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Fusobacterium nucleatum regulation of neutrophil transcription

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Background and Objective: Abnormal neutrophil responses have been observed in periodontitis patients, including hyper-reactivity in terms of production of reactive oxygen species (ROS) following exposure to the key quorum-sensing plaque bacterium, *Fusobacterium nucleatum*. This study was designed to characterize the transcriptional response of neutrophils to *F. nucleatum*.

Material and Methods: Peripheral blood neutrophils were exposed to *F. nucleatum*, and gene expression was analysed using high-throughput transcriptomics.

Results: Microarray technology demonstrated differential expression of 208 genes (163 increased and 43 decreased relative to control genes), which identified regulation of several ontological classes, including signal transduction (13%), transcription regulation (7%) and ROS response (14%). Individual gene expression analysis of selected transcripts, including *CSF*, *CXCL3*, *FOS*, *HMOX1*, *HSP40*, *SOD2*, *NFKB2* and *GP91*, in individual and pooled RNA samples from control and *F. nucleatum*-exposed neutrophils corroborated microarray data. Analysis of ROS generation, combined with transcript analysis, in response to a panel of proinflammatory stimuli (*F. nucleatum*, *Porphyromonas gingivalis*, *Escherichia coli* lipopolysaccharide and opsonized *Staphylococcus aureus*) identified significant differences in ROS and transcript regulatory control. Further analyses of neutrophils from periodontitis patients and periodontally healthy control subjects stimulated with *F. nucleatum* indicated significant differential induction of several ROS response-related transcripts.

Conclusion: These data demonstrate that neutrophils are transcriptionally active in response to the periodontal pathogen *F. nucleatum* and that these changes in gene expression are likely to affect neutrophil function. The differential response of neutrophils to a range of stimuli combined with data demonstrating differences between patient and control neutrophils indicate the importance of this cell and its interaction with the local tissue environment in the pathogenesis of periodontitis.

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Chronic periodontitis is an inflammatory disease driven by microbial plaque accumulation. The microflora that accumulate within the gingival crevice illicit an inflammatory/immune response, which current evidence indicates is dysregulated in predisposed individuals and leads to a failure to eliminate causative pathogens and a nonresolving inflammation (1,2). The oral microflora is diverse, comprising over 1200 species (3), although only a few are implicated as being key to progression of periodontitis. Studies have reported that the principal cultivable bacteria associated with periodontitis are *Porphymonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Treponema denticola*, *Capnocytophaga* spp. and *Fusobacterium* spp., including *F. nucleatum* (4). *F. nucleatum* is a gram-negative anaerobe present at high frequency and number in the subgingival plaque biofilm in periodontal

lesions. Notably, it is a key quorum-sensing microorganism that is able to aggregate with other plaque bacteria to form a bridge between early and late colonizers of the tooth surface. Potentially, this transition is central to periodontal disease pathogenesis as the microflora develops from a non-pathogenic to a pathogenic state (5). In addition, *F. nucleatum* can internalize within oral keratinocytes and fibroblasts and can facilitate this process for other periodontal bacteria (6,7). Whilst pre-inocculation with *F. nucleatum* can cause reduced levels of serum antibodies to *P. gingivalis* (8), it also stimulates production of proinflammatory mediators, including interleukin (IL)-1, IL-6, IL-8 tumour necrosis factor α (TNF-α) and reactive oxygen species (ROS) from neutrophils (9). Host cells and tissues are able to detect *F. nucleatum* via Toll-like receptors (TLRs)-2 and -4 (10–12), and *in vitro* data using gingival epithelial cells have demonstrated that TLR-2 activation by *F. nucleatum* results in the up-regulation of the antimicrobial peptides β-defensin-2 and -3 (13).

Neutrophils are the primary host immune cell involved in the defense of the periodontium against invading plaque bacteria. They are the most abundant leukocyte within the periodontal environment, comprising approximately 50% of the inflammatory cell infiltrate within the junctional epithelium and 90% of the infiltrate within gingival crevicular fluid (14). Neutrophils reportedly express all known TLRs, with the exception of TLR-3 (15), and *F. nucleatum* is known to stimulate neutrophils by increasing their release of elastase, cytokines and ROS (9,16–18). Host neutrophil hyperreactivity is likely to be a key determinant of periodontal disease progression as excessive or prolonged local release of cytokines, enzymes and ROS in response to plaque bacteria leads to collateral host-tissue damage. Indeed, recent data indicate that neutrophils from periodontitis patients exhibit a hyper-reactive phenotype following *F. nucleatum* exposure in terms of their ROS production (18). The ROS produced subsequently cause lipid peroxidation and oxidative molecular damage within the neutrophils themselves as well as having a similar effect on adjacent cell and tissues (9,19). Production of ROS in neutrophils can be induced in response to a range of stimuli, including *E. coli* lipopolysaccharride (LPS), opsonized bacteria or zymosan, phorbol 12-myristate 13-acetate, *N*-formyl-Met-Leu-Phe, oxidized low-density lipoprotein and advanced glycation end-products (via complementary receptor ligands). Notably, these stimulants use a number of different pathways of activation (16,17). Interestingly, it has been reported that the level of ROS generated by neutrophils in response to *F. nucleatum* is greater than that observed for *P. gingivalis* or *Aggregatibacter actinomycetemcomitans* activation (17).

Recent work has demonstrated that whole bacteria or their components (e.g. LPS, CpG DNA) stimulate significant transcriptional changes within neutrophils (20–22). Differential effects with regard to gene expression activation have also been noted between bacteria, and this may also correlate with their degree of pathogenicity (23–25). It is therefore important that studies regarding the effect of individual bacteria on cells key to the progression of periodontitis are performed (26), as well as biofilm studies. As *F. nucleatum* may, in part, be responsible for the aberrant immune response observed in patients with periodontitis and as there are currently no data on the transcriptional response of neutrophils to *F. nucleatum*, the aim of the present study was to perform high-throughput gene expression analysis to identify regulatory changes that may be important in the immunobiology of periodontitis pathogenesis.

Material and methods

Study populations

Patients with chronic periodontitis (n = 5; two males and three females; mean age, 45.6 ± 6.1 years; range, 36–53 years) were recruited from the population of patients referred to the periodontal department of Birmingham's Dental Hospital. Chronic periodontitis was defined as previously reported (27), i.e. the presence of at least two nonadjacent sites per quadrent with probing pocket depths ≥ 5 mm, which bled on gentle probing, demonstrated radiographic bone loss ≥ 30% of the root length and were not first molar or incisor sites. Periodontally healthy subjects (n = 14; eightmales and six females; mean age, 44.9 ± 10.3 years; range, 31–60 years), which included five age- and sex-matched controls to the periodontitis patients, were recruited from staff of the Dental Hospital. All volunteers were systemically healthy, and exclusion criteria included a course of nonsteroidal anti-inflammatory drugs or antimicrobial drugs within a 3 month period prior to enrolment, pregnancy, use of mouthwashes or vitamin supplements within the previous 3 months. All patients had never been smokers, had a negative history of current or previous recreational drug use and had no special dietary requirements. This population has previously been investigated in both cross-sectional and longitudinal studies regarding the effects of nonsurgical periodontal therapy upon peripheral blood neutrophil hyper-responsiveness (18,28) and local and systemic antioxidant levels (27,29). Ethical approval for this work was granted by South Birmingham Local Research Ethics Committee (LREC 5643). Informed consent to participate was initially obtained, followed by the completion of a medical questionnaire.

Collection of venous blood

Venous blood was collected in 8mL aliquots from patients and corresponding age- and sex-matched control subjects (simultaneously) from the antecubital fossa into Vacutainer™ (Becton, Dickinson U.K. Limited, Oxford, UK) lithium heparin (17 IU/mL) tubes following an overnight fast when subjects were also asked to refrain from drinking (except water) or chewing gum. These blood samples were used to provide neutrophils for stimulation with *F. nucleatum* and subsequent RT-PCR analysis to determine any differences in stimulated and unstimulated gene expression between patients and control subjects.

Venous blood was collected in a similar manner from periodontally

healthy control subjects ($n = 9$) to provide neutrophils for treatment with various stimuli and subsequent microarray, RT-PCR and enhanced chemiluminescence (ECL) analysis.

Bacterial culture and suspensions

The periodontopathogens *F. nucleatum* (ATCC 10953) and *P. gingivalis* (ATCC 33277) were grown anaerobically at 37°C as described previously (30). Bacteria were isolated from broth cultures by centrifugation, washed three times in sterile phosphate-buffered saline (PBS) and heat treated (100°C for 10 min) prior to dilution with sterile PBS to give a final stock suspension of 4×10^8 cells/mL, which was stored at −30°C. Opsonized *Staphylococcus aureus* (NCTC 6571) was prepared and stored as described previously (18).

Preparation of neutrophils

Neutrophils were isolated from venous blood (lithium heparin) as previously described (18,28) using a discontinuous Percoll gradient (delta = 1.079:1.098) followed by erythrocyte lysis (0.83% NH_4Cl containing 1% $KHCO_3$, 0.04% $Na_2EDTA.2H_2O$ and 0.25% bovine serum albumen). Isolated cells were resuspended in PBS supplemented with glucose (1 mM) and cations (1 mM $MgCl_2$ and 1.5 mM $CaCl_2$) at 1×10^6 cells/mL. Cell viability, typically > 98%, was determined using dye exclusion (trypan blue).

Enhanced chemiluminescence assay

All assays were performed as previously described (18,28). In brief, neutrophils (1×10^5) were placed in preblocked (PBS containing 1% bovine serum albumin, overnight at 4°C) white microwells (Microlite2, Dynex, Huntingdon, UK) with supplemented PBS (35 μL) and luminol (3 mmol/L; 30 μL), for detection of total ROS production, or isoluminol (3 mmol/L; 60 μL) with 6 U horseradish peroxidase, for detection of extracellular ROS release. Following equilibration in the microplate reader (37°C, Berthold microplate-luminometer, LB96v, Berthold Technologies (U.K.) Ltd., Harpenden, UK) for 30 min, cells were stimulated with 25 μL *F. nucleatum* (100 bacteria per neutrophil), *P. gingivalis* (100 bacteria per neutrophil), opsonized *S. aureus* (300 bacteria per neutrophil), LPS (1 μg per 10^5 neutrophils; serotype 026:B6; Sigma, Dorset, UK), or PBS as a control. All analyses were performed in triplicate. Light emission in relative light units (RLUs) was recorded both pre- and post-stimulation (150 min) and the peak signal determined.

Stimulation of neutrophils prior to RNA extraction

Five hundred microlitres of supplemented PBS containing either *F. nucleatum* (2×10^5 bacteria/μL; 100 bacteria per neutrophil), *P. gingivalis* (2×10^5 bacteria/μL; 100 bacteria per neutrophil), opsonized *S. aureus* (6×10^5 bacteria/μL; 300 bacteria per neutrophil), LPS (20 ug/mL; 1 μg per 10^5 neutrophils) or PBS was carefully mixed with 1 mL of isolated neutrophils (1×10^6 cells) in 2 mL Eppendorf tubes. Mixtures were then incubated, exposed to air at 37°C, for 3 h. Following stimulation, cells were pelleted by centrifugation (2 min, 100*g*), and the pellet resuspended in 1 mL of TRIzol (Sigma). After phenol–chloroform extraction (Sigma, Gillingham, UK), the aqueous phase was combined with 70% ethanol and added to an RNeasy mini-column (Qiagen, Crawley, UK). Subsequent purification and DNase treatment were performed as recommended by the manufacturer (Qiagen). The RNA was eluted in 30 μL of sterile water, and concentrations were determined from absorbance values at 260 nm using a BioPhotometer (Eppendorf, Cambridge, UK). Integrity of the RNA was verified by visual inspection of samples on 1% nondenaturing agarose gels stained with SYBR Gold (Molecular Probes, Paisley, UK).

Microarray target preparation, hybridization and analysis

Total RNA was isolated from pairs of *F. nucleatum*-stimulated and unstimulated cells from healthy control subjects ($n = 4$). Samples were pooled to give stimulated and unstimulated RNA preparations, which were analysed using human Affymetrix HG_U133A oligonucleotide arrays, as described at http://www.affymetrix.com/products/arrays/specific/hgu133.affx. Total RNA from each sample was used to prepare biotinylated target RNA, according to the manufacturer's instructions (http://www.affymetrix.com/support/technical/manual/expression_manual.affx).

Briefly, DNase-digested total RNA (5 μg) was used to generate double-stranded cDNA using Super-Script reagents (Life Technologies, Paisley, UK) and a T7-linked oligo(dT) primer. Then cRNAs were synthesized using the EnzoBioarray High Yield RNA transcript labelling kit (Affymetrix, Maidenhead, UK), and resulting biotinylated labelled cRNA was subsequently fragmented into 35–200 bp lengths using Fragmentation buffer (Affymetrix). As recommended by the manufacturers, RNA, cDNA and cRNA quality and size distribution were visually confirmed by 1.5% agarose gel electrophoresis. Spike controls B2, bio-B, bio-C, bio-D and Cre-x were added to the hybridization cocktail before overnight hybridization at 45°C for 16 h. Arrays were stained and washed on the Fluidics Station 400 (Affymetrix) using the EukGE-WS2 protocol (dual staining) before being scanned twice on the GeneChip Scanner 3000 at an excitation wavelength of 488 nm. The integrity and quality of prepared samples was confirmed using Affymetrix Test3 GeneChips. HG_U133A microarrays (Affymetrix) were subsequently hybridized with the cRNA samples. Analysis of control parameter data (as described for Test3 GeneChip analysis) confirmed hybridization success (data not shown), and scaling factors were within Affymetrix recommended guidelines.

Microarray data analysis

For analysis of hybridization results, which all met minimum requirements for data quality and distribution, raw data files were exported from Affymetrix microarray suite 5.0 software (MAS 5.0; Affymetrix) into GeneSpring 5.1 software (Silicon Genetics, San Carlos, CA, USA). Values were normalized to

the median signal values for each array. Based on previous experiences, Affymetrix and GeneSpring recommendations and published literature (31,32), genes with at least a twofold change in expression level and with a signal intensity value of > 100 on either the control or test arrays were classified as being differentially expressed. The remaining genes were considered informative and were subjected to Student's paired *t*-test using a global error model with the variance statistic derived from replicates. Finally, to reduce false-differential gene expression, a Bonferroni multiple testing correction filter was applied (33). Microarray analyses were Minimum Information About a Microarray Experiment compliant, and raw data files are available in the Gene Expression Omnibus database under the series number GSE20151 (http://www.ncbi.nlm.nih.gov/geo/).

Synthesis of cDNA and semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR was performed on samples of the pooled RNA preparations used for microarray analysis and on individual RNA samples extracted from healthy control neutrophils ($n = 9$), with and without pretreatment with *F. nucleatum*. In addition, RT-PCR was performed on pooled RNA samples extracted from healthy control neutrophils ($n = 5$), with and without pretreatment with *F. nucleatum*, *P. gingivalis*, opsonized *S. aureus*, LPS or PBS and on individual RNA isolated from *F. nucleatum*-stimulated and unstimulated patient ($n = 5$) and age- and sex-matched control neutrophils ($n = 5$).

For cDNA synthesis, 2 μg of total RNA was used for oligo-dT (Ambion, Huntingdon, UK) reverse transcription to generate single-stranded cDNA (Omniscript kit; Qiagen). Concentrations of cDNA were determined using a Biophotometer (Eppendorf). Semi-quantitative RT-PCR analysis for the genes chosen was performed using the housekeeping gene *GAPDH* as a control. Typically, 50 ng of cDNA were used to seed 50 μL REDTaq PCR mixes (Sigma) and subjected to between 25 and 40 cycles. A typical amplification cycle of 95°C for 20 s, 60/61°C for 20 s and 72°C for 20 s was performed using a Mastercycler thermal cycler (Eppendorf). Following the designated number of cycles, 7 μL of the reaction was removed and PCR products separated and visualized on a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide. Scanned gel images were imported into AIDA image analysis software (Fuji, Sheffield, UK), and the volume density of amplified products was calculated and normalized against *GAPDH* control values. Primer sequences for the genes analysed and cycling conditions used are provided in Table 1. Primers were designed from the Affymetrix probe target ID sequences using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_ http://www.cgi).

Data handling and statistical analysis

Chemiluminescent data were recorded automatically and collected into Excel spreadsheets (Microsoft, Redmond, WA, USA). Manipulation of data was performed in Excel and statistical evaluation performed using Instat 3.2 (GraphPad, Aberystwyth, UK). Differences in gene expression and neutrophil chemiluminescence were performed by Wilcoxon rank sum test. A level of $p < 0.05$ was employed for assigning statistical significance.

Results

Gene expression analysis in *F. nucleatum*-stimulated neutrophils

Initial assessment revealed that while granulocyte colony-stimulating factor receptor (GCSFR), CD45 and liver/bone/kidney alkaline phosphatase transcripts were detected at high signal levels, the macrophage colony-stimulating factor receptor transcript was

Table 1. Details of primer sequences and semi-quantitative RT-PCR conditions

Gene	Symbol	Sequence	Product	Cycle no.
Colony-stimulating factor 1 (macrophage)	*CSF1*	Forwards: GCACTAATTGGGTCCCAGAA Reverse: GATGC AGGGAGTGGAGAAGA	159 bp	33
Chemokine (C-X-C motif) ligand 3	*CXCL3*	Forwards: TAAATGACAGGGTGGGGAAC Reverse: GCATTATGCCCTACAAGCAA	224 bp	42
Nuclear factor-κB 2	*NFKB2*	Forwards: CGTACCGACAGACAACCTCA Reverse: CCGTACGCACTGTCTTCCTT	187 bp	27
Heme oxygenase 1	*HMOX1*	Forwards: AACCTCCAAAAGCCCTGAGT Reverse: CACCC CAACCCTGCTATAAA	207 bp	33
Superoxide dismutase 2	*SOD2*	Forwards: TGGAGGCATCTAGTGGAAAAA Reverse: CCCAGTCTCTCCCCATTACA	186 bp	27
Heat shock protein 40	*HSP40*	Forwards: TACAGGAGCACTGTGGAACG Reverse: AGGTCTGAGCACTGGACTGG	192 bp	30
Cytochrome *b*-245/phox gp91	*GP91–phox*	Forwards: GCTGTTGAATGCTTGTGGCT Reverse: TCTCCTCATCATGGTGCACA	325 bp	33
FOS	*FOS*	Forwards: CCTCTTCCGGAGATGTAGCA Reverse: TCCAGCACCAGGTTAATTCC	184 bp	30
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	Forwards: TCTAGACGGCAGGTCAGGTCC Reverse: CCACCCATGGCAAATTCCATG	391 bp	26

All DNA sequences are shown in the 5′ to 3′ orientation.
F, forward primer; R, reverse primer.

absent, indicating that there was no detectable monocytic contamination of the isolated neutrophil RNA samples.

Pairwise analysis of hybridization data indicated that of the 5680 genes detected as being present in both targets, 208 genes (3.7% of detected genes) were twofold or greater differentially expressed between *F. nucleatum*-stimulated and unstimulated neutrophil samples. Of this dataset, 165 were more highly expressed (> twofold) in neutrophils stimulated with *F. nucleatum* (Table 2), whilst 43 transcripts were more abundant (> twofold) in unstimulated neutrophils (Table 3). Genes more abundant in stimulated neutrophils (n = 165) mapped to a range of biological processes and molecular functions, covering several ontological classes, including signal transduction (13%), transcription regulation (7%) and ROS response (14%; > 2.5-fold changes in ROS-related genes highlighted in Table 2).

To investigate the gene expression data obtained by microarray analysis, eight differentially expressed genes, representing a range of ontological groups and fold changes, were selected and analysed further by RT-PCR. The gene expression data confirmed that transcript levels for the six genes *CSF*, *CXCL3*, *FOS*, *HMOX1*, *HSP40* and *SOD2* were demonstrably increased upon *F. nucleatum* stimulation, whilst two genes, *NFKB2* and $GP91^{PHOX}$, were decreased, in both pooled RNA and individual samples, relative to unstimulated control samples (Fig. 1). Results also demonstrated the heterogeneity of gene expression levels in individuals (Fig. 1C).

Analysis of neutrophil responses to stimuli

Due to reported differences in stimulated ROS production in peripheral blood neutrophils in periodontitis patients (18,28) along with the induction of ROS-dependent genes by stimulation with *F. nucleatum* (Tables 2 and 3), a range of stimuli reported to have differing effects on gene expression and/or ROS production (17,20,24) were assessed for their comparative effects upon ROS and gene expression in neutrophils from healthy individuals. Data indicated differential effects of stimuli with regards to total and extracellular ROS production (Fig. 2). Notably, opsonized *S. aureus* induced similar total and extracellular levels of ROS to *F. nucleatum* exposure, whilst *P. gingivalis* and *E. coli* LPS exposure induced similar but lower levels of total and extracellular ROS compared with *F. nucleatum* and opsonized *S. aureus* exposure. Gene expression analysis indicated that all *F. nucleatum*-stimulated neutophils responded as previously reported by regulating transcript levels. Consistent with the ROS analysis, opsonized *S. aureus* exposure affected gene expression in a similar manner to that of *F. nucleatum* (Fig. 2C). In contrast, neutrophils exposed to *E. coli* LPS exhibited increased expression of all eight genes studied, whilst *P. gingivalis* stimulation increased *CFS*, *CXCL3*, *FOS* and *HMOX* gene expression but either inhibited or had no effect on all other genes analysed.

Patient and control neutrophil transcriptional responses

Owing to the reported important role of *F. nucleatum* in the pathogenesis of periodontitis and potentially in altered patient immune responses, transcript level analysis was performed in neutrophils from periodontitis patients and pair-matched healthy control subjects. Gene expression analysis of the eight selected transcripts highlighted several significant differences between patients and healthy control subjects either at baseline or after stimulation with *F. nucleatum* (Fig. 3). Notably, *CXCL3*, *HMOX1*, *HSP40* and *SOD2* transcript levels were significantly increased in stimulated cells from periodontitis patients, whereas only *CXCL3* and *SOD2* transcripts were significantly increased in stimulated control cells. Patient neutrophils exhibited a significant decrease in Gp91-*phox* transcript levels, whereas in control subjects the decreased gene expression was not significant. Control neutrophils had a significant decrease in *NFKB2* transcript levels, whereas this was not evident in patient neutrophils. Levels of *NFKB2* also appeared significantly lower in unstimulated patient neutrophils compared with control neutrophils.

Discussion

Neutrophils are clearly important in establishing the initial lesion; however, their abnormal response of hyperactivity and hyper-reactivity in terms of ROS production probably contributes to chronic periodontal tissue destruction and disease progression (18,28). The composition of the dental plaque is an essential factor in the pathogenic process (4), and therefore the interaction between the neutrophil and components of the plaque microflora is of particular relevance. Whilst, due to their relatively short lifespan and rapid response to stimulation, neutrophils were originally thought to be transcriptionally inactive, recent data have shown that bacteria and their components are able to modify the gene expression profile of neutrophils (20–22). For the first time, we report data demonstrating that neutrophils are transcriptionally active in response to the periodontal pathogens *F. nucleatum* and *P. gingivalis*, in addition to other stimuli, and that the transcriptional activity detected varies between stimuli, as does ROS production detected by chemiluminescence.

It is important to consider that autocrine signalling events may be important modulators of the neutrophil transcriptional response to periodontal pathogens. Indeed, the rapid induction and relatively high levels of ROS induced by *F. nucleatum* have the potential to modify gene expression via redox-regulated gene transcription factors in addition to the bacterially induced signalling response. Notably, the gene expression data presented here demonstrate a large number of 'stress-response' genes, such as *HSPA1A*, *HSPH1* and *HSPA8* (Table 2), in neutrophils following exposure to *F. nucleatum*. In support of our data, others have also demonstrated elevated levels of stress-response and *HSP* transcripts in neutrophils exposed to bacteria, and this result may reflect the

Table 2. Genes more abundantly expressed in stimulated compared with unstimulated neutrophils as determined using GeneSpring software

Affymetrix ID	Fold change	*p*-Value	Gene	Gene symbol
207850_at	19.1	0.000827	Chemokine (C-X-C motif) ligand 3	*CXCL3*
205020_s_at	14.38	0.0471	ADP-ribosylation factor-like 4A	*ARL4A*
204326_x_at	9.373	0.0129	Metallothionein 1L	*MT1L*
210387_at	8.172	0.00204	*Homo sapiens* cDNA clone IMAGE:2989839, with apparent retained intron	*H2BFA*
200790_at	7.364	0.00409	Ornithine decarboxylase 1	*ODC1*
207082_at	7.065	0.0285	Colony stimulating factor 1 (macrophage)	*CSF1*
209189_at	7.061	0.0117	v-fos FBJ murine osteosarcoma viral oncogene homologue	*FOS*
208581_x_at	6.246	0.0428	Metallothionein 1X	*MT1X*
202581_at	5.957	0.00432	Heat shock 70 kDa protein 1B	*HSPA1B*
212225_at	5.895	0.0305	Putative translation initiation factor	*SUI1*
214637_at	5.724	0.0106	Oncostatin M	*OSM*
202340_x_at	5.61	0.0432	Nuclear receptor subfamily 4, group A, member 1	*NR4A1*
200666_s_at	5.606	0.00154	DnaJ (Hsp40) homologue, subfamily B, member 1	*DNAJB1*
203665_at	5.591	0.0209	Heme oxygenase (decycling) 1	*HMOX1*
204170_s_at	5.526	0.000189	CDC28 protein kinase regulatory subunit 2	*CKS2*
200664_s_at	5.308	0.00653	DnaJ (Hsp40) homologue, subfamily B, member 1	*DNAJB1*
222329_x_at	5.189	0.0402	Transcribed sequence with weak similarity to protein sp:P39188 (*H. sapiens*) ALU1_HUMAN Alu subfamily J sequence contamination warning entry	
200800_s_at	5.128	0.00733	Heat shock 70 kDa protein 1A	*HSPA1A*
206976_s_at	5.061	0.0244	Heat shock 105 kDa/110 kDa protein 1	*HSPH1*
209716_at	5.013	0.00886	Colony-stimulating factor (CSF-1) precursor; Human macrophage-specific colony-stimulating factor (CSF-1) mRNAC, cSoFm1p; lMetCe ScFd;s.	*MGC31930*
209681_at	4.621	0.00787	Solute carrier family 19 (thiamine transporter), member 2	*SLC19A2*
201566_x_at	4.483	0.00734	Inhibitor of DNA binding 2, dominant negative helix–loop–helix protein	*ID2*
211527_x_at	4.209	0.0153	Vascular endothelial growth factor	*VEGF*
214542_x_at	4.154	0.0487	Histone 1, H2ai	*HIST1H2AI*
206461_x_at	4.022	0.0164	Metallothionein 1H	*MT1H*
209803_s_at	3.966	0.0492	Pleckstrin homology-like domain, family A, member 2	*PHLDA2*
203590_at	3.93	0.0464	Dynein, cytoplasmic, light intermediate polypeptide 2	*DNCLI2*
205586_x_at	3.854	0.0112	VGF nerve growth factor inducible	*VGF*
210512_s_at	3.653	0.00163	Vascular endothelial growth factor	*VEGF*
217957_at	3.599	0.00975	Likely orthologue of mouse gene trap locus 3	*GTL3*
214657_s_at	3.496	0.000934	MRNA; cDNA DKFZp686L01105 (from clone DKFZp686L01105)	
213566_at	3.441	0.0299	Ribonuclease, RNase A family, k6	*RNASE6*
219082_at	3.411	0.0119	CGI-14 protein	*CGI-14*
200697_at	3.407	0.0362	Hexokinase 1	*HK1*
212665_at	3.294	0.00185	TCDD-inducible poly(ADP-ribose) polymerase	*TIPARP*
202843_at	3.291	0.00525	DnaJ (Hsp40) homologue, subfamily B, member 9	*DNAJB9*
215567_at	3.252	0.0386	Chromosome 14 open reading frame 111	*C14orf111*
117_at	3.223	0.0235	Unnamed protein product; heat-shock protein HSP70B; Human heat-shock protein HSP70B' gene	*HSPA6*
201235_s_at	3.213	0.00698	BTG family, member 2	*BTG2*
210513_s_at	3.208	0.00767	Vascular endothelial growth factor	*VEGF*
217831_s_at	3.146	0.036	NSFL1 (p97) cofactor (p47)	*NSFL1C*
205040_at	3.144	0.0072	Orosomucoid 1	*ORM1*
211969_at	3.14	0.00182	Heat shock 90 kDa protein 1, α	*HSPCA*
209122_at	3.119	0.0186	Adipose differentiation-related protein	*ADFP*
200730_s_at	3.083	0.0375	602135085F1 NIH_MGC_81 *H. sapiens* cDNA clone IMAGE:4290141 5′, mRNA sequence	*PTP4A1*
208909_at	3.08	0.0412	Ubiquinol–cytochrome *c* reductase, Rieske iron-sulphur polypeptide 1	*UQCRFS1*
222173_s_at	3.08	0.0409	TBC1 domain family, member 2	*TBC1D2*
212185_x_at	3.078	0.00581	Metallothionein 2A	*MT2A*
200961_at	3.032	0.00514	Selenophosphate synthetase 2	*SEPHS2*
201044_x_at	2.964	0.00847	Dual specificity phosphatase 1	*DUSP1*
209774_x_at	2.913	0.00233	Chemokine (C-X-C motif) ligand 2	*CXCL2*

Table 2. (Continued)

Affymetrix ID	Fold change	*p*-Value	Gene	Gene symbol
215078_at	2.895	0.0139	Superoxide dismutase 2, mitochondrial	*SOD2*
205133_s_at	2.87	0.0343	Heat shock 10 kDa protein 1 (chaperonin 10)	*HSPE1*
214522_x_at	2.857	0.00951	Histone 1, H3d	*HIST1H3D*
203068_at	2.851	0.0137	KIAA0469 gene product	*KIAA0469*
221804_s_at	2.816	0.0244	Similar to uncharacterized hypothalamus protein HT011	*LOC404636*
219237_s_at	2.798	0.0334	Hypothetical protein FLJ14281 DnaJ (Hsp40) homologue, subfamily B, member 14	*FLJ14281*
204470_at	2.781	0.0313	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, α)	*CXCL1*
212171_x_at	2.764	0.0264	Vascular endothelial growth factor	*VEGF*
205193_at	2.709	0.0268	v-maf musculoaponeurotic fibrosarcoma oncogene homologue F (avian)	*MAFF*
204970_s_at	2.671	0.00314	v-maf musculoaponeurotic fibrosarcoma oncogene homologue G (avian)	*MAFG*
208687_x_at	2.665	0.0372	Heat shock 70 kDa protein 8	*HSPA8*
203394_s_at	2.664	0.0054	Hairy and enhancer of split 1, (*Drosophila*)	*HES1*
202382_s_at	2.654	0.043	Glucosamine-6-phosphate deaminase 1	*GNPDA1*
204828_at	2.646	0.0436	RAD9 homologue A (*S. pombe*)	*RAD9A*
213506_at	2.617	0.0107	601659282R1 NIH_MGC_70 *H. sapiens* cDNA clone IMAGE:3895653 3′, mRNA sequence	*F2RL1*
217858_s_at	2.609	0.00737	Synonyms: ALEX3, MGC12199; arm protein lost in epithelial cancers, X chromosome, 3; 1200004E24Rik; *H. sapiens* armadillo repeat containing, X-ARMCX3; ALEX3; MGC12199	
208886_at	2.589	0.00544	H1 histone family, member 0	*H1F0*
209674_at	2.589	0.00477	Cryptochrome 1 (photolyase-like)	*CRY1*
202464_s_at	2.579	0.00765	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	*PFKFB3*
215147_at	2.574	0.000788	CDNA FLJ35626 fis, clone SPLEN2011086	
212998_x_at	2.562	0.0464	Major histocompatibility complex, class II, DQ β1	*HLA-DQB1*
213145_at	2.561	0.00718	F-box and leucine-rich repeat protein 14	*FBXL14*
209355_s_at	2.546	0.0413	Phosphatidic acid phosphatase type 2B	*PPAP2B*
205000_at	2.534	0.0223	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	*DDX3Y*
213980_s_at	2.521	0.0292	C-terminal binding protein 1	*CTBP1*
204621_s_at	2.516	0.0351	Nuclear receptor subfamily 4, group A, member 2	*NR4A2*
215779_s_at	2.516	0.033	601140646F1 NIH_MGC_9 *H. sapiens* cDNA clone IMAGE:3049883 5′, mRNA sequence. HISTONE	*HIST1H2BG*
212722_s_at	2.5	0.0111	Phosphatidylserine receptor	*PTDSR*

A > 2.5-fold increase is shown for clarity; raw data files are available in the gene expression omnibus database, GSE20151.
The ROS-related genes are highlighted.

cells' response to their own ROS production (25). Further support for this hypothesis comes from data that demonstrate the induction of several heat shock proteins (HSPs), including HSP40, HSPCA, HSPCB and HSPF1, in neutrophils directly exposed to ROS (25). Whilst the primary role of the HSP molecular chaperones is to stabilize proteins denatured by ROS activity (34), these molecules may also be important to the overall immune response. Data demonstrate that HSPs can act as molecular chaperones during antigen presentation (35) and that neutrophil-derived HSPs can modulate macrophage activity (36). Indeed, in a periodontitis study, data demonstrated that peripheral blood mononuclear cells from patients exhibited a decreased proliferative response to HSPs compared with healthy control subjects and resulted in decreased macrophage phagocytic activity of dead or dying neutrophils (37). The disruption of the clearance mechanism by HSPs has the potential to lead to a failure in resolution of the inflammatory lesion, which may thereby contribute to the chronic inflammation observed within the periodontal tissues.

Other oxidative stress-response-related transcripts increased in neutrophils induced by *F. nucleatum* exposure included *SOD2*, an enzyme involved in the removal of superoxide free radicals, metallothioneins, which scavenge ROS (38,39), and heme oxygenase (*HMOX*), an important endogenous antioxidant which degrades heme and leads to the formation of the more powerful antioxidant, bilirubin (40). Notably, bilirubin is known to be particularly important in other tissues in the defence against oxidative-stress-induced damage that arises during the inflammatory process (41,42). Interestingly, our group has also found, in fully adjusted models, a significant negative association between periodontitis prevalence and

Table 3. Genes less abundantly expressed in stimulated compared with unstimulated neutrophils as determined using GeneSpring software

Affymetrix ID	Fold change	*p*-Value	Gene	Gene symbol
209950_s_at	8.894	0.00233	Villin-like	*VILL*
209728_at	3.563	0.0402	Major histocompatibility complex, class II, DR β3	*HLA-DRB3*
218507_at	3.425	0.022	Hypoxia-inducible protein 2	*HIG2*
201269_s_at	3.284	0.0455	KIAA1068 protein	*KIAA1068*
220686_s_at	3.206	0.00526	piwi-like 2 (*Drosophila*)	*PIWIL2*
202308_at	2.869	0.00362	Sterol regulatory element binding transcription factor 1	*SREBF1*
203702_s_at	2.794	0.016	Tubulin tyrosine ligase-like family, member 4	*TTLL4*
202509_s_at	2.758	0.0449	Tumour necrosis factor α-induced protein 2	*TNFAIP2*
203673_at	2.738	0.00348	Thyroglobulin	*TG*
205599_at	2.644	0.00329	TNF receptor-associated factor 1	*TRAF1*
220941_s_at	2.616	0.00291	Chromosome 21 open reading frame 91	*C21orf91*
220054_at	2.567	0.00842	Interleukin 23, α subunit p19	*IL23A*
202150_s_at	2.523	0.0303	Neural precursor cell expressed, developmentally down-regulated 9	*NEDD9*
221867_at	2.494	0.00998	Hypothetical protein FLJ31821	*FLJ31821*

A >2.5-fold decrease is shown for clarity; raw data files are available in gene expression omnibus database, GSE20151.
The ROS-related genes are highlighted.

plasma bilirubin concentrations in the NHANES III dataset, implying a potentially protective effect for bilirubin in periodontitis (29). In agreement with the data presented here, others have also shown bacterial induction of the *SOD2* transcript in neutrophils whilst *Phox* genes, including GP91-*phox* and *RAC1*, which encode components of the NADPH oxidase complex, were down-regulated (18,25,43). Decreased expression of these component molecules may represent a self-protective mechanism, whereby cells limit their ROS production in conditions of chronic stimulation.

F. nucleatum-stimulated neutrophils expressed increased transcript levels for intercellular signalling molecules, including the cytokines *CXCL-1*, *CXCL-2*, *CXCL-3*, *CSF* and *OSM*, all of which can act as neutrophil chemoattractants, activators and prosurvival factors (44,45). Notably, members of the CXCL chemokine family are related to IL-8 and are potent chemotactic agents for neutrophils (46,47). Indeed, *in vivo* studies in several tissues have demonstrated that blockade of CXCL1 and CXCL2 signalling decreases neutrophil infiltration in response to bacterial infection or antigen administration (48,49). Whilst structural cells resident in the periodontium, such as epithelial cells and fibroblasts, can up-regulate their release of neutrophilic cytokines/chemokines in response to periodontal bacteria (24,50), it is also interesting to note that neutrophils themselves, when recruited to the site of infection, may also contribute further to the proinflammatory milieu. Combined, the release of these factors would potentially contribute to the chronic inflammatory process and subsequent collateral damage of periodontal tissues in response to the subgingival biofilm.

The gene expression levels of several transcription factors was also altered in neutrophils exposed to *F. nucleatum*, indicating the role of transcriptional regulation in the neutrophils' cellular response. Notably, in this category of genes, there was up-regulation of nuclear hormone receptor *NR4A*, *MAFF* and *EGR1*, which have all previously been implicated in the inflammatory and prosurvival responses of other cell types (51–54). In contrast, components of the nuclear factor κB signalling pathway, NFκB2 and IκBϵ, were down-regulated. As this pathway is key to the inflammatory response, due to its regulatory role in cytokine and ROS production as well as in regulating apoptosis (24,55), the down-regulation of these key pathway components may represent modulation of the neutrophils' proinflammatory and survival responses. Other genes involved in the regulation of apoptosis were also up-regulated in *F. nucleatum*-treated neutrophils and included *BCL2A1*, *MCl1*, *PPIF* and *TNFAP3*. As these genes are usually associated with decreased apoptosis, it is conceivable that their up-regulation may not simply be a direct response to the bacterial stimulation but may be due to autocrine feedback mechanisms in response to the elevated cytokines previously discussed (25). Nevertheless, decreased apoptosis would lead to increased tissue levels of neutrophils during chronic inflammation.

Neutrophil ROS and selected transcript levels following exposure to a range of stimuli were also investigated due to reported variations in molecular and cellular responses. Notably, the stimuli selected were known to interact with different receptors, singularly or in combinations, for example by combinations of TLRs or via the Fcγ receptor. Comparable to previous studies (17), there was variation in the level of ROS production induced by the different stimuli tested. Similar trends in neutrophil gene expression responses were evident between stimuli, and the data obtained for *E. coli* LPS were consistent with other published studies using stimulated neutrophils, LPS, *E. coli* and *N*-formyl-Met-Leu-Phe (26). Our data therefore support the requirement for individual analysis of different aetiological agents, including disease-associated bacteria, in relevant model systems.

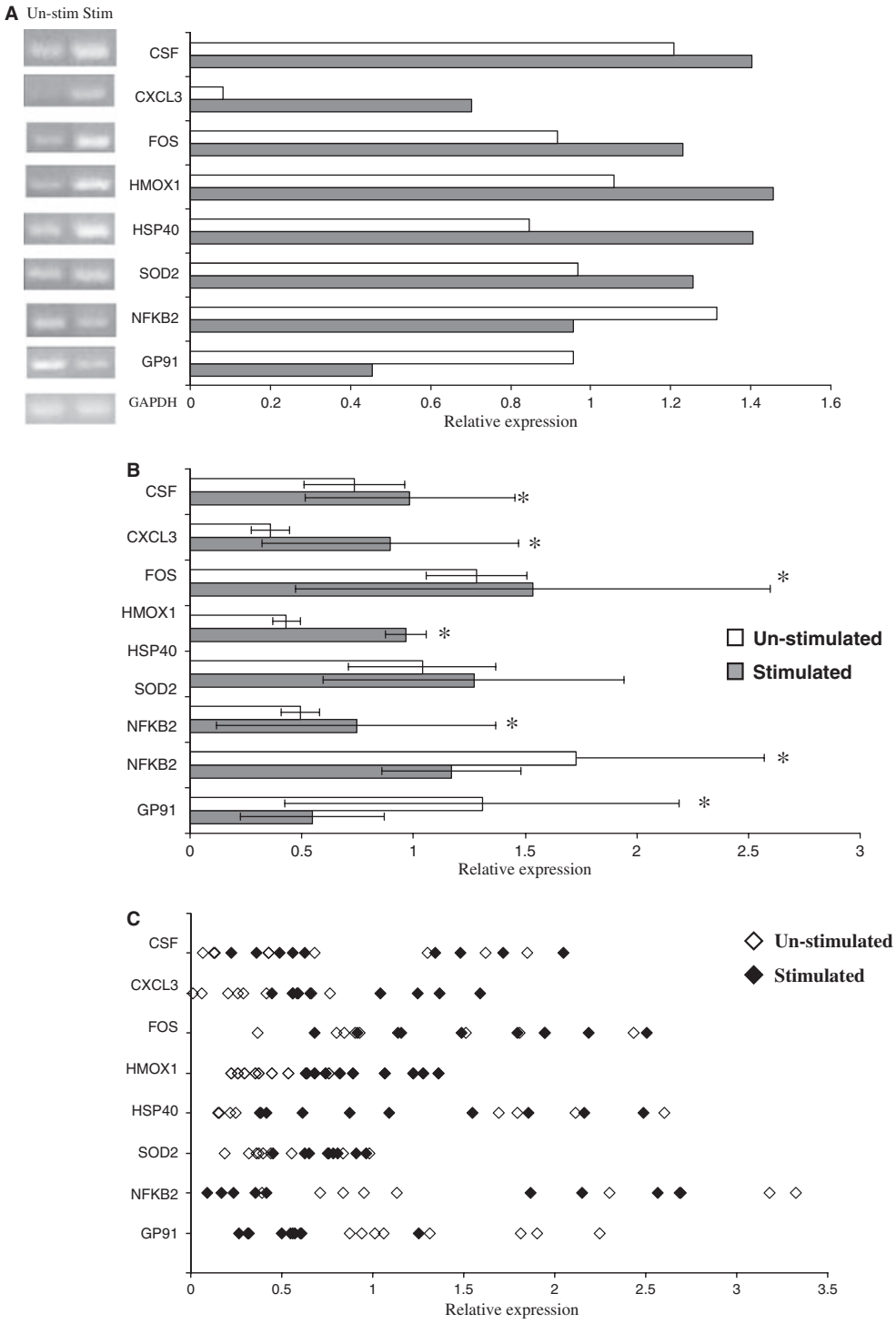

Fig. 1. Relative gene expression levels for *CSF*, *CXCL3*, *FOS*, *HMOX1*, *HSP40*, *SOD2*, *NFKB2* and *GP91PHOX* in *F. nucleatum*-stimulated and unstimulated neutrophils from healthy control donors (shown as ratio to *GAPDH* levels). (A) Analysis of pooled ($n = 4$) RNA samples of stimulated and unstimulated neutrophils. (B) Mean ($\pm$ SEM) gene expression in RNA from stimulated and unstimulated neutrophils from healthy individual volunteers ($n = 9$, $*p < 0.02$, paired Wilcoxon test). (C) Individual gene expression data in RNA from stimulated and unstimulated neutrophils from healthy individual volunteers ($n = 9$).

Analysis of the response of neutrophils from periodontitis patients, previously shown to be hyper-reactive with respect to *F. nucleatum*-stimulated ROS production (18), indicated that this may be accompanied by a modified transcriptional profile. For example, neutrophils from patients exhibited significantly greater increases

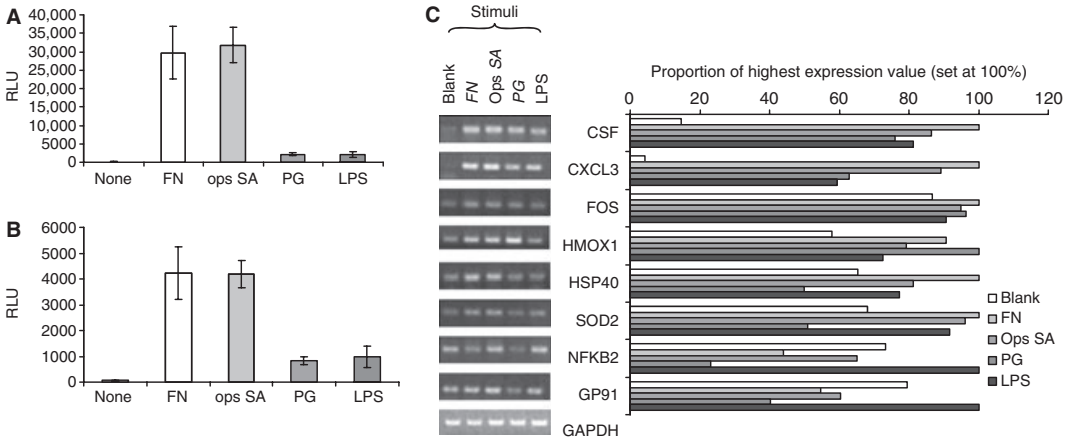

Fig. 2. Analysis of reactive oxygen species (ROS) and gene expression by stimulated and unstimulated neutrophils in healthy control subjects. Mean (± SEM) luminol-dependent total (A) and isoluminol-dependent extracellular chemiluminescence (B) generated by peripheral neutrophils unstimulated and stimulated with opsonized *S. aureus* (ops SA), *F. nucleatum* (FN), *P. gingivalis* (PG) or *E. coli* LPS (LPS). (C) Gene expression analysis in pooled RNA from healthy control neutrophils stimulated with opsonized *S. aureus*, *F. nucleatum*, *P. gingivalis* or *E. coli* LPS. Gel images and densitometric analysis are shown. (Blank is PBS).

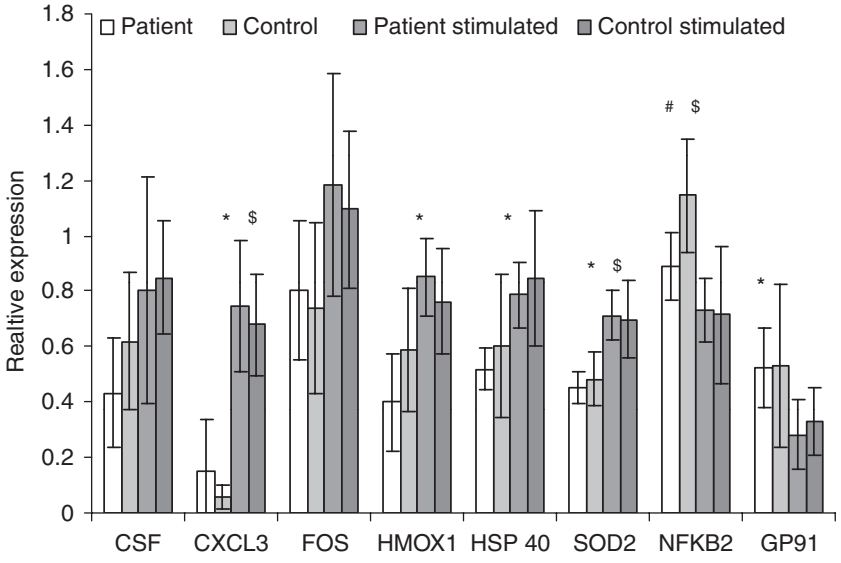

Fig. 3. Comparison of transcript levels in stimulated and unstimulated patient and control neutrophils. Mean (± SD) gene expression levels are shown for periodontitis patients and periodontally healthy matched control subjects, for *F. nucleatum*-stimulated and unstimulated neutrophils ($n = 5$). (For patient vs. patient stimulated, $*p > 0.032$; for control vs. control stimulated, $\$p > 0.032$; for patient vs. control, $\#p > 0.032$; Wilcoxon paired test).

in *HSP40* and *HMOX1* transcript levels, due to *F. nucleatum* stimulation, than did control cells. Whilst these changes may be subtle, it is conceivable that accumulation of dysregulated levels of a range of proinflammatory and neutrophil pro-survival molecules could lead to the chronic inflammatory response within the local tissue environment observed in periodontitis. Our recent studies have indicated that peripheral blood neutrophils from periodontitis patients are in a primed state with regard to gene expression and ROS production and that elevated levels of circulating cytokines, such as IFNα, may be responsible for this phenomenon (18,28,33). Whilst it is unclear as to the source of this priming agent, our data continue to support the key role of the neutrophil in periodontitis progression and that therapeutics targeted at modifying aberrant neutrophil responses may ultimately have clinical benefit.

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