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A challenge with *Porphyromonas gingivalis* differentially affects the osteoclastogenesis potential of periodontal ligament fibroblasts from periodontitis patients and non-periodontitis donors

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Abstract

Aim: *Porphyromonas gingivalis* (*Pg*) may cause an immune-inflammatory response in host cells leading to bone degradation by osteoclasts. We investigated the osteoclast-inducing capacity of periodontal ligament fibroblasts from periodontitis patients and non-periodontitis donors after a challenge with viable *Pg*.

Materials and methods: PDLFs from periodontitis patients ($n = 8$) and non-periodontitis donors ($n = 7$) were incubated for 6 h with or without viable *Pg* and subsequently co-cultured with osteoclast precursors from peripheral blood mononuclear cells (PBMCs). The number of multinucleated tartrate-resistant acid phosphatase-positive cells was determined at 21 days. Expression of osteoclastogenesis-associated genes was assessed after infection of PDLFs mono-cultures and in PDLFs-PBMCs co-cultures. Resorption activity was analysed on bone slices.

Results: *Pg* induced the expression of osteoclastogenesis-associated genes by PDLFs. After bacterial challenge the formation of osteoclast-like cell was decreased in co-cultures of PBMCs with non-periodontitis PDLFs, but not with PDLFs from periodontitis patients.

Conclusion: PDLFs from sites free of periodontitis respond to an infection with *Pg* by tempering formation of osteoclast-like cells, probably promoting clearance of the infection. PDLFs from periodontitis sites are desensitized to a *Pg* challenge in terms of their osteoclast-inducing capacity.

The periodontal ligament is the soft connective tissue, rich in vessels and cells, that encloses the roots of the teeth and connects the root cementum to the alveolar bone (Beertsen et al. 1997). The predominant cell type of the periodontal ligament is the periodontal ligament fibroblast (PDLF), which occupies 25% of the periodontal ligament volume (Beertsen et al. 1997). PDLFs are multipotent cells that contribute to the maintenance of the bone-cementum attachment apparatus (Ten Cate et al. 1976), the formation of acellular cementum (Groeneveld et al. 1993, 1995), local

modulation of the immune-cell behaviour (Buckley et al. 2001) and alveolar bone formation and resorption (Roberts et al. 1981, Lekic & McCulloch 1996, Kanzaki et al. 2001).

Bone resorption is mediated via the action of osteoclasts, which are tartrate-resistant acid phosphatase (TRACP)-positive multinucleated cells (Teitelbaum 2007) originating from the monocyte/macrophage lineage (Udagawa et al. 1990). PDLFs can regulate osteoclast formation and activity via the selection and attraction of osteoclast precursors and by subsequent binding them with cell–cell adhesion molecules, like intercellular adhesion molecule-1 (ICAM-1) (Bloemen et al. 2009). Once the osteoclast precursors bind to the PDLFs, a drastic up-regulation is induced of osteoclastogenesis stimulatory molecules, such as macrophage-colony stimulating factor (M-CSF), receptor activator of nuclear factor κ-B (RANKL) and tumour necrosis factor-alpha (TNF-α) (Bloemen et al. 2010). Finally, PDLFs retract and osteoclast precursors migrate to the bone surface where they fuse to form TRACP$^+$ multinucleated cells (Bloemen et al. 2011). PDLFs can also inhibit osteoclast formation by production of osteoprotegerin (OPG), which is decoy receptor for RANKL (Kanzaki et al. 2001).

In periodontitis, the presence of subgingival bacteria causes an immune-inflammatory response in the periodontal tissues. As a consequence, tissue is degraded, ultimately leading to alveolar bone loss through osteoclast-mediated bone resorption (Pihlstrom et al. 2005). *Porphyromonas gingivalis* (*Pg*) is an important periodontal pathogen (Socransky et al. 1998, van Winkelhoff et al. 2002, Hajishengallis et al. 2012). PDLFs are able to recognize *Pg* via pattern recognition receptors (PRRs) like Toll-like receptors (TLRs) and CD14 (Sun et al. 2010). The recognition of *Pg* components, like lipopolysacharide (LPS), by PPRs of PDLFs leads to activation of transcription of nuclear factor kappa B (NF-κB), which upon activation translocates to the nucleus and may result in an increased expression of osteoclastogenesis-associated molecules (Carmody & Chen 2007, Kawai & Akira 2007).

Several *in vitro* studies have investigated the effect of *Pg* or its components on the expression by PDLFs of osteoclastogenesis-associated molecules. A significant up-regulation of osteoclastogenesis-associated chemokines and cytokines has been observed after challenge of PDLFs with either viable *Pg* for 6 h (Scheres et al. 2010) or 24 h (Yamamoto et al. 2006) or *Pg* LPS (Krajewski et al. 2009, Sun et al. 2010, Park et al. 2012) or heat-killed *Pg* (Sun et al. 2010) or supernatant from viable *Pg* cultures (Belibasakis et al. 2007).

On the basis of the evidence that *Pg* challenge of PDLFs leads to production of cytokines beneficial for osteoclastogenesis, we further investigated the effect of a *Pg* stimulus on osteoclast formation. In this study, we used co-cultures of PDLFs with peripheral blood mononuclear cells (PBMCs) – as a source of osteoclast precursors – to investigate the osteoclast inducing capacity of periodontal ligament fibroblasts from non-periodontitis donors and periodontitis patients after a 6 h challenge with viable *Pg*.

Materials and Methods

Fibroblast donors

Periodontal ligament fibroblasts – from eight periodontitis patients and seven non-periodontitis donors – were obtained during a previous study (Scheres et al. 2011b). Periodontitis patients underwent tooth extraction as part of periodontal treatment and displayed deepened probing pocket depths (PPDs) and alveolar bone loss detectable in intra-oral radiographs around the extracted teeth. Non-periodontitis donors underwent extraction as part of treatment for other reasons than periodontitis and did not present deepened PPDs and alveolar bone loss due to periodontitis around the extracted teeth. The characteristics of the donors and clinical parameters are described in Table 1. None of the donors suffered from systemic diseases or was pregnant.

Bacterial samples were collected from the subgingival biofilm of all donors with the paperpoint method and were analysed for the presence of *Pg* by anaerobic culture. All donors were recruited at the Centre for Implantology and Periodontology Amstelveen, The Netherlands. All donors had given written informed consent.

Fibroblast isolation

PDLFs were recovered from fragments of periodontal ligament obtained by scraping exclusively the middle third of extracted teeth (Scheres et al. 2011b). Experiments were performed with cells from passages 5–6. In previous experiments, fibroblasts expressed high levels of *S100A4*, a marker for PDLFs (Scheres et al. 2011b).

Bacterial strain and culture

Porphyromonas gingivalis W83 was cultured anaerobically (80% N_2, 10% H_2 and 10% CO_2) until log-growth-phase in Brain-Heart-Infusion (BHI)-broth supplemented with

Table 1. Characteristics and clinical parameters of fibroblast donors

	Non-periodontitis donors ($n = 7$)	Periodontitis patients ($n = 8$)
Age (years; mean $\pm$ SD)	53.1 $\pm$ 19.1	57.1 $\pm$ 11.3[ns]
Gender (F)	5	8[ns]
Smoking (Smokers)	1	2[ns]
Bone loss (% $\pm$ SD)	0.43 $\pm$ 0.79	65 $\pm$ 26.73**
Pocket depth (mm; mean $\pm$ SD)	2.57 $\pm$ 1.13	7.63 $\pm$ 3.2**
Pg carriers (nr)	2	4[ns]

Bone loss: percentage of alveolar bone loss around extracted teeth. Pocket depth: probing pocket depth around extracted teeth. *Pg* carriers: nr of donors in whose subgingival biofilm *Pg* was detected by anaerobic culture. **$p < 0.01$ significant differences between the patients group and non-periodontitis group; ns: no significant difference.

hemin (5 mg/l) and menadione (1 mg/l). Purity was checked with Gram staining.

Viable *Pg* was isolated by centrifugation. Bacterial pellets were washed twice in sterile phosphate buffered salt solution (PBS) and re-suspended in antibiotic-free DMEM with 10% FCS. The optical density was measured at 690 nm to establish the number of colony forming units (CFUs). A suspension of 2×10^8 CFU/ml was used to challenge the PDLFs (approximately 7000 *Pg*/PDLF).

Human peripheral blood mononuclear cells (PBMCs) isolation

PBMCs from buffy coats (Sanquin, Amsterdam, the Netherlands) were isolated as previously described (de Vries et al. 2006).

Co-cultures

PDLFs (1.5×10^4 per well) were seeded in 48-well plates either on plastic or on 650 μm thick bovine cortical bone slices for 24 h. Hereafter, the culture medium was removed and replaced with either 0.5 ml of a *Pg* W83 suspension of 2×10^8 CFU/ml in antibiotic-free DMEM with 10% FCS, or only DMEM with 10% FCS.

PDLFs were incubated with *Pg* or medium for 6 h in a humidified aerobic atmosphere with 5% CO_2 at 37°C. After challenge, PDLFs were washed three times in DMEM with 10% FCS and 2% antibiotics and fibroblast morphology was checked for abnormalities or cell-detachment by phase contrast microscopy (Olympus CK2, Olympus, Japan). In previous experiments, it was shown that viability of the fibroblasts was not

affected by this treatment (Scheres et al. 2010).

Subsequently, 0.5×10^6 PBMCs per well were seeded on top of the PDLFs. Co-cultures were performed for additional 7 days (RNA analysis in co-cultures on plastic) and 21 days (RNA analysis in co-cultures on plastic and osteoclast-like cell formation on bone and plastic) in DMEM with 10% FCS and 1% antibiotics (0.4 ml per well). Culture medium was refreshed every 3 or 4 days (Bloemen et al. 2010).

Mono-cultures

PDLFs (1.5×10^4 cells per well) were seeded analogously to the co-cultures. Cells on plastic were challenged with *Pg* for 6 h and samples were taken after these 6 h and after 7 days for RNA analysis.

After the culture period of the RNA analysis assays, both mono-cultures and co-cultures were washed twice with PBS and stored in lysis buffer (Qiagen, Hilden, Germany) containing 1% β-mercaptoethanol at −80°C until RNA extraction.

TRACP staining and osteoclast quantification

TRACP staining was performed as previously described (de Vries et al. 2006). Nuclei were stained with diamidino-2phenylindole dihydrochloride (DAPI).

Micrographs of co-cultures on plastic were taken from five justified positions per well, with a digital camera (Leica, Wetzlar, Germany) and analysed for the number of TRACP$^+$ MNCs containing three or more nuclei. On bone, the whole surface was analysed for the number of

TRACP$^+$ MNCs. The number of TRACP$^+$ MNCs on bone slices was corrected for the bone area.

RNA analysis and real-time quantitative polymerase chain reaction (Q-PCR)

RNA from cultured cells was isolated as previously described (de Vries et al. 2006).

Real-time PCR primers were designed using Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA, USA; Table 2). To avoid amplification of genomic DNA, each amplicon spanned at least one intron. Real-time PCR was performed on the ABI PRISM 7000 (Applied Biosystems) as described previously (de Vries et al. 2006). Expression of house keeping gene porphobilinogen deaminase (PBGD) was not affected by the experimental conditions. Samples were normalized for the expression of PBGD by calculating the ΔC_t ($C_{t,\text{gene of interest}} - C_{t,\text{PBGD}}$) and expression of the different genes is expressed as $2^{-(\Delta C_t)}$.

TRACP enzyme activity in tissue culture supernatant

TRACP enzyme activity was measured in supernatant from co-cultures at 21 days. Ten microlitre supernatant was incubated with 90 μl of incubation medium containing 4.2 mg/ml P-nitrophenylphosphate (PnPP) diluted in 88.8% buffer sodium acetic acid, 10% potassium chloride solution, 1% sodium potassium tartrate solution, 0.1% ascorbic acid solution and 0.1% $FeCl_3$ solution in a 96-well plate. The reaction was stopped with 100 μl 0.3 M sodium hydroxide and the TRACP enzyme

Table 2. Real-time PCR primer sequences

Gene	Primer sequence 5′-3′ Fw	Primer sequence 5′-3′ Rv
Carbonic anhydrase II	TggACTggCCgTTCTAggTATT	TCTTgCCCTTTgTTTTAATggAA
ICAM-1	TgAgCAATgTgCAAgAAgATAgC	CCCgTTCTggAgTCCAgTACA
IL-1β	CTTTgAAgCTgATggCCCTAAA	AgTggTggTCggAgATTCgT
IL-6	ggCACTggCAgAAAACAACC	ggCAAgTCTCCTCATTgAATCC
MCP-1	CAgCCAgATgCAATCAATgC	TgCTgCTggTgATTCTTCTATAgCT
M-CSF	CCgAggAggTgTCggAgTAC	AATTTggCACgAggTCTCCAT
OPG	CTgCgCgCTCgTgTTTC	ACAgCTgATgAgAggTTTCTTCgT
PBGD	TgCAgTTTgAAATCATTgCTATgTC	AACAggCTTTTCTCTCCAATCTTAgA
RANKL	CATCCCATCTggTTCCCATAA	gCCCAACCCCgATCATg
RANTES	CATCTgCCTCCCCATATTCCT	TgCCACTggTgTAgAAATACTCCTT
TNF-α	CCCAgggACCTCTCTCTAATCA	gCTTgAgggTTTgCTACAACATg
TRACP	CACAATCTgCAgTACCTgCAAgAT	CCCATAgTggAAgCgCAgATA

activity was assessed at 405 nm in a plate reader (Biotek, Winooski, VT, USA).

Actin staining and resorption

F-actin was stained using Alexa 488-conjugated phalloidin (Molecular Probes, Eugen, OR, USA) based on the method previously described (de Vries et al. 2009). Collagen I was visualized as described before (Vermeer et al. 2013) with rabbit anti-collagen type I (Abcam, Cambridge, UK) and goat anti-rabbit Alexa 647 (Molecular Probes). Nuclei were stained with propidium iodide. Image stacks were generated using confocal laser scanning microscopy (Leica) using an Argon laser (Alexa 488 and propidium iodide) and a Helium/Neon laser (Alexa 647).

Statistical analysis

Comparisons of the number of TRACP$^+$ MNCs and the level of TRACP enzyme activity between co-cultures of PDLFs that had or had not been challenged with *Pg* were tested with paired *t*-tests in cases of normal distribution of the data, or Wilcoxon signed rank test if data were not normally distributed. Normality of the data distribution was checked with the Shapiro–Wilk test. The results are expressed as medians or means ± SEM. Comparisons of the characteristics and clinical parameters of fibroblast donors were tested with Fisher's exact test for qualitative data and Mann–Whitney U-test for quantitative data. Linear regression analysis was performed to investigate the relationship between the number of TRACP$^+$ MNCs with the clinical characteristics of the extracted teeth (PPDs and bone loss) from each donor. Differences were considered significant at *p* < 0.05. Analysis was performed with Graph-Pad Prism software (version 5, GraphPad Software Inc., La Jolla, CA, USA).

Results

Pg induces expression of osteoclastogenesis-associated genes by PDLFs

A 6 h *Pg*-challenge of PDLFs from both non-periodontitis donors and periodontitis patients resulted in induction of mRNA expression of all the studied genes. This effect was no longer present at 7 days for ICAM-1, RANTES (regulated upon activation, normal T-cell expressed and secreted), monocyte chemotactic protein-1 (MCP-1), interleukin 1-beta (IL-1β), interleukin-6 (IL-6) and TNF-α.

A reverse trend was observed for M-CSF and OPG, where mRNA expression was higher at 7 days both in non-periodontitis and periodontitis derived PDLFs that had not received a *Pg* challenge (Fig. 1).

Pg challenge caused an increase in RANKL expression at 6 h after infection, but was hardly detectable after 7 days (Fig. 1). In contrast, OPG was highly expressed in PDLF monocultures just after infection as well as at 7 days. OPG expression exceeded RANKL at least 1000-fold throughout the experiment (Fig. 1).

Pg decreases osteoclastogenesis by control PDLFs, but not periodontitis PDLFs

After 21 days co-culture of PDLFs and PBMCs the number of TRACP$^+$ MNCs was assessed on plastic and bone. Examples of TRACP$^+$ MNCs on plastic and on bone are presented in Fig. 2(a,c). When non-periodontitis PDLFs were incubated for 6 h with viable *Pg*, significantly lower numbers of TRACP$^+$ MNCs were formed after 21 days of co-culture with PBMCs compared to non-challenged PDLFs. This was found both on plastic (Fig. 2b) and on bone (Fig. 2d). In contrast, the *Pg* challenge of PDLFs from periodontitis patients did not affect their osteoclast inducing capacity, neither on plastic nor on bone (Fig. 2b,d).

These results indicate that PDLFs from periodontitis patients are insensitive to a *Pg* challenge in terms of the modifying effect on osteoclastogenesis. Possibly, these cells have encountered a bacterial challenge *in vivo* and responded differently as a result. Therefore, we next compared the osteoclast-like cell forming capacity of PDLFs from *Pg* carriers (controls+periodontitis patients, who carried *Pg* in their subgingival plaque) with non-carriers (controls+periodontitis patients, who did not carry *Pg* in their subgingival plaque). Interestingly, osteoclastogenesis

was decreased after a *Pg* challenge only in the non-carriers group, both on plastic (*p* = 0.019) and on bone (*p* = 0.007).

Moreover, our analysis regarding the relationship between the clinical characteristics (PPDs and bone loss) of the extracted teeth and the formation of TRACP$^+$ MNCs did not show any significant relationship for the patients group.

Pg infection nor health status of PDLFs leads to actively resorbing osteoclasts in PDLF-PBMCs co-cultures

We next assessed whether a challenge with *Pg* could activate TRACP$^+$ MNCs on bone. Thus, we investigated whether PDLFs from periodontitis patients and controls were able to form actin rings in co-cultures with PBMCs using confocal microscopy. No actin rings and no exposed collagen I adjacent to multi-nucleated cells as a sign of bone resorption were seen in 40 multinucleated cells assessed on bone slices (*n* = 5 non-periodontitis donors without or after *Pg* challenge; *n* = 5 periodontitis patients without or after *Pg* challenge; Fig. 3).

These results indicate that neither disease state nor brief exposure to *Pg* prior to the experiment resulted in active, bone resorbing osteoclasts.

Gene expression of TRACP and carbonic anhydrase II increases over time and is lower in *Pg* challenged co-cultures of PDLFs and PBMCs

The osteoclastic nature of the TRACP$^+$ MNCs was further investigated by analysing the gene expression of the osteoclast-related molecules TRACP and carbonic anhydrase II (CAII) at 7 and 21 days. The incubation with viable *Pg* for 6 h resulted in severely lower expression of TRACP and CAII both at 7 and 21 days in PDLFs from both non-periodontitis donors and periodontitis patients (Fig. 4).

Secreted TRACP enzyme activity in co-cultures of PDLFs and PBMCs

The osteoclastic character of the TRACP$^+$ MNCs was assessed by measuring secreted TRACP enzyme activity in supernatant from the
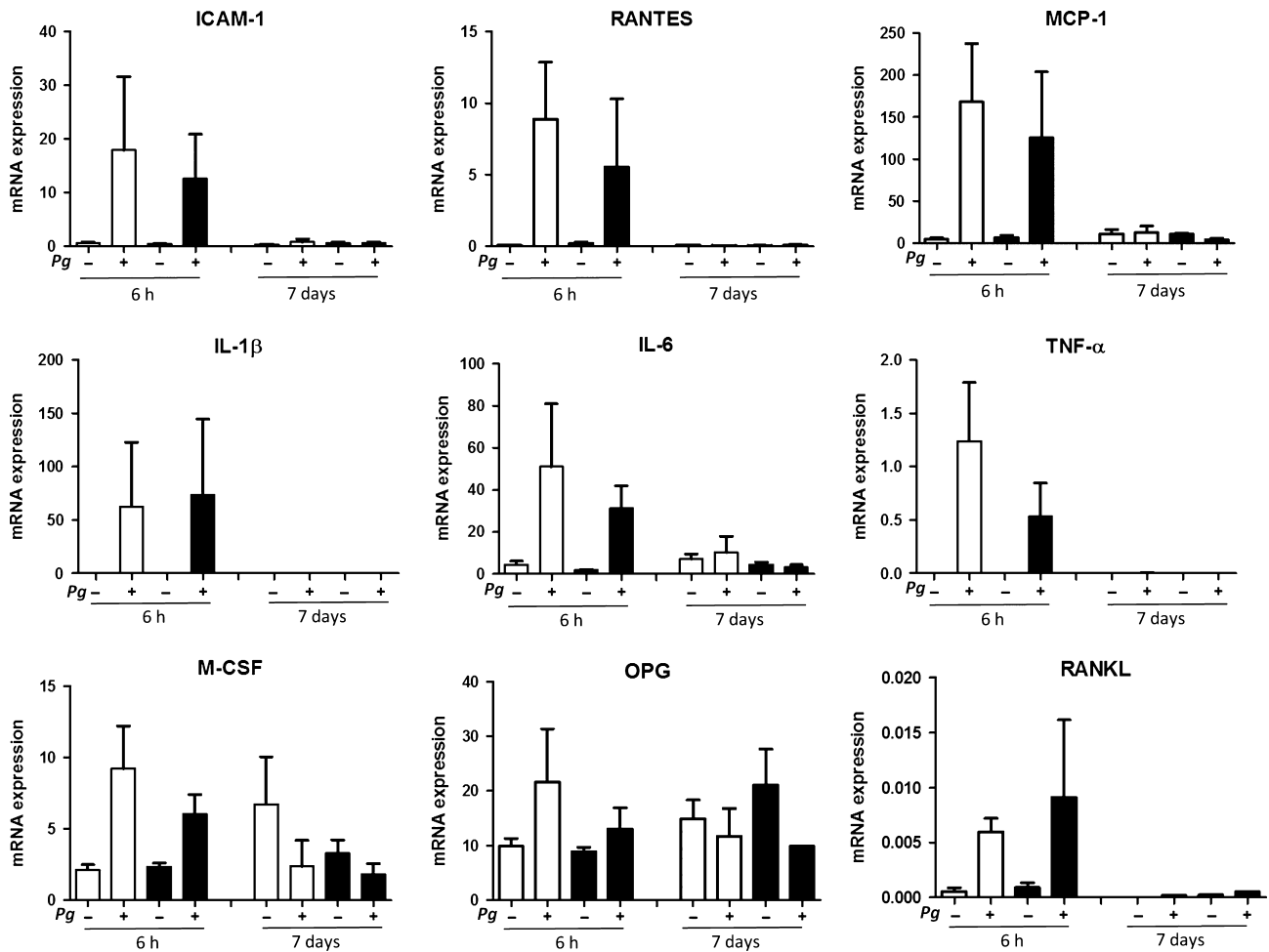
Fig. 1. mRNA expression of osteoclastogenesis-associated molecules at 6 h and 7 days from PDLFs of non-periodontitis donors (white bars) and periodontitis patients (black bars) challenged with *Pg* for 6 h or non-challenged. Bars represent mean ± SEM. *n* = 5 or 6 donors at *t* = 6 h and *n* = 1–6 donors, at *t* = 7 days. Data are shown from donors that either both not (−) or were challenged (+) with *Pg*.

co-cultures of PDLFs and PBMCs on plastic and on bone surfaces at 21 days. No significant differences were observed in any of the media analysed (Fig. 5).

Discussion

In this study, we observed that a short challenge of periodontal ligament fibroblasts with viable *Pg* resulted in a reduction of their capacity to induce osteoclast-like cell formation in co-cultures by the PDLFs of non-periodontitis donors, but not by PDLFs from periodontitis patients. The decreased osteoclast formation by PDLFs of non-periodontitis donors suggests a protective role on bone resorption in response to infection, which is in accordance with findings of Belibasa-

kis et al. (2007). In that model, *Pg* initially induced an increased RANKL/OPG ratio from PDLFs of a healthy donor, however, longer *Pg* challenge of PDLFs resulted in a significantly lower RANKL/OPG ratio, suggesting a protective adaptation of PDLFs in terms of osteoclast formation. Previous studies that investigated the effect of LPS on osteoclast precursors have shown inhibition of osteoclastogenesis via stimulation of the TLRs. This inhibition was attributed to "re-route" the osteoclast precursors from becoming osteoclasts towards enhancement of their immune response for removal of bacterial infection (Takami et al. 2002, Ji et al. 2009). Therefore, a similar "re-route" of PDLFs from non-periodontitis donors cannot be excluded, especially taking into

account the multi-potent character of these cells.

The lack of effect of a *Pg* challenge in PDLFs from periodontitis patients could partially be attributed to a previous encounter of PDLFs to a bacterial challenge *in vivo*, either directly (e.g. PDLFs came in contact with *Pg*), or indirectly (e.g. PDLFs were altered due to responding gingival/immune cells that encountered *Pg*), since we also found that a *Pg* challenge *inhibited* the osteoclastogenesis potential of PDLFs from *Pg* non-carriers, but had a variable effect in PDLFs from *Pg* carriers. Scheres et al. (2011b) observed that PDLFs from *Pg* carriers, both non-periodontitis and periodontitis donors, responded more strongly to viable *Pg* than non-carriers with regard to the expression of
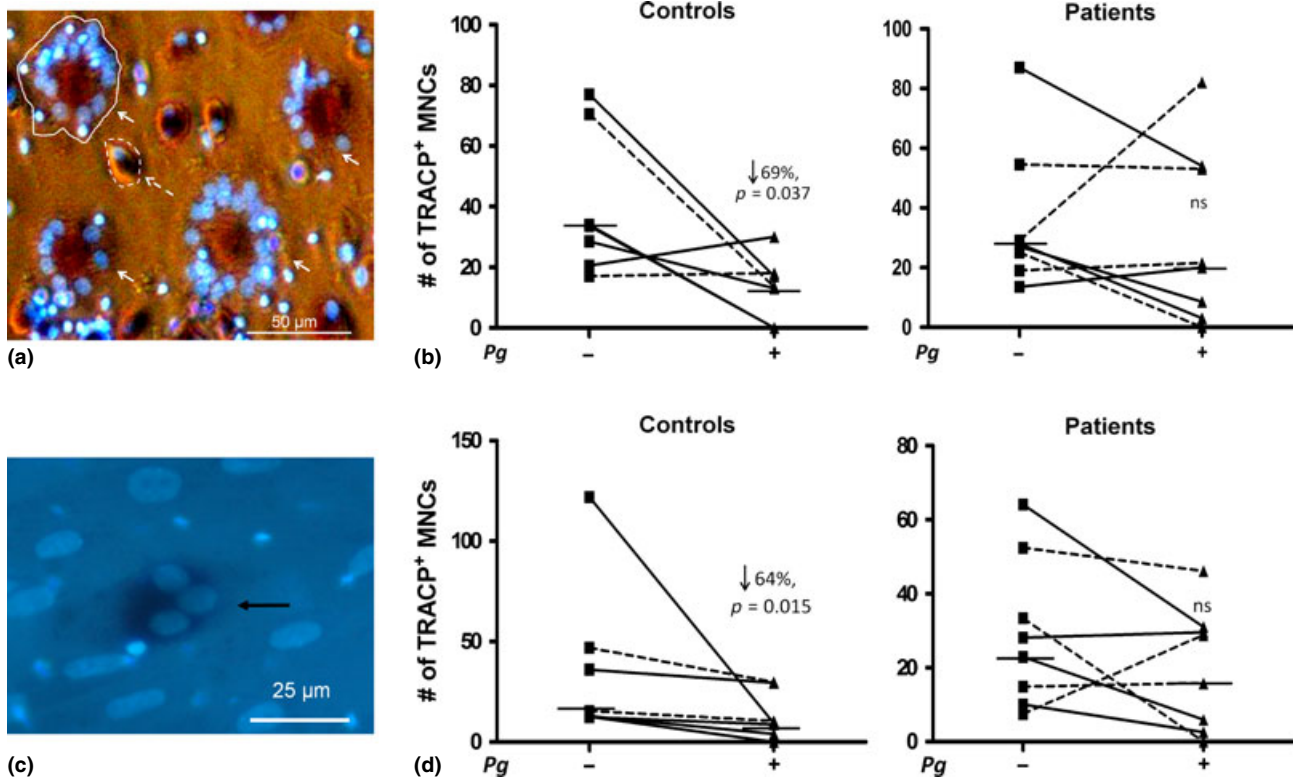
Fig. 2. Pg inhibits osteoclast-like cell formation by control PDLFs, but not by patient PDLFs. (a) Example of TRACP$^+$ MNC on plastic, solid arrows: TRACP+ MNC, interrupted arrows: mononuclear TRACP+ cell. Area of one mononuclear and one multinu-cleated cell is shown. (b) Results on plastic. (c) example of TRACP$^+$ MNC on bone. Black arrow show TRACP$^+$ MNCs. (d) results on bone (per cm^2). Squares represent formation of osteoclast-like cell by PDLFs without *Pg* challenge, triangles with. Dashed lines represent *Pg* carriers. Short horizontal lines in the graph represent median number of TRACP+ MNC. Percentages represent relative reduction of osteoclast-like cell formation between co-cultures of PDLFs non-challenged and challenged with *Pg*. *n* = 7 non-periodontitis donors and eight periodontitis patients.

pro-inflammatory mediators and receptors. A possible explanation for this alteration may be that previous exposure of PDLFs to bacterial and inflammatory stressors could affect gene activation and cell phenotype via epigenetic alterations (Barros & Offenbacher 2009). This modified PDLF phenotype could be maintained *in vitro* after multiple passages (El-Awady et al. 2010) and conse-quently lead to variable response to *Pg*. It cannot be excluded that the observed differences in the osteoclas-togenic behaviour between the PDLFs from healthy donors and periodontitis patients are due to dif-ference in their susceptibility to periodontal pathogens such as *Pg*. Especially, considering the significant differences in all the clinical indices between the two groups, such a pos-sibility cannot be ignored. However, when we tried to explain the observed inter-individual variable

osteoclastogenic response to *Pg* chal-lenge with the clinical indices of the donors, such a significant relation-ship was not found, maybe due to the small number of participants. Another possible explanation for the results could be that there are differ-ences in invasion of *Pg*, thus result-ing in differential effects. Nevertheless, the specific mechanism for the variable response of PDLFs from periodontitis donors to *Pg* regarding osteoclastogenesis needs further elucidation.

Here, a *Pg* challenge initially induced the expression of various osteoclastogenesis-associated genes tested in both PDLF populations. In line with this, Scheres et al. (2011b) found that PDLFs from controls and patients reacted similarly to via-ble *Pg* in terms of cytokine response. Although in that study PDLFs from periodontitis patients displayed higher levels of TLRs and lower lev-

els of NF-κBIL1 (nuclear factor kappa B-inhibitor-like protein 1) compared to non-periodontitis donors, in both studies PDLFs reacted comparable to the initial bacterial challenge via pathogen rec-ognition receptors and transcription factors irrespective of their disease status, leading eventually to increased expression of osteoclasto-genesis-associated molecules. This can be attributed to the short dura-tion of the *Pg* challenge (6 h). It could be that differences in cytokine expression become apparent at longer infection. However, *Pg* can-not survive in longer challenge peri-ods, due to aerobic environment. Assessment of the different osteocl-astogenesis-associated molecules at the protein level could provide addi-tional information regarding the PDLFs behaviour, although such an assessment could be influenced from the proteolytic action of viable *Pg*,
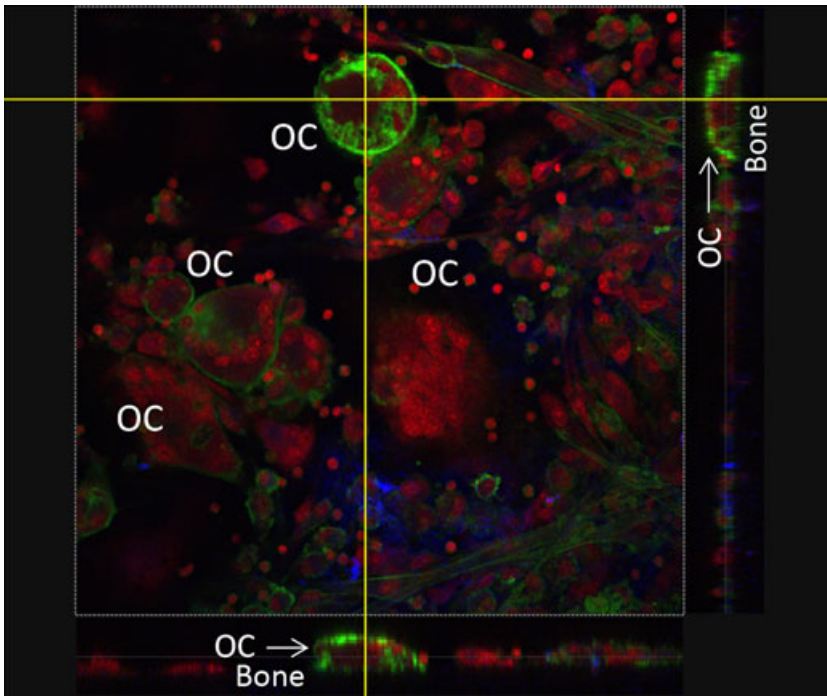
Fig. 3. Example of osteoclasts (OCs) that formed on bone surface after 21 days of co-culture of PDLFs from non-periodontis donor and PBMCs without *Pg* challenge. Neither actin rings nor exposed collagen are obvious adjacent to the MNCs. XZ and YZ projection of OCs show that osteoclasts stay on top of the bone surface, never in lacunae, indicating that no resorption has taken place. Actin: green; Collagen I: blue; nuclei: red.
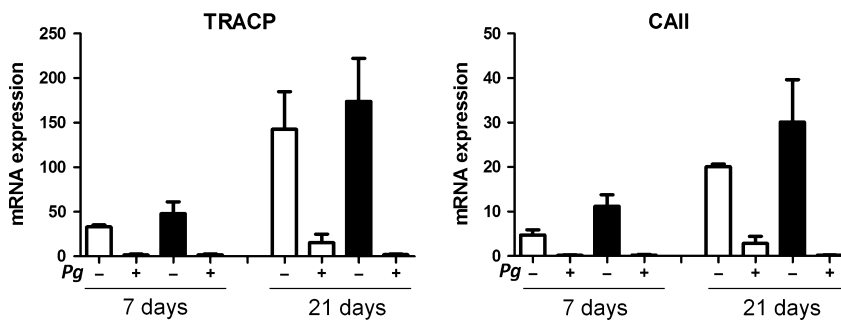

Fig. 4. *Pg* reduces the mRNA expression of TRACP and CAII in the co-cultures of PDLFs, from non-periodontitis donors (white bars) and periodontitis patients (black bars), with PBMCs on plastic, at 7 and 21 days. Bars represent mean ± SEM. $n = 2$ non-periodontitis donors and three periodontitis patients. Data are shown from donors that either both not (−) or were challenged (+) with *Pg*.
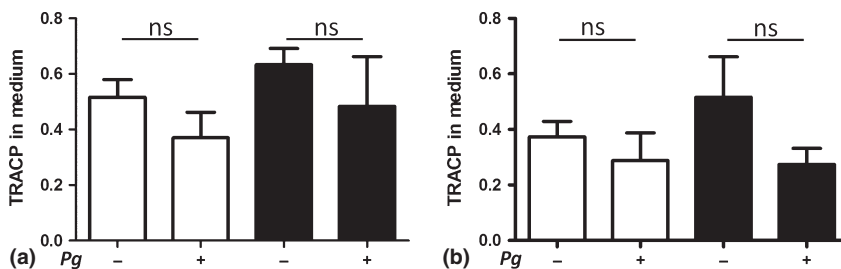

Fig. 5. TRACP enzyme activity in co-cultures of PDLFs from non-periodontitis donors (white bars) and periodontitis patients (black bars) with PBMCs, after 21 days of co-culture. (a) Results on plastic. (b) Results on bone. Bars represent mean ± SEM. $n = 4$ non-periodontitis donors and five periodontitis patients.

as has been shown from Scheres et al. (2010). Given the reduced osteoclastogenesis by control PDLFs after *Pg* challenge, it is clear that the balance between osteoclastogenesis stimulatory (for instance RANKL, TNF-α, IL-1β) and osteoclastogenesis inhibiting molecules (IL-10, OPG) is shifted compared to osteoclastogenesis cultures from infected patient PDLFs.

Nevertheless, the initial stimulatory effect of *Pg* was diminished at 7 days for all the genes investigated, except for M-CSF and OPG, which were expressed higher in non-challenged PDLFs. This implies that their expression follows different intracellular pathways than the other molecules. It could also infer an adaptation of the cells to the previous *in vitro*, but no longer existing bacterial challenge.

The initial increase in the expression of osteoclastogenesis-associated molecules after *Pg* challenge was not translated in more osteoclasts in a later stage. Moreover, we were not able to detect actively resorbing osteoclasts in co-cultures of PDLFs from non-periodontitis donors or periodontitis patients with PBMCs. It is possible that the presence of stimulatory factors early in culture is not sufficient to enhance osteoclastogenesis. For instance, Kim et al. (2009) observed inability of IL-1 to enhance osteoclastogenesis in cultures of bone marrow-derived macrophages when added early in culture. Likely, addition of M-CSF and RANKL in the co-cultures could overcome the lack of bone resorption, as shown previously (de Vries et al. 2006). However, Scheres et al. (2011a) mimicked a long term infection by culturing osteoclast precursors in the presence of *Pg* conditioned fibroblast medium. They also found inhibition of osteoclast formation in the presence of M-CSF and RANKL. This inhibitory effect has been attributed in other studies to the action of *Pg* LPS or the haemoglobin receptor protein of *Pg* (Nagasawa et al. 2002, Zou & Bar-Shavit 2002, Fujimura et al. 2006). Nevertheless, until now no specific cellular mechanism has been proposed to explain the interactions of *Pg* with host cells in the presence or absence of M-CSF or RANKL.

Interestingly, the osteoclast markers TRACP and carbonic anhydrase II

were minimally expressed in co-cultures of PBMCs and PDLFs from either non-periodontitis or periodontitis donors after *Pg* challenge. This is partially in agreement with less TRACP$^+$ MNCs being formed after *Pg*-challenge in PDLFs from non-periodontitis donors. The effect on gene expression, however, was more severe than the effect on the formation of TRACP$^+$ MNCs. This could mean that the TRACP$^+$ MNCs that were formed in the co-cultures of PDLFs from periodontitis patients and PBMCs on plastic were not fully matured osteoclasts or were multinucleated cells of a different category such as foreign body multinucleated giant cells (Brodbeck & Anderson 2009).

It is likely that *in vivo*, gingival fibroblasts rather than PDLFs are the resident cells that provide initial osteoclastogenesis signals, since these cells are anatomically located coronally of the alveolar bone. Also, it cannot be ignored that in the inflamed periodontal microenvironment, immune cells and osteoblasts may have a prominent role in alveolar bone resorption. However, it has been shown that gingival fibroblasts induce less osteoclasts in co-cultures with PBMCs compared to PDLFs (de Vries et al. 2006). On the basis of this finding and due to the extensiveness of the current study, we decided to investigate *Pg*'s effects on PDLFs. Also, it is technically not feasible to perform experiments of inflammatory cells isolated from the inflamed tissue in conjunction with the PDLFs that need propagation before they can be used in such an experiment.

In conclusion, a 6 h challenge of PDLFs with viable *Pg* caused a decrease in the capacity of PDLFs from non-periodontitis donors to induce osteoclast-like cell formation, whereas *Pg* had no significant effect on the osteoclast-inducing capacity of PDLFs from periodontitis patients. Thus, we speculate that PDLFs in sites free of inflammation respond to an infection with *Pg* by maintaining bone homeostasis and promoting clearance of the infection. In contrast, PDLFs from periodontitis sites seem to have lost this response to the periodontal pathogen *Pg*, possibly due to earlier direct or indirect encounter of periodontal pathogens before the teeth were

extracted or difference in their susceptibility to periodontal pathogens.

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Clinical Relevance

Scientific rationale for the study: Although *Porphyromonas gingivalis* is associated with periodontitis, thus far, little is known whether infection with this bacterium influences osteoclastogenesis induced by periodontal ligament fibroblasts. Here, we mimicked *in vivo* conditions by challenging fibroblasts from periodontitis patients and non-periodontitis donors with viable *Pg* and co-culturing them with osteoclast precursor cells.

Principal findings: *Pg* did not affect osteoclast-like cell formation in the periodontitis group. Unexpectedly, osteoclastogenesis was reduced in the non-periodontitis group.

Practical implications: Lack of responsiveness of periodontal ligament fibroblasts from periodontitis patients to *Pg* indicates that the disease environment has modified the phenotype of these cells.