Spontaneous and induced osteoclastogenic behaviour of human peripheral blood mononuclear cells and their CD14⁺ and CD14[−] cell fractions

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Abstract

Objectives: Osteoclasts are descended from the CD14⁺ monocyte/macrophage lineage, but influence of other haematopoietic cells on osteoclastic commitment of their precursors has remained poorly understood. In this study, osteoclastogenic behaviour of peripheral blood mononuclear cells (PBMC) and their CD14⁺ and CD14[−] subpopulations has been accessed, in the absence or presence of M-CSF and RANKL.

Materials and Methods: Cell cultures were characterized for presence of actin rings and vitronectin and calcitonin receptors, TRAP activity and calcium phosphate resorbing activity, expression of osteoclast-related genes and secretion of M-CSF and RANKL.

Results: In the absence of growth factors, PBMC and CD14⁺ cultures had some degree of cell survival, and some spontaneous osteoclastogenesis was observed, only on cultures of the former. Supplementation with M-CSF and RANKL significantly increased osteoclastogenic behaviour of cell cultures, particularly CD14⁺ cell cultures. Nevertheless, PBMC derived a higher degree of osteoclastogenesis, either as absolute values or after normalization by protein content. It was observed that unlike CD14⁺ cells, PBMC were able to express M-CSF and RANKL, which increased following growth factor treatment. Also, expression of TNF- α , GM-CSF, IL-1 β , IL-6 and IL-17 was higher in PBMC cultures.

Finally, CD14[−] cultures exhibited limited cell survival and did not reveal any osteoclast features.

Conclusions: Results show that although osteoclastic precursors reside in the CD14⁺ cell subpopulation, other populations (such as CD14[−] cells) derived from PBMC, have the ability to modulate osteoclastogenesis positively.

Introduction

Osteoclast development and activation are complex processes that require numerous types of crosstalk between osteoclast precursors and several other cell types (1–5). Osteoclasts are formed through multiple fusions of their mononuclear precursors that have haematopoietic origin and are derived from the monocyte/macrophage lineage (6,7). It has been observed that osteoclasts can be generated from colony-forming unit granulocyte macrophage progenitors (8,9) and osteoclastic cells can also be obtained from monocytes present in peripheral blood (10). Monocytes are a heterogeneous population (11) that comprises of, in addition to osteoclastic progenitors, precursors of several other cell types, such as macrophages (12), endothelial cells (13) and dendritic cells (14). It is not known whether all monocyte subpopulations are able to differentiate into osteoclasts, however, it has been shown that several monocyte subpopulations, namely, CD14⁺, CD11b⁺, CD61⁺, CD15⁺ and CD169⁺, have different types of osteoclastogenic behaviour (7). Currently, the best characterized (and probably the main) osteoclast precursor monocyte subpopulation is CD14⁺ CD16[−] (10,15,16). CD14 is a surface marker expressed by monocytes, macrophages and osteoclasts, and it has been reported that its expression is essential for osteoclastogenesis (17). Interestingly, regardless of identity of osteoclast precursors, their presence in peripheral blood seems to be low (18–22).

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Taken together, the precise osteoclast lineage, and mainly mechanisms involved in commitment of osteoclast precursors to the osteoclastogenic process, as well as relationships between osteoclasts and their precursors with other haematopoietic cells, largely remain to be elucidated. It is known that some blood cells express osteoclastogenic molecules, for example, RANKL expression by B and T lymphocytes (23,24). Understanding whether, and how, osteoclast precursors are influenced towards osteoclastic differentiation by other haematopoietic cells, even before arriving in the bone microenvironment, is a very important topic for comprehending bone metabolism. Recently, we have observed that human peripheral blood mononuclear cells (PBMC) treated with recombinant M-CSF and RANKL exhibited osteoclast differentiation in a pattern similar to their osteoclast precursor cell subpopulation (CD14⁺ cells). However, in the absence of any osteoclastogenic factor, only PBMC cultures displayed any spontaneous osteoclast formation (2). Considering this observation, the present study aimed to investigate this behaviour in more detail and to understand whether the PBMC population would have any particular feature that could assist in differentiation of its osteoclast-precursor population. To accomplish this, adherent PBMC and their subpopulations of CD14⁺ and CD14⁻ cells were compared with regard to the osteoclastogenic response observed, in absence or in presence of recombinant M-CSF and RANKL, the two classic *in vitro* osteoclastogenic inducers (16). Adherent cultures were evaluated for total protein content throughout a 21-day incubation period, as an indicator of cell survival/cell density, and osteoclast-related markers, including calcium phosphate resorbing ability. In addition, cultures were characterized for expression of osteoclastogenic modulators M-CSF, RANKL, TNF- α , GM-CSF, IL-1 β , IL-6 and IL-17.

Materials and methods

Isolation of human PBMC, CD14⁺ and CD14⁻ cells

Cells were isolated from blood of healthy male donors aged 25–35 years, as described previously (1). Briefly, blood was diluted with PBS (1:1) and applied on Ficoll-Paque™ PREMIUM (GE Healthcare Bio-Sciences, Piscataway, USA). After centrifugation at 400 g for 30 min, PBMC were collected and washed twice in PBS. Typically for each 100 ml of processed blood, in the order of 70×10^6 PBMC were obtained. CD14⁺ and CD14⁻ cells were isolated from PBMC using magnetic cell sorting (MACS; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After incubation of PBMC with MACS Microbeads conjugated with mouse monoclonal antibody raised against human CD14 protein, CD14⁻ cells were

recovered by washing PBMC-labelled cells in PBS. CD14⁺ cells were eluted in PBS after magnetic detachment of the column. Cells were resuspended in culture medium. On average, CD14⁺ cells comprised 10% of PBMC.

PBMC, CD14⁺ and CD14⁻ cell cultures

PBMC, at 3×10^6 and 1.5×10^6 cells/cm², and corresponding yields of CD14⁺ and CD14⁻ cells were cultured in α -MEM supplemented with 30% (V/V) human serum (same donors from whom the cells had been obtained), 2 mM L-glutamine, 100 IU/ml penicillin, 2.5 μ g/ml streptomycin, 2.5 μ g/ml amphotericin B. Cell cultures were performed in these conditions (base medium) and in presence of recombinant M-CSF (25 ng/ml) and RANKL (40 ng/ml) (16). Cultures were washed in PBS on first medium change to remove non-adherent cells, and were maintained for 21 days at 37 °C in a 5% CO₂ humidified atmosphere. Culture medium was replaced once a week. Adherent PBMC, CD14⁺ and CD14⁻ cultures were characterized at days 7, 14 and 21 as described below.

Protein quantification. Quantification of total protein content of cell cultures (days 7, 14 and 21) was performed by Bradford's method (25), using bovine serum albumin as standard. Cell cultures were briefly washed in PBS, solubilized with 0.1 M NaOH and treated using Coomassie® Protein Assay Reagent (Fluka, Milwaukee, USA). After incubation for 2 min at room temperature, 600 nm absorbance was determined using an ELISA plate reader (Synergy HT; Biotek, Winooski, USA). Considering that the majority of cells present in PBMC populations are non-adherent, and CD14⁺ monocytes are not able to proliferate, evaluation of total protein content provides information, with some reservations, on cell density/survival in each condition.

TRAP activity. TRAP activity was quantified by the *para*-nitrophenolphosphate (pNPP) hydrolysis assay, at days 7, 14 and 21. After being washed twice in PBS, cell cultures were solubilized with 0.1% (V/V) Triton X-100. Following incubation with 12.5 mM pNPP in 0.04 M tartaric acid and 0.09 M citrate (pH 4.8) for 1 h at 37 °C, the reaction was stopped with 5 M NaOH, and 405 nm absorbance of samples was measured using an ELISA plate reader (Synergy HT; Biotek). Results were normalized with total protein content of cultures and expressed as nmol/min/mg protein.

Number of TRAP-positive multinucleate cells. Cell cultures were washed twice in PBS and fixed in 3.7% formaldehyde for 10 min, at day 21. Then cultures were rinsed

in distilled water and stained for TRAP using an acid phosphatase, leucocyte (TRAP) kit (Sigma-Aldrich, Missouri, USA), according to the manufacturer's instructions. Briefly, cells were incubated in naphthol AS-BI 0.12 mg/ml, in the presence of 6.76 mM tartrate and 0.14 mg/ml fast garnet GBC, at 37 °C for 1 h in the dark. Then, cells were washed and stained in haematoxylin. Multinucleate cells positive for TRAP were counted.

Confocal laser scanning microscopy (CLSM) visualization of actin, vitronectin receptor and calcitonin receptor. At day 21, cell cultures were washed twice in PBS and fixed in 3.7% (V/V) *para*-formaldehyde for 15 min. Cells were permeabilized with 0.1% (V/V) Triton X-100 for 5 min and stained for actin with 5 U/ml Alexa Fluor® 647-Phalloidin (Invitrogen, California, USA), and for vitronectin receptor (VNR) and calcitonin receptor (CTR) with 50 µg/ml mouse IgGs anti-VNR and IgGs anti-CTR (R&D Systems, Minneapolis, USA) respectively. Detection of anti-VNR and anti-CTR IgGs was performed with 2 µg/ml Alexa Fluor® 488-Goat anti-mouse IgGs.

Calcium phosphate resorption assay. Cells were cultured for 21 days using BD BioCoat™ Osteologic™ Bone Cell Culture Plates (BD Biosciences, New Jersey, USA). Cells were removed by incubation with 6% NaOCl and 5.2% NaCl, according to the manufacturer's instructions. Calcium phosphate layers were visualized by phase contrast light microscopy. Image analysis of resorpted areas was performed using IMAGEJ 1.41 software (IMAGEJ, National Institutes of Health, USA).

RT-PCR analysis. PBMC cultures, performed in absence or presence of M-CSF and RANKL, and CD14⁺ cell cultures supplemented with the growth factors, were analysed by RT-PCR, at day 21. CD14⁺ cell cultures maintained in base medium and CD14⁻ cell cultures, did not yield suffi-

cient amounts of mRNA to perform RT-PCR, at day 21. RNA was extracted from PBMC cultures using Rneasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was quantified by UV spectrophotometry at 260 nm and expression of GAPDH, osteoclast-related genes *c-myc*, *c-src*, *TRAP*, *CATK* and *CA2*, and osteoclastogenic-inductive factors M-CSF, RANKL, TNF-α, GM-CSF, IL-1, IL-6 and IL-17 were analysed by RT-PCR. For that, 0.5 µg of RNA was reverse transcribed and amplified (25 cycles) using the Titan One Tube RT-PCR System (Roche, Basel, Switzerland), with annealing temperature of 55 °C. Primers used are listed in Table 1. RT-PCR products were separated on 1% (w/V) agarose gel, and densitometric analyses were performed with IMAGEJ 1.41 software. Values were normalized for the corresponding GAPDH value of each experimental condition.

In addition to the above procedures, in a preliminary experiment, it had been observed that a culture established with equal proportions of CD14⁺ and CD14⁻ cells, behaved identically to PBMC cultures (data not shown), suggesting that magnetic purification of CD14⁺ cells did not significantly interfere with their ability to generate osteoclasts. In that context, PBMC populations might be regarded as representing the sum of CD14⁺ and CD14⁻ cell fractions.

M-CSF and RANKL quantification on culture media

M-CSF and RANKL quantification was performed using Human M-CSF Quantikine ELISA Kit (R&D Systems) and sRANKL (total) Human ELISA (Osteoprotegerin Ligand) (BioVendor, Brno, Czech Republic) respectively, following the manufacturers' instructions. After detection, absorbance of samples was measured at 450 nm using an ELISA plate reader (Synergy HT; Biotek). Results were expressed as ng/ml.

Table 1. Primers used in RT-PCR analysis of PBMC cultures

Gene	5' Primer	3' Primer
GAPDH	5'-CAGGACCAGGTTACCAACAAGT-3'	5'-GTGGCAGTGATGGCATGGACTGT-3'
TRAP	5'-ACCATGACCACCTTGGAATGTCTC-3'	5'-ATAGTGAAGCGCAGATAGCCGT-3'
CATK	5'-AGGTTCTGCTGCTACCTGTGGTGAG-3'	5'-CTTGCAATCAATGGCCACAGAGACAG-3'
CA2	5'-GGACCTGAGCACTGGCATAAGGACT-3'	5'-AAGGAGGCCACGAGGATCGAAGTT-3'
c-myc	5'-TACCCTCTCAACGACAGCAG-3'	5'-TCTTGACATTCTCCTCGGTG-3'
c-src	5'-AAGCTGTTCCGAGGCTTCAA-3'	5'-TTGGAGTAGTAGGCCACCAG-3'
M-CSF	5'-CCTGCTGTTGTTGGTCTGTC-3'	5'-GGTACAGGCAGTTGCAATCA-3'
RANKL	5'-GAGCGCAGATGGATCCTAAT-3'	5'-TCCTCTCCAGACCGTAACCT-3'
TNF-α	5'-TCAGATCATCTTCTCGAACC-3'	5'-CAGATAGATGGGCTCATACC-3'
GM-CSF	5'-CTGAGTAGAGACACTGCTGCTG-3'	5'-TGCCTGTATCAGGGTCAGTGTG-3'
IL-1	5'-ATGGCAGAAGTACCTAAGCTCGC-3'	5'-ACACAAATTGCATGGTGAAGTCAGTT-3'
IL-6	5'-ATGAACCTCTTCTCCACAAG-3'	5'-GTGCCTGCAGCTTCGTCAGCA-3'
IL-17	5'-TAGACTATGGAGAGCCGACC-3'	5'-TACCCCAAAGTTATCTCAGG-3'

Statistical analysis

Data presented in this work were obtained from three separate experiments using cell cultures from six different donors. Analyses were performed with three replicates. Groups of data were evaluated using two-way analysis of variance (ANOVA) and no significant differences in patterns of cell behaviour were seen. Statistical differences found between control and experimental conditions were determined by Bonferroni's method. Values of $P \leq 0.05$ were considered significant.

Results

Total protein content

In base medium, PBMC cultures displayed slow reductions in total protein content over culture time, with a tendency for stabilization after the first week. CD14⁺ and CD14⁻ cell cultures showed significant decreases in number until day 14, with low and approximately stable values during the third week (Fig. 1). Supplementation with M-CSF and RANKL induced significant increase in protein content in all cell populations, particularly in CD14⁺ cells. Cultures maintained a pattern similar to that observed in base medium. In both conditions, protein content of PBMC cultures was significantly higher than that of CD14⁺ cell cultures, which had a higher response than CD14⁻ cells, particularly at day 21. In addition, for each cell population, protein content was similar regardless of initial cell plating density.

TRAP activity

In base medium, PBMC cultures had low TRAP activity at day 7, but significantly higher values by days 14 and

21, whereas CD14⁺ and CD14⁻ cells revealed negligible enzyme activity throughout culture times (Fig. 2). Following supplementation with M-CSF and RANKL, PBMC presented 2-fold increase in TRAP activity. However, in CD14⁺ cultures, TRAP activity was substantially induced, from negligible values to values only slightly lower than those observed in PBMC cultures. In addition, enzyme activity increased significantly from days 7 to 14, and remained constant after that. CD14⁻ cells displayed low TRAP activity at day 7, and the enzyme was not detected afterwards. Seeding density did not affect responses of the three cell populations.

Presence of TRAP-positive multinucleated cells

In base medium (Fig. 3a), PBMC cultures revealed low numbers of multinucleate cells positively expressing TRAP, whereas CD14⁺ cell cultures had only few osteoclast-like cells; no multinucleate cells were observed in CD14⁻ cultures. In the presence of M-CSF and RANKL (Fig. 3b), PBMC and CD14⁺ cultures revealed a significant increase in numbers of multinucleate cells positively expressing TRAP, in the order of 5.2 and 23 times higher, respectively, than those found in cultures performed in base medium. Nevertheless, PBMC cultures displayed a response that was more than 2.3 times higher than that obtained for CD14⁺ cell cultures. Normalization to total protein content of cultures (Fig. 3c,d) approximated those values. No TRAP-positive multinucleate cells were observed in CD14⁻ cell cultures. Once again, initial cellular seeding density did not affect results in the cultures.

CLSM visualization of PBMC and CD14⁺ cell cultures

PBMC and CD14⁺ cell cultures supplemented with M-CSF and RANKL, observed on day 21, displayed

Figure 1. Total protein content of cell cultures. PBMC, CD14⁺ and CD14⁻ cell cultures, performed in base medium (a) or in presence of recombinant M-CSF and RANKL (b). Cellular protein was quantified by Bradford's method.

Figure 2. TRAP activity of cell cultures. PBMC, CD14⁺ and CD14⁻ cell cultures, performed in base medium (a) or in presence of recombinant M-CSF and RANKL (b). TRAP activity was assessed by pNPP hydrolysis assay.

Figure 3. Histochemical TRAP staining of PBMC, CD14⁺ and CD14⁻ cell cultures. After 21 days in base medium (a, c) or in presence of M-CSF and RANKL (b, d), multinucleate cells positive for TRAP were counted (a, b) and values were normalized by total protein content of cell cultures (c, d).

presence of cells with actin rings and vitronectin and calcitonin receptors (Fig. 4). Furthermore, numbers of osteoclasts in culture were significantly higher in PBMC cultures. CD14⁻ cultures did not have adherent cells with these features (data not shown).

Calcium phosphate resorbing activity

In base medium, PBMC, but not CD14⁺ cells, showed some ability to resorb calcium phosphate (Fig. 5a,b), which was significantly higher following supplementation with M-CSF and RANKL. CD14⁺ cells supplemented with the growth factors also showed some resorbing

activity. Nevertheless, PBMC cultures displayed percentages of resorption in the region of 3.5 times higher (Fig. 5b). However, normalization by total protein content approximated the percentage of resorption of PBMC and CD14⁺ cell cultures (Fig. 5c). Over the tested culture period (21 days), CD14⁻ cells were not observed and no resorption lacunae were identified (data not shown).

RT-PCR characterization of cell cultures and quantification of M-CSF and RANKL of culture media

PBMC cultured in base medium expressed osteoclast-related genes *c-myc*, *c-src*, *TRAP*, *CATK* and *CA2* and in

Figure 4. CLSM visualization of PBMC and CD14⁺ cell cultures. (a) Representative images: Osteoclast from PBMC cultures stained blue for actin and green for VNR or CTR. (b) PBMC and CD14⁺ cells were cultured for 21 days in presence of M-CSF and RANKL and visualized by CLSM. White bars represent 50 μm (a) or 100 μm (b). Negative control represents staining of cell cultures with secondary anti-mouse antibody alone.

addition, the two classic osteoclastogenic genes for M-CSF and RANKL, Fig. 6a,b. Supplementation with the growth factors increased expression of all genes, especially differentiation and activation genes, *c-myc* and *c-src*, and also of *RANKL*. CD14⁺ cultures supplemented with M-CSF and RANKL also expressed osteoclast-related genes in a way slightly lower than supplemented PBMC cultures. However, CD14⁺ cells cultures did not express the osteoclastogenic genes M-CSF and RANKL. Regarding the quantification of M-CSF and RANKL on culture medium (Fig. 6c,d), it was observed that in the absence of exogenous supplementation with growth factors, PBMC and CD14[−] cells were able to secrete both M-CSF and RANKL, although the latter population exhibited almost negligible values (Fig. 6c); CD14⁺ cell culture medium did not reveal their presence. Following growth factor treatment, all culture media revealed presence of M-CSF and RANKL, at values similar to concentrations used for exogenous supplementation (Fig. 6d). Nevertheless, PBMC culture medium had slightly higher values.

PBMC cultures (either maintained in base medium or treated with M-CSF and RANKL) and CD14⁺ cells cultured in presence of both recombinant growth factors were also assessed for expression of several haematopoietic osteoclastogenic molecules other than M-CSF and RANKL, that is, TNF-α, GM-CSF, IL-1β, IL-6 and IL-17 (Fig. 7).

All genes for these proteins were expressed by the three cell populations (that of TNF-α was significantly more highly expressed than the others), with the one exception being for IL-1β, whose expression was only detected on PBMC cultures treated with M-CSF and RANKL, and at only almost undetectable levels. In a general way, PBMC cultures maintained in presence of the growth factors displayed highest expression levels, followed by PBMC maintained in base medium, and, finally, CD14⁺ cells. However, in the case of IL-6, CD14⁺ population revealed a higher response than PBMC cultured in base medium.

Discussion

Osteoclasts are derived from the monocyte/macrophage lineage found in peripheral blood (6). They are formed through multiple fusions of osteoclast CD14⁺ precursor cells (6,10,15,16); however information regarding the relationship between osteoclast precursors and other haematopoietic cells, mainly with respect to their commitment to osteoclast differentiation, is still scarce. In this study, a non-fractionated adherent peripheral blood mononuclear cell population, PBMC, and sub-populations CD14⁺ and CD14[−] cells, were compared for their ability to differentiate into osteoclasts, over time. Cells were cultured in absence (base medium) and in presence of recombinant M-CSF and RANKL (16), to obtain information concerning spontaneous osteoclastogenic potential of the populations, and also their sensitivity to osteoclast-inducing conditions. Results of the two seeding densities were similar, which is most probably related to low numbers of osteoclast precursors found in peripheral blood (16).

PBMC cultured in base medium had high and stable protein content after the first week, suggesting high cell density in long-term cultures, while CD14⁺ and CD14[−] cells exhibited lower levels of survival, particularly CD14[−] cells. Supplementation with recombinant M-CSF and RANKL increased protein content in all three cell populations, but specially in the CD14⁺ cell fraction. In these conditions, at day 7, protein content of CD14⁺ and PBMC cultures were similar, which might be related to high numbers of seeded cells, possibly saturating well surfaces and yielding identical values in both cell cultures. However, at days 14 and 21, both in base medium and in presence of M-CSF and RANKL, protein content of PBMC cultures was higher than sums of protein content of CD14⁺ and CD14[−] cultures, suggesting increased survival of osteoclasts, or their precursors (CD14⁺ cell fraction), and/or of non-osteoclast precursor cell population (CD14[−] cell fraction).

In base medium, PBMC gave rise to cells with osteoclastic features, which increased following supplementation with M-CSF and RANKL. On the other hand,

Figure 5. PBMC and CD14⁺ cell cultures were performed on calcium phosphate layers for 21 days. After cell removal, formation of resorption lacunae was analysed (a). Resorpted areas were quantified and expressed as percentage of total area (b). Percentage of resorption was normalized with total protein content (c). BM, base medium; M+R, Supplementation with M-CSF + RANKL.

Figure 6. RT-PCR analysis of cell cultures. (a) PBMC cultures performed in absence or presence of recombinant M-CSF and RANKL and CD14⁺ cell cultures treated with both growth factors. Cell layers were assessed for expression of GAPDH (1), M-CSF (2), RANKL (3), TRAP (4), CATK (5), CA2 (6), c-src (7) and c-myc (8). (b) RT-PCR products were subjected to a densitometric analysis and were normalized to value obtained for GAPDH. (c and d) Quantification of M-CSF and RANKL present in culture medium of PBMC, CD14⁺ and CD14⁺ cultures, performed in base medium (c) or in presence of recombinant M-CSF and RANKL (d). BM, base medium; M + R, Supplementation with M-CSF + RANKL.

osteoclast formation from CD14⁺ cells was totally dependent on presence of M-CSF and RANKL, as expected from a homogeneous cell population of osteoclast precursors in presence of M-CSF and RANKL (2,5). Nevertheless, CD14⁺ cell cultures yielded low absolute values of osteoclast-related parameters, compared to PBMC cultures. Normalization of the osteoclastogenic response to total protein content minimized the differences, which was expected, assuming similar numbers of osteoclast precursors in PBMC and CD14⁺ cell cultures, according to

the experimental protocol used. However, PBMC still presented slightly higher (15–20%) normalized values for most tested osteoclast parameters. Considering the limited number of CD14⁺ cells that survived at later incubation times did not reveal osteoclast markers, results suggest that the higher osteoclastogenic behaviour of PBMC, compared to CD14⁺ cells, was not related to presence of higher numbers of osteoclast precursors.

Behaviour of PBMC revealed that this unfractionated cell population was able to support some osteoclast

Figure 7. Expression profile of TNF- α , GM-CSF, IL-1 β , IL-6 and IL-17 by cell cultures. (a) PBMC cultures maintained in base medium (1) or treated with recombinant M-CSF and RANKL (2), or by CD14⁺ cells treated with M-CSF and RANKL. (b) Densitometric analysis of RT-PCR products. BM, base medium; M + R, Supplementation with M-CSF + RANKL.

survival and differentiation. PBMC compose a heterogeneous population, which, after Ficoll isolation, is composed approximately of monocytes (10–15%), T-lymphocytes (66%), B cells (9%) and small numbers of other blood-cell types (16,20); even among peripheral blood monocytes, there are distinct subpopulations with specialized functions (26–29). Thus, it is tempting to hypothesize that there would be PBMC subpopulation(s) that have an effective role in modulation of osteoclastogenesis. These subpopulations, apparently, do not comprise osteoclast precursors – the present results show that CD14[–] cells did not display any ability to originate osteoclastic cells. In this context, it is important to note that T-lymphocytes are known to have an important role in modulation of osteoclastogenesis, through expression of RANKL and other osteoclastogenic cytokines (30,31). However, in the present work, data were collected from adherent cell cultures. T-lymphocytes are non-adherent cells, and there might be other adherent haematopoietic CD14[–] cells that also have the ability to modulate osteoclast development. Absence of such accessory subpopulations in CD14⁺ cultures might explain the lower degree of osteoclastogenesis of this fraction, compared to PBMC cultures. Regarding this, we have shown that PBMC, but not CD14⁺ cells, produced M-CSF and RANKL, and expression of these factors increased following supplementation with M-CSF and RANKL. It is known that M-CSF is a very important growth factor for osteoclastic/macrophage precursor cell survival (5,32) and that RANKL is mainly involved in later steps of osteoclast development (5,33), which can explain the observed behaviour of PBMC cultures. Previous reports (16,34–37) have also shown expression of these factors by haematopoietic cells in certain experimental conditions. Expression of M-CSF has been observed in PBMC maintained in presence of foetal bovine serum after exogenous stimulation, but not in absence of any stimuli

(34,35). Also, it has been shown that PBMC expressed RANKL after incubation with M-CSF (16). In a further study, RANKL expression by PBMC was detected at low levels after 5 days of culture, but it declined progressively and after 18 days, was not detected (38). Besides differences between the present work and others, it seems that relevant osteoclastogenic-related information, that is, osteoclastic precursors and osteoclastogenic modulators such as M-CSF and RANKL, are present in PBMC population. Furthermore, there are several other osteoclastogenic factors, in addition to M-CSF and RANKL, which are produced by haematopoietic cells – such as TNF- α , GM-CSF, IL-1, IL-6 and IL-17 (39), that might be important players in the osteoclastogenic response of PBMC. In that context, analysis of expression of genes coding for these proteins revealed that all were expressed by PBMC and CD14⁺ cells. The only exception was IL-1 β , which was only detected at very low levels in PBMC cultures treated with M-CSF and RANKL. The observed expression profile is consistent with previous reports where it was seen that PBMC have the ability to express the tested molecules, although some only at low levels (40–43). In addition, it has been demonstrated elsewhere that monocytes express low levels of those genes (44,45).

Previous studies have reported mixed results for osteoclast formation from PBMC and CD14⁺ cells. It has been shown elsewhere that CD14⁺ populations displayed osteoclastic features about 1.6–8 fold higher than PBMC cultures, when cultured in the presence of foetal calf serum, M-CSF, RANKL, TNF- α and dexamethasone, but it was also suggested that osteoclasts could be induced from PBMC more simply, efficiently and rapidly (7). The same study reported that CD14[–] populations, under culture conditions described above, had some osteoclast formation capacity (7), although authors have argued that it was probably a consequence of some monocyte contamination in the purified fractions. In a further report, it was suggested that osteoclastogenesis from CD14⁺ cells was more efficient than that from PBMC, in the presence of foetal bovine serum, M-CSF and RANKL (16). Also, it was shown that preparations of differentiated cells from monocyte/macrophage lineage, such as CD14⁺ cells, display very low efficiency for generating osteoclasts (8–10,16,21,22), and it was observed that PBMC treated with M-CSF and RANKL generated large numbers of osteoclastic cells (18). Significant differences in experimental protocols regarding culture supplementation, culture time and measured osteoclast parameters, along with substantial variations observed with different blood donors (16), render it difficult to establish a pattern regarding osteoclastogenic behaviour of PBMC and CD14⁺ cells. In addition, in the present work, cell cultures were

maintained with a high concentration of autologous human serum (instead of bovine or calf sera), which might be an advantage, as it contributes to creating a more representative culture environment when comparing behaviour of osteoclastic precursor cell cultures, but might elicit a different profile of effects from that of bovine or calf sera. Thus, the present results appear to be consistent with some previous pieces of work (8–10,16,21,22), but not with those reporting higher osteoclastogenic response of CD14⁺ cell fractions (7,16). However, this might not be conflicting because although PBMC cultures have shown slightly higher normalized osteoclastic features, the CD14⁺ cell fraction exhibited higher responsiveness to osteoclastogenic inducers.

Utilization of PBMC and CD14⁺ cell cultures as representative models in osteoclast-related studies might serve distinct purposes. PBMC are easy to isolate and to obtain in large numbers, being essentially a co-culture of osteoclast precursors and accessory haematopoietic cells, which provide a simple, efficient and rapid way to establish osteoclast-rich cultures. Such a model might be interesting for running screening assays, as it would be advantageous to have a large and stable pool of PBMC to work with. Also, as PBMC display some degree of spontaneous osteoclast formation, this can be explored as a clinical diagnostic assay in some bone disorders, to assess risk of bone loss and response to therapy (46). On the other hand, CD14⁺ cells are more difficult to isolate, involving some time-consuming cell manipulations, namely during labelling and magnetic separation. However, they are a homogeneous osteoclast-precursor cell population that is highly sensitive to osteoclastogenic-inducing conditions, being a good tool to analyse intracellular mechanisms and to detect subtle effects of osteoclast differentiation and activation.

In conclusion, human PBMC and the CD14⁺ and CD14[−] cell fractions fed autologous serum revealed significant differences regarding spontaneous and induced osteoclast formation. The behaviour of PBMC might be related, at least partially, to their ability to express high levels of M-CSF and TNF- α and, also, some expression of RANKL, GM-CSF, IL-1 β , IL-6 and IL-17. Such expression profiles are not shared with CD14⁺ cells, or at least observed only at lower extents, suggesting that the non-precursor cell population (CD14[−]) has an effective role in osteoclastogenesis modulation.

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References

- Costa-Rodrigues J, Fernandes MH (2011) Paracrine-mediated differentiation and activation of human hematopoietic osteoclast precursor cells by skin and gingival fibroblasts. *Cell Prolif.* **44**(3), 264–273.
- Costa-Rodrigues J, Teixeira CA, Sampaio P, Fernandes MH (2010) Characterisation of the osteoclastogenic potential of human osteoblastic and fibroblastic conditioned media. *J. Cell. Biochem.* **109**, 205–216.
- Datta HK, Ng WF, Walker JA, Tuck SP, Varanasi SS (2008) The cell biology of bone metabolism. *J. Clin. Pathol.* **61**, 577–587.
- Zaidi M (2007) Skeletal remodeling in health and disease. *Nat. Med.* **13**, 791–801.
- Boyle WJ, Simonet WS, Lacey DL (2003) Osteoclast differentiation and activation. *Nature* **423**, 337–342.
- Vaananen HK, Laitala-Leinonen T (2008) Osteoclast lineage and function. *Arch. Biochem. Biophys.* **473**, 132–138.
- Husheem M, Nyman JK, Vaaranemi J, Vaananen HK, Hentunen TA (2005) Characterization of circulating human osteoclast progenitors: development of in vitro resorption assay. *Calcif. Tissue Int.* **76**, 222–230.
- Ciraci E, Barisani D, Parafioriti A, Formisano G, Arancia G, Bottazzo G *et al.* (2007) CD34 human hematopoietic progenitor cell line, MUTZ-3, differentiates into functional osteoclasts. *Exp. Hematol.* **35**, 967–977.
- Hodge JM, Kirkland MA, Aitken CJ, Waugh CM, Myers DE, Lopez CM *et al.* (2004) Osteoclastic potential of human CFU-GM: biphasic effect of GM-CSF. *J. Bone Miner. Res.* **19**, 190–199.
- Massey HM, Flanagan AM (1999) Human osteoclasts derive from CD14-positive monocytes. *Br. J. Haematol.* **106**, 167–170.
- Grage-Griebenow E, Flad HD, Ernst M (2001) Heterogeneity of human peripheral blood monocyte subsets. *J. Leukoc. Biol.* **69**, 11–20.
- Nagy L, Tontonoz P, Alvarez JG, Chen H, Evans RM (1998) Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. *Cell* **93**, 229–240.
- Fernandez Pujol B, Lucibello FC, Gehling UM, Lindemann K, Weidner N, Zuzarte ML *et al.* (2000) Endothelial-like cells derived from human CD14 positive monocytes. *Differentiation* **65**, 287–300.
- Komi J, Lassila O (2000) Nonsteroidal anti-estrogens inhibit the functional differentiation of human monocyte-derived dendritic cells. *Blood* **95**, 2875–2882.
- Komano Y, Nanki T, Hayashida K, Taniguchi K, Miyasaka N (2006) Identification of a human peripheral blood monocyte subset that differentiates into osteoclasts. *Arthritis Res. Ther.* **8**, R152.
- Nicholson GC, Malakellis M, Collier FM, Cameron PU, Holloway WR, Gough TJ *et al.* (2000) Induction of osteoclasts from CD14-positive human peripheral blood mononuclear cells by receptor activator of nuclear factor kappaB ligand (RANKL). *Clin. Sci. (Lond)* **99**, 133–140.
- Suda K, Woo JT, Takami M, Sexton PM, Nagai K (2002) Lipopolysaccharide supports survival and fusion of preosteoclasts independent of TNF-alpha, IL-1, and RANKL. *J. Cell. Physiol.* **190**, 101–108.
- Matsuzaki K, Udagawa N, Takahashi N, Yamaguchi K, Yasuda H, Shima N *et al.* (1998) Osteoclast differentiation factor (ODF) induces osteoclast-like cell formation in human peripheral blood mononuclear cell cultures. *Biochem. Biophys. Res. Commun.* **246**, 199–204.
- Quinn JM, Neale S, Fujikawa Y, McGee JO, Athanasou NA (1998) Human osteoclast formation from blood monocytes, peritoneal macrophages, and bone marrow cells. *Calcif. Tissue Int.* **62**, 527–531.

- 20 Shalhoub V, Elliott G, Chiu L, Manoukian R, Kelley M, Hawkins N *et al.* (2000) Characterization of osteoclast precursors in human blood. *Br. J. Haematol.* **111**, 501–512.
- 21 Fujikawa Y, Quinn JM, Sabokbar A, McGee JO, Athanasou NA (1996) The human osteoclast precursor circulates in the monocyte fraction. *Endocrinology* **137**, 4058–4060.
- 22 Udagawa N, Takahashi N, Akatsu T, Tanaka H, Sasaki T, Nishihara T *et al.* (1990) Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. *Proc. Natl. Acad. Sci. USA* **87**, 7260–7264.
- 23 Kawai T, Matsuyama T, Hosokawa Y, Makihira S, Seki M, Karimbux NY *et al.* (2006) B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease. *Am. J. Pathol.* **169**, 987–998.
- 24 Colucci S, Brunetti G, Cantatore FP, Oranger A, Mori G, Quarta L *et al.* (2007) Lymphocytes and synovial fluid fibroblasts support osteoclastogenesis through RANKL, TNF α , and IL-7 in an in vitro model derived from human psoriatic arthritis. *J. Pathol.* **212**, 47–55.
- 25 Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
- 26 Passlick B, Flieger D, Ziegler-Heitbrock HW (1989) Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood* **74**, 2527–2534.
- 27 Sunderkotter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA *et al.* (2004) Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J. Immunol.* **172**, 4410–4417.
- 28 Wang SY, Mak KL, Chen LY, Chou MP, Ho CK (1992) Heterogeneity of human blood monocyte: two subpopulations with different sizes, phenotypes and functions. *Immunology* **77**, 298–303.
- 29 Geissmann F, Jung S, Littman DR (2003) Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* **19**, 71–82.
- 30 Gillespie MT (2007) Impact of cytokines and T lymphocytes upon osteoclast differentiation and function. *Arthritis Res. Ther.* **9**, 103.
- 31 O'Gradaigh D, Compston JE (2004) T-cell involvement in osteoclast biology: implications for rheumatoid bone erosion. *Rheumatology (Oxford)* **43**, 122–130.
- 32 Pixley FJ, Stanley ER (2004) CSF-1 regulation of the wandering macrophage: complexity in action. *Trends Cell Biol.* **14**, 628–638.
- 33 Boyce BF, Xing L (2008) Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch. Biochem. Biophys.* **473**, 139–146.
- 34 Rambaldi A, Young DC, Griffin JD (1987) Expression of the M-CSF (CSF-1) gene by human monocytes. *Blood* **69**, 1409–1413.
- 35 Lindemann A, Riedel D, Oster W, Ziegler-Heitbrock HW, Mertelsmann R, Herrmann F (1989) Granulocyte-macrophage colony-stimulating factor induces cytokine secretion by human polymorphonuclear leukocytes. *J. Clin. Invest.* **83**, 1308–1312.
- 36 Wada T, Nakashima T, Hiroshi N, Penninger JM (2006) RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol. Med.* **12**, 17–25.
- 37 Crotti TN, Smith MD, Weedon H, Ahern MJ, Findlay DM, Kraan M *et al.* (2002) Receptor activator NF- κ B ligand (RANKL) expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthritis, osteoarthritis, and from normal patients: semiquantitative and quantitative analysis. *Ann. Rheum. Dis.* **61**, 1047–1054.
- 38 Faust J, Lacey DL, Hunt P, Burgess TL, Scully S, Van G *et al.* (1999) Osteoclast markers accumulate on cells developing from human peripheral blood mononuclear precursors. *J. Cell. Biochem.* **72**, 67–80.
- 39 Atkins GJ, Haynes DR, Geary SM, Loric M, Crotti TN, Findlay DM (2000) Coordinated cytokine expression by stromal and hematopoietic cells during human osteoclast formation. *Bone* **26**, 653–661.
- 40 Bernhardt TM, Burchardt ER, Welte K (1993) Assessment of G-CSF and GM-CSF mRNA expression in peripheral blood mononuclear cells from patients with severe congenital neutropenia and in human myeloid leukemic cell lines. *Exp. Hematol.* **21**, 163–168.
- 41 Hasan N, Yusuf N, Toossi Z, Islam N (2006) Suppression of Mycobacterium tuberculosis induced reactive oxygen species (ROS) and TNF- α mRNA expression in human monocytes by allicin. *FEBS Lett.* **580**, 2517–2522.
- 42 Johnson JL, Shiratsuchi H, Toossi Z, Ellner JJ (1997) Altered IL-1 expression and compartmentalization in monocytes from patients with AIDS stimulated with Mycobacterium avium complex. *J. Clin. Immunol.* **17**, 387–395.
- 43 Pertosa G, Grandaliano G, Gesualdo L, Ranieri E, Monno R, Schena FP (1998) Interleukin-6, interleukin-8 and monocyte chemotactic peptide-1 gene expression and protein synthesis are independently modulated by hemodialysis membranes. *Kidney Int.* **54**, 570–579.
- 44 Frankenberger M, Sternsdorf T, Pechumer H, Pforte A, Ziegler-Heitbrock HW (1996) Differential cytokine expression in human blood monocyte subpopulations: a polymerase chain reaction analysis. *Blood* **87**, 373–377.
- 45 Fujino S, Andoh A, Bamba S, Ogawa A, Hata K, Araki Y *et al.* (2003) Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* **52**, 65–70.
- 46 Xing L, Schwarz EM (2005) Circulating osteoclast precursors: a mechanism and a marker of erosive arthritis. *Curr. Rheumatol. Rev.* **1**, 21–28.