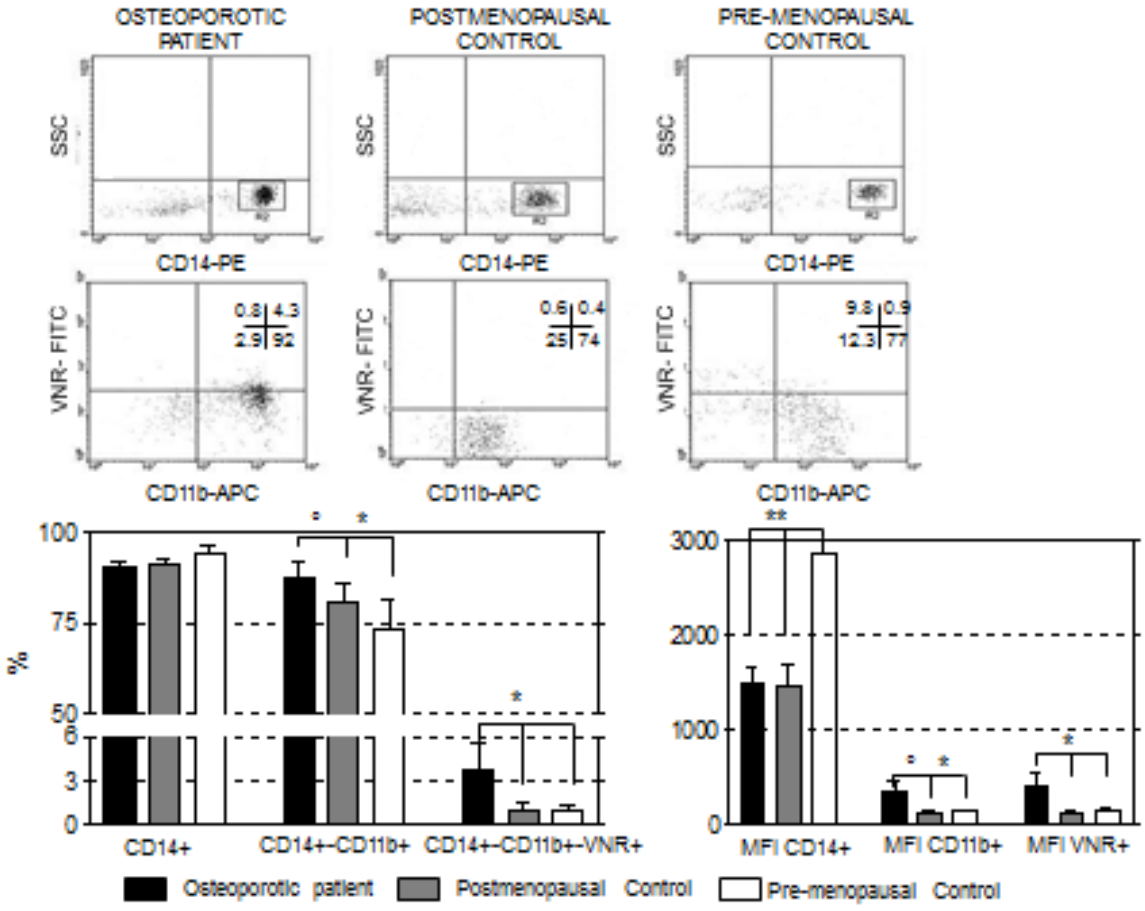

Figure 3. Western Blot analyses performed with the ELISA antibody (upper panel) and with the antibody specific for Western Blot (lower panel). The images show the detection of the purified RANKL in low doses (0.25, 0.5 and 1 pg) on the left and the linear expression of RANKL with increasing doses of PBMC supernatant (from 100 to 600 pg). RANKL appears ad two bands at 49 and 29 kDa.

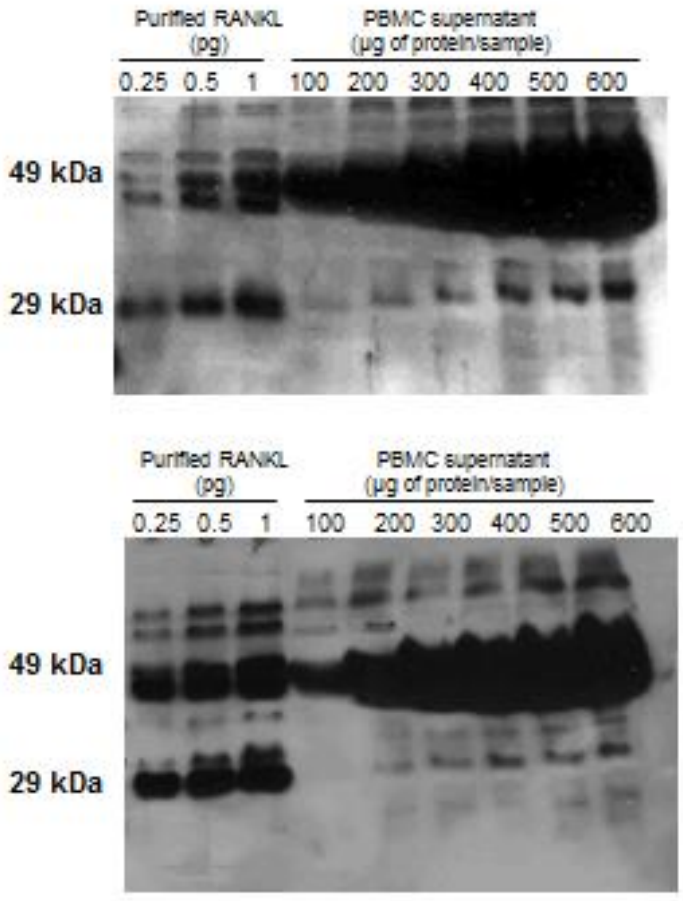

Figure 4. RANKL mRNA expression by PBMC, monocytes and T cells. Cells were analysed at baseline or after stimulus with PHA [10 ng/ml] for 24 h. RANKL mRNA expression was determined by real-time quantitative PCR. The results were normalized for the expression of S14 . (A) The RANKL mRNA expression in PBMC from osteoporotic 5 patients 5 postmenopasual and 5 pre-menopausal controls at baseline and after PHA stimulus. (B) The RANKL mRNA expression in T cells and

monocytes from 3 patients and 3 menopausal controls at baseline. Bar represents mean ± SD, p values were obtained by one way ANOVA.

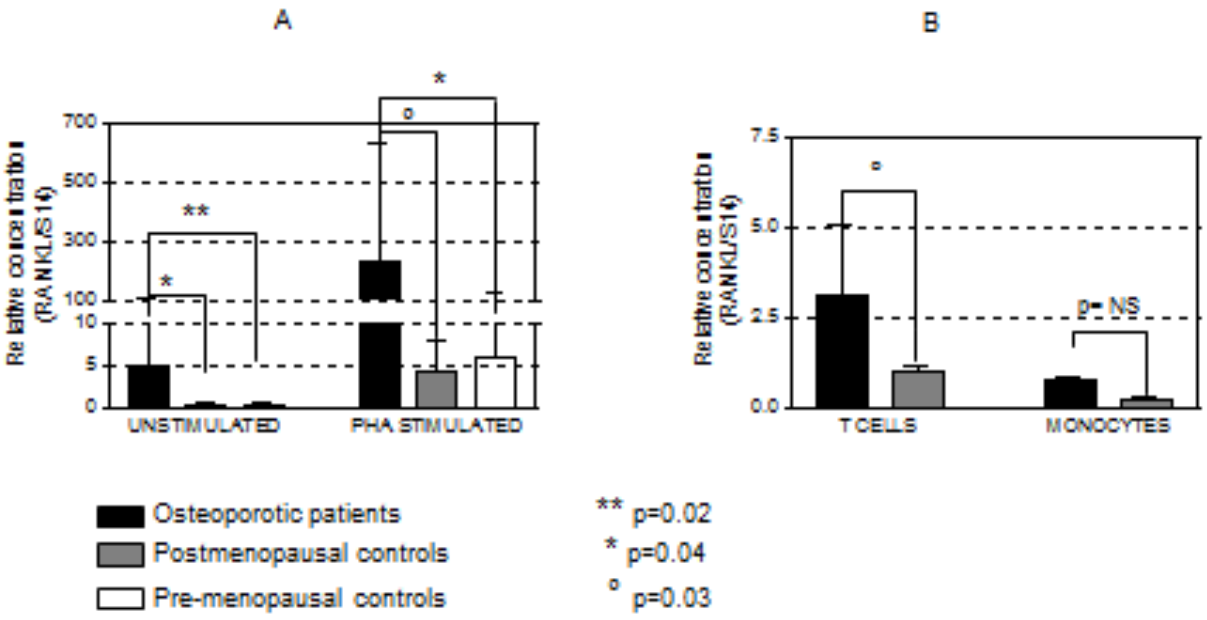

Figure 5. TNF α production by PBMC. FACS analysis of PBMC at baseline labelled with PerCP-conjugated anti-CD3, APC-conjugated anti-CD14, PE-conjugated anti TNF mAbs or with the corresponding isotype controls in an osteoporotic woman, a postmenopausal control and a pre-menopausal control. The shaded curve represent the positive cells, while the empty curve represent the negative control, percentages of positive cells are indicated.

A. Histograms with TNF producing CD3+ cells (T cells) gated on PBMC in three subjects studied at baseline.

B. Histograms with TNF producing CD14+ cells (monocytes) gated on PBMC in three subjects studied at baseline.

C. Graph showing the percentage of CD3+TNF+, CD14+TNF+ at baseline and after stimulus with PHA for 24h. The bars show the mean and SD for all the subjects (25 osteoporotic patients, 23 postmenopausal and 10 pre-menopausal controls). P values were calculated with one way ANOVA, the significant values are indicated.

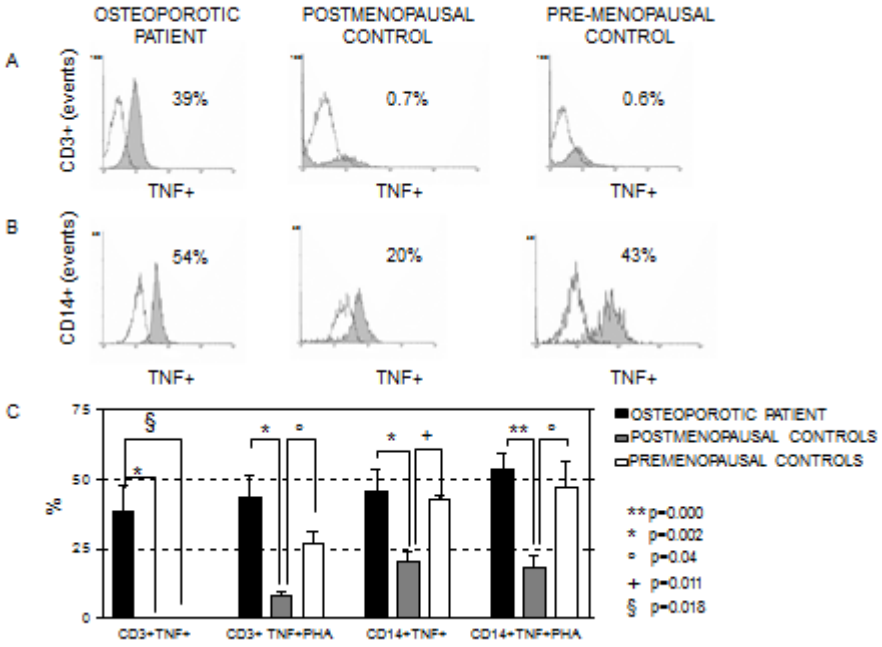

Figure 6. Co-cultures of monocytes and lymphocytes, obtained from an osteoporotic patient, with and without growth factors (25 ng/ml M-CSF, 30 ng/ml RANKL). TRAP staining (10 x) of monocytes without (A) and with (D) M-CSF and RANKL, co-cultures of monocytes and B cells without (B) and with (E) M-CSF and RANKL, and co-cultures of monocytes and T cells without (C) and with (F) M-CSF and RANKL. The OCs are indicated with arrows.

(B) Graph showing the number of osteoclast with and without growth factors, in PBMC, monocytes, monocytes plus B cells and monocytes plus T cells cultures. The bars show the mean and SD obtained for 5 experiments in duplicate. P values calculated with paired Students T-test: * $p<0.001$

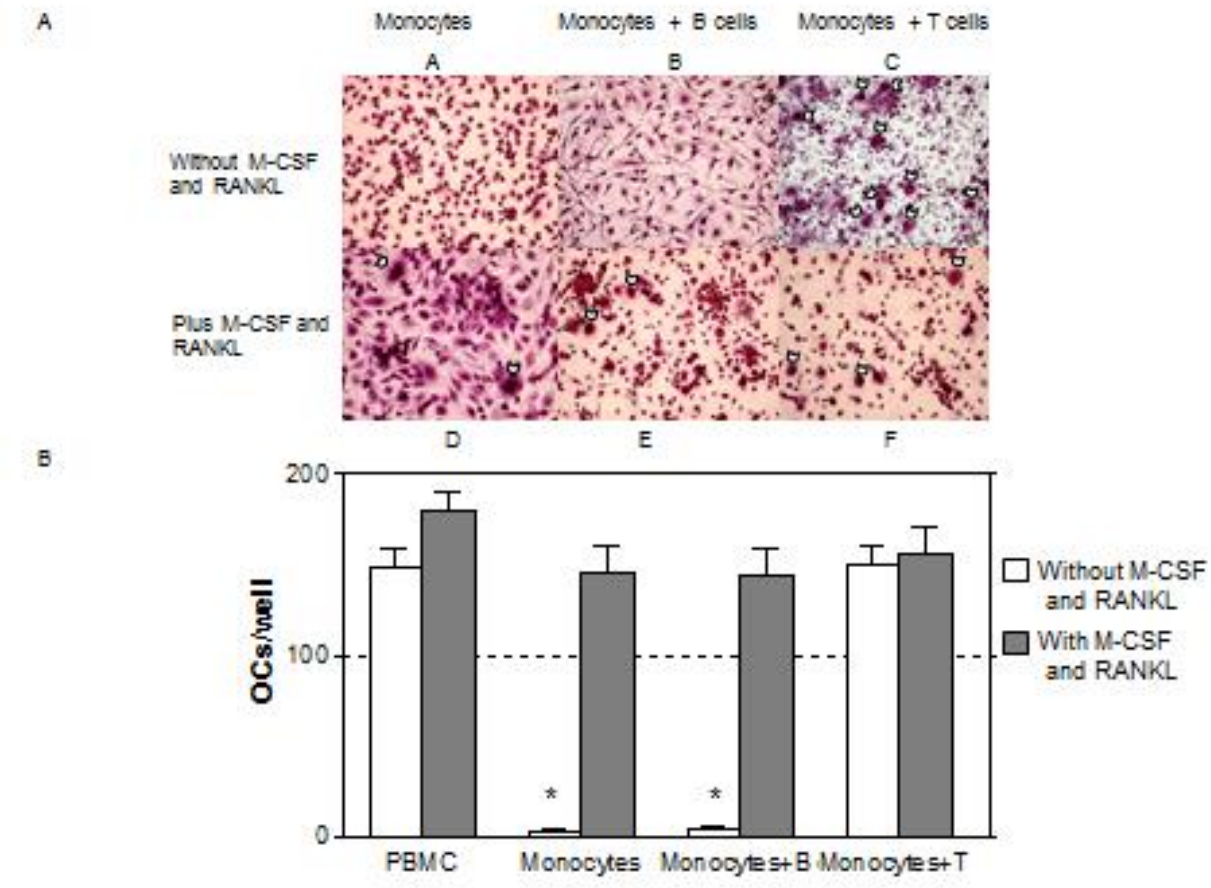

Figure 7. Inhibition of TNF α and RANKL reduces osteoclast formation. Upper panels: TRAP staining (10 x) of cultures of PBMC from an osteoporotic patient treated with anti TNFα mAb at different concentrations. Lower panels: TRAP staining (10 x) of cultures of PBMC from the same patient treated with anti RANKL mAb at different concentrations. The OCs are indicated with arrows.

The graphs illustrate the reduction of OC formation after addition of anti-TNFα (A) or anti-RANKL (B) mAbs, with means ± SD. The exponential best-fit curves and their equations are also shown. The experiments were performed in duplicate with PBMC from 3 osteoporotic patients whose RANKL and TNF levels correspond to the mean of the analysed patients.