Gene xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Induction of osteoclastogenesis in an *in vitro* model of Gaucher disease is mediated by T cells *via* TNF-α

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ARTICLE INFO

Article history:
Accepted 30 July 2012
Available online xxxx

Keywords:
Gaucher disease
Glucocerebrosidase deficiency
Bone disease
Macrophages
TNF-α
T cells

ABSTRACT

Gaucher disease is a lysosomal storage disorder caused by deficiency of glucocerebrosidase enzymatic activity leading to accumulation of its substrate glucocerebrosidase mainly in macrophages. Skeletal disorder of Gaucher disease is the major cause of morbidity and is highly refractory to enzyme replacement therapy. However, pathological mechanisms of bone alterations in Gaucher disease are still poorly understood. We hypothesized that cellular alteration in Gaucher disease produces a proinflammatory milieu leading to bone destruction through enhancement of monocyte differentiation to osteoclasts and osteoclasts resorption activity. Against this background we decided to investigate in an *in vitro* chemical model of Gaucher disease, the capacity of secreted soluble mediators to induce osteoclastogenesis, and the mechanism responsible for this phenomena. We demonstrated that soluble factors produced by CBE-treated PBMC induced differentiation of osteoclasts precursors into mature and active osteoclasts that express chitotriosidase and secrete proinflammatory cytokines. We also showed a role of TNF-α in promoting osteoclastogenesis in Gaucher disease chemical model. To analyze the biological relevance of T cells in osteoclastogenesis of Gaucher disease, we investigated this process in T cell-depleted PBMC cultures. The findings suggest that T cells play a role in osteoclast formation in Gaucher disease. In conclusion, our data suggests that *in vitro* GCASE deficiency, along with concomitant glucosylceramide accumulation, generates a state of osteoclastogenesis mediated in part by pro-resorptive cytokines, especially TNF-α. Moreover, T cells are involved in osteoclastogenesis in Gaucher disease chemical model.

1. Introduction

Gaucher disease (OMIM ID: 230800) is a lysosomal storage disorder caused by mutations in the gene encoding β-glucocerebrosidase (GCase) (E.C. 3.2.1.45). Deficient GCase enzymatic activity leads to accumulation of its substrate glucocerebrosidase mainly in macrophages (Grabowski and Beutler, 2009), resulting in lipid-laden activated macrophages referred as "Gaucher cells". Phenotypically Gaucher disease could manifest in 3 different clinical types, based on the presence of neurological manifestations: I, II and III. Type I or non-neuronopathic Gaucher disease is the most frequent form, and is characterized by hepatosplenomegaly, anemia, thrombopenia and skeletal alterations. Bone manifestations include bone pain, bone crises, osteopenia, osteoporosis, avascular necrosis and pathological fractures. Skeletal disorder of Gaucher disease is the major cause of morbidity and is highly refractory to enzyme replacement therapy (Deegan et al., 2011; Goker-Alpan, 2011). The pathological mechanisms of bone alterations in Gaucher disease are still poorly understood (Cox, 2010).

Macrophages are a heterogeneous group of cells, whose morphology and phenotype differ depending on the tissue/organ and stimuli. They participate in tissue remodelling, host defense, and many disease processes, and can secrete both anti or proinflammatory cytokines. Although an infinite number of potential phenotypes can be suggested, macrophages could be associated with two main types: classical or alternative. Activation of macrophages occurs at 3 stages: first stage or differentiation depends on growth factors such as M-CSF. The second one is the priming, and involves the induction

Abbreviations: TNF-α, Tumor necrosis factor alpha; CBE, Conduritol β Epoxide; PBMC, Peripheral blood mononuclear cell; GCase, β-glucocerebrosidase; TGF-β, Transforming growth factor beta; IL, Interleukin; CCL, Chemokine (C–C motif) ligand; CD, Cluster of differentiation; TRAP, Tartrate-resistant acid phosphatase; MMP, Matrix metalloproteinases; M-CSF, Macrophage colony stimulating factor; RANK, Receptor activator of nuclear factor k B; RANKL, RANK ligand; OPG, Osteoprotegerin; IFN, Interferon; LPS, Lipopolysaccharide; CM, Conditioned media; THP-1, Human acute monocytic leukemia cell line; TIMP, Tissue inhibitors of metalloproteases; CHIT, Chitotriosidase; α-MEM, α-minimum essential medium.

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http://dx.doi.org/10.1016/j.gene.2012.07.071

of the specific phenotype, classical or alternative, depending on the predominant cytokine in the environment, IFN-γ or IL-4/IL-13, respectively. Finally, the stimulus for full activation of classical or alternative macrophages is delivered by a TLR or analogous receptor (Gordon and Martinez, 2010). Gaucher cells resemble alternative activated macrophages (Boven et al., 2004), characterized by expression of chitotriosidase and CCL18.

In different experimental models of bone disease, cytokines and chemokines are usually released within the inflammatory environment, implying interplay between immune system and bone in the pathophysiology (Takayanagi, 2010a). In particular in Gaucher disease, various reports have shown significant increase of serum concentrations of cytokines and chemokines such as TGF-β, IL-18, CCL18, CCL4, IL-8, CCL5 (Boven et al., 2004; Campeau et al., 2009; Yoshino et al., 2007; Pavlova et al., 2011). Moreover, high levels of expression of bone disease markers such as Cathepsin K, tartrate-resistant acid phosphatase (TRAP) and matrix metalloproteinase-2 (MMP-2) are detected in serum from patients (Moran et al., 2000). MMPs are proteinases that participate in extracellular matrix degradation (Brinckerhoff and Matrisian, 2002). Enhanced production of MMPs in chronic inflammation may contribute to bone resorption associated to various disease processes (Burrage and Brinckerhoff, 2007).

Bone disease results from a disruption of the fine balance between osteoblastic bone formation and osteoclastic bone resorption. Osteoclasts originate from the fusion of precursors belonging to the monocyte/macrophage lineage, originating multinucleated cells. Macrophage colony stimulating factor (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 (Leibbrandt and Penninger, 2008). This process is inhibited by the presence of osteoprotegerin (OPG). Although RANK(L) signaling is essential for osteoclastogenesis, cross talks with other signaling molecules are important equally. RANKL is also expressed on activated T cells, suggesting that bone resorption is influenced by T cells. Although RANKL is the major cytokine that regulates osteoclast differentiation (Takayanagi, 2010b) it is notable that most of the T-cell cytokines, including IFN-γ, IL-4 and IL-10, inhibit osteoclastogenesis. However, IL-17 induces RANKL on osteoclastogenesis-supporting cells such as osteoblasts (Okamoto and Takayanagi, 2011). IL-17 also enhances local inflammation and increases the production of inflammatory cytokines, which further promote osteoclastogenic activity. Under proinflammatory conditions, RANKL and TNF-α cooperate to enhance osteoclastogenesis (Zhang et al., 2001). Osteoclast differentiation could also occur through a mechanism that is independent of RANKL-RANK interaction but mediated by TNF-α (Kim et al., 2005).

Theories have been postulated to explain the disruption of homeostatic balance of bone in Gaucher disease, implying dysfunction of osteoclasts, osteoblasts and mesenchymal cells (Campeau et al., 2009; Mistry et al., 2010; van Dussen et al., 2011). In our work, we hypothesized that cellular alteration in Gaucher disease produces a proinflammatory milieu leading to bone destruction through enhancement of monocytes differentiation to osteoclasts and osteoclasts resorption activity. Against this background we decided to investigate in an *in vitro* chemical model of Gaucher disease, the capacity of secreted soluble mediators to induce osteoclastogenesis, and the mechanism responsible for this phenomena.

2. Materials and methods

2.1. Ethics statement

Human PBMCs were isolated from healthy blood donors in accordance with the guidelines of the Ethical Committee of AADELFA (Medical Association for Study of Lysosomal Disorders and Fabry disease in Argentina) according to provisions of the Declaration of Helsinki in 1995. The nature and purpose of the study were explained to all volunteers and all patients gave their informed consent prior to participation in this study.

2.2. Conditioned media preparation

Peripheral blood samples (10 ml) were collected 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. PBMCs were cultivated in AIM-V media (Invitrogen, Carlsbad, CA, USA) at 37 °C in a 5% CO_2 atmosphere to a number of 10^6 cells/ml for 72 h in the presence or absence of CBE 500 mM (Sigma, St Louis, MO, USA). LPS 500 ng/ml was added in the last 24 h of the culture. After the 72 h culture conditioned media (CM) was obtained by centrifugation of the cultures.

2.3. Glucosylceramide accumulation

After culture PBMCs were collected. Glycolipids were extracted according to the method of Vance and Sweeley (1967) with modifications. Briefly, 3 ml of chloroform:methanol 2:1, v/v and 0.8 ml of water were added to the cell pellet, mixed by vortex for 30 s and incubated on ice 15 min. One milliliter of chloroform and 1 ml of water were added and mixed by vortex again for 30 s. The tube was centrifugated to separate the phases, the upper aqueous phase was removed, and the lower phase was brought to dryness. One milliliter of methanol and 0.1 ml 1.0 mol/l NaOH were added to the dried residue, and incubated at 37 °C overnight. After the addition of 2 ml of chloroform and 0.5 ml water and separation of the phases, the upper phase was removed. The lower phase, corresponding to the glycolipid extract, was brought to dryness. Glycolipids were then dissolved in chloroform:methanol 2:1, v/v and applied in a Silicagel 60 thin layer chromatography (TLC) plate. TLC was performed using chloroform:methanol:water 70:30:4, v/v/v and glycolipids were detected with orcinol spray reagent. Three micrograms of pure glucosylceramide (Matreya, Pleasant Gap, PA) was used as standard.

2.4. Osteoclast formation assay

THP-1 cells 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 medium) and 30 ng/ml of recombinant human macrophage colony stimulating factor (M-CSF) (R&D, Minneapolis, MN, USA) 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 CM and complete medium in a 1:1 ratio supplemented with M-CSF and 2 μg/ml of Polymyxin-B sulfate to avoid LPS inhibition of osteoclast formation (Mistry et al., 2010) for 7 days replacing the media every 48 h. In the last 48 h of culture the media was replaced with complete media alone and the supernatant was harvested to asses for the presence of cytokines and Gaucher disease biomarkers. As positive control of osteoclast formation cultures received 50 ng/ml of human RANKL (Millipore, Billerica, MA, USA). To evidence the role of TNF-α in osteoclastogenesis the osteoclast formation assay was performed in the presence of an anti-TNF-α antibody at 2 ng/ml (BD Pharmingen, San Diego, CA) to neutralize this cytokine or an isotype control (BD Pharmingen, San Diego, CA). To analyze the involvement of T cells in osteoclastogenesis, the osteoclast formation assay was performed using CM from CD3 depleted PBMC. CD3 depletion of PBMC was carried out by the use of RosetteSep® Human CD3 Depletion Cocktail (Stem cell Technologies, Vancouver, Canada) according to manufacturer's protocol. To identify

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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.

2.5. 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). CD51-positive multinucleated (more than 3 nuclei) cells were defined as osteoclasts.

2.6. Pit formation assay

THP-1 cells (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.

2.7. Cytokines assays

Concentrations of IL-1β, IL-6, TNF-α, transforming growth factor (TGF)-β, IL-4, IL-12, IFN-γ (BD Pharmingen, San Diego, CA), IL-13 and IL10 (eBiosciences, San Diego, USA) in culture supernatants were determined by capture ELISA.

2.8. Zymography

MMP levels were assayed by zymography according to the method of Hibbs et al. (1985). 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.

2.9. Gelatinase activity under native conditions

Gelatinase activity in unprocessed culture supernatants (native conditions) was measured by using a gelatinase/collagenase fluorometric assay kit (EnzChek; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The EnzChek kit contains DQ gelatin, a fluorescein-conjugated gelatin so heavily labeled with fluorescein that fluorescence is quenched. When this substrate is digested by gelatinases or collagenases it yields highly fluorescent peptides, and fluorescence increase is proportional to proteolytic activity. Collagenase purified from *Clostridium histolyticum* provided in the assay kit serves as a control enzyme. Plates were read in a fluorescence plate reader (Victor3; Perkin-Elmer, Waltham, MA).

2.10. Statistical analysis

Statistical analysis was performed with one-way ANOVA, followed by *post hoc* Tukey Test using GraphPad Prism 4.0 software. Data are represented as mean ± SD.

3. Results

3.1. Glucosylceramide accumulation in the in vitro chemical model

In order to study the impact of GCase deficiency and glucosylceramide accumulation, we used an already described chemical model of Gaucher disease induced by CBE, a specific inhibitor of GCase activity (Kacher and Futerman, 2009; Schueler et al., 2004; Yatziv et al., 1988). The optimal dose of CBE able to inhibit GCase activity was found to be 500 μM. In this condition, only 2% of the original GCase activity remained. PBMC exposed to CBE accumulate glucosylceramide after 3 days of culture as detected by TLC (Fig. 1).

This *in vitro* chemical model of Gaucher disease has been developed by addition of CBE. Moreover, we also tested the effect of the addition of LPS in the last 24 h of culture, after early stages of activation of macrophages. Addition of LPS could resemble the situation of a concomitant infection in a Gaucher disease patient.

3.2. Cytokine determination in supernatants from normal control PBMC exposed to CBE/LPS

PBMC exposed to CBE alone did not secrete significant amounts of proinflammatory cytokines. However, addition of both CBE plus LPS increased significantly proinflammatory cytokines secretion TNF-α ($p=0.001$), IL-1β, IL-12 and IL-6 compared to the secretion induced by LPS stimulation. A tendency towards a reduction in the secretion of the T cell derived cytokines IFN-γ, IL-13, IL-10 and TGF-β was observed (Fig. 2). However, exposure to PBMC to CBE induced an increase production of IL-4 that could reflect the differentiation of macrophages into an alternative phenotype.

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

In order to test whether soluble factors secreted by cells from this Gaucher disease *in vitro* chemical model are capable to induce osteoclast formation from human acute monocytic leukemia cell line (THP-1), these cells were stimulated with M-CSF in conjunction with CM.

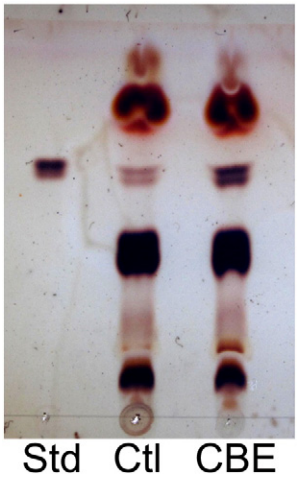

Fig. 1. Thin layer chromatography of glycolipids from PBMC exposed to CBE (CBE) or untreated (Ctl). Glucosylceramide was used as standard (Std).

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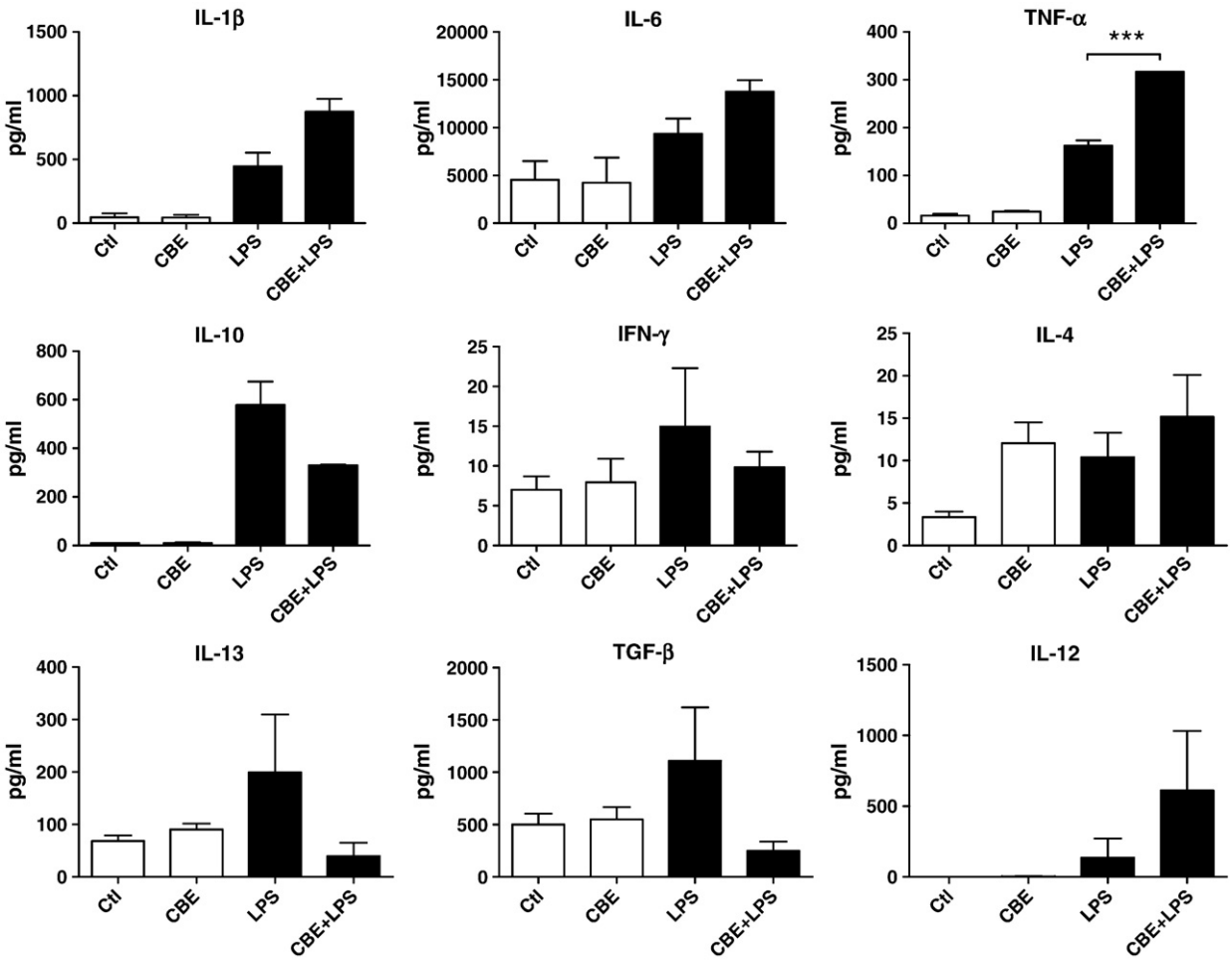

Fig. 2. Cytokine levels in supernatants from PBMC exposed to CBE or CBE/LPS in comparison to their respective controls (untreated or LPS, respectively). IL-1β, IL-6, TNF-α, IL-10, IFN-γ, IL-4, IL-13, TGF-β, and IL-12 were determined in CM by ELISA. Bars express the mean ± sem of duplicates. Data shown are from a representative experiment of five performed. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ *versus* control.

Addition of CM from PBMC exposed to CBE (PBMC/CBE) induced *ex vivo* osteoclastogenesis as was determined by the generation of $TRAP^{+}$ multinucleated cells from precursors. This effect was increased when CM from PBMC exposed to CBE + LPS (PBMC/CBE + LPS) was used; suggesting an increased differentiation of precursors into osteoclasts (Fig. 3a).

Since differentiated osteoclasts also secret MMP-9 we decided to analyze whether these $TRAP^{+}$ multinucleated cells were capable to produce and secrete matrix metalloproteinases (MMPs). The production of MMPs was determined by zimography in culture supernatants (Fig. 3b). A significant increase in the production of MMP-9 was detected when precursors cells were exposed to CM obtained from PBMC/CBE + LPS as compared to its control. This increment in MMP production could result in an increment in degradation of extracellular matrix; however, the net effect will result from a balance between MMP and their inhibitor TIMPs. For this reason, we decided to determine the net gelatinolytic activity, by adding CM to a nonfluorescent gelatin-fluorescein conjugate, and the fluorescence unmasked as a consequence of gelatin degradation was measured in a fluorometer. An increment in net gelatinolytic activity was revealed in supernatants from cells exposed to CM from PBMC/CBE alone or PBMC/CBE + LPS as compared to their respective controls (Fig. 3c).

Furthermore, to corroborate that $TRAP^{+}$ multinucleated cells were osteoclasts, we analyzed the presence of CD51 on multinucleated cells (Fig. 3d). The number of multinucleated CD51 positive cells was significantly higher when precursors were incubated with CM from PBMC/CBE alone or PBMC/CBE + LPS as compared to their respective controls.

3.4. Osteolytic activity

Our premise is that Gaucher disease induces a proinflammatory microenvironment that would promote the generation of osteoclasts, leading to bone loss. Thus, we assessed the functional activity of Gaucher induced osteoclast like cells by their ability to resorb dentine. To this end, osteoclastogenesis experiments were performed on a synthetic bone matrix. Number of resorption areas was increased when osteoclasts precursors were cultured in the presence of CM from PBMC/CBE. This effect was accentuated with CBE + LPS (Fig. 4).

3.5. Functional osteoclasts secrete cytokines and markers of Gaucher cells

We aimed to analyze whether activated osteoclasts were capable to induce itself a proinflammatory millieu by secreting cytokines. To test this hypothesis, the presence of IL-1β, TNF-α and IL-6 was analyzed in supernatants from differentiated osteoclasts by ELISA (Fig. 5a). Osteoclasts secreted IL-1β and TNF-α, when stimulated with CM from PBMC/CBE, and the effect was statistically significant when stimulated with CM from PBMC/CBE + LPS in comparison to its control.

It has been demonstrated that the enzyme chitotriosidase (CHIT) (Hollak et al., 1994) is produced by Gaucher cells, and is a marker of alternative macrophage activation. Measurement of CHIT activity

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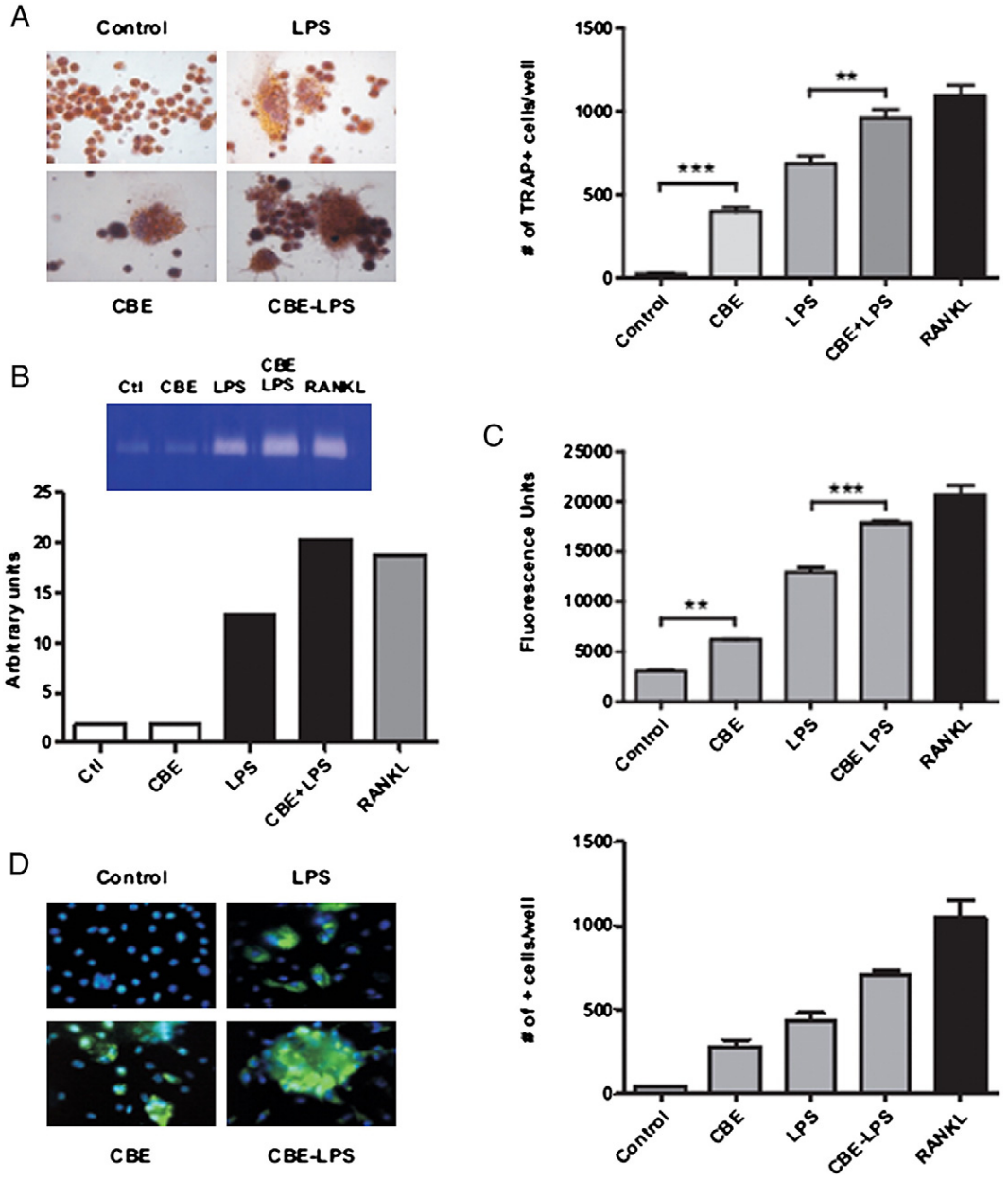

Fig. 3. Induction of differentiation of precursors by CM. Precursors cells exposed to CM from PBMC/CBE or PBMC/CBE + LPS were analyzed for following markers of osteoclasts. 3A: $TRAP^+$ cells showing ≥3 nuclei. 3B: MMP-9 levels by zimography. 3C: net gelatinolotic activity by fluorometry. 3D: expression of vitronectin receptor by immunofluorescence. Bars express the mean ± sem of duplicates. Data shown are from a representative experiment of five performed. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ *versus* control.

demonstrated an induction of the production of this enzyme by osteoclasts differentiated by the addition of CM from PBMC/CBE or PBMC/CBE + LPS as compared to each control (Fig. 5b).

3.6. Role of TNF-α in osteoclastogenesis

The differentiation of new osteoclasts is dependent on the balance between the expression of RANKL and that of its decoy receptor, osteoprotegerin, in osteoblasts (Kong et al., 1999; Suda et al., 2001). During chronic inflammatory bone diseases, cellular recruitment contributes to bone loss. RANKL and proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, have been shown to be important for disease progression and bone loss (Haynes, 2004; Kotake et al., 1996; Merkel et al., 1999; Nair et al., 1996; Wei et al., 2005).

We hypothesized that osteoclastogenesis in this chemical *in vitro* model of Gaucher disease could be independent of RANKL but dependent on proinflammatory cytokines. In particular, TNF-α is the key cytokine implicated in RANKL independent osteoclastogenesis (Azuma et al., 2000; Kobayashi et al., 2000).

To test this hypothesis, osteoclastogenesis experiments were performed in the presence of a TNF-α blocking antibody or its isotype control (Fig. 6). Incubation of precursors with CM along with anti-TNF-α significantly reduced both $TRAP^+$ multinucleated cells and resorptive areas, whereas an isotype control had no effect. Moreover incubation of osteoclasts precursors with recombinant TNF-α alone induced osteoclastogenesis.

3.7. Involvement of T cells in osteoclastogenesis

To evaluate involvement of T cells in chemical model-induced osteoclastogenesis, we immunodepleted mature CD3 T cells from PBMC cultures Fig. 7. As shown in Fig. 7a depletion of T cells significantly

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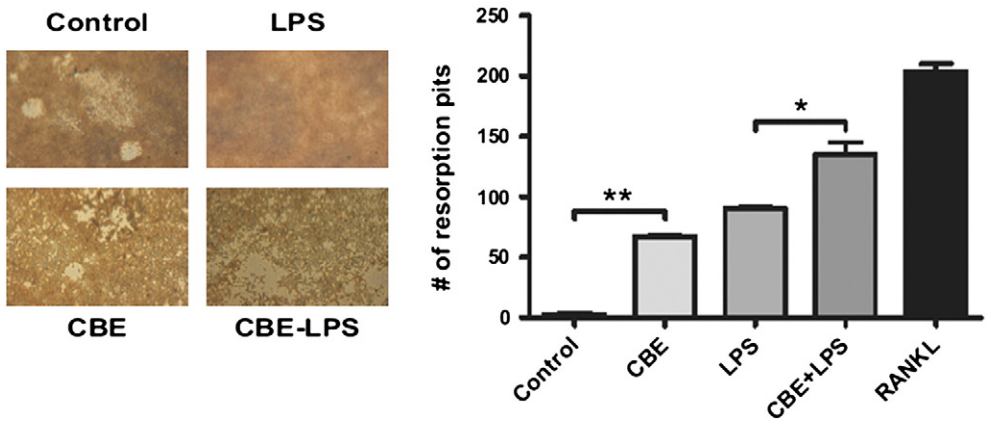

Fig. 4. Resorption activity of TRAP$^+$ multinucleated cells. Functional activity of CM induced osteoclasts was determined by their ability to resorb dentine. Osteoclastogenesis experiments were performed on dentine discs under the same conditions as described above. After 5 days, cells were removed, and dentine resorption was determined by light microscopy, and the number of resorption pits was counted. Bars express the mean ± sem of duplicates. Data shown are from a representative experiment of five performed. *P<0.05; **P<0.01; ***P<0.001 *versus* control.

reduced the secretion of proinflammatory cytokines in response to CBE and CBE + LPS stimulation. Moreover, the ability of CM from CBE and CBE/LPS stimulated T cell depleted PBMC to induce osteoclast differentiation was reduced by 2-fold respect to not depleted PBMC (Fig. 7b)

4 . Discussion

Since the pathophysiology of bone disorder observed in Gaucher disease has not yet been fully understood (Mikosch, 2011), we hypothesized that cellular alteration caused by GCase deficiency and concomitant glucosylceramide accumulation in Gaucher disease may induce a proinflammatory milieu leading to an imbalance in normal bone physiology which results in an increment in bone resorption. To search this supposition, we aimed to study the capacity of soluble mediators secreted by a chemical model of Gaucher disease to induce osteoclastogenesis. We demonstrated that soluble factors produced by CBE-treated PBMC induced differentiation of osteoclasts precursors into mature and active osteoclasts. These osteoclasts secreted high levels of MMP with net gelatinolitic activity and presented functional osteolytic activity. In accordance with our results, by using a similar approach, Lecourt et al. (2012) have shown CBE-treated mesenchymal stem cells CM induced an increase in resorption areas when added to monocyte precursors. On the other hand, they did not find any stimulation by CBE itself, suggesting

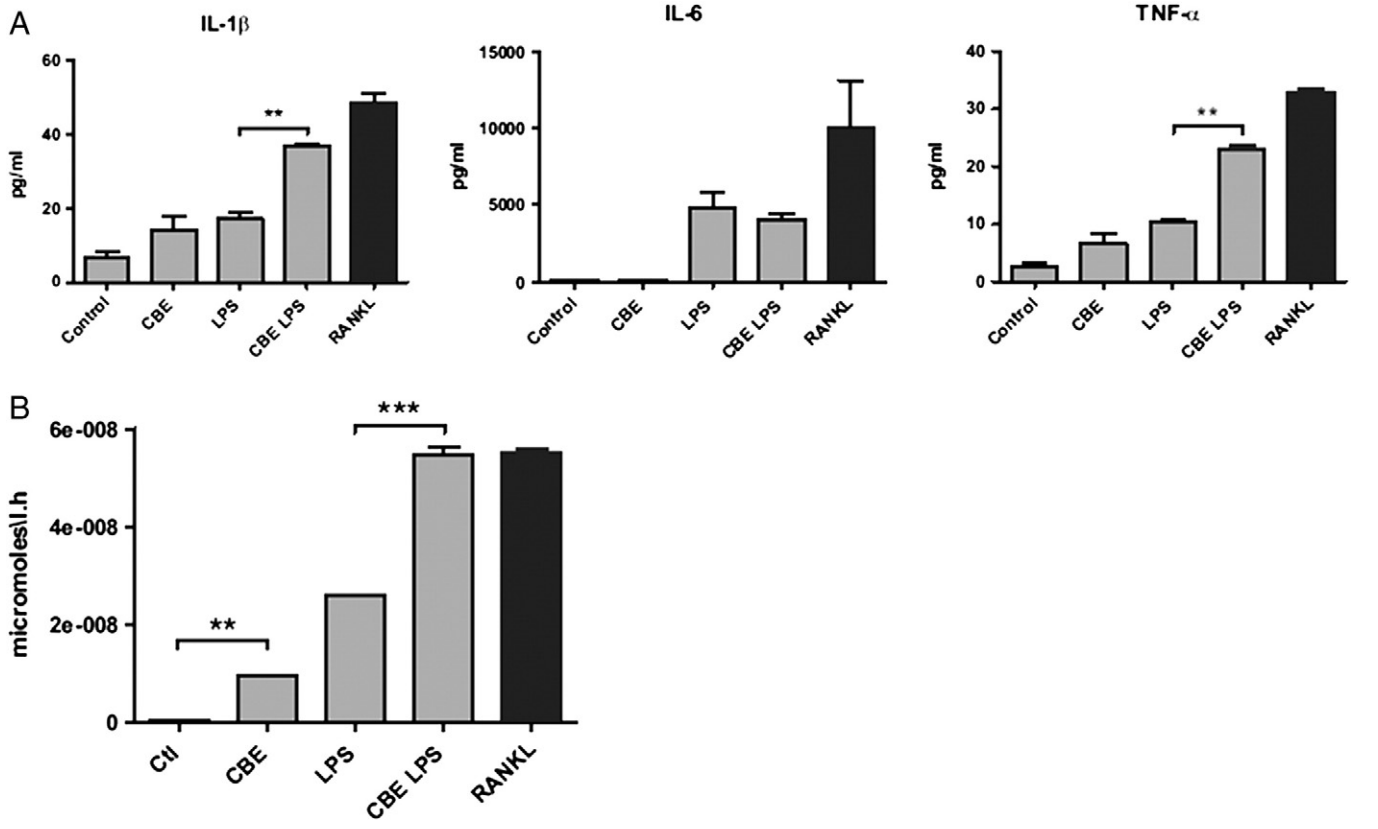

Fig. 5. Characterization of secretion of cytokines and Gaucher-disease biomarkers from functional osteoclasts generated by exposition of CM from PBMC/CBE or PBMC/CBE + LPS. 5A: Cytokine (IL-1β, IL-6, TNF-α) levels in supernatants from functional osteoclasts were determined in CM by ELISA. 5B: chitotriosidase activity as determined by fluorometry. Bars express the mean ± sem of duplicates. *P<0.05; **P<0.01; ***P<0.001 *versus* control.

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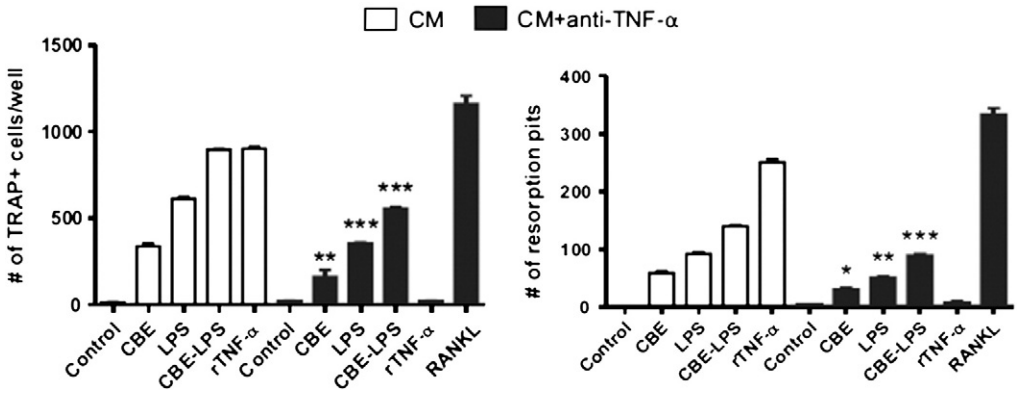

Fig. 6. Osteoclastogenesis in Gaucher disease is TNF-α-dependent. Measurement of $TRAP^{+}$ multinucleated cells and resorption areas after the addition of CM from PBMC/CBE or PBMC/CBE + LPS to precursors in the presence of anti-TNF-α or isotype control. Bars express the mean ± sem of duplicates. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ *versus* control.

osteoclasts activation observed in Gaucher disease likely results from extrinsic stimulation.

Chitotriosidase is a molecule produced by Gaucher cells, and is currently used as biomarker in Gaucher disease (Hollak et al., 1994). This molecule correlated strongly with disease symptoms and is used to monitor the efficacy of therapy (Deegan et al., 2005). Osteoclasts produced by the addition of CM in our work have revealed the expression of this characteristic marker. Chitotriosidase production is also reflecting alternative activation of macrophages that is a hallmark of Gaucher cells (Boven et al., 2004). Moreover, these cells secrete proinflammatory cytokines itself, amplifying the inflammatory state.

In order to analyze the effect on bone pathology in the situation of an infection in a Gaucher disease patient, we carried out the same set of experiments but in the presence of LPS. We observed an enhancement of osteoclastogenesis and resorption when the cells were exposed to LPS. This result could imply a worsening of bone pathology during an

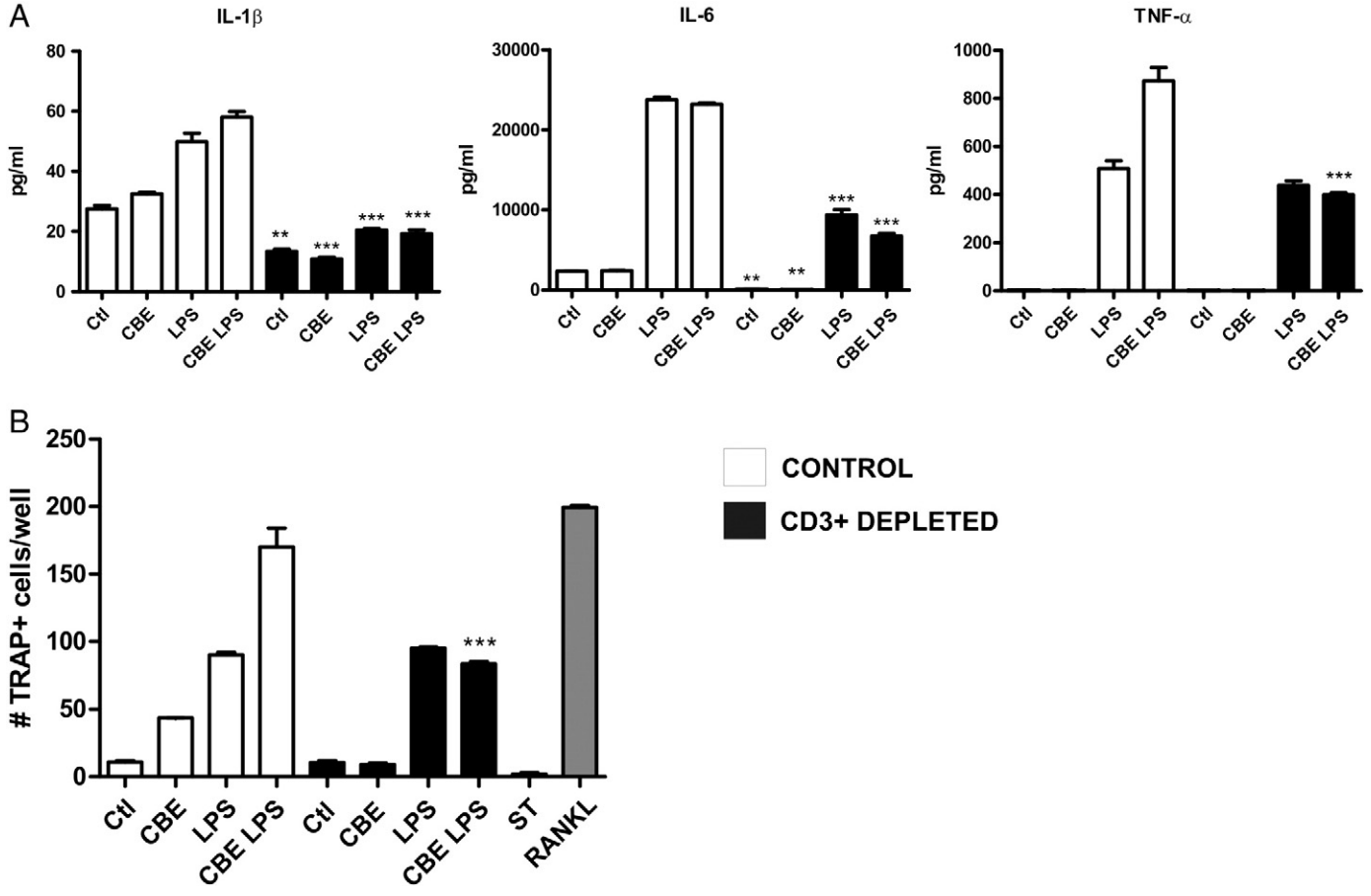

Fig. 7. Involvement of T cells in osteoclastogenesis in Gaucher disease chemical model. Cytokine (IL-1β, IL-6, and TNF-α) levels in supernatants (7A) and TRAP determination (7B) from mature osteoclasts obtained by stimulation with CM from PBMC or CD3 depleted PBMC exposed to CBE or CBE/LPS in comparison to their respective controls (untreated or LPS stimulated, respectively). Bars express the mean ± sem of duplicates. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ *versus* control.

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infection. Clinically, it could be related to the predisposition to bone infections and complications that are hard to treat in GD (Finkelstein et al., 1992; Margalit et al., 2002).

Osteoclastogenesis is physiologically induced by the axis RANK–RANKL–OPG. On the other hand, in pathologic states this process could be independent of this axis, but induced by cytokines like TNF-α (Zhang et al., 2001). Studies in Gaucher disease patients suggest this axis probably does not predict bone mineral density or osteoporosis (Magal et al., 2006). In this study, production of cytokines was induced. Indeed, osteoclasts differentiation and its osteolytic activity were decreased (but not totally eliminated) when cells were exposed to anti-TNF-α. This result suggests a role of TNF-α in promoting osteoclastogenesis in Gaucher disease chemical model, although other soluble factors may also be contributing.

T cells have the capacity to support osteoclastogenesis by RANKL dependent and independent mechanisms (Kotake et al., 2001). Moreover, T cells are known to be capable to secrete RANKL and proinflammatory and proosteoclastogenic cytokines such as TNF-α and IL-17 (Roggia et al., 2004; Sato et al., 2006). It has long been recognized that infection, inflammation, and autoimmune disorders are associated with systemic and local bone loss. Yet, only recently it has been shown that T lymphocytes and their products are key regulators of osteoclast and osteoblast activity. It is well established that RANKL and M-CSF are essential for physiologic osteoclast renewal. On the other hand, in a variety of conditions such as inflammation, hyperparathyroidism, and estrogen deficiency, other cytokines produced by T cells, are implicated in enhancement of osteoclast activity. One such factor is TNF-α, which enhances osteoclast formation and the responsiveness of osteoclast precursors to RANKL (Roggia et al., 2001; Weitzmann and Pacifici, 2005). Indications of T cell compromise associated to bone pathology in Gaucher disease have been recognized for a long time (Lacerda et al., 1999). To analyze the biological relevance of T cells in osteoclastogenesis of Gaucher disease, we investigated osteoclastogenesis in T cell-depleted PBMC cultures. This experiment revealed that CBE-induced exacerbation of proinflammatory cytokine secretion and osteoclastogenesis in LPS stimulated PBMC are dependent on T cells. These findings suggest that T cells, at least in part, play a role in osteoclast formation in Gaucher disease. This model was also valuable to analyze the impact of an infection on GD-bone pathology. In concordance with these results, high levels of cytokines derived from T cells and significant osteopenia have been revealed in GCase gene deleted murine model of Gaucher disease. Although reduction in bone formation has been associated to bone pathology, the implication of osteoclast in this process has not been ruled out (Mistry et al., 2010).

5. Conclusions

In conclusion, our data suggests that *in vitro* GCase deficiency, and concomitant glucosylceramide accumulation, generate a state of osteoclastogenesis mediated in part by pro-resorptive cytokines, especially TNF-α. Moreover, T cells are involved in osteoclastogenesis in Gaucher disease chemical model.

Role of funding source

This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica, Argentina [PICT01070 to PR]. The finding source had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication

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.

Other authors have no conflict of interest to declare.

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