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# Peripheral blood monocytes show increased osteoclast differentiation potential compared to bone marrow monocytes

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## Abstract

Bone marrow (BM) and peripheral blood (PB) derived mononuclear cells are precursors of *in vitro* osteoclast differentiation. However, few studies have compared the phenotypic and functional properties of osteoclasts generated from these sources and the effects of different growth factors on osteoclastogenesis. Both cell types differentiated into functional osteoclasts, but culturing the cells with or without transforming growth factor beta (TGF- $\beta$ ) and dexamethasone revealed differences in their osteoclastogenic capacity. When receptor activator for nuclear factor  $\kappa$ B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) were used for differentiation, we did not observe differences in bone resorption activity or expression of osteoclastogenic genes *calcitonin receptor (CR)* and *nuclear factor of activated T-cells (NFATc1)* between the osteoclasts formed from the two sources. Addition of TGF- $\beta$  and dexamethasone led to

higher number of nuclei in multinuclear cells and increased expression of tartrate resistant acid phosphatase (TRACP) 5a and 5b, *CR* and *NFATc1* in PB- derived osteoclasts depicting the higher osteoclastogenic potential and responsiveness to TGF- $\beta$  and dexamethasone in PB monocytes. These results conclude that the choice of the osteoclast precursor source as well as the choice of osteoclastogenic growth factors are essential matters in determining the phenotypic characteristics of heterogeneous osteoclast populations.

Keywords: Cell biology, Stem cell research

## 1. Introduction

The multinuclear bone resorbing cells, osteoclasts, are differentiated by fusion of monocyte/macrophage lineage cells originating from the hematopoietic stem cell population [1, 2, 3, 4, 5]. These cells are present in bone marrow (BM) as well as circulating in peripheral blood (PB), and are widely used as osteoclast precursors in *in vitro* studies of bone biology [6, 7, 8, 9]. A population of the circulating monocytes is assigned as cell cycle-arrested quiescent osteoclast precursors (QOPs), which begin their differentiation initially in hematopoietic tissues, thereafter circulate transiently in the bloodstream, and finally migrate to bone surfaces for the last stages of osteoclastogenesis [10, 11, 12, 13].

After isolation, mononuclear cells from BM or PB can be readily differentiated into osteoclasts, since the differentiation requires only two growth factors, receptor activator for nuclear factor  $\kappa$ B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [3, 14, 15]. Although these two growth factors are regularly used for differentiation, there are also studies which show that addition of transforming growth factor beta (TGF- $\beta$ ) and dexamethasone can enhance the osteoclast-forming potential of the precursors and the resorptive activity of the generated osteoclasts [16, 17, 18].

It has recently been proposed that there might actually be more than just one type of osteoclast. Sprangers and co-workers [19] suggested that different monocyte subpopulations can differentiate into distinct types of osteoclasts depending on the prevailing physiological condition. They propose that in physiological homeostasis the main osteoclast precursor is the classical (CD14<sup>++</sup>CD16<sup>-</sup>) monocyte, whereas in inflammatory conditions the intermediate (CD14<sup>++</sup>CD16<sup>+</sup>) monocytes could differentiate into osteoclasts which have an increased ability to resorb bone. In this regard, it is interesting that the major monocyte type in blood, the classical monocyte, has also been shown to be the primary osteoclast precursor cell [20, 21, 22, 23, 24, 25, 26], whereas bone marrow contains mainly intermediate monocytes [27].

We hypothesized that osteoclast precursors derived from BM and PB exhibit different osteoclastogenic potential and responsiveness to TGF- $\beta$ /glucocorticoids. There are few studies comparing the osteoclasts differentiated from BM and PB, and they mainly concentrate on comparing the osteoclast precursor sources rather than studying the differentiation process i.e. osteoclastogenesis or the functional differences between the osteoclasts [28, 29]. We have previously shown that gap junctional communication is one of the mechanisms in the cell fusion during osteoclastogenesis from BM and PB monocytes [30]. Here, we have compared multinuclear osteoclast-like cell formation and the effects of different growth factor cocktails on it with human BM and PB mononuclear cells. To our knowledge, this is the first study comparing osteoclastogenesis, bone resorption activity, sensitivity to TGF- $\beta$ /dexamethasone, and osteoclast-specific marker expression in human osteoclasts differentiated from BM and PB monocytes.

## 2. Materials and methods

### 2.1. Osteoclastogenesis from human BM mononuclear cells

The isolation and culture protocol were modified from [18]. BM samples were received from hip replacement surgery patients in Oulu University Hospital. Patients were 52–77-year-old men and women who gave a written informed consent. The total number of patients participating in the study was 12, but the single experiments were carried out with 3 separate patient samples due to the low number of cells obtained from one patient. The patient samples used for different experiments are listed in Table 1. The study was approved by the Ethical Committee of The Northern Ostrobothnia Hospital District. All experiments in this study were performed in

**Table 1.** Patient samples used in each experiment.

Donor	Sex	Age	Samples	Experiment
1	F	64	BM + PB	Cell and nuclei number counting, resorption pit analysis, ICTP analysis
2	M	57	BM + PB	
3	M	52	BM + PB	
4	M	67	BM + PB	TRACP ELISA
5	M	66	BM + PB	
6	M	63	BM + PB	
7	M	67	BM + PB	qRT-PCR
8	M	69	BM + PB	
9	M	63	BM + PB	
10	F	77	BM	TRACP immunostaining
11	F	59	PB	
12	M	71	BM + PB	

accordance with the relevant guidelines and regulations. BM sample was first cultured in  $\alpha$ -MEM (Corning Life Sciences, Tewksbury, MA) containing 10 % FBS, 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin and 24 mM Hepes buffer (Sigma-Aldrich, St. Louis, MO) at +37 °C (5 % CO<sub>2</sub>, 95 % air) for 1–2 days. After this, media containing the non-adherent cells was collected, diluted 1:1 in PBS and layered over (1:1) Ficoll-Paque Premium solution (GE Healthcare, Little Chalfont, UK). The samples were centrifuged at 400  $\times$  g for 35 minutes following the manufacturer's protocol. Mononuclear cell layer was collected and centrifugated twice at 190  $\times$  g for 10 minutes in PBS, and finally suspended in  $\alpha$ -MEM (Sigma-Aldrich). 300 000 cells ( $9.4 \times 10^5$  cells/cm<sup>2</sup>) were layered on sonicated human cortical bone slices (0.28 cm<sup>2</sup>) in 96-well plates (Costar; Corning Life Sciences). The cell seeding density was optimized for osteoclastogenesis from our cell sources. The slices were cut from anonymous bone samples acquired from clinical bone bank held in Oulu University Hospital, city of Oulu, Finland. Special National Supervisory Authority for Welfare and Health (Valvira) granted a permission for use of aged cadaver specimens for research purposes, decision 8.5.2009, diary number 2240/05.01.00.06/2009. Cells were cultured in  $\alpha$ -MEM containing 10 % FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin (Sigma-Aldrich). Osteoclastogenesis was induced with 20 ng/ml RANKL (PeproTech EC) and 10 ng/ml M-CSF (R&D Systems) according to [31] or with 50 ng/ml RANKL, 25 ng/ml M-CSF, 5 ng/ml TGF- $\beta$  (R&D Systems) and 1  $\mu$ M dexamethasone (Sigma-Aldrich) according to [18]. Half of the medium was changed every 3–4 days (100  $\mu$ l). At one week time point, all medium was changed (200  $\mu$ l/well). Cells were cultured at +37 °C (5 % CO<sub>2</sub>, 95 % air) for 12 days when all growth factors were used and 14 days when only RANKL and M-CSF were used.

## 2.2. Osteoclastogenesis from human PB mononuclear cells

PB mononuclear cells were collected from whole blood sample from the hip replacement surgery patients shortly before the operation. The patients gave a written informed consent before donating the samples. Ficoll-Paque Premium gradient centrifugation was performed for the diluted (1:1; PBS) blood sample as described earlier. Cell culture and differentiation were done equally to the BM sample.

## 2.3. Counting of multinuclear cells and the number of nuclei

After culturing the cells were fixed with 4 % PFA in PBS. The actin cytoskeleton was stained with Alexa 488-conjugated phalloidin (200 U/ml stock diluted 1:100 in PBS; Invitrogen Europe, Paisley, UK) for 20 minutes at +37 °C. Nuclei were stained with Hoechst 33258 (1 mg/ml stock diluted 1:800 in PBS; Sigma-Aldrich) for 10 minutes at room temperature. Staining for osteoclast-specific enzyme TRACP was carried out with a commercial acid phosphatase leukocyte kit (Sigma-Aldrich) for 20

minutes at +37 °C. The samples were mounted in 70 % glycerol-PBS and viewed in a Nikon Eclipse E600 fluorescence microscope (Tokyo, Japan) and Plan 10 × objective. Multinuclear cells with three or more nuclei were counted from each bone slice from five randomly chosen microscope fields, bone slice  $n \geq 3$ . The number of nuclei were counted from 4 multinuclear cells from 5 randomly chosen areas. Images were taken with QImaging MicroPublisher 5.0 RTV camera and QCapture 2.90.1 software (QImaging, Surrey, Canada). Confocal images were taken with LSM 510 META confocal microscope combined with an Axiovert 200 M inverted microscope (Carl Zeiss, Oberkochen, Germany) with 20 x Plan Neofluar objective (Carl Zeiss).

#### **2.4. Field Emission Scanning Electron Microscopy and measurement of resorption pit areas**

Resorption pit areas were measured from Field Emission Scanning Electron Microscopy (FESEM) images with Merz grid analysis. After multinuclear cell counting, cells were detached from the slices by brushing, samples were dehydrated in ascending ethanol series and dried with a critical point drying equipment K850 (Quorum technologies, UK). Samples were coated with 5 nm platinum by Q150T ES sputter coater (Quorum Technologies) and viewed with Sigma HD VP FESEM (Carl Zeiss Microscopy GmbH, Germany). FESEM images were taken from three fields (voltage 5.0 kV, magnification 50×, area 0,035 cm<sup>2</sup>) from each bone slice,  $n = 3$ . The morphometric analysis of the pits was performed with ImageJ 1.49t software (NIH, USA) by superimposing a Merz grid with 80–88 points in semicircular lines over the image. Points in pits were counted and the proportion of resorption pits versus intact bone surface was counted. The proportion of resorbed area was normalized to multinuclear cell number (counting described above), and the average area resorbed by one cell was calculated.

#### **2.5. Measurement of C-terminal telopeptide of type I collagen (ICTP)**

As bone is degraded during resorption, ICTP fragments are released into culture medium indicating the level of bone resorption. ICTP levels were measured with UniQ ICTP radioimmunoassay kit (Orion Diagnostica, Espoo, Finland). The media were collected on day 12 or 14 before fixation. ICTP was measured according to the kit instructions in duplicates from 3 cultures made from independent patient samples. The ICTP levels were normalized to cell number (counting described above).

#### **2.6. TRACP immunostaining**

For TRACP immunostaining 500 000 mononuclear cells from BM and PB samples were grown on glass slides (0.20 cm<sup>2</sup>;  $2.5 \times 10^6$  cells/cm<sup>2</sup>). After fixation with 4 %

PFA in PBS the cells were permeabilized with 0.1 % Triton X-100 for 10 minutes and blocked with 1 % BSA (Sigma-Aldrich) for 30 minutes at room temperature. TRACP 5a was stained with 1:100 diluted rabbit serum anti-TRACP 5a for 1 hour at +37 °C. TRACP 5a and 5b were stained similarly with rabbit serum anti-TRACP, which binds to both 5a and 5b isoforms. TRACP antibodies were produced as described earlier [32]. Secondary antibody was goat anti-rabbit Alexa 488 (2 mg/ml stock diluted 1:100 in PBS; Invitrogen) incubated for 30 minutes at +37 °C. Actin cytoskeleton was stained with TRITC-conjugated phalloidin (0.1 mg/ml stock diluted 1:100 in PBS; Sigma-Aldrich) for 20 minutes at +37 °C. Nuclei were stained with Hoechst 33258 (1 mg/ml stock diluted 1:700 in PBS; Sigma-Aldrich) for 10 minutes at room temperature. Samples were mounted in Immount (Thermo Fisher Scientific, Waltham, MA) and viewed in LSM 510 META confocal microscope combined with an Axiovert 200 M inverted microscope (Carl Zeiss, Oberkochen, Germany) with 10x Plan Neofluar objective (Carl Zeiss).

## 2.7. Analysis of TRACP isoforms from cell culture medium

After the 12 or 14 days culture the media were removed and stored in –80 °C. TRACP levels in media were measured with a sandwich ELISA for TRACP 5a and TRACP 5b separately. Briefly, for capturing of TRACP 5a, media and recombinant TRACP 5a standards, diluted 1:20 in 0.1% BSA (Sigma-Aldrich, Stockholm, Sweden) in TBST (25 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween-20), were added to 96-well half-volume ELISA plates (Costar, Chicago, IL) coated with 5 µg/ml mouse anti-TRACP 5a mAb 46 (Mabtech, Nacka, Sweden) at 4 °C overnight. The samples were incubated at 4 °C overnight. mAb 46 is developed to recognize only TRACP 5a [33]. The supernatant was diluted to final dilution 1:100 and was transferred to 96-well half-volume ELISA plates (Costar) coated with 5 µg/ml mouse anti-TRACP (5a and 5b) mAb 25.44 (Mabtech) at 4 °C overnight for the capture of TRACP 5b. All samples were incubated for 2 h at room temperature (RT). After two hours the plates (both the mAb 46 coated plate with captured TRACP 5a and the mAb 25.44 plate with the captured TRACP 5b) were washed 3 times in TBST. The plates were incubated with biotinylated mouse anti-TRACP (5a and 5b) mAb 12.56 (Mabtech) 0.25 µg/ml for 1 h at RT. After 1 h the plates were washed 3 times in TBST followed by incubation in Streptavidin HRP (Mabtech) diluted 1:1000 in 0.1% BSA in TBST. After incubation the plates were washed 3 times in TBST. Lastly plates were washed and developed with K-Blue Substrate (TMB) (Neogen, Lansing, MI) for 20 min and absorbance was measured at 450 nm in BioTek's PowerWave HT microplate spectrophotometer (BioTek, Winooski, VT). Quantification of TRACP 5a was done using the TRACP 5a standard curve on the mAb 46 coated plate. Quantification of TRACP 5b was done using the newly

added TRACP 5a standards and the absorbance of the supernatant on the plate coated with mAb 25.44. The transferred standards of TRACP 5a from mAb 46 to mAb 25.44 plate were used to correct for any carry-over of TRACP 5a from plate mAb 46 to plate mAb 25.44.

## 2.8. Quantitative reverse transcription PCR (qRT-PCR)

After 12 or 14 days' culture, total RNA was isolated from the bone slices with a commercial RNA isolation kit (Macherey-Nagel, Düren, Germany). Media were removed from the wells and lysis buffer with 20 mM DTE was pipetted back and forth in the wells. Isolation was completed with the NucleoSpin RNA column purification. Lysis buffer from 3 parallel wells were combined in one column. After isolation, RNA concentration was measured with Nano-Drop ND 1000 spectrophotometer. Thermo Scientific First Strand cDNA Synthesis kit with both Oligo (dT)18 and D(N)6 primers was used for the reverse transcription of the RNA (Thermo Scientific, Waltham, MA). The PCR reactions were performed with 2  $\mu$ l cDNA in total volume of 20  $\mu$ l of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) containing SYBR Green I dye, hot-start iTaq DNA polymerase, dNTPs and MgCl<sub>2</sub>. QRT-PCR reaction was performed on Bio-Rad CFX Connect device according to the protocol described in [34]: activation step (10 min, 94 °C) and 40 cycles of two-step PCR (95 °C for 15 seconds, 60 °C for 1 minute). For *TATA-box binding protein (TBP)* housekeeping gene a three-step protocol was performed (95 °C for 10 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds). *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* and *TBP* housekeeping genes were used as endogenous controls for normalizing each sample. The sample values were normalized to the average expression of the two housekeeping genes and the relative expression was determined by 2<sup>- $\Delta$ CT</sup>-method [34]. The housekeeping genes were shown to be stable using Bio-Rad CFX Manager Gene Study, version 3.1. The following (at least one intron spanning) primers for the housekeeping genes and targets (*calcitonin receptor; CR*, and *nuclear factor of activated T-cells; NFATc1*) were designed according to [34] and synthesized by Metabion International (Planegg, Germany): *GAPDH* (Accession number *ENSG00000149397*): Forward primer 5'-ATGGGGAAGGTGAAGGTCG-3'; reverse 5'-TAAAAGCAGCCCTGGTGACC-3', *TBP* (*ENSG00000112592*): forward 5'-GGTCTGGGAAAATGGTGTGC-3'; reverse 5'-GCTGGAAAACCCAACCTTCTG-3', *CR* (*ENSG0000004948*): forward 5'-GCATACCAAGGAGAAGGTCCATAT-3'; reverse 5'-ATACTC-CAGCCGGTGTGTCAT-3' *NFATc1* (*ENSG00000131196*): forward 5'-AGCA-GAGCACGGACAGCTATC-3'; reverse 5'-GGTCAGTTTTTCGCTTCCATCTC-3'. Unspecific PCR products were tested by subjecting all genes to melt curve analysis. QRT-PCR analysis was performed for cultures obtained from 3 independent donors.

## 2.9. Statistical analysis

All experiments were done with groups of  $n \geq 3$  and repeated with at least 3 independent patient samples. Statistical analyses were performed using SPSS statistics program version 22 (SPSS Inc., Chicago, IL). The normality of the response variables was tested with Kolmogorov-Smirnov or Shapiro-Wilk test (for experiments with  $n < 50$ ) and histogram visualization. If the response variables were normally distributed, statistical differences between the test groups were evaluated using one-way ANOVA, and comparison between groups was done with two-sample t-test. If the response variables were not normally distributed, statistical differences between the test groups were evaluated using Kruskal-Wallis test, and comparison between groups was done with Mann-Whitney U-test. The graphical presentation of the results was created with OriginPro 9.1 software (OriginLab, Northampton, MA).  $p < 0.05$  was considered significant. Data are shown as means  $\pm$  SEM.

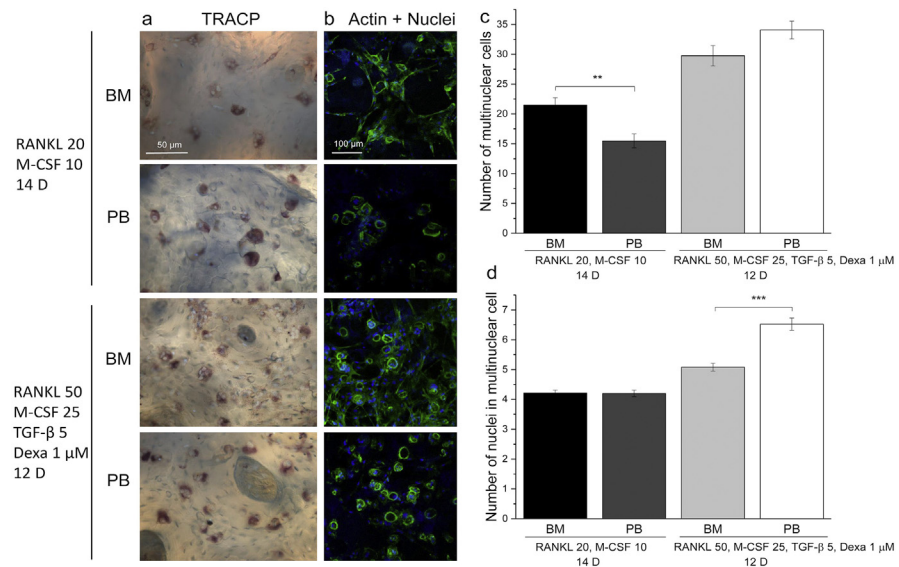
## 3. Results

### 3.1. Multinuclear cells developed in bone marrow and peripheral blood mononuclear cell cultures

Osteoclastogenesis in human BM and PB monocytes on bone slices was induced with two different growth factor sets: RANKL and M-CSF only [31] or RANKL, M-CSF, TGF- $\beta$  and dexamethasone [18]. Both cell types differentiated into tartrate-resistant acid phosphatase (TRACP)-positive multinuclear cells with visible actin rings regardless of the growth factors used (Fig. 1a and b). A significant proportion of cells in BM cultures were fibroblast-shaped stromal-like cells, which were almost absent in PB cultures. Fig. 2 illustrates the presence of stromal-like cells in these cultures. The morphology of the elongated and spindle-like stromal cells differs clearly from the more spherical appearance of the monocytes. Furthermore, although some TRACP-positive spherical monocytes can be regularly seen in the cultures, the stromal-like cells are always TRACP-negative (data not shown), depicting that they presumably represent a mesenchymal origin. Therefore, we here refer to them as stromal cells. The different culture protocols did not seem to affect the number of the stromal cells in the BM cultures.

When multinuclear cell formation from BM and PB were compared at day 14, the number of multinuclear cells differentiated from PB with RANKL and M-CSF was significantly lower than from BM ( $p < 0.01$ ) (Fig. 1c). With all growth factors and dexamethasone, there were no significant differences in the number of multinuclear cells between the two mononuclear cell sources. However, with all growth factors and dexamethasone, the PB-derived multinuclear cells had significantly more nuclei per cell compared to BM derived cells ( $p < 0.001$ ) (Fig. 1d). The difference was not seen in cells cultured with RANKL and M-CSF only.

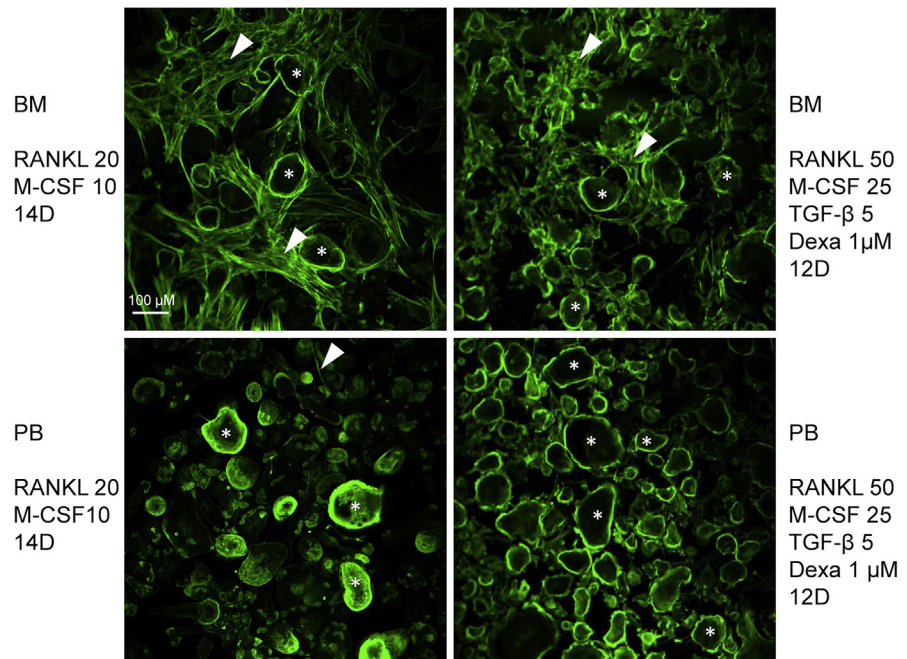




**Fig. 1.** Osteoclasts differentiated from BM and PB cultures with RANKL and M-CSF or RANKL, M-CSF, TGF- $\beta$  and dexamethasone. Osteoclastogenesis was induced for BM or PB monocytes on bone slices and the cells were stained with Hoechst for nuclei (blue), Alexa 488-conjugated phalloidin for actin (green) and acid phosphatase leukocyte kit to visualize TRACP (red). The multinuclear TRACP-positive cells are shown in (a) and the actin rings are illustrated in (b). When the cells were differentiated with RANKL and M-CSF, the number of multinuclear cells was higher in BM derived cultures (c). The difference was not seen when all growth factors and dexamethasone were used. The number of nuclei in multinuclear cell was similar in BM and PB derived osteoclasts when only RANKL and M-CSF were used, but with all growth factors and dexamethasone the PB derived osteoclasts contained significantly more nuclei (d). Concentrations of the growth factors are shown as ng/ml, except for dexamethasone (1  $\mu$ M). 14D: Cell culture time 14 days. 12D: Cell culture time 12 days. The data in graphs is pooled from 3 patients and shown as mean  $\pm$  SEM. Images (representative data from one patient) (a) were taken with combined light and fluorescence microscope and 20  $\times$  objective. Images (b) were taken with confocal microscope and 20  $\times$  objective. \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.

### 3.2. Both bone marrow and peripheral blood derived multinuclear cells resorbed bone

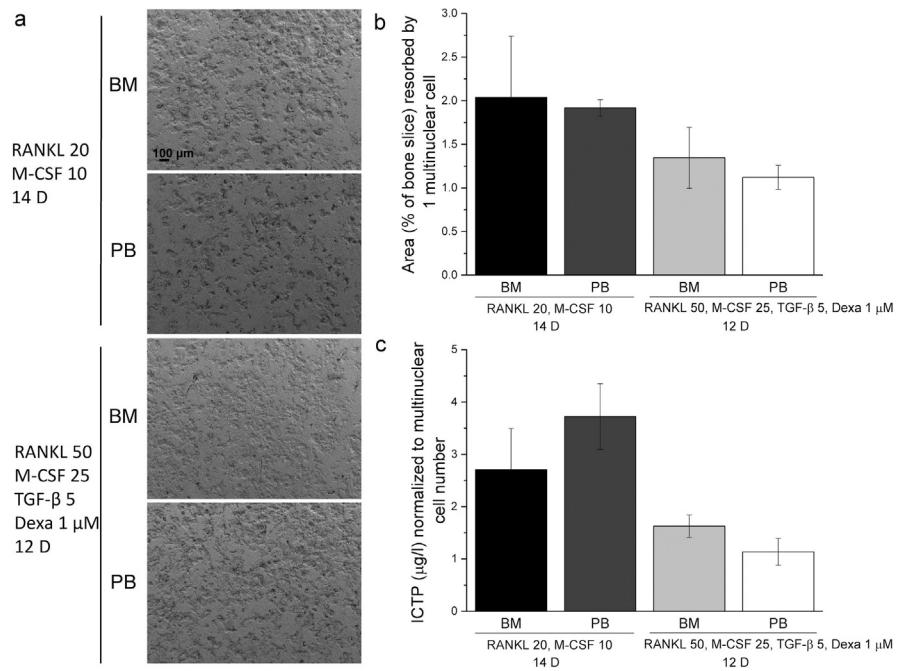
Osteoclasts from the different sources and with different growth factor sets were able to form resorption pits on the human bone slices on which they were cultured (Fig. 3a). All osteoclasts were rather active in bone resorption, since the area resorbed by one cell ranged from 1.1 % to 2.0 % of the total resorption area. In all groups 30–40 % of the total bone slice area was resorbed. There were no differences in bone resorption activity between the cell types with either culture protocol (Fig. 3b). Similarly, there were no differences in the cells' ability to release ICTP from bone (Fig. 3c). Slightly more ICTP was released from bone by PB-derived osteoclasts when cultured with RANKL and M-CSF, but the effect was not statistically significant.



**Fig. 2.** Presence of stromal cells in BM and PB cultures. Osteoclastogenesis was induced for BM or PB monocytes on glass slides and the cells were stained with Alexa 488-conjugated phalloidin for actin (green). A high number of fibroblast-shaped stromal cells (arrow heads) growing in patches are present in BM samples, whereas the PB samples contain very few stromal cells among the multinuclear cells (asterisks). Concentrations of the growth factors are shown as ng/ml, except for dexamethasone (1  $\mu$ M). 12D: Cell culture time 12 days. 14D: Cell culture time 14 days. Images (representative data from one patient) were taken with fluorescence microscope and 10  $\times$  objective.

### 3.3. Peripheral blood derived osteoclasts showed higher expression levels of TRACP 5a and 5b

TRACP 5a can be visualized without interference of isoform 5b using an antibody generated towards the 5a-specific loop domain, which is missing in TRACP 5b. TRACP 5a and 5b i.e total TRACP were detected using another polyclonal antibody raised towards the full-length TRACP 5a protein [32, 35]. Confocal microscopy revealed that TRACP 5a was expressed more intensely in BM and PB cultures which were differentiated only with RANKL and M-CSF (Fig. 4a). TRACP 5a was detected mainly in mononuclear cells with a weaker signal in multinuclear cells. When treated with RANKL and M-CSF, the PB cultures contained more mononuclear cells than the BM cultures. With this culture protocol, it seemed that the most intense staining was observed in PB cultures, corresponding to the increased number of mononuclear cells positive for TRACP5a. This was supported by ELISA for the different TRAP isoforms, which also showed that the highest level of TRACP 5a was observed in media from PB culture with RANKL and M-CSF, although the effect was not significant when compared to BM cultures (Fig. 4c). In TRACP 5b



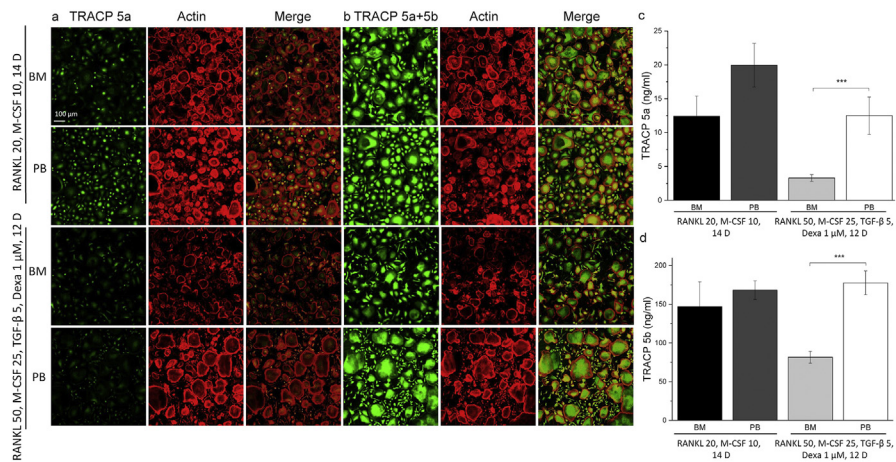
**Fig. 3.** Bone resorption activity of osteoclasts differentiated from bone marrow and peripheral blood. Both BM and PB derived multinuclear cells formed resorption pits on human bone slices (a), and there were no statistical differences in bone resorption activity between the cell types or growth factors used (b). In addition, ICTP levels were similar in all groups (c). Concentrations of the growth factors are shown as ng/ml, except for dexamethasone (1 μM). 12D: Cell culture time 12 days. 14D: Cell culture time 14 days. Images from the pits (representative data from one patient) were taken with FESEM with 50 × magnification and the resorption pit area was calculated with a superimposed Merz grid with ImageJ software. The data in graphs is pooled from 3 patients and shown as mean ± SEM.

analysis, no significant differences were observed between the two cell types in secretion of TRACP 5b in the culture medium (Fig. 4d).

Interestingly, TRACP 5a and 5b staining revealed that the lowest levels of TRACP were expressed in BM cultures with all growth factors and dexamethasone (Fig. 4b). ELISA analysis of TRACP isoforms revealed that these cultures secreted significantly lower amounts of both TRACP 5a and TRACP 5b in cell culture medium compared to PB cultures differentiated with the same protocol (Fig. 4c and d), corresponding to the lower number of multinuclear cells formed in BM cultures (Fig. 1c). The lower secretion level of TRACP 5b might correspond to the lower number of osteoclasts in BM cultures (Fig. 1c), and the lower level of TRACP 5a corresponds to the fewer mononuclear preosteoclasts present in BM cultures.

### 3.4. Peripheral blood derived osteoclasts expressed higher levels of calcitonin receptor and NFATc1

The relative expression of two genes expressed in mature osteoclasts, *calcitonin receptor* (CR) and *nuclear factor of activated T-cells* (NFATc1), were analyzed with



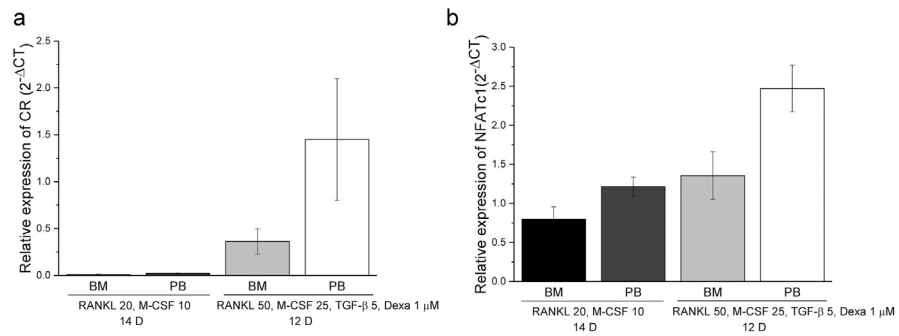
**Fig. 4.** TRACP 5a and 5b levels in osteoclasts differentiated from BM and PB. (a) TRACP 5a staining showed that TRACP 5a was expressed more intensely in BM and PB cultures differentiated with RANKL and M-CSF. The most intense staining was observed in PB cultures with RANKL and M-CSF. (b) TRACP 5a + 5b staining showed that with all growth factors and dexamethasone the immunofluorescence signal of both TRACP isoforms was lower in BM cultures. (c) In TRACP 5a ELISA of the culture media the highest level of TRACP 5a was observed in PB culture with RANKL and M-CSF. (d) TRACP 5b levels did not differ between the cell types when only RANKL and M-CSF were used. With all growth factors and dexamethasone both secreted TRACP 5a (C) and 5b (D) levels were lower in BM cultures compared to PB cultures. Concentrations of the growth factors are shown as ng/ml, except for dexamethasone (1 μM). 14D: Cell culture time 14 days. 12D: Cell culture time 12 days. The data in graphs is pooled from 3 patients and shown as mean ± SEM. Images from one representative patient sample were taken with confocal microscope and 10 x objective. \*\*\*p < 0.001.

qRT-PCR. When cultured with only RANKL and M-CSF, *CR* expression in both cell types was at the same level with the expression of housekeeping genes *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* and *TATA-box binding protein (TBP)* (Fig. 5a). When cultured with all growth factors and dexamethasone, the expression of *CR* increased, but no statistical differences were seen between BM and PB cultures.

The expression of *NFATc1* seemed to be slightly higher than the expression of the housekeeping genes *GAPDH* and *TBP* in the groups cultured with RANKL and M-CSF (Fig. 5b). Similar to *CR* expression, also *NFATc1* expression was upregulated with the addition of TGF-β and dexamethasone, but there were no statistical differences between the cell types. As *NFATc1* is a transcription factor crucial for osteoclastogenesis, the results indicate that all types of cells studied had started to differentiate towards osteoclasts. Although not statistically significant, the tendency for the higher expression levels of osteoclast specific genes in PB cultures might depict a more complete osteoclast differentiation in these cells.

#### 4. Discussion

This study originated from the question whether different culture conditions have varying effects on osteoclastogenesis from BM and PB monocytes. Although BM



**Fig. 5.** Relative expression of *CR* and *NFATc1* in osteoclasts differentiated from BM and PB. *CR* was expressed in low amounts in both BM and PB cultures differentiated with RANKL and M-CSF, but when TGF- $\beta$  and dexamethasone were added, the expression was increased (a). The highest level of *CR* expression was observed in PB cultures differentiated with all growth factors and dexamethasone. *NFATc1* was expressed in both BM and PB osteoclasts differentiated with RANKL and M-CSF, and similarly increased with TGF- $\beta$  and dexamethasone (b). However, no statistical differences were seen between the cell types with either differentiation protocol. The results are shown as relative expression compared to the average expression of two housekeeping genes *GAPDH* and *TBP*. Concentrations of the growth factors are shown as ng/ml, except for dexamethasone (1  $\mu$ M). 12D: Cell culture time 12 days. 14D: Cell culture time 14 days. The data is pooled from 3 patients and shown as mean  $\pm$  SEM.

and PB mononuclear cells are widely used as osteoclast precursors in *in vitro* studies, little is known about the differences in the osteoclastogenic potential between these two sources. We show here that functional osteoclasts can be differentiated from both sources, but the osteoclasts generated by different protocols vary in expression levels of common osteoclast markers.

When only RANKL and M-CSF were used for differentiation, more multinuclear cells formed from BM derived monocytes, but there were no differences in bone resorption capability or osteoclastogenic gene expression compared to PB- derived cultures.

As previously shown [16, 17, 18], addition of TGF- $\beta$  and dexamethasone increased the multinuclear cell and nuclei number, as well as the expression of osteoclast-related genes *CR* and *NFATc1* in both BM and PB derived osteoclast cultures. With this protocol the PB derived osteoclasts contained more nuclei, secreted more of both TRACP isoforms 5a and 5b to the cell culture medium and expressed higher levels of osteoclastogenic genes *CR* and *NFATc1* compared to BM derived osteoclasts, although the latter differences were not statistically significant. Since *CR* expression is considered to be relatively specific for osteoclasts, it is likely that in cells treated with all growth factors and dexamethasone, the osteoclastogenic differentiation was more complete in regard to the gene expression profile. Interestingly, *CR* levels differ between the groups potentially reflecting differences in calcitonin-mediated regulation of the cells.

These results highlight the importance of the combination of growth factors and concentrations of them for osteoclastogenesis from these mononuclear precursors. The

concentrations and culture duration we used were chosen from the regularly applied protocols for BM and PB cultures. The growth factors for PB cultures in our laboratory were originally only RANKL (20 ng/ml) and M-CSF (10 ng/ml), and culture time 14 days. For bone marrow cultures we used all growth factors (RANKL; 50 ng/ml, M-CSF; 25 ng/ml, TGF- $\beta$ ; 5 ng/ml) and dexamethasone (1  $\mu$ M), and the culture time was 12 days. It is possible that the lower levels of TRACP 5a secreted from cultures treated with all growth factors and dexamethasone correspond to the lower abundance of TRACP 5a positive mononuclear cells in this setting. The addition of TGF- $\beta$  and dexamethasone supposedly promotes the differentiation towards osteoclasts and thereby diminishes the TRACP 5a signal from mononuclear cells. This differentiation-promoting effect of the additional growth factors is also indicated by the higher CR expression from these cultures. Another explanation might be in the cell culture duration, since with all four growth factors the culture time was 12 days instead of 14 days which was the duration with only RANKL and M-CSF. The different culture times in the two protocols is a limitation in this study, so we have only statistically compared the cell types to each other within one experimental protocol.

The group showing the highest level of osteoclastogenic potential was the PB cell culture with all growth factors and dexamethasone, albeit having lower bone resorption capability. It has been shown that osteoclastogenesis and bone resorption can be distinctly regulated. For instance, Fuller *et al.* found that TGF- $\beta$ , which strongly stimulates osteoclastogenic differentiation, did not have an effect on bone resorption [36]. The PB monocytes appeared to be more responsive to TGF- $\beta$  and dexamethasone than BM-derived monocytes. This is in line with the concept of the circulating osteoclast precursors [10, 11, 12, 13] and the fact that the majority of monocytes in PB are the classical monocytes, which are the main osteoclast precursors [20, 21, 22, 23, 24, 25, 26, 27]. It seems that the addition of TGF- $\beta$  and dexamethasone for PB monocytes intensely boosts their osteoclastogenesis, which could indicate a higher sensitivity of PB monocytes to TGF- $\beta$ . TGF- $\beta$  has been shown to be a co-stimulator for RANKL in promoting differentiation and survival of osteoclast precursors and to inhibit osteoclastogenesis suppressor molecules [16, 17]. Similarly, dexamethasone has been reported to stimulate the initial phases of osteoclastogenesis synergistically with TGF- $\beta$  and to decrease the expression of certain suppressor molecules, such as interferon beta (IFN- $\beta$ ) [37].

Essential in interpreting these results is also the fact that the BM cultures contain a large number of stromal cells, which might secrete osteoclastogenic and osteoclast regulatory molecules, such as TGF- $\beta$  [38, 39]. The stromal cells were almost completely absent in PB cultures. This could explain why BM cultures with only RANKL and M-CSF contained less multinuclear cells but expressed more osteoclast-related TRACP 5b and had an equal bone resorbing activity as the group differentiated with all growth factors. It is possible that the stromal cells produce

osteoclast-activating substances compensating for shortage of the other growth factor molecules. We have previously shown that the stromal cells possibly participate in the gap junctional mediated fusion of the osteoclast precursors by secreting the osteoclastogenesis promoting factors [30]. Moreover, the complex regulatory effects of the stromal cells was examined by Karst *et al.* [40], who showed that a high concentration of TGF- $\beta$  inhibits osteoclastogenesis from bone marrow precursors if they are cultured with stromal cells. On the contrary, a low concentration stimulated osteoclastogenesis through an increased RANKL/OPG ratio produced by the stromal cells. It is possible that in our study the additional TGF- $\beta$  supplemented to the cultures along with the secretion of it from the stromal cells led to a surplus amount of it leading to diminution of osteoclastogenesis in BM cultures compared to PB cultures with the same culture protocol. As the PB cultures contained only few stromal cells, the net effect could be the observed stimulation of osteoclastogenesis with a lower concentration of TGF- $\beta$  in the culture.

Another issue worth consideration when drawing conclusions from our results is that the blood and bone marrow donating patients were middle-aged or older. Aging commonly leads to bone loss [41], which has been shown to be mediated by increased RANKL/OPG ratio and enhanced osteoclastogenesis [42]. Therefore, our results might not represent the situation in younger individuals, but instead illustrate the biology of the bone cells of the group in the higher risk of age-related bone diseases. Due to the global aging of people, the size of this population is continuously growing leading to generalization of these diseases and increased costs. Therefore, it is important to study bone biology especially within the elder population.

In conclusion, this data shows that phenotypically different osteoclasts in regard to number of nuclei, TRACP isoform secretion and osteoclast specific gene expression can be generated from BM and PB monocytes by using different growth factor cocktails. It is possible that the osteoclasts observed in PB cultures with all growth factors and dexamethasone are mainly derived from classical monocytes, which are most prone for osteoclastogenesis, and their differentiation represents osteoclastogenesis taking place in homeostasis. Moreover, monocytes from BM and PB seem to have altered osteoclastogenic potential, as PB monocytes were more responsive to TGF- $\beta$  and dexamethasone. It is also possible that osteoclastogenesis from these two sources proceeds at a different pace. Various timepoints for the cell fusion and acquisition of the resorptive capability during osteoclastogenesis have been reported. Lader *et al.* [43] showed that osteoclasts appeared by day 3–4 in both stromal cell-free and stromal cell-rich monocyte cultures. On the contrary, Merrild *et al.* [44] found that PB -derived osteoclasts were faster in initiating bone resorption compared to BM -derived osteoclasts. In addition, several studies have reported that the fusion of PB -derived monocytes begins approximately at day 3 [20, 45, 46], whereas in BM cultures osteoclasts appear at days 5–10

depending on the culture conditions [45]. In our study, it seems that the BM monocytes differentiate quicker into osteoclasts based on higher multinuclear cell number and the lower level of TRACP 5a and thus fewer mononuclear cells in BM cultures. Since TRACP 5a is more abundantly expressed in mononuclear pre-osteoclasts, and the expression of TRACP 5a is lower in BM cultures than in PB cultures following addition of TGF- $\beta$  and dexamethasone, it is possible that in this setting the mononuclear cells had fused more efficiently into multinuclear cells and thus differentiation was promoted more effectively. The increased expression of *CR* and *NFATc1* upon addition of TGF- $\beta$  and dexamethasone are also in line with this contention. However, the finding that bone resorption activity in cultures with TGF- $\beta$  and dexamethasone was decreased might reflect a different osteoclastogenic phenotype of the cells cultured with this protocol. These results conclude that the choice of the osteoclast precursor source as well as the choice of osteoclastogenic growth factors are essential matters in determining the phenotypic characteristics of heterogeneous osteoclast populations. In addition, the results might reflect that the cytokine requirements for osteoclastogenesis are other than for the activation of bone resorption.

## Declarations

### Author contribution statement

Elina Kylmäoja, Miho Nakamura, Christina Patlaka: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sanna Turunen, Juha Tuukkanen: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Göran Andersson: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Petri Lehenkari: Analyzed and interpreted the data; Wrote the paper.

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### Competing interest statement

The authors declare no conflict of interest.



## Additional information

No additional information is available for this paper.

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