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Porphyromonas gingivalis lipopolysaccharide alters atherosclerotic-related gene expression in oxidized low-density-lipoprotein-induced macrophages and foam cells

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Background and Objective: The molecular mechanism linking atherosclerosis formation and periodontal pathogens is not clear, although a positive correlation between periodontal infections and cardiovascular diseases has been reported. The aim of this study was to determine whether stimulation with *Porphyromonas gingivalis* lipopolysaccharide (LPS) affected the expression of atherosclerosis-related genes, during and after the formation of foam cells.

Material and Methods: Macrophages from human THP-1 monocytes were treated with oxidized low-density lipoprotein (oxLDL) to induce the formation of foam cells. *P. gingivalis* LPS was added to cultures of either oxLDL-induced macrophages or foam cells. The expression of atherosclerosis-related genes was assayed by quantitative real-time PCR, and the production of granulocyte–macrophage colony-stimulating factor, monocyte chemotactic protein-1, interleukin (IL)-1β, IL-10 and IL-12 proteins was determined using ELISA. Nuclear translocation of nuclear factor-kappaB (NF-κB) P_{65} was detected by immunocytochemistry, and western blotting was used to evaluate inhibitory kappa B-α (IκB-α) degradation to confirm activation of the NF-κB pathway.

Results: P. gingivalis LPS stimulated atherosclerosis-related gene expression in foam cells and increased the oxLDL-induced expression of chemokines, adhesion molecules, growth factors, apoptotic genes and nuclear receptors in macrophages. Transcription of the proinflammatory cytokines *IL1*β and *IL12* was elevated in response to LPS in both macrophages and foam cells, whereas transcription of the anti-inflammatory cytokine, *IL10*, was not affected. Increased activation of the NF-κB pathway was also observed in macrophages costimulated with LPS + oxLDL.

Conclusion: P. gingivalis LPS appears to be an important factor in the development of atherosclerosis by stimulation of atherosclerosis-related gene expression in both macrophages and foam cells via activation of the NF-κB pathway.

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Periodontal disease is a condition that affects the supporting tissues surrounding the teeth, and which, in severe cases, can result in tooth loss. There is a growing body of epidemiological evidence to suggest that pathogenic periodontal bacterial species may play a role in the development of cardiovascular diseases (1,2). For example, $ApoE^{-/-}$ atherosclerosis-prone mice infected, by blood or oral inoculation, with *Porphyromonas gingivalis,* a principal pathogen in periodontitis, showed signs of accelerated atherosclerosis, and *P. gingivalis* DNA was found in the aortic tissues of infected mice (3,4). Furthermore, chronic infusion of *P. gingivalis* lipopolysaccharide increased atherosclerosis formation in $ApoE^{-/-}$ mice (5). The molecular mechanisms linking LPS from *P. gingivalis* and atherosclerosis are not clear, although evidence points to a correlation between atherosclerosis and periodontal infection.

Atherosclerosis is a chronic inflammatory disease characterized by the early and persistent presence of macrophages in the arterial wall (6). Scavenger receptors mediate the uptake, by macrophages, of oxidized low-density lipoprotein (oxLDL particles), which leads to intracellular cholesterol accumulation and the formation of foam cells (7). Upon entering the blood circulatory system, live *P. gingivalis,* its outer membrane vesicles, or its LPS in the presence of LDL, can also promote foam cell formation (8,9), possibly by the interaction of LPS with cell constituent structures, such as Toll-like receptor (TLR)-2 and TLR-4 and the glycosylphosphatidylinositol-linked protein, CD14, on macrophages located at lipid-laden sites. This leads to an inflammatory response (10), which in turn leads to the modified lipid metabolism observed in the activated macrophages (8).

To our knowledge there are no reports addressing the global effects of *P. gingivalis* LPS on atherosclerosis-related gene expression in macrophages/foam cells in the presence of oxLDL. In this study we therefore tested the hypothesis that *P. gingivalis* LPS could alter the global expression profile of atherosclerosis-related genes in macrophages and foam cells. We investigated the gene profile of macrophages incubated in the presence of oxLDL to tease out the role of *P. gingivalis* LPS on macrophages during and after foam cell formation. Our results indicate that LPS from *P. gingivalis* may stimulate atherosclerosis-related gene expression in macrophages and foam cells in the presence of oxLDL through the enhanced activation of the nuckear factor-kappaB (NF-κB) pathway.

Material and methods

Lipoprotein preparation

LDL (d = 1.019–1.063 g/mL) was isolated by sequential ultracentrifugation of sera from five healthy donors who had fasted for 12–14 h. Informed consent was obtained from the subjects to take part in the study, and the study protocol was approved by the Institutional Review Board of Fujian Medical University, China. The LDL fraction was oxidized by a 12-h treatment with $CuSO_4$, as described previously (11). The extent of lipid oxidation was assessed using a thiobarbituric acid-reactive substance assay to detect malondialdehyde-like compounds. The amount of thiobarbituric acid-reactive substance in our sample was 40 ± 5 nmol malondialdehyde equivalents/mg of protein. The endotoxin content of all LDL preparations was measured using the *Limulus* amebocyte lysate kit (Houshiji, Xiamen, China) and was always ≤ 5 pg/mL of LDL protein. The LDL and the oxLDL were stored under N_2 at 4°C for up to 2 wk.

Cell culture and stimulation

Human THP-1 monocytes (American Type Tissue Culture Collection, Camden, NJ, USA) were placed in the wells of 24- or 6-well plates with glass coverslips, then grown in suspension at 37°C and 5% CO_2 in bicarbonate-buffered RPMI 1640 supplemented with 10% fetal bovine serum (v/v), 100 U/mL of penicillin and 100 μg/mL of streptomycin (GIBCO-BRL Life Technologies, Guangzhou, China). The THP-1 monocytes were induced to differentiate into macrophages by 36 h of culture in the presence of 160 nM phorbol 12-myristate,13-acetate (Sigma, St Louis, MO,USA), then cultured in serum-free medium for 24 h before exposure to further stimuli. The effects of LPS on foam cell formation were studied by culturing macrophages under the following conditions: RPMI 1640 alone; RPMI 1640 + 50 μg/mL of oxLDL; and RPMI 1640 + 50 μg/mL of oxLDL + 1 μg/mL of LPS. To study the effects of LPS after foam cell formation, macrophages were first incubated with 50 μg/mL of oxLDL for 48 h to induce the formation of foam cells, and then cultured under the following conditions: RPMI 1640 + 50 μg/mL of oxLDL or RPMI 1640 + 50 μg/mL of oxLDL + 1 μg/mL of LPS. The LPS was sourced from the *P. gingivalis* strain ATCC 33277 (InvivoGen, San Diego, CA, USA). The potency of the *P. gingivalis* LPS used in this study was $> 1.25 \times 10^4$ endotoxin units/mg (as determined using the *Limulus* amebocyte lysate assay), and the purity of this LPS was > 95%.

Oil Red O staining and lipid analysis

Foam cells formation by macrophages, following incubation for 36 or 48 h with 50 μg/mL of oxLDL, was determined by Oil Red O staining. The total and free cholesterol in macrophages was assayed using a biochemical kit (Merck, Darmstadt, Germany) described by Gamble *et al*. (12). The ratio of cholesterol esters to total cholesterol was further assessed to confirm the formation of foam cells.

RT^2 Profiler™ PCR array

The expression of atherosclerosis genes, following stimulation with oxLDL and LPS, was assayed using an atherosclerosis-specific real-time PCR array (OHS-037; SuperArray SABiosciences, Frederick, MD, USA) according to the manufacturer's instructions. Total RNA was isolated from three individual cell cultures in the same treatment using 1 mL of Trizol reagent, as described by the manufacturer (Invitrogen Life Technologies, Carlsbad,

CA, USA). All RNA samples were treated with DNase and purified using the RNeasy kit (QIAGEN Inc., Valencia, CA, USA). The quality of total RNA was assessed by visualization of intact 18S and 28S ribosomal bands on a 1.2% formaldehyde agarose gel. One microgram of total RNA was used to synthesize template cDNA using the SuperArray RT^2 First Strand Synthesis kit according to the manufacturer's protocol. The template cDNA was mixed with $2 \times RT^2$ qPCR Master Mix and 25 μL of the cocktail was aliquoted into each well on the PCR array plate, which contained predispensed gene-specific primer sets. Each plate consisted of a panel of 96 primer sets of 84 atherosclerosis-related genes, plus five housekeeping genes and three RNA and PCR quality controls. The reactions were carried out using a 7300 real-time PCR System (Applied Biosystems, Foster City, CA, USA). PCR amplification followed a two-step cycling programme: a 10-min activation step at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. Details of the RT^2 PCR arrays are available at http://www.sabiosciences.com/. Genes were considered as differentially expressed if there was a twofold difference with statistical significance in their expression values.

Table 1. Primers for real-time PCR

Genes	Primers for real-time PCR	Deactivation temperature (°C)	Amplicon size (bp)
GAPDH	F: 5′-GGGAAACTGTGGCGTGAT-3′ R: 5′-GAGTGGGTGTCGCTGTTGA-3′	59	299
IL1β	F: 5′-ATGATGGCTTATTACAGTGGCA-3′ R: 5′-CTGTAGTGGTGGTCGGAGATT-3′	59	143
IL10	F: 5′-CGCTGTCATCGATTTCTTCC-3′ R: 5′-CAAACTCACTCATGGCTTTGTAGA-3′	59	112
IL12 P40	F: 5′-AGGGGACAACAAGGAGTATGAG-3′ R: 5′-AGGGAGAAGTAGGAATGTGGAG-3′	59	270

Real-time PCR

The RT^2 Profiler™ PCR kit did not include the important inflammatory cytokines interleukin (IL)-1β, IL-10 and IL-12, so expression of the *IL1*β, *IL10* and *IL12* genes was assayed separately by real-time PCR.

Briefly, total RNA was extracted from cells using Trizol reagent and 1 μg was used to synthesize template cDNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Warrington, Cheshire, UK). Gene-specific primers were designed using Taqman Primer Express Software V5.0 (Applied Biosystems,Warrington, UK) and are shown in Table 1. Real-time PCR was performed in a 25-μL reaction volume containing 12.5 μL of SYBR green Master Mix (Applied Biosystems), 2.5 μL (10 μM) of each forward and reverse primer for each gene of interest at a final concentration of 20 pmol, 2.5 μL of cDNA template and 5 μL of RNase-free water. The reactions were performed in triplicate and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a control. The reaction was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) and the PCR amplification followed one cycle of 30 min at 48°C, one cycle of 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. Melting curve analysis was performed to validate that the specific amplicons were free from genomic DNA contamination. The relative expression levels for each gene were normalized by the cycle threshold (C_t) value of the housekeeping gene and calculated using the ΔC_t method.

ELISA

The concentrations of granulocyte–macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein-1 (MCP-1), IL-1β, IL-10 and IL-12 were assayed using an ELISA kit (GM-CSF, IL-10 and IL-12; R&D Systems, Minneapolis, MN, USA; MCP-1 and IL-1β; Jingmei Biotech, Beijing, China), according to the manufacturer's instructions, from supernatants of conditioned medium collected after the cells had been treated with LPS and oxLDL, as described above.

Western blot

Altered expression of nuclear receptor genes detected in the PCR array was further probed by studying activation of the NF-κB pathway by western blot analysis of inhibitory kappa B-α (IκB-α) protein expression. A total of 3×10^6 macrophages in 5 mL of culture medium were cultured in 60-mm discs. The cells were treated with oxLDL and/or LPS for 2 h before being harvested in a lysis buffer. Fifty micrograms of total protein from each sample was denatured, separated an 8% sodium dodecyl sulphate polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was then blocked with 5% (w/v) bovine serum albumin for 1 h at room temperature and incubated with polyclonal rabbit anti-human IκB-α Ig serum (1 : 1000 dilution; Upstate Biotech, Lake Placid, NY, USA), then with a secondary antibody of goat anti-rabbit horseradish peroxidase-linked IgG at 2 μg/mL (Kangchen, Shanghai, China). Actin was used as a loading control using a rabbit anti-actin antibody at1 μg/mL (Kangchen). The bands were visualized using enhanced chemiluminescence. Finally, the signal strength was estimated using the NIH IMAGE J system (http://rsbweb.nih.gov/ij/index.html).

Immunocytochemistry

Activation of the NF-κB pathway was confirmed by probing for the nuclear translocation of NF-κB p65 subunits using immunocytochemistry. Macrophages, grown on glass coverslips, were collected at 0 and 30 min, and at 1, 2 and 4 h, after the addition of oxLDL and LPS. The cellular components were treated according to the manufacturer's protocol and probed with anti-RelA P_{65} (1 : 100 dilution; Santa Cruz Biotech, Santa Cruz, CA, USA),

then incubated with secondary antibodies (Dako, Glostrup, Denmark). The antibody binding was visualized using diaminobenzidine (Dako) in 0.06% hydrogen peroxide, and the number of nuclear-positive cells, out of 400, was counted to determine the rate of positive nuclear translocation.

Statistical analysis

The data shown in all the figures are expressed as means ± standard deviation of at least three repeated experiments. Comparisons of multiple groups were performed by analysis of variance (ANOVA) and a Student-Newman-Keuls post-ANOVA multiple comparison test when the ANOVA was significant. The two-tailed Mann–Whitney *U*-test was applied when two mean values were compared. A p-value of < 0.05 was considered statistically significant.

Results

Analysis of foam cell formation

The rates of cholesterol ester to total cholesterol in the macrophages all exceeded 50% following incubation with 30, 50 or 80 μg/mL of oxLDL for 48 h (Fig. 1A). Obvious formation of intracellular lipid droplets was observable when macrophages were incubated with 50 μg/mL of oxLDL for 48 h (Fig. 1B), which further confirmed foam cell formation.

P. gingivalis *LPS enhanced expression of atherosclerotic genes in oxLDL-induced macrophages*— The expression of about 40 of the 87 atherosclerotic genes was altered following treatment with ox-LDL and LPS, and many of the same genes differentially regulated by oxLDL were also influenced by *P. gingivalis* LPS. Interestingly, most of the genes up-regulated by oxLDL were further up-regulated by LPS, and similarly, genes that were down-regulated by oxLDL were further down-regulated by LPS; *P. gingivalis* LPS therefore enhanced the change in gene expression caused by oxLDL in macrophages (Table 2).

The expression of cell-adhesion molecules, chemokines and growth factors was also up-regulated by oxLDL alone or with LPS. MCP-1 (CCL2), RANTES (regulated on activation, normal, T-cell expressed, and secreted/CCL5), vascular cell adhesion molecule-1 (VCAM-1) and GM-CSF expression was increased by 1.22-, 3.88-, 1.15- and 18.92- fold, respectively, in the oxLDL treatment groups, and increased by 2.87-, 8.55-, 30.48- and 225.91- fold, respectively, in the oxLDL + LPS group. Platelet-derived growth factor-α expression was down-regulated by oxLDL and further down-regulated by the addition of LPS (Table 2).

The apoptotic gene, BCL2-related protein A1 (*BCL2A1*), was up-regulated by stimulation with oxLDL, and further up-regulated by LPS. Genes involved in lipid transport and metabolism were also affected: for example, the expression of ATP-binding cassette, sub-family A-1 (*ABCA1*) and of apolipoprotein A-1 (*ApoA-1*) were increased by 3.50- and 2.17- fold, respectively, in the oxLDL groups and by 3.62- and 1.06- fold in the oxLDL + LPS groups, respectively, whereas the low-density lipoprotein receptor (*LDLR*) was attenuated by 1.96-fold in the oxLDL group and by 2.23-fold in the oxLDL + LPS group. The expression of the transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) was lower in both the oxLDL and oxLDL + LPS groups, by 2.56- and 5.26-fold, respectively (Table 2).

Effects of P. gingivalis *LPS on oxLDL-induced foam cells*— Incubating macrophages with oxLDL for 48 h resulted in even greater changes in gene expression. Twenty-eight genes were either up-regulated or down-regulated in oxLDL-treated macrophages, and 33 genes were either up-regulated or down-regulated in macrophages treated with oxLDL + LPS. The corresponding numbers in foam cells were 42 and 45, respectively, suggesting a different biological response to LPS between macrophages and foam cells (Table 3).

The expression of atherosclerosis-related gene expression in foam cells in response to stimulation with *P. gingivalis* LPS was quite different from that of macrophages: a total of 11 genes were up-regulated and only *IL5*

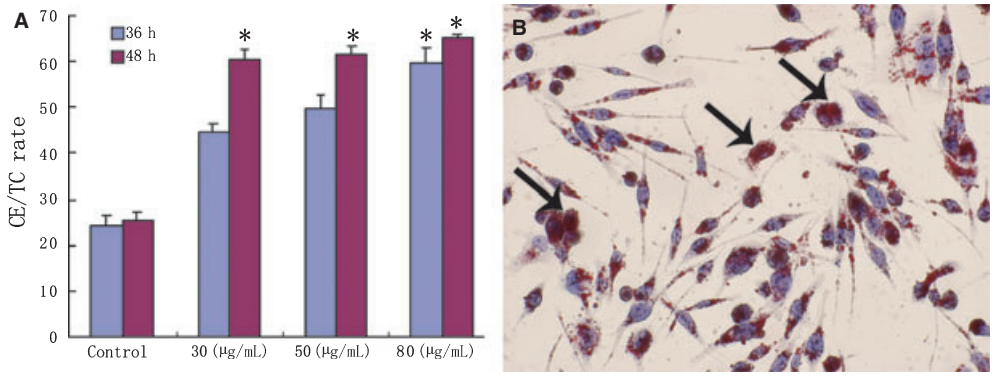

Fig. 1. Foam cell formation after stimulation with oxidized low-density lipoprotein (oxLDL). (A) The ratio of cholesterol ester to total cholesterol (CE/TC rate) was analyzed in macrophages after 36 and 48 h of treatment with 30, 50 or 80 μg/mL of oxLDL. At 48 h, the ratio of cholesterol ester to total cholesterol exceeded 50% in all oxLDL groups (*). (B) At 48 h, numerous lipid droplets aggregated in the cytoplasm in macrophages treated with 50 μg/mL of oxLDL, as shown by Oil Red O staining.

Table 2. Effects of *Porphyromonas gingivalis* lipopolysaccharide (LPS) and oxidized low-density lipoprotein (oxLDL) on atherosclerotic gene expression in macrophages

Gene name	Symbol	Fold changes/control		Functional groups
		oxLDL 2h	oxLDL + LPS 2 h	
Chemokine (C-C motif) ligand 2	*CCL2*	**1.23 ± 0.17**	**2.87 ± 0.24**	Cellular adhesion molecules and chemokines
Chemokine (C-C motif) ligand 5	*CCL5*	**3.88 ± 0.52**	**8.55 ± 0.95**	
CD44 molecule (Indian blood group)	*CD44*	4.17 ± 0.45	7.53 ± 0.23	
Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	*ICAM1*	3.86 ± 0.19	2.94 ± 0.26	
Vascular cell adhesion molecule 1	*VCAM1*	**1.15 ± 0.25**	**30.48 ± 3.02**	
Selectin E (endothelial adhesion molecule 1)	*SELE*	4.63 ± 0.56	4.72 ± 0.35	
Colony stimulating factor 2 (granulocyte-macrophage)	*CSF2*	**18.92 ± 3.56**	**225.91 ± 14.25**	Growth factors
Connective tissue growth factor	*CTGF*	**114.8 ± 13.65**	**28.02 ± 5.35**	
Platelet-derived growth factor beta polypeptide	*PDGFB*	2.46 ± 0.41	1.49 ± 0.45	
Transforming growth factor, beta 1	*TGFB1*	2.08 ± 0.45	1.41 ± 0.24	
Platelet-derived growth factor alpha polypeptide	*PDGFA*	**−1.69 ± 0.56**	**−10.00 ± 11.23**	
B-cell CLL/lymphoma 2	*BCL2*	1.27 ± 0.35	2.18 ± 0.12	Apoptotic genes
BCL2-related protein A1	*BCL2A1*	**11.95 ± 1.25**	**26.22 ± 2.15**	
Baculoviral IAP repeat-containing 3	*BIRC3*	6.14 ± 0.45	10.86 ± 1.25	
CASP8 and FADD-like apoptosis regulator	*CFLAR*	3.18 ± 1.02	3.75 ± 0.65	
Peroxisome proliferator-activated receptor gamma	*PPARG*	**−2.56 ± 0.53**	**−5.26 ± 1.23**	Nuclear receptors and transcriptional factors
Nuclear receptor subfamily 1, group H, member 3	*NR1H3*	**2.25 ± 0.53**	**12.54 ± 2.15**	
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	*NFKB1*	1.44 ± 0.42	2.15 ± 0.35	
ATP-binding cassette, sub-family A (ABC1), member 1	*ABCA1*	3.5 ± 0.38	3.62 ± 0.63	Lipid metabolism-related genes
Apolipoprotein A-I	*APOA1*	**2.17 ± 0.45**	**1.06 ± 0.35**	
Low density lipoprotein receptor (familial hypercholesterolemia)	*LDLR*	−1.96 ± 0.14	−2.23 ± 0.32	
Macrophage scavenger receptor 1	*MSR1*	−2.22 ± 0.65	−1.89 ± 0.61	
Interleukin 1, alpha	*IL1A*	8.53 ± 1.25	7.95 ± 0.35	Cytokines and other genes
Interleukin 1 receptor, type I	*IL1R1*	2.44 ± 0.23	2.78 ± 0.46	
Tumour necrosis factor (TNF superfamily, member 2)	*TNF*	3.22 ± 0.35	3.61 ± 0.64	
Leukaemia inhibitory factor (cho linergic differentiation factor)	*LIF*	**12.6 ± 2.35**	**6.18 ± 1.35**	
Prostaglandin-endoperoxide synthase 1	*PTGS1*	**−2.78 ± 0.25**	**−6.67 ± 1.25**	
Serpin peptidase inhibitor, clade B (ovalbumin), member 2	*SERPINB2*	**−5.88 ± 1.52**	**−100 ± 8.57**	

Genes induced by more than two-fold, compared with the blank control, by stimulation with either oxLDL alone or oxLDL + LPS, are listed according to their function. Data are representative of three experiments.
The values shown in bold indicate changes of more than two-fold between oxLDL alone and oxLDL + LPS groups. Minus values indicate down-regulation.

Table 3. Numbers of genes down-regulated or up-regulated by more than two-fold between groups

	ox 2 h/Ctr	ox + LPS 2 h/Ctr	ox 50 h/Ctr	ox 48 h + LPS 2 h/Ctr	ox 50 h/ ox 2 h	ox 48 h + LPS 2 h/ox 50 h
Up-regulated (*n*)	22	26	27	31	13	11
Down-regulated (*n*)	6	7	15	14	22	1

Ctr, control; LPS, lipopolysaccharide; ox, oxidized low-density lipoprotein.

Table 4. Effects of *Porphyromonas gingivalis* lipopolysaccharide (LPS) and oxidized low-density lipoprotein (oxLDL) on atherosclerotic gene expression in foam cells

Gene name	Symbol	Fold difference relative to the control	
		oxLDL 50 h	oxLDL 48 h + LPS 2 h
Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	*ACE*	−3.12 ± 0.17	1.08 ± 0.35
B-cell CLL/lymphoma 2	*BCL2*	−1.33 ± 0.25	2.90 ± 0.35
BCL2-related protein A1	*BCL2A1*	3.68 ± 0.35	7.56 ± 1.32
Colony stimulating factor 2 (granulocyte-macrophage)	*CSF2*	151.69 ± 15.2	323.35 ± 12.3
Connective tissue growth factor	*CTGF*	7.70 ± 1.20	35.03 ± 3.32
Early growth response 1	*EGR*	2.32 ± 0.75	24.12 ± 2.53
Kinase insert domain receptor (a type III receptor tyrosine kinase)	*KDR*	3.43 ± 1.05	10.45 ± 1.42
Kruppel-like factor 2 (lung)	*KLF2*	4.87 ± 0.35	12.81 ± 1.01
Leukemia inhibitory factor (cholinergic differentiation factor)	*LIF*	11.23 ± 2.12	34.25 ± 2.32
Selectin E (endothelial adhesion molecule 1)	*SELE*	24.87 ± 3.53	58.53 ± 5.12
Nuclear receptor subfamily 1, group H, member 3	*NR1H3*	3.97 ± 0.53	22.96 ± 2.35
Interleukin 5 (colony-stimulating factor, eosinophil)	*IL5*	−2.38 ± 0.48	−5.88 ± 0.43

Genes that were up-regulated or down-regulated by more than two-fold by stimulation with LPS when compared with oxLDL alone are included in the table. Data are representative of three experiments. Minus values indicate down-regulation.

was down-regulated, by 2.5-fold, compared with oxLDL stimulation alone (Table 4). The most strikingly up-regulated gene was *GM-CSF* which was up-regulated by 151.69-fold in cells stimulated with oxLDL alone and by a massive 323.35-fold in cells stimulated with oxLDL + LPS, compared with unstimulated macrophages. Selectin E was up-regulated by 24.87-fold in cells stimulated with oxLDL and by 58.53-fold in cells stimulated with oxLDL + LPS (Table 4).

Effects of P. gingivalis *LPS on expression of the inflammatory cytokines IL1*β*, IL10 and IL12*— The atherosclerosis-specific real-time PCR array kit (RT^2 Profiler™ PCR) does not contain the important inflammatory cytokines IL-1β, IL-10 and IL-12. The expression of *IL1*β, *IL10* and *IL12* genes was therefore studied separately by quantitative real-time PCR, and the results are shown in Fig. 2. Treatment with *P. gingivalis* LPS resulted in significant up-regulation of *IL1*β in both macrophages and

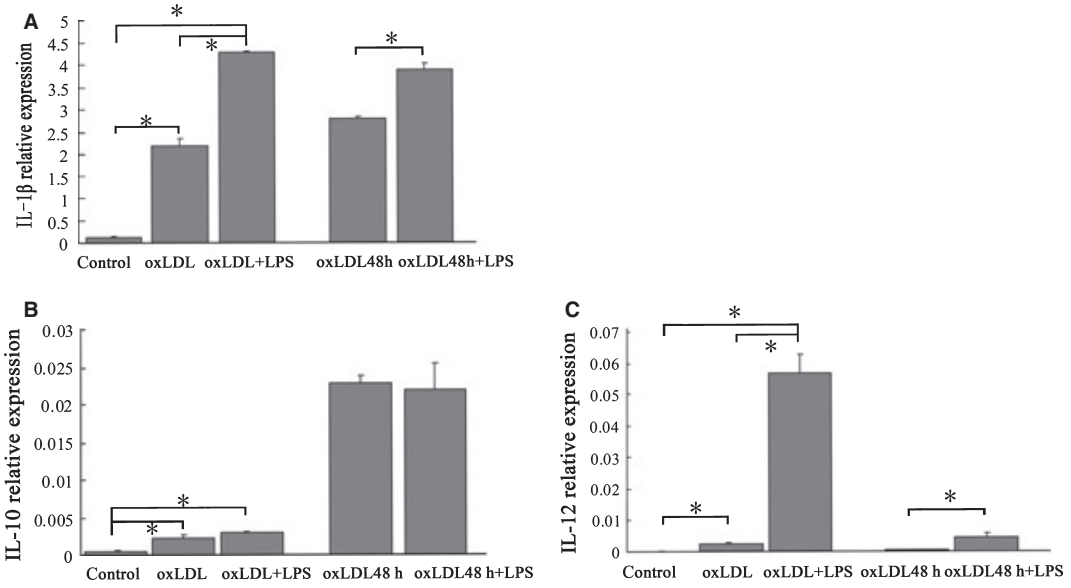

Fig. 2. Effects of *Porphyromonas gingivalis* lipopolysaccharide (LPS) on the transcription of inflammatory cytokines. Cells were incubated with 50 μg/mL of oxidized low-density lipoprotein (oxLDL) alone (oxLDL group) or with 50 μg/mL of oxLDL + 1 μg/mL of LPS (oxLDL + LPS group) for 2 h to examine the effects of LPS on macrophages incubated with oxLDL. Other macrophages were incubated with 50 μg/mL of oxLDL for 48 h in order to induce the formation of foam cells. These cells were then stimulated with or without 1 μg/mL of LPS (oxLDL48 h + LPS and oxLDL48 h respectively) for a further 2 h. The transcription of *IL1*β (A), *IL10* (B) and *IL12* (C) was evaluated using real-time PCR. The results shown in the graphs are representative of three experiments. Error bars represent the standard deviation. *$p < 0.05$.

foam cells, with an increase of more than two-fold in macrophages but of only 0.4-fold in foam cells compared with the cells cultured without LPS (Fig. 2A). The expression of *IL12* increased by a robust 26.84-fold in macrophages and by 16.34-fold in foam cells (Fig. 2B) as a result of stimulation with LPS, whereas *IL10* was not significantly influenced by LPS stimulation compared with oxLDL pre-incubated macrophages and foam cells (Fig. 2C).

Validation of the mRNA expression of selected genes induced by P. gingivalis *LPS by ELISA assay*— The concentrations of GM-CSF, MCP-1, IL-1β, IL-10 and IL-12 protein in the supernatants at 2-, 6- and 12-h time-points during incubation with oxLDL and oxLDL + LPS were determined to confirm the mRNA data obtained in both the real-time PCR and PCR array studies. LPS from *P. gingivalis* enhanced the production of GM-CSF during the formation of foam cells, and enhanced the secretion of MCP-1 in both oxLDL-induced macrophages and foam cells at 6 and 12 h (Fig. 3A

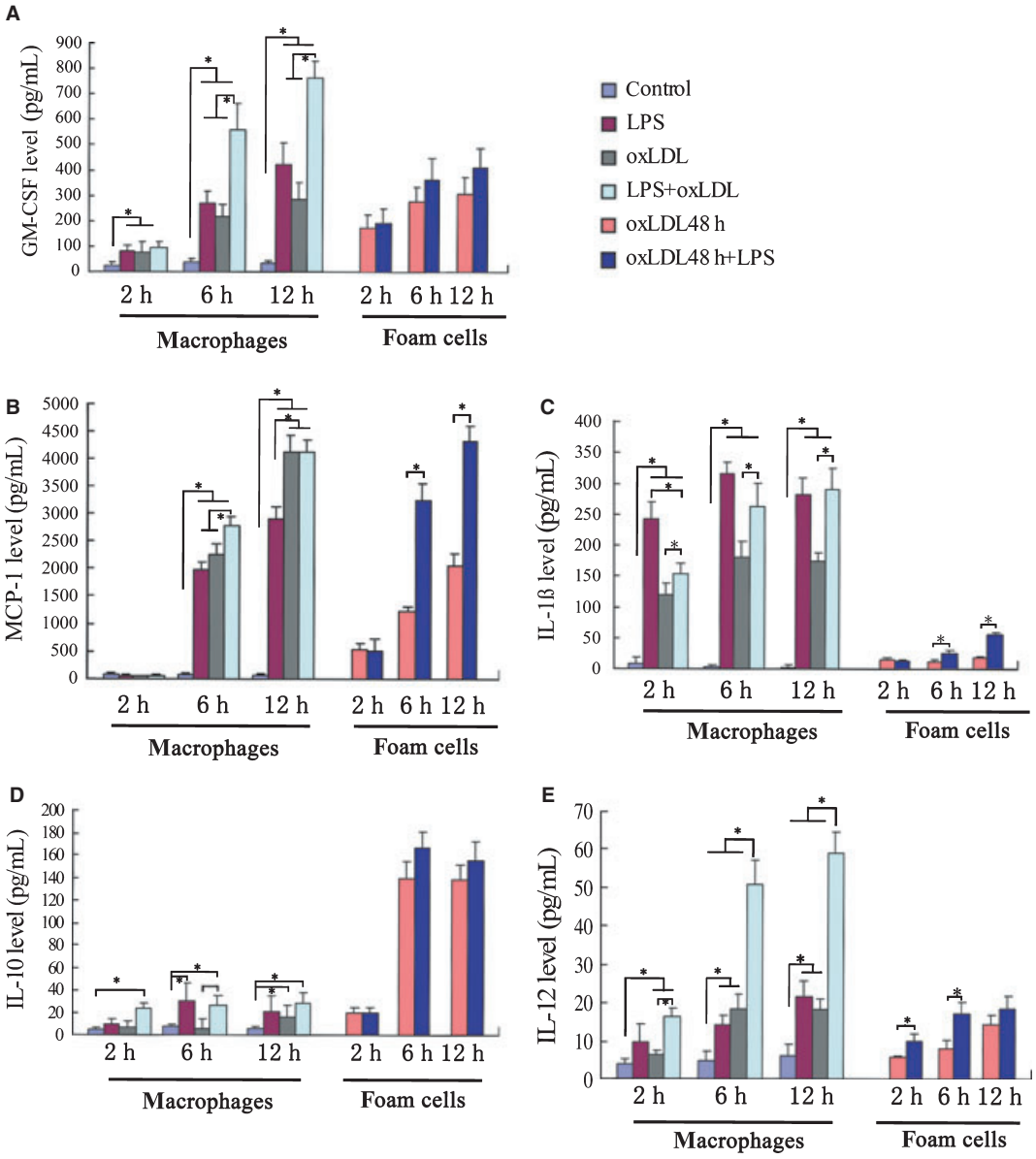

Fig. 3. Concentrations of granulocyte–macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein-1 (MCP-1), interleukin (IL)-1β, IL-10 and IL-12 in the supernatants of the macrophage and the foam cell cultures. Macrophages and foam cells were loaded with 50 μg/mL of oxidized low-density lipoprotein (oxLDL) and 1 μg/mL of *P. gingivalis* lipopolysaccharide (LPS) for 2, 6 and 12 h, and the concentrations of GM-CSF (A) MCP-1 (B), IL-1β (C), IL-10 (D) and IL-12 (E) were evaluated by ELISA to prove the transcriptional data Tables 2 and 4 (GM-CSF and MCP-1), and Fig. 2 (IL-1β, IL-10 and IL-12). The graphs are representative of three independent experiments. $^*p < 0.05$. Error bars represent the standard deviation.

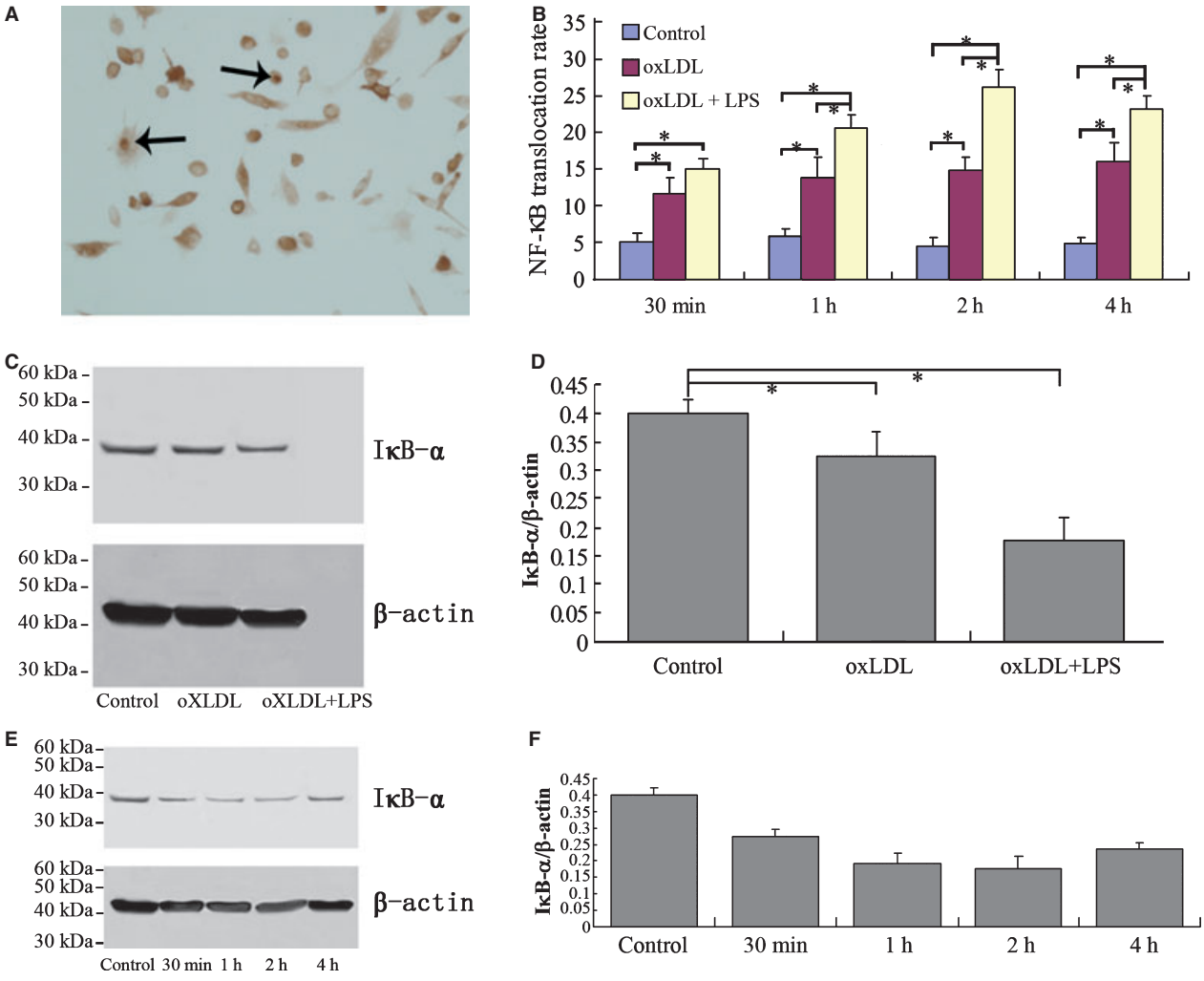

Fig. 4. Nuclear factor-kappaB pathway activation in macrophages treated with 50 μg/mL of oxidized low-density lipoprotein (oxLDL) and 1 μg/mL of lipopolysaccharide (LPS). (A) Immunocytochemistry was used to show nuclear translocation-positive macrophages (arrows). (B) Calculation of the ratio of nuclear translocation-positive cells. (C, D) Levels of inhibitory kappa B-α (IκB-α) in macrophages treated with oxidized low-density lipoprotein (oxLDL) alone or with LPS was evaluated by western blotting, 2 h after the start of treatment. (E, F) Concentration of IκB-α in the group stimulated with 50 μg/mL of oxLDL + 1 μg/mL of LPS. The graphs are representative of three experiments. Error bars represent the standard deviation. $*p < 0.05$.

and 3B). The concentrations of the proinflammatory cytokines IL-1β and IL-12 were significantly higher following stimulation with *P. gingivalis* LPS + oxLDL compared to stimulation with LDL alone, during and after the formation of foam cells (Fig. 3C and 3E). However, the concentration of the prominent anti-inflammatory cytokine, IL-10, was not significantly influenced by stimulation with LPS compared to macrophages and foam cells incubated with oxLDL alone (Fig. 3D). These data suggest that *P. gingivalis* LPS had a predominantly additive effect on proinflammatory cytokines in oxLDL-induced macrophages and foam cells.

NF-κB pathway activation

NF-κB P_{65} translocation was seen as early as 15 min in the experimental groups (Fig. 4A), but was not detectable in the controls (result not shown). NF-κB P_{65} translocation was significantly greater in the oxLDL + LPS group compared with the oxLDL-only group (Fig. 4A and 4B). Cytoplasmic IκB-α levels at 2 h decreased proportionally with the increased NF-κB P_{65} translocation, the levels of IκB-α in the cytoplasm showed a decrease at 2 h (Fig. 4C and 4D) and a time-dependent decrease was seen in the oxLDL + LPS group (Fig. 4E and 4F), which confirmed NF-κB activation. The influence of *P. gingivalis* LPS on the NF-κB translocation rate and IκB-α expression also appeared to be concentration-dependent.

Discussion

P. gingivalis is an important aetiological agent of chronic adult periodontitis and may be associated with systemic diseases, including atherosclerosis. LPS is a major structural component of the outer cell membrane of gram-negative bacteria, such as *P. gingivalis* (13). LPS from *P. gingivalis* induces an inflammatory response, mainly through

activation of TLR2, and affects a less potent response from the innate immune system compared with LPS derived from other gram-negative bacteria, such as *Escherichia coli* (14). ApoE-deficient mice develop significantly increased artherosclerotic lesions in response to blood-infused *P. gingivalis* LPS (5). In the present study we were able to demonstrate that *P. gingivalis* LPS plays an important role by modulating atherosclerosis-related gene expression in macrophages and in foam cells.

P. gingivalis LPS has been shown to have greater ability than live *P. gingivalis* to induce the production of IL-8, MCP-1, RANTES, VCAM-1 and vascular endothelial growth factor, suggesting that LPS from *P. gingivalis* may exert a more profound atherogenic role than does live *P. gingivalis* (15). In this study we demonstrated that *P. gingivalis* LPS up-regulated, in macrophages, the expression of adhesion molecules such as CD44, VCAM-1, Selectin E and intercellular adhesion molecule 1; chemokines such as MCP-1 (CCL2) and RANTES (CCL5); and growth factors such as granulocyte-macrophage colony-stimulating factor (GM-MSF). Moreover, *P. gingivalis* LPS also increased the expression of Selectin E, GM-MSF and connective tissue growth factor in oxLDL-induced foam cells. Increased expression of these molecules may enhance the aggregation of additional monocytes, smooth muscle cells and T lymphocytes into the lipid-laden atherosclerotic lesions, and thereby participate in the progression of atherosclerosis.

It is now well established that atherosclerosis, besides being a lipid disorder, is also symptomatic of a state of chronic inflammation (6). Macrophages, when activated, produce both pro- and anti-inflammatory cytokines, which affect surrounding cells as well as having an autocrine effect (14). Our PCR results suggest that, in oxLDL-induced macrophages and foam cells, *P. gingivalis* LPS enhances the inflammatory process by up-regulating transcription of the proinflammatory factors IL-1β and IL-12. The inflammatory response is a tightly regulated system, the activation of which is under the control of autocrine feedback mechanisms. IL-10 is a major anti-inflammatory cytokine, and our results showed that IL-10 was more strongly up-regulated in foam cells than in macrophages, suggesting that when exposed to a prolonged inflammatory challenge the host immune cells may try to suppress the inflammatory process by increasing the expression of IL-10. These results contrast to those reported by Groeneweg *et al.* (16) who found lower expression of IL-10 in foam cells than in macrophages**.** Interestingly, we did not find that the expression of IL-10 in foam cells was affected by *P. gingivalis* LPS, and together these data suggest that *P. gingivalis* LPS exacerbates the inflammatory response in host cells through a process of increased proinflammatory cytokine expression rather than by inhibiting the anti-inflammatory pathway.

Apoptosis may be another potential mechanism through which oxLDL influences the inflammatory response. This is all the more possible given that the expression of pro-apoptotic genes, such as B-cell CLL/lymphoma 2 (*BCL-2*), BCL2-related protein A1 (*BCL2A1*), Baculoviral IAP repeat containing 3 (*BIRC-3*), and anti-apoptotic genes, such as CASP8 and FADD-like apoptosis regulator (*CFLAR*), were affected by oxLDL stimulation. The resulting enhanced apoptotic gene responses indicated that the balance between pro-apoptotic and anti-apoptotic gene expression was disturbed in macrophages and foam cells; a TdT-mediated biotin–dUTP nick-end labelling (TUNEL) assay could be used to detect whether *P. gingivalis* LPS leads to increased DNA fragmentation, confirming activation of the apoptotic pathway.

The PCR array data suggest that *P. gingivalis* LPS have an additive effect on oxLDL-regulated genes, further activating the nuclear receptor subfamily 1, group H, member 3 (*NR1H3*) and inhibiting *PPAR*γ. This supports the notion that LPS functions as a negative regulator of PPARγ in macrophages, and that this may, in fact, accelerate the formation of foam cells (17). Changes such as these, to nuclear receptors and transcriptional factors, lead to extensive changes to the transcription of downstream genes. *ABCA1*, an integral membrane protein that transports intracellular cholesterol out of the membrane (18), which is influenced by the PPARγ level, was up-regulated by stimulation with oxLDL or with oxLDL + LPS. It may be that such effects are part of a feedback mechanism used by the host cells to reduce the increased intracellular lipid extent. Whereas we saw an increased transcription of *ABCA1*, Ma *et al.* (19) report that *ABCA1* expression is decreased in foam cells isolated from smooth muscle tissues. It appeared that, as a response to increased intracellular lipid extent, macrophages transcribed fewer LDL receptors and macrophage scavenger receptor-1 (*MSR1*) genes; this would have the effect of decreasing inward lipid transport, as well as increasing outward lipid transport.

The PCR array data showed enhanced NF-κB pathway activation in response to *P. gingivalis* LPS and oxLDL stimulation. The transcriptional factor NF-κB is an important player in gene regulation during inflammatory reactions, immune reactions and cell apoptosis (20). Activated NF-κB has been identified in atherosclerotic lesions (e.g. in monocytes/macrophages or endothelial cells) (21). NF-κB exists as a dimer that is trapped in the cytosol in an inactive state by inhibitory proteins, including IκB-α. The activation of NF-κB is facilitated by the IκB-kinase (IKK) complex, which consists of IKK-α, -β and -γ. Activation of IKK leads to IκB phosphorylation, which is subsequently degraded by the proteasome, followed by nuclear translocation of NF-κB (22). An increased NF-κB nuclear translocation rate with a simultaneously decreased concentration of IκB-α in the cytosol was observed in the oxLDL + LPS stimulation group; thus, our results indicate a possible role for NF-κB in mediating the expression of pro-atherosclerotic genes and cytokines, thus leading to foam cell formation, a crucial event in atherogenesis.

Alterations in the NF-κB pathway gave us a clue towards understanding

the possible mechanism of *P. gingivalis* LPS on the role of oxLDL in foam cells. *P. gingivalis* LPS and oxLDL may together interact with cell constituent structures, such as TLR-2 and TLR-4, and the glycosylphosphatidylinositol-linked protein, CD14, on macrophages, and therefore activate the same pathway, such as the NF-κB pathway investigated in this study. This synergistic effect of oxLDL and LPS from *P. gingivalis* generated pleiotropic roles, which in turn led to altered cell function and morphology in foam cells.

Here, we report some of the cellular responses of oxLDL-induced macrophages and foam cells to *P. gingivalis* LPS. The particular focus of this study was the effects of LPS on ox-LDL-induced macrophages during and after foam cell formation. For this reason, for the most part we did not include a treatment group of *P. gingivalis* LPS alone; however, we did include an LPS control group with the ELISAs for the purpose of exploring the inflammatory effect of LPS and the time-dependent response. Groeneweg *et al*. (16) reported that prior exposure to oxLDL enhanced *E. coli* LPS-induced gene expression in murine macrophages. Our experimental model revealed a different gene-expression profile by *P. gingivalis* LPS in oxLDL-induced macrophages and foam cells.

It is known, from previous publications, that LPS from *P. gingivalis* and *E. coli* activates different TLRs and intracellular signalling pathways. *P. gingivalis* LPS acts mainly through the TLR2–JNK pathway to induce cytokine production in THP-1 macrophages, whereas *E. coli* LPS acts mainly via the TLR4–p38MAPK pathway to induce cytokines (23). Thus, *P. gingivalis* LPS may generate an atherosclerotic gene profile that is different from that of *E. coli* LPS; however, future work is needed to discriminate whether the differential expression of atherosclerotic gene expression is a general phenomenon under LPS stimulation or species dependent. One more limitation of this study is that we only used a commercially available LPS from *P. gingivalis* ATCC 33277). As *P. gingivalis* produces different isoforms of LPS under different growth conditions (24), it will be interesting to investigate whether other isoforms of LPS from *P. gingivalis* will have a similar effect on the alteration of atherosclerotic genes in macrophages and foam cells. One more limitation of the current study is that we did not include a live strain of *P. gingivalis*. As common gentle daily activities, such as tooth brushing, are a potential source of bacteraemia, it will be very interesting to compare the effect, on atherosclerosis, of live *P. gingivalis* with LPS from *P. gingivalis* or other periodontal pathogens.

Based on the atherosclerosis-related genes examined in this study, albeit a limited number, we propose these tentative conclusions: (i) *P. gingivalis* LPS appears to enhance the expression of genes related to cell chemotactic activity, adhesion and growth, as well as genes related to apoptosis in both macrophages and foam cells; and (ii) *P. gingivalis* LPS up-regulates nuclear receptor expression and enhances the activation of the NF-κB pathway, which leads to pleiotropic changes in gene expression in response to LPS stimulation in these cells.

A better understanding of the molecular mechanisms involved in periodontal pathogen infection and atherogenesis should give us a clearer view of the correlation that exists between periodontal infection and atherosclerosis.

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