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Effects of smoking on the *ex vivo* cytokine production in periodontitis

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Background and Objective: Smoking is associated with increased severity of periodontitis. The underlying mechanisms of this phenomenon are not well understood. The purpose of the present study was to compare the monocyte-derived T cell directing (Th1/Th2) response and pro-inflammatory cytokine production in *ex vivo* whole blood cell cultures (WBCC) of smoking and non-smoking chronic periodontitis patients.

Material and Methods: Venous blood was collected from 29 periodontitis patients (18 non-smokers and 11 smokers) receiving supportive periodontal treatment, and diluted 10-fold for WBCC. The WBCC were stimulated for 18 h with *Neisseria meningitidis* lipo-oligosaccharide (LOS) or *Porphyromonas gingivalis* sonic extract (Pg-SE). The production of the T cell directing cytokines interleukin (IL)-12 p40 and IL-10, as well as the pro-inflammatory cytokines IL-1β, IL-6 and IL-8, was measured in the culture supernatants.

Results: After LOS stimulation of WBCC, smokers showed a lower IL-12 p40/IL-10 ratio than non-smokers ($P < 0.05$). Interleukin-1β production was significantly lower in smokers compared with non-smokers after stimulation with either LOS or Pg-SE ($P < 0.05$). Interleukin-6 and IL-8 production was similar in WBCC from both smokers and non-smokers, for both LOS and Pg-SE.

Conclusion: A more pronounced Th2 response in smoking periodontitis patients may be related to increased severity of the disease.

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Periodontitis is a chronic, multifactorial, infectious disease of the supporting tissue of the teeth. Periodontitis patients suffer from gradual loss of tooth attachment in the jaw bone, leading to periodontal pockets, receding gums, loose teeth and eventually tooth exfoliation (1). The onset and progression of periodontitis is due to an imbalance of the interaction between bacterial pathogens and host immunity. Host immunity is greatly influenced by both genetic susceptibility and environmental risk factors (2). Although several specific periodontopathogens have been implicated in the disease, two gram-negative bacteria, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, have the strongest association (3). Cigarette smoking is considered to be one of the most important environmental risk factors in periodontitis (4), since more clinical attachment loss and bone loss have been observed in smoking than in non-smoking patients (5,6). Moreover, smoking may be responsible for a less favourable outcome after periodontal treatment and may more frequently cause disease progression despite a strict periodontal maintenance care programme (7).

Monocytes play a crucial role in orchestrating the host immune system. When triggered by whole bacteria, as well as bacterial components, monocytes produce cytokines (monokines) which direct both innate and adaptive immunity (8). Monokines, such as the pro-inflammatory interleukin (IL)-1β, IL-6, IL-8 and IL-12 and the anti-inflammatory IL-10, have been shown

to be part of the inflammatory response in periodontitis and most probably may determine the host susceptibility and thereby variation in periodontal destruction (8–11).

Cytokines, derived mainly from dendritic cells, monocytes and macrophages, play a pivotal role in directing lymphocytic differentiation of non-committed precursors, CD4+ T cells, into either T-helper (Th)1 or Th2 cells (12). In human infections, an imbalance of Th1 and Th2 cytokine production may be related to disease progression (12). The role of T-helper cells in amplifying immune responsiveness is well established. In periodontitis, the nature of the lymphocytic infiltrate seems to be crucial in disease progression (13). A Th1 cytokine profile is the major mediator in the early/stable lesion, while the dominance of B-cells/plasma cells in the advanced/progressive lesion would suggest a role for Th2 cells. Therefore, it is likely that in those later stages, changes in cytokine profiles that modulate the Th1/Th2 balance may affect the susceptibility to or the course of the periodontal infection (10). Previous studies have shown that periodontitis patients display a monocytic cytokine profile which may favour a Th2 immune response. Indeed, periodontitis is currently regarded as a Th2-type disease; therefore, the Th2-monocytic promoting phenotype may be an important risk factor (14,15).

Differences in monocytic cytokine production between smoking and non-smoking periodontitis patients have been scarcely investigated. However, clinical data demonstrate that, compared with non-smokers, patients who smoke show a more severe disease and relapse during supportive periodontal treatment (SPT). In other inflammatory diseases, it has been shown that tobacco smoke may exacerbate disease progression through 'priming' of immune cells towards a Th2 phenotype (16,17). In several inflammatory conditions, including periodontitis, *ex vivo* stimulation assays of peripheral blood cells with lipopolysaccharide (LPS) stimulant have been used as a measure of the host immune capacity (18–21). Monocyte responsiveness, both in isolated monocyte cell cultures and in whole blood cell cultures (WBCC), is regarded as a reliable measure for the *in vivo* situation (22).

The aim of the present study was to compare the *ex vivo* production of IL-1β, IL-6, IL-8, IL-10 and IL-12 p40 in WBCC of smoking and non-smoking periodontitis patients positive for *Porphyromonas gingivalis* (*P. gingivalis*), after stimulation with lipo-oligosaccharide from *Neisseria meningitidis* (LOS) and a sonic extract of *P. gingivalis* (Pg-SE).

Material and methods

Patients

The study population consisted of patients referred to our clinic [Department of Periodontology at the Academic Center for Dentistry Amsterdam (ACTA)] for the treatment of periodontal disease. Patients were selected from a pool of 900 consecutive patients that were sampled for bacteriological investigation at the intake before periodontal treatment. The selection criteria included: (1) Western European Caucasian descent; (2) diagnosis of chronic adult periodontitis at intake; (3) receiving SPT after non-surgical and surgical treatment had been completed; (4) age between 40 and 60 years; (5) presence of ≥20 permanent teeth; (6) periodontal bone loss of ≥ one-third of the total root length of ≥ two teeth as visible on peri-apical radiographs; and (7) subgingival presence of *P. gingivalis*. Exclusion criteria were: (1) presence of any systemic condition that may affect the periodontal status; (2) pregnancy; (3) use of antibiotics within the last 6 months preceding the study; and (4) use of any medicine that may interfere with the periodontal health. This selection resulted in 48 potentially eligible patients. Of the 48 patients, 29 volunteered to participate in the present study. Subsequently, patients were classified into two groups according to the reported smoking status: (1) non-smokers, who had never smoked or had ceased smoking more than 10 years before entering the study; and (2) smokers, who were current smokers and had been smoking for at least 10 years with a consumption of ≥10 cigarettes/day.

The final study population included 18 non-smokers and 11 smokers. For each subject, all teeth were radiographically examined for interproximal bone loss at the mesial and distal aspects, using cemento-enamel junction (CEJ) of the tooth and the bone crest as reference points. With the use of a translucent plastic ruler (Schei ruler technique), the percentage of bone loss at the deepest proximal site of each tooth was measured.

Approval for this study was obtained by the Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam. Participants were informed both verbally and in writing about the purpose of the study, and provided signed informed consent.

Stimuli

Lipo-oligosaccharide (LOS) as used previously (23) was purified from *Neisseria meningitidis*, strain H44/76 (a kind gift from Dr J. Poolman, Rijksintituut voor Volksgezondheid en Milieu, Bilthoven, The Netherlands). *Porphyromonas gingivalis* strain 381 was grown in brain heart infusion broth enriched with hemin (5 mg/L) and menadione (1 mg/L) in an anaerobic atmosphere (80% N_2, 10% H_2, 10% CO_2) for 48 h at 37°C. The *P. gingivalis* bacteria were harvested in the log phase, pelleted by centrifugation (8000 ***g***), washed three times in phosphate-buffered saline (PBS), and resuspended in PBS at a concentration of optical density $(OD)_{690} = 1$, corresponding to approximately 7×10^8 colony forming units per mL. Aliquots (500 μL) of resuspended bacteria were disrupted using a sonifier in a sonifying vessel on ice (Soniprep MSE 150, amplitude 18, 4 min, 5 s intervals).

The degree of disruption of the bacteria was assessed by phase-contrast microscopy and with gram staining by light microscopy. Sonicates were stored at 4°C until use. Before use,

P. gingivalis sonicates were centrifuged (8000 ***g***, 1 min) and used in WBCC as described in the subsection below.

A mouse monoclonal antibody raised against human CD3 (anti-CD3, CLB-T3/4.E) was from CLB, Amsterdam, The Netherlands and has been previously described (24).

Whole blood cell cultures

Preliminary experiments were performed to determine the most optimal LOS and Pg-SE concentration for the WBCC. Whole blood samples of periodontally healthy donors were used for this purpose. From each subject, venous blood was collected by venipuncture from the antecubital fossae in a sterile pyrogen-free blood collection tube (Vacuette, Greiner, Alphen a/d Rijn, The Netherlands) containing sodium heparin, and diluted 10-fold in pyrogen-free Iscove's modified Dulbecco's medium (IMDM, Bio Whittaker, Verviers, Belgium) supplemented with 0.1% fetal calf serum (FCS, Bodinco, Alkmaar, The Netherlands), 100 IU/mL penicillin, 100 μg/mL streptomycin (Gibco, Merelbeke, Belgium) and 15 IU/mL sodium heparin (Leo Pharmaceutical Products BV, Weesp, The Netherlands). Diluted whole blood in 200 μL flat-bottomed microtitre culture plates (Nunc, Roskilde, Denmark) was stimulated for 18 h with different LOS concentrations (1000, 250, 62 and 16 pg/mL; 23) or Pg-SE dilutions (1:100, 1:400, 1:1600 and 1:64 000), and cytokine production of IL-1β, IL-6, IL-8, IL-10 and IL-12 p40 was measured. The most optimal LOS and Pg-SE concentrations for WBCC stimulation were 1000 pg/mL and 1:100, respectively (data not shown). Especially for IL-10, the capacity of each stimulant alone to elicit cytokine production at the indicated concentrations was still weak. To increase IL-10 production, we decided to combine monocyte stimulation with a T cell stimulus. In the 18 h incubation period, anti-CD3 alone did not lead to measurable cytokine production. However, when combined with monocyte stimuli, such as LOS or Pg-SE, anti-CD3 led to strongly increased production of cytokines, such as IL-10 and IL-12 (data not shown).

From each subject of the final study population (29 subjects), diluted whole blood as described previously was stimulated with LOS at a final concentration of 1000 pg/mL or with Pg-SE at 1:100 dilution, in the presence of anti-CD3 at 1 μg/mL. Unstimulated diluted whole blood served as a negative control. Supernatants were harvested and stored at −20°C until cytokine measurements.

Venous blood of each participant was also collected in an EDTA (K_3)-containing tube (Becton Dickinson Vacutainer System Europe, Meylan, France) for the determination of the total number of leukocytes and for leukocyte differentiation (neutrophils, eosinophils, basophils, lymphocytes and monocytes) with standard automated procedures (Cell-Dyn 4000, Hematology Analyzer, Abbott Laboratories, Park, IL, USA) operated in the clinical chemistry laboratory of the Slotervaart Hospital, Amsterdam, The Netherlands.

Toll-like receptor-transfected human embryonic kidney 293 (HEK 293) cell cultures

Human embryonic kidney 293 (HEK 293) cells stably transfected with CD14, CD14–Toll-like receptor (TLR)2 or CD14–TLR4 were a kind gift from Drs D. Golenbock and E. Latz, Division of Infectious Diseases, University of Massachusetts Medical School, Worcester, MA, USA. Transfected HEK 293 cells were cultured in IMDM supplemented with 5% heat-inactivated FCS, 100 IU/mL penicillin, 100 μL/mL streptomycin, 50 μM 2-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany) and 5 μg/mL puromycin (Sigma-Aldrich). For stimulation experiments, cells were seeded at 5×10^5 cells per well in 96-well flat-bottomed microtitre plates (Nunc, Roskilde, Denmark), and stimulated the next day with the appropriate preparation. The HEK 293–CD14–TLR4 cells were stimulated in the presence of 5% human serum as described elsewhere (25). After 16–20 h incubation, supernatants were harvested for determination of IL-8 production as a marker for nuclear factor-kappa B activation, i.e. cell activation.

Assays for cytokines

Cytokine levels of IL-1β, IL-6, IL-8, IL-10 and IL-12 p40 were measured in the supernatants of WBCC using commercially available enzyme-linked immunosorbent assay (ELISA) kits (PeliKine Compact^{TM} human ELISA kits, CLB, Amsterdam, The Netherlands) as previously described (21). The plates were read in an ELISA reader (Labsystems Multiskan Multisoft, Helsinki, Finland) at 450 nm, with 540 nm as a reference. Cytokine production in WBCC supernatants was adjusted per 10^6 monocytes.

Statistical analysis

The SPSS package version 11.0 for Windows (SPSS, Chicago, IL, USA) was used for descriptive data, data analysis and box plot generation. Differences between smokers and non-smokers were analysed by Mann–Whitney *U*-test. Differences in number of subjects per group were tested by Fisher exact tests.

Results

Subject background characteristics, age, gender and educational level are presented in Table 1. Cigarette smoking consumption reported by current smokers showed a mean of 14 cigarettes/day for 35 years, with a range of 10–20 cigarettes/day. The levels of total white blood cells were significantly higher in smokers than in non-smokers (7.42×10^9 and 5.78×10^9/L, respectively, $p = 0.001$). This was mainly due to increased numbers of neutrophils in smokers compared with their non-smoking counterparts. Values for eosinophils, basophils, lymphocytes and monocytes did not differ between the two groups (Table 1). Dental radiographic analysis of subjects at the moment of intake is presented in Table 2. Smokers had, on average, 16.1 teeth with ≥30% bone loss and 5.1 teeth with ≥50% bone loss, while non-smoking patients had

Table 1. Characteristics of the study population (non-smoking and smoking periodontitis patients)

	Non-smokers ($n = 18$)	Smokers ($n = 11$)	p-value
Age (years)	54 ± 5.3	51 ± 7.2	0.310
Gender			
Female	11 (61%)	7 (64%)	1.000
Male	7 (39%)	4 (36%)	—
Education level			
≤ High school	6 (33%)	2 (18%)	0.671
> High school	12 (67%)	9 (82%)	—
Smoking habits			
Number of cigarettes/day	0	14.2 ± 4.4	—
Number of years smoking	0	35 ± 6.9	—
Total WBC (10^9/L)	5.78 ± 1.36	7.42 ± 1.11	0.001
Neutrophils (10^9/L)	3.22 ± 1.11	4.31 ± 0.81	0.005
Eosinophils (10^9/L)	0.14 ± 0.12	0.19 ± 0.12	0.280
Basophils (10^9/L)	0.02 ± 0.07	0 ± 0	0.261
Lymphocytes (10^9/L)	1.90 ± 0.46	2.21 ± 0.77	0.079
Monocytes (10^9/L)	0.46 ± 0.13	0.50 ± 0.13	0.485

Values are means ± SD or number (%) of subjects.

Table 2. Dental radiographic characteristics of the study population at intake (non-smoking and smoking periodontitis patients)

	Non-smokers ($n = 18$)	Smokers ($n = 11$)	p-value
Number of teeth	26.4 ± 2.7	26.2 ± 3.2	0.964
Teeth ≥ 30% bone loss	12.1 ± 7.5	16.1 ± 5.6	0.120
Teeth ≥ 50% bone loss	3.7 ± 3.3	5.1 ± 4.3	0.420

Values are means ± SD.

12.1 and 3.7, respectively (differences not significant).

After stimulation of WBCC with LOS, supernatants were analysed for the cytokines IL-12 p40 and IL-10 (Fig. 1A,B). Regarding IL-12 p40, no differences could be assessed between smokers and non-smokers. The median of IL-10 for non-smokers was 14300 ng/10^6 monocytes, whereas in smokers the median was 21300 ng/10^6 monocytes. However, the values were not significantly different ($p = 0.217$). To explore the balance of Th1/Th2, we calculated the IL-12 p40/IL-10 ratio (Fig. 1C). Results showed a significantly lower ratio in smokers when compared with non-smokers ($p = 0.022$), which may indicate a more pronounced Th2 response in this group. Production of IL-1β, IL-6 and IL-8 after LOS stimulation is presented in Fig. 1D,E and F, respectively. The IL-1β production was significantly higher in non-smokers than in smokers ($p = 0.012$), whereas no differences were found for IL-6 and IL-8 cytokine production between the two groups.

In addition to the LOS stimulation experiments, Pg-SE was similarly used to explore the cytokine production of WBCC when challenged with a periodontal pathogen. Results are displayed next to the LOS box plots in Fig. 1. No difference in IL-12 p40 production was found between smokers and non-smokers (Fig. 1A). The levels of IL-10 and the calculated IL-12 p40/IL-10 ratio showed no difference between smokers and non-smokers (Fig. 1B,C). Pro-inflammatory cytokine IL-1β, as it occurred after LOS stimulation, was significantly higher in non-smokers than in smokers ($p = 0.015$, Fig. 1D), while IL-6 and IL-8 did not differ between the groups (Fig. 1E,F).

Smokers and non-smokers produced higher amounts of IL-8 after stimulation with Pg-SE than after LOS ($P < 0.001$, Fig. 1F) showing a clear difference in the levels of cytokine production dependent on the stimulation used. It is well known that Toll-like receptors (TLR) have the ability of transmitting LPS signalling across the cell membrane. It has been previously shown that the activation of either TLR2 or TLR4 may influence the Th1/Th2 balance. Therefore, in an effort to explain the observed differences between LOS and Pg-SE, it was decided to test the specificity of LOS and Pg-SE used in our experiments for both TLR2 and TLR4 on transfected HEK 293 cells. After stimulation of the cells with either LOS or Pg-SE, IL-8 production was measured in harvested supernatants. We observed that LOS stimulated HEK 293–CD14–TLR4 but not HEK 293–CD14–TLR2 to produce IL-8. Conversely, when Pg-SE was used, only HEK 293–CD14–TLR2 cells were able to produce IL-8 (one set of graphs representative of three experiments is shown in Fig. 2). Thus, in line with expectations, we observed that LOS signalled through TLR4, whereas Pg-SE stimulated TLR2.

Discussion

A body of evidence indicates that both prevalence and severity of periodontal disease is increased in smokers compared with non-smokers and that smokers in SPT show faster relapse and/or progression of disease (25–27). To date, however, no comparative study of stimulated cytokine profile in smoking and non-smoking periodontitis patients exists in the literature. The present study constitutes a first attempt to explore the effects of smoking on the monocytic cytokine profile in chronic adult periodontitis patients. In previous studies, a common and highly purified LPS from *Escherichia coli* (*E. coli*) has been mostly used for WBCC stimulation purposes. However, the use of *E. coli* may be critised. Firstly, in the *in vivo* situation and during infection, LPS is surely not the only bacterial component interacting with immune cells. Secondly, *E. coli* is not a periodontal pathogen. In order to overcome these problems to some extent, we selected periodontitis patients who before treatment were subgingival positive for *P. gingivalis*, a major periodontal

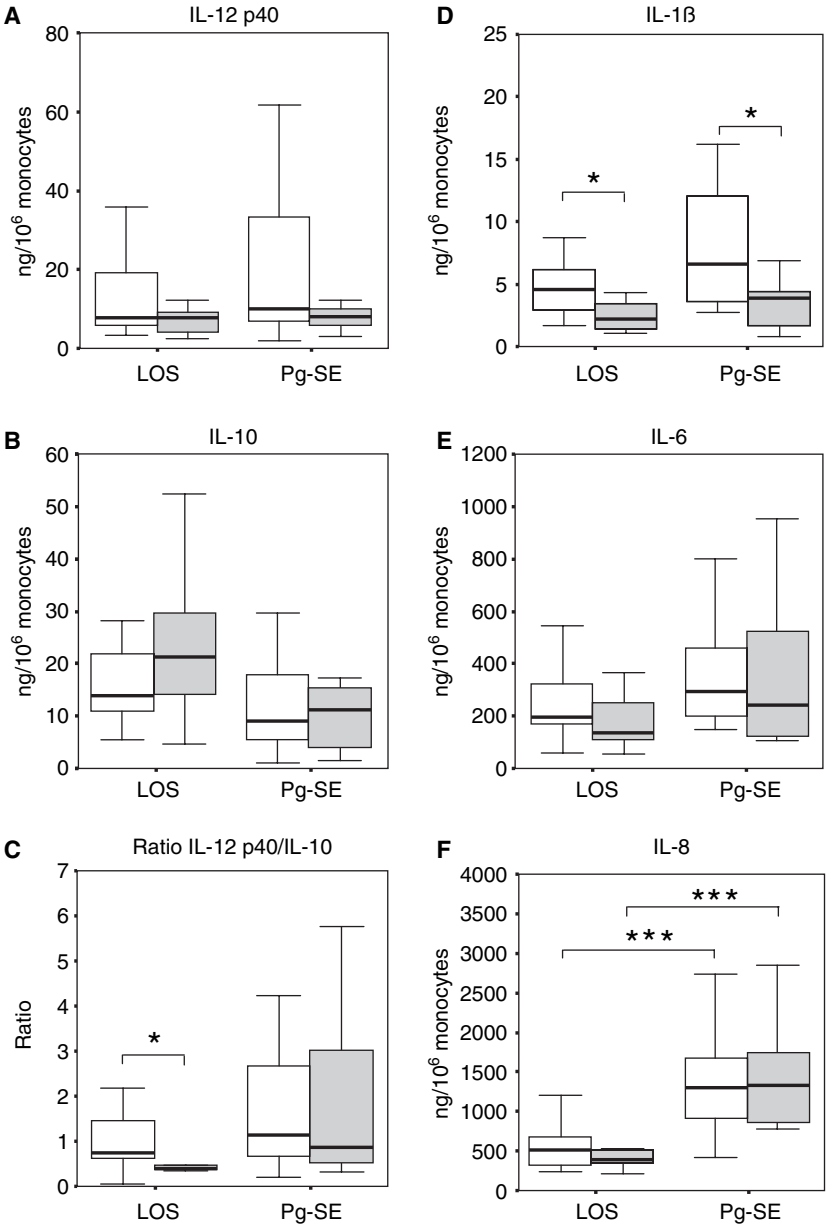

Fig. 1. Boxplots for T cell directing monocytic cytokines IL-12 p40 and IL-10, and the IL-12 p40/IL-10 ratio (A, B and C, respectively), and pro-inflammatory cytokines IL-1β, IL-6, IL-8 (D, E and F, respectively) expressed as ng/10^6 monocytes of whole blood cell cultures from non-smoking (open bar) and smoking periodontitis patients (shaded bar) after 18 h stimulation with *Neisseria meningitidis* LOS (LOS) and *Porphyromonas gingivalis* sonicate extract (Pg-SE), in the presence of anti-CD3 (1 μg/mL). *$P < 0.05$ and ***$P < 0.001$.

pathogen, and used for comparison with *N. meningitidis* LOS, a sonic extract of *P. gingivalis* for WBCC stimulation. The addition of anti-CD3 to the WBCC enhanced the monocytic cytokine production elicited by the single use of either LOS or Pg-SE. Via stimulation of the T cell, anti-CD3 leads to co-stimulation of TLR-triggered monocytes, possibly by upregulation of CD40-ligand (28). It could be argued that a potential mechanism of co-stimulation would be multivalent immunoglobulin (Ig) exposure to low-affinity Fcγ receptors (FcγR) or complement activation. However, to avoid such complications we made use of an anti-CD3 monoclonal antibody of the IgE isotype which is incapable of interacting with FcγR and with complement (29).

In our experiments, the decision to measure IL-12 p40 and not IL-12 p70 is supported by previous observations. Firstly, IL-12 p40 subunit can be produced in large excess over the heterodimer IL-12 p70, favouring IL-12 p40 detection in the supernatants, since IL-12 p70 could be practically undetectable (30,31). Secondly, the production of IL-12 p40 and IL-12 p70 by LPS- and interferon-γ (IFN-γ)-stimulated macrophages is affected to the same degree by smokeless tobacco, suggesting that both cytokines may be similarly influenced by tobacco (32). Moreover, others have used the measurement of either p40 mRNA or p40 alone as indicators of total IL-12 production in humans (33,34). The Th1-promoting activity of IL-12 p40 has been demonstrated, and this cytokine is considered to be an indicator of Th1 differentiation (35–38).

Cytokine production has usually been studied in cultures of peripheral blood mononuclear cells. However, the study of cytokine production in WBCC has some advantages over separated cultures. Firstly, the whole blood culture system reduces the likelihood of endotoxin contamination since there is minimal handling of the cells. Secondly, the risk of cellular activation resulting from the isolation procedures is reduced. Thirdly, the WBCC system may represent more closely the natural environment, with the presence of various immunomodulating and pro- and anti-inflammatory mediators in whole blood. Fourthly, disturbances in the ratios of different cell types resulting from purification procedures are avoided (21,39). Therefore, the integrity of the cellular interactions is maintained as best possible, although the whole blood is diluted 1:10. It has been extensively studied and shown in parallel cultures of whole blood and freshly isolated monocytes as well as in kinetics that WBCC stimulated with LPS specifically reflect the behaviour of the monocytes (23,40,41).

The present data show that smokers suffering from periodontitis have a lower IL-12 p40/IL-10 ratio after LOS

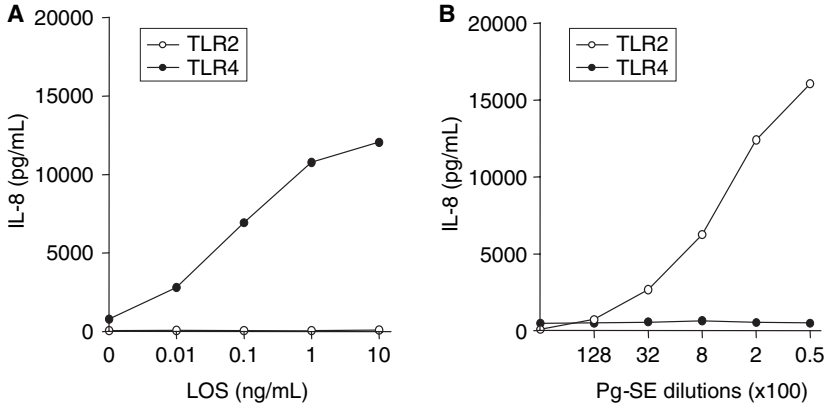

Fig. 2. Interleukin-8 cytokine production of TLR2 and TLR4 in transfected HEK 293 cell cultures induced by LOS and Pg-SE. The HEK 293 cell lines were stimulated with *Neisseria meningitidis* LOS (LOS) at concentrations of 0.01, 0.1, 1 and 10 pg/mL (A) and *Porphyromonas gingivalis* sonicate extract (Pg-SE) at serial dilutions (B).

stimulation and lower IL-1β production after LOS and Pg-SE stimulation than their non-smoking counterparts. Recently, it was reported that tobacco smoke leads to an increase in cAMP levels in human oral epithelial cell line (42). In T cells, cAMP elevations lead to a greatly decreased IL-12 p40/IL-10 ratio (23). The lower IL-1β production in smokers is in agreement with *in vitro* experiments that demonstrated that tobacco smoke can inhibit cytokine production by peripheral blood monocytes, including production of IL-1β (43). Furthermore, it has been shown that IL-1 is involved in the upregulation of IFN-γ production by Th1 cells and downregulation of IL-4 production by Th2 cells (44–46). Since we found in smokers lower levels of IL-1β production and a lower IL-12 p40/IL-10 ratio, it may be suggested that in periodontitis, smoking influences the Th1/Th2 balance into a more pronounced Th2 profile. This may result in continuous polyclonal B cell activation with less protective antibody production (47), which could partly explain the clinical finding of more severe periodontitis in smokers.

The present study also showed that more IL-8 was produced in response to stimulation with Pg-SE compared with LOS, both in smoking and in non-smoking periodontitis patients. The finding that Pg-SE stimulates via TLR2, whereas LOS is recognized by TLR4, might provide an explanation for the difference in IL-8 production. This is in line with a body of evidence demonstrating striking differences in cytokine gene transcription in TLR2 and TLR4 activation (48,49). Alternatively, the difference in IL-8 levels might be caused by differences in the cell types involved in IL-8 production. Since TLR2, but not TLR4, is substantially expressed on the surface of human neutrophils, it is likely that Pg-SE activates both monocytes and neutrophils, whereas after LOS stimulation only monocytes are activated (50). It is clear that the role of TLRs in periodontal pathogen recognition and subsequent cytokine production is not yet completely understood, and that the use of different microbial stimuli may be an important consideration for the interpretation of past and future data.

In summary, the results showed differences between smoking and non-smoking periodontitis patients in *ex vivo* cell culture cytokine production. Our findings suggest that the Th2 pattern in periodontitis may be accentuated by smoking. This hypothesis is consistent with the contention that a strong innate immune response is associated with a protective Th1 pattern, favouring inflammatory responses that contain the infection with periodontal pathogens. Therefore, a more pronounced Th2 phenotype may accelerate disease progression and account for the relapse during SPT that is frequently observed in smoking periodontitis patients. Our findings may serve as another step in revealing generalized inflammatory patterns in smoking periodontitis patients.

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