Journal of
PERIODONTAL RESEARCH

J Periodont Res 2010; 45: 239–245

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2009.01225.x

Enhanced monocyte migration and pro-inflammatory cytokine production by *Porphyromonas gingivalis* infection

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Pollreisz A, Huang Y, Roth GA, Cheng B, Kebschull M, Papapanou PN, Schmidt AM, Lalla E. Enhanced monocyte migration and pro-inflammatory cytokine production by Porphyromonas gingivalis *infection. J Periodont Res 2010; 45: 239–245. © 2009 The Authors. Journal compilation © 2009 Blackwell Munksgaard*

Background and Objective: Porphyromonas gingivalis, a major periodontal pathogen, has been reported to be involved in atherogenesis. In order to further understand this pathogen's link with systemic inflammation and vascular disease, we investigated its influence on murine monocytes and macrophages from three different sources.

Material and Methods: Concanavalin A-elicited peritoneal macrophages, peripheral blood monocyte-derived macrophages and WEHI 274.1 monocytes were infected with either *P. gingivalis* 381 or its non-invasive fimbriae-deficient mutant, DPG3.

Results: Infection with *P. gingivalis* 381 markedly induced monocyte migration and significantly enhanced production of the pro-inflammatory cytokines, tumor necrosis factor-α and interleukin-6. Consistent with a role for this pathogen's major fimbriae and/or its invasive capacity, infection with DPG3 had a minimal effect on both monocyte attraction and pro-inflammatory cytokine production.

Conclusion: Since monocyte recruitment and activation are important steps in the development of vascular inflammation and atherosclerosis, these results suggest that *P. gingivalis* infection may be involved in these processes.

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Key words: *Porphyromonas gingivalis*; infection; periodontal disease; atherosclerosis

Accepted for publication March 13, 2009

Porphyromonas gingivalis, a gram-negative anaerobic bacterium of the subgingival dental plaque, is involved in the etiopathogenesis of periodontitis, which is a chronic infection of the supporting structures of the teeth (1). Recent evidence has drawn special attention to the association between *P. gingivalis* and atherosclerotic vascular disease because the pathogen can gain access into the systemic circulation and has also been isolated from human atherosclerotic plaques (2–4). Furthermore, *P. gingivalis* has been shown to invade endothelial cells (5–7), to induce its uptake by human macrophages and to promote foam cell formation (8,9).

The local host response to periodontal bacteria consists of the recruitment of monocytes/macrophages and neutrophils. Monocytes are part of the mononuclear phagocytic system and reside in the circulation before entering

the tissue, where they become macrophages (10). The migration of monocytes is fundamental to various *in vivo* processes and has been studied extensively. Attachment to the vessel wall followed by transmigration via diapedesis represents an important step in atherogenesis, where monocytes both initiate and enhance the immune response by releasing various cytokines and chemotactic molecules to attract other monocytes and inflammatory cells (11).

We have shown that infection of human aortic endothelial cells with *P. gingivalis* 381 leads to a significantly enhanced adhesion of human U-937 monocytes (7), a crucial step in the initiation and development of the inflammatory processes associated with atherogenesis (12,13). Other important factors are the secretion of pro-inflammatory cytokines and the expression of adhesion molecules, which are also upregulated upon *P. gingivalis* infection (7,14,15).

Taking these findings into consideration, in the present study we investigated the influence of *P. gingivalis* 381 on monocytes and macrophages from different sources to further understand the involvement of this pathogen in vascular inflammation and the development of atherosclerosis.

Material and methods

Bacterial strains and growth conditions

Porphyromonas gingivalis FDC381 was grown on blood agar plates (Anaerobe Systems, Morgan Hill, CA, USA) in anaerobic chambers at 37°C. Its fimbriae-deficient mutant, DPG3, constructed by insertional inactivation of the *fimA* gene (16), was grown on blood agar plates supplemented with erythromycin (Anaerobe Systems) in anaerobic chambers at 37°C. The antibiotic ensures selective growth of the erythromycin-resistant mutant, as opposed to the erythromycin-susceptible wild-type strain. Bacterial suspensions were prepared in phosphate-buffered saline without Mg^{2+}/Ca^{2+} (PBS) using established growth curves and spectrophotometric analysis.

Murine peripheral blood monocyte-derived macrophages

All animal studies were performed in accordance with the policies of the Institutional Animal Care and Use Committee at Columbia University. Male C57BL/6, and also for the cytokine assessments (described below) hypercholesterolemic ApoE−/− mice, purchased from The Jackson Laboratory (Stock 5052; Bar Harbor, ME, USA) and maintained in specific-pathogen-free conditions with full access to food and water, were used at the age of 8 wk. Ketamine (50 mg/kg) and xylazine (5 mg/kg) were administered intraperitoneally until mice were areflexic. Blood was collected from the inferior vena cava and diluted 1:2 with sterile PBS. The diluted blood was then transferred to a leukocyte separation tube (Greiner, Frickenhausen, Germany) preloaded with 15 mL Histopaque 1083 (Sigma-Aldrich, St Louis, MO, USA) and centrifuged for 15 min at 800 *g* at room temperature with brakes off. After centrifugation, the enriched cell fraction containing lymphocytes/peripheral blood mononuclear cells was gently harvested and washed with PBS. After another wash with MACS buffer (2 mM EDTA and 0.5% bovine serum albumin in PBS), cells were counted and magnetic bead separation was performed using CD11b MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The magnetically labeled cells were flushed out of the column, resuspended in RPMI medium (ATCC, Manassas, VA, USA) supplemented with 10% fetal bovine serum, and seeded in 8-well chamber slides (Nunc, Rochester, NY, USA). Three hours later, non-adherent cells were removed by change of medium.

The purity of the cell suspension was determined with the use of multiparameter flow cytometry, according to the method described by Lagasse *et al.* (17). In brief, single cell suspensions were stained with Mac-1, GR-1, CD3 and B220 antibodies, and analyzed on a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Cell viability was determined using 7-amino actinomycin D. The CD3 and B220 antibodies were used to gate out lymphocytes. Cells with Gr1 neg/lo and Mac1 hi expression were considered to be macrophages. Purity and cell viability of magnetically labeled cells was above 90%.

WEHI 274.1 murine monocyte cell line

The WEHI 274.1 monocytes were purchased from ATCC (Rockville, MD, USA) and grown in Dulbecco's modified Eagle's medium (ATCC) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin and 0.05 mM mercaptoethanol (Gibco, Grand Island, NY, USA).

Concanavalin A-elicited murine peritoneal macrophages

Concanavalin A (Sigma-Aldrich) at a concentration of 80 μg/mL was injected intraperitoneally in C57BL/6 mice, and macrophages were harvested 3 d later by peritoneal lavage, as previously described (18). Cells were cultured in Dulbecco's modified Eagle's medium medium supplemented with 10% fetal bovine serum and 20% L-cell conditioned medium for 48 h, at which point they were typically at ~90% confluency.

Infection with *P. gingivalis* 381 or DPG3

Peripheral monocyte-derived macrophages, WEHI 274.1 cells, or concanavalin A-elicited peritoneal macrophages were plated in 8-well chamber slides (1.5×10^5 per well) in antibiotic-free medium for 24 h and then co-incubated with *P. gingivalis* 381 or DPG3 at a multiplicity of infection of 100 for 90 min or left untreated (non-infected control) at 37°C, 5% CO_2. In order to approximate *in vivo* conditions, the bacteria were not centrifuged onto the monocytes/macrophages to promote intimate contact. For all experiments, multiplicity of infection was calculated based on the number of macrophages per well when seeded. After the incubation

period, cells were washed with PBS and maintained in the appropriate medium without antibiotics for 8 or 24 h. The cell culture supernatant was collected, centrifuged (1,400*g* for 10 min at 4°C) and stored at −70°C until used in experiments.

Determination of cytotoxicity

Cytotoxicity in cultures of infected or uninfected WEHI 274.1 cells, concanavalin A-elicited peritoneal macrophages or peripheral monocyte-derived macrophages was measured by determining the release of lactate dehydrogenase (LDH) into the cell culture supernatant using a Cytotoxicity Detection Kit (Roche, Mannheim, Germany). Supernatants were collected by centrifugation 8 or 24 h after infection. By lysing non-infected cells with mammalian protein extraction reagent (M-PER; Pierce, Rockford, IL, USA) the maximal amount of LDH release was determined. The percentage of cytotoxicity, calculated according to the formula [(optical denisity of sample minus optical denisity of medium)/(optical denisity of maximal LDH release minus optical denisity of medium)] × 100 showed no significant difference between infected and control groups in all three cell types assessed.

Migration assay

Cell culture supernatant was collected 8 or 24 h after infection. These supernatant samples were then placed as a chemoattractant in the bottom wells of a cell migration chamber (Millipore, Billerica, MA, USA). Fresh RPMI with 10% fetal bovine serum served as a control stimulus. The WEHI 274.1 cells (1.5×10^5 per well) were added to the 5 μm pore-size migration chamber and incubated for 12 h at 37°C in a CO_2 incubator. The total number of migrated cells was then determined by measurement of fluorescence. The results are expressed as chemotactic index (CI); that is, the number of cells migrating in the presence of the attractant divided by the number of cells migrating in the presence of fresh medium alone.

Production of pro-inflammatory cytokines

Levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were quantified in cell culture supernatants from peripheral monocyte-derived macrophages by ELISA, using commercially available kits (TNF-α, Bender MedSystems, Vienna, Austria; IL-6, R&D Systems, Minneapolis, MN, USA).

Data and statistical analysis

All experiments were performed in duplicate wells for each set of conditions and repeated three or four times. Data are presented as means ± SD, and n represents the number of experiments. Unless otherwise stated, statistical analysis of data was performed by means of a two-way ANOVA with the type of infection (no infection, *P. gingivalis* 381 or DPG3) and the time point (8 or 24 h) as the two independent variables (factors). For each of the two time points, differences among the three experimental groups were assessed using a one-way ANOVA. The interaction between type of infection and the source of the peripheral blood monocytes (C57BL/6 or ApoE−/− mice) by time point was assessed in a similar manner. When the one-way ANOVA result was significant, we proceeded with *post hoc* student's *t*-tests for pairwise comparisons. Under the Bonferroni adjustment for multiple (three) *post hoc* comparisons, an individual $p < 0.017$ is necessary to achieve statistical significance at the 5% level.

Results

Supernatant of *P. gingivalis* 381-infected WEHI 274.1 monocytes attracts WEHI 274.1 monocytes

To assess the ability of supernatant from WEHI 274.1 moncytes infected with *P. gingivalis* 381 to induce monocyte migration, we performed chemotaxis assays. As shown in Fig. 1A, supernatant from *P. gingivalis* 381-infected WEHI 274.1 monocytes attracted 60% more WEHI 274.1 cells than supernatant from non-infected cells ($p < 0.0001$, Student's *t*-test; $n = 4$). This result suggests the presence of chemotactic substances released by WEHI 274.1 monocytes into the cell culture supernatant upon infection with the periodontal pathogen.

Supernatant of *P. gingivalis* 381-infected peritoneal macrophages attracts WEHI 274.1 monocytes

We next investigated whether the effect of attracting monocytes is also present when using supernatant from concanavalin A-elicited mouse peritoneal macrophages in the same experimental conditions. In addition to the non-infected control group, the non-invasive fimbriae-deficient mutant DPG3 was used. As shown in Fig. 1B, the number of monocytes that migrated through the membrane was higher in chambers containing supernatant from *P. gingivalis* 381-infected peritoneal macrophages (CI: 1.37 ± 0.16) compared with supernatant from non-infected control (CI: 1.08 ± 0.12), $p = 0.0341$, or DPG3-infected cells (CI: 1.15 ± 0.1), $p = 0.0808$ ($n = 3$ for both).

Supernatant from peripheral monocyte-derived macrophages promotes WEHI 274.1 monocyte migration

Since supernatants from *P. gingivalis* 381-infected WEHI 274.1 and peritoneal macrophages were found to have chemoattractant properties, we next investigated whether peripheral monocyte-derived macrophages have the same ability. Considering the particular importance of these unprimed primary cells, we also included an earlier time point (8 h after infection). As shown in Fig. 2, there was an increase in migration of WEHI 274.1 cells when supernatant from *P. gingivalis* 381-infected peripheral monocyte-derived macrophages was used, at both 8 and 24 h postinfection (CI: 2.76 ± 0.04 at 8 h and 2.67 ± 0.05 at 24 h) compared with supernatant from the DPG3 (CI: 2.58 ± 0.02 at 8 h and 2.52 ± 0.04 at 24 h) or the uninfected control group (CI: 2.6 ± 0.02 at 8 h and 2.55 ± 0.05 at 24 h). The interaction between infection type and time point was not significant ($p = 0.5620$). (*P. gingivalis* 381 vs. non-infected at 8 and 24 h,

$p = 0.0005$ and $p = 0.0211$, respectively; *P. gingivalis* 381 vs. DPG3 at 8 and 24 h, $p = 0.0003$ and $p = 0.0109$, respectively; DPG3 vs. non-infected at 8 h and 24 h, $p = 0.3919$ and $p = 0.6107$, respectively; $n = 3$.)

Infection of peripheral monocyte-derived macrophages from C57BL/6 or ApoE−/− mice with *P. gingivalis* 381 enhances production of TNF-α and IL-6

Since pro-inflammatory cytokines are important chemotactic factors and serve to attract monocytes and other leukocytes, levels of TNF-α were determined in supernatant of monocyte-derived macrophages from C57BL/6 mice (Fig. 3A) and also from ApoE−/− mice (Fig. 3B). The latter were employed to investigate the effect of the periodontal pathogen on macrophages from hypercholesterolemic mice, prone to atherosclerosis. Infection of macrophages from C57BL/6 and ApoE−/− mice with *P. gingivalis* 381 led to a significantly enhanced production of TNF-α 8 h later, 67.43 ± 2.03 and 62.82 ± 2.02 pg/mL, respectively, compared with 2.52 ± 0.77 and 1.42 ± 0.37 pg/mL in the non-infected control and to 21.36 ± 4.72 and 14.65 ± 1.5 pg/mL in the DPG3 group ($p < 0.0001$ for all, $n = 3$). Similar results from C57BL/6 and ApoE−/− mice were obtained at the 24 h time point upon *P. gingivalis* 381 infection (61.6 ± 12.2 and 58.83 ± 2.02 pg/mL, respectively) when compared with DPG3 (18.41 ± 1.7 and 22.15 ± 2.45 pg/mL), $p < 0.0003$ and $p < 0.0001$, respectively, or the untreated control (1.44 ± 0.58 and 1.27 ± 0.46 pg/mL), $p < 0.0001$ for both; $n = 3$.

IL-6 production by peripheral monocyte-derived macrophages from C57BL/6 (Fig. 4A) and ApoE−/− mice (Fig. 4B) was also markedly increased by infection with *P. gingivalis* 381 at the 8 h time point; 10.19 ± 3.2 and 9.28 ± 1.76 pg/mL, respectively, compared with 1.37 ± 0.81 and 0.69 ± 0.55 pg/mL in the untreated control and 2.67 ± 0.27 and 5.11 ± 1.05 pg/mL in the DPG3 group ($p < 0.005$ for all, $n = 4$). Finally, there was also a statistically significant increase of IL-6 levels in supernatant of *P. gingivalis* 381-infected cells from C57BL/6 and ApoE−/− mice 24 h later (16.01 ± 5.62 and 23.21 ± 0.73 pg/mL, respectively) compared with non-infected (2.07 ± 1.46 and 5.77 ± 3.9 pg/mL) or DPG3-infected cells (5.67 ± 0.77 and 10.29 ± 1.87 pg/mL), $p < 0.0001$ for all; $n = 4$.

Discussion

We previously demonstrated that *P. gingivalis* 381 promotes mononuclear cell adhesion to human aortic endothelial cells and increases endothelial cell secretion of pro-inflammatory cytokines (IL-6, IL-8 and monocyte chemoattractant protein-1) and expression of adhesion molecules (vascular cell adhesion molecule-1, intercellular adhesion molecule-1 and E-selectin) (7). Furthermore, we were able to show that *P. gingivalis* 381 leads to procoagulant responses in human aortic endothelial cells (19). These findings point to the systemic burden produced by this common pathogen.

In the present study, we investigated the effect of *P. gingivalis* on three different types of murine monocytes/macrophages. We used WEHI 274.1 monocytes, concanavalin A-elicited peritoneal macrophages and, to the best of our knowledge for the first time, peripheral blood monocyte-derived macrophages isolated from both wild-type and atherosclerosis-prone ApoE−/− mice. The migration of WEHI 274.1 monocytes was increased when supernatant from all three types of *P. gingivalis* 381-infected cells was used as an attractant. Furthermore, higher levels of TNF-α and

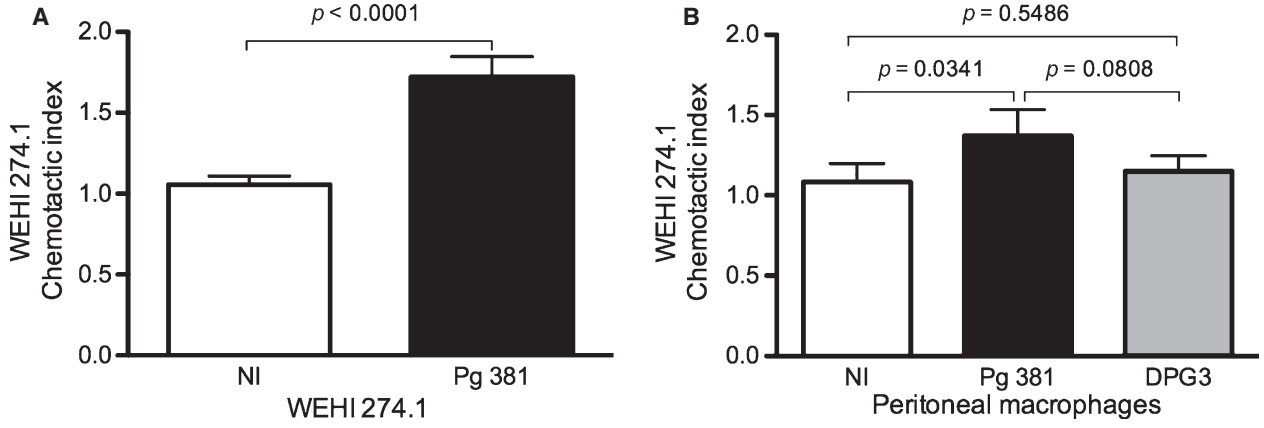

Fig. 1. (A) WEHI 274.1 monocytes were infected with *P. gingivalis* 381 (Pg 381) for 90 min, and supernatants were collected 24 h after infection. Supernatants from *P. gingivalis* 381-infected cells attracted significantly more WEHI 274.1 monocytes [chemotaxis index (CI): 1.72 ± 0.13] than the non-infected group (NI) (CI: 1.06 ± 0.05); $n = 4$. (B) Concanavalin A-elicited peritoneal macrophages were infected with *P. gingivalis* 381 or DPG3 or were left uninfected, and 24 h later the supernatants were collected and used in the migration assays. An increased number of WEHI 274.1 cells was attracted from supernatants of *P. gingivalis* 381-infected cells (CI: 1.37 ± 0.16) compared with those attracted by either the DPG3-infected (CI: 1.15 ± 0.1) or the non-infected group (CI: 1.08 ± 0.12), but the differences did not reach statistical significance under the Bonferroni adjustment; $n = 3$. Data are shown as mean CI values ± SD.

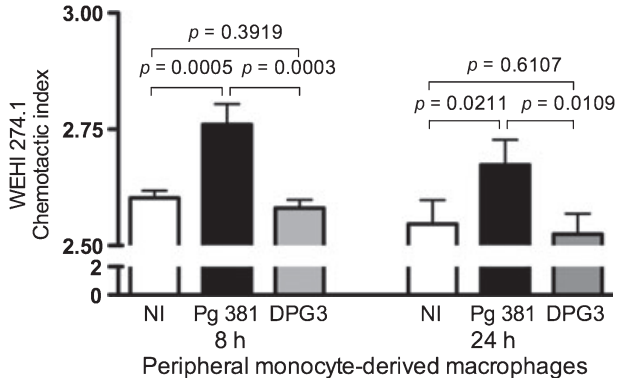

Fig. 2. Peripheral blood monocyte-derived macrophages were infected with *P. gingivalis* 381 (Pg 381) or DPG3 or were left uninfected (NI). Significantly more WEHI 274.1 cells were attracted in the presence of supernatants from *P. gingivalis* 381-infected cells [chemotaxis index (CI): 2.76 ± 0.04 at 8 h and 2.67 ± 0.05 at 24 h] compared with DPG3 (CI: 2.58 ± 0.02 at 8 h and 2.52 ± 0.04 at 24 h) or to the non-infected control (CI: 2.6 ± 0.02 at 8 h and 2.55 ± 0.05 at 24 h); $n = 3$. Data are shown as mean CI values ± SD.

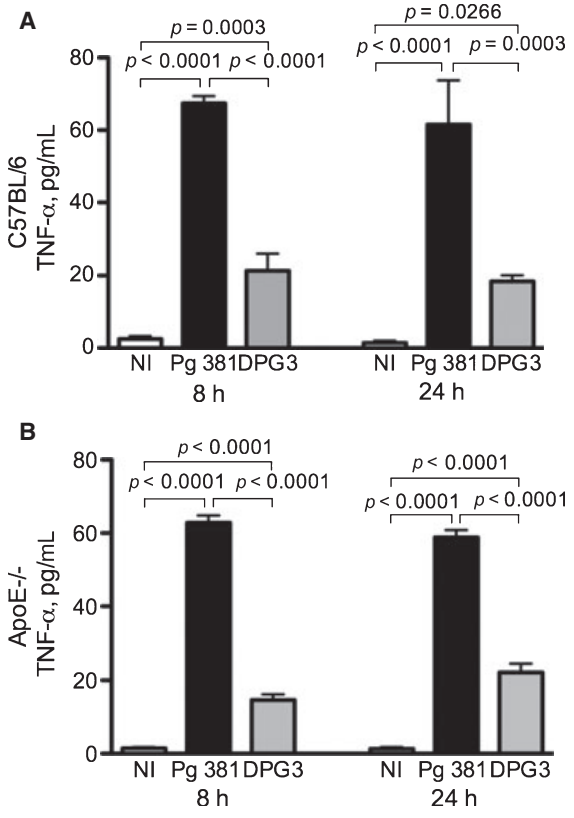

Fig. 3. Production of tumor necrosis factor-α as determined by ELISA was significantly increased in *P. gingivalis* 381-infected peripheral monocyte-derived macrophages (Pg 381) at 8 and 24 h postinfection compared with the non-infected control cells (NI) and DPG3-infected cells. (A) Results obtained in C57BL/6 mice ($n = 3$). (B) Results obtained in ApoE−/− mice ($n = 4$). Data are shown as means ± SD.

IL-6 were found in supernatants of peripheral monocyte-derived macrophages infected with *P. gingivalis* 381 compared with those infected with the fimbriae-deficient mutant, DPG3, or the non-infected control cells. These results are consistent with the concept that major fimbriae and/or the invasive capacity of *P. gingivalis* 381 are involved in provoking inflammatory responses.

In other studies, *P. gingivalis* fimbriae protein and *P. gingivalis* lipopolysaccharide have been shown to enhance migration of human monocytes by chemotaxis (20). Furthermore, d'Empaire *et al.* (21) observed a vigorous polymorphonuclear leukocyte migration in response to culture fluids from macrophages stimulated with different types of *P. gingivalis* capsular polysaccharides in migration assays. We obtained similar results with supernatant of monocytes challenged with whole *P. gingivalis* 381, namely a significant increase of migrated monocytes in a chemotaxis assay compared with supernatant from the fimbriae-deficient mutant DPG3 or the non-infected control groups.

It has been reported that the functional responses of monocytes/macrophages obtained from different sources (murine peritoneal or alveolar macrophages and human peripheral monocytes) differ when exposed to the same stimulus (22–24). Thioglycollate-elicited murine peritoneal macrophages secreted TNF-α in the culture supernatant when stimulated with *P. gingivalis* lipopolysaccharide, whereas in macrophages elicited by intraperitoneal injection of heat-killed *P. gingivalis* the cytokine was not detectable. At the mRNA level, TNF-α was expressed by both cell types (25). In the chemotaxis assays performed with concanavalin A-elicited peritoneal macrophages in our study, we observed that the ability of supernatant to attract monocytes did not reach statistical significance (under the Bonferroni adjustment) when fluids from *P. gingivalis* 381-infected cells were compared with DPG3 or the non-infected control cells. These findings are consistent with a concanavalin A-associated priming effect of monocytes *in vivo*. Given that, based on their origin, peripheral monocyte-derived macrophages are more relevant in the study of *P. gingivalis* effects on systemic inflammation and atherogenesis and in order to limit any factors affecting the activation state of monocytes, these peripheral

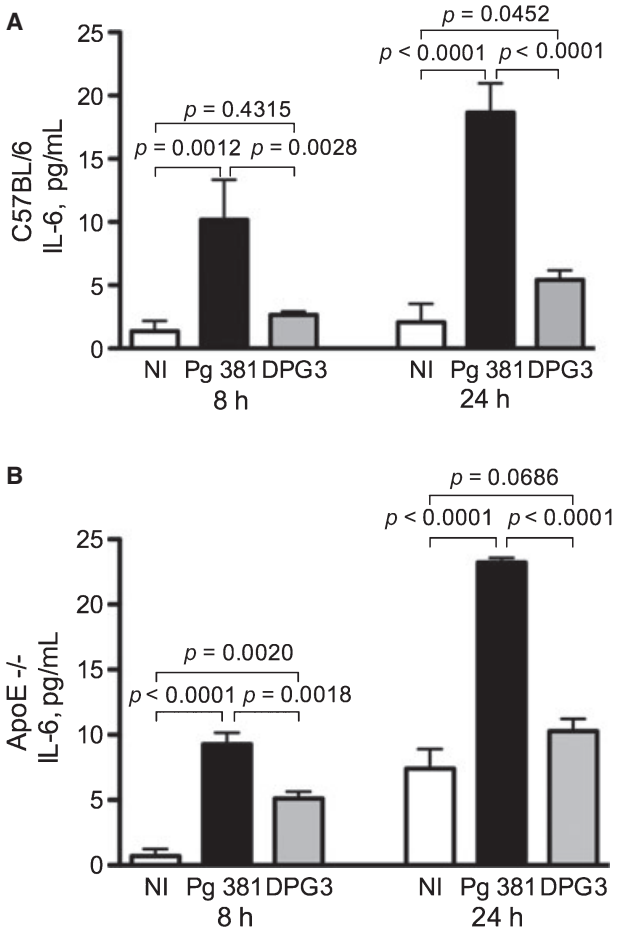

Fig. 4. Infection with *P. gingivalis* 381 (Pg 381) led to a significantly enhanced secretion of interleukin-6 at 8 and 24 h postinfection of peripheral monocyte-derived macrophages compared with the non-infected control (NI) and DPG3-infected macrophages. (A) Results obtained in C57BL/6 mice. (B) Results obtained in ApoE−/− mice. Data are shown as means ± SD; $n = 4$.

blood-derived cells were used for subsequent experiments. In previous reports, the most common type of macrophages used were of peritoneal origin, possibly because the challenge of obtaining adequate numbers of peripheral blood cells from mice constitutes a barrier to their being studied more extensively. Despite the use of three different types of monocytes/macrophages, the present results derive from an *in vitro* model system of *P. gingivalis* infection and have limited clinical implications at this point.

Furthermore, evidence suggests that *P. gingivalis* 381 is involved in the development of inflammatory atherosclerotic processes by activating proadhesive pathways in human monocytes (7,8,26,27), leading to an enhanced transendothelial migration of *P. gingivalis* 381-infected monocytes compared with cells infected with non-fimbriated isogenic mutants. To assess the effects of *P. gingivalis* 381 in an atherosclerosis-prone state, we employed ApoE−/− mice, which are affected by hypercholesterolemia and development of atherosclerotic lesions. Since cytokine expression upon infection has been described extensively in different studies (28,29) using other sources of monocytes/macrophages, but not peripheral blood-derived cells, we investigated whether these cells show similar properties. Tumor necrosis factor-α, an important mediator of inflammation involved in several inflammatory diseases, was markedly upregulated in *P. gingivalis* 381-infected supernatants from monocyte-derived macrophages harvested from C57BL/6 or ApoE−/− mice compared with cells infected with DPG3 or the non-infected control cells. Another pro-inflammatory cytokine that has been described to be strongly upregulated upon infection with *P. gingivalis* 381 is IL-6 (28). We observed increased levels of IL-6 after 24 h, with significantly higher secretion from *P. gingivalis* 381-infected cells compared with DPG3 or the non-infected control cells, suggesting that the major fimbriae and/or the invasive capacity of *P. gingivalis* 381 are involved in these responses. Certainly, the role of other bacterial components/molecules (including lipopolysaccharide) in this setting cannot be completely ruled out. Indeed, on some occasions, DPG3-infected cells produced higher levels of the cytokines (especially in the case of TNF-α) compared with the non-infected cells.

The interaction between type of infection (no infection, *P. gingivalis* 381 or DPG3) and the source of the peripheral blood monocytes (C57BL/6 or ApoE−/− mice) did not reach statistical significance for either cytokine or time point with this number of individual experiments. Thus, the source of the peripheral blood monocytes does not appear to significantly modify either the relationship or the magnitude of the differences observed among the three infection groups. Interestingly, and consistent with an additional priming effect of hypercholesterolemia in this setting, ApoE−/− macrophages secreted significantly higher levels of IL-6 at the later time point compared with C57BL/6 macrophages across all three infection groups ($p < 0.0001$, two-way ANOVA without interaction).

Taken together, the present findings contribute to our understanding of the systemic, pro-inflammatory and pro-atherogenic effects of *P. gingivalis* and suggest that the invasive ability of the pathogen may be involved in these processes.

Acknowledgements

This work was supported by NIH R01 grant DE14575. The authors wish to thank Ms Romanita Celenti for her valuable technical assistance.

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