Immunological aspects of the pathophysiology of periodontitis

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The possible role of neutrophils in the induction of osteoclastogenesis
ABSTRACT

The ligand of the receptor activator of NF-kB (RANKL) is a key molecule in the formation of osteoclasts, the key cells that cause the disease-associated alveolar bone resorption in periodontitis. We hypothesized that polymorphonuclear leukocytes (PMNs), found as the most prominent cells of inflamed periodontal tissues, could play an important role in providing signals to trigger osteoclastogenesis and thus activating pathological bone resorption in periodontitis. RANKL expression was investigated on circulatory PMNs (cPMNs) and oral PMNs (oPMNs) taken from both controls and periodontitis patients. On average, 2.3% and 2.4% RANKL expression was detected on the cPMNs and oPMNs from periodontitis patients, which did not differ significantly from healthy controls. Since cPMNs may acquire a more osteoclastogenesis-facilitating phenotype whilst migrating into the inflamed periodontium, we next investigated whether stimulated (with LPS, TNF-α, or IL-6) cPMNs have the capacity to contribute to osteoclastogenesis. Enduring surface expression of RANKL for short-lived cells as cPMNs was achieved by fixing stimulated cPMNs. RANKL expression on stimulated cPMNs, as assessed by flow cytometry and immunohistochemistry, was limited (6.48 ± 0.72%, mean expression ±SEM) after 24 and 48 hours of stimulation with LPS. Likewise, stimulation with TNF-α and IL-6 resulted in limited RANKL expression levels. These limited levels of expression did not induce osteoclastogenesis when cocultured with pre-osteoclasts for 10 days. We report that, under the aforementioned experimental conditions, neither cPMNs nor oPMNs directly induced osteoclastogenesis. Further elucidation of the key cellular players and immune mediators that stimulate alveolar bone resorption in periodontitis will help to unravel its pathogenesis.
INTRODUCTION

Periodontitis is a chronic inflammatory disease of the tooth-supporting tissues (e.g. the periodontium). The chronic inflammatory cell infiltration of the periodontal soft tissues is accompanied by osteoclast-induced alveolar bone resorption, the hallmark of periodontitis progression (1,2). Osteoclasts are derived from monocyte/macrophage precursors and regulate bone resorption. Monocyte differentiation into osteoclasts requires the activation of their RANK receptors that recognize activator NF-kappa B-ligand (RANKL) (3). Additionally, macrophage-colony stimulating factor (M-CSF) is needed to trigger differentiation in osteoclast cultures (4). To differentiate into (pre-)osteoclasts, monocytes likely receive their RANKL differentiation signal from cell-cell interactions (5). Expression of RANKL has been reported on a wide variety of cells of the periodontium, including T cells, B cells (6), and periodontal ligament and gingival fibroblasts (7). Alveolar bone osteocytes also express RANKL, and it has recently been demonstrated that especially osteocyte-expressed RANKL could be crucial in the initiation of periodontitis as demonstrated in a RANKL knock-out mouse model with a targeted disruption of RANKL in osteocytes (8), reviewed by De Vries and Huesa (9). RANKL in humans is expressed in three different forms: the primary secreted soluble form sRANKL, the cell membrane-bound and transmembrane RANKL (mRANKL), and a truncated ectodomain moiety cleaved from the cell-bound form (10).

The host inflammatory response in periodontitis is induced by the constant interaction occurring between host cells and the biofilm present at the roots of the teeth. An aberrant host response creates a shift in the ecosystem where Gram-negative bacteria can thrive, resulting in a dysbiotic microflora, reviewed by Lamont and Hajishengallis (11). LPS is a cell wall component of Gram-negative bacteria and is widely considered to be a potent stimulator of innate host defenses. One of the major pathogens associated with periodontitis is Porphyromonas gingivalis. Even at low colonization levels, provided that the ecosystem is favorable, P. gingivalis can disrupt the homeostasis of the commensal dental biofilm and can enhance a dysbiotic microflora (11). This shift in the microfloral environment can aggravate inflammatory immune responses, including the production of pro-inflammatory cytokines, in a range of host cells such as gingival fibroblasts, gingival epithelial cells, monocytes, macrophages and polymorphonuclear leukocytes (PMNs) (12–15). Several pro-inflammatory cytokines that are elevated in periodontal disease, such as tumor necrosis factor alpha (TNF-α), and interleukin (IL-) 6 have been shown to stimulate osteoclastogenesis (16–19). These pro-inflammatory cytokines can, in turn, promote monocyte differentiation into pre-osteoclasts and eventually trigger the activation of osteoclasts independently of the RANKL pathway (19). Elevated RANKL release or expression is possibly caused by bacterial products such as LPS, which on its own can also enhance RANKL expression (20). LPS as an initial stimulator can evoke inflammatory responses and can subsequently stimulate
or enhance osteoclast formation, leading to both elevated numbers of osteoclasts and increased osteoclastic activity. In periodontitis, this potentially leads to irreversible alveolar bone resorption and, eventually, tooth loss.

Our group described that bacterial priming of the osteoclastogenesis-inducing cells residing in the periodontium, such as the periodontal ligament fibroblasts, alters the potential for osteoclast formation in vitro (14,21). Furthermore, we also found that gingival fibroblasts play a crucial role in osteoclastogenesis when cultured with monocytes. Next to their role in osteoclastogenesis, they also facilitate the survival, retention and selective proliferation of lymphocytes (22). Dutzan et al. confirmed the distinct cellular composition of periodontitis lesions when compared to uninflamed healthy gingiva (23). As such, periodontal lesions show a substantial infiltration of innate immune responders, i.e. PMNs.

PMNs originate in the bone marrow and are found in circulating blood (further referred to as circulatory PMNs [cPMNs]) in numbers between 2.5-7.5 ×10⁹/L. These cell numbers can increase in a chronic inflammatory state such as in periodontitis, morbid obesity, diabetes mellitus and atherosclerotic vascular disease (24–29). Although resting cPMNs have a short lifespan (6-8 hours in circulation), stimulated cPMNs have been shown to have an extended lifespan (several days) and are capable of synthesizing considerable amounts of proteinaceous and lipid immune mediators, which are important in inflammatory processes (30,31). Although high numbers of PMNs have been found at sites of bone erosion (32), their impact on the differentiation of monocytes into pre-osteoclasts and mature osteoclasts remains unclear.

PMNs are also found both in the oral cavity and saliva (further referred to as oral PMNs [oPMNs]). The gingival crevice (sulcus) is identified as the main point of entrance for oPMNs transiting towards the oral cavity. However, their transmigration through all other mucosal tissues has also been found (33). Under healthy conditions, approximately 30,000 oPMNs per minute have been shown to enter the oral cavity through the crevices around the teeth, however, the number of oPMNs entering the oral cavity increases by a factor of 4 in cases of gingival inflammation (i.e. gingivitis) or periodontitis (34). In contrast to cPMNs which exist in the almost-sterile circulatory system, the extracellular environment of oPMNs consists of salivary factors, oral bacteria, shed epithelial cells, and cell debris. Accordingly, oPMNs were shown to have exhausted capacity for efficient chemotaxis which may be the result of migration through the oral tissues into the oral cavity and they produce more ROS and NETs than cPMNs (34–36). To date, RANKL expression on oPMNs has not yet been investigated. Interestingly, the mRANKL expression has been reported on cPMNs and synovial fluid-derived PMNs from rheumatoid arthritis patients (37–39). Moreover, the expression of mRANKL in these cPMNs appears upregulated in the presence of bacterial lipopolysaccharide (LPS). By fixing cPMNs and adding them to live osteoclast precursors, the transmembranic RANKL was shown to induce differentiation of these pre-osteoclasts (37).
PMNs are one of the most prominent cells in periodontitis lesions where they are often activated or in a hyperactive state (40,41). Therefore, PMNs could conceivably play an important role in providing signals to trigger osteoclastogenesis activating pathological bone resorption in periodontitis. Through this study, we attempted to validate the aforementioned hypothesis in two ways. In part A, we investigated whether oPMNs, as a model representing the activated PMNs from periodontitis lesions express RANKL and whether they can be primed and activated in response to the continuous presence of extracellular stimulants (saliva, oral bacteria, shed epithelial cells, and cell debris) that are present in the gingival sulcus and oral cavity. To accomplish this, RANKL expression was investigated in the cPMNs and oPMNs of both healthy controls and periodontitis patients. In part B of this study, we investigated whether cPMNs, after activation by the immunological modulators LPS, IL-6, or TNF-α, have the capacity to contribute to osteoclast formation via RANKL expression as previously published by Chakravarti et al. (37).

MATERIALS AND METHODS

Study design
This study consisted of two parts. Part A of this study, investigating RANKL expression on cPMNs and oPMNs, was carried out at the Department of Periodontology at the Academic Centre for Dentistry Amsterdam (ACTA), Amsterdam, the Netherlands. In part B of this study, the in vitro capacity of cPMNs to induce osteoclastogenesis was investigated at the Department of Medicine, Université of Laval (Québec, Canada) and experiments were performed as previously described (37).

Part A

Study population
Control subjects (n = 13) without periodontitis were recruited among individuals scheduled for regular dental check-ups at the educational practice at ACTA according to the following criteria: (i) a maximum DPSI-score (Dutch Periodontal Screening Index (42)) of 3− (corresponding to a maximum pocket depth of 4-5 mm without recession), (ii) no alveolar bone loss visible on recent (< 1 year ago) bite-wing radiographs; and (iii) at least 25 years of age.

Periodontitis patients (n = 9) in this part of the study were recruited among those who were referred to the Department of Periodontology at ACTA for diagnosis and treatment. Periodontitis was defined based on the criteria for periodontitis as previously agreed upon (43); the presence of proximal attachment loss of at least 3 mm in at least 2 non-adjacent teeth and the presence of proximal attachment loss of at least 5 mm in at least 30% of teeth.
present. Alveolar bone loss was confirmed on recent x-rays (vertical bite-wings or periapical radiographs less than 1 year old). Periodontitis patients had to be at least 36 years of age and had not received periodontal treatment in the year preceding the study.

The following exclusion criteria applied to the whole study population; American Society of Anaesthesiologists (ASA) classification of ≥2, indicating that the person is a normal healthy patient (e.g. a nonsmoking, fit, non-obese [BMI under 30 kg/m²] person with a good exercise tolerance) (44), pregnancy and lactation currently or in the past year, systemic disease, autoimmune disease or immunodeficiency, use of antibiotics or immune-influencing medication in the past year, acute bacterial or viral infections, oral wounds, and current or past chemotherapy.

The study was approved by the Medical Ethical Committee of the Amsterdam University Medical Center, The Netherlands (2012-210#B2012406). Written informed consent and a questionnaire were obtained from all participants and all experiments were conducted according to Dutch law.

cPMN collection and isolation for part A of this study
Isolation of cPMNs was performed as previously described (36). Venous blood (2 x 10 mL) from controls (n = 13) and periodontitis patients (n = 9) was obtained in lithium heparin tubes (Vacuette ® Heparin tubes, Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Blood was diluted 1:1 in 1% PBS citrate (pH 7.4). Subsequently, 25 mL of the diluted blood was carefully layered on top of 15 mL Lymphoprep (Axis-shield Po CAS, Oslo, Norway). After centrifugation (800 RCF, 30 min, RT, no brake), the supernatant above the red cell layer was discarded, after which remaining erythrocytes were lysed in cold lysis buffer (NH₄Cl [1.5 M], NaHCO₃ [100 mM], disodium EDTA [1 mM], all Sigma-Aldrich, Merck, Darmstadt, Germany, 10 x diluted in sterile Milli-Q [MQ] water). Immediately after erythrocyte lysis, the cPMN pellet was washed twice in cold PBS (Gibco, Thermo Fischer Scientific, Paisley, Scotland, UK), and recovered in culture medium (phenol-red free, Roswell Park Memorial Institute [RPMI] 1640, Gibco). All samples were handled on the same day without delay.

oPMN collection and isolation
oPMNs were isolated as previously described (45,46). Controls (n = 13) and periodontitis patients (n = 9) rinsed the oral cavity 4 times with 10 mL 0.9% NaCl solution (Versylene®, Fresenius Kabi, Sèvres, France) for 30 seconds with 4 minute intermission periods. Per subject, the collected samples were pooled, centrifuged (500 RCF, 10min, 4°C), and finally, the pellet was recovered in 40 mL PBS. The filtration protocol consisted of 4 filtrations with 70.0, 40.0 (Greiner Bio-one), 31.5, and 10.5 micrometers (µm) nylon meshes (Vlint, Nedfilter, Almere, The Netherlands) to exclude epithelial cells and cell debris. The filtrated fraction was centrifuged (500 RCF, 10 min, 4°C), washed in cold PBS and suspended in phenol-red free culture medium. All samples were handled on the same day without delay.
Flow cytometry analysis

The expression of RANKL on cPMNs and oPMNs from controls and periodontitis patients was analyzed using flow cytometry. Directly after isolation, PMNs were stained with either the mouse anti-human surface RANKL (PE-conjugated, clone 12A380, R&D Systems, Minneapolis, MN, USA) or the isotype control IgG1 (PE-conjugated, BD Biosciences, Franklin Lakes, NJ, USA). All cells were stained with anti-human CD16 (APC conjugated, clone 3G8, BD Biosciences) and anti-human CD66b (FITC conjugated, clone G10F5, BD Biosciences) as PMN marker to assess PMN purity (46). Flow cytometric analysis was performed on the Accuri C6 flow cytometer (BD Biosciences), where at least 1,000 cells were analyzed. The gating strategy employed is shown in Supplementary Figure 1. The PMNs were gated according to their relative size (Forward Scatter, FSC) and granularity (Side Scatter, SSC) and characteristic CD16 and CD66b expression (46). In the live gating (encircled in red, Supplementary Figure 1), RANKL expression was quantified. This expression was corrected for the non-specific binding of isotype control antibodies (IgG1-PE).

Part B

cPMN collection and isolation

Volunteers were recruited among subjects attending the blood donation facility at the Centre de Recherche du Centre Hospitalier de l’Université Laval (Québec, Canada). The study was approved by the institutional review board of the Université Laval, Québec, Canada. Volunteers signed a written informed consent in accordance with the Declaration of Helsinki.

cPMNs were isolated from systemically healthy blood donors (n = 24 in total), all non-smokers with an average age of 43 ± 10 years. Isolation of cPMNs was performed as previously described (37). Venous blood (500 mL) from was collected in 10 mL citrate-coated tubes (Thermo Fischer Scientific, Eugene, Oregon, USA). Per subject, blood was distributed in 50 mL tubes and after centrifugation (300 RCF, 10 min, at room temperature (RT), acceleration 7, deceleration 7), platelet-rich plasma was removed. After 30 minutes of red blood cell sedimentation with dextran (10 mL 2% dextran from Leuconostoc spp., 1M HEPES, 0.16M CaCl₂, 10% Hanks Balanced Salt solution [HBSS, 10x, without phenol red, sodium bicarbonate or calcium and magnesium, Multicell by Wisent Inc., St. Bruno, Québec, Canada] in sterile MQ, pH 7.4) the supernatant was transferred to a new tube and layered on Ficoll-Paque (Multi-cell, Wisent Inc.). After density gradient centrifugation (800 RCF, 30 min, no brake, at room temperature), the supernatant containing serum and Ficoll was removed, PBMCs were transferred to another tube for further isolation (see below), and contaminating erythrocytes were lysed by hypotonic lysis using sterile MQ water. After maximally 20 seconds of lysis in MQ, cells were recovered in 40 mL 10x HBSS. Finally, cPMNs...
were washed and suspended in culture medium (RPMI 1640, + 10% fetal bovine serum, 1% penicillin-streptomycin, all Wisent Inc.). Cell counts were routinely assessed by trypan blue staining using a hemocytometer.

**Isolation of monocytes from PBMCs**

In parallel to cPMN isolation, PBMCs were isolated from the same blood donor, on the same day and without delay. PBMCs were collected after Ficoll-Paque density gradient centrifugation. The percentage of monocytes present (approximately 10 - 30%) was determined by flow cytometry (BD FACsverse™) after anti-human CD14 staining (clone M5E2, eBioscience inc. by Thermo Fischer). PBMCs were seeded in plastic culture flasks at a density of 1 x 10^6 monocytes/mL (polystyrene, non-pyrogenic cell culture flasks, Falcon®, Corning, New York, NY, USA). This allowed the attachment of monocytes (37°C, 5% CO₂). Since peripheral blood lymphocytes (PBLs) are non-adhering cells, PBLs were removed by aspiration of the medium after 2 hours. Finally, monocytes were collected after detachment with accutase (Wisent Inc.) and counted by trypan blue using a hemocytometer.

**cPMN stimulation**

To measure RANKL expression, the cPMNs were stimulated with LPS, TNF-α, or IL-6. cPMNs (5 x 10^6 cells/mL) were incubated in 12-well plates with a range of concentrations of LPS (100, 500, and 1000 ng/mL, *Escherichia coli*, 0111: B4; Sigma-Aldrich, Oakville, ON, Canada), IL-6 (10, 100, 500, or 1000 ng/mL, R&D systems) or TNF-α (recombinant human, *E. coli*-derived, 10, 50, or 100 ng/mL, R&D systems, Oakville, ON, Canada). Prior to the addition, LPS was sonicated for 5 minutes and kept at room temperature until use. Granulocyte-macrophage colony-stimulating factor (GM-CSF, 1 nM, Peprotech, Rocky Hill, NJ, USA) was added to all conditions in order to increase cPMN viability.

**cPMN enrichment**

After 24 and 48 hours of stimulation, viable cPMNs were enriched by discontinuous Percoll (1.1309 gram/mL, GE Healthcare, Biosciences, Mississauga, ON, Canada) density gradient centrifugation as previously described (47). Briefly, equal gradients of 31%, 42% and 51% Percoll in 10x RPMI medium (RPMI-1640 containing L-glutamine, and phenol red, supplemented with 1.19 mM sodium bicarbonate, 1.2 mM HEPES, and 1% BSA, all Wisent Inc.) were layered and cell suspensions were carefully layered on top of these gradients. All gradient solutions were kept at 4⁰C. After centrifugation (610 RCF, 28 minutes, 4°C), the pellet containing viable cPMNs was collected. Finally, cPMNs were washed in PBS, recovered in culture medium and counted with a hemocytometer.
Fixation of cPMNs
After stimulation, cPMNs were fixated for 15 minutes at room temperature with 2% paraformaldehyde (0.67 M PFA in PBS, Wisent Inc.) and washed three times in PBS (37).

Immunohistochemical RANKL staining
CPMNs, either stimulated with LPS (100 ng/mL in PBS, 30 minutes 37°C) or unstimulated, were centrifuged onto glass slides using a Cytospin-2 centrifuge (Shandon Southern Products, Astmoor, U.K.). Image-iT® FX Signal Enhancer (Invitrogen, ThermoFisher Scientific inc.) was used to block nonspecific binding sites. After washing with PBS, cells were incubated with the primary anti-human antibody RANKL (Human TRANCE/TNFSF11/ RANKL Monoclonal Mouse IgG2a, Clone 70525, R&D systems), or mouse IgG2a control (clone MPC11, R&D Systems) for 1 hour at room temperature. This was followed by a secondary antibody staining (Alexa Fluor 488 goat anti-human IgG, Invitrogen). After 1 hour of incubation in the dark, slides were washed twice with PBS. Nuclei of the cells were stained with propidium iodide (PI) containing a fluorescent vectashield (Vectashield with PI, H-1300, Vector Laboratories Inc. CA, Burlingame, CA, USA). The analysis was performed using confocal microscopy with a 20 x objective and a 4 x zoom (Leica Microsystems TCS, SP2, Wetzlar, Germany) for multidimensional imaging of the cells. Leica confocal analysis software program (version 2.6, Leica Microsystems) was used for imaging.

Flow cytometric analysis
RANKL expression of cPMNs was analyzed using flow cytometry as described before (37), comparing the expression of unstimulated and stimulated cPMNs. After blocking (Human Trustain FcX™, Biolegend, San Diego, CA, USA), cPMNs were stained with either the mouse anti-human surface RANKL (PE-conjugated, clone 12A380, Santa Cruz Biotechnology, Mississauga, ON, Canada), or the isotype control IgG1 (PE-conjugated, BD Biosciences, Mississauga, ON, Canada). All cells were stained with anti-human CD66b (FITC conjugated, clone G10F5, eBioscience inc. by Thermo Fischer) as cPMN marker to assess PMN purity (46). Flow cytometric analysis was performed using a flow cytometer FACSverse™ (BD Biosciences) where at least 5,000 cells were analyzed. The gating strategy employed for flow cytometric analysis is shown in Supplemental Figure 1. The cells were gated according to their relative size (Forward Scatter, FSC) and granularity (Side Scatter, SSC). RANKL expression was analyzed for live, CD66b positive cells and was corrected for isotype IgG2B expression. Flow cytometry data were analyzed using FACSuite software (version 6.1.3, BD Biosciences).

Osteoclastogenesis
Osteoclastogenesis assays with activated, fixed cPMNs in coculture with pre-osteoclasts was performed as previously described (37). Briefly, monocytes (250,000 per well) were seeded in duplicate and allowed to attach for 2 days in 48-well plates in triplicate. Monocytes were cultured in osteoclastogenic RPMI medium containing 25 ng/mL M-CSF (Human M-CSF
recombinant protein, eBioscience by Invitrogen) and 80 ng/mL RANKL (Human sRANKL ligand, Peprotech). After 2 days, the cytokine or LPS activated and fixed cPMNs (5 x 10^5 per well) were cocultured with monocytes in the presence of 25 ng/mL M-CSF. Control conditions contained 80 ng/mL RANKL. Cultures were refreshed every 3-4 days and maintained for 10 days (37°C, 5% CO₂).

**Osteoclast staining using the fluorescence ELF-97 TRACP stain**

After 10 days of coculturing, osteoclast formation was studied by tartrate-resistant acid phosphatase (TRACP) staining according to the manufacturer’s instructions (Enzyme-Labeled Fluorescence, ELF-97 Endogenous phosphatase detection kit, Thermo Fischer) (48). After fixation, cells were incubated for 15 minutes at 37⁰C with ELF-97 phosphatase substrate working solution (7.4 mM tartrate, 1.1 mM sodium nitrite, 110 mM acetate solution and 150 μM ELF-97, all from Thermo Fischer). After one wash with PBS, cells were counterstained with the permeable nuclear dye 4’,6-diamidino-2-fenylindool (DAPI, 300 nM, Thermo Fisher) and washed again. Finally, micrographs were taken with a fluorescence microscope (excitation 360-370, emission 420, Leica DFC320; Leica Microsystems). Cells were considered to be osteoclasts when they were TRACP-positive and contained at least three nuclei.

**Statistics**

All study population data analyses of part A of this study were performed using SPSS software (Version 25, IBM, Armonk, NY, USA). The study population data were checked for normality using the Kolmogorov-Smirnov test, and some data sets were found to be not normally distributed. Therefore, statistical analysis was further performed using non-parametric testing. Possible differences were tested between groups (controls vs periodontitis patients) for continuous variables using the Mann-Whitney U test and Chi-square tests were performed for categorical variables. All flow cytometry data were analyzed using GraphPad Prism software (version 6.07, La Jolla, CA, USA). Data sets were compared with paired one-way ANOVA and presented as means ± standard error of the mean (SEM). Differences were considered significant at p < 0.05.

**RESULTS**

**RANKL expression on the cPMNs and oPMNs from healthy controls and periodontitis patients**

In part A of our study, we investigated RANKL expression by cPMNs and oPMNs from healthy controls and periodontitis patients. Detailed information about the study population is provided in Supplementary Table 1. We measured RANKL expression on cPMNs from
healthy controls and found 3.9 ± 1.5% expression (mean ± SEM, Fig. 1, white bar). RANKL expression by cPMNs from periodontitis patients (grey bar) was 2.3 ± 0.8%, which did not significantly differ from controls.

Next, we studied the role of oPMNs that have migrated through the periodontium and oral mucosal tissues. It is known that oPMNs have a hyperactive phenotype due to their transmigration through the oral mucosal tissues (35,36). We, therefore, hypothesized that oPMNs could express RANKL as they may have been activated by bacteria in the sulcus and oral cavity (49). We measured RANKL expression on oPMNs from controls and periodontitis patients and found that on average, 2.4 ± 1.3% and 2.4 ± 1.0% RANKL was measured on oPMNs from controls and periodontitis patients respectively (Fig. 1). No statistically significant difference in RANKL expression was found on oPMNs between these groups.

**Figure 1: RANKL expression on (unstimulated) PMNs from controls and periodontitis patients.** RANKL expression was measured on circulatory (cPMNs) and oral PMNs (oPMNs) from healthy controls (white bars) and periodontitis patients (grey bars). Bars represent the percentage of RANKL expression corrected for IgG isotype control expression on CD66b positive PMNs. Whiskers demonstrate standard errors of means. The employed gating strategy is shown in Supplementary Figure 1. No significant differences were found between conditions. n =13 controls, n = 9 periodontitis patients.

**LPS induces limited RANKL expression on cPMNs**

Based on previous research (37) and to further substantiate the findings from part A of our study, the experiments conducted in part B of this study were designed to further investigate the possible role of cPMNs in the induction of osteoclastogenesis. We investigated whether LPS-stimulated cPMNs expressed RANKL and thus could stimulate osteoclastogenesis. Immunohistochemical staining was performed to visualize RANKL expression on unstimulated and stimulated cPMNs. After stimulation with LPS (100 ng/mL, 24h), cPMNs were stained for RANKL. Nuclear staining with PI was performed to locate cPMNs. Accordingly, typical PMN lobule nuclei are shown in red (Fig. 2). Unstimulated cPMNs did not show any RANKL expression (Fig. 2A, green signal absent) and RANKL expression was absent for the IgG2b isotype control staining of LPS-stimulated cPMNs (Fig.
1B, green signal absent). As shown in the micrographs of Figure 2C, LPS stimulation induced low levels of RANKL expression of the observed cPMNs (green signal, indicated by arrows, Fig. 2C). Since RANKL expression was not homogeneously expressed on stimulated cPMNs, we next quantified RANKL expression using flow cytometry.

**Figure 2**: Immunohistochemical visualization of RANKL expression on LPS-stimulated cPMNs. (A) Unstimulated and (B, C) LPS-stimulated cPMNs were stained with the nuclear stain propidium iodide (PI, visualized in red) and RANKL (visualized in green) (A). Unstimulated PMNs do not express RANKL. (B) Isotype staining of stimulated cPMNs did not show any green signal. (C) Minimal RANKL expression is observed on stimulated cPMNs (indicated with arrows in Figure C). Scale bars represent 37.5 µm. Here, representative micrographs of 3 independent experiments are shown. PI is visualized as red fluorescence and RANKL is visualized as green fluorescence.

cPMNs express limited levels of RANKL after LPS stimulation

After 24 and 48 hours of stimulation with different concentrations of LPS, the percentages of cPMNs expressing RANKL were measured using flow cytometry. Percentages of RANKL expression by unstimulated and stimulated cPMNs after 24 and 48 hours are shown in Figure 3. After stimulation, no significant increase in the percentage of RANKL expression was found for cPMNs stimulated with 100, 500, or 1000 ng/mL of LPS (Fig. 3). This was the case for both 24 and 48-hour stimulation.

LPS-stimulated cPMNs do not stimulate osteoclastogenesis

Despite the low levels of RANKL expression after LPS stimulation, it remains possible that these LPS activated and fixated cPMNs could stimulate osteoclastogenesis. Therefore, we studied osteoclastogenesis directly by coculturing (stimulated) fixated cPMNs with monocytes as pre-osteoclasts. Control conditions (monocytes cultured with M-CSF and RANKL) showed multinucleated and TRACP-positive cells (indicated with arrows, Fig. 4A). Next, unstimulated cPMNs were cultured with M-CSF treated monocytes (Fig. 4B), showing TRACP positive mononuclear cells. Neither unstimulated (Fig. 4B) nor LPS-stimulated cPMNs (Fig. 4C) lead to the formation of osteoclasts without the addition of RANKL. Noteworthy,
the condition containing LPS-stimulated cPMNs (Fig. 4C) shows elongation of monocytes with relatively big nuclei in comparison to the control condition with unstimulated cPMNs (Fig. 4B).

![LPS expression graph]

**Figure 3: RANKL expression on LPS-stimulated cPMNs.** Percentages of RANKL expression by cPMNs after 24 (white bars) and 48 hours (grey bars). Different stimulatory conditions are shown on x-axes. Percentages (mean ± standard error of means, n = 3) of RANKL expression on live, CD66b positive cells are shown on y-axes. Overall, no significant differences (paired one-way ANOVA) were observed between unstimulated and stimulated conditions. The employed gating strategy is shown in Supplementary Figure 1.

![Figure 4: LPS-activated cPMNs do not stimulate osteoclastogenesis in cocultures with osteoclast precursors.](image)

**Figure 4: LPS-activated cPMNs do not stimulate osteoclastogenesis in cocultures with osteoclast precursors.** Cells were stained for tartrate-resistant acid phosphatase (TRACP, green) and counterstained with the permeable nuclei dye 4',6-diamidino-2-fenylindool (DAPI, blue). (A) Pre-osteoclasts (monocytes) were cultured for 10 days with M-CSF and RANKL. Formed osteoclasts are indicated with arrows. (B) Pre-osteoclasts (monocytes) were cultured for 10 days with M-CSF and unstimulated cPMNs. No osteoclasts were formed in this condition. (C) Pre-osteoclasts (monocytes) were cocultured with LPS-activated (100 ng/mL, 48 hours) cPMNs. In this condition, no osteoclasts were observed. Representative micrographs of three independent experiments are shown. Scale bars represent 100 µm.

**Minimal RANKL expression by cPMNs after stimulation with IL-6 and TNF-α**

Since LPS induced RANKL expression in cPMNs to a very limited extent, we tested whether pro-inflammatory cytokines IL-6 and TNF-α, known to induce RANKL expression (16,18), could contribute to an increased RANKL expression of cPMNs (38,50). cPMNs were tested
for RANKL expression after 24 and 48 hours of stimulation with different concentrations of IL-6 and TNF-α. Percentages of RANKL expression by cPMNs after IL-6 stimulation are shown in Figure 5A. On average, between 0.5 and 2.2% (min-max) cPMNs expressed RANKL after 24 and 48 hours of stimulation with IL-6. This was not significantly different from unstimulated conditions, irrespective of the different concentrations IL-6 used. Percentages of RANKL expression by cPMNs after TNF-α stimulation are shown in Figure 5B. Stimulation of cPMNs with 50 ng/ml TNF-α induced significantly higher (p = 0.0364, paired one-way ANOVA) RANKL expression after 24 hours (Fig. 5B). However, other conditions and concentrations did not differ statistically from unstimulated conditions.

**Figure 5: RANKL expression on IL-6 or TNF-α-stimulated cPMNs.** Percentages of RANKL expression by cPMNs stimulated with (A) IL-6 or (B) TNF-α after 24 (white bars), and 48 (grey bars) are shown on y-axes. Concentrations of stimulants are shown on x-axes. Percentages (mean + standard error of means, n = 3) of RANKL expression on live, CD66b positive cells are shown on y-axes. Overall, no significant differences (paired one-way ANOVA) were observed between unstimulated and IL-6 stimulated conditions. The significant difference (*p < 0.05) was compared (paired one-way ANOVA) to the unstimulated condition. The employed gating strategy is shown in Supplementary Figure 1.

**IL-6 and TNF-α stimulated cPMNs do not stimulate osteoclastogenesis**

We hypothesized that cytokine-stimulated cPMNs would induce osteoclastogenesis. In order to test our hypothesis, TNF-α or IL-6 stimulated cPMNs were cocultured with monocytes as pre-osteoclasts in order to investigate osteoclastogenesis. Cocultures of stimulated cPMNs and monocytes after 10 days are shown in Figure 6. Control conditions contained osteoclasts cultured for 10 days with M-CSF and RANKL (Fig. 6A, indicated with arrows). All other conditions were cultured with M-CSF and without RANKL. Hypothetically, osteoclastogenesis stimuli could originate from stimulated activated cPMNs. Thus, monocytes were cocultured with unstimulated cPMNs (Fig. 6B), IL-6- stimulated cPMNs (100
ng/mL, Fig. 6C), or TNF-α stimulated cPMNs (50 ng/mL for 24 hours [Fig. 6D], 50 ng/mL for 48 hours [Fig. 6E]), in addition to M-CSF which was present in all conditions. No osteoclasts were observed in conditions with either stimulated (Fig. 6C-E) or unstimulated (Fig. 6B) cPMNs. TNF-α stimulated cPMNs gave rise to more elongated monocytes when cultured together with M-CSF (Fig. 6D and E). In conclusion, our results demonstrate that stimulated cPMNs express RANKL in a limited fashion, however, under the current conditions, no osteoclastogenesis was induced.

Figure 6: Activated cPMNs do not stimulate osteoclastogenesis in cocultures with osteoclast precursors. Nuclei are stained with DAPI (visualized in blue) and TRACP expression is shown in green. Pre-osteoclasts (monocytes) were cultured for 10 days with RANKL and M-CSF. (A) TRACP-positive, multinucleated cells were formed (depicted by arrows). (B) Monocytes were cultured with unstimulated cPMNs. (C) Monocytes were cultured with stimulated cPMNs (IL-6, 100 ng/mL for 48 hours) and did not differentiate into TRACP-positive multinucleated cells. (D) Monocytes were cocultured with TNF-α stimulated cPMNs (50 ng/mL for 24 hours) and did not differentiate into osteoclasts. (E) Monocytes were cocultured with TNF-α stimulated cPMNs (50 ng/mL, 48 hours) and did not differentiate into osteoclasts. Representative micrographs of three independent experiments are shown. Scale bars represent 200 µm.

DISCUSSION

Osteoclast-mediated resorption of the tooth-adjacent alveolar bone, the hallmark of periodontitis progression, is accompanied by chronic inflammatory cell infiltration of the periodontal soft tissues. PMNs are the predominant innate immune responders that infiltrate inflammatory lesions and they have been shown to play key roles in chronic inflammatory conditions and in the regulation of immune responses (51). Part A of this study aimed to investigate the cellular expression of RANKL by cPMNs and oPMNs taken from both controls and periodontitis patients. Of note, previous studies have shown that periodontal bacteria
in intimate contact with the ulcerated epithelium can infiltrate the bloodstream (52,53). We, therefore, hypothesized that cPMNs from periodontitis patients express more RANKL than those sourced from controls. However, we found that RANKL expression was not higher in cPMNs from periodontitis patients with chronically inflamed gingival tissues. These data show that cPMNs from periodontitis patients are not per se primed to contribute to osteoclastogenesis via the RANKL pathway.

In cases of chronic inflammation of the periodontium, such as in periodontitis, an increased influx of oPMNs extravasate to the oral cavity (35). The oral cavity, a microorganism-rich ecosystem, harbors over 700 different species of colonizing bacteria, which possibly prime and activate oPMNs (54). oPMNs were shown to have an hyperactive phenotype by their hyperactive ROS and NET production (36,55). Evidently, oPMNs are more mature cells than cPMNs with exhausted chemotactic capacities due to their transendothelial extravasation, oral transepithelial migration, and exposure to the oral biofilm (36,40). Moreover, LPS has been shown to induce RANKL expression (20). As such, we hypothesized that oPMNs could express more RANKL than cPMNs which originate from the nearly sterile circulatory system (54). Notwithstanding this, the aforementioned differences did not impact the minimal levels of RANKL expression in both the cPMNs and oPMNs from healthy controls. In periodontitis, a pathogenic imbalance of the oral ecosystem occurs (56). This, coupled with a persistent immune activation maintained by their inability to eliminate pathogens, causes an aberrant inflammatory response triggering the secretion of important molecular mediators of inflammation, including inflammatory cytokines (such as TNF-α and IL-6). These inflammatory cytokines, in turn, can activate oPMNs to express more RANKL. We, therefore, hypothesized that levels of RANKL expression would be higher in oPMNs from periodontitis patients. However, no significant difference was found between the RANKL expression levels of oPMNs and cPMNs originating from either the patient or control groups. Collectively, our results demonstrate that neither contact with bacteria (in oPMN samples) nor chronic gingival inflammation (in periodontitis patients) induces mRANKL expression on cPMNs or oPMNs.

Part B of this study was performed to further investigate and refine the findings of Chakravarti et al. (37). Chakravarti et al. demonstrated that LPS-stimulated cPMNs have the potential to express RANKL and thereby induce osteoclastogenesis (37). Despite performing exactly the same protocols (using corresponding chemicals and antibodies), in the same laboratory with the same equipment as described by Chakravarti et al., we were not able to reproduce these findings. They reported that surface RANKL was expressed by less than 5% of unstimulated cPMNs, while on average 23 ± 7% of the LPS-activated cPMNs expressed RANKL. In the current study, we demonstrate that cPMNs expressed lower levels (6.48 ± 0.72%, mean expression ± SEM) of RANKL after 24 or 48 hours of stimulation with LPS. Flow cytometry data is commonly reported as the percentage of cells expressing a certain molecule of interest. In the current case, it is of importance to determine the intensity
of this expression by reporting the geometric mean fluorescence intensity (gMFI) of this population given that the intensity of expression can differ between the same number of cells in different populations (57). However, in our studies, the number of events (i.e. PMNs) expressing RANKL, and thus the size of our population of interest, was too small to determine the gMFI. Therefore, the quantification of RANKL expression was solely reported as a percentage of the whole PMN population.

Although RANKL is expressed in three different forms in humans, we solely investigated mRANKL expression by PMNs since it was previously shown that supernatants of cultured activated cPMNs did not contain any sRANKL (detection limit: 15 pg/mL) and no resorption was observed after coculturing pre-osteoclasts with conditioned medium of activated PMNs which demonstrates that (stimulated) cPMNs are incapable of producing sRANKL (37). Nevertheless, it could still be possible that PMNs secrete sRANKL via extracellular vesicles which are membrane-derived vesicles produced in response to various inflammatory stimuli during inflammatory processes (58). Since it is known that cPMNs do not release sRANKL, effective cell surface interactions require direct cell-cell contact between pre-osteoclasts and cPMNs. cPMNs have been shown to interact with monocytes (59) and they adhere to osteoclasts after stimulation with LPS (37). In our study, we did not observe adherence of cPMNs to monocytes, therefore, a direct cell-cell contact, needed for PMN-mRANKL presentation to monocyte-RANK, probably hardly occurred. This, as well as the low levels of RANKL, could be one of the conceivable reasons why no osteoclasts were detected in our coculture experiments.

In our experimental setup, cPMNs were incapable of inducing osteoclastogenesis when fixed and cocultured with pre-osteoclasts for 10 days. This correlated with a limited RANKL expression, suggesting that the stimuli LPS, TNF-α, and IL-6 over the range used, were insufficient to activate osteoclastogenesis, either through RANKL or other osteoclastogenesis pathways. Also, it could be that the fixed RANKL was incapable of interacting with the RANK present on osteoclast precursor cells. RANKL dependency was assessed by others by adding OPG to these assays, which was shown to inhibit osteoclast formation (38,50). However, the main finding of part B of our study was that cPMNs stimulated with LPS, IL-6, or TNF-α, did not induce osteoclastogenesis. Since no osteoclasts were formed, the usefulness to perform such experiments with OPG are lacking.

PMNs, T, and B cells populate inflamed periodontal lesions (23). However, the role of PMNs in osteoclastogenesis remains unclear. Riegel and colleagues reported that cPMNs contain preformed RANK, stored in secretory vesicles and specific granules, which can be translocated to the cells’ membrane after 24 hours of stimulation with LPS or TNF-α (60). These RANK positive PMNs, in turn, can be activated via RANKL, in a likely autocrine manner as others have demonstrated that cPMNs express RANKL (37). However, Riegel et al. investigated the stimulation of cPMNs in whole blood cell cultures. Thus, in the latter study,
cPMNs were stimulated in the presence of other immune cells such as monocytes, B, and T cells, which could potentially influence the induction of RANK. To support this affirmation, similar results have been shown in other non-pure culture systems such as monocytes in the presence of tooth-associated fibroblasts, in which case the presence of other leukocytes induced the production of (pro-) inflammatory cytokines (5,22).

PMNs are short-living cells with an estimated half-life of 6-8 hours, which remain in the circulatory system for a few hours before they extravasate into surrounding tissues (61). Poubelle and colleagues demonstrated that cPMNs incubated for 3 days with medium containing TNF-α, expressed RANKL and maintained their viability (38). Despite the use of GM-CSF, a cytokine which improves PMN viability (62), the majority of cPMNs went into apoptosis after 24 to 48 hours of incubation in the present study. To overcome this problem, discontinuous Percoll gradient centrifugation was performed in our experiments to enrich viable cPMNs prior to fixation and culturing with pre-osteoclasts (47). Despite the selection of viable cPMNs before fixation, we were not able to find an effect on osteoclast formation.

**CONCLUSIONS**

In conclusion, we report that, in contrast to the study of Chakravarti et al. (37), stimulated cPMNs did not directly stimulate osteoclastogenesis. Furthermore, RANKL expression was not significantly higher on cPMNs and oPMNs originating from periodontitis patients than from controls. Based on our current results, it remains unclear whether PMNs play a role in providing signals to trigger monocytes into the formation of osteoclasts and thus directly activate pathological bone resorption such as present in periodontitis.

**ACKNOWLEDGMENTS**

The authors are grateful to Koen Hermes for his technical assistance during part A of this study. They also thank Isabelle Allaeyes for technical assistance during the initial experimental phases of part B of this study.
REFERENCES


### SUPPLEMENTARY DATA

**Supplementary Table 1:** Characteristics of the study population for part A of this study.

<table>
<thead>
<tr>
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<th>Controls (n = 13)</th>
<th>Periodontitis (n = 9)</th>
<th>p-value</th>
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<tr>
<td>Age (years)</td>
<td>45 ± 12</td>
<td>49 ± 13</td>
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<tr>
<td>Gender (male/female)</td>
<td>6/7</td>
<td>5/4</td>
<td>0.665</td>
</tr>
<tr>
<td>Ethnicity (European/non-European)</td>
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<td>6/3</td>
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<td>Smoking (10+ per month/10- per month/not last year)</td>
<td>1/2/10</td>
<td>6/0/3</td>
<td>0.079</td>
</tr>
<tr>
<td>Medication (currently using/not currently using)</td>
<td>10/3</td>
<td>2/7</td>
<td>0.027*</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>23.3 ± 2.1</td>
<td>26.9 ± 3.4</td>
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</tr>
<tr>
<td>Number of teeth</td>
<td>28 ± 3</td>
<td>25 ± 4</td>
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<tr>
<td>#teeth with &gt; 50% bone loss</td>
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<td>Sites with plaque (%)</td>
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<tr>
<td>Sites with bleeding on probing (%)</td>
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<td>Pockets ≥5 mm (%)</td>
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</table>

Data are means ± standard deviation or number of subjects. P-values calculated with the Mann-Whitney U test or Chi-square test. * Statistically significant different (p < 0.05).
Supplementary Figure 1: Gating strategy for flow cytometry experiments. The antibody combination for flow cytometric experiments was anti-RANKL-PE/CD66b-FITC. Acquisition and analysis were performed on a BD FACSverse flow cytometer using associated FACSuite software. The gating tree was set as follows: (A) forward scatter/sideward scatter representing the distribution of cells in the light scatter based on size and intracellular composition, respectively, where the live population is encircled in red. In the [live] population, (B,C) RANKL, (D,E) CD66b+ cells were identified. (A) The RANKL+ gating (green) was set in unstimulated cPMN samples. (D) The CD66b+ gating (blue) was set in unstained cPMN samples. Here, representative plots of unstimulated (B) and stimulated (C, D, E) cPMNs are shown.