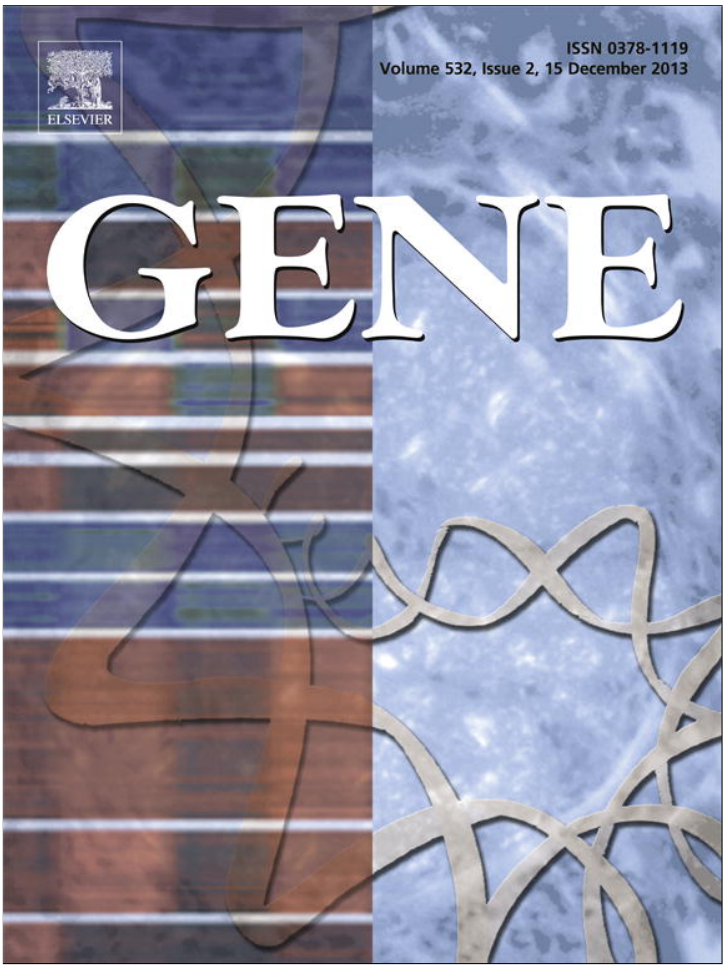

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Uncoupling of osteoblast–osteoclast regulation in a chemical murine model of Gaucher disease

Juan M. Mucci [a], Florencia Suqueli García [a], Pablo N. de Francesco [a], Romina Ceci [a], S. Di Genaro [b], Carlos A. Fossati [a], M. Victoria Delpino [c], Paula A. Rozenfeld [a,*]

[a] LISIN, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata 1900, Argentina
[b] Laboratorio de Inmunopatologia y Citometria de Flujo, Universidad Nacional de San Luis, San Luis, Argentina
[c] Instituto de Inmunología, Genética y Metabolismo (INIGEM), Hospital de Clínicas "José de San Martín", Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

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ABSTRACT

Gaucher disease (GD) is caused by mutations in the GBA gene that confer a deficient level of activity of glucocerebrosidase (GCase). This deficiency leads to accumulation of the glycolipid glucocerebroside in the lysosomes of cells of monocyte/macrophage system. Type I GD is the mildest form and is characterized by the absence of neuronopathic affection. Bone compromise in Gaucher disease patients is the most disabling aspect of the disease. However, pathophysiological aspects of skeletal alterations are still poorly understood.

The homeostasis of bone tissue is maintained by the balanced processes of bone resorption by osteoclasts and formation by osteoblasts. We decided to test whether bone resorption and/or bone formation could be altered by the use of a chemical in vitro murine model of Gaucher disease.

We used two sources of cells from monocyte/macrophages lineage isolated from normal mice, splenocytes (S) and peritoneal macrophages (PM), and were exposed to CBE, the inhibitor of GCase (S-CBE and PM-CBE, respectively). Addition of both conditioned media (CM) from S-CBE and PM-CBE induced the differentiation of osteoclasts precursors from bone marrow to mature and functional osteoclasts. TNF-α could be one of the factors responsible for this effect. On the other hand, addition of CM to an osteoblast cell culture resulted in a reduction in expression of alkaline phosphatase and mineralization process. In conclusion, these results suggest implication of changes in both bone formation and bone resorption and are consistent with the idea that both sides of the homeostatic balance are affected in GD.

1. Introduction

Gaucher disease (GD), the most prevalent inherited lysosomal storage disease, is caused by mutations in *GBA* gene that confer a deficient level of activity of glucocerebrosidase (GCase). This deficiency leads to accumulation of the glycolipid glucocerebroside in the lysosomes of cells of monocyte/macrophage system. Glucocerebroside accumulation results in engorged cells called "Gaucher cells". Symptoms and pathology of Gaucher disease result from the accumulation of Gaucher cells in various organ systems. There are 3 main clinical phenotypes of Gaucher disease, being type I the most prevalent one (Beutler, 1995).

Type I GD is the mildest form and is characterized by the absence of neuronopathic affection. Affected organs include spleen, liver, lung, kidney, bone and bone marrow, and patients may exhibit hepatosplenomegaly, anemia, thrombocytopenia and skeletal and bone marrow pathology. Bone compromise in GD patients is the most disabling aspect of the disease, causing high morbidity and a significant decrease in quality of life. However, pathophysiological aspects of skeletal alterations are still poorly understood (Cox, 2010).

Bone pain is common among patients with GD, and can manifest as acute episodes or being chronic. An alteration in remodeling of the metaphyseal region of the femur results in the typical but not pathognomonic "Erlenmeyer flask deformity". Reduced bone mineral density is observed in most patients, and imposes an increased risk of fractures. Osteosclerosis and osteonecrosis can also occur (Deegan et al., 2011; Wenstrup et al., 2002).

The homeostasis of bone tissue is maintained by the balanced processes of bone resorption by osteoclasts and formation by osteoblasts. Imbalance can give rise to a broad spectrum of skeletal pathologies, of

Abbreviations: GD, Gaucher disease; GBA, glucocerebrosidase gene; GCase, glucocerebrosidase; TNF-α, tumor necrosis factor alpha; TNFRp55, cell surface receptor of TNF-α; TNFRKO, Knock out mice for cell surface receptor of TNF-α; PM, peritoneal macrophages; S, Splenocytes; CBE, Conduritol-beta-epoxide; CM, Conditioned media; M-CSF, macrophage colony stimulating factor; α-MEM, α-minimum essential medium; MMP, Matrix metalloproteinases; RANKL, Receptor activator of nuclear factor kappa-B ligand; TRAP, tartrate-resistant acid phosphatase; SDS, sodium dodecyl sulfate; MC3T3, Murine osteoblasts cell line; ARS, Alizarin Red S.

* Corresponding author at: LISIN, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 y 115, La Plata 1900, Argentina. Tel.: +54 221 4250497; fax: +54 221 4226947.
E-mail address: paurozen@biol.unlp.edu.ar (P.A. Rozenfeld).

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which osteoporosis, characterized by a decrease in bone density, is the best known.

Causes of disruption of bone homeostasis in Gaucher disease have not been elucidated yet, however, some bibliographic reports revealed alterations in function of both osteoclasts and osteoblasts. One of the hypotheses to explain diminished bone density is an augmented bone resorption by osteoclasts. Increased osteoclastogenesis has been recently shown by different authors, and TNF-α could be implicated in this process (Mucci et al., 2012; Reed et al., 2013). On the other side, osteopenia could be associated to a reduced osteoblastic bone formation. In fact, a recent type I GD murine model presented with diminished bone acquisition during growth (Mistry et al., 2010).

Taking into consideration that both sides of the homeostatic balance have been shown to be altered using different experimental models, we decided to test weather bone resorption and/or bone formation could be altered by the use of a chemical *in vitro* murine model of GD.

2. Materials and methods

2.1. Animals

Six- to 8-week-old female C57Bl/6 mice and TNFRp55 KO mice (on the C57BL/6 background) (TNFRKO) were provided by University of La Plata and University of San Luis (Argentina), respectively. Animals were housed in groups of five, under controlled temperature (22 °C) and artificial light under a 12-h cycle period. Mice were provided with sterile food and water ad libitum.

2.2. Cells and conditioned media preparation

Thioglycolate-elicited peritoneal macrophages (PM) were isolated as described previously (Giambartolomei et al., 2004) from C57BL/6 WT mice. Splenocytes (S) from C57BL/6 WT were obtained by homogenization of spleen. These cells were cultured in AIM-V media (Invitrogen, Carlsbad, CA, USA) at 37 °C in a 5% CO_2 atm to a number of 10^6 cells/ml for 72 h in the presence or absence of conduritol-beta-epoxide (CBE) 500 mM (Sigma, St Louis, MO, USA). The effect of CBE was tested, demonstrating the inhibition of glucocerebrosidase activity and glucocerebroside accumulation, as previously described (Mucci et al., 2012). Conditioned media (CM) was obtained by centrifugation of the cultures.

2.3. Osteoclast formation assay

Bone marrow-derived monocytes from C57Bl/6 mice or TNFRp55 KO mice were seeded at 5×10^5 cells/ml and cultured at 37 °C in 5% CO_2 atm in α-minimum essential medium (α-MEM) supplemented with 2 mM L-glutamine, 10% heat inactivated fetal bovine serum (Gibco-BRL, Life Technologies, Grand Island, NY), 100 U of penicillin per ml and 100 μg of streptomycin per ml (complete medium) and 30 ng/ml of recombinant murine macrophage colony stimulating factor (M-CSF) (R&D, Minneapolis, MN, USA) for 48 h. Non-adherent cells were washed out and adherent cells were used for the osteoclast formation assays. For all the assays the adherent cells were cultured in CM and complete medium in a 1:1 ratio supplemented with M-CSF for 7 days replacing the media every 48 h. The supernatant was harvested to assess for the presence of MMP. As positive control of osteoclast formation cultures received 50 ng/ml of murine RANKL or TNF-α (Millipore, Billerica, MA, USA). As a negative control cultures received CBE in complete medium. To identify osteoclasts, cells were fixed in 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP; Sigma Aldrich). TRAP-positive multinucleated (more than 3 nuclei) cells were defined as osteoclasts, and the number was determined by microscopic counts.

2.4. Assessment of vitronectin receptor expression

Vitronectin receptor (CD51) expression was determined by fluorescent microscopy using a FITC-labeled anti-human CD51 (BD Pharmingen, San Diego, CA). DAPI was used to stain for nuclei. CD51-positive multinucleated (more than 3 nuclei) cells were defined as osteoclasts.

2.5. Pit formation assay

Bone marrow-derived monocytes (2×10^4 cells/0.25 ml/well) were plated on dentine disks (BD BioCoat™ Osteologic™, San Diego, CA) in 96-well culture dishes and cultured in CM and complete medium in a 1:1 ratio containing M-CSF (30 ng/ml) for 6 days. Media and all reagents were replaced every day to avoid acidification of medium. After culture with cells, dentine disks were washed with 1 M NH_4OH to remove adherent cells. After rinsing with water, dentine disks were visualized by light microscopy to determine resorption lacunae.

2.6. Zymography

MMP levels were assayed by zymography according to the method of Hibbs et al. [22]. Briefly, 20 μl of osteoclast conditioned medium mixed with 5 μl of sample buffer (0.25 M Tris [pH 6.8], 50% glycerol, 5% sodium dodecyl sulfate [SDS], and bromophenol blue crystals) were loaded per lane on 10% SDS-PAGE gels containing 0,1% of gelatin (Sigma-Aldrich, Argentina)/ml. After electrophoresis, the gels were washed with 50 mM Tris–HCl (pH 7.5)–2.5% Triton X-100 for 30 min and with 50 mM Tris–HCl (pH 7.5)–2.5% Triton X-100–5 mM $CaCl_2$–1 μM $ZnCl_2$ for 30 min and then incubated with 50 mM Tris–HCl (pH 7.5)–2.5% Triton X-100–10 mM $CaCl_2$–200 mM NaCl for 48 h at 37 °C. This denaturation/renaturation step promotes MMP activity without proteolytic cleavage of pro-MMP-9. Gelatin activity was visualized by staining gels with 0.5% Coomassie blue. Unstained bands indicated the presence of gelatinase activity, and their position indicated the molecular weight of the enzymes involved.

2.7. Osteoblast culture

Murine osteoblasts cell line MC3T3 was cultured at 37 °C in 5% CO_2 atm in complete medium and CM 1:1, containing osteogenic supplements, 1 mM sodium glycerophosphate, 50 mM L-ascorbate, and, in most cases, 10^{-8} M dexamethasone (all Sigma-Aldrich), with changes of medium every 3–4 days. As a negative control cultures received CBE in complete medium.

2.8. Quantification of mineralization by Alizarin Red S (ARS)

Monolayers in 6-well plates (10 cm2/well) were washed with PBS and fixed in 10% (v/v) formaldehyde (Sigma–Aldrich) at room temperature for 15 min. The monolayers were then washed twice with excess dH_2O prior to addition of 1 mL of 40 mM ARS (pH 4.1) per well. The plates were incubated at room temperature for 20 min with gentle shaking. After aspiration of the unincorporated dye, the wells were washed four times with 4 mL dH_2O while shaking for 5 min.

800 μL 10% (v/v) of acetic acid was added to each well, and the plate was incubated at room temperature for 30 min with shaking. The monolayer, now loosely attached to the plate, was then scraped from the plate with a cell scraper (Fisher Lifesciences) and transferred with 10% (v/v) acetic acid to a 1.5-mL microcentrifuge tube. After vortexing for 30 s, the slurry was overlaid with 500 μL mineral oil (Sigma-Aldrich), heated to exactly 85 °C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 20,000 *g* for 15 min and 500 μL of the supernatant was removed to a new 1.5-mL microcentrifuge tube. Then 200 μL of 10% (v/v) ammonium hydroxide was added to neutralize the acid. In

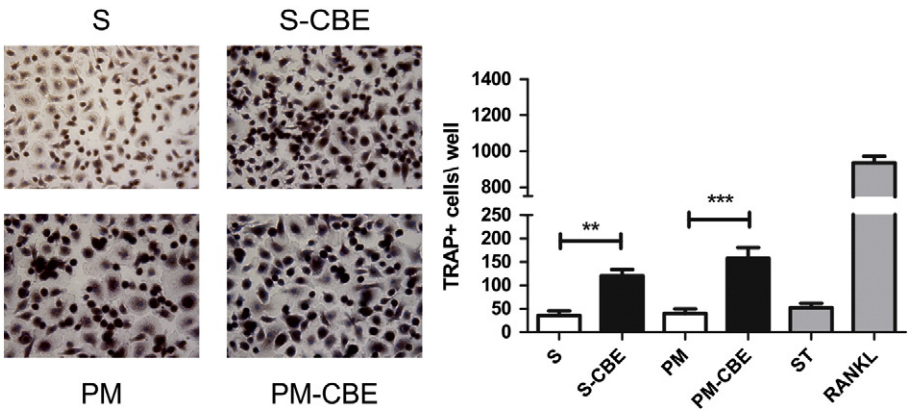

Fig. 1. Induction of differentiation of precursor cells by CM. Precursor cells exposed to CM from S, S/CBE or PM, PM/CBE were analyzed by TRAP staining and TRAP + cells showing ≥3 nuclei were counted. Bars express the mean ± sem, n = 5. $^{**}p < 0.01$, $^{***}p < 0.001$ *t*-test.

some cases, the pH was measured at this point to ensure that it was between 4.1 and 4.5. Aliquots (150 μL) of the supernatant were read in triplicate at 405 nm.

2.9. Sirius Red

Osteoblasts cultured on 24 well culture plates were washed 3 times with PBS and fixed using 250 μl of Bouin fluid for 1 h, Bouin fluid was prepared at the moment of use by mixing 15 ml of saturated picric acid with 5 ml of formaldehyde 35% v/v. After fixation, cells were washed 3 times with excess of distilled water and allowed to dry for several minutes. 1 ml of a 0.1% Sirius Red (Sigma-Aldrich) in picric acid solution was added and incubated at room temperature for 18 h with gentle shaking. After the incubation cells were washed 5 times with 0.01 N HCl to remove excess dye. For quantification 200 μl of 0.1 N NaOH were added and incubated for 30 min with shaking to dissolve the dye and the solution absorbance was measured at 550 nm.

2.10. Alkaline phosphatase

Osteoblast monolayer cultured on 24 well plates, were washed 4 times with TBS covered with 200 μl of BCIP/NBT and incubated for 10–15 min at room temperature. During incubation the color generation was followed by phase microscopy so that color saturation point was not reached. The reaction was stopped by the addition of distilled water. For quantification the ratio between the colored area and the total area occupied by cells was assayed by an image processing software.

2.11. Statistical analysis

Statistical analysis was performed with *t*-test or one-way ANOVA, followed by post hoc Tukey test using GraphPad Prism 4.0 sofware. Data are presented as mean ± SD.

3. Results

3.1. Induction of osteoclasts differentiation by conditioned media (CM)

We used two sources of cells from monocyte/macrophage lineage isolated from normal mice, splenocytes (S) and peritoneal macrophages (PM). To obtain an *in vitro* model of Gaucher disease, they were exposed to CBE, the inhibitor of GCase (S-CBE and MP-CBE, respectively). After the culture the conditioned media (CM) was collected. CM from CBE

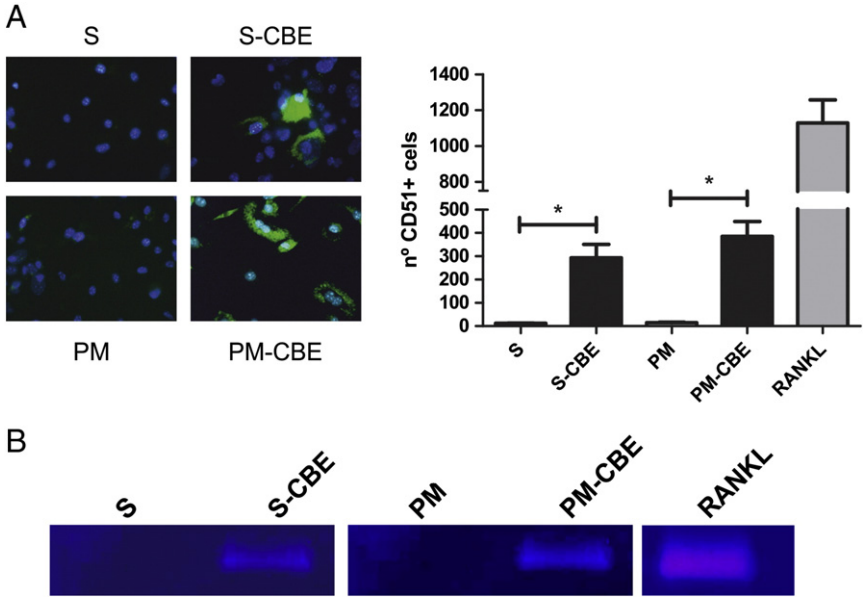

Fig. 2. Osteoclast markers on differentiated precursor cells by CM. Precursor cells exposed to CM from S, S/CBE or PM, PM/CBE were analyzed for expression of vitronectin receptor (A) and MMP-9 levels by zymography on supernatants (B). Bars express the mean ± sem, n = 5. $^{*}p < 0.05$ *t*-test.

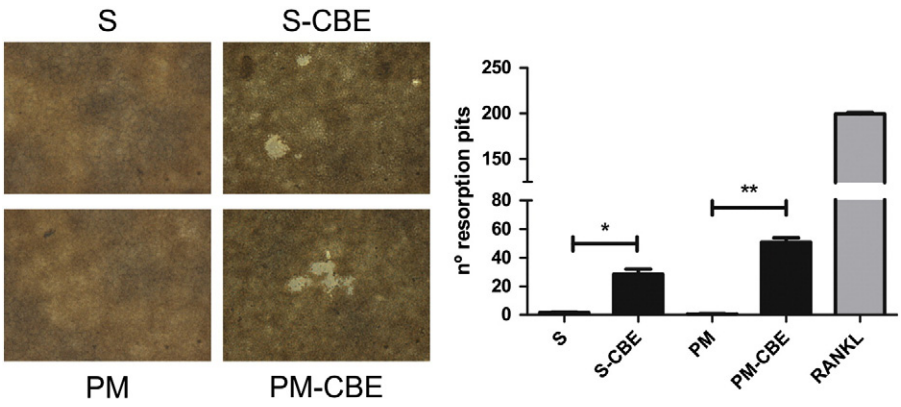

Fig. 3. Resorption activity of TRAP+ multinucleated cells. Functional activity of CM induced osteoclasts was determined by their ability to resorb dentine. Osteoclastogenesis experiments were performed on dentine disks under the same conditions as described above. After 5 days, cells were removed, and dentine resorption was determined by light microscopy, and the number of resorption pits was counted. Bars express the mean ± sem, n = 5. *p < 0.05, **p < 0.01 *t*-test.

treated (S-CBE, PM-CBE) or untreated (S, PM) cells was added to a culture of murine bone marrow cells with the aim to analyze if the cells from the models secrete factors that induce osteoclastogenesis.

Addition of both CM from S-CBE and PM-CBE induced the differentiation of osteoclast precursors presented in bone marrow to osteoclasts, as evidenced by the presence of higher numbers of multinucleated $TRAP^+$ cells (Fig. 1) in those cultures.

3.2. Assessment of markers of osteoclasts

We then decided to characterize markers of these multinucleated $TRAP^+$ cells, to analyze if they behave as mature and functional osteoclasts. Mature osteoclasts produce and secrete MMP, enzymes that degrade extracellular matrix. Production of MMP by these cells was tested by zymography. Results shown in Fig. 2 reveal the presence of higher levels of MMP in supernatant from bone marrow cells exposed to CM from S-CBE and MP-CBE, as compared to their controls. The molecular weight of the band corresponds to MMP-9, the typical MMP secreted by osteoclasts.

Vitronectin receptor is a typical marker of osteoclasts in bone. Staining of vitronectin receptor in cells exposed to CM was analyzed by immunofluorescence. A higher number of positive cells were obtained when they were differentiated by the addition of CM from S-CBE and MP-CBE as compared to controls (Fig. 2).

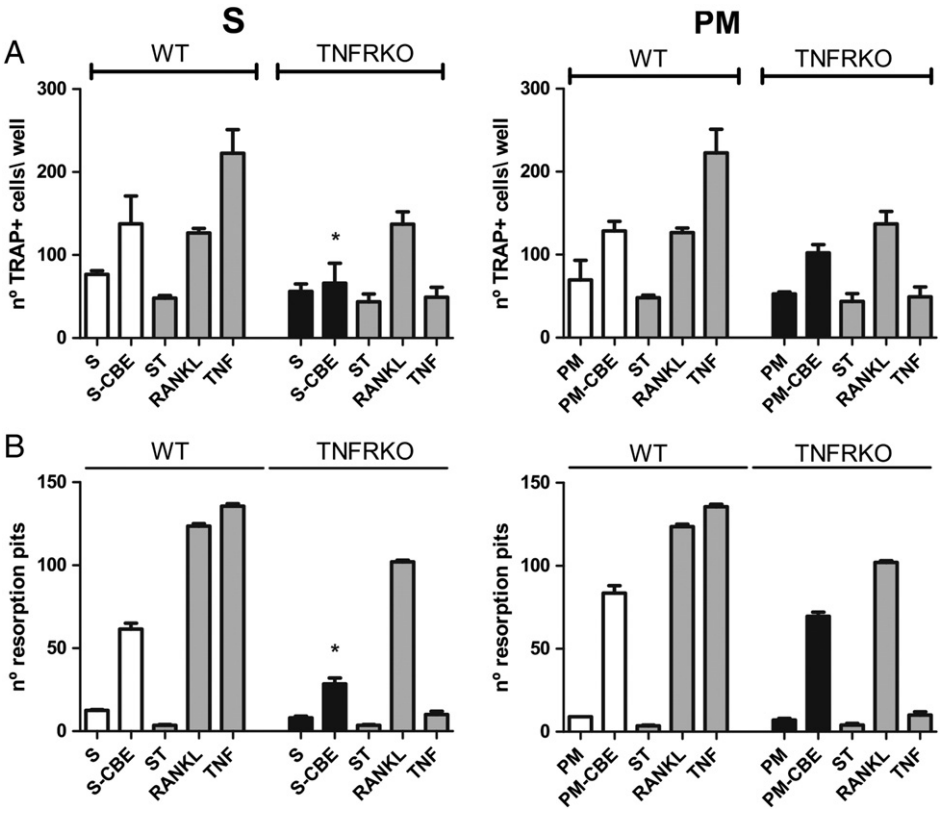

Fig. 4. TNF-α involvement on CMinduced osteoclastogenesis.Measurement of TRAP+ multinucleated cells and resorption areas after the addition of CM fromS, S/CBE or PM, PM/CBE on precursor cells from TNFR-KO mice orWT littermates. Bars express the mean ± sem, n = 5. *p < 0.05 one-way Anova.

3.3. Osteolytic activity

To complete the characterization of multinucleated TRAP cells as functional osteoclasts, we carried out osteolysis assays. Bone marrow cells were cultured in the presence of CM on an artificial bone matrix, and the number of resorption pits was counted. As can be seen in Fig. 3, the number of resorption pits increased when the cells were exposed to CM from CBE treated cells.

3.4. Role of TNF-α in osteoclastogenesis

Gaucher disease is accompanied by an underlying inflammatory chronic condition. Due to several reports showing the contribution of proinflammatory cytokines in osteoclastogenesis, we hypothesized that cytokines such as TNF-α could be involved in enhanced osteoclastogenesis observed in this model. For this reason we tested the contribution of TNF-α by the use of a TNF-α receptor knockout mouse (TNFRKO).

When we used bone marrow cells from TNFRKO, a decrease in the differentiation to mature osteoclasts, as assayed by TRAP and osteolysis, was observed as compared to WT (Fig. 4).

3.5. Osteoblast growth and mineralization

Changes in osteoblast physiology by the addition of CM was studied in three different ways. We analyzed the expression of alkaline phosphatase, a protein associated with bone cell phenotype. Furthermore we studied the mineralization process, by analyzing levels of collagen and inorganic material deposition by Sirius Red and Alizarin Red S stainings respectively. The incubation of osteoblasts with CM from PM-CBE treated cells resulted in a significant reduction in all three markers tested (Fig. 5).

4. Discussion

The concept of Gaucher disease as a disorder with cytopenias and visceromegalias has changed in recent years. GD is now principally thought to be a skeletal disorder with complications on bone structure that could be, in some cases, refractory to therapy (Deegan et al., 2011). Until now, the pathophysiological mechanisms are poorly understood, and so, specific targeted treatments to bone in GD could not be established.

Osteopenia in GD1 could be attributed to increased bone resorption or reduced bone formation. Although GD1 patients display normal levels of bone formation markers before treatment (Ciana et al., 2005), restoration of enzyme replacement therapy has been shown to increase osteocalcin and bone specific alkaline phosphatase concentration. Moreover, levels of bone resorption markers are frequently elevated at baseline and decreased with therapy (Sims et al., 2008). Mineral density assessment in patients on ERT revealed improvements in this parameter, but not generally to normal levels (Stirnemann et al., 2010).

These results suggest implication of changes in both bone formation and bone resorption and are consistent with the idea that both sides of the homeostatic balance are compromised.

For above reasons we decided to test bone formation and resorption using the same chemical *in vitro* model. In this work, we aimed to analyze if monocyte/macrophages from two different sources upon inhibition of GCase secrete factors that affect bone formation and resorption.

We were able to show that osteoclastogenesis is enhanced by factors released by cells from the chemical model. Differentiation of precursors to mature and functional osteoclasts was augmented. A similar result was obtained by using a human *in vitro* model of GD (Lecourt et al., 2011; Mucci et al., 2012). Moreover, experiments using mononuclear cells from GD patients revealed the secretion of factors that promote differentiation to osteoclasts (Reed et al., 2013).

In proinflammatory disorders alterations in bone metabolism is a common phenomenon. It is well established that proinflammatory cytokines, such as TNF-α, can induce the osteoclastogenesis process. And Gaucher disease is associated with production of cytokines. For this reason, we tested the influence of TNF-α in this process. By using bone marrow precursors from TNFR knockout mice, we were able to reveal that TNF-α could be one of the factors responsible for the increase in differentiation to osteoclasts.

Osteoblasts could also be the target of factors secreted by Gaucher cells, that may influence their functional activity of bone formation. We tested this hypothesis, by adding CM to a culture of an osteoblastic cell line. This experiment resulted in a reduction of production of bone matrix when the cells were exposed to factors secreted by CBE-treated cells. These results are concordant with the one from Mistry et al. The type I GD murine model developed by this group, displayed a dramatic reduction in bone formation, caused by a defect in osteoblastogenesis.

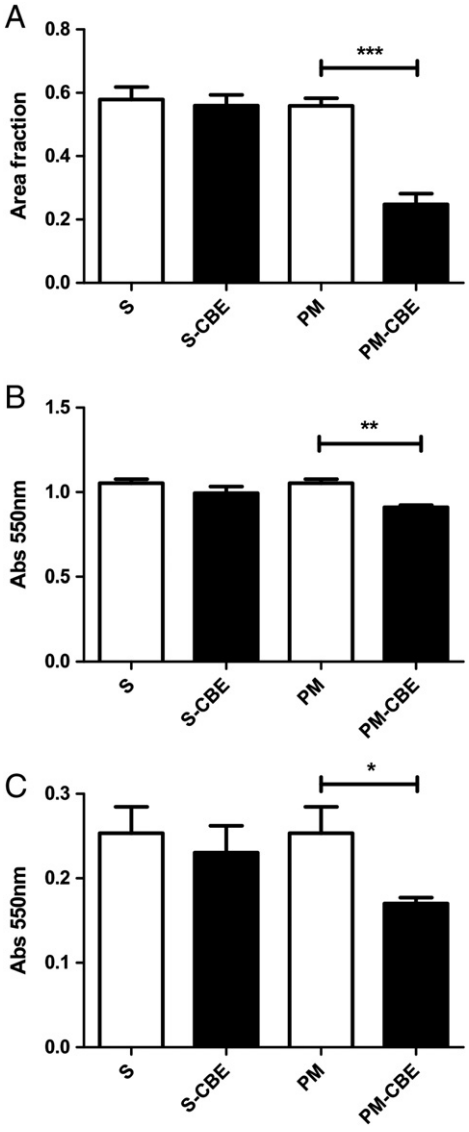

Fig. 5. Osteoblast growth and mineralization. MC3T3 cells exposed to CM from S, S/CBE or PM, PM/CBE were analyzed for alkaline phosphatase activity (A), collagen production by Sirius Red staining (B) and mineralization activity by Alizarin Red staining (C). Bars express the mean ± sem, n = 5. *p < 0.05, **p < 0.01, ***p < 0.001 *t*-test.

5. Conclusions

In conclusion, this work provides evidence that bone formation and resorption activities are affected in Gaucher disease, both contributing to reduced bone mineral density characteristic of this disorder.

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Conflict of interest statement

PR has received research grants from Shire HGT and acts as a consultant for Shire HGT. However, this arrangement would not have any influence on this work.

All other authors have no conflict of interest to declare.

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