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Cigarette smoke modifies neutrophil chemotaxis, NET formation and inflammatory response-related gene expression

Short title: Cigarette smoke modifies neutrophil function

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ABBREVIATIONS

CS: cigarette smoke

CSE: cigarette smoke extract

CXCL8: C-X-C motif chemokine ligand 8

CYBB: cytochrome B-245 beta chain

DNAJB1: DnaJ heat shock protein family (Hsp40) member B1

fMLP: *N*-formyl-methionyl-leucyl-phenylalanine

HOCl: hypochlorous acid

HSP40: heat shock protein 40

IL-8: interleukin 8

NCF1: neutrophil cytosolic factor 1

NCF2: neutrophil cytosolic factor 2

NETs: neutrophil extracellular traps

NFKBIE: nuclear factor-kappa-b inhibitor epsilon

PMA: phorbol 12-myristate 13-acetate

ROS: reactive oxygen species

SCN-: thiocyanate

YWHAZ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

ABSTRACT

Background and Objectives: Cigarette smoking is a major risk factor for periodontitis, and smoking perturbs neutrophil reactive oxygen species (ROS) production. This study tested the hypothesis that cigarette smoke extract (CSE) and its components/metabolites nicotine, cotinine and thiocyanate (SCN-), may influence neutrophil functions.

Material and Methods: Chemotaxis was assessed in neutrophils pre-treated with CSE using real-time video microscopy. NET release in response to CSE, nicotine, cotinine, SCN- as well as to phorbol 12-myristate-13-acetate (PMA) and hypochlorous acid (HOCl) following pre-treatment with CSE, nicotine, cotinine or SCN- was assessed using fluorescence-based assays. The impact of CSE and SCN- treatment on neutrophil respiratory burst- and inflammation-related gene expression (NFKBIE, DNAJB1, CXCL8, NCF1, NCF2, CYBB) was determined by real-time polymerase chain reaction.

Results: Both CSE and SCN- pre-treatment inhibited PMA-stimulated NET release. Additionally, SCN- inhibited HOCl-stimulated NET formation, while SCN- alone stimulated NET release. Overall, neutrophils pre-treated with CSE exhibited reduced speed, velocity and directionality relative to untreated neutrophils. Although CSE and SCN- promoted DNAJB1 expression, increased redox-related gene expression was only detected in response to SCN-.

Conclusion: These results suggest that CSE can alter *ex vivo* neutrophil activation by mechanisms independent of SCN- and nicotine, and SCN- may contribute to the perturbed innate immune responses observed in smokers.

INTRODUCTION

Periodontitis is a prevalent chronic disease that is characterised by destructive and non-resolving inflammation of the periodontal tissues. Periodontitis results from a dysregulated host inflammatory-immune response elicited by microbial dysbiosis within the subgingival plaque biofilm (1). The initial inflammatory response is protective, driving the chemotactic recruitment and activation of neutrophils, and subsequent activation of the acquired cellular and humoral immune responses. However, in periodontitis, abnormal neutrophil behaviour reportedly contributes to disease progression and its chronicity. It is noteworthy that whilst neutrophil hyperactivity and hyper-reactivity likely exacerbates periodontal tissue destruction, impaired neutrophil activity can also increase disease severity (2).

Cigarette smoking is considered a component cause of periodontitis and delayed periodontal wound healing (3, 4). Tobacco smoking is also associated with a wide range of systemic pathologies, including cardiovascular disease (5, 6). Smoking has also been found to adversely affect neutrophil function, indeed, cigarette smoke extract (CSE) has been shown to promote neutrophil degranulation, which may cause an exaggerated response to invading bacteria and induce local tissue damage (7). We have reported previously that CSE can affect neutrophil reactive oxygen species (ROS) production (8, 9) and it is possible that exposure to cigarette smoke (CS)-derived chemicals may perturb neutrophil responses to periodontal bacteria if ROS species are not being generated appropriately.

Cigarette smoke is estimated to consist of more than 7,000 different chemical compounds (10). The first widely accepted tobacco biomarkers as indicators of exposure were salivary and serum thiocyanate (SCN-) as well as nicotine and its metabolite cotinine, and they have remained in use for exposure studies until today. SCN- is the metabolite of hydrogen cyanate, which is present in CS in concentrations exceeding those approved by the US Food and Drug Administration. Nicotine is present in high concentrations in CS relative to other CS components, thus leading to medically significant systemic cotinine levels (10, 11).

In addition to the previously reported altered neutrophil ROS responses to CSE (8, 9), changes in chemotaxis and formation of neutrophil extracellular traps (NETs; NETosis), may also represent a mechanistic link between smoking and periodontitis. Moreover, the production of NETs, which possess antimicrobial as well as pro- and auto-inflammatory properties, may also be perturbed by CS due to their dependence on ROS generation (12). However, to date, there are no published data on the direct effects of CSE on NET formation. Therefore, this study was conducted to better understand the impact of CSE, nicotine, cotinine, and SCN- upon NET release, expression of inflammatory response-related genes as well as on chemotaxis.

MATERIALS & METHODS

Neutrophil isolation

Neutrophils were isolated from the peripheral venous blood of periodontally and systemically healthy non-smoking volunteers (University of Birmingham Ethics

Reference: ERN_13-0325) using discontinuous Percoll gradients (GE Healthcare, Amersham, UK) as previously described (13, 14). Cell viability and purity were confirmed by trypan blue exclusion and flow cytometry, respectively, and was typically >98%.

Extraction and preparation of cigarette smoke extract (CSE)

CSE was extracted and prepared as previously described (8). In brief, CSE was collected by drawing CS through filters and bubbled through PBS. The resultant extract represented 83.6 µg/ml of nicotine and was stored in liquid nitrogen until further use. Reported levels of nicotine in saliva and gingival crevicular fluid (GCF) after smoking one cigarette are 1.82 ± 0.61 µg/ml and 5.96 ± 0.77 µg/ml, respectively (mean ± SE) (15). CSE was diluted with PBS to final concentrations of 1%, 5% and 10%, which is equivalent to 0.84 µg/ml, 4.2 µg/ml and 8.4 µg/ml of nicotine, respectively. Notably, neutrophils enter the oral cavity through the gingival crevice and are thus exposed to the CS component/metabolite concentrations found in GCF and saliva. Nicotine, cotinine and SCN- were obtained from Sigma Aldrich (Dorset, UK).

SCN- levels in CSE

SCN- was assessed by colorimetric quantification of the red coloured complex ion formed by Fe(III) chloride (Sigma Aldrich, Dorset, UK).

Quantification and visual assessment of NET-DNA

Quantification of NET-DNA was performed as described previously (13). Neutrophils were pre-treated for 30 min with either PBS (pre-treatment control), CSE (1%, 5%

and 10%), nicotine, cotinine (both 1 µg/ml, 5 µg/ml or 10 µg/ml) or SCN- (50 µM, 100 µM or 150 µM), washed three times and subsequently stimulated with phorbol 12-myristate-13-acetate (PMA, 50nM*),* hypochlorous acid (HOCl, 0.75 mM) or PBS (unstimulated control). Samples were stimulated for 4 h at 37°C in 5% CO_2.

For NET visualisation, neutrophils ($1x10^5$) were added to a clear non-treated 24-well plate previously coated with 1% BSA for 30 min, treated with the appropriate stimuli and stained with Sytox Green (Invitrogen, Paisley, UK). NETs were visualised with an epi-fluorescence microscope (Nikon Eclipse TE300). Images were captured with a QImaging camera (Retiga 2000R) in Micro-Manager and analysed in Fiji (ImageJ, National Institutes of Health, Bethesda, MD).

Assessment of neutrophil apoptosis

Neutrophil apoptosis in response to CSE, nicotine, cotinine or SCN- was measured by determining caspase-3 and -7 activity using the Caspase-Glo® 3/7 assay (Promega, Southampton, UK) according to the manufacturer's instructions. In addition, neutrophil adenosine triphosphate (ATP) production was measured by chemiluminescence (Berthold Tristar2 plate reader, Harpenden, UK) using CellTiter-Glo® (Promega, Southampton, UK) according to the manufacturer's instructions. The amount of ATP measured is indicative of neutrophil metabolic activity and therefore proportional to the number of viable cells (16).

Neutrophil chemotaxis assay

Chemotaxis assays were conducted as previously described (17) using the Insall chamber (18). To determine the effect of CSE on neutrophil chemotaxis, neutrophil

migration of 5 different donors was assessed. Isolated neutrophils were pre-incubated with PBS (negative control) or CSE (1%, 5% and 10%) for 30 mins and then washed three times with RPMI. Chemoattractants were *N*-formyl-methionyl-leucyl-phenylalanine (fMLP, 10 nM) and interleukin-8 (IL-8, 200 ng/mL) (both from Sigma Aldrich, Dorset, UK) and RPMI medium alone served as a negative control. The movement of 15 randomly chosen cells was tracked over a time-course of 20 min through each frame (MtrackJ plug-in, version 1.5.1), which is an established method for *ex vivo* neutrophil tracking (19). It allows to calculate speed (the average speed of a neutrophil in any direction), velocity (the average speed of a cell in its most prominent direction) and the resultant vector length (the directional accuracy of neutrophil chemotaxis towards the chemoattractant) (20).

Gene expression of CSE and SCN- treated neutrophils

Quantitative real-time polymerase chain reaction (PCR) analysis was employed to compare neutrophil inflammatory and redox related gene expression profiles in response to CSE or SCN- treatment. After incubation with CSE and SCN- for 4 h, neutrophils were centrifuged for 6 mins at 500 rcf, after which the supernatant was carefully removed and discarded. Cell pellets were resuspended in 1mL of Tri-reagent (Sigma Aldrich, Dorset, UK) and RNA was isolated using phenol/chloroform extraction from duplicate samples, pooled onto a Qiagen RNeasy extraction column (Qiagen, Manchester, UK) and extracted according to the manufacturer's instructions. RNA from 10 individuals was pooled after which cDNA synthesis was performed using the Bioline Tetro cDNA synthesis kit (Tetro Bioline, Luckenwalde,

Germany). The samples were equally pooled across the treatment conditions based on the amount of RNA in each sample.

Reference and target gene primer sequences (**Table 1**) were purchased from Invitrogen (Paisley, UK). NCF1, NCF2 and CYBB genes were assessed to examine the effect of CSE and SCN- on the NADPH oxidase. DNAJB1 and CXCL8 genes were used as markers of inflammation, and NFKBIE as a determinant of immune suppression. Real-Time PCR was performed using the LightCycler® 480 (Roche Life Science, Burgess Hill, UK) in a 96-well microtitre plate in a final volume of 20 µL containing 2 µL of cDNA, 10 µL LightCycler® 480 SYBR green PCR mix, forward and reverse primers at 0.5µM and RNase-free water. Cycling conditions were as follows: 95°C for 10 mins, 45 cycles of 95°C 20 secs, 60°C 20 secs and 72°C 30 secs followed by melt curve analysis. All samples were amplified in duplicate and two “no-template” controls per primer pair were included in every run. Three technical replicates were performed. Gene expression levels were obtained from crossing point (Cp) values by fit point analysis, as computed by the LightCycler® 480 software (Roche Diagnostics version 1.5) with standard settings and following the manufacturer’s instructions. PCR efficiency for each primer pair was determined using dilutions of sample cDNA (1:1-1:1000). Fold-changes in gene expression were calculated as a ratio of a reference gene. The statistical algorithm “BestKeeper” was employed to determine the most appropriate reference gene (21, 22). Based on these results, the YWHAZ reference was considered the most consistent and subsequently used as a reference to quantify the expression of the genes of interest. The YWHAZ gene encodes the 14-3-3 protein zeta/delta, which is a central hub protein for many signal transduction pathways (23).

Statistical analysis

All statistical analyses were performed in GraphPad Prism 6 software package (San Diego, CA, USA, version 6.07). The distribution of data was determined by Shapiro-Wilks normality tests. Statistical tests employed in this study were at a significance of 0.05. One-way ANOVA and Dunnett's post-hoc tests were used for NET quantification and viability assays, where different parameters were compared with a control. One-way ANOVA and Bonferroni post-hoc tests were used for chemotaxis and PCR data, where pairwise comparisons were applied.

RESULTS

SCN- is not present in CSE

Cotinine is known to be both a metabolite of nicotine and a component of tobacco leaves (24). Whilst SCN- is a metabolite generated in the liver from hydrogen cyanide present in CS (25), there are no reports regarding the possible presence of SCN- in CS or tobacco products. Although many plants are thought to generate SCN- (26), we found no detectable SCN- in our CSE, as the measured concentrations were below the lower limit of detection of <10 nM. This suggests that SCN- is generated within the host after CS ingestion only.

Effect of CSE, nicotine, cotinine and SCN- on NET release in the absence of subsequent stimulation

The effect of CSE (at concentrations of 1%, 5% or 10%) on NET release did not differ significantly from PBS-treated cells (negative control) (*p=0.99,* **Figure 1-1A**). Similarly, nicotine and cotinine treatment (1, 5 or 10 µg/ml), as well as exposure to SCN- at lower concentrations (50 and100 µM), did not alter NET production compared with PBS-treated control cells (**Figures 1-1B–D**). In contrast, treatment of neutrophils with 150 µM SCN- resulted in a significant increase in NET release (*p<0.05*).

Effect of CSE, nicotine, cotinine and SCN- pre-treatment on PMA and HOCl stimulated NET release

CSE pre-treatment and subsequent stimulation with PMA was associated with significantly decreased NET production at concentrations of 5% and 10% CSE (*p<0.05* and *p<0.0001* for 5% and 10% CSE, respectively; **Figure 1-2A**). By contrast, when cells were pre-treated with CSE and subsequently stimulated with HOCl, no differences in NET production due to CSE pre-treatment were observed (*p=0.90*; **Figure 1-3A**). Nicotine and cotinine pre-treatment and subsequent stimulation with PMA and HOCl was found to have no effect on NET release (**Figures 1-2B,C** and **1-3B,C**), whereas SCN- pre-treatment inhibited both PMA and HOCl induced NET release compared with PBS-pre-treated neutrophils (**Figures 1-2D** and **1-3D**). This was observed at all concentrations of SCN- (*p<0.001*). These results were confirmed microscopically, where SCN- as well as CSE at concentrations of 5% and 10% did not lead to NET formation subsequent to PMA stimulation (**Figure 1E**).

Cell viability following treatment with CSE, nicotine, cotinine and SCN-

For NET visualisation, neutrophils and NETs were stained with the fluorescent DNA-binding dye Sytox green. However, this staining method does not differentiate between DNA derived from NETs and DNA released by other cell processes, such as necrosis. Therefore, to determine whether reduced DNA production may be due to neutrophil cell death induced by CSE, nicotine, cotinine or SCN-, their effect on neutrophil viability was compared with PBS treated cells. Following 4 h of incubation, trypan blue exclusion revealed that all four components at the highest concentrations used did not significantly affect neutrophil viability (*p=0.23*; data not shown). Data were consistent with those of Matthews et al. (8).

Analysis of migration of CSE-treated neutrophils in response to chemoattractants

As expected, cells incubated with PBS (untreated controls) displayed directional movement towards the source of the chemotactic signals, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and interleukin 8 (IL-8). This directionality was progressively reduced in cells incubated with 1 and 5% CSE, and cells incubated with 10% CSE showed minimal movement in any direction (**Figures 2A** and **B**). The speed (μm/minute) of neutrophil chemotaxis in response to fMLP significantly increased following incubation with 5% CSE, compared with cells incubated with PBS (*p<0.001*). However, treatment with 10% CSE resulted in significantly decreased speed of neutrophil migration in response to fMLP (*p<0.001* **Figure 2C**). Migration speed in response to IL-8 (10 nM) following 10% CSE treatment was significantly lower than that of PBS controls (*p<0.001,* **Figure 2D**).

Velocity of neutrophil migration was significantly decreased in response to fMLP and IL-8 following treatment with 1%, 5% and 10% CSE, compared with those treated with PBS (**Figures 2E** and **F**). Notably, the speed and velocity of 10% CSE-treated neutrophils in response to fMLP and IL-8 were not significantly different from cells moving in response to the negative control (RPMI). Accordingly, 5% and 10% of CSE caused significant decreases in resultant vector length (a measure of directional accuracy) in response to fMLP and IL-8 compared to cells incubated with PBS (**Figures 3A** and **B**). Representative angular histograms and resultant vector plots of neutrophils treated with PBS or 10% CSE in response to fMLP and IL-8 showed a lack of directional movement in 10% CSE-treated cells towards both chemotactic agents (**Figure 3C** and **D)**, compared to the chemotaxis in cells treated with PBS.

Gene expression following CSE and SCN- treatment

As NET formation and chemotaxis are influenced by ROS and cytokines, the impact of CSE and SCN- treatment on inflammatory and redox-regulated gene expression was assessed. Neutrophil gene expression of nuclear factor-kappa-b inhibitor epsilon (NFKBIE), heat shock protein (HSP) 40 (DNAJB1), IL-8 (CXCL8), P-47phox (NCF1), P-67phox (NCF2) and GP-91phox (CYBB) was quantified by real-time PCR following CSE treatment (1%, 5% or 10%) (**Figure 4A**) and SCN- treatment (50μM, 100 μM or 150 μM) (**Figure 4B**). Data are expressed as a ratio of the reference gene (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta gene (YWHAZ)), and RNA expression of cells treated with PBS was normalised to 1.

In response to CSE treatment, HSP40 expression significantly increased at concentrations of 5% and 10% CSE (*p=0.0001*). No changes were found in NFKBIE, CXCL8, NCF1, NCF2 and CYBB expression in the samples containing CSE. In response to all concentrations of SCN- treatment, DNAJB1 expression significantly increased (*p=0.0001*), whereas increases in NCF1 expression were only statistically significant following 150 μM SCN- treatment (*p=0.0001*). NCF2 expression was elevated following treatment with 100 μM and 150 μM SCN- (*p<0.0001*). NFKBIE, CXCL8 and CYBB expression were unaffected by SCN-.

DISCUSSION

This is the first study to investigate the effects of CSE, nicotine, cotinine and SCN- on *ex vivo* neutrophil chemotaxis, NET formation and inflammatory and redox gene expression. Overall, our functional assay data demonstrate that NET release was affected by SCN- and by CSE, and that CSE inhibited neutrophil chemotaxis. Furthermore, SCN- had the ability to directly stimulate NETosis. These altered cell functions were accompanied by increased HSP40 gene (DNAJB1) expression.

Treatment of neutrophils for 30 minutes with CSE, cotinine and nicotine did not trigger NET release. Extended periods of neutrophil treatment with CSE containing approximately 25 mg/ml nicotine (27) and application of nicotine concentrations ranging from 1.62 – 500 mg/mL have been reported to induce NETosis (28, 29). However, stable levels of the nicotine metabolite cotinine in saliva and serum of individuals smoking up to 50 cigarettes are <700 ng/ml (30), thus the concentrations

used in these studies may not be applicable to *in vivo* conditions. The data presented here, together with our previously published reports showing that nicotine and cotinine have no effect on the neutrophil respiratory burst (8, 9), suggest that these two CS components do not affect neutrophil function when applied in physiologically relevant concentrations, and that CSE alone is not at NET trigger.

CSE exhibited differential effects upon *ex vivo* stimulated NET release, with CSE pre-treatment inhibiting PMA-induced NET production, but having no effect on HOCl-induced NET formation. Two reports demonstrated that CSE inhibits PMA-induced neutrophil ROS production (15, 31), being in accordance with the findings of our study. PMA is a potent agonist for protein kinase C (PKC), which triggers neutrophil NADPH-oxidase activation, subsequent ROS production and ROS-dependent NET release. It is recognised that smoke exposure can perturb PKC signalling and therefore influence cell inflammatory responses (32, 33). Reduced neutrophil total and extracellular ROS and superoxide formation following CSE pre-treatment have been observed in response to a variety of stimuli including the periodontal pathogen *Fusobacterium nucleatum* (8, 9). This suggests that smoking may subvert the successful elimination of periodontal bacteria by neutrophils.

No differences in HOCl-induced NET production following CSE pre-treatment were detected in this study, supporting a mechanism involving perturbed PKC signalling, as unlike PMA, HOCl activates neutrophil NET formation downstream of PKC (12). Notably, the current data demonstrate that the inhibitory effects of CSE on neutrophil DNA/NET release are not due to cytotoxic effects, as neutrophil viability was demonstrated by trypan blue dye exclusion. This is in accordance with our previous

studies demonstrating that neutrophil viability was not affected by CSE (1-100%), nicotine (1-10 mg/ml or cotinine (1-10 mg/ml) (8). Thus, in future studies, those compounds of CSE, which are responsible for the inhibition of PKC-mediated NET release, need to be unravelled in comprehensive screening assays. They should involve all classes of chemicals present in CS as well as in the host after metabolic breakdown of these chemicals, such as aldehydes, phenols, amines and *N*-nitrosamines, heavy metals and hydrocarbons (10).

Salivary SCN- is implicated in the delayed wound healing observed in smokers (34). SCN- pre-treatment reduced PMA- and HOCl-induced NET production, but resulted in a concentration-dependent increase in otherwise unstimulated NET release. SCN- is the preferred substrate of neutrophil myeloperoxidase (MPO), which catalyses a reaction resulting in the formation of either hypothiocyanous acid (HOSCN) or homocitrulline, a citrulline homologue (35). As citrullination of the nuclear DNA is a crucial step for NET formation, the generation of homocitrulline could be a possible way by which SCN- directly induces NETosis (36). The reaction of SCN- and myeloperoxidase can thus also inhibit PMA-induced NET release, as it occurs downstream of PKC signalling (37). HOSCN, on the other hand, selectively reacts with thiols such as glutathione (GSH) (38), and thus causes increased depletion of antioxidant thiols. Interestingly, increases in HOSCN, following a switch from HOCl production induced by increased SCN-, is believed to afford protection against HOCl-mediated tissue damage (39). In order to benefit from increased levels of HOSCN, it is believed that adequate thiol repair systems are required. If compromised, for example in smokers who reportedly have decreased antioxidant capacity (40), increased levels of HOSCN may exacerbate tissue damage via GSH depletion (41).

Neutrophils pre-treated with CSE exhibited reduced speed, velocity and directional chemotaxis relative to untreated neutrophils, in response to fMLP and IL-8. Importantly, neutrophils were washed following pre-treatment with CSE in our studies, suggesting that the continued presence of CSE is not required to exert its inhibitory effects on chemotaxis and thus transient CS exposure may have long-standing effects. This is consistent with our previous findings on the inhibitory effect of CSE pre-treatment on stimulation of neutrophil superoxide release (9). During chemotaxis, neutrophils form pseudopods in the direction of the chemotactic gradient. It is postulated that ROS are involved in modulating neutrophil directionality by destroying pseudopods that are not in the direction of the chemoattractant (42, 43). Therefore, reduced ROS as a result of CSE treatment (8, 9) may contribute to the impaired directional chemotactic accuracy observed. Whilst smoking may associate with reduced chemotaxis, the severity of periodontitis is positively correlated with neutrophil counts in dental plaque, saliva and GCF (44). This suggests that whilst chemotactic accuracy may be compromised in periodontitis, neutrophils still reach the infected tissues, albeit with longer tissue transit times, which may be associated with increased neutrophil-mediated tissue damage.

Our data demonstrate that at least two CS-derived chemicals, SCN- and one or multiple unknown components of our CSE preparation caused a concentration-dependent increase in neutrophil DNAJB1 (HSP40) expression. HSPs are highly conserved molecular chaperones that are involved in the protection of other proteins (45). HSP40 is believed to regulate processes performed by HSP70, which confers cell protection by inhibiting caspase-dependent and independent apoptosis (7, 46).

Guzik *et al.* demonstrated that HSP70 significantly increases immediately after CSE treatment, being consistent with our previous findings that neutrophil caspase-3 and -7 activity was reduced following CSE treatment (8). Elevations in DNAJB1 expression in response to CSE and SCN- treatment shown in the present study may therefore suppress apoptosis by interactions with HSP70. In the periodontal tissues of smokers, this may associate with neutrophil longevity, contributing to exacerbated inflammation and tissue damage (47).

Following treatment with SCN-, NCF1, NCF2 but not CYBB gene expression levels were significantly increased. All three encode NADPH-oxidase subunits that play a critical role in the activation of this enzyme, suggesting that SCN- may interfere with its assembly. CSE treatment of neutrophils did not result in differences in NCF1 and NCF2 expression, highlighting that this effect was mediated by SCN- alone, which may thus contribute to the aberrant neutrophil responses observed in periodontitis. The possible interference of SCN- with the assembly and activity of the NADPH-oxidase needs to be targeted in future studies in order to clarify whether this could be an underlying mechanism of the disrupted NET formation seen here. This may be accomplished by investigating cell signalling, protein complexes and protein-protein interactions using affinity tagging, co-immunoprecipitation and peptide array techniques.

The methods employed in this study measured *ex vivo* neutrophil functions and have therefore several limitations. *In vivo*, neutrophil functions can be influenced by the complex interactions of (signalling) molecules and cell-to-cell communication. Chemotaxis can be altered by further bioactive molecules present in the tissues and

saliva such as chemoattractants, cytokines, bacteria and variable environmental conditions. No *in vitro* models currently exist to account for such influence factors. Moreover, neutrophils *in vivo* may already become primed systemically by circulating toxins and antigens. It is also possible that lower levels of CS-derived chemicals may act as priming agents, thus leading to a more pronounced neutrophil response towards subsequent stimuli such as bacteria. In our study, we used CSE and CS component/metabolite concentrations previously reported in the saliva of smokers. Although the concentrations of cotinine are very similar in blood and saliva (48), nicotine and SCN- levels are up to 20x higher in saliva than in blood after smoking (49, 50). Thus, future studies may be directed at investigating the effect of low-level circulating CS components on neutrophil priming. Interestingly, CS-derived compounds may also be degraded more rapidly in the oral cavity in the presence of specific bacterial strains (51), possibly leading to lower compound concentrations and unknown degradation products.

Nicotine, cotinine and SCN- as well as the role of oxidants and antioxidants were not tested regarding their effects on neutrophil chemotaxis. Previous studies have shown that CS can alter actin filament arrangement and kinetics in neutrophils, gingival fibroblasts and macrophages (52-54). Assessing the effects of CS components and metabolites as well as of oxidants and antioxidants on cytoskeleton dynamics and pseudopod formation may lead to a better understanding of the mechanism by which CS perturbs neutrophil function. A further limitation is the use of a chemotaxis model (Insall chamber), which does not account for local factors in the tissue environment influencing chemotaxis, such as physical barriers (extracellular matrix, stromal cells) or additional mediators that promote or inhibit chemotaxis and migration. To date,

however, no *ex vivo* models mimicking the periodontal tissue environment exist. A bias may also have arisen from possible outliers among the pooled RNA samples in our gene expression analysis, which was performed in order to obtain an average level of expression across all samples. However, such bias was minimised by involving a larger number of individuals in this analysis, moreover, the differences in gene expression in response to CSE and SCN- were distinct, suggesting that these are true influence factors.

Smoking frequently leads to the onset and progression of severe forms of periodontitis (55). In 2015, over 1.1 billion people smoked tobacco worldwide and while this number is declining in many countries, the prevalence of tobacco smoking appears to be increasing in some other regions (56). Thus, smoking remains a relevant risk factor for oral and systemic diseases. The data presented here, despite of the limitations of this study, suggest that CSE and SCN- can alter neutrophil activation, and thus can contribute to a loss of equipoise in neutrophil function *in vivo* in smokers. Inhibition of NET production and loss of neutrophil mobility within periodontal tissues as a result of smoking may indirectly associate with reduced bacterial clearance and thus contribute to periodontitis. Future studies should be directed at investigating the effects of further potentially harmful CS metabolites on neutrophil function. Moreover, mechanistic studies may elucidate cellular processes involved in cigarette smoke-mediated neutrophil perturbance.

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CONFLICT OF INTEREST

The authors declare no commercial or financial conflict of interest.

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FIGURE LEGENDS

Figure 1

Effect of CSE, nicotine, cotinine and SCN- on NET production. NET release in response to stimulation with increasing concentrations of CSE (n=10 in triplicate) (**A**), CS component nicotine (n=5 in triplicate) (**B**), and metabolites cotinine (n=5 in triplicate) (**C**) and SNC- (n=5 in triplicate) (**D**), compared with PBS-treated cells (unstimulated negative control). Fluorometrically measured results after 4h of direct stimulation with CSE, nicotine, cotinine and SCN- (**1**), after 30 min of pre-treatment with these components prior to stimulation with 50nM PMA (**2**) and after 30 min of pre-treatment with these components prior to stimulation with 0.75mM HOCl (**3**) are shown. Statistical significance was calculated using 1way ANOVA and Dunnett's post-tests (*$p \leq 0.05$, ***$p \leq 0.001$, ****$p < 0.0001$, n.s.=not significant). Data are presented as AFU (arbitrary fluorescence units) and are expressed as mean ± SD. **E**: NETs in response to CSE, nicotine, cotinine and SCN- were visualised with Sytox Green (20x magnification), following stimulation with PMA (50nM), or pre-treated for 30 mins with different concentrations of CSE, nicotine, cotinine or SCN- prior to PMA stimulation. Images are representative of 2 experiments in duplicate. Scale bar represents 100µm.

Figure 2

Analysis of migration of CSE-treated neutrophils in response to chemoattractants. A and **B**: Spider plots showing the tracking of individual neutrophils (n=15 cells from n=5 experiments) from a central reference point (0, 0) in

response to fMLP (100nM) and IL-8. Cells were incubated with PBS (control) or CSE (1%, 5% or 10%) prior to the addition of fMLP or IL-8. The top of the Y-axis represents the source of the chemoattractant. Whilst PBS-treated neutrophils generally moved towards the source of the chemoattractant signal, CSE-treated neutrophils exhibited less directional movement. Lower panel: Neutrophil speed (μm/min) following incubation with CSE (0 [PBS], 1%, 5% or 10%) in response to 100nM fMLP (**C**) or 10nM IL-8 (**D**). RPMI was employed as a negative control. Neutrophil velocity (μm/minute) following incubation with CSE (0 [PBS], 1%, 5% or 10%) in response to fMLP (**E**) or IL-8 (**F**) or the negative control (RPMI). Statistical significance was calculated using 1way ANOVA and Bonferroni post hoc testing (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, n.s.=not significant). Data were calculated from the tracking of 15 cells (n=5) and are expressed as box- and whisker plots (min to max with median, first and third quartile; blue crosses show the mean values).

Figure 3

Resultant vector length and directional accuracy of CSE-treated neutrophil migration. Resultant vector lengths following incubation with CSE (0 [PBS], 1%, 5% or 10%) were determined in response to fMLP (100nM) (**A**), IL-8 (10nM) (**B**) or RPMI (negative control). Statistical significance was calculated using 1way ANOVA and Bonferroni post-tests (*$p<0.05$, **$p<0.01$, ***$p<0.001$, n.s.=not significant). Data calculated from the tracking of 15 cells and expressed as box- and whisker plots (min to max with median, first and third quartile; blue crosses show the mean values). **C** and **D**: Representative angular histograms (top row) and resultant vector

plots (bottom row) of neutrophils treated with PBS (control) or 10% CSE in response to fMLP (100nM) or IL8 (10nM). The angle and width of the histogram bars indicates the direction and the proportion of cells migrating in that direction, respectively. The resultant vector plots indicate the strength and directionality of the cells in response to the chemoattractant. The red line denotes the mean resultant vector and the dashed line shows the variation (95% confidence intervals) within the entire cohort of cells. The length of the red line is indicative of the strength of cell directionality. The blue circles along the circumference of the plot represent individual neutrophil migration end-points. For both plots, 90° at the top of both plots represents the source of the chemoattractant. The plots show that neutrophil directional chemotaxis is reduced in 10% CSE-treated neutrophils compared with PBS-treated control cells, evidenced by the distribution and strength of cell directionality.

Figure 4

Gene expression following cigarette smoke extract or SCN- treatment.
Neutrophil gene expression following treatment with CSE (**A**) or SCN- (**B**) is expressed as a ratio to the reference gene, YWHAZ, and RNA expression in cells treated with PBS (unstimulated negative control) is normalised to 1. Statistical significance was calculated using 1way ANOVA and Bonferroni post hoc testing (****$p<0.0001$). Data are shown as mean ± SD (n=10 pooled biological repeats and n=3 technical repeats in triplicate).

Table 1. Reference and target gene primer sequences, accession numbers and amplicon sizes**.**

Gene (encoded protein)	Primer sequence (5' – 3')	Accession number	Amplicon size (bp)
Reference genes			
YWAHZ (14-3-3 protein zeta/delta)	(F) ACTTTTGGTACATTGTGGCTTCAA (R) CCGCCAGGACAAACCAGTAT	XM_005251063	94
GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)	(F) CTCCTGTTCGACAGTCAG (R) GCCCAATACGACCAAATC	NM_001289745	111
RPL13 (Ribosomal protein L13)	(F) CCTGGAGGAGAAGAGGAAAGAGA (R) TTGAGGACCTCTGTGTATTTGTCAA	NM_001270491	126
HPRT1 (Hypoxanthine phosphoribosyltransferase 1)	(F) GACCAGTCAACAGGGGACAT (R) AACACTTCGTGGGGTCCTTTTC	NM_000194	195
Target genes			
NFKBIE (Nuclear factor-kappa-b inhibitor epsilon)	(F) GTGAAGCCTGTTTGCCTCTC (R) AGGGGTCCTCAACAGCAAGAA	NM_004556	172
DNAJB1 (Heat shock protein 40)	(F) TACAGGAGCACTGTGGAAACG (R) AGGTCTGAGCACTGGACTGG	XM_006722734	192
CXCL8 (Interleukin 8)	(F) TAGCAAAATTGAGGCCAAGG (R) GGACTTGTGGATCCTGGCTA	NM_000584	204
NCF1 (P-47phox)	(F) ACCCAGCCAGCACTATGTGT (R) AGTAGCCTGTGACGTCGTCT	NM_000265	767
NCF2 (P-67phox)	(F) CGAGGGAACCAGCTGATAGA (R) CATGGGAACACTGAGCTTCA	XM_011509581	726
CYBB (GP-91phox)	(F) GCTGTTCAATGCTTGTGGCT (R) TCTCCTCATCATGGTGCACA	NM_000397	403

Figure 1

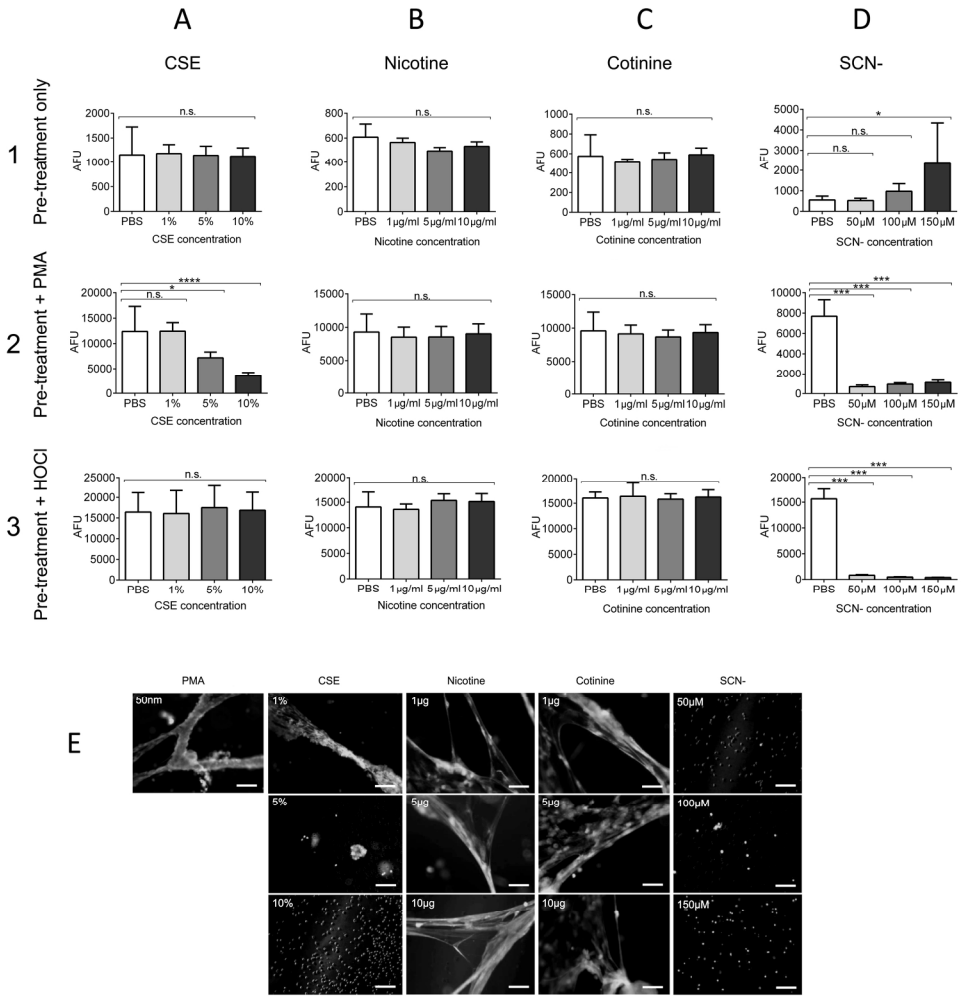
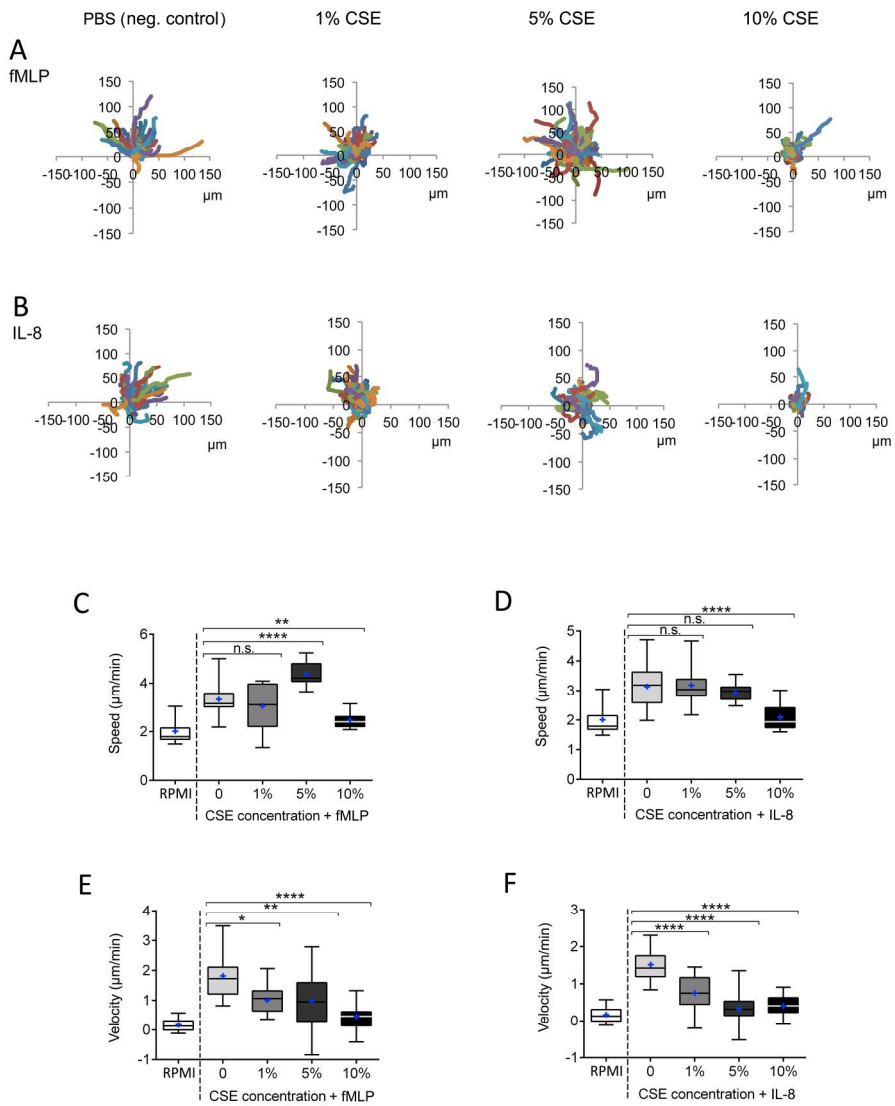


Figure 1. Effect of CSE, nicotine, cotinine and SCN- on NET production. NET release in response to stimulation with increasing concentrations of CSE (n=10 in triplicate) (A), CS component nicotine (n=5 in triplicate) (B), and metabolites cotinine (n=5 in triplicate) (C) and SNC- (n=5 in triplicate) (D), compared with PBS-treated cells (unstimulated negative control). Fluorometrically measured results after 4h of direct stimulation with CSE, nicotine, cotinine and SCN- (1), after 30 min of pre-treatment with these components prior to stimulation with 50nM PMA (2) and after 30 min of pre-treatment with these components prior to stimulation with 0.75mM HOCl (3) are shown. Statistical significance was calculated using 1way ANOVA and Dunnett's post-tests (*p≤0.05, ***p≤0.001, ****p<0.0001, n.s.=not significant). Data are presented as AFU (arbitrary fluorescence units) and are expressed as mean ± SD. E: NETs in response to CSE, nicotine, cotinine and SCN- were visualised with Sytox Green (20x magnification), following stimulation with PMA (50nM), or pre-treated for 30 mins with different concentrations of CSE, nicotine, cotinine or SCN- prior to PMA stimulation. Images are representative of 2 experiments in duplicate. Scale bar represents 100µm.

203x218mm (300 x 300 DPI)

Figure 2



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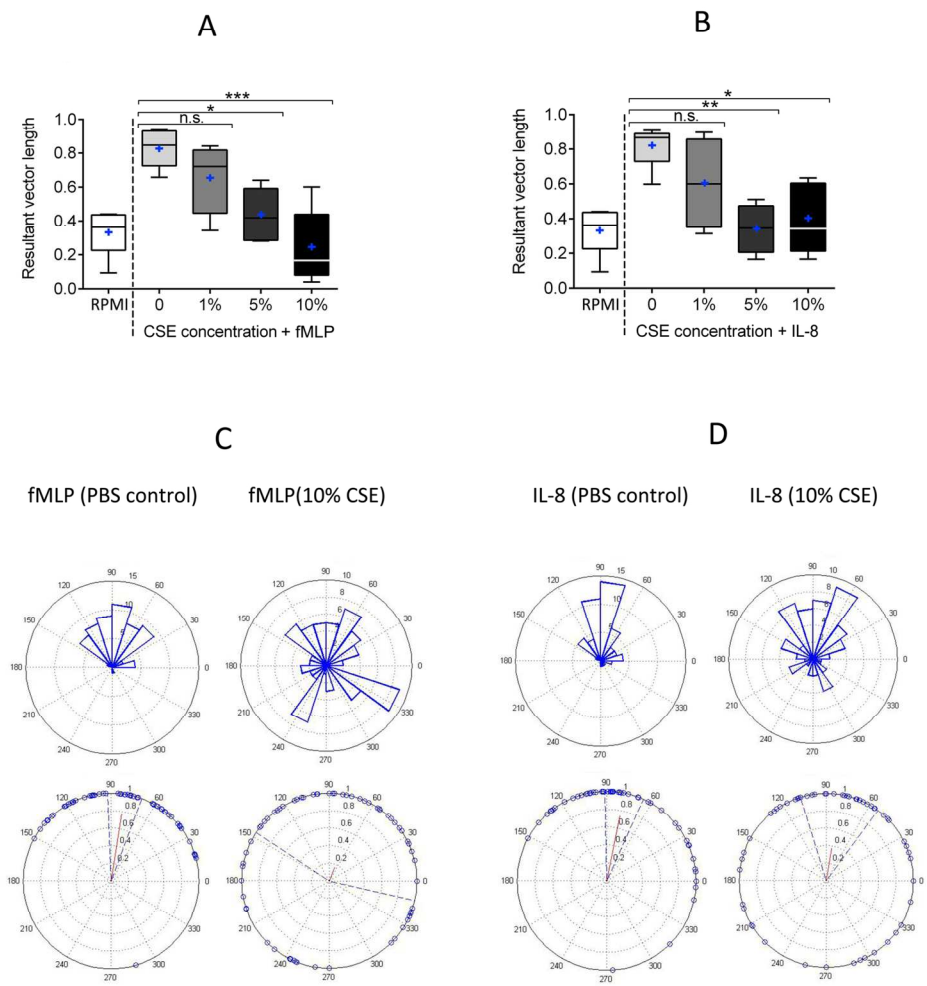
208x260mm (300 x 300 DPI)

Figure 3



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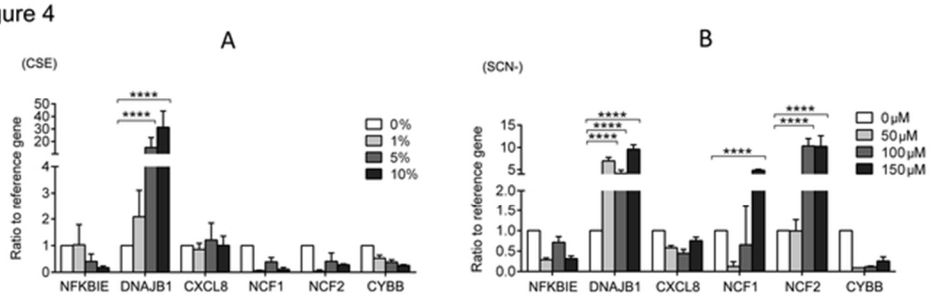
146x157mm (300 x 300 DPI)

Figure 4



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58x18mm (300 x 300 DPI)