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# Modulation of neutrophil extracellular trap (NET) and reactive oxygen species (ROS) release by periodontal bacteria

**Running title: NET and ROS release in response to oral bacteria**

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**ABSTRACT**

**Background:** Oral bacteria are the main trigger for the development of periodontitis and some species are known to modulate neutrophil function. This study aimed to explore the release of neutrophil extracellular traps (NETs), associated antimicrobial proteins and reactive oxygen species (ROS) in response to periodontal bacteria, as well as the underlying pathways.

**Methods:** Isolated peripheral blood neutrophils were stimulated with 19 periodontal bacteria. NET and ROS release as well as the expression of NET-bound antimicrobial proteins, elastase, myeloperoxidase and cathepsin G, in response to these species were measured using fluorescence-based assays. NET and ROS release were monitored after the addition of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase pathway modulators and inhibitors of Toll-like receptors (TLRs). Moreover, bacterial entrapment by NETs was visualised microscopically and bacterial killing was assessed by bacterial culture.

**Results:** Certain microorganisms, e.g. *Veillonella parvula* and *Streptococcus gordonii*, stimulated higher ROS and NET release than others. NETs were found to entrap, but not kill, all periodontal bacteria tested. NADPH-oxidase pathway modulators decreased ROS but not NET production in response to the bacteria. Interestingly, TLR inhibitors did not impact on ROS and NET release.

**Conclusions:** These data suggest that the variability in neutrophil response towards different bacteria may contribute to the pathogenesis of periodontal diseases by mechanisms such as bacterial avoidance of host responses and activation of neutrophils. Moreover, our results indicate that bacteria-stimulated NET release may

75    in part arise via NADPH oxidase-independent mechanisms. The role of TLR

76    signalling in bacteria-induced ROS and NET release needs to be further elucidated.

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## INTRODUCTION

Periodontitis is initiated by the accumulation of microbial biofilms at and below the gingival margin. Indeed it has been estimated that ~700 oral bacterial species and ~1,200 predominant phylotypes exist (1-3). Of these bacterial species, 5 major bacterial complexes (red, orange, yellow, green and purple) have been identified by Socransky *et al.* using DNA probes (4). The clustering and ordination analysis allowed them to assign microbial species to a colour complex dependent upon the strength of association with each other and the clinical staging of periodontitis. The biofilms, which develop during disease, are orchestrated to maximise their adherence, communication and survival. The accumulation of bacterial species within the biofilm enables its development and perseverance, and certain bacteria, such as *Fusobacterium nucleatum* (*F. nucleatum*), are key orchestrators of biofilm formation and maturation (5).

In susceptible individuals, dysbiosis and an aberrant host-microbial equilibrium can result in the onset of disease (6), where the microbial biofilm thrives by exploiting the host inflammatory response. This process fuels a vicious cycle of bacterial accumulation, inflammation and subsequent tissue destruction. The acute inflammatory reaction is predominantly mediated by neutrophils and is initially protective, via activation of innate neutrophil-derived defence mechanisms and also the activation of the acquired cellular and humoral immune system. In periodontitis, however, the aberrant neutrophil response is reputed to contribute to collateral tissue damage and formation of disease-associated molecular patterns, which perpetuate the inflammation leading to chronicity (7). Furthermore, the inflammatory state itself

125 supplies nutrients to pathogenic bacteria such as *Porphyromonas gingivalis* (*P.*

126 *gingivalis)*, e.g. iron from heme, supporting its survival and proliferation (8).

127

128 An exaggerated immune activity is also observed in peripheral blood neutrophils

129 from both chronic and aggressive periodontitis patients. These neutrophils are

130 reportedly hyper-reactive in response to a microbial challenge in terms of their

131 release of reactive oxygen species (ROS), but also hyperactive in the absence of an

132 exogenous stimulus (9-11). In addition, excessive neutrophil-driven proteolytic

133 activity and pro-inflammatory cytokine production have been observed in

134 periodontitis and associate with pathogenicity (7). One of the mechanisms by which

135 neutrophils combat microorganisms through the production of neutrophil extracellular

136 traps (NETs), whereby decondensed DNA is released into the extracellular

137 environment to immobilise and potentially kill invading bacteria. NET release is

138 reported to be dependent on the production of ROS, such as hydrogen peroxide

139 ($H_2O_2$), via superoxide generation by the enzyme NADPH oxidase (12).

140

141 Little is known about the differential interactions between oral bacteria and

142 neutrophils, however there is evidence that certain species and strains can evoke

143 different neutrophil responses (13-16). This study aimed to elucidate the ability of

144 bacterial species and strains frequently isolated from the oral cavity of healthy and

145 diseased individuals to activate ROS and NET responses in neutrophils. The ability

146 of NETs to entrap and kill bacteria, along with the expression of the antimicrobial and

147 NET-associated proteins neutrophil elastase (NE), myeloperoxidase (MPO) and

148 cathepsin G (CG) were also analysed.

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**RESULTS**

**Neutrophil ROS release in response to periodontal bacteria**

The production of total ROS, extracellular ROS and superoxide in response to 19 periodontal bacteria and *S. aureus* (**Table 1**) were determined. Certain bacteria elicited higher total ROS production in neutrophils, which was measured by luminol chemiluminescence. This was statistically significant for *P. acnes, S. anginosus* and *C. rectus* as well as the positive control opsonised *S. aureus* (**Figure 1A**). Consistent with the data expressed as total peak ROS production, time-course ROS production expressed as "area under the curve" demonstrated that ROS production was highest in response to opsonised *S. aureus* followed by *S. anginosus*. Notably, the increase in total ROS in response to opsonised *S. aureus* was more rapid than following direct stimulation with periodontal bacteria, as illustrated by the sharp elevation of the curve immediately following stimulation. Neutrophil extracellular ROS production was subsequently analysed by isoluminol chemiluminescence. Phorbol 12-myristate 13-acetate (PMA; positive control) and *S. gordonii* induced significantly higher extracellular ROS than PBS treatment (negative control) (**Figure 1B**). The steep time-course curve in response to PMA indicates a rapid neutrophil response. Neutrophil extracellular superoxide production was measured using lucigenin. PMA and opsonised *S. aureus* did not induce significantly higher superoxide production relative to the PBS control. However, some periodontal bacteria increased extracellular superoxide production in neutrophils, which was statistically significant for *S. anginosus*, *C. suptigena,* and *F. nucleatum subsp. nucleatum* (**Figure 1C**).

**Quantification of NET production in response to periodontal bacteria**

NET release in response to the bacterial challenge was quantified. Some bacteria led to an enhanced NET-DNA production, which was statistically significant for *P. acnes*, *V. parvula,* and *S. gordonii* compared with the PBS control (**Figure 2A**). NET-bound NE, MPO and CG were quantified colorimetrically and data demonstrated that certain periodontal bacteria elicited an increased production of NET-bound proteins relative to PBS (**Figure 2B**-D). Similarly, stimulation with PMA and opsonised *S. aureus* (positive controls) induced statistically significant elevations in MPO and CG expression (**Figures 2C** and **D**).

**NET entrapment of bacteria does not associate with Socransky complexes or with bacterial cell death**

For clinical relevance, data are presented by grouping periodontal bacteria according to the Socransky complexes (4) (**Figure 3A**). Non-Socransky complex: *I. noxia and V. parvula* were found to be entrapped within NET structures in higher numbers compared to the negative controls (unstimulated neutrophils or degraded NETs) However, neither *A. actinomycetemcomitans* (serotype b) nor *P. acnes* or *A. viscosus* were significantly associated with NET entrapment. Yellow complex: *S. anginosus* and *S. gordonii* were significantly entrapped within NETs. However, the other yellow complex bacteria assayed, *S. sanguinis, S. oralis* and *S. intermedius,* were not found within NET structures. Green complex: none of the green complex bacteria assayed appeared within NETs at a significant level. Orange complex: *C. rectus, C. showae* and *F. nucleatum* subsp. *polymorphum* were significantly entrapped within NETs relative to the negative controls, whereas *S. constellatus* and *F. nucleatum* subsp. *nucleatum* were not. Red complex: *P. gingivalis* was

significantly associated with NET structures compared with bacteria incubated with

unstimulated neutrophils or degraded NET structures. SEM images of unstimulated

neutrophils demonstrated spherical cells with no NET structures evident, whereas

neutrophils incubated with *A. actinomycetemcomitans* serotype a, *V. parvula* and *A.*

*viscosus* revealed the release of NET structures (**Figure 3B**). The strand-like

filaments between the neutrophils appeared to associate with bacteria, for example,

*A. actinomycetemcomitans* (serotype a) clustered along NET structures. The

bacterial killing assays employed to detect the microbicidal properties of NETs

revealed that the viablitiy of the 6 periodontal bacteria tested was unaffected by NET

trapping (**Figure 3C**).


**Effect of NADPH-oxidase pathway modulating agents on ROS and NET**
**production**

Components of the NADPH-oxidase signalling pathway were targeted in order to

assess whether NADPH-oxidase is essential for neutrophil ROS and NET production

in response to periodontal bacteria. The data show that diphenyleneiodonium (DPI;

NADPH-oxidase inhibitor), N-acetyl-cysteine (NAC; $H_2O_2$ scavenger) and taurine

(HOCl scavenger) treatment resulted in a reduction in total ROS release in response

to all stimuli. This was statistically significant for PMA, opsonised *S. aureus*, *S.*

*gordonii*, *C. rectus*, *F. nucleatum* subsp. *polymorphum* and *S. noxia* (**Figure 4A**).

NET production was, with the exception of PMA, not significantly affected by these

inhibitors, however, a moderate reduction of NET release was visible in all samples

(**Figure 4B**).


**Effect of Toll-like receptor (TLR) inhibition on ROS and NET production**

225 The role of TLR signalling in neutrophil ROS and NET responses to periodontal

226 bacteria was investigated by using specific inhibitors. Chloroquine (TLR3, 7 and 9

227 inhibitor) and oxidised 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine

228 (OxPAPC; (TLR2 and 4 inhibitor) treatment as well as treatment with both

229 components did not reduce ROS production by neutrophils. However, a significant

230 increase in ROS release from neutrophils treated with OxPAPC and opsonised S.

231 aureus was seen (**Figure 5A**). Similarly, NET release in response to bacterial

232 stimulation was not affected by the TLR inhibitors (**Figure 5B**).

233

234

235 **DISCUSSION**

236

237 Neutrophil ROS production is a vital component of the innate immune response,

238 which enables killing and clearance of pathogens. Neutrophils are the predominant

239 immune cell in periodontitis (17), and the results presented here support that their

240 stimulation with periodontal bacteria promotes extracellular, intracellular and

241 superoxide ROS release; however data indicate that this may be species specific.

242 Indeed, some species consistently elicited higher neutrophil ROS production while

243 other bacteria, such as *P. gingivalis* or *S. sanguinis,* were not found to significantly

244 promote ROS release. Bacteria like *P. gingivalis, F. nucleatum* and oral streptococci

245 can scavenge neutrophil-derived ROS production, which is attributed to a range of

246 oxidative stress response genes encoding proteins like rubrerythrin, glutathione

247 peroxidase, glutaredoxin, NADH oxidase and superoxide dismutase (18-22). It is

248 possible that these bacterial defence mechanisms may function to afford protection

249 to other biofilm organisms that are less resistant to ROS.

250

251 Periodontitis is known to arise from an exaggerated inflammatory response to

252 microbial plaque (6). Whilst it is recognised that ROS facilitate microbial killing, ROS

253 do not discriminate between pathogens and host tissues, and therefore tissue injury

254 can arise from excess plaque-induced extracellular ROS release. ROS are reported

255 to contribute to periodontitis progression by direct and indirect mechanisms,

256 including tissue damage (23, 24), lipid peroxidation (25), DNA strand breakage (26),

257 increased osteoclast differentiation (27) and initiation of a self-perpetuating cycle that

258 activates chronic immune cell-derived ROS production (28). Notably, Matthews *et al.*

259 showed an increased ROS production by peripheral blood neutrophils in chronic

260 periodontitis (9, 10). In patients susceptible to the deleterious effects of ROS, a

261 discordance between oxidant and antioxidant levels may also play a role. This is

262 supported by Chapple *et al.*, who demonstrated that total antioxidant activity is lower

263 in the saliva of periodontitis patients (29). It has also been reported that neutrophil

264 chemotaxis is compromised in chronic periodontitis, and that these patients'

265 neutrophils produce the chemoattractant interleukin-8 in excess when stimulated,

266 potentially creating distracted chemotaxis (30). Such processes may increase

267 neutrophil tissue transit times and thereby potentially exacerbating ROS-mediated

268 collateral tissue damage (17).

269

270 Quantification of NET-DNA and NET-bound antimicrobial proteins demonstrated

271 differential NET production in response to the periodontal bacteria tested. DNA is

272 released during other forms of neutrophil cell death, such as necrosis, and the

273 quantification of NET-bound components (NE, MPO and CG) therefore provides a

274 DNA-independent measure of NETs. It is noteworthy that differences between

275    individuals have been reported, such as neutrophil responsiveness to stimuli, which

276    can also affect NET quantification results regardless of the analytical method

277    employed (31, 32). Significant NET production in response to individual periodontal

278    bacteria, however, indicates these events likely occur *in vivo*. Notably, NETs have

279    previously been shown to exist in purulent exudate from periodontal pockets, where

280    they are postulated to entrap invading microbes and prevent their dissemination (33,

281    34). Recently, many periodontal bacterial species have been shown to release

282    DNAses, which in addition to regulating biofilm formation (35), can potentially

283    disassemble NET structures to enable NET evasion (36). Thus, bacterial DNAse

284    expression may explain why some periodontal species showed less entrapment,

285    such as *S. constellatus,* which reportedly releases large quantities of DNAse (36).

286

287    Following bacterial entrapment, the high local concentration of antimicrobial proteins

288    associated with NETs is thought to disable and kill pathogens (37). In the present

289    study, the incubation of NETs with periodontal bacteria did not impede bacterial

290    growth or survival, being in accordance with data reported by Menegazzi *et al.* (38).

291    Cytochalasin B was applied in our study to exclude the possibility of bacterial killing

292    through phagocytosis and this inhibition occurs via blocking of actin polymerization

293    (39). As functional actin filaments may play a role in NET formation (40), it is

294    possible that cytochalasin B interfered with NET and antimicrobial protein release in

295    our study and thus prevented bacterial killing. However, other known inhibitors of

296    phagocytosis and endocytosis such as Latrunculin A or CK666 also exert their

297    effects by disturbing actin polymerisation (41, 42). Future experiments may be

298    directed at investigating differences among such inhibitors regarding their

299    interference with NET release.

300

301 Treatment of neutrophils with the NADPH-oxidase inhibitor DPI, glutathione

302 peroxidase precursor substrate NAC and the HOCl scavenger taurine abrogated

303 total ROS release, being in accordance with data previously reported (43-45). NET

304 release was only inhibited marginally in response to the bacterial challenge. At the

305 same time, NET production was significantly affected by the inhibitors in neutrophils

306 stimulated with PMA. This may be explained by the fact that PMA induces NETs via

307 protein kinase C, which then activates the NADPH oxidase, and thus can elicit NETs

308 only via the generation of ROS (43). These findings indicate that NADPH-oxidase-

309 independent NET formation may play a role in host defence against periodontal

310 bacteria (46, 47).

311

312 Further experiments aimed to establish the role of TLR activation in NET production.

313 Pre-treatment of neutrophils with the intracellular TLR3, 7, 8 and 9 inhibitor

314 chloroquine and with the TLR2 and 4 inhibitor OxPAPC separately or combined, did

315 not lead to significant reductions in ROS or NET release. Previous findings have

316 suggested that ROS release is both TLR2 and 4 dependent (48), however, Gould *et*

317 *al.* recently demonstrated that blocking TLR2 and 4 did not abolish NET release (49),

318 being in line with the findings of this study. Notably, neutrophils are not responsive to

319 TLR3 ligands (50), therefore, the involvement of TLR7, 8 and 9 was investigated by

320 using chloroquine. Similarly, to our results, Salmon *et al.* found that chloroquine had

321 no effect on the oxidative metaboslism in neutrophils (51). Thus, a lack of inhibition

322 of ROS and NET generation by chloroquine and OxPAPC indicates that other

323 signalling pathways may have played a role in this study. For example, C-type lectin

324 receptors and NOD-like receptors can be activated by bacterial triggers, and both

325 have been reported to induce immune activation in neutrophils, including the release

326 of ROS (52, 53). Moreover, signalling via TLR co-receptors may have bypassed the

327 inhibited pathways (54). Importantly, although widely used as a TLR inhibitor,

328 chloroquine is thought to directly interfere with multiple physiological cell functions

329  including chemotaxis, phagocytosis and ROS release, by alkalising lysosomes and

330 phagolysosomes (55, 56). Our results do not support an inhibitory effect of

331 chloroquine on these functions in the concentrations applied in the present study, as

332 no significant differences were seen between neutrophils treated with chloroquine

333 and negative controls. However, results from functional cell assays using

334 chloroquine as an inhibitor should be interpreted with care.

335

336 Future experiments should target these receptors to further elucidate their specific

337 role in ROS and NET release. Interestingly, in OxPAPC-treated neutrophils

338 stimulated with opsonised *S. aureus*, a significant increase in ROS was seen.

339 Previous studies reported that OxPAPC has the potential to increase ROS release in

340 endothelial cells via activating the NADPH oxidase (57, 58). Moreover, Fc gamma

341 receptor (FcγR) signalling is known to trigger ROS release (59). It is therefore

342 possible that OxPAPC may act as a co-trigger of FcγR-mediated ROS release in

343 neutrophils challenged with opsonised bacteria, however, further experiments are

344 required to confirm this hypothesis.

345

346 As a limitation of this study, planktonic single-species preparations were used to

347 stimulate neutrophils. *In vivo*, however, neutrophils are challenged by multi-species

348 biofilms. These biofilms produce metabolites and extracellular matrix components

349 that may lead to a different response pattern compared to that observed under our

350 experimental conditions. Variability in these extracellular products generated by

351 naturally or artificially grown biofilms is high and reproducibility of experiments

352 involving such biofilms is difficult (13, 60). Moreover, natural dental biofilms are

353 highly variable in their composition, and it is difficult to attribute their activation of

354 neutrophils to certain species or biofilm components. Therefore, little is know about

355 the interactions between host cells and mixed species biofilms. Further efforts aimed

356 at creating a reproducible neutrophil-biofilm interaction model *in vitro* are currently

357 being carried out by our group. Nevertheless, in order to understand the interaction

358 of neutrophils with oral bacterial species, such microorganisms playing key roles in

359 neutrophil activation need to be identified and investigated separately. Insights from

360 these experiments may subsequently allow for better understanding of neutrophil

361 responses to oral biofilms.

362

363 As a further limitation, heat-killed microorganisms were employed in our study.

364 Although heat-killing may lead to the denaturation of surface antigens and pathogen-

365 associated molecular patterns (PAMPs), this is thought to be reversible at

366 temperatures below 80°C (61). Moreover, previous studies using live bacteria (*A.*

367 *actinomycetemcomitans* serotype b or *S. gordonii*, *F. nucleatum* subsp.

368 *polymorphum* and *V. parvula*) showed similar NET formation outcomes regarding

369 AFU measurements or relative differences in NET production, respectively (14, 62).

370 Another restriction in our study is the limited number of different bacterial species

371 used to investigate neutrophil activation. Future studies may need to include further

372 species, particularly of the red complex, such as *Treponema denticola* and

373 *Tannerella forsythia.*

374

375    In the present study, neutrophils from periodontally and systemically healthy donors

376    were used. Overall, these neutrophils were responsive to some health-associated

377    species and opportunistic pathogens rather than disease-associated species. By

378    contrast, our previous investigations of periodontally diseased patients have shown

379    that their neutrophils are hyperactive and hyper-reactive towards *F. nucleatum* and

380    *P. gingivalis* in terms of ROS and cytokine release. On the other hand, NET

381    production in response to various stimuli was not altered and was similar in

382    periodontitis patients and non-periodontitis controls. However, these studies did not

383    compare the effect of health- and disease-associated bacteria on neutrophils (9, 30,

384    63, 64). It is possible that neutrophils from periodontitis patients may show a higher

385    reactivity towards periodontal bacteria than those from healthy subjects, as these

386    neutrophils may be primed in the circulation by bacterial components, such as LPS,

387    accessing the blood stream through periodontal microlesions (65, 66). Further

388    studies examining responses of neutrophils from healthy and periodontally diseased

389    individuals to different oral bacteria may shed light on possible mechanisms of

390    immune tolerance in health and disease.

391

392    In summary, the data presented here demonstrate variability between periodontal

393    bacteria in their ability to stimulate neutrophil ROS production and NET responses.

394    This may contribute to the pathogenesis of periodontitis by mechanisms such as

395    bacterial avoidance of host defence mechanisms and thus persistence of infection,

396    or excess ROS release with associated tissue damage. Moreover, our results

397    indicate that innate immune receptors other than the TLRs investigated here may be

398    involved in bacteria-triggered ROS and NET release, and that NADPH oxidase-

399    independent NET formation may occur in response to periodontal pathogens.

400 Comprehensive studies are required to fully elucidate the role of NETs and ROS in

401 periodontitis, in particular with regard to the receptors, activation pathways and

402 intracellular responses triggered by different bacteria. Also, investigating the possible

403 activation of protective mechanisms, such as glutathione up-regulation, or of anti-

404 inflammatory signalling routes by these bacteria may improve our understanding of

405 their differential effects seen in this study.

406

407

408 **MATERIALS AND METHODS**

409

410 **Neutrophil isolation**

411 Neutrophils were isolated from the peripheral venous blood of periodontally and

412 systemically healthy volunteers (University of Birmingham Ethics Reference:

413 ERN_13-0325) using discontinuous Percoll gradients (GE Healthcare, Amersham,

414 UK) as previously described (67). The medical history was taken from each donor

415 and periodontal examinations were conducted to ensure periodontal and systemic

416 health. Cell viability and purity were confirmed by trypan blue exclusion and flow

417 cytometry, respectively, and this was typically >98%.

418

419 **Bacterial culture**

420 A panel of 19 periodontal bacteria and opsonised *S. aureus* were employed to

421 stimulate neutrophils. Bacterial stocks were originally obtained from the Forsyth

422 Institute (Boston, MA, USA) or purchased from the American Type Culture Collection

423 (ATCC). Blood agar plates (Base no. 2 with 7% horse blood) were purchased from

424 Oxoid (Basingstoke, UK) and used for growing most bacterial strains. *P. gingivalis*

17

425  (strain W83) was cultured on anaerobic 20% blood agar plates (Wilkins Chalgren,

426  Oxoid) and *S. aureus* was cultured on tryptone soya agar (TSA) plates. Trypticase

427  soy broth (TSB), brain heart infusion broth (BHI) (both from Oxoid) or fastidious

428  anaerobe broth (Lab M, Heywood, UK) were used for planktonic growth of the

429  microorganisms. Bacterial cell suspensions were measured spectrophotometrically

430  at an optical density of 600 nm to estimate bacterial numbers and bacteria were

431  heat-killed at 80°C for 30 min. Bacterial cells were washed with PBS, centrifuged and

432  the pellet resuspended to produce a stock solution, which was stored at -20°C prior

433  to use. The bacteria used, their growth conditions and assignment to Socransky

434  complexes are listed in **Table 1**.

435

436  **Opsonisation of *S. aureus***

437  *S. aureus* was grown planktonically in TSB. Following 48h of aerobic growth,

438  bacteria were washed and pelleted by centrifugation for 15 min at 1800 rcf and 4°C.

439  Bacteria were opsonised with Vigam liquid (5 mg/mL IgG, Bio Products Laboratory,

440  Borehamwood, UK). This mixture was agitated overnight at room temperature and,

441  after washing, stored at -20°C until needed.

442

443  **Stimuli employed to activate neutrophils**

444  Neutrophils were stimulated using a range of stimuli. PMA targets NET production

445  via the activation of protein kinase C (PKC). Our previous findings demonstrated that

446  the concentration of PMA required for NET release is at 50 nM, whereas 25 nM are

447  sufficient to stimulate ROS production (43). Both Gram-positive and -negative

448  bacteria were used to activate neutrophils via TLR2 and 4 in ROS and NET assays.

449  In addition, ROS and NETs were produced in response to stimulation with opsonised

450     *S. aureus*, which activates neutrophils via the FcγR. For some supplementary

451     assays, a smaller panel of bacteria was employed, where the selection was made

452     based on variable characteristics of the microorganisms: *Gram*-positive and *Gram*-

453     negative, aerobic, anaerobic and microaerophilic bacteria from different complexes

454     with FcγR and TLR activation properties were chosen, and both health and disease-

455     associated bacteria were represented. Moreover, variable DNase production was a

456     selection criterion, as DNAses have the ability to disassemble NETs (e.g., *F.*

457     *nucleatum* and *S. aureus* are known to produce DNase, whereas *A. viscosus* and *V.*

458     *parvula* produce little or no DNase, respectively) (36). In addition, bacteria reported

459     to interfere with ROS scavenging were included: *F. nucleatum* and *S. noxia*, which

460     can metabolise the antioxidants glutathione and L-cysteine (68), as well as *A.*

461     *viscosus*, which produces the ROS scavenger catalase (69).

462

463     **Quantification of NET production in response to periodontal bacteria**

464     NET release was determined using a NET quantification assay previously described

465     (70). Neutrophils were stimulated with the positive controls PMA (50 nM) and

466     opsonised *S. aureus* as well as with 19 heat-killed periodontal bacteria (multiplicity of

467     infection [MOI] of 1,000 (71)) after being equilibrated for a 30 min baseline period.

468     Unstimulated neutrophils (PBS) were employed as negative controls. Neutrophils

469     from 10 periodontally and systemically healthy donors were used to perform NET

470     quantification in triplicate wells per donor.

471

472     **Chemiluminescence protocol for ROS assay**

473     ROS production in response to the periodontal bacteria (MOI of 1,000) was

474     determined using enhanced chemiluminescence. Neutrophils ($1 \times 10^5$) from five

475 different donors were added to a 96-well plate (using triplicate wells per donor) pre-

476 coated with 1% bovine serum albumin (BSA). ROS release following exposure to

477 PBS (unstimulated negative control), PMA (25 nM, positive control) and opsonised

478 *S. aureus* (MOI of 500, positive control) was quantified. Neutrophils were stimulated

479 after being equilibrated for a 30 min baseline period and then ROS was measured

480 over the subsequent 100 min. To measure total ROS, extracellular ROS and

481 superoxide, luminol (3 mM), isoluminol (3 mM) with 1.5 units of horseradish

482 peroxidase (HRP), and lucigenin (0.25 mg/mL), respectively, were added to the

483 samples and the light output was read for 130 min in a luminometer (Berthold

484 Tristar2, Berthold Technologies, Harpenden, UK). All readings were expressed as

485 relative light units (RLUs) and read at 37°C (MikroWin2000, Informer Technologies,

486 Madrid, Spain). All reagents for chemiluminescence were purchased from Sigma

487 Aldrich (Dorset, UK).

488

489 **NET entrapment and quantification of NET-mediated killing of periodontal**

490 **bacteria**

491 To assess the ability of NETs to immobilise periodontal bacteria, fluorescein

492 isothiocyanate (FITC)-stained live bacteria (MOI of 100) were incubated for 1h with

493 unstimulated neutrophils, intact NETs (produced by prior 0.75 mM HOCl stimulation

494 with a subsequent washing step (43)) or NET structures degraded with micrococcal

495 nuclease (MNase, New England Biolabs, Hitchin, UK) in a 96-well plate pre-coated

496 with 1% BSA, using five different donors and triplicate wells per donor. *In vitro* NETs

497 are formed within 2-3h, therefore, a relevant induction of NET release from otherwise

498 unstimulated neutrophils by bacteria could be excluded. Following multiple wash

499 steps to remove any unbound bacteria, the amount of bacteria entrapped was

500    fluorometrically quantified and normalised to FITC-stained bacteria incubated with

501    PBS (cell-free control). To determine whether NETs are capable of killing entrapped

502    bacteria, six strains (*F. nucleatum* subsp. *polymorphum, S. intermedius*, *S.*

503    *sanguinis*, *A. viscosus*, *V. parvula* and *C. gingivalis*) were incubated at a MOI of 100

504    with PBS (negative control), unstimulated neutrophils, intact NETs or degraded

505    NETs from five different donors in triplicate wells per donor. Additionally, samples

506    containing neutrophils were treated with the phagocytosis inhibitor cytochalasin B

507    (Sigma Aldrich, Harpenden, UK) at a concentration of 10 μg/mL (72). Following 1h of

508    incubation bacteria were released from NETs by MNase digestion, diluted and

509    inoculated onto agar plates and cultured for 24h prior to performing colony counts.

510

511    **Effect of NADPH-oxidase pathway modulating agents on ROS and NET**

512    **production**

513    To further understand the importance of NADPH-oxidase and downstream products

514    in bacteria-induced ROS and NET production, specific components of the NADPH-

515    oxidase signalling pathway were targeted. Isolated neutrophils from three different

516    donors were incubated with DPI (25 μM), an inhibitor of NADPH-oxidase, NAC

517    (10 mM), a synthetic glutathione precursor that scavenges $H_2O_2$, or taurine

518    (100 mM), which scavenges HOCl to produce taurine chloramine (duplicate wells per

519    donor). Neutrophil total ROS and NET production were measured following pre-

520    incubation with the modulating agent for 30 min prior to stimulation with PMA

521    (50 nM), opsonised *S. aureus* (MOI of 500) and 8 selected bacteria (MOI of 1000; *S.*

522    *aureus, V. parvula, F. nucleatum* subsp. *nulceatum, F. nucleatum* subsp.

523    *polymorphum, S. gordonii, C. rectus, A. viscosus* and *S. noxia*). NET-DNA was

524    quantified with Sytox Green following enzymatic degradation of NET structures with

MNase. All reagents were purchased from Sigma Aldrich (Dorset, UK).

**Effect of TLR inhibition on ROS and NET production**

To better understand the signalling involved in ROS and NET activation, the effect of
TLR inhibitors was investigated. Isolated neutrophils from three different donors were
incubated in duplicate wells per donor with chloroquine (100 µM, Invivogen,
Toulouse, France), an intracellular inhibitor of endosomal TLR 3, 7, 8 and 9, or
OxPAPC (30µg/ml, Invivogen, Toulouse, France), which inhibits intracellular
signalling of activated TLR 2 and 4, or both TLR inhibitors were used simultaneously.
Neutrophil total ROS and NET production were measured following pre-incubation
with the inhibitor for 30 min prior to stimulation with PMA (50 nM), opsonised *S.
aureus* (MOI of 500) and 8 selected bacteria (MOI of 1000; *S. aureus, V. parvula, F.
nucleatum* subsp. *nulceatum, F. nucleatum* subsp. *polymorphum, S. gordonii, C.
rectus, A. viscosus* and *S. noxia*).

**Statistical analysis**

All statistical analyses were performed in GraphPad Prism 5 software package for
Windows (San Diego, CA, USA). The distribution of data, and thus whether data
were considered parametric or non-parametric, was determined by Kolmogorov-
Smirnov tests. Statistical tests employed for the purpose of this study were at a
significance of 0.05. The level of significance is indicated as follows: *, **, *** and ****
denotes <0.05, <0.01, <0.001 and <0.0001, respectively. Kruskal-Wallis and Dunn's
multiple comparison tests were performed for quantification of ROS and NET
release. One-way ANOVA and Dunnett's post-hoc tests were employed for NET
entrapment assays. Two-way ANOVA and Bonferroni post-hoc tests were applied to

550 calculate significances of pathway modulation and inhibition assays. All quantitative

551 data are shown as mean values ± standard deviations and all statistical tests were

552 performed comparing different donors.

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555 **ACKNOWLEDGMENTS**

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**FIGURE LEGENDS**

**Figure 1**

**Neutrophil ROS production in response to periodontal bacteria.** Neutrophil total

reactive oxygen species (ROS) (**A**), extracellular ROS (**B**) and superoxide production

(**C**) were quantified and time-course production assayed over 130 min in response to

periodontal bacteria using luminol, isoluminol and lucigenin enhanced

chemiluminescence, respectively. ROS release in response to PBS (unstimulated

negative control), phorbol 12-myristate 13-acetate (PMA) (25 nM, positive control)

and opsonised *S. aureus* (positive control) was also quantified. Data are presented

as relative light units (RLU) and represent neutrophils of five different donors

assessed in triplicate wells.

**Figure 2**

**Quantification of NET production in response to periodontal bacteria.**

Neutrophil extracellular trap (NET) production was quantified in response to

periodontal bacteria and to PBS (unstimulated negative control), phorbol 12-

myristate 13-acetate (PMA) (50 nM, positive control) and opsonised *S. aureus*

(positive control). NET-DNA was quantified using Sytox Green assay (**A**) and NET-

bound neutrophil elastase (**B**), myeloperoxidase (**C**) and cathepsin G (**D**) were

quantified colorimetrically. Data are presented as arbitrary fluorescence units (AFU),

U/mL or mU/mL and represent neutrophils of ten different donors assessed in

triplicate wells.

**Figure 3**

819  **NET entrapment of periodontal bacteria. A**: Neutrophil extracellular trap (NET)

820  entrapment of bacteria that were not assigned to a Socransky complex (white, grey,

821  black), as well as purple, yellow, green, orange, red and blue complex bacteria.

822  Results are normalised to fluorescein isothiocyanate-stained bacteria in PBS.

823  Statistical significance of bacterial entrapment in NETs is shown relative to bacteria

824  entrapped by unstimulated neutrophils and degraded NETs (n.s.=not significant).

825  Data are presented as arbitrary fluorescence units (AFU) **B**: Representative images

826  of bacterial entrapment by NETs. Neutrophils (yellow arrows) incubated with PBS

827  (control), live *A. actinomycetemcomitans* serotype a, *V. parvula* or *A. viscosus* were

828  visualised by scanning electron microscopy. Blue arrows indicate NET strand

829  structures and NET-associated bacteria are indicated with green arrows.

830  Representative images of three experiments are shown, the scale bar represents

831  10µm. **C**: Bacterial survival after exposure to neutrophils, NETs, degraded NETs and

832  neutrophils with cytochalasin B (n.s.=not significant). All results shown represent

833  neutrophils of five different donors assessed in triplicate wells.

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835  **Figure 4**

836  **Effect of NADPH-oxidase pathway modulating agents on ROS and NET**

837  **production.** Total reactive oxygen species (ROS) (**A**) and Neutrophil extracellular

838  trap (NET) (**B**) production by neutrophils was quantified in response to selected

839  periodontal bacteria, as well as to phorbol 12-myristate 13-acetate (PMA) (50 nM)

840  and opsonised *S. aureus* (positive controls) following pre-incubation (30 mins) with

841  diphenyleneiodonium (DPI) (25 µM), N-acetyl-cysteine (NAC) (10 mM) and taurine

842  (100 mM). Data are presented as relative light units (RLU) and arbitrary fluorescence

843  units (AFU). Experiments were conducted in duplicate using three different donors.

845 **Figure 5**

846 **Effect of Toll-like receptor inhibition on ROS and NET production.** Total reactive

847 oxygen species (ROS) (**A**) and Neutrophil extracellular trap (NET) (**B**) production by

848 neutrophils was quantified in response to selected periodontal bacteria, as well as to

849 phorbol 12-myristate 13-acetate (PMA) (50 nM) and opsonised *S. aureus* (positive

850 controls) following pre-incubation (30 mins) with chloroquine (100 µM), oxidised 1-

851 palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC) (30 µg/mL) or

852 chloroquine and OxPAPC. Experiments were conducted in duplicate wells using

853 three different donors (n.s.=not significant).

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869 **Table 1**

870 Bacteria used, their assignment to Socransky complexes and growth conditions.

| Bacterial strain | ATCC number | Socransky complex | Growth conditions |
|---|---|---|---|
| Actinomyces viscosus (naeslundii genospecies 2) | 43146 | blue | anaerobic |
| Aggregatibacter actino-mycetemcomitans serotype a | 29523 | green | anaerobic |
| Aggregatibacter actino-mycetemcomitans serotype b | 43718 | white | anaerobic |
| Campylobacter rectus | 33238(371) | orange | anaerobic |
| Campylobacter showae | 51146 | orange | anaerobic |
| Capnocytophaga gingivalis | 33624(27) | green | anaerobic |
| Capnocytophaga sputigena | 33612(4) | green | anaerobic |
| Fusobacterium nucleatum subsp. nucleatum | 25586 | orange | anaerobic |
| Fusobacterium nucleatum subsp. polymorphum | 10953 | orange | anaerobic |
| Porphyromonas gingivalis | W83 | red | anaerobic |
| Propionibacterium acnes | 11827 | white | anaerobic |
| Selenomonas noxia | 43541 | white | anaerobic |
| Staphylococcus aureus (opsonised) | 9144 | N/A | aerobic |
| Streptococcus anginosus | 33397 | yellow | 5% $CO_2$ |
| Streptococcus constellatus | 27823(M32b) | orange | 5% $CO_2$ |
| Streptococcus gordonii | 10558 | yellow | 5% $CO_2$ |
| Streptococcus intermedius | 27335 | yellow | 5% $CO_2$ |
| Streptococcus oralis | 35037 | yellow | 5% $CO_2$ |
| Streptococcus sanguinis | 10556 | yellow | 5% $CO_2$ |
| Veillonella parvula | 10790 | purple | anaerobic |

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Figure 1
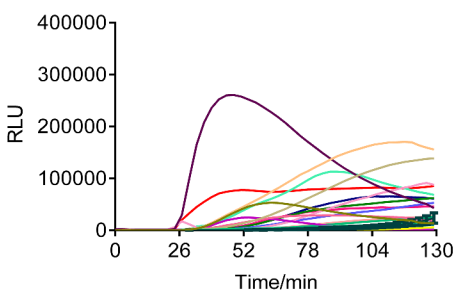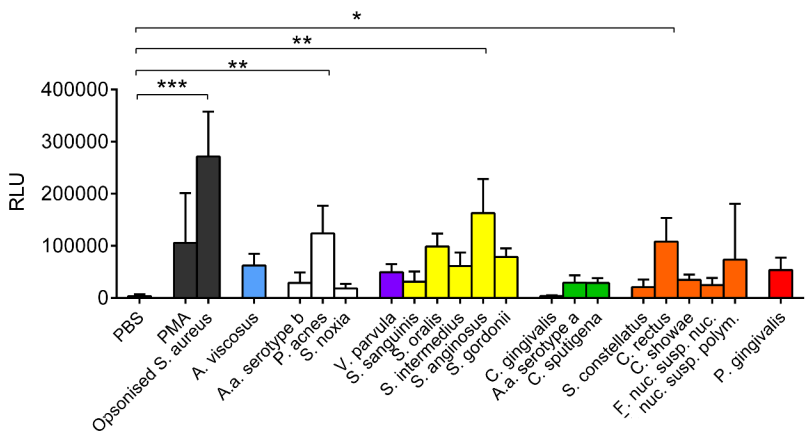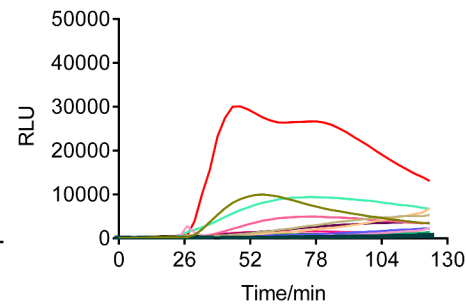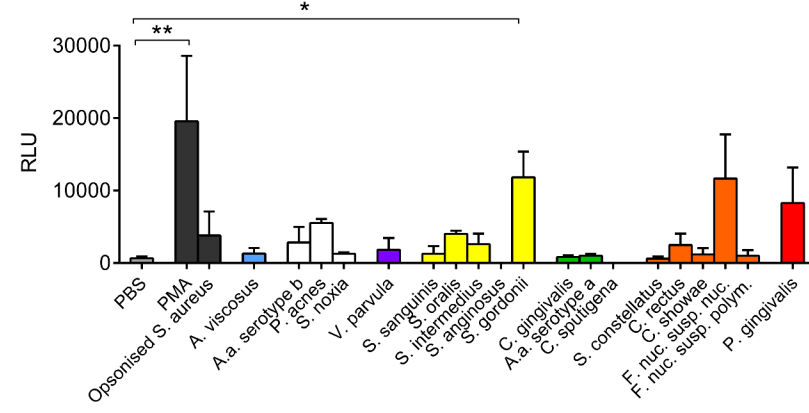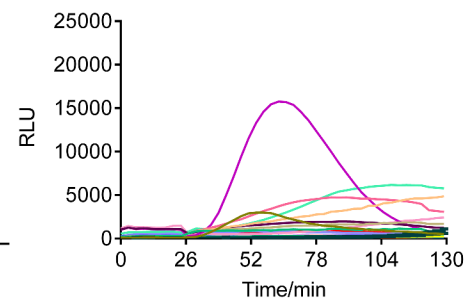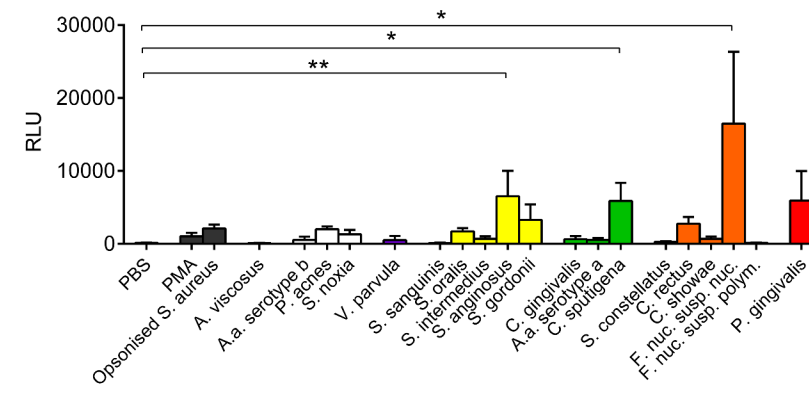
Figure 2

# Figure 3

## A



## B



## C

# Figure 4

# Figure 5