

## PAPER

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# Oleuropein and its peracetylated derivative negatively regulate osteoclastogenesis by controlling the expression of genes involved in osteoclast differentiation and function

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During chronic inflammation, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) have well established effects on gene networks that stimulate osteoclastogenesis, which is the culprit of several bone diseases. In this study, we investigated the anti-osteoclastogenic effects *in vitro* of oleuropein (OL) and its peracetylated derivative (Per-OL) by exploring the expression level of key hub genes involved in fate decision and lineage commitment, differentiation, and function of human blood monocyte-derived osteoclasts. Monocytes were purified from peripheral blood mononuclear cells of healthy individuals using commercial antibodies coated with magnetic beads and treated with M-CSF/RANKL in the presence or absence of OL or Per-OL (25 and 50  $\mu$ M) for 6 days. We demonstrated that OL and especially Per-OL impair transcriptional gene circuits able to support osteoclastogenesis from human blood monocytes. Our results indicate that OL and notably Per-OL are promising candidates to control osteoclastogenesis.

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## Introduction

Bone homeostasis is a dynamic complex process involving equilibrium of opposite driving forces for bone resorption by osteoclasts and bone formation by osteoblasts. The disruption of bone homeostasis in favour of osteoclasts leads to manifestation of several pathological conditions, such as osteoporosis, rheumatoid arthritis, lytic bone metastases, and Paget's bone disease.<sup>1–3</sup> Osteoclastogenesis is primarily mediated by cells of the monocyte/macrophage lineage, which act as upstream precursors of osteoclasts upon stimulation with the macrophage colony-stimulating factor (M-CSF) and the ligand for the receptor activator of nuclear factor- $\kappa$ B (RANKL). M-CSF/RANKL sig-

nalling dictates the transcriptional activation of genes involved in the commitment of pre-osteoclasts, including the *TNFRSF11A* gene that encodes the receptor for RANKL (RANK) providing an autocrine loop to amplify the input signal of RANKL for further differentiation into mononucleated pre-osteoclasts and then into mature and functional multinucleated osteoclasts.<sup>4,5</sup> Therefore, osteoclasts are target cells to arrest their differentiation and/or function for the treatment of osteoclastogenic disorders.

Antiresorptive medication is often associated with many safety concerns.<sup>6</sup> Owing to these limitations, natural products, mainly phytochemicals are becoming an important research field to explore new therapeutic choices for bone diseases. Oleuropein (OL) is a secoiridoid glycoside that typically represents the most prominent hydrophilic phenolic compound in olives and olive leaves. By enzymatic or chemical hydrolysis, OL can degrade to yield its aglyconic form and hydroxytyrosol, which may be found naturally in olive oils.<sup>7</sup> On the other hand, full acetylated or peracetylated OL (Per-OL) has been recently shown to be a safe and biologically active semi-synthetic stable derivative with better bioavailability and sometimes even with better biological activity than OL.<sup>8</sup> After consumption, this approach designed to preserve OL integrity

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allows the OL to be released in the surrounding extracellular milieu or inside cells upon extensive deacetylation by secreted or cytosolic esterases.<sup>9</sup> Pharmacological effects of OL such as antioxidant, antihypertensive, hypoglycaemic, cardioprotective, neuroprotective, antimicrobial, and anti-inflammatory, and as adjuvant in multitarget anti-cancer treatments have already been outlined.<sup>10–13</sup> OL also elicits osteoprotective effects by promoting differentiation of human bone marrow mesenchymal stem cells into osteoblasts<sup>14,15</sup> and by reducing tartrate-resistant acid phosphatase (TRAP) activity in osteoclasts formed from mouse spleen cells.<sup>16</sup> In addition, Per-OL has been confirmed to lessen carrageenan-induced paw oedema in rats<sup>17</sup> and metabolic abnormalities in a mouse model of high-fat induced obesity,<sup>18</sup> and also to exhibit antioxidant and antiproliferative effects in human breast<sup>19</sup> and thyroid<sup>20</sup> cancer cell lines. However, the effects of OL and Per-OL on any stage of osteoclast development of the human monocyte/macrophage lineage have not yet been reported. In the present study, Per-OL was prepared by environmentally friendly protocols, and the effects of OL and Per-OL on osteoclast differentiation and function were investigated. We put the focus on the transcriptional activity of genes related to the differentiation process from committed pre-osteoclasts to mature and functional osteoclasts.

## Materials and methods

### Chemicals

OL was extracted from olive leaves according to the reported literature<sup>21</sup> and then purified using silica gel column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 10:1 to 5:1 v/v) to give a yellow solid (Scheme 1A). For obtaining Per-OL, OL (1 g, 1.85 mmol) was solved in pyridine/acetic anhydride (1:1 v/v) at 0 °C for 10 min and then the reaction was kept at room temperature overnight. After hydrolysing the acetic anhydride, the solution was concentrated to dryness and the crude residue was purified using silica gel column chromatography (ethyl acetate–hexane, 1:1). Spectroscopy data of the resultant brown solid (Scheme 1B) was identical to that reported in literature.<sup>22</sup>

### Human monocytes

This study was conducted according to Good Clinical Practice Guidelines and in line with the principles outlined in the Helsinki Declaration of the World Medical Association. Study subjects were recruited at Virgen del Rocio University Hospital

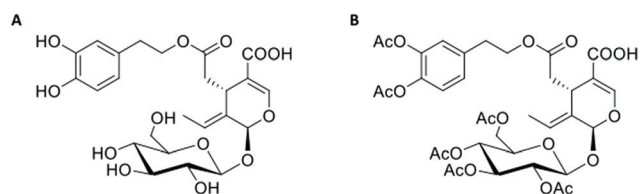
(VRUH, Seville, Spain). Informed consent for the study was obtained. All protocols were approved by the local institutional review board (Ethics Committee of the VRUH, #OLNAMS 08/11). Venous blood samples were obtained from healthy adult volunteers (<35 years old) non-smokers and not taking any medication. Donors were recognized as healthy, according to medical history and routine laboratory tests. Peripheral blood mononuclear cells (MNCs) were isolated by centrifugation over a Ficoll-Histopaque (Sigma-Aldrich, St Louis, MO, USA) gradient.<sup>23</sup> Monocytes were isolated from peripheral blood MNCs using CD14 microbeads and LS columns on a midiMACS system (Miltenyi Biotec, Madrid, Spain). Monocyte (CD14<sup>+</sup>) purity was routinely >90% by flow cytometry analysis (FACSCanto II flow cytometer and FACSDiva software; Becton Dickinson, San Jose, CA, USA) and cell viability >95% by trypan blue exclusion (Sigma-Aldrich). The monocytes were seeded in 24-well culture plates at a density of  $1 \times 10^6$  cells per mL and cultured in ultra-low attachment flasks in RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin, and 10% heat-inactivated foetal bovine serum (complete culture medium).

### Osteoclast differentiation and viability

Monocytes were induced to differentiate into osteoclasts for 6 days with human recombinant M-CSF (50 ng mL<sup>-1</sup>) and RANKL (50 ng mL<sup>-1</sup>) in the absence or presence OL or Per-OL (25 and 50 μM). These concentrations were within the range of concentrations consistent with the *in vitro* beneficial effects of oleuropein or Per-OL on different cell types.<sup>16,24–27</sup> Complete culture medium was replaced every 2 days with fresh medium, the cytokines, and OL or Per-OL. For cell viability, monocytes were seeded in 96-well plates ( $1 \times 10^5$  cells per well) and differentiated into osteoclasts as indicated above, in the absence or presence of OL or Per-OL (in the range of 12.5–50 μM). At day 6, methylthiazolyldiphenyl-tetrazolium bromide (MTT) solution (Sigma-Aldrich) was added to cells for 2 h until a purple precipitate was visible. MTT-formazan crystals were then solubilized with DMSO (Sigma-Aldrich) and measured with a microplate reader at 570 nm corrected to 650 nm. Cell survival was expressed as the percentage of absorbance compared with that of control, nontreated cells.

### TRAP staining and activity assay

TRAP staining was performed using an acid phosphatase leukocyte kit (Sigma-Aldrich) in osteoclasts fixed with 4% paraformaldehyde. TRAP-positive multinuclear cells containing more than three nuclei were scored as osteoclasts and examined using an Olympus microscope (40×) (Model IX71, Olympus, Center Valley, PA, USA). TRAP activity was determined using a TRAP assay kit (Takara Bio, Shiga, Japan). Briefly, 50 μL of cell extract and 50 μL of *p*-nitro-phenyl phosphate (*p*NPP) were mixed with a sodium tartrate solution and incubated at 37 °C for 25 min. The reaction was stopped with 0.5 N NaOH and the absorbance was measured at 405 nm. A solution containing acid phosphatase and *p*NPP, but not the sodium tartrate solution, was used as a control.



**Scheme 1** (A) Chemical structure of oleuropein (OL) and (B) of peracetylated-oleuropein (Per-OL).



### RNA isolation and quantitative PCR

Total RNA was extracted by using Trisure Reagent (Bioline, London, UK). RNA quality was assessed by  $A_{260}/A_{280}$  ratio in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Greenville, SC, USA). Briefly, RNA (250 ng) was subjected to reverse transcription (iScript, Bio-Rad, Madrid, Spain). An amount of 20 ng of the resulting cDNA was used as a template for real-time PCR amplifications. The mRNA levels for specific genes were determined in a CFX96 system (Bio-Rad). For each PCR reaction, cDNA template was added to Brilliant SYBR green QPCR Super-mix (Bio-Rad) containing the primer pairs for either gene or for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene (Table 1). All amplification reactions were performed in triplicate and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The

magnitude of change of mRNA expression for candidate genes was calculated by using the standard  $2^{-(\Delta\Delta Ct)}$  method. All data were normalized to endogenous reference (GAPDH) gene content and expressed as relative fold-change of control.

### Cytokine quantification

Tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-6, and IL-10 were determined by ELISA, following the indications of the manufacturer (Diaclone, Besançon, France). Cytokine concentration was expressed in pg mL<sup>-1</sup>, as calculated from the calibration curves from serial dilution of human recombinant standards in each assay.

### Statistical analysis

All values are expressed as mean  $\pm$  SD. Data were evaluated with GraphPad Prism 5.01 (GraphPad Software, San Diego,

**Table 1** Primers used for quantitative PCR

Target	Accession no.	Direction	Sequence (5' $\rightarrow$ 3')
ACP5	NM_001111034	Forward	CTTTCTACCGCTGCACTTC
		Reverse	GTTTCTTGAGCCAGGACAGC
ATP5PO	NM_001697.3	Forward	TCCTGAAGGAACCCAAAGTG
		Reverse	ATCGACCATTTTCAGCAAGC
ATP6V0D2	NM_152565.1	Forward	TGGCACTGAATTGAGCAAAG
		Reverse	CACTGCCACCTACAGCTTCA
CTSK	NM_000396	Forward	TTCTGCTGCTACCTGTGGTG
		Reverse	CCAGGTGGTTCATAGCCAGT
CYCS	NM_018947.5	Forward	TTGGCAATCCGTCATCAGTA
		Reverse	CCCGACAGTGCCTAGAAGAG
DCSTAMP	NM_030788.4	Forward	CACTTGAAACTGCACGGAGA
		Reverse	AGGACAACAGTCCCAGCATC
FOS	NM_005252.4	Forward	CTTCCTGTTCCAGCATCAT
		Reverse	GTACAGGTGACCACCGGAGT
GAPDH	NM_001289746	Forward	CACATGGCCTCCAAGGAGTAAG
		Reverse	CCAGCAGTGAGGGGTCTCTCT
GNA13	NM_006572.6	Forward	TCCGTGAAATCATGCCTAGTA
		Reverse	CGCCTTAAAAATGATGGGAGA
IL10	NM_000572	Forward	CTGACATCAAGGAGCAGCTG
		Reverse	GGCTTTGTAGACACCCCTCT
IL1B	NM_000576	Forward	AAGGAGAGCTCTTTCCACC
		Reverse	GCCTCTGGTCTCCTTGGATT
IL6	NM_000600_	Forward	TAACCATCCCTGTCCCAACC
		Reverse	AGTTACATGCCAGTGGACA
ITGB3	NM_000212.2	Forward	GCAATGGGACCTTTGAGTGT
		Reverse	GTGGCAGACACATTGACCAC
MAFB	NM_005461.5	Forward	GCCTGCGCTAATTGTAGGAG
		Reverse	CGCACTTGAAAGTTGCAAAA
NFATC1	NM_172390.3	Forward	AGAAAGCGAAGCCAGTACCA
		Reverse	CGGTCTCACTAACGGGACAT
NFE2L2	NM_001145412.3	Forward	GCGACGGAAGAGTATGAGC
		Reverse	GTTGGCAGATCCACTGGTTT
NRF1	NM_001293163.2	Forward	GTCCAGATCCCTGTGAGCAT
		Reverse	CAATGTCACCACCTCCACAG
OSCAR	NM_001282349	Forward	CCCAGCTTCATACCACCCTA
		Reverse	GAAGAGAAGGGGAGCGATCT
PPARGC1B	NM_133263.4	Forward	GGAGACTGAACCTGAGCTG
		Reverse	GTAGGAGCTGCTGGTCTTGG
TFAM	NM_001270782.1	Forward	GTGGGAGCTTCTACTCTGG
		Reverse	TAGGGCTTTTCTCTGCAA
TNF	NM_000594	Forward	TCCTTCAGACACCCTCACC
		Reverse	AGGCCCCAGTTTGAATTCTT
TNFRS11A	NM_001270949	Forward	GGTGACGCTCTAACTCCTG
		Reverse	TTGAGACCAGGCTGGGTAAC
TNFRS11B	NM_002546	Forward	GGCAACACAGCTCACAAGAA
		Reverse	CTGGGTTTGCATGCCTTTAT



USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA); following Newman-Keuls' test for multiple comparisons. Differences between groups were considered to be significant at a  $p$  value of  $<0.05$ .

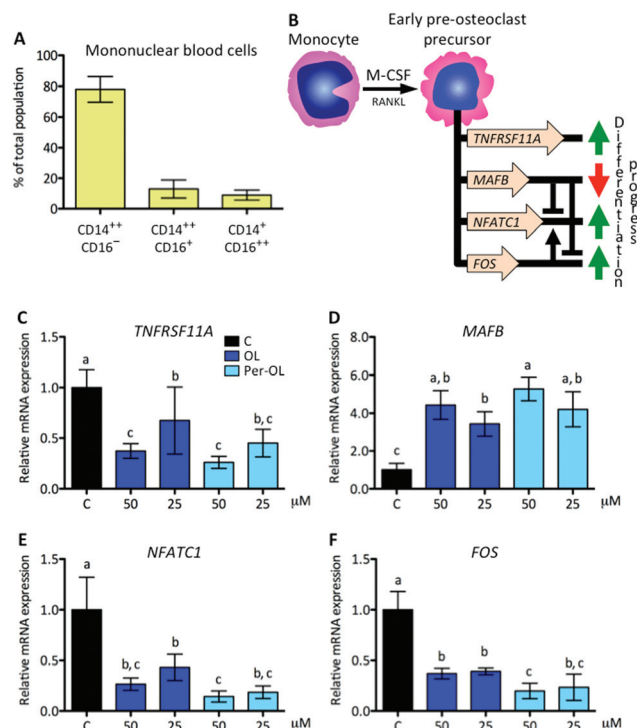
## Results and discussion

It has been well established that osteoclastogenesis plays fundamental roles in the development and progression of bone diseases. Hence, identification of new molecules effective on targets involved in the control of osteoclast differentiation and function would be crucial for maintenance of bone health and better-tailored treatments of disorders affecting the bone. Here, we designed a hypothesis-driven approach to probe if OL and Per-OL have any role in regulating transcriptional activity of selected genes that are required for commitment, differentiation, and function of osteoclasts from M-CSF/RANKL-treated human blood monocytes. This rationale was, in part, guided by previous evidence suggesting bone-protecting effects of OL in ovariectomized rats<sup>28</sup> and antiresorptive effects of OL in TRAP positive cells derived from mouse spleen.<sup>16</sup> A strength of our study stems from the inclusion of Per-OL, which is an OL derivative with potential enhanced ability to penetrate cell membrane.<sup>24,29</sup>

### OL and Per-OL disturb the instruction of M-CSF for the commitment of human blood monocytes to the osteoclast fate

In this study, monocytes were isolated from the blood of healthy volunteers and the surface expression of CD14 and CD16 antigens was determined by FACS. As expected, classical ( $CD14^{++}CD16^{-}$ ) and intermediate ( $CD14^{++}CD16^{+}$ ) monocytes were the most abundant subsets ( $\sim 78\%$  and  $\sim 13\%$ , respectively), with non-classical monocytes ( $CD14^{+}CD16^{++}$ ) remaining at  $\sim 9\%$  (Fig. 1A). In previous studies, all of these three subsets were reported to give rise to osteoclasts.<sup>30</sup> More recently, a systematic literature review concluded that specific features of the clinical course of bone disease can have a significant impact on commitment of human circulating monocyte subsets, with prominence of those rich in CD14, towards a bone-resorbing phenotype.<sup>31</sup>

At the beginning of the differentiation process of monocytes to pre-osteoclasts, M-CSF is a cytokine that primes monocytes to gain osteoclastogenic activity by the reprogram of different target genes, which are required for a rapid response to RANKL and for successive differentiation stages. The *TNFRSF11A* gene is critical to sustain the RANK/RANKL loop and the transcripts of V-maf musculoaponeurotic fibrosarcoma oncogene homolog B gene (*MAFB*) are associated with the escape of monocytes to M-CSF-induced proliferative and osteoclast differentiation signals *via* repression of the master nuclear factor of activated T-cells cytoplasmic 1 (*NFATC1*) and *FOS* genes.<sup>32</sup> *FOS* is also involved in monocyte commitment to pre-osteoclasts *via* its cooperation for transcriptional activity of *NFATC1* gene.<sup>33</sup> This view is depicted in Fig. 1B. In the pres-



**Fig. 1** Human monocytes and expression of genes that instruct osteoclast fate by M-CSF. Monocytes were isolated from the blood of healthy volunteers and the surface expression of CD14 and CD16 antigens was determined by FACS (A). Cells were induced to differentiate for 6 days with human recombinant M-CSF ( $50 \text{ ng mL}^{-1}$ ) and RANKL ( $50 \text{ ng mL}^{-1}$ ) in the absence (control cells) or presence of OL and Per-OL (25 and  $50 \mu M$ ). (B) Schematic representation of M-CSF induced commitment of monocyte into early pre-osteoclast precursor and set of principal genes involved. Levels of (A) *TNFRSF11A*, (B) *MAFB*, (E) *NFATC1*, and (F) *FOS* mRNA abundance normalized to GAPDH were determined by quantitative PCR. Data were generated in sextuplicate. Results are shown as mean  $\pm$  SD and those marked with different letters were significantly different ( $p < 0.05$ ) as calculated by one-way ANOVA following the Newman-Keuls' test.

ence of OL and Per-OL, the transcriptional activity of *TNFRSF11A* gene was found to be inhibited in M-CSF/RANKL-treated human blood monocytes (Fig. 1C). In addition, the expression of *MAFB* gene was dramatically upregulated (Fig. 1D) while that of *NFATC1* (Fig. 1E) and *FOS* (Fig. 1F) genes was downregulated. Differences between OL and Per-OL at  $50 \mu M$ , having Per-OL higher effect than OL, were observed for *FOS* gene expression. However, we did not detect any dose-dependent effect of OL or Per-OL. None of the concentrations used herein (up to  $50 \mu M$ ) of OL and Per-OL reduced cell viability as per MTT assay (data not shown). Deficient expression of *TNFRSF11A* gene has been shown to impede osteoclastogenesis and to induce osteopetrosis in mice.<sup>34</sup> A similar phenotype has been documented for patients with *TNFRSF11A* gene mutations associated with autosomal recessive osteopetrosis, who exhibited loss-of-function in both the extracellular and intracellular domains of RANK, and whose monocytes were unable to completely overcome the commitment to an osteoclast fate.<sup>35</sup> In a context of OL and Per-OL inducing the repres-



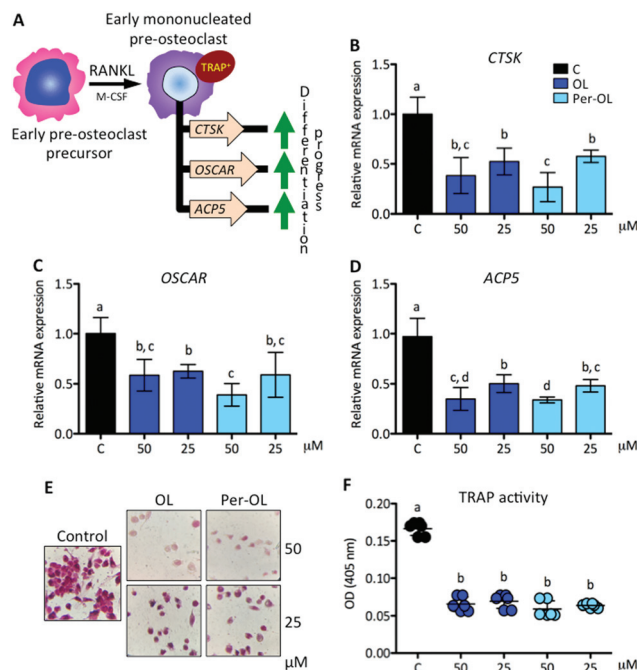


sion of *TNFRSF11A* gene, the contrariwise effect occurring on the transcriptional activity of *MAFB* gene agrees with previous research showing that expression of *MAFB* gene is negatively regulated by the RANKL-RANK axis in M-CSF/RANKL-treated murine bone marrow monocytes.<sup>32</sup> Gain-of-function experiments with murine bone marrow hematopoietic stem cells have also led to the identification of *MAFB* gene with ability to limit M-CSF instructions for commitment.<sup>36</sup> Therefore, our findings suggest that OL and Per-OL might induce defective osteoclastogenesis during early stages in commitment of human monocyte/macrophage lineage into pre-osteoclasts.

### OL and Per-OL disturb the course of M-CSF/RANKL-mediated osteoclast differentiation of human blood monocytes

The expansion of mononucleated pre-osteoclasts from those primed with M-CSF is a consequence of RANKL making an entry into the differentiation process upon interaction with its receptor RANK. This intensifies the rise of expression of *NFATC1* and *FOS* genes resulting in a transcriptional program of early differentiation marker genes, such as cathepsin K (*CTSK*),<sup>37</sup> osteoclast-associated immunoglobulin-like receptor (*OSCAR*),<sup>38</sup> and TRAP type 5 (*ACP5*),<sup>39</sup> with mononucleated pre-osteoclasts ready to exhibit TRAP activity.<sup>40</sup> This view is depicted in Fig. 2A. In the presence of OL and Per-OL, the transcriptional activity of *CTSK* (Fig. 2B), *OSCAR* (Fig. 2C), and *ACP5* (Fig. 2D) genes was found to be inhibited in M-CSF/RANKL-treated human blood monocytes. In addition, TRAP-positive cells were reduced (Fig. 2E). No differences between OL and Per-OL at the same concentration were observed. However, there was a dose-dependent effect of OL on *ACP5* gene and of Per-OL on *CTSK* and *ACP5* genes. Previous studies have been reported that OL decreases the formation of TRAP in osteoclasts from mouse spleen cells<sup>16</sup> and in periodontium of rats with experimentally induced periodontal inflammation.<sup>41</sup> Interestingly, and coincident to what was seen above, overexpression of *MAFB* gene also inhibits the formation of TRAP and attenuates the expression of pro-osteoclastogenic *OSCAR* and *ACP5* genes in M-CSF/RANKL-treated murine bone marrow monocytes.<sup>32</sup> Furthermore, mice with deficient expression of *CTSK*<sup>42</sup> and *APC5*<sup>43</sup> genes have been shown to suffer from osteopetrosis due to impaired osteoclastogenesis.

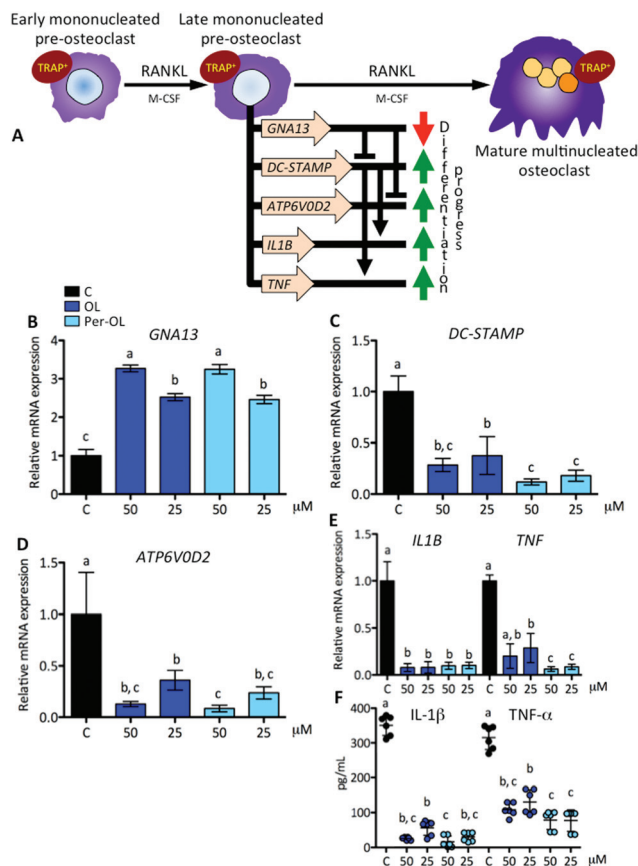
With the progress of differentiation, RANKL drives mononucleated pre-osteoclasts to a fusion-competent state. In this late stage of osteoclast precursors, they can fuse with one another to form multinucleated osteoclasts after the transcription of master fusogenic genes, such as dendritic cell-specific transmembrane protein (*DC-STAMP*) and vacuolar H<sup>+</sup> ATPase V0 subunit d2 (*ATP6V0D2*).<sup>44</sup> The fusion process can also be enhanced by the expression and release of IL-1 $\beta$  and TNF- $\alpha$  functioning in an autocrine mode,<sup>45</sup> which may depend on expression of *DC-STAMP* gene.<sup>46</sup> Interestingly, recent evidence has shown that guanine nucleotide-binding protein subunit  $\alpha$ 13 gene (*GNA13*), a member of the G12 subfamily of the heterotrimeric G proteins, is critically integrated in the late stage of pre-osteoclast differentiation *via* repression of *DC-STAMP*



**Fig. 2** Expression of genes that instruct differentiation of early pre-osteoclast precursors by RANKL. Monocytes from the blood of healthy volunteers were induced to differentiate for 6 days with human recombinant M-CSF (50 ng mL<sup>-1</sup>) and RANKL (50 ng mL<sup>-1</sup>) in the absence (control cells) or presence of OL and Per-OL (25 and 50  $\mu$ M). (A) Schematic representation of RANKL induced differentiation of early pre-osteoclast precursors into early mononucleated pre-osteoclasts and set of principal genes involved. Levels of (B) *CTSK*, (C) *OSCAR*, and (D) *ACP5* mRNA abundance normalized to GAPDH were determined by quantitative PCR. (E) Representative micrographs of cells examined by TRAP staining. (F) Relative TRAP activity. Data were generated in sextuplicate. Results are shown as mean  $\pm$  SD and those marked with different letters were significantly different ( $p < 0.05$ ) as calculated by one-way ANOVA following the Newman-Keuls' test.

and *ATP6V0D2* genes for the regulation of cell-cell fusion and the maintenance of a balanced differentiation program.<sup>47</sup> This view is depicted in Fig. 3A. In the presence of OL and Per-OL, the transcriptional activity of *GNA13* gene (Fig. 3B) was found to be upregulated in M-CSF/RANKL-treated human blood monocytes. In addition, the expression of *DC-STAMP* (Fig. 3C) and *ATP6V0D2* (Fig. 3D) genes was dramatically downregulated. Similar effects were observed on expression of *IL1B* and *TNF* genes (Fig. 3E) and on release of IL-1 $\beta$  and TNF- $\alpha$  (Fig. 3F). We detected differences between OL and Per-OL at 25  $\mu$ M, having Per-OL higher effects than OL on *DC-STAMP* and *TNF* gene expression and on release of TNF- $\alpha$ . There was also a dose-dependent effect of OL and Per-OL on *GNA13* and *ATP6V0D2* genes. As a negative regulator of osteoclastogenesis, overexpression of a constitutively active form of *GNA13* using lentivirus has been shown to drastically block fusion, formation of TRAP, and resorption activity of late mononucleated pre-osteoclasts derived from several M-CSF/RANKL-treated myeloid cells including murine bone marrow cells and those of the macrophage-like cell line RAW264.7.<sup>48</sup> The authors also found that gain-of-function of *GNA13* using adenoviral trans-





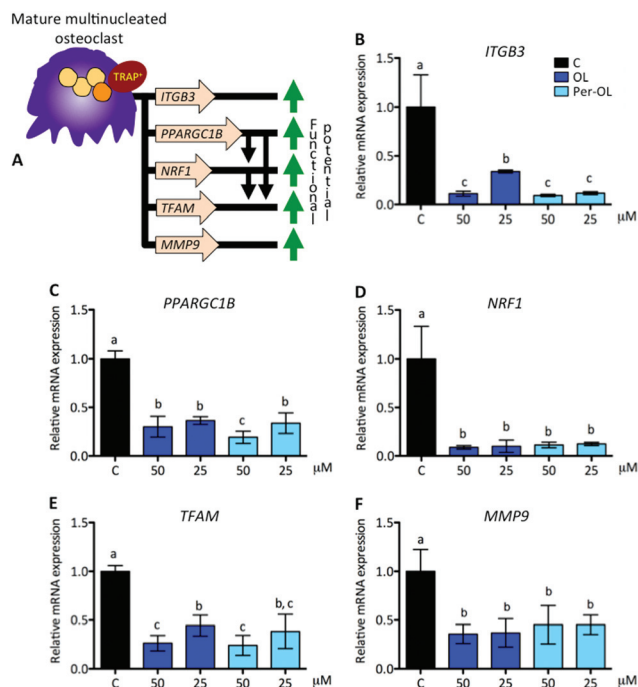
**Fig. 3** Expression of genes that instruct differentiation of early mononucleated pre-osteoclasts to a fusion-competent state by RANKL. Monocytes from the blood of healthy volunteers were induced to differentiate for 6 days with human recombinant M-CSF (50 ng mL<sup>-1</sup>) and RANKL (50 ng mL<sup>-1</sup>) in the absence (control cells) or presence of OL and Per-OL (25 and 50 μM). (A) Schematic representation of RANKL induced differentiation of early mononucleated pre-osteoclasts into late mononucleated pre-osteoclasts, including the end stage of mature multinucleated osteoclast, and set of principal genes involved. Levels of (B) *GNA13*, (C) *DC-STAMP*, (D) *ATP6V0D2*, and (E) *IL1B* and *TNF* mRNA abundance normalized to GAPDH were determined by quantitative PCR. (F) Levels of IL-1β and TNF-α proteins released into the culture medium. Data were generated in sextuplicate. Results are shown as mean ± SD and those marked with different letters were significantly different ( $p < 0.05$ ) as calculated by one-way ANOVA following the Newman-Keuls' test.

fection was protective for bone loss in different animal models of bone disease. The relevance of *DC-STAMP* gene in the fusion step in osteoclastogenesis has been well established *in vitro* using an osteoclast precursor clone from RAW264.7 cells when exposed to RANKL<sup>49</sup> and *in vivo* using *DC-STAMP*<sup>-/-</sup> mice in which multinucleation of osteoclast was entirely abolished.<sup>50</sup> High-resolution microcomputed tomography studies in *ATP6V0D2*<sup>-/-</sup> mice have also revealed a direct relationship between the extremely low mRNA levels of *ATP6V0D2* and the mineral density of bones, mainly explained by reduced cell-cell fusion and thereby lowered formation of mature multinucleated osteoclasts in late stages of osteoclast differentiation.<sup>51</sup> The inhibitory effects of OL on release of IL-1β and TNF-α have been previously described in human neutrophils<sup>52</sup> and syno-

vial fibroblasts.<sup>26</sup> Per-OL was also shown to inhibit the concentrations of IL-1β and TNF-α in the supernatants of LPS-treated murine peritoneal macrophages.<sup>24</sup> Therefore, our findings underscore the notion that OL and Per-OL might induce defective osteoclastogenesis during late stages of differentiation of human blood monocyte-derived pre-osteoclasts.

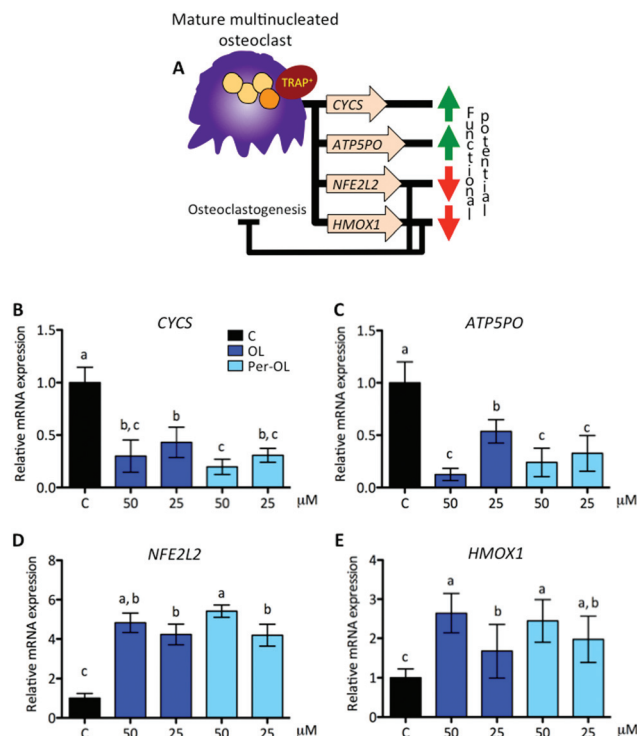
### OL and Per-OL disturb the function of human blood monocyte-derived osteoclasts

Functional mature multinucleated osteoclasts can attach to mineralized surfaces for which they are given novel cytoskeletal organization that requires mitochondrial biogenesis probably due to high energetic needs. Expression of adhesion receptor αβ3 integrin-encoding gene (*ITGB3*)<sup>53</sup> and of peroxisome proliferator-activated receptor gamma coactivator 1-beta (PGC1β) gene (*PPARGC1B*)<sup>54</sup> is considered essential in the organization of osteoclast cytoskeleton for mineralized matrix recognition. This is of interest because *PPARGC1B* gene is also a master regulator of mitochondrial biogenesis *via* stimulation gene expression of nuclear respiratory factor 1 (*NRF1*)<sup>55</sup> and then of mitochondrial transcription factor A (*TFAM*).<sup>56</sup> In this scenario of cytoskeletal rearrangements, the matrix metalloproteinase 9 gene (*MMP9*) has been shown to be entailed in osteoclast motility and bone resorption activity,<sup>57</sup> and in efficient transcription of key osteoclastogenic genes.<sup>58</sup> This view is depicted in Fig. 4A. In the presence of OL and Per-OL, the transcriptional activity of *ITGB3* (Fig. 4B), *PPARGC1B* (Fig. 4C), *NRF1* (Fig. 4D), *TFAM* (Fig. 4E), and *MMP9* (Fig. 4F) genes was found to be dramatically downregulated in M-CSF/RANKL-treated human blood monocytes. We detected differences between OL and Per-OL at 50 μM, having Per-OL higher effects than OL on *PPARGC1B* gene expression. There was also a dose-dependent effect of OL on *ITGB3* and *TFAM* genes, and of Per-OL on *PPARGC1B* gene. M-CSF/RANKL-treated bone marrow cells from mice with deficient expression of *ITGB3* gene have been reported to exhibit apparent normal differentiation, but severe morphological and functional abnormalities that led to diminished bone resorptive capacity.<sup>59</sup> Similar results of a role for PGC1β in osteoclast function but not in osteoclast differentiation irrespective of the presence of M-CSF and RANKL have been recently shown in bone marrow cells from *PPARGC1B*-deficient mice.<sup>54</sup> In keeping the notion that *ITGB3* and *PPARGC1B* genes mediate osteoclast cytoskeleton organization in a canonical and non-canonical manner, respectively, OL and Per-OL could therefore be acting to interfere both pathways during cytoskeleton remodelling of osteoclast from human blood monocytes. The inhibitory effects of OL and Per-OL on expression of *NRF1* and *TFAM* genes also suggest a dampening of mitochondrial function. This ability of OL to induce mitochondrial dysfunction has been observed in human bone marrow mesenchymal stem cells during adipogenic differentiation.<sup>14</sup> Also importantly, engineered mice lacking the *MMP9* gene were shown to have abnormal ossification<sup>60</sup> and IL-1β-treated chondrocytes of patients with osteoarthritis pre-incubated with OL were shown to have lower mRNA levels of *MMP1* and *MMP3* genes than control cells.<sup>25</sup>



**Fig. 4** Expression of genes that drive cytoskeletal organization and mitochondrial biogenesis of mature multinucleated osteoclasts. Monocytes from the blood of healthy volunteers were induced to differentiate for 6 days with human recombinant M-CSF (50 ng mL<sup>-1</sup>) and RANKL (50 ng mL<sup>-1</sup>) in the absence (control cells) or presence of OL and Per-OL (25 and 50 μM). (A) Schematic representation of M-CSF/RANKL induced genes involved in cytoskeletal organization and mitochondrial biogenesis of mature multinucleated osteoclasts. Levels of (B) *ITGB3*, (C) *PPARGC1B*, (D) *NRF1*, (E) *TFAM*, and (F) *MMP9* mRNA abundance normalized to GAPDH were determined by quantitative PCR. Data were generated in sextuplicate. Results are shown as mean ± SD and those marked with different letters were significantly different ( $p < 0.05$ ) as calculated by one-way ANOVA following the Newman-Keuls' test.

The pattern of excessive mitochondrial biogenesis in functional osteoclasts is associated with high expression of oxidative phosphorylation genes, such as cytochrome c (*CYCS*) and ATP synthase subunit O (*ATP5PO*).<sup>47</sup> Likewise, a disproportionate oxidative phosphorylation produces intramitochondrial reactive oxygen species and free radicals, which are essential for osteoclastogenesis and osteoclast function.<sup>61</sup> To protect against oxidative stress and deregulation of cellular redox homeostasis, the nuclear factor-erythroid 2-related factor 2 gene (*NFE2L2*) is involved in the transcription of powerful anti-oxidant genes including heme oxygenase-1 (*HMOX1*). *NFE2L2* and *HMOX1* genes also take part in osteoclastogenesis, the first as negative regulator of early pre-osteoclasts by its role in the upregulation of *MAFB* gene,<sup>62</sup> the second as negative regulator of late pre-osteoclasts by its role in the downregulation of RANKL-dependent osteoclastogenic genes.<sup>63</sup> This view is depicted in Fig. 5A. In the presence of OL and Per-OL, the transcriptional activity of *CYCS* (Fig. 5B) and *ATP5PO* (Fig. 5C) genes was found to be downregulated in M-CSF/RANKL-treated human blood monocytes. In addition, the expression of *NFE2L2* gene (Fig. 5D) and that of its downstream target



**Fig. 5** Expression of genes that drive oxidative phosphorylation and oxidative-stress-response of mature multinucleated osteoclasts. Monocytes from the blood of healthy volunteers were induced to differentiate for 6 days with human recombinant M-CSF (50 ng mL<sup>-1</sup>) and RANKL (50 ng mL<sup>-1</sup>) in the absence (control cells) or presence of OL and Per-OL (25 and 50 μM). (A) Schematic representation of M-CSF/RANKL induced genes involved in oxidative phosphorylation and resistance to reactive oxygen species of mature multinucleated osteoclasts. Levels of (B) *CYCS*, (C) *ATP5PO*, (D) *NFE2L2*, and (E) *HMOX1* mRNA abundance normalized to GAPDH were determined by quantitative PCR. Data were generated in sextuplicate. Results are shown as mean ± SD and those marked with different letters were significantly different ( $p < 0.05$ ) as calculated by one-way ANOVA following the Newman-Keuls' test.

*HMOX1* gene (Fig. 5E) were upregulated. Differences between OL and Per-OL at 25 μM, having Per-OL higher effect than OL, were observed for *ATP5PO* gene expression. There was also a dose-dependent effect of OL on *ATP5PO* and *HMOX1* genes, and of Per-OL on *NFE2L2* gene. Consistent with these findings, recent studies have documented that OL suppresses oxidative stress by targeting the transcriptional activity of renal *NFE2L2* and *HMOX1* genes in mice with experimentally-induced acute kidney injury.<sup>64</sup>

Interestingly, osteoclasts themselves can limit osteoclastogenesis and osteoclast function through the activation of TNFRSF11B gene, which encodes osteoprotegerin (OPG).<sup>65</sup> OPG is a member of the superfamily of TNF receptors that interferes with the RANK/RANKL system, and inhibits bone-resorbing activity and promotes apoptosis of mature osteoclasts. Fully differentiated osteoclasts have also been shown to exhibit a cytokine phenotype that resembles that of non-classically activated M2 macrophages, with an inverse modulation in the secretion of IL-6 and IL-10.<sup>66</sup> IL-6 is pro-osteoclastogenic pre-





sumably by the upregulation of *TNFRS11A* gene,<sup>67</sup> whereas IL-10 has the opposite effect by different mechanisms, among which the upregulation of *TNFRS11B* gene.<sup>68</sup> This view is depicted in Fig. 6A. In the presence of OL and Per-OL, the transcriptional activity of *TNFRS11B* gene (Fig. 6B) was found to be dramatically upregulated in M-CSF/RANKL-treated human blood monocytes. In addition, the expression of *IL6* gene (Fig. 6C) was downregulated, whereas *IL10* gene (Fig. 6D) was upregulated. Similar effects were observed on release of IL-6 (decreasing) and IL-10 (increasing) (Fig. 6E). We detected differences between OL and Per-OL, having Per-OL higher effects than OL on *IL10* gene expression (at 50  $\mu$ M) and on release of IL-10 (at 25  $\mu$ M). There was also a dose-dependent effect of OL and Per-OL on *IL6* gene, and of Per-OL on *IL10* gene. Previous studies have addressed the ability of OL and Per-OL to decrease the production and release of IL-6 in LPS-treated murine peritoneal macrophages,<sup>26</sup> and of OL to increase the concentration of IL-10 in the colon tissue with experimentally-induced colitis.<sup>69</sup> These findings and those

highlighted above are indicative that OL and Per-OL might induce dysfunction in human blood monocyte-derived osteoclasts.

Taken together, this study demonstrates for the first time that OL and Per-OL (Per-OL > OL) strongly interfered with M-CSF/RANKL-induced osteoclast commitment, differentiation, and function from human blood monocytes, suggesting a novel inhibitory role of OL and Per-OL in multiple stages of osteoclastogenesis. Given that OL and Per-OL may be subjected to phase I and phase II hepatic metabolism, a major limitation of our study is that included only *in vitro* experiments of direct interactions between of OL or Per-OL and monocytes/osteoclasts. Previous studies have shown that intact OL may circulate in blood after administration of a single oral dose in humans.<sup>70,71</sup> It is possible that treatment with OL or Per-OL maintained for long periods could have cumulative effects in specific anatomical sites. Further studies on this topic and on novel administration routes,<sup>72</sup> and on protection strategies to avoid intense exposure to metabolic detoxification pathways<sup>73,74</sup> of OL and Per-OL are needed to substantiate our findings. This will pave the way for novel strategies to protect bone health, opening up new directions in research against the development and progression of osteoclast-related diseases.

## Conflicts of interest

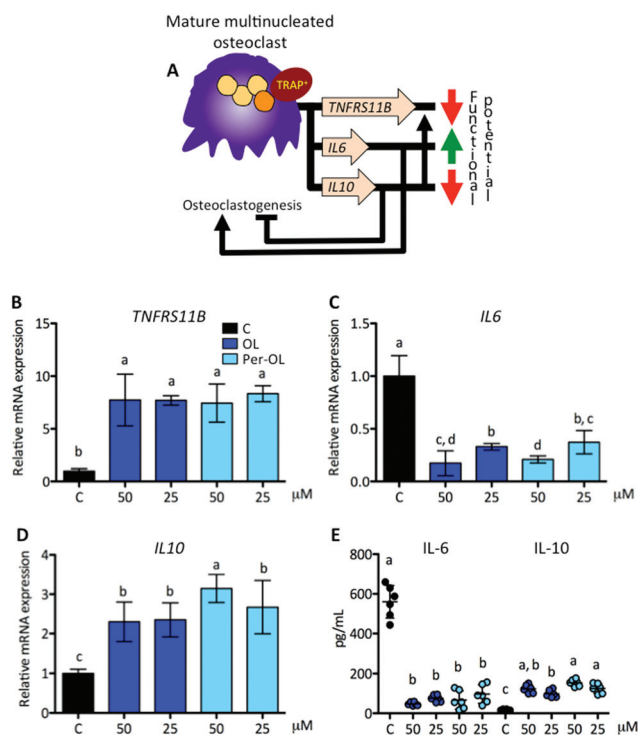
There are no conflicts to declare.

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**Fig. 6** Expression of genes that drive the functional end of mature multinucleated osteoclasts. Monocytes from the blood of healthy volunteers were induced to differentiate for 6 days with human recombinant M-CSF (50 ng mL<sup>-1</sup>) and RANKL (50 ng mL<sup>-1</sup>) in the absence (control cells) or presence of OL and Per-OL (25 and 50  $\mu$ M). (A) Schematic representation of M-CSF/RANKL induced genes involved in limiting the duration of function of mature multinucleated osteoclasts. Levels of (B) *TNFRS11B*, (C) *IL6*, and (D) *IL10* mRNA abundance normalized to GAPDH were determined by quantitative PCR. (E) Levels of IL-6 and IL-10 proteins released into the culture medium. Data were generated in sextuplicate. Results are shown as mean  $\pm$  SD and those marked with different letters were significantly different ( $p < 0.05$ ) as calculated by one-way ANOVA following the Newman-Keuls' test.





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