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Impaired bone remodeling in children with osteogenesis imperfecta treated and untreated with bisphosphonates: the role of DKK1, RANKL and TNFα

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MINI-ABSTRACT

In this study we investigated the bone cell activity in patients with osteogenesis imperfecta (OI) treated and untreated with neridronate. We demonstrated the key role of DKK1, RANKL and TNFα in regulating bone cell of untreated and treated OI subjects. These cytokines could represent new pharmacological targets for OI.

ABSTRACT

Purpose. Bisphosphonates are widely used in the treatment of children with osteogenesis imperfecta (OI) with the objective of reducing the risk of fractures. Although bisphosphonates increase bone mineral density in OI subjects, the effects on fracture incidence are conflicting. The aim of this study was to investigate the mechanisms underlying bone cell activity in subjects with mild untreated forms of OI and in a group of subjects with severe OI treated with cycles of intravenous neridronate.

Methods. Sclerostin, DKK1, TNFα, RANKL, OPG and bone turnover markers were quantified in serum of 18 OI patients (12 females, mean age 8.86 ± 3.90), 8 of which were receiving cyclic intravenous neridronate, and 21 sex- and age-matched controls. The effects on osteoblastogenesis and OPG expression of media conditioned by the serum of OI patients and anti-DKK1 neutralizing antibody were evaluated. Osteoclastogenesis was assessed in cultures from patients and controls.

Results. DKK1 and RANKL levels were significantly increased both in untreated and treated OI subjects respect to controls. The serum from patients with high DKK1 levels inhibited both osteoblast differentiation and OPG expression *in vitro*. High RANKL and low OPG mRNA levels were found in lymphomonocytes from patients. High amounts of TNFα were expressed by monocytes, and an elevated percentage of circulating CD11b-CD51/CD61+ osteoclast precursors was observed in patients.

Conclusions. Our study demonstrated the key role of DKK1, RANKL and TNFα in regulating bone cell activity of subjects with OI untreated and treated with bisphosphonates. These cytokines could represent new pharmacological targets for OI patients.

Keywords: Osteogenesis imperfecta; osteoblasts; osteoclasts; RANKL; DKK1.

Conflict of Interest: Giacomina Brunetti, Francesco Papadia, Albina Tummolo, Rita Fischetto, Francesco Nicastro, Laura Piacente, Annamaria Ventura, Giorgio Mori, Angela Oranger, Isabella Gigante, Silvia Colucci, Maria Ciccarelli, Maria Grano, Luciano Cavallo, Maurizio Delvecchio, and Maria Felicia Faienza declare that they have no conflict of interest.

INTRODUCTION

Osteogenesis Imperfecta (OI) is a heterogeneous heritable connective tissue disorder occurring in 1/15–20,000 births [1] in which the major causative defect is directly related to type I collagen (COLL I), the key extracellular matrix component of bone and other connective tissue [2].

The clinical features of OI commonly include low bone mass and reduced bone strength, resulting in bone fragility and easy susceptibility to fractures, bone deformity and growth deficiency. About 90% of patients with a clinical diagnosis of OI display a dominant pattern of inheritance, with mutations in *COL1A1* or *COL1A2*, which alter the structure or quantity of COLL I and cause a skeletal phenotype ranging from subclinical to lethal [3]. Recessive forms of OI with lethal to moderate phenotypes are caused by defects in genes whose products are involved in the assembly, processing and maturation of collagen type I (CRTAP, P3H1, PPIB, SERPINH1, FKBP10, BMP1), or in the formation and homeostasis of bone tissue (SERPINF1, SP7) [4]. Moreover, histological studies performed on OI patients have showed a high bone turnover [5, 6]. Intravenous bisphosphonates, anti-resorptives drugs, have been suggested as treatment to improve bone fragility and to reduce fracture risk in children with severe OI [7]. There are evidences that bisphosphonates increase bone mineral density (BMD) in OI patients, but conflicting results have been reported with regard to the effect on fractures [8]. Moreover, many studies showed that bisphosphonates do not improve clinical status (pain, growth and functional mobility) in subjects with OI [8]. Prolonged bisphosphonate use can also impair metaphyseal modeling in children [9]. Moreover, some genetic forms of OI, as the type VI, have shown a poor response to bisphosphonate treatment, thus requiring alternative therapy. The use of anabolic agents to increase bone mass and size in children with severe forms of OI is an attractive theoretical option, but these drugs generally increase bone turnover, making the development of deformity more likely. Thus, a better understanding of the mechanism/s underlying bone cell activity in OI patients will be useful to identify alternative therapeutic strategies.

Crucial pathways regulating bone cell activity are represented by the axis receptor activator of nuclear factor-κB (RANK)/ RANK ligand (RANKL)/osteoprotegerin (OPG) and Wnt/β-catenin signaling [10]. The former regulates OC formation and the latter osteoblast (OB) differentiation. Sclerostin and Dickkopf-1 (DKK1) are secreted Wnt-signaling inhibitors which prevent the formation of the Wnt-Frizzled-LRP5 complex by internalization of the LRP5/6 coreceptor and competitive binding to LRP5, respectively [11, 12]. Sclerostin

and DKK1 are critical regulator of bone mass [13], and their role in some bone disease has been established [10, 14, 15]. DKK1 blocks the maturation of OBs, decreases OPG levels and increases RANKL expression in OBs, thereby shifting the OPG/RANKL ratio in favor of bone resorption. DKK1 levels have been linked to another pro-osteoclastogenic cytokine TNFα [16]. In the present study, we analyzed the serum levels of sclerostin, DKK1, TNFα, RANKL and OPG in a group of children and adolescents with moderate to severe forms of OI, some of which under intravenously neridronate treatment, as well as the possible involvement of these cytokines in the altered bone cell activity associated to the disease. The purpose of this study derives from the need to identify new pharmacological targets for OI patients.

SUBJECTS AND METHODS

Subjects. Patients were recruited between March and December 2013 from the Unit of Metabolic and Genetic diseases and Unit of Pediatric Endocrinology of the Giovanni XXIII Hospital (Bari, Italy). A total of 18 patients with OI (12 females, mean age 8.86 ± 3.90) were enrolled into the study after signing an informed consent form. The local ethic committee approved the study. The study was conducted in accordance to the criteria of the declaration of Helsinki. Patients were classified according to the classic Sillence criteria [17] in type I (12 subjects), III (3 subjects) and IV (3 subjects). All patients had a daily intake of vitamin D of at least 400 IU/d, and of calcium of at least 600 mg/d, and underwent specific and individualized physiotherapy. Eight of 18 OI patients (3 with type III, 3 with type IV and 2 with severe type I) were receiving cyclic neridronate at the dose of 2 mg/kg diluted in 250 ml of saline solution infused intravenously in 30 minutes every 3 months (mean period of treatment 5.1±1.3 years; range 4.8-6.5 years) [18]. All patients had fractures throughout their life, but no one had presented fractures in the last 12 months previous the laboratory assessment.

Results of sequence analysis of *COL1A1* and *COL1A2* revealed disease-causing mutations in 15 of the 18 OI patients (83%) enrolled for this study. In the remaining 3 patients, where *COL1A1* and *COL1A2* sequence analyses were negative, the diagnosis of OI was clinically asserted by the association of frequent fractures and/or positive family history, low bone mass, blue sclera or dentinogenesis imperfecta.

A control group of 21 subjects (12 female, mean age 8.23 ± 3.19) was recruited on a voluntary basis in the outpatient clinic. It comprised children with the same age range of the OI patients and not taking any medicament, referred to our hospital for minor surgery or electrocardiographic screening.

After an overnight fast, venous peripheral blood samples were taken in opportune tubes for serum separation as well as in EDTA tubes for cell cultures and flow cytometry from patients and controls. For serum separation, the samples were opportunely centrifuged and immediately frozen at −80°C until the determination was performed.

Anthropometric measurements. Height was measured using a Harpenden stadiometer (Holtain, Crymych, UK). Weight was determined using mechanical scales (Healthometer, Bridgeview, IL). Height and weight measurements were converted to age- and sex-specific z-scores on the basis of reference data published by the Centers for Disease Control and Prevention [19].

Biochemical measurements. Serum total calcium, phosphate, and alkaline phosphatase were measured using colorimetric methods (Monarch; Instrumentation Laboratories Inc., Lexington, MA). Serum active intact PTH (fragments 1–84, Diasorin, Stillwater, MN) and 25-OH vitamin D (Osteo SP; Incstar Corp., Stillwater, MN) were measured by RIA. TNFα (R&D Systems, Minneapolis, MN) and C-terminal telopeptide of collagen type 1 (CTX) (Biomedica, Vienna, Austria) was measured in the sera using commercially available ELISA kits according to the manufacturer's instructions.

Dual-energy x-ray absorptiometry. BMD was measured at lumbar spine L2–L4 by dual energy X-ray absorptiometry (DPX) (ACN Unigamma X-Ray Plus; L'ACN Scientific Laboratories) and converted to SD scores (Z-scores) in relation to age and sex-matched normal population. Height also was measured at the time of BMD evaluation.

Bone cytokine assessment. The cytokine analysis was performed with the Luminex technology, which combines the principle of a sandwich immunoassay with fluorescent bead-based technology, thus allowing individual and multiplex analysis of different analytes in a single microtiter well [20]. Capture antibodies directed against the desired biomarker are covalently coupled to fluorescently dyed magnetic microspheres, each with a distinct color code or spectral address to permit discrimination of individual tests within a multiplex suspension. Coupled beads react with the sample containing the biomarker of interest. After a series of washes

to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin-phycoerytrin (SA-PE) conjugate. Phycoerytrin serves as fluorescent indicator, or reported.

The assay for OPG, SOST, DKK1 and RANKL (EMD Millipore Corporation, Billerica, MA USA) was carried out at Bioclarma srl, Torino, Italy. Human serum samples were analyzed in 96-well microplates accordingly to the recommendations of manufacturers and the contents of each well were drawn up into the Bio-Plex 100 System array reader (Bio-Rad Laboratories Bio-Rad Laboratories, Hercules California, USA), which identifies and quantifies each specific reaction based on bead color and fluorescent signal intensity. The data were finally processed using Bio-Plex Manager software (version 6.1) using five-parametric curve fitting and converted in pg/mL.

Osteoblastogenesis. Mesenchymal stem cells (MSCs), isolated as previously described [21] were plated in six well plates at the density of $10x10^4$/well, using a medium composed of α-Minimal Essential Medium (α-MEM) supplemented with 50 ng/ml Bone Morphogenetic Protein 2 (BMP2, R&D Systems, Minneapolis, MN, USA) and 10% Foetal Calf Serum (FCS, Gibco Life Technologies, Milan, Italy) or human serum obtained from controls or OI patients, in the presence or absence of 5μg/ml anti-DKK1 monoclonal Ab (mAb) (R&D Systems) or an anti-IgG Ab. After 48 hours of culture, cells were subjected to RNA extraction. In some experiments MSCs were also cultured in the presence of 50 ng/ml BMP2 and 50 ng/ml DKK1 (R&D Systems).

Osteoclastogenesis. OCs were obtained from peripheral blood mononuclear cells (PBMCs) of OI patients and controls. PBMCs were isolated by centrifugation of peripheral blood samples over Histopaque 1077 density gradient (Sigma Chemical, St. Louis, MO), diluted at 1×10^6 cells/ml in α-MEM (Life Technologies, Inc. Ltd, Uxbridge, UK) and supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Life Technologies,). To obtain fully differentiated human OCs, the PBMCs were then cultured for about 20 days in the presence or absence of 25 ng/ml recombinant human MCSF and 30 ng/ml RANKL (R&D Systems). In some experiments, PBMCs were cultured in the presence of 100 ng/ml RANK-Fc or 10 ng/ml TNF-R1-Fc (both from R&D Systems).

At the end of the culture period, mature OCs were identified as tartrate-resistant acid phosphatase-positive (TRAP+) multinucleated cells (Sigma Aldrich, Milan, Italy) containing three or more nuclei. The photomicrographs were obtained using a Leica DMIL Led (Leica Microsystems Srl, Italy) equipped with Leica Plan 10×/0.22 dicl. The microscope was connected with a Leica digital camera ICC50HD.

Flow cytometry analysis. Fresh peripheral blood samples from OI patients and controls were stained with suitable conjugated antibody to evaluate RANKL expression (PE-RANKL, R&D Systems) and to characterize OC precursors [*i.e.* PerCP-CD14, PE-CD51/61, (Beckmann Coulter, Milan, Italy) and FITC-CD11b (Miltenyi Biotec, Milan, Italy)]. Cells were incubated for 15 min at room temperature and for other 10 minute with VersaLyse reagent (Beckmann Coulter) to lyse red blood cells. TNFα positive cells were stained using TNFα-PE secretion kit, according manufacturer's instruction (Miltenyi Biotec S.r.l., Italy) and further characterized using PerCP-CD14 or FITC-CD3 (R&D Systems). Data were acquired using BD Accuri™ C6 flow cytometer (Becton Dickinson Immunocytometry System, Mountain View, CA, USA). Positivity area was determined using an isotype-matched mAb, and a total of 2000 events for each cell sub-population was acquired.

RNA isolation and real-time polymerase chain reaction (PCR) amplification. RNA extraction and reverse-transcription as well as Real-Time PCR amplification were performed, as previously described [22]. The following appropriate primer pairs were used: RANKL S: CGTTGGATCACAGCACAT, RANKL AS: GCTCCTCTTGGCCAGTC (NM_003701.3); TNF-α S: ATCTACTCCCAGGTCCTC, TNF-α AS: GATGCGGCTGATGGTGT (NM_000594.3); COLL-I S: CGTGGCAGTGATGGAAGTG, COLL-I AS AGCAGGACCAGCGTTACC (NM_000089.3); RUNX2 S GGAATGCCTCTGCTGTTATG, RUNX2 AS TTCTGTCTGTGCCTTCTGG (NM_001024630.3); OPG S: GACCACTACTACACAGACAG, OPG AS: AAGCAGAACTCTATCTCAAGG (NM_002546.3); GAPDH S: TCATCCCTGCCTCTACTG, GAPDH AS: TGCTTCACCACCTTCTTG (NM_002046.5).

Statistical analyses. For statistical analysis, the Statistical Package for the Social Sciences (SPSS) for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA) was used. Results are presented as means ± standard deviations (SDs). The Kolmogorov-Smirnov test was applied to test for the normality of the distribution of the

studied parameters. In parameters with normal distribution, mean values were compared using unpaired Student t-test, whereas linear correlations were calculated with the Pearson's correlation coefficient. In parameters with skewed distribution, significance was assessed with Mann-Whitney test and Spearman's correlation coefficient respectively. The limit of statistical significance was set at 0.05.

RESULTS

Clinical characteristics. The most relevant characteristics of the study population are listed in Table 1. The healthy controls were significantly taller and heavier as compared to patients**.** The gender distribution was similar among groups**.** The time to last fracture ranged in all patients from 12 to 18 months. Lower BMD-Z-score was found in OI patients compared to controls ($P<0.05$). Regarding bone marker values we found that 25-OH Vitamin D, PTH, calcium, phosphorus, CTX and bone alkaline phosphatase were comparable to that found in controls (Table 2). Although, CTX levels were lower in treated compared to untreated OI patients, this difference did not reach the statistically significance. Only osteocalcin levels were higher in OI patients respect to controls ($P<0.01$).

Bone cytokine serum levels. Significant elevated DKK1 levels were measured both in untreated and treated OI patients respect to controls (Table 2). Regarding sclerostin serum levels, we found a difference not statistically significant between treated and untreated patients, and between all OI patients and controls (Table 2). Higher levels of RANKL statistically significant were found in OI patients than in controls, while OPG levels were not significantly different between OI patients and controls (Table 2).

Interestingly, in all OI patients DKK1 serum levels significantly correlated with OPG ($r=0.588$, $p=0.01$), RANKL ($r=0.571$, $p=0.02$), CTX ($r=0.464$, $p=0.05$), and osteocalcin ($r=0.684$, $p=0.04$) serum concentrations as well as with RANKL/OPG ratio ($r=0.555$, $p=0.05$) and height-SDS ($r=-0.567$, $p=0.02$). RANKL levels correlated with CTX ($r=0.700$, $p=0.002$) and sclerostin ($r=0.661$, $p=0.003$), whereas OPG with sclerostin ($r=0.661$, $p=0.004$).

Effect of Serum From OI Patients on Osteoblast Differentiation In Vitro. To assess whether the high serum levels of DKK1 would be able to inhibit OB differentiation in untreated and treated OI patients, we

cultured human MSCs from dental follicle in the basal condition (medium + 10% FCS), in conditioned media with sera obtained from patients or controls, and in the presence or absence of anti-DKK1-neutralizing antibody or an anti-IgG. To promote OB differentiation, these cells were cultured in the presence of BMP2, which can stimulate OB differentiation through a mechanism that involves Wnt/β-catenin signaling [23], as we also found (Fig.1A-B). In fact, the treatment of MSCs with 50 ng/ml BMP-2 for 48 hours induced them to differentiate towards OBs expressing higher levels of the osteogenic transcription factor 329144 9160 *RUNX2* and *COLL-I* compared to untreated cells. This differentiation was inhibited in the same cultures that were concomitantly cultured with BMP-2 and 50 ng/ml recombinant human DKK1 (Fig. 1A), as demonstrated by the down-regulation of *COLL-I* and *RUNX2* expression (Fig. 1B).

In the cultures treated with BMP2 and serum from controls, the presence or absence of the anti-DKK1-neutralizing mAb did not exert effects on the expression of *COLL-I* and *RUNX2* (Fig 1C-1D). Conversely, we found that conditioned medium containing the serum from OI patients and anti-DKK1-neutralizing mAb significantly increased the *COLL-I* and *RUNX2* expression in the differentiated cells after 48 h of culture. No effect was found using anti-IgG antibody.

On the basis of the knowledge that OPG and RANKL expression can be affected by Wnt inhibitors [14] and we also showed for OPG (Fig. 2A), we investigated whether in our culture system the serum from OI patients could affect, through DKK1 production, the expression of these cytokines. In MSCs cultured with BMP2 and the serum from patients, the anti-DKK1-neutralizing mAb strongly up-regulated OPG expression (Fig 2B). No effect was exerted by anti-IgG antibody. The anti-DKK1 antibody did not affect RANKL expression (data not shown).

OPG and RANKL expression on leukocytes from OI patients. The obtained results prompted us to evaluate OPG and RANKL expression on circulating leukocytes as additional source of these cytokines. Interestingly, by real time PCR we demonstrated that OPG mRNA levels were significantly reduced (3 fold) both in untreated and treated OI patients compared to controls, whereas RANKL mRNA levels were significantly higher (2 fold) in OI patients than in controls (Fig. 3A-3B). The high RANKL expression in OI patients was also showed by flow cytometry analysis (Fig. 3C). OI patients also showed higher TNFα (2 fold) mRNA levels compared to controls (Fig. 3Di), which flow cytometry analysis revealed to be mainly expressed on monocytes (Fig 3Dii-

Div). OI patients also presented an elevated percentage of circulating OC precursors identified as CD14+CD11b+CD51/CD61, respect to the controls (Fig 4, A-B).

These results let us to evaluate OC formation in cultures of PBMCs from both untreated and treated OI patients and controls. A high number of large multinucleated (nuclei > 3) $TRAP^+$ OCs were identified in the unstimulated PBMC cultures from OI patients (Fig. 4E) respect to the few OCs in the cultures from controls (Fig. 4D). The addition of M-CSF and RANKL to PBMC cultures from patients did not significantly change the OC number (Fig. 4H). Conversely, M-CSF and RANKL significantly affected osteoclastogenesis in PBMCs of controls (Fig. 4G). No statistical significant differences in OC formation were found between treated and untreated patients as well as among the different types of OI.

Furthermore, we demonstrated an inhibitory effect exerted by RANK-Fc and TNF-R1-Fc on osteoclastogenesis in unfractionated PBMC cultures from OI patients. This inhibition was much more evident when the two molecules were simultaneously added to the culture media (Fig. 4L).

DISCUSSION

The results of the present study highlighted the elevated levels of DKK1 and RANKL in the sera of OI children, despite intravenous neridronate treatment, and the possible involvement of these cytokines in the altered bone cell activity associated to this disease. CTX levels were lower in treated than untreated patients, although it was reported that bisphosphonates modify levels of CTX. However, this difference did not reach the statistically significance probably due to the different types of OI and the different duration of treatment (range 4.8-6.5 years) [24]. Moreover, a significant increase in osteocalcin serum levels was observed in our OI patients, in particular in those with the most severe form of the disease (OI type III and IV), according to previous studies [25]. High osteocalcin levels were also found in children with Haemophilia A, who have reduced BMD and are at risk for developing osteoporosis and fractures later in life [26]. Our OI patients showed reduced BMD associated to a high number of fractures throughout their life. In OI patients as well as in haemophilics it would seem to be a compensatory increase in the bone formation rate demonstrated by high osteocalcin levels [25,26].

DKK1 is a critical regulator of bone mass [13], and its role as bone-remodeling regulator in pathological conditions has been established [10, 14, 15]. It inhibits Wnt signaling, thus resulting in the decrease of bone

formation and increase of bone resorption [22]. Consistently, we found that the sera obtained both from OI children untreated with bisphosphonates and OI subjects treated with intravenous neridronate can directly inhibit OB differentiation *in vitro* as well as OPG expression, and these effects were neutralized by the addition of an anti-DKK1 antibody in the same cultures. The possible key role of DKK1 in the regulation of bone formation and resorption in OI children is further sustained by our findings demonstrating that the high serum levels of DKK1 significantly correlated with those of OPG, RANKL and CTX. Previous studies have demonstrated the relationship between DKK1 and RANKL levels in osteosarcoma [27], glucocorticoid induced osteoporosis [22], as well as in *in vitro* studies using vascular progenitor cells [28] and murine mesenchymal stem cells [29]. The relationship between DKK1 and OPG has also been demonstrated in OB precursors [14]. In agreement with our findings, it was demonstrated that high DKK1 serum levels correlate with CTX amounts in multiple myeloma, in chronic glucocorticoid treatment [22, 30], in mastocytosis [31] and in rheumatoid arthritis [32]. In patients with OI bone turnover is generally increased but an increase also in RANKL/OPG ratio and its positive correlation with DKK1 could confirm *in vivo* the prevalence of bone resorption showed *in vitro* and the possible role of DKK1.

Interestingly, the pharmacological treatment does not seem significantly affect the levels of these cytokines. In detail, the serum levels of sclerostin were not significantly increased in treated OI patients compared to untreated, in contrast to that reported in postmenopausal women [24]. This difference could be due to the different disease and to the use of another ELISA kit.

Interestingly, we also found that lymphomonocytes from OI children expressed high amounts of RANKL and low levels of OPG, thus supporting the high osteoclastogenic potential of PBMCs from these patients. In fact, the neutralization of RANKL through RANK-Fc inhibited osteoclastogenesis. Additionally, we found that in OI children the high osteoclastogenesis potential of PBMCs is sustained by the elevated percentage of circulating OC precursors and TNFα. The major source of these cytokines is represented by monocytes.

Although our study included only 18 patients, and a limitation is that the results on osteoblastogenesis were not referred to OBs isolated from the patients, our results are in agreement with literature data. In detail, previous studies on OI performed both in patients and murine models (*oim/oim, Brtl*) demonstrated the altered activity of both OBs and OCs [33-37]. In particular, a high bone turnover, with increased OB and OC surfaces, increased bone formation rate, and decreased mineral apposition rate have been observed in pediatric OI (type

III and IV) [5]. In addition, an increased osteoclastic activity was demonstrated in children with OI in bone biopsies [5, 33-35] and *Brtl* model [36]. The high OC formation in OI models seems to be sustained by OBs as well as to intrinsic properties of OC precursors, thus supporting at least in part our results [37]. In particular, the formation of a greater number of OCs occurred in co-cultures of OB lineage cells derived from *oim/oim* mice with bone marrow macrophages [37]. Li et al. revealed that less mature OB lineage cells exhibit an increased OC inductive potential due to an higher RANKL/OPG ratio compared to differentiated OBs [37]. The same authors demonstrated an increase of TNFα expression in oim/oim OBs compared with wild type OBs [37], as well as a partial inhibition of osteoclastogenesis using a TNFα antibody in the co-culture with *oim/oim* OBs [37]. Therefore, the higher number of OCs and their enhanced resorptive activity in co-culture with *oim/oim* OBs can be explained by the synergistic effect of TNFα, highly expressed by immature OBs, along with RANKL and M-CSF also produced by OBs. In addition, it has been hypothesized that the defective collagen present in OI may also trigger the secretion of inflammatory cytokines [36]. This hypothesis is also supported by the increase of PGE_2 [38] that other authors found in OI, and this permits us to explain our results which show the "commitment" of monocytes from OI patients to become OCs, as well as the alteration of the RANKL/OPG ratio in circulating cells.

In agreement with our results, the "commitment" of monocytes has also been demonstrated in the *Brtl* model of OI. In particular, Uveges et al. reported the increased RANK levels in *Brtl* mice, although a normal RANKL/OPG ratio is maintained. They also found that TRAP+ precursors are markedly elevated in Brtl marrow cultures and form more OCs, suggesting that OC increases arise from more RANK-expressing precursors. These literature data support our results demonstrating the high percentage of circulating OC precursor in OI children.

All these findings prompted some researchers to neutralize RANKL both in OI murine models and patients [39-45]. In detail, first studies showed that the alendronate increased BMD, alter geometric and biomechanical properties of *oim/oim* bone and reduce fractures in these mice [39-41], whereas RANKL inhibition also increased BMD and alter geometric and biomechanical properties [41, 42], but has no discernible effect on fracture incidence [43]. It was hypothesized that the lack of fracture reduction with RANKL inhibition was due to a relatively late start of treatment (6 weeks of age) and a high baseline number of fractures [43, 44]. Recently, Bargman et al. compared bisphosphonate therapy and RANKL inhibition in neonatal *oim/oim* and

wild-type mice and assessed that they are equally effective in decreasing fracture incidence and improving bone properties [45]. Furthermore, successful results have been obtained treating patients with type VI OI with anti-RANKL antibody (Denosumab).

In conclusion, our results demonstrate that OI children have high levels of RANKL, despite the treatment with bisphosphonates, further sustaining the importance of neutralizing RANKL in OI patients, and simultaneously highlight the key role of DKK1 and the beneficial effect that could derive from its neutralization. We cannot exclude that the neutralization of DKK1 could simultaneously improve OB and OC activity in OI.

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FIGURE LEGENDS.

Figure 1. Effect of serum from OI patients on osteoblastic differentiation of mesenchymal stem cells in *vitro*. Human Mesenchymal stem cells were cultured with 50 ng/ml BMP2 in basal condition (medium + 10% FCS) in presence or in absence of 50 ng/ml DKK1 (A-B) as well as in conditioned media with sera from controls or patients in the presence or in absence of 5μg/ml anti-DKK1 neutralizing antibody (C-D). In all the described condition the expression of collagen I (COLL-I) and RUNX2 was evaluated by real time PCR. The histograms represent the mean values ± S.E. of six independent experiments performed using 6 controls, 6 untreated and 6 six treated OI patients.

Figure 2. Effect of serum from OI patients on the OPG expression in mesenchymal stem cells differentiated toward osteoblast. Human Mesenchymal stem cells were cultured with BMP2 in basal condition (medium + 10% FCS) in presence or in absence of 50 ng/ml DKK1 (A) as well as in conditioned media with sera from patients or controls, in presence or in absence of 5μg/ml anti-DKK1 neutralizing antibody (B). In all the described condition OPG expression was evaluated by real time PCR. The histograms represent the mean values ± S.E. of six independent experiments performed using 6 controls, 6 untreated and 6 six treated OI patients.

Figure 3. RANKL, OPG and TNFα expression on lymphomonocytes from OI patients. mRNA levels of RANKL (A) and OPG (B) in lymphomonocytes from all controls and OI patients. Flow cytometry analysis of RANKL on PBMCs from controls and OI patients (Panel C). mRNA levels TNFα in lymphomonocytes from all controls and OI patients were reported (Di). Flow cytometry dot plots showed TNF-α expression on $CD14^+$ monocytes, from a representative OI patient (Dii) and a representative control (Diii). The histograms reported the results arising from the evaluation of TNFα levels in all OI patients and controls by flow cytometry (Div).

Figure 4. Osteoclast precursors and osteoclastognesis in OI patients. A representative flow cytometry dot plots showed the percentage of circulating osteoclast (OC) precursors, identified as CD14+/CD11b+/CD51-61+ cells, in controls (A) and OI patients (B). The histograms reported the results arising from the evaluation of circulating OC precursors in all OI patients and controls by flow cytometry (C).

Tartrate-resistant acid phosphatase-positive ($TRAP^+$) and multinucleated cells with three or more nuclei, identified as OCs, were obtained under different culture conditions from peripheral blood mononuclear cells (PBMCs) from controls and OI patients. Few small OCs were observed in unstimulated PBMC cultures from

a representative control (D). The formation of numerous multinucleated $TRAP^+$ cells can be observed in unstimulated PBMCs from a representative OI patient control (E). The graph represents the results arising for the unstimulated PBMC cultures performed from all the enrolled subjects (F). In parallel, in unfractioned PBMCs from the controls, the formation of OCs can be observed after the addition of M-CSF and RANKL (G). Unfractionated PBMCs from OI patients were stimulated with M-CSF and RANKL (H) leading to the formation of multinucleated $TRAP^+$ cells. The graph represents the results arising for the stimulated PBMC cultures performed from all the enrolled subjects (I). The formation of multinucleated $TRAP^+$ OCs was evaluated in unstimulated PBMCs from 5 untreated and 4 treated OI patients cultured in the absence or presence of 100 ng/ml RANK-Fc and/or 10 ng/ml TNF-R1-Fc (L).

Table 1. Anthropometric, clinical and densitometric data of study population.

	Controls N=21	All OI patients N=18	OI type I* N=12	OI type III* N=3	OI type IV* N=3
Gender (male/female)	9/12	6/12	4/8	1/2	1/2
Age (yr)	8.23 ± 3.19	8.86 ± 3.90	9.17 ± 0.38	9.68 ± 5.71	6.70 ± 2.43
Height SDS	0.36 ± 1.02	-1.54 ± 1.80§	-0.89 ± 0.97§	-5.16 ± 0.23§	-2.77 ± 0.42§
Weight SDS	0.43 ± 0.87	-0.67 ± 1.57§	-0.02 ± 0.93§	-3.75± 0.56§	-2.15 ± 0.74§
BMI-SDS	0.25 ± 0.78	0.24 ± 0.85	0.36 ± 0.93	-0.01 ± 0.81§	-0.35 ± 0.64§
BMD-Z-score	0.13 ± 0.80	-1.26 ± 1.04	-2.5 ± 0.78§	-1.85 ± 0.98§	-1.20 ± 1.03§
Blue sclera		11	11	0	0
Dentinogenesis imperfecta		2	1	1	0
Number of fractures (0/-10/+10)	21/0/0	4/10/4	4/6/2	0/1/2	0/3/0
Limited mobility	-	5	1	3	1
Scoliosis	-	9	4	3	2
Leg deformities	-	9	4	3	2

SDS: standard deviation score; BMI: body mass index; BMD: bone mineral density.

*According to Sillence et al. [17]

§ p <0.05 referred to controls

Table 2. Biochemical results of the study population.

	Controls N=21	All OI patients N=18	Untreated OI patients	Treated OI patients	OI type I* N=12	OI type III* N=3	OI type IV* N=3
25-OH Vitamin D (ng/ml)	38.6 ± 14.7	31.7 ± 6.4	29.3 ± 7.2	33.6 ± 2.3	35.0 ± 9.3	30.0 ± 4.4	36.8 ± 7.7
Osteocalcin (ng/ml)	38.3 ± 19.2	68.6 ± 22.8§	67.2 ± 28.3§	53.5 ± 12.7§	54.4 ± 27.7§	75.8 ± 15.9§	71.8 ± 17.5§
PTH (pg/ml)	43.0 ± 15.1	44.1 ± 17.1	44.6 ± 18.9	42.6 ± 17.7	42.2 ± 12.1	49.5 ± 15.6	38.9 ± 13.2
Ca (mg/dl)	9.71 ± 0.40	9.74 ± 0.46	9.83 ± 0.47	9.45 ± 0.35	10.0 ± 0.1	9.42 ± 0.32	10.1 ± 0.6
P (mg/dl)	4.54 ± 1.40	4.75 ± 0.58	4.81 ± 0.67	4.53 ± 0.01	5.27 ± 0.41	4.65 ± 0.16	5.06 ± 1.12
B-ALP (µg/l)	109.8 ± 39.2	98.3 ± 56.1	110.9 ± 58.1	86.1 ± 30.1	112.3 ± 47.3	99.8 ± 59.9	93.9 ± 37.3
CTX (ng/ml)	1.57 ± 0.46	1.28 ± 0.77	1.64 ± 0.61	1.05 ± 0.67	1.42 ± 0.81	1.10 ± 0.25	1.20 ± 0.45
DKK1 (pg/ml)	3521 ± 1173	4516 ± 890§	4431 ± 785§	4367 ± 1127§	4304 ± 804§	4806 ± 649§	4165 ± 1100§
Sclerostin (pg/ml)	1365 ± 267	1915 ± 984	1719 ± 867	1896 ± 1325	1942 ± 955	1231 ± 529	1299 ± 820
RANKL (pg/ml)	87.2 ± 17.8	113.0 ± 79.5§	107.5 ± 13.1§	167.2 ± 89.6§	125.2 ± 83.7§	108.2 ± 15.2§	114.2 ± 14.4§
OPG (pg/ml)	436.7 ± 95.5	500.2 ± 75.1	497.3 ± 82.6	506.8 ± 83.1	491.0 ± 85.0	510.7 ± 50.3	506.0 ± 49.0

PTH: parathyroid hormone; Ca: calcium; P: phosphorus; B-ALP: bone alkaline phosphatase; CTX: C-terminal telopeptide of type I collagen; DKK1: dickkopf-1; RANKL: receptor activator of nuclear factor kappa-B ligand; OPG: osteoprotegerin.

*According to Sillence et al. [17]

§ p <0.01 referred to controls

Figure 1

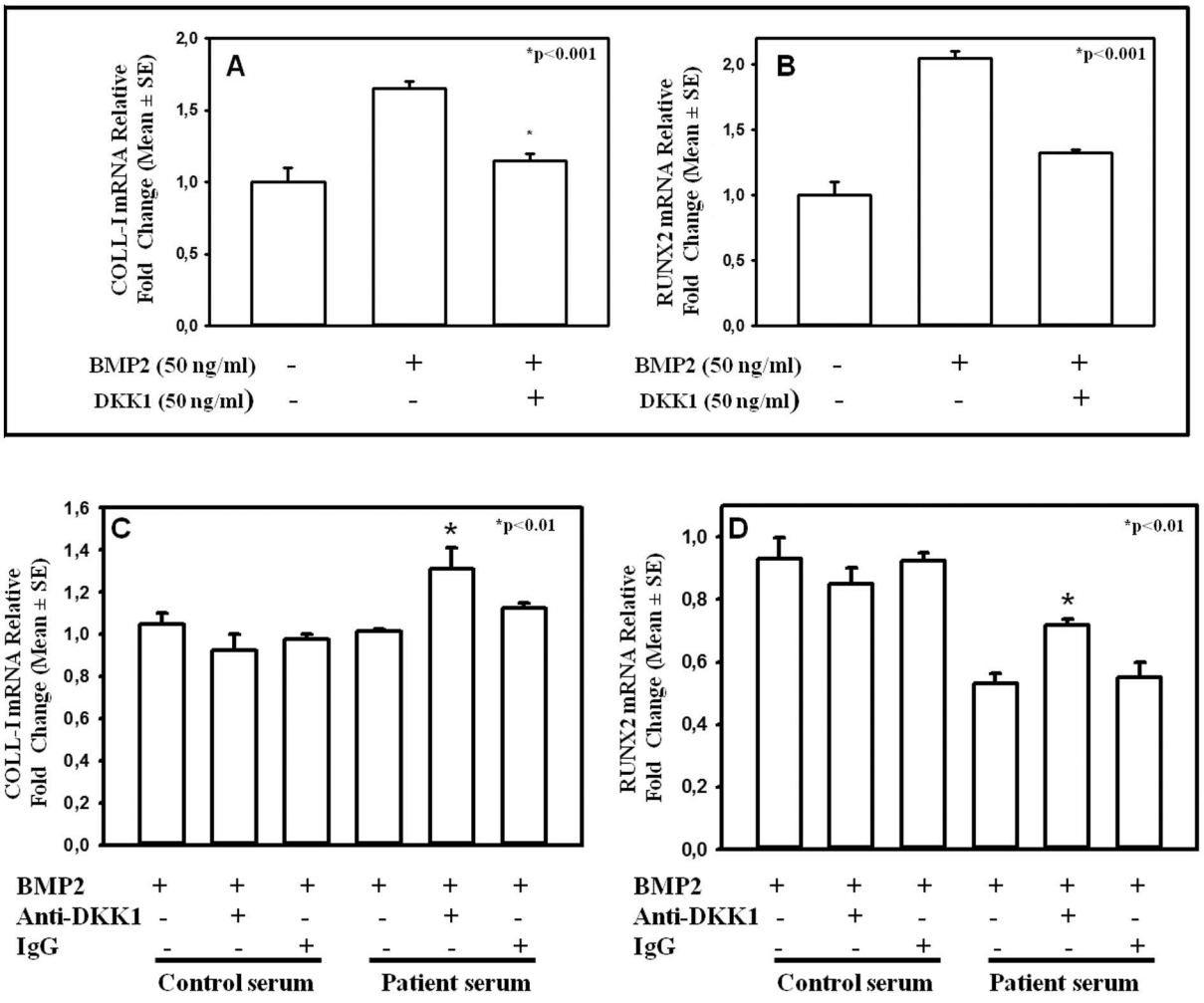

A
*p<0.001
COLL-I mRNA Relative Fold Change (Mean ± SE)
2,0
1,5
1,0
0,5
0,0
*
BMP2 (50 ng/ml) - + +
DKK1 (50 ng/ml) - - +
B
*p<0.001
RUNX2 mRNA Relative Fold Change (Mean ± SE)
2,0
1,5
1,0
0,5
0,0
BMP2 (50 ng/ml) - + +
DKK1 (50 ng/ml) - - +
C
*p<0.01
COLL-I mRNA Relative Fold Change (Mean ± SE)
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
*
BMP2 + + + + + +
Anti-DKK1 - + - - + -
IgG - - + - - +
Control serum
Patient serum
D
*p<0.01
RUNX2 mRNA Relative Fold Change (Mean ± SE)
1,0
0,8
0,6
0,4
0,2
0,0
*
BMP2 + + + + + +
Anti-DKK1 - + - - + -
IgG - - + - - +
Control serum
Patient serum

Figure 2

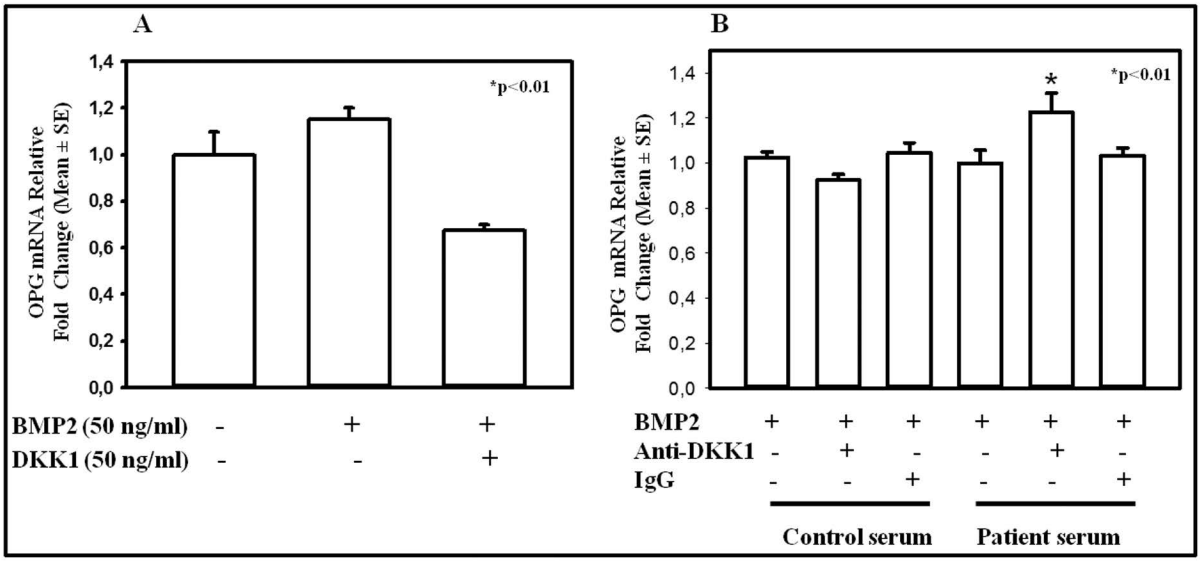

A
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
OPG mRNA Relative
Fold Change (Mean ± SE)
*p<0.01
BMP2 (50 ng/ml) - + +
DKK1 (50 ng/ml) - - +
B
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
OPG mRNA Relative
Fold Change (Mean ± SE)
*p<0.01
*
BMP2 + + + + + +
Anti-DKK1 - + - - + -
IgG - - + - - +
Control serum
Patient serum

Figure 3

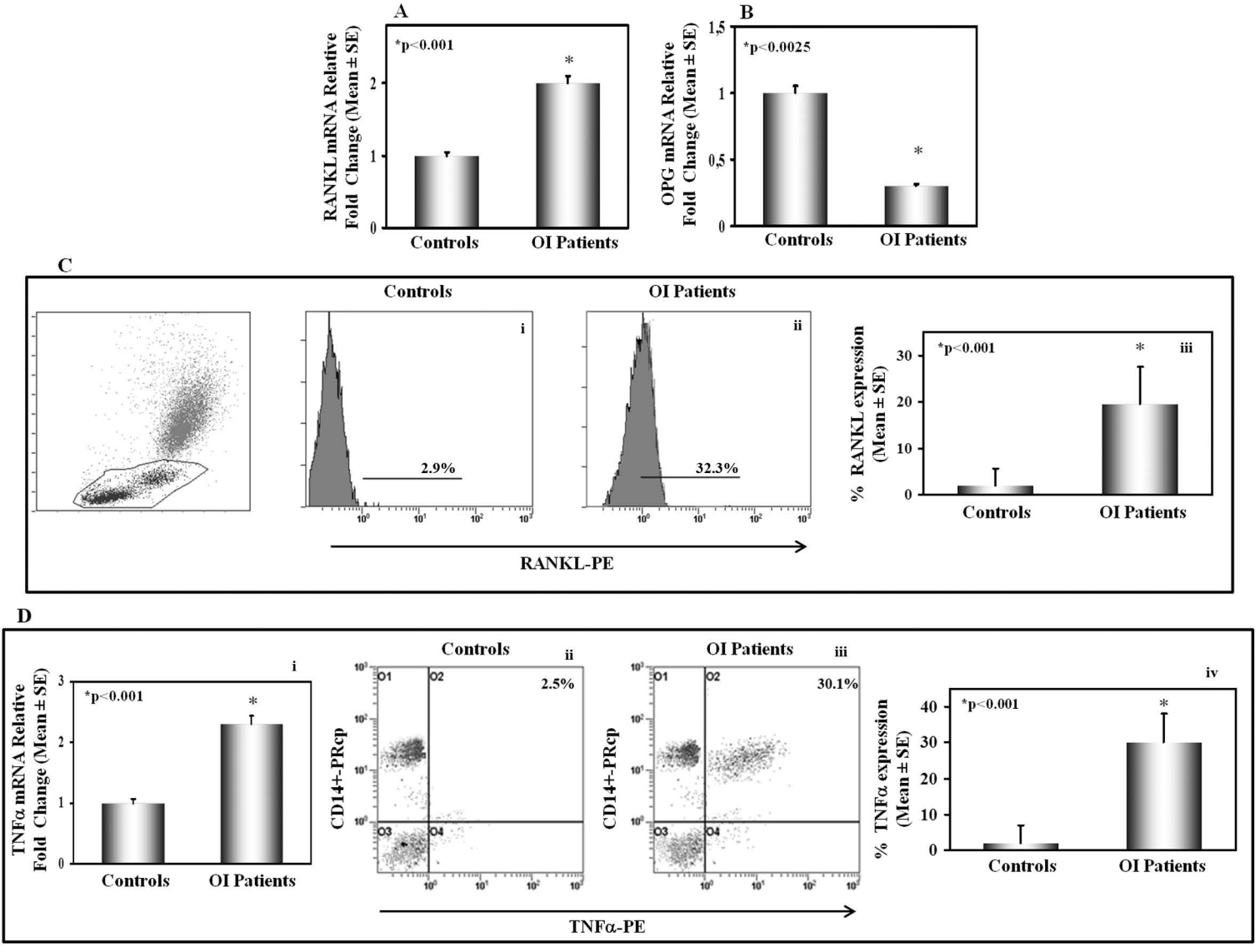
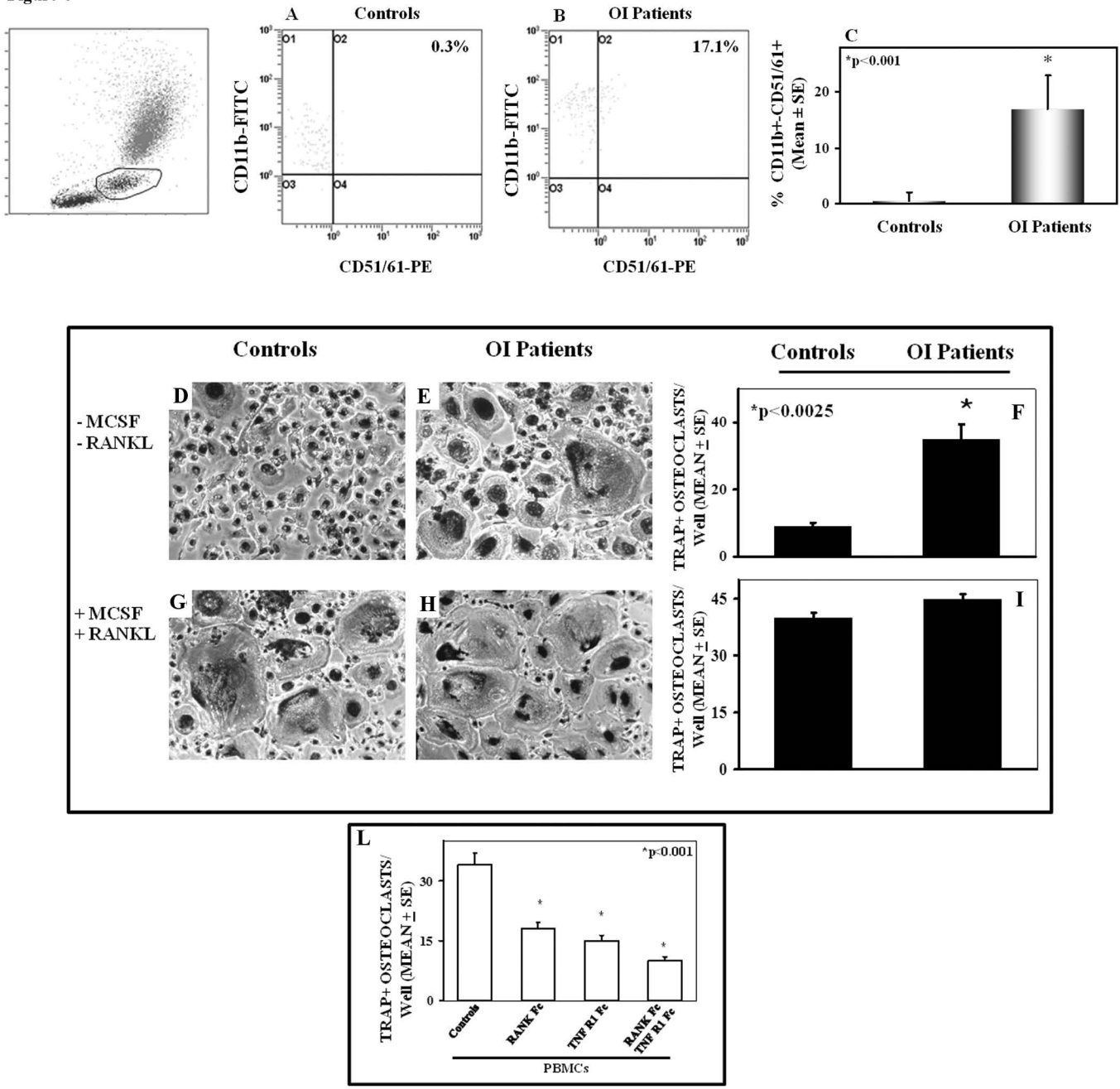

A
RANKL mRNA Relative Fold Change (Mean ± SE)
*p<0.001
Controls
OI Patients
B
OPG mRNA Relative Fold Change (Mean ± SE)
*p<0.0025
Controls
OI Patients
C
Controls
OI Patients
i
ii
2.9%
32.3%
RANKL-PE
% RANKL expression (Mean ± SE)
*p<0.001
iii
Controls
OI Patients
D
TNFα mRNA Relative Fold Change (Mean ± SE)
*p<0.001
i
Controls
OI Patients
CD14+-PRcp
TNFα-PE
ii
2.5%
iii
30.1%
% TNFα expression (Mean ± SE)
*p<0.001
iv
Controls
OI Patients

Figure 4


A
Controls
0.3%
B
OI Patients
17.1%
CD11b-FITC
CD51/61-PE
C
*p<0.001
% CD11b+-CD51/61+ (Mean ± SE)
Controls
OI Patients
D
E
F
G
H
I
- MCSF
- RANKL
+ MCSF
+ RANKL
*p<0.0025
TRAP+ OSTEOCLASTS/ Well (MEAN ± SE)
L
*p<0.001
Controls
RANK Fc
TNF R1 Fc
RANK Fc TNF R1 Fc
PBMCs