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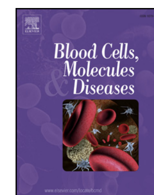

Proinflammatory and proosteoclastogenic potential of peripheral blood mononuclear cells from Gaucher patients: Implication for bone pathology

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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 the accumulation of the glycolipid glucocerebroside in the lysosomes of cells of monocyte/macrophage system. Bone compromise in Gaucher disease patients is the most disabling aspect of the disease. However, pathophysiological aspects of skeletal alterations are still poorly understood. On the other hand it is well known that inflammation is a key player in GD pathology. In this work, we revealed increased levels of the proinflammatory $CD14^{+}CD16^{+}$ monocyte subset and increased inflammatory cytokine production by monocytes and T cells in the circulation of GD patients. We showed increased levels of osteoclast precursors in PBMC from patients and a higher expression of RANKL in the surface of T cells. PBMC from patients presented higher osteoclast differentiation compared to healthy controls when cultured in the presence of M-CSF alone or in combination with RANKL. In vitro treatment with Velaglucerase reduced osteoclast levels to control levels. On the other hand THP-1 derived osteoclast precursors cultured in the presence of conditioned media from PBMC of GD patients presented higher differentiation to active osteoclasts. This induction involved TNF-α and RANKL.

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1. Introduction

Gaucher disease (GD) (OMIM ID: 230800) is an autosomal recessive lysosomal storage disorder caused by mutations in the gene encoding β-glucocerebrosidase (GCase) (E.C. 3.2.1.45), causing a deficiency in GCase enzymatic activity. This alteration leads to the accumulation of its substrate glucocerebrosidase mainly in macrophages [1], resulting in lipid-laden activated macrophages referred as "Gaucher cells". Phenotypes can be classified depending on the presence or not of neuronopathic manifestations in types II/III or I, respectively. Type I GD is the most frequent form, and is characterized by hepatosplenomegaly, anemia, thrombocytopenia and skeletal alterations. After more than 20 years of the introduction of enzyme replacement therapy for Gaucher disease, experts view this disorder as a principally skeletal disorder with high morbidity, and with some of the effects refractory to therapy [2]. Bone manifestations include bone pain, bone crises, osteopenia, osteoporosis, osteolytic lesions and osteonecrosis [3]. The pathological mechanisms of bone alterations in Gaucher disease are still not completely understood [4].

The bone is a dynamic tissue undergoing continuous remodeling. Skeletal disease results from a disruption of the fine balance between osteoblastic bone formation and osteoclastic bone resorption. Osteoclasts originate from the fusion of osteoclast precursors belonging to the monocyte/macrophage lineage, originating multinucleated cells. M-CSF is a crucial cytokine for the proliferation and survival of osteoclastic precursor cells. Osteoblasts express RANKL at cell surface and binding to its receptor RANK results in the activation of signaling cascades controlling lineage commitment and activation of osteoclasts [5]. This process is inhibited by the presence of osteoprotegerin (OPG), the RANKL neutralizing soluble decoy receptor. RANKL could also be expressed on dendritic cells, T and B cells, suggesting that osteoclastic bone resorption could be influenced by these cells. Osteoclastogenesis was reported to occur in cocultures of monocytes and T cells [6]. Activated T cells in inflammatory conditions lead to T cell production of osteoclastogenic cytokines such as RANKL and TNF-α. TNF-α was also reported to induce the formation of osteoclastic cells independently of the RANKL/RANK/OPG axis as it could support osteoclastogenesis in

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the presence of OPG or when bone marrow hematopoietic precursors from RANK null mice where cultured in vitro with this cytokine [7–9]. Alternatively, in the presence of TNF-α, osteoclast formation is induced by low concentrations of RANKL, and TNF-α increases the effects of RANKL [10]. Most of the T-cell cytokines, including interferon (IFN)-γ, IL-4, and IL-10, inhibit osteoclastogenesis [11]. However, among T cell subsets, Th17 cells have been suggested to be the osteoclastogenic T cell subset. Recently, IL-17 has been suggested to be involved in the upregulation of osteoclast precursor formation in inflammation by increasing the release of RANKL, which may synergize with IL-1β and TNF-α [12]. On the other hand, Treg inhibits osteoclast differentiation and function [13]. Treg subset is involved in tolerance maintenance and immunological homeostasis countering the inflammatory effects of the Th1/Th17 cells [14]. This subset has been shown to be altered in pathologies with an inflammatory involvement.

Higher numbers of circulating osteoclast precursors have been reported in several bone diseases associated with bone loss [15]. In bone loss-associated diseases, those osteoclast precursors are recruited from peripheral blood mononuclear cells (PBMCs) [16] and osteoclast formation occurs through stimulation by RANKL and macrophage colony-stimulating factor (M-CSF) [17–19]. In diseases with inflammatory conditions, osteoclastogenic cytokines necessary for osteoclast formation are produced by PBMCs themselves [16]. In a previous work it was shown that monocytes from GD patients cultured in the presence of M-CSF and RANKL gave rise to an increased level of mature and active osteoclasts which was reverted by treatment with Imiglucerase [20].

Inflammation is a key factor in the pathogenesis of GD and increased bone resorption markers have been described in patients. Moreover, we had previously shown that cellular alteration in GD, as modeled by in vitro GCase inhibition, produces bone destruction through the enhancement of macrophages differentiation to osteoclast and osteoclast resorption activity [18–20]. The aim of this study is to evaluate the presence and number of circulating proinflammatory cells and osteoclast precursors from Gaucher patients. Moreover, we aimed to analyze the spontaneous osteoclast formation by PBMC from Gaucher patients and to analyze the factors involved in this mechanism.

Table 1
Demographic and clinical data of patients included. Clinical parameters are presented as percentage of the number of patients that presented the symptom over the total of patients tested for that symptom.

Age range (years old)	32,16 ± 19,66	(10–77)		
Female (n)	11			
Male (n)	13			
Prior splenectomy (n)	4			
Genotype (n)				
c.1448 T > C (L444P)/c.1226A > G (N370S)	2			
c.1226A > G/c.481C > T	1			
c.1226A > G/RecNciI	4			
c.245C > T/unknown	2			
c.1226A>G/c.1226A>G	4			
RecNciI/c.1348T>A	1			
c.1226A > G/c.703 T > C	1			
c.1342G > C/c.1348 T > A	2			
RecNciI/c.1226A > G	1			
RecNciI/c.1504C > T	1			
ERT	Velaglucerase		Imiglucerase	
(n)	5		19	
Range (years)	10,79 ± 5,89	(1–20)		
Clinical parameters (%)	At diagnosis		Present	
Anemia	70,83	(17/24)	8,70	(2/23)
Thrombocytopenia	62,50	(15/24)	26,09	(6/23)
Splenomegaly	94,44	(17/18)	27,78	(5/18)
Hepatomegaly	91,30	(21/23)	27,27	(6/22)
Chitotriosidase elevated	80,00	(12/15)	25,00	(4/16)
Bone involvement	79,17	(19/24)	61,90	(13/21)
Parameter improvement (%)				
Anemia	93,75	(15/16)		
Thrombocytopenia	64,28	(9/14)		
Splenomegaly	68,75	(11/16)		
Hepatomegaly	70,00	(14/20)		
Chitotriosidase elevated	90,00	(9/10)		
Bone involvement	25,00	(4/16)		

2. Materials and methods

2.1. Ethics statement

This study was approved by the Ethical Committee of IBYME (Instituto de Biología y Medicina Experimental, CONICET, Argentina) according to provisions of the Declaration of Helsinki in 1995. Human PBMCs were isolated from patients and healthy blood donors in accordance with the guidelines provided by the ethical committee. The nature and purpose of the study were explained to all volunteers and all patients gave their written informed consent prior to participation in this study. In the case of minors/children written informed consent was obtained from the next of kin, caretakers, or guardians.

2.2. Patient demographics

Twenty four Gaucher patients (11 females and 13 males, median age: 32.16 ± 19.66 years old) were included in the present study. All the patients were under enzyme replacement therapy (ERT) with either Imiglucerase (I) (Cerezyme®, Genzyme, a Sanofi Company, Cambridge, USA) or Velaglucerase (V) (VPRIV®, Shire, Lexington, USA). Patient demographic and clinical data are summarized in Table 1.

Anemia was considered when hemoglobin concentration was below 1 g/dl below lower limit of normal for age and gender at the local laboratory. Thrombocytopenia was assigned positive when platelet count was below 90,000. The presence of splenomegaly or hepatomegaly was evaluated by quantitative abdominal magnetic resonance imaging. Bone involvement was defined positive when the patient presented at least one of the following manifestations: bone pain, bone crisis, osteopenia/osteoporosis, and osteonecrosis. Clinical data is presented as a percentage calculated as the number of affected patients over the total number of patients evaluated for each clinical parameter. The parameter improvement percentage was calculated as the number of patients that presented an improvement after ERT treatment over the number of patients that presented the clinical parameter before ERT treatment for each parameter evaluated. As shown in Table 1 bone involvement was the clinical parameter that presented the lowest improvement after ERT. Twenty four healthy individuals (mean age: 31.93 ± 21.10; range: 15–65 years old) matched for age and sex served as controls.

2.3. Cell isolation

Peripheral blood samples from patients or healthy controls were collected 24 h before ERT infusion by venipuncture in heparin as anticoagulant and immediately processed. Mononuclear cells from whole blood (PBMC) were isolated by Ficoll Hypaque (Sigma, St Louis, MO, USA) gradient separation.

2.4. Flow cytometry

PBMCs from patients with Gaucher disease and healthy controls were incubated in PBS containing 10% of normal human serum for 20 min at 4 °C after which were centrifuged and resuspended in 50 μl of PBS and 10% normal human serum containing different conjugated antibodies. In all cases cells were incubated for 25 min at 4 °C washed with PBS and analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA). The following antibodies were used: CD51-FITC, CD16-PE and CD14-PECy5.5 to define osteoclast precursors as $CD51^+CD16^+CD14^+$ cells. CD3-FITC, RANKL-PE and CD20-PerCP to assess the levels of RANKL in T cells ($CD3^+RANKL^+CD20^-$) and B cells

($CD3^-RANKL^+CD20^+$) and Lin1-FITC (a cocktail of antibodies containing CD3, CD14, CD16, CD19, CD20 and CD56), RANKL-PE and HLA-DR-PECy7 to analyze the levels of RANKL in dendritic cells ($RANKL^+Lin1^-HLA\text{-}DR^+$ cells). The antibodies were supplied by BD Biosciences, San Diego, CA. In the case of intracytoplasmic staining after performing the corresponding staining of the surface molecules as detailed above, cells were fixed and permeabilized using the kit Citofix/Citoperm (BD Biosciences, San Diego, CA) according to the manufacturer specifications. After permeabilization step intracytoplasmic staining was performed using the following specific antibodies: Foxp3-PE, IL-1β-APC, IL-6-APC, TNF-α-APC (BD Biosciences, San Diego, CA) in 50 μl Wash buffer/Perm included in the kit. Staining was performed at 4 °C for 25 min, after which were washed twice with PBS and analyzed in the flow cytometer.

2.5. Conditioned media preparation

PBMCs (10^6 cells/ml) were cultivated in AIM-V media (Invitrogen, Carlsbad, CA, USA) at 37 °C in a 5% CO_2 atmosphere for 72 h. Culture conditioned media was obtained by centrifugation of the cultures.

2.6. Osteoclast formation assay

PBMCs from patients or healthy donors were seeded at 5×10^5 cells/ml and cultured at 37 °C in 5% CO_2 atmosphere 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 media) and 30 ng/ml of recombinant human macrophage colony stimulating factor (M-CSF) (R&D, Minneapolis, MN, USA) alone or in combination with 50 ng/ml of human RANKL (Millipore, Billerica, MA, USA) for 7 days replacing the media every 48 h. To analyze the effect of in vitro treatment with recombinant enzyme osteoclast formation assays were performed in the presence of 1.5 U/ml of Velaglucerase (the concentration of Velaglucerase was calculated based on the quantity of enzyme infused to the patients and the plasma volume at the time of infusion) (VPRIV™, Shire, USA). To identify osteoclasts, cells were fixed in 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP; Sigma Aldrich, St Louis, MO, USA). TRAP-positive multinucleated (more than 3 nuclei) cells were defined as osteoclasts, and the number was determined by microscopic counts on a whole well.

For testing induction of osteoclast formation by the addition of conditioned media from PBMC, THP-1 cells were seeded at 5×10^5 cells/ml and cultured at 37 °C in 5% CO_2 atmosphere in complete media and 30 ng/ml of recombinant human M-CSF for 72 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 conditioned media and complete media in a 1:1 ratio supplemented with M-CSF for 7 days replacing the media every 48 h. As positive control of osteoclast formation cultures received 50 ng/ml of human RANKL. To evidence the role of TNF-α in osteoclastogenesis, the osteoclast formation assay was performed in the presence of an anti-TNF-α neutralizing antibody at 2 ng/ml or an isotype control (BD Pharmingen, San Diego, CA). To analyze the involvement of RANKL in osteoclastogenesis, the osteoclast formation assay was performed in the presence of 50 ng/ml of recombinant OPG (Millipore, Billerica, MA, USA) the soluble decoy receptor for RANKL. To identify osteoclasts, cells were fixed in 4% paraformaldehyde and stained for TRAP. TRAP-positive multinucleated (more than 3 nuclei) cells were defined as osteoclasts, and the number was determined by microscopic counts.

2.7. Pit formation assay

PBMCs from patients or healthy controls (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 the presence of M-CSF (30 ng/ml) alone or in combination with 50 ng/ml of human RANKL for 6 days. Media and all reagents were replaced every day to avoid acidification of medium. To analyze the effect of in vitro treatment with recombinant enzyme osteoclast formation assays were performed in the presence of Velaglucerase as previously described. 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.

THP-1 cells (2×10^4 cells/0.25 ml/well) were plated on dentine disks in 96-well culture dishes and cultured in CM and complete media 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. To evidence the role of TNF-α and RANKL on this process pit formation assays were performed in the presence of an anti-TNF-α antibody or OPG respectively as previously described. 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 and the resorption lacunae were counted on the whole dentine disk.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 program applying t-test. For studies of cytometric mean fluorescence levels one sample t-test was used, comparing the data with a hypothetical value of 1. Data are presented as mean ± SD.

3. Results

3.1. Patient demographics

Patient demographics are summarized in Table 1. Each clinical parameter is presented as a percentage of the number of patients that presented the symptom over the total of patients tested for that symptom.

In our study 24 patients were included (11 females and 13 males) with a mean age of 32,16 ± 19,66 years (10–77 years range). All patients included were under ERT treatment either with Imiglucerase (I) (Genzyme Corporation, Cambridge, USA) or Velaglucerase (V) (VPRIV®), (Shire, Lexington, USA). Clinical parameters from patients were evaluated at the time of diagnosis and at the moment of inclusion in this study, when all the patients were on ERT (Table 1). Better improvement rates in clinical parameters after ERT were observed for anemia, thrombocytopenia, splenomegaly and hepatomegaly, and chitotriosidase measurement followed a similar trend. However, bone involvement, present in 79% of patients, remained in 61,9% of them.

3.2. Proinflammatory profile

Several reports have described increased levels of proinflammatory molecules in sera of patients with Gaucher disease [22]. On the other hand it is well known that the monocyte/macrophage lineage is the most affected in Gaucher disease [23]. On this basis we decided to study the percentages of two distinct monocyte populations in PBMC from patients with Gaucher disease by flow cytometry. We studied the $CD14^+CD16^-$ "classical" monocyte subset and the proinflammatory $CD14^+CD16^+$ monocyte subset [24]. Patients showed a decreased $CD14^+CD16^-$ monocyte percentage (Fig. 1A) while the $CD14^+CD16^+$ subset was significantly ($p < 0{,}001$) increased in patients (Fig. 1B).

In order to continue with the study of the involvement of circulating subsets in the proinflammatory state in Gaucher disease patients we decided to evaluate the production of IL-1β, IL-6 and TNF-α by several cell populations in patients PBMC. The production of these cytokines was evaluated by intracellular staining followed by flow cytometry

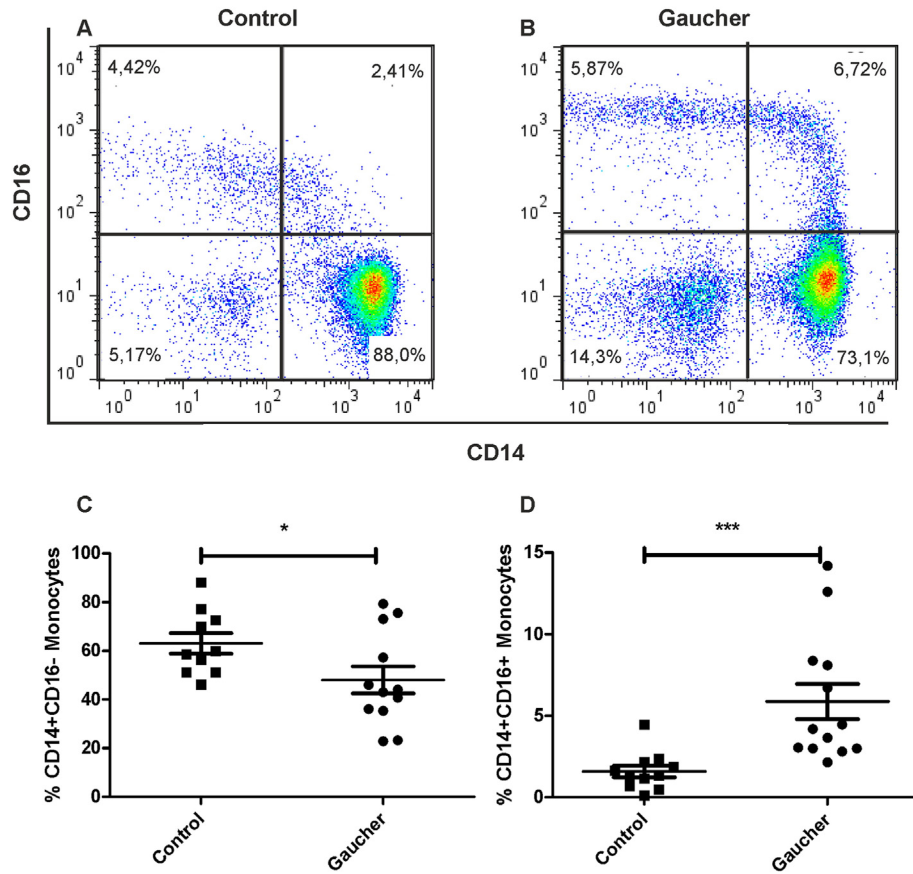

Fig. 1. Monocyte subsets from PBMC of Gaucher disease patients evaluated by flow cytometry is shown as the percentage of $CD14^+CD16^-$ (C) and $CD14^+CD16^+$ (D) monocytes. Representative dot plots of $CD14^+CD16^+$ from control (A) and Gaucher (B) patients are shown as example. *p < 0,05, ***p < 0,001, t-test n = 10.

analysis. Surface markers were stained to identify T cells (T), dendritic cells (DC) and $CD14^+CD16^-$ or $CD14^+CD16^+$ monocytes. Results are presented as percentage of positive cells or as relative increase of the median fluorescence intensity (MFI) for each cytokine on Fig. 2A and B respectively. Statistically ($p < 0,05$) increased production of IL-6 and TNF-α by T cells from patients was evidenced by a higher MFI for these cytokines (Fig. 2B). Percentage of IL-6 and TNF-α positive monocytes was increased ($p < 0,05$) (Fig. 2A) for the $CD14^+CD16^-$ subset. These results would show the involvement of monocytes and T cells in the increased levels of cytokines observed in sera of Gaucher disease patients.

Based on the information Treg is involved in proinflammatory disorders, we decided to evaluate Treg numbers in PBMC from Gaucher disease patients. This subset was evaluated by flow cytometry by intracellular staining of the key transcription factor Foxp3 together with the staining of surface molecules CD4 and CD25. Treg levels in PBMC from Gaucher disease patients did not differ from healthy donors (Fig. 3). This implies that Treg cells wouldn't be participating in the suppression of the response to the proinflammatory state observed in patients, although further studies would be required regarding the production of anti-inflammatory cytokines produced by this subset in patients with GD.

3.3. Proosteoclastogenic profile

Bone turnover in physiological conditions is maintained by a highly regulated dynamic equilibrium between the action of bone generating osteoblasts and bone resorbing osteoclasts. The osteoclastogenesis process is regulated mainly by the RANK/RANKL/OPG axis in which RANKL along with M-CSF induces differentiation of osteoclasts [5]. Besides being expressed in bone cells, RANKL can be expressed on the surface of several immune cell types including T, B and dendritic cells [25]. Therefore we decided to study RANKL expression on the surface of these three cell types in PBMC from patients with Gaucher disease by flow cytometry. Patients showed higher levels of RANKL on the surface of T cells (Fig. 4) while no differences were observed for the expression on B cells or DC.

In physiological conditions osteoclast precursors generated in bone marrow migrate to the circulation and then return to bone microenvironment where they complete the differentiation process into mature osteoclasts with resorption activity [26]. Bone loss in several diseases has been related to an increased number of circulating osteoclast precursors [27]. We proposed to evaluate the presence of osteoclast precursors, defined as previously described [14] as $CD14^+CD16^+CD51^+$ cells in PBMC by flow cytometry. Gaucher patients showed significantly ($p < 0,001$) higher levels of circulating osteoclast precursors as compared to healthy donors (Fig. 5).

Based on this finding we sought to analyze the potential of circulating osteoclast precursors to differentiate into active osteoclasts. To this end we performed osteoclastogenesis differentiation assays seeding PBMC from patients or healthy donors in the presence of M-CSF or M-CSF and RANKL. In addition we wanted to study the possible effect of Velaglucerase in vitro on the differentiation of osteoclast precursors. Culture of PBMC from Gaucher disease patients either with M-CSF or M-CSF and RANKL induced a significantly ($p < 0,001$) higher osteoclast differentiation as determined by TRAP positive multinucleated cells and they were functional by their ability to resorb dentine compared to PBMC from healthy donors (Fig. 6A and B). The addition of Velaglucerase in vitro significantly reduced osteoclast differentiation and activity to control levels (Fig. 6A and B). These results indicate that osteoclast precursors present in the circulation of patients with Gaucher disease have the potential to differentiate into mature and

active osteoclasts. On the other hand we have shown that in vitro treatment of PBMC from patients with Velaglucerase reduced the osteoclastogenic potential of the osteoclast precursors to control levels which could be related to the improvement on bone involvement observed in patients under ERT treatment with Velaglucerase.

3.4. Soluble mediators in osteoclasts differentiation

Thereafter, we hypothesized that there could be factors secreted by mononuclear cells from patients that mediate and induce differentiation of precursors. To test this hypothesis we cultured PBMC during three days and harvested the conditioned media that was then added to an osteoclast precursor culture as obtained by treatment of THP-1 cells with M-CSF. Osteoclastogenesis and resorption assays were performed to evaluate the induction of osteoclast differentiation by soluble factors present in conditioned media secreted by PBMC from patients. The treatment of osteoclast precursors with conditioned media from patients induced higher numbers of both multinucleated TRAP positive cells (Fig. 6C) and resorption pits (Fig. 6D). This result could be revealing the presence of soluble mediators secreted by patients' PBMC that induced the differentiation of precursors into mature and active osteoclasts.

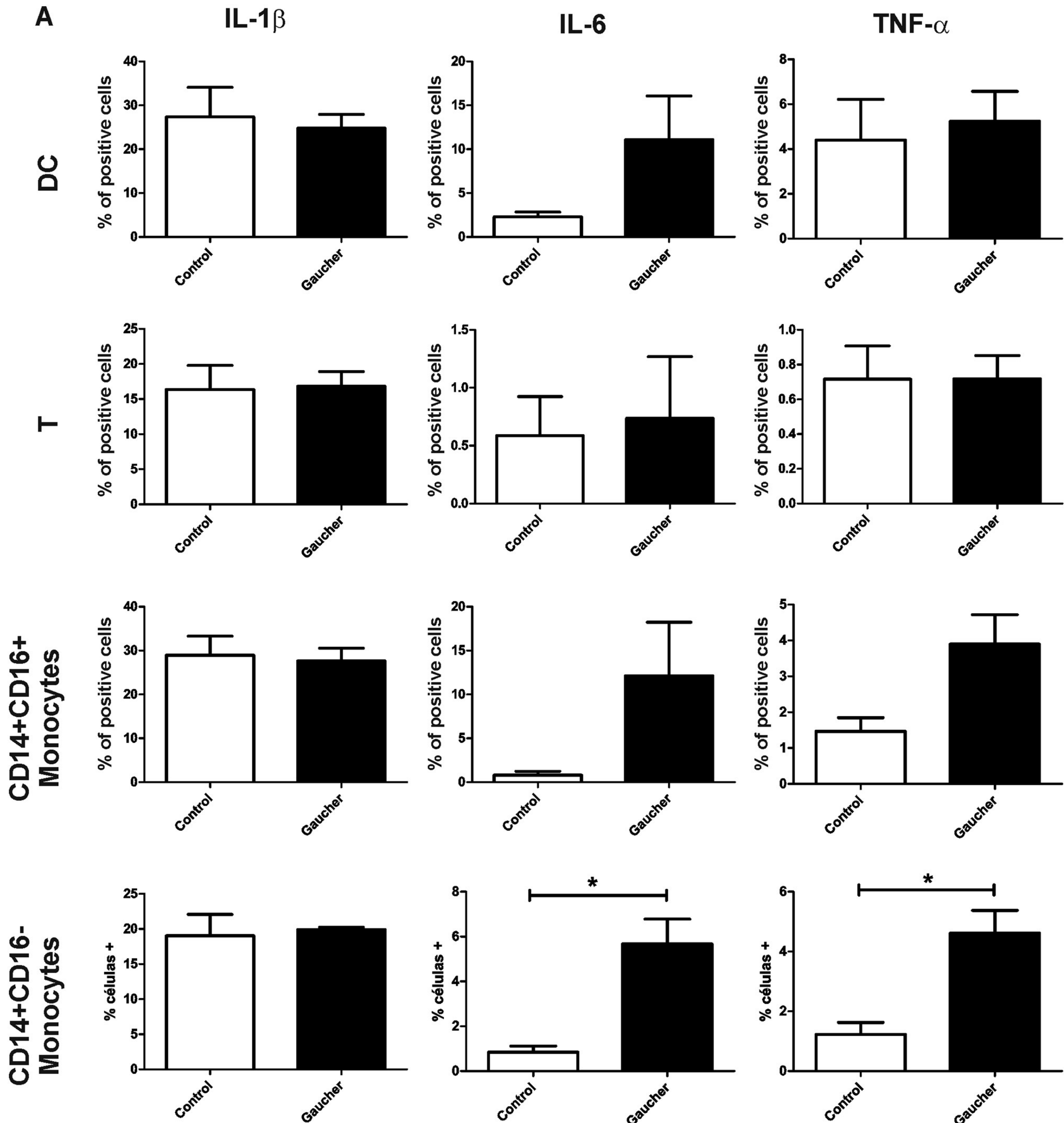

Fig. 2. Flow cytometric analysis of cytokine production by subsets from PBMC from Gaucher disease patients. The production of IL-1β, IL-6 and TNF-α by dendritic cells (DC), T cells (T) and $CD14^{+}CD16^{+}$ or $CD14^{+}CD16^{-}$ monocytes was evaluated by intracellular staining. Results are shown as percentage of positive cells (A) or relative increase of the median fluorescence intensity (B). *p < 0,05, t-test (A) n = 4, *p < 0,05 one sample t-test (B) n = 4.

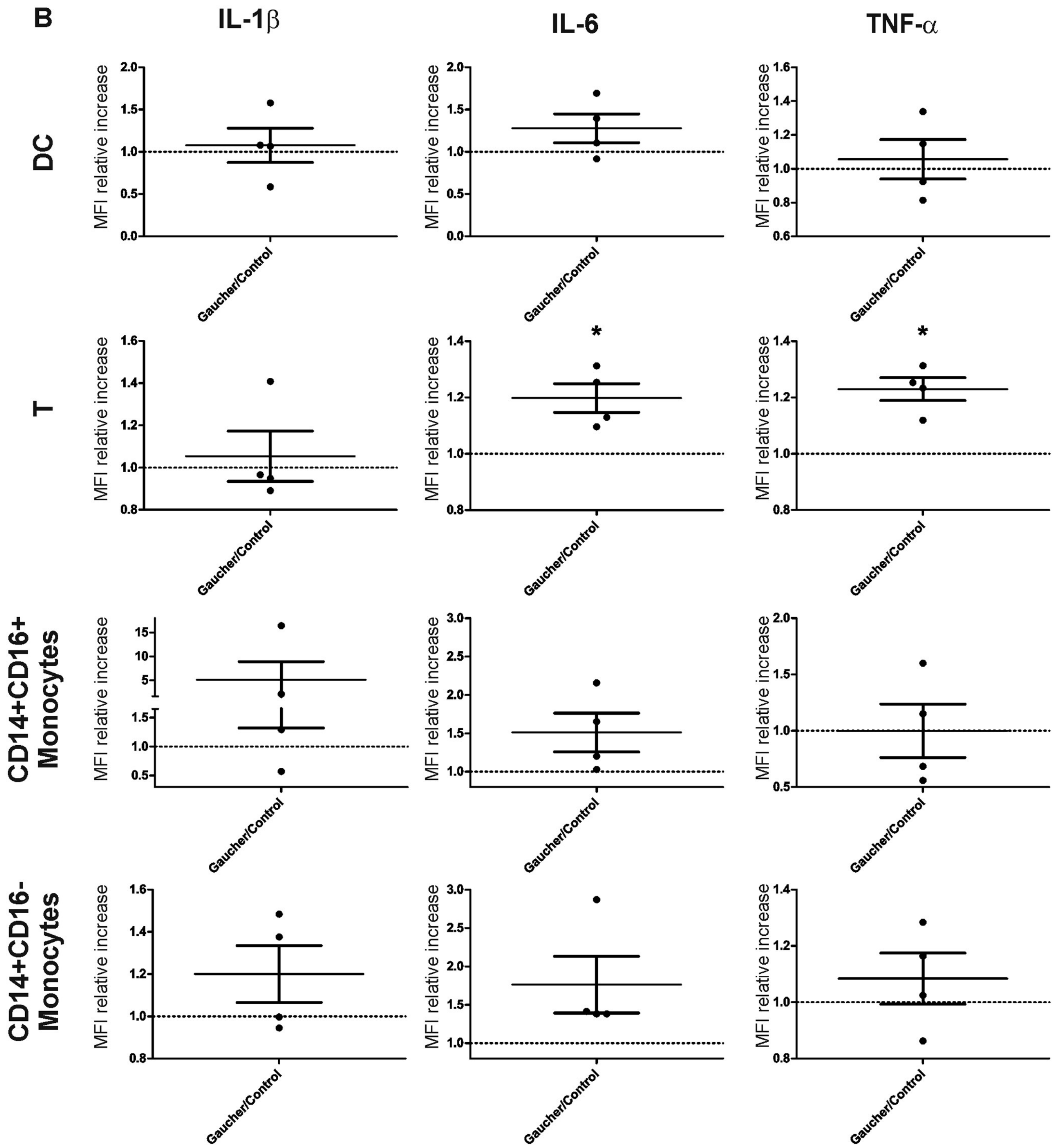

Fig. 2 (*continued*).

Then we sought to analyze the possible involvement of TNF-α and RANKL in the induction of osteoclastogenesis by soluble mediators. To this end osteoclastogenesis and resorption assays were performed in the presence of a neutralizing antibody specific for TNF-α and/or OPG (the soluble decoy receptor for RANKL). The presence of either the neutralizing antibody or OPG resulted in a decreased number of TRAP positive cells and resorption pits to control levels (Fig. 6C and D). The treatment with both neutralizing molecules together did not show further differences as compared to the treatment with each molecule alone (data not shown). In conclusion TNF-α and RANKL would be key molecules involved in this process.

4. Discussion

In the present study, we demonstrated that Gaucher patients PBMC display a proinflammatory and proosteoclastogenic potential. By different tests, we were able to show that inflammation is activated in Gaucher disease patients. The percentage of 'inflammatory' non-classical monocytes is overrepresented in the CD14 population as compared to the numbers observed in healthy controls. The production of proinflammatory cytokines by $CD14^+$ cells from Gaucher patients was also increased. It was demonstrated that $CD14^+CD16^+$ monocytes promotes expansion of the Th17 cell population [28], and Th17 subset of

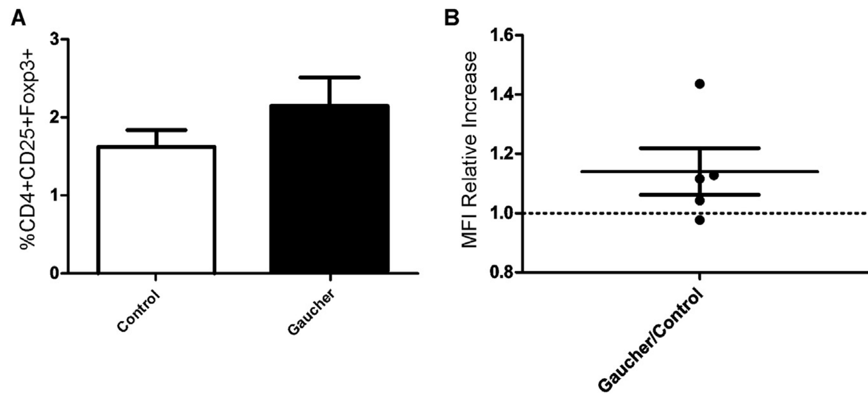

Fig. 3. Percentage of Treg cells in PBMC from Gaucher disease patients assayed by flow cytometry. Results are presented as cell percentage (A) or relative increase of the median fluorescence intensity for Foxp3 (B). n = 5.

CD4 cells is defined as the proosteoclastogenic one [29]. The role of $CD14^+CD16^+$ monocytes could be central in pathological osteoclastogenesis in Gaucher disease. Along with monocytes, we also detected production of proinflammatory cytokines by T cells and DC from Gaucher patients, implying involvement of these cells in this state. Another reason in favor of inflammation activation is the finding of normal levels of Tregs, suggesting an absence of a regulatory process by these cells that in physiological conditions could have being controlling/alleviating inflammation [30].

We also demonstrated a proosteoclastogenic potential of mononuclear cells from Gaucher patients. One of the evidences is the presence of increased levels of RANKL in T cells from patients. A role of T cells in osteoclastogenesis has been extensively studied. Osteoclastogenesis dependence on T cells has been detected in many conditions [31]. It has been demonstrated that T cells express RANKL and are capable of directly triggering osteoclastogenesis. In our previous article, we revealed T cell contribution in osteoclastogenesis in GD chemical model [21]. Moreover, T cells are the ones from the mix of mononuclear

Fig. 4. RANKL levels on the surface of T cells (T), B cells (B) and dendritic cells (DC) were evaluated by flow cytometry in circulating cells from Gaucher patients and normal controls. A representative histogram is shown and results were expressed as relative increase of the median fluorescence intensity for RANKL. *p < 0,05 one sample test n = 5.

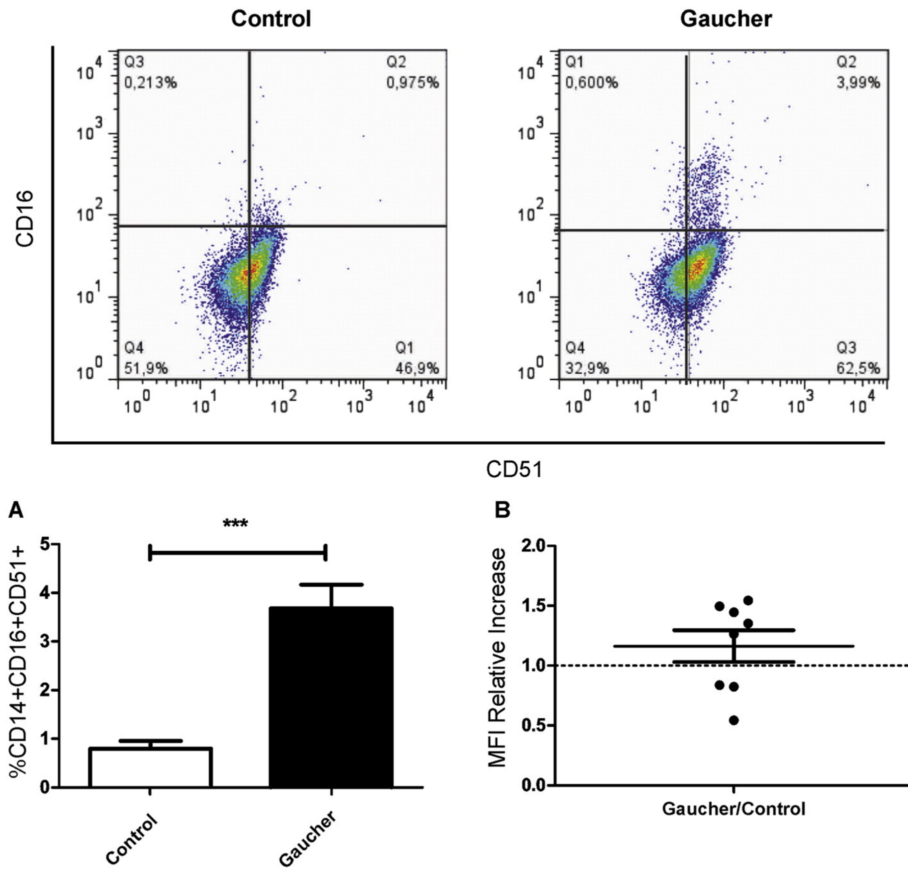

Fig. 5. Osteoclast precursor levels in PBMC from Gaucher disease patients, defined as $CD14^+CD16^+CD51^+$ cells were evaluated by flow cytometry. The gating strategy included the definition of the monocyte subset based on size and granularity and the $CD14^+$ subset based on the isotype control. Representative dot plots of $CD16^+CD51^+$ from control and Gaucher patients are shown as example. Results are presented as percentage of osteoclast precursors (A) and relative increase of the median fluorescence intensity for CD51 on $CD14^+CD16^+$ cells (B). ***$p < 0{,}001$, t-test $n = 8$.

cells shown to induce spontaneous osteoclastogenesis when cultured with monocytes [32,6]. Based on these findings, T cell cytokines have been suggested to be essential drivers of osteoclastogenesis. In favor of this is the finding in this study of production of high levels of IL-6 and TNF-α by T cells from Gaucher patients. In summary, these results would reveal a proosteoclastogenic environment present in circulation of patients with Gaucher disease and the probable involvement of T cells in osteoclast precursor priming.

Bone degradation in bone loss-associated diseases occurs through increased differentiation of osteoclast precursors into mature, multinucleated osteoclasts, and those precursors are recruited from circulating blood [16]. Different pathological conditions, such as phenylketonuria [15] and psoriatic arthritis [33], characterized by bone loss are mediated by the presence of increasing numbers of osteoclast precursors. We were able to show in this study that a similar behavior is present in Gaucher disease. Gaucher patients presented increased circulating levels of osteoclast precursors evaluated by flow cytometry. Moreover, these cells exhibited a significant increase of in vitro osteoclast formation and resorption when compared to PBMCs from healthy controls. This result is in agreement with the study performed by Reed et al. [20], although the main difference between the two experimental designs relies on the fact that in our work osteoclast formation was induced in the presence of the whole PBMC subsets including lymphocytes in contrast to the induction of osteoclastogenesis on monocytes alone reported in the work by Reed et al. This is an important difference as we have shown a possible involvement of lymphocytes on the proosteoclastogenic state on patients with GD.

This increased tendency of osteoclastogenesis from Gaucher cells revealed here could be mediated by secreted factors from mononuclear cells of patients. Indeed conditioned media from patients' cells induced differentiation of precursors to functional osteoclasts. Several reports have shown that surface RANKL–RANK interaction is not the sole pathway that leads osteoclast progenitors to differentiate into osteoclasts in vitro. Different soluble factors were discovered that can trigger osteoclastogenesis [34,35], mainly in inflammatory diseases [36]. From all these factors, we detected that at least, both, TNF and RANKL would be key molecules involved in this process of GD enhanced osteoclastogenesis.

Enzyme replacement therapy, the standard of care for Gaucher disease patients improves the major symptomatic manifestations of skeletal disease, such as bone pain and crisis. The prospective study of the use of Imiglucerase showed a decrease in the worsening of bone pain and reduction in bone crisis; and the appearance of incidents was more likely to occur in patients with pre-existent skeletal complications [37]. The first prospective study of experience of treatment with Velaglucerase showed significant improvement of bone mineral density and bone marrow burden among patients with significant bone disease at baseline. The observed improvements were not only statistically significant but were also reflected in clinically meaningful transitions in the patients' WHO bone classifications [38,39]. Substrate reduction therapy showed similar trends [40,41]. However, bone alterations are not completely eliminated, suggesting some degree of refractoriness of bone tissue to therapy.

All the patients included in this study were under ERT, and among clinical manifestations presented in patients at diagnosis, skeletal alterations are the ones that persist with the highest prevalence. It seems that ERT is able to correct bone alterations from Gaucher patients, but not completely, however, the cause of this problem is still unknown. In an attempt to underscore a possible mechanism of ERT on osteoclastogenesis in Gaucher disease, we studied the in vitro effect

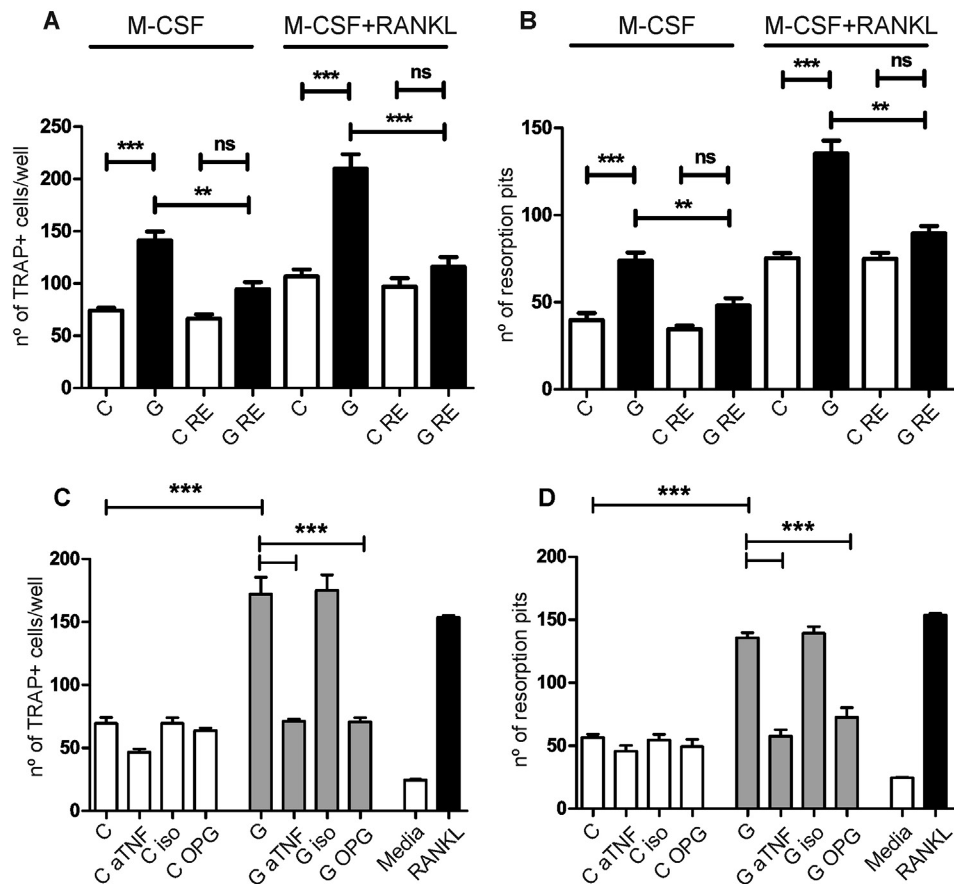

Fig. 6. Osteoclastogenesis in Gaucher (G) disease patients or normal controls (C). The potential of osteoclast precursors from PBMC of patients to generate mature and active osteoclasts was assayed by PBMC culture in the presence of M-CSF or M-CSF + RANKL and evaluation of the number of TRAP positive multinucleated cells (A) and number of resorption pits (B) in the absence or presence of Velaglucerase (recombinant enzyme, RE). Evaluation of soluble mediators that induced osteoclastogenesis was evaluated by treatment of THP-1 cells with M-CSF and conditioned media. The number of TRAP positive multinucleated cells (C) and the number of resorption pits (D) were analyzed in the presence of a TNF-α neutralizing antibody or OPG. **p < 0,01, ***p < 0,001, t-test n = 6 (A and B), n = 8 (C and D).

of the addition of Velaglucerase, and we were able to show that this enzyme reduces osteoclast differentiation and resorption activity of circulating mononuclear cells. A similar result was obtained with other specific therapies approved for Gaucher disease [20]. This effect would be of benefit because it diminishes proosteoclastogenic potential of circulating precursors that would migrate to the bone, and may explain, at least in part, the improvements in bone disease from patients on therapy. The cause of the incomplete resolution of bone problems in Gaucher disease is still not known and not completely studied. Probable problems would be the accessibility of the enzyme into the bone microenvironment generating a reduced effective concentration and/or existence of niches where inflammatory state persists despite ERT that perpetuates proosteoclastogenic state in the tissue.

5. Conclusions

The elevated expression levels of pro-inflammatory cytokines reflect a higher activation state of monocytes and T cells from Gaucher patients. This proinflammatory environment may have induced an expansion and/or partial differentiation of osteoclast precursors already in the circulation and might explain the increased tendency of mononuclear cells from patients to mature into functional osteoclasts. Therefore, our results provide more insight in the mechanism for Gaucher associated bone loss. Osteoclastogenesis is increased in Gaucher disease and probably mediated by the interaction of CD14CD16 and T cells in circulation, that might trigger increased extravasation of activated osteoclast precursors to the bone where they mature into functional osteoclasts that resorb the bone. Key cytokines in this process are, at least, TNF and RANKL. Recombinant glucocerebrosidase is able to reduce osteoclastogenesis in vitro, by reducing the potential of precursors to mature, however bone tissue special histology and physiology might be responsible for reduced efficiency of enzyme replacement to cure skeletal pathology.

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