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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

in part arise via NADPH oxidase-independent mechanisms. The role of TLR signalling in bacteria-induced ROS and NET release needs to be further elucidated.

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

supplies nutrients to pathogenic bacteria such as *Porphyromonas gingivalis* (*P. gingivalis)*, e.g. iron from heme, supporting its survival and proliferation (8).

An exaggerated immune activity is also observed in peripheral blood neutrophils from both chronic and aggressive periodontitis patients. These neutrophils are reportedly hyper-reactive in response to a microbial challenge in terms of their release of reactive oxygen species (ROS), but also hyperactive in the absence of an exogenous stimulus (9-11). In addition, excessive neutrophil-driven proteolytic activity and pro-inflammatory cytokine production have been observed in periodontitis and associate with pathogenicity (7). One of the mechanisms by which neutrophils combat microorganisms through the production of neutrophil extracellular traps (NETs), whereby decondensed DNA is released into the extracellular environment to immobilise and potentially kill invading bacteria. NET release is reported to be dependent on the production of ROS, such as hydrogen peroxide (H_2O_2), via superoxide generation by the enzyme NADPH oxidase (12).

Little is known about the differential interactions between oral bacteria and neutrophils, however there is evidence that certain species and strains can evoke different neutrophil responses (13-16). This study aimed to elucidate the ability of bacterial species and strains frequently isolated from the oral cavity of healthy and diseased individuals to activate ROS and NET responses in neutrophils. The ability of NETs to entrap and kill bacteria, along with the expression of the antimicrobial and NET-associated proteins neutrophil elastase (NE), myeloperoxidase (MPO) and cathepsin G (CG) were also analysed.

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

The role of TLR signalling in neutrophil ROS and NET responses to periodontal bacteria was investigated by using specific inhibitors. Chloroquine (TLR3, 7 and 9 inhibitor) and oxidised 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC; (TLR2 and 4 inhibitor) treatment as well as treatment with both components did not reduce ROS production by neutrophils. However, a significant increase in ROS release from neutrophils treated with OxPAPC and opsonised S. aureus was seen (**Figure 5A**). Similarly, NET release in response to bacterial stimulation was not affected by the TLR inhibitors (**Figure 5B**).

DISCUSSION

Neutrophil ROS production is a vital component of the innate immune response, which enables killing and clearance of pathogens. Neutrophils are the predominant immune cell in periodontitis (17), and the results presented here support that their stimulation with periodontal bacteria promotes extracellular, intracellular and superoxide ROS release; however data indicate that this may be species specific. Indeed, some species consistently elicited higher neutrophil ROS production while other bacteria, such as *P. gingivalis* or *S. sanguinis,* were not found to significantly promote ROS release. Bacteria like *P. gingivalis, F. nucleatum* and oral streptococci can scavenge neutrophil-derived ROS production, which is attributed to a range of oxidative stress response genes encoding proteins like rubrerythrin, glutathione peroxidase, glutaredoxin, NADH oxidase and superoxide dismutase (18-22). It is possible that these bacterial defence mechanisms may function to afford protection to other biofilm organisms that are less resistant to ROS.

Periodontitis is known to arise from an exaggerated inflammatory response to microbial plaque (6). Whilst it is recognised that ROS facilitate microbial killing, ROS do not discriminate between pathogens and host tissues, and therefore tissue injury can arise from excess plaque-induced extracellular ROS release. ROS are reported to contribute to periodontitis progression by direct and indirect mechanisms, including tissue damage (23, 24), lipid peroxidation (25), DNA strand breakage (26), increased osteoclast differentiation (27) and initiation of a self-perpetuating cycle that activates chronic immune cell-derived ROS production (28). Notably, Matthews *et al.* showed an increased ROS production by peripheral blood neutrophils in chronic periodontitis (9, 10). In patients susceptible to the deleterious effects of ROS, a discordance between oxidant and antioxidant levels may also play a role. This is supported by Chapple *et al.*, who demonstrated that total antioxidant activity is lower in the saliva of periodontitis patients (29). It has also been reported that neutrophil chemotaxis is compromised in chronic periodontitis, and that these patients' neutrophils produce the chemoattractant interleukin-8 in excess when stimulated, potentially creating distracted chemotaxis (30). Such processes may increase neutrophil tissue transit times and thereby potentially exacerbating ROS-mediated collateral tissue damage (17).

Quantification of NET-DNA and NET-bound antimicrobial proteins demonstrated differential NET production in response to the periodontal bacteria tested. DNA is released during other forms of neutrophil cell death, such as necrosis, and the quantification of NET-bound components (NE, MPO and CG) therefore provides a DNA-independent measure of NETs. It is noteworthy that differences between

individuals have been reported, such as neutrophil responsiveness to stimuli, which can also affect NET quantification results regardless of the analytical method employed (31, 32). Significant NET production in response to individual periodontal bacteria, however, indicates these events likely occur *in vivo*. Notably, NETs have previously been shown to exist in purulent exudate from periodontal pockets, where they are postulated to entrap invading microbes and prevent their dissemination (33, 34). Recently, many periodontal bacterial species have been shown to release DNAses, which in addition to regulating biofilm formation (35), can potentially disassemble NET structures to enable NET evasion (36). Thus, bacterial DNAse expression may explain why some periodontal species showed less entrapment, such as *S. constellatus,* which reportedly releases large quantities of DNAse (36).

Following bacterial entrapment, the high local concentration of antimicrobial proteins associated with NETs is thought to disable and kill pathogens (37). In the present study, the incubation of NETs with periodontal bacteria did not impede bacterial growth or survival, being in accordance with data reported by Menegazzi *et al.* (38). Cytochalasin B was applied in our study to exclude the possibility of bacterial killing through phagocytosis and this inhibition occurs via blocking of actin polymerization (39). As functional actin filaments may play a role in NET formation (40), it is possible that cytochalasin B interfered with NET and antimicrobial protein release in our study and thus prevented bacterial killing. However, other known inhibitors of phagocytosis and endocytosis such as Latrunculin A or CK666 also exert their effects by disturbing actin polymerisation (41, 42). Future experiments may be directed at investigating differences among such inhibitors regarding their interference with NET release.

Treatment of neutrophils with the NADPH-oxidase inhibitor DPI, glutathione peroxidase precursor substrate NAC and the HOCl scavenger taurine abrogated total ROS release, being in accordance with data previously reported (43-45). NET release was only inhibited marginally in response to the bacterial challenge. At the same time, NET production was significantly affected by the inhibitors in neutrophils stimulated with PMA. This may be explained by the fact that PMA induces NETs via protein kinase C, which then activates the NADPH oxidase, and thus can elicit NETs only via the generation of ROS (43). These findings indicate that NADPH-oxidase-independent NET formation may play a role in host defence against periodontal bacteria (46, 47).

Further experiments aimed to establish the role of TLR activation in NET production. Pre-treatment of neutrophils with the intracellular TLR3, 7, 8 and 9 inhibitor chloroquine and with the TLR2 and 4 inhibitor OxPAPC separately or combined, did not lead to significant reductions in ROS or NET release. Previous findings have suggested that ROS release is both TLR2 and 4 dependent (48), however, Gould *et al.* recently demonstrated that blocking TLR2 and 4 did not abolish NET release (49), being in line with the findings of this study. Notably, neutrophils are not responsive to TLR3 ligands (50), therefore, the involvement of TLR7, 8 and 9 was investigated by using chloroquine. Similarly, to our results, Salmon *et al*. found that chloroquine had no effect on the oxidative metaboslism in neutrophils (51). Thus, a lack of inhibition of ROS and NET generation by chloroquine and OxPAPC indicates that other signalling pathways may have played a role in this study. For example, C-type lectin receptors and NOD-like receptors can be activated by bacterial triggers, and both

have been reported to induce immune activation in neutrophils, including the release of ROS (52, 53). Moreover, signalling via TLR co-receptors may have bypassed the inhibited pathways (54). Importantly, although widely used as a TLR inhibitor, chloroquine is thought to directly interfere with multiple physiological cell functions including chemotaxis, phagocytosis and ROS release, by alkalising lysosomes and phagolysosomes (55, 56). Our results do not support an inhibitory effect of chloroquine on these functions in the concentrations applied in the present study, as no significant differences were seen between neutrophils treated with chloroquine and negative controls. However, results from functional cell assays using chloroquine as an inhibitor should be interpreted with care.

Future experiments should target these receptors to further elucidate their specific role in ROS and NET release. Interestingly, in OxPAPC-treated neutrophils stimulated with opsonised *S. aureus*, a significant increase in ROS was seen. Previous studies reported that OxPAPC has the potential to increase ROS release in endothelial cells via activating the NADPH oxidase (57, 58). Moreover, Fc gamma receptor (FcγR) signalling is known to trigger ROS release (59). It is therefore possible that OxPAPC may act as a co-trigger of FcγR-mediated ROS release in neutrophils challenged with opsonised bacteria, however, further experiments are required to confirm this hypothesis.

As a limitation of this study, planktonic single-species preparations were used to stimulate neutrophils. *In vivo*, however, neutrophils are challenged by multi-species biofilms. These biofilms produce metabolites and extracellular matrix components that may lead to a different response pattern compared to that observed under our

experimental conditions. Variability in these extracellular products generated by naturally or artificially grown biofilms is high and reproducibility of experiments involving such biofilms is difficult (13, 60). Moreover, natural dental biofilms are highly variable in their composition, and it is difficult to attribute their activation of neutrophils to certain species or biofilm components. Therefore, little is know about the interactions between host cells and mixed species biofilms. Further efforts aimed at creating a reproducible neutrophil-biofilm interaction model *in vitro* are currently being carried out by our group. Nevertheless, in order to understand the interaction of neutrophils with oral bacterial species, such microorganisms playing key roles in neutrophil activation need to be identified and investigated separately. Insights from these experiments may subsequently allow for better understanding of neutrophil responses to oral biofilms.

As a further limitation, heat-killed microorganisms were employed in our study. Although heat-killing may lead to the denaturation of surface antigens and pathogen-associated molecular patterns (PAMPs), this is thought to be reversible at temperatures below 80°C (61). Moreover, previous studies using live bacteria (*A. actinomycetemcomitans* serotype b or *S. gordonii*, *F. nucleatum* subsp. *polymorphum* and *V. parvula*) showed similar NET formation outcomes regarding AFU measurements or relative differences in NET production, respectively (14, 62). Another restriction in our study is the limited number of different bacterial species used to investigate neutrophil activation. Future studies may need to include further species, particularly of the red complex, such as *Treponema denticola* and *Tannerella forsythia*.

In the present study, neutrophils from periodontally and systemically healthy donors were used. Overall, these neutrophils were responsive to some health-associated species and opportunistic pathogens rather than disease-associated species. By contrast, our previous investigations of periodontally diseased patients have shown that their neutrophils are hyperactive and hyper-reactive towards *F. nucleatum* and *P. gingivalis* in terms of ROS and cytokine release. On the other hand, NET production in response to various stimuli was not altered and was similar in periodontitis patients and non-periodontitis controls. However, these studies did not compare the effect of health- and disease-associated bacteria on neutrophils (9, 30, 63, 64). It is possible that neutrophils from periodontitis patients may show a higher reactivity towards periodontal bacteria than those from healthy subjects, as these neutrophils may be primed in the circulation by bacterial components, such as LPS, accessing the blood stream through periodontal microlesions (65, 66). Further studies examining responses of neutrophils from healthy and periodontally diseased individuals to different oral bacteria may shed light on possible mechanisms of immune tolerance in health and disease.

In summary, the data presented here demonstrate variability between periodontal bacteria in their ability to stimulate neutrophil ROS production and NET responses. This may contribute to the pathogenesis of periodontitis by mechanisms such as bacterial avoidance of host defence mechanisms and thus persistence of infection, or excess ROS release with associated tissue damage. Moreover, our results indicate that innate immune receptors other than the TLRs investigated here may be involved in bacteria-triggered ROS and NET release, and that NADPH oxidase-independent NET formation may occur in response to periodontal pathogens.

Comprehensive studies are required to fully elucidate the role of NETs and ROS in periodontitis, in particular with regard to the receptors, activation pathways and intracellular responses triggered by different bacteria. Also, investigating the possible activation of protective mechanisms, such as glutathione up-regulation, or of anti-inflammatory signalling routes by these bacteria may improve our understanding of their differential effects seen in this study.

MATERIALS AND METHODS

Neutrophil isolation

Neutrophils were isolated from the peripheral venous blood of periodontally and systemically healthy volunteers (University of Birmingham Ethics Reference: ERN_13-0325) using discontinuous Percoll gradients (GE Healthcare, Amersham, UK) as previously described (67). The medical history was taken from each donor and periodontal examinations were conducted to ensure periodontal and systemic health. Cell viability and purity were confirmed by trypan blue exclusion and flow cytometry, respectively, and this was typically >98%.

Bacterial culture

A panel of 19 periodontal bacteria and opsonised *S. aureus* were employed to stimulate neutrophils. Bacterial stocks were originally obtained from the Forsyth Institute (Boston, MA, USA) or purchased from the American Type Culture Collection (ATCC). Blood agar plates (Base no. 2 with 7% horse blood) were purchased from Oxoid (Basingstoke, UK) and used for growing most bacterial strains. *P. gingivalis*

(strain W83) was cultured on anaerobic 20% blood agar plates (Wilkins Chalgren, Oxoid) and *S. aureus* was cultured on tryptone soya agar (TSA) plates. Trypticase soy broth (TSB), brain heart infusion broth (BHI) (both from Oxoid) or fastidious anaerobe broth (Lab M, Heywood, UK) were used for planktonic growth of the microorganisms. Bacterial cell suspensions were measured spectrophotometrically at an optical density of 600 nm to estimate bacterial numbers and bacteria were heat-killed at 80°C for 30 min. Bacterial cells were washed with PBS, centrifuged and the pellet resuspended to produce a stock solution, which was stored at -20°C prior to use. The bacteria used, their growth conditions and assignment to Socransky complexes are listed in **Table 1**.

Opsonisation of *S. aureus*

S. aureus was grown planktonically in TSB. Following 48h of aerobic growth, bacteria were washed and pelleted by centrifugation for 15 min at 1800 rcf and 4°C. Bacteria were opsonised with Vigam liquid (5 mg/mL IgG, Bio Products Laboratory, Borehamwood, UK). This mixture was agitated overnight at room temperature and, after washing, stored at -20°C until needed.

Stimuli employed to activate neutrophils

Neutrophils were stimulated using a range of stimuli. PMA targets NET production via the activation of protein kinase C (PKC). Our previous findings demonstrated that the concentration of PMA required for NET release is at 50 nM, whereas 25 nM are sufficient to stimulate ROS production (43). Both Gram-positive and -negative bacteria were used to activate neutrophils via TLR2 and 4 in ROS and NET assays. In addition, ROS and NETs were produced in response to stimulation with opsonised

S. aureus, which activates neutrophils via the FcγR. For some supplementary assays, a smaller panel of bacteria was employed, where the selection was made based on variable characteristics of the microorganisms: *Gram*-positive and *Gram*-negative, aerobic, anaerobic and microaerophilic bacteria from different complexes with FcγR and TLR activation properties were chosen, and both health and disease-associated bacteria were represented. Moreover, variable DNase production was a selection criterion, as DNAses have the ability to disassemble NETs (e.g., *F. nucleatum* and *S. aureus* are known to produce DNase, whereas *A. viscosus* and *V. parvula* produce little or no DNase, respectively) (36). In addition, bacteria reported to interfere with ROS scavenging were included: *F. nucleatum* and *S. noxia*, which can metabolise the antioxidants glutathione and L-cysteine (68), as well as *A. viscosus*, which produces the ROS scavenger catalase (69).

Quantification of NET production in response to periodontal bacteria

NET release was determined using a NET quantification assay previously described (70). Neutrophils were stimulated with the positive controls PMA (50 nM) and opsonised *S. aureus* as well as with 19 heat-killed periodontal bacteria (multiplicity of infection [MOI] of 1,000 (71)) after being equilibrated for a 30 min baseline period. Unstimulated neutrophils (PBS) were employed as negative controls. Neutrophils from 10 periodontally and systemically healthy donors were used to perform NET quantification in triplicate wells per donor.

Chemiluminescence protocol for ROS assay

ROS production in response to the periodontal bacteria (MOI of 1,000) was determined using enhanced chemiluminescence. Neutrophils ($1x10^5$) from five

different donors were added to a 96-well plate (using triplicate wells per donor) pre-coated with 1% bovine serum albumin (BSA). ROS release following exposure to PBS (unstimulated negative control), PMA (25 nM, positive control) and opsonised *S. aureus* (MOI of 500, positive control) was quantified. Neutrophils were stimulated after being equilibrated for a 30 min baseline period and then ROS was measured over the subsequent 100 min. To measure total ROS, extracellular ROS and superoxide, luminol (3 mM), isoluminol (3 mM) with 1.5 units of horseradish peroxidase (HRP), and lucigenin (0.25 mg/mL), respectively, were added to the samples and the light output was read for 130 min in a luminometer (Berthold Tristar2, Berthold Technologies, Harpenden, UK). All readings were expressed as relative light units (RLUs) and read at 37°C (MikroWin2000, Informer Technologies, Madrid, Spain). All reagents for chemiluminescence were purchased from Sigma Aldrich (Dorset, UK).

NET entrapment and quantification of NET-mediated killing of periodontal bacteria

To assess the ability of NETs to immobilise periodontal bacteria, fluorescein isothiocyanate (FITC)-stained live bacteria (MOI of 100) were incubated for 1h with unstimulated neutrophils, intact NETs (produced by prior 0.75 mM HOCl stimulation with a subsequent washing step (43)) or NET structures degraded with micrococcal nuclease (MNase, New England Biolabs, Hitchin, UK) in a 96-well plate pre-coated with 1% BSA, using five different donors and triplicate wells per donor. *In vitro* NETs are formed within 2-3h, therefore, a relevant induction of NET release from otherwise unstimulated neutrophils by bacteria could be excluded. Following multiple wash steps to remove any unbound bacteria, the amount of bacteria entrapped was

fluorometrically quantified and normalised to FITC-stained bacteria incubated with PBS (cell-free control). To determine whether NETs are capable of killing entrapped bacteria, six strains (*F. nucleatum* subsp. *polymorphum, S. intermedius*, *S. sanguinis*, *A. viscosus*, *V. parvula* and *C. gingivalis*) were incubated at a MOI of 100 with PBS (negative control), unstimulated neutrophils, intact NETs or degraded NETs from five different donors in triplicate wells per donor. Additionally, samples containing neutrophils were treated with the phagocytosis inhibitor cytochalasin B (Sigma Aldrich, Harpenden, UK) at a concentration of 10 µg/mL (72). Following 1h of incubation bacteria were released from NETs by MNase digestion, diluted and inoculated onto agar plates and cultured for 24h prior to performing colony counts.

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

To further understand the importance of NADPH-oxidase and downstream products in bacteria-induced ROS and NET production, specific components of the NADPH-oxidase signalling pathway were targeted. Isolated neutrophils from three different donors were incubated with DPI (25 µM), an inhibitor of NADPH-oxidase, NAC (10 mM), a synthetic glutathione precursor that scavenges H_2O_2, or taurine (100 mM), which scavenges HOCl to produce taurine chloramine (duplicate wells per donor). Neutrophil total ROS and NET production were measured following pre-incubation with the modulating agent 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*). NET-DNA was 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

calculate significances of pathway modulation and inhibition assays. All quantitative data are shown as mean values ± standard deviations and all statistical tests were performed comparing different donors.

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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

NET entrapment of periodontal bacteria. A: Neutrophil extracellular trap (NET) entrapment of bacteria that were not assigned to a Socransky complex (white, grey, black), as well as purple, yellow, green, orange, red and blue complex bacteria. Results are normalised to fluorescein isothiocyanate-stained bacteria in PBS. Statistical significance of bacterial entrapment in NETs is shown relative to bacteria entrapped by unstimulated neutrophils and degraded NETs (n.s.=not significant). Data are presented as arbitrary fluorescence units (AFU) **B**: Representative images of bacterial entrapment by NETs. Neutrophils (yellow arrows) incubated with PBS (control), live *A. actinomycetemcomitans* serotype a, *V. parvula* or *A. viscosus* were visualised by scanning electron microscopy. Blue arrows indicate NET strand structures and NET-associated bacteria are indicated with green arrows. Representative images of three experiments are shown, the scale bar represents 10µm. **C**: Bacterial survival after exposure to neutrophils, NETs, degraded NETs and neutrophils with cytochalasin B (n.s.=not significant). All results shown represent neutrophils of five different donors assessed in triplicate wells.

Figure 4

Effect of NADPH-oxidase pathway modulating agents on ROS and NET production. Total reactive oxygen species (ROS) (**A**) and Neutrophil extracellular trap (NET) (**B**) production by neutrophils was quantified in response to selected periodontal bacteria, as well as to phorbol 12-myristate 13-acetate (PMA) (50 nM) and opsonised *S. aureus* (positive controls) following pre-incubation (30 mins) with diphenyleneiodonium (DPI) (25 µM), N-acetyl-cysteine (NAC) (10 mM) and taurine (100 mM). Data are presented as relative light units (RLU) and arbitrary fluorescence units (AFU). Experiments were conducted in duplicate using three different donors.

Figure 5

Effect of Toll-like receptor inhibition on ROS and NET production. Total reactive oxygen species (ROS) (**A**) and Neutrophil extracellular trap (NET) (**B**) production by neutrophils was quantified in response to selected periodontal bacteria, as well as to phorbol 12-myristate 13-acetate (PMA) (50 nM) and opsonised *S. aureus* (positive controls) following pre-incubation (30 mins) with chloroquine (100 μM), oxidised 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC) (30 μg/mL) or chloroquine and OxPAPC. Experiments were conducted in duplicate wells using three different donors (n.s.=not significant).

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

871

872

Figure 1

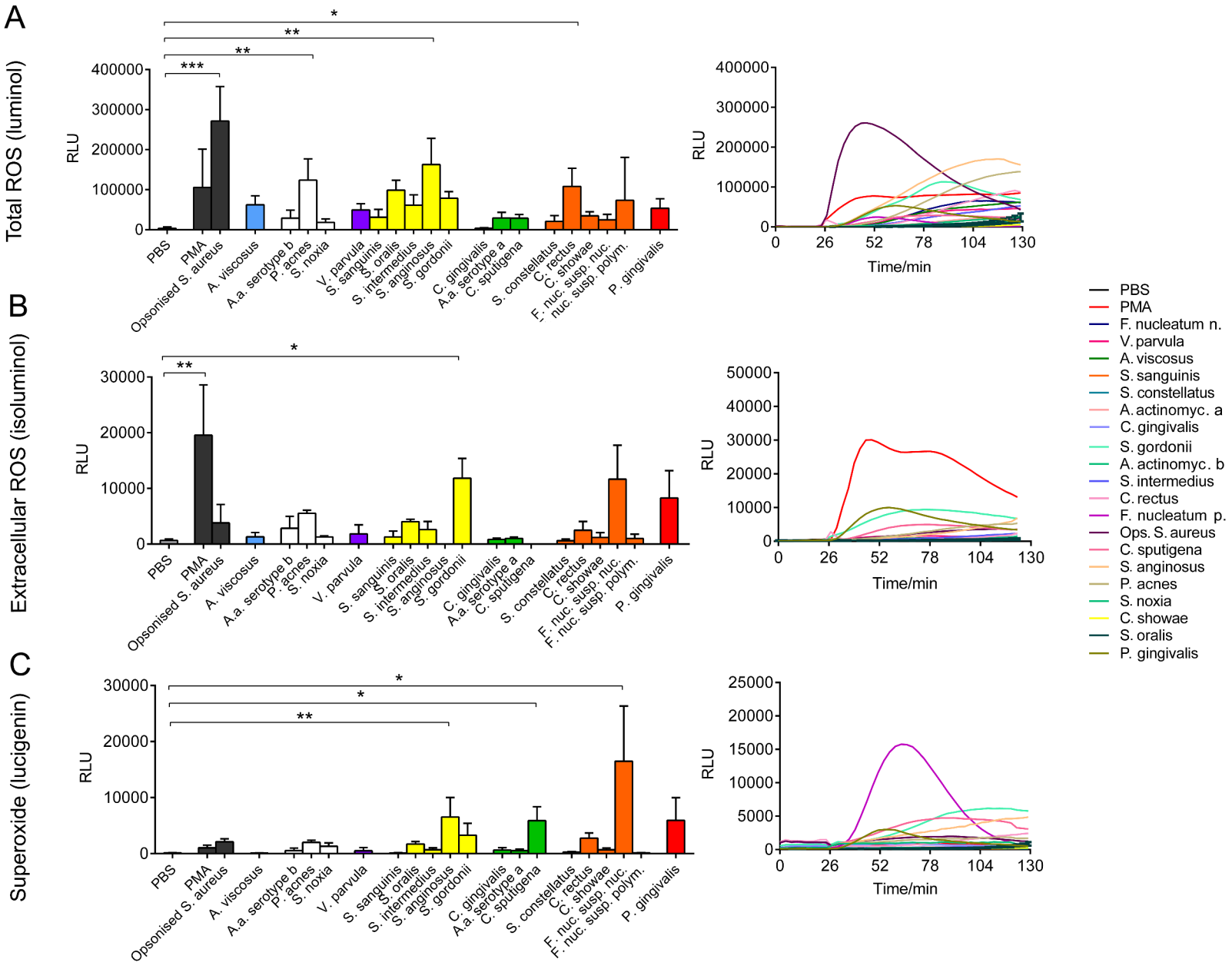

A
Total ROS (luminol)
RLU
400000
300000
200000
100000
0
*
**
**
***
PBS
PMA
Opsonised S. aureus
A. viscosus
A.a. serotype b
P. acnes
S. noxia
V. parvula
S. sanguinis
S. oralis
S. intermedius
S. anginosus
S. gordonii
C. gingivalis
A.a. serotype a
C. sputigena
S. constellatus
C. rectus
C. showae
F. nuc. susp. nuc.
F. nuc. susp. polym.
P. gingivalis
RLU
400000
300000
200000
100000
0
0
26
52
78
104
130
Time/min
B
Extracellular ROS (isoluminol)
RLU
30000
20000
10000
0
*
**
PBS
PMA
Opsonised S. aureus
A. viscosus
A.a. serotype b
P. acnes
S. noxia
V. parvula
S. sanguinis
S. oralis
S. intermedius
S. anginosus
S. gordonii
C. gingivalis
A.a. serotype a
C. sputigena
S. constellatus
C. rectus
C. showae
F. nuc. susp. nuc.
F. nuc. susp. polym.
P. gingivalis
RLU
50000
40000
30000
20000
10000
0
0
26
52
78
104
130
Time/min
PBS
PMA
F. nucleatum n.
V. parvula
A. viscosus
S. sanguinis
S. constellatus
A. actinomyc. a
C. gingivalis
S. gordonii
A. actinomyc. b
S. intermedius
C. rectus
F. nucleatum p.
Ops. S. aureus
C. sputigena
S. anginosus
P. acnes
S. noxia
C. showae
S. oralis
P. gingivalis
C
Superoxide (lucigenin)
RLU
30000
20000
10000
0
*
*
**
PBS
PMA
Opsonised S. aureus
A. viscosus
A.a. serotype b
P. acnes
S. noxia
V. parvula
S. sanguinis
S. oralis
S. intermedius
S. anginosus
S. gordonii
C. gingivalis
A.a. serotype a
C. sputigena
S. constellatus
C. rectus
C. showae
F. nuc. susp. nuc.
F. nuc. susp. polym.
P. gingivalis
RLU
25000
20000
15000
10000
5000
0
0
26
52
78
104
130
Time/min

Figure 2

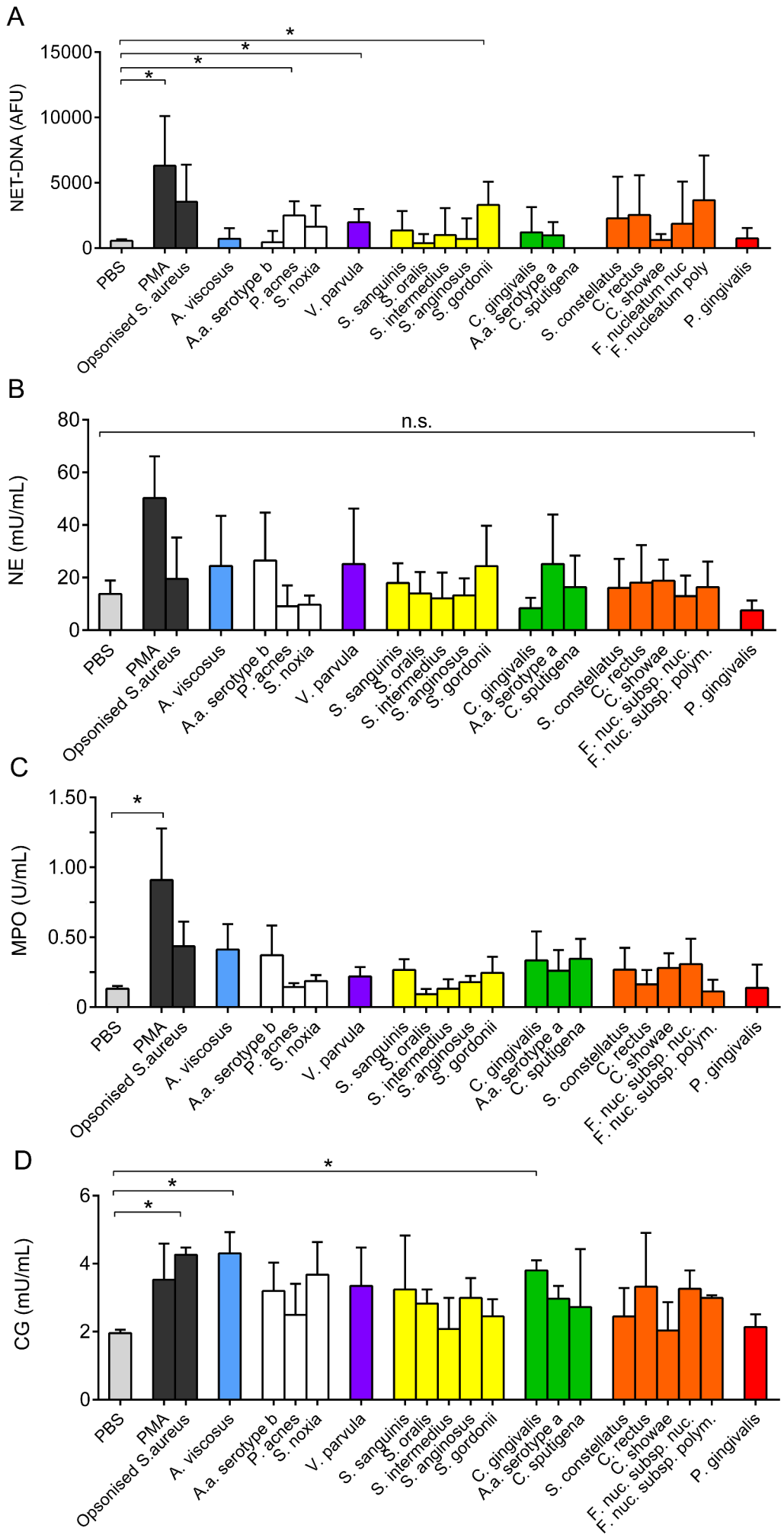

A
NET-DNA (AFU)
15000
10000
5000
0
*
*
*
*
*
B
NE (mU/mL)
80
60
40
20
0
n.s.
C
MPO (U/mL)
1.50
1.00
0.50
0
*
D
CG (mU/mL)
6
4
2
0
*
*
*
PBS
PMA
Opsonised S.aureus
A. viscosus
A.a. serotype b
P. acnes
S. noxia
V. parvula
S. sanguinis
S. oralis
S. intermedius
S. anginosus
S. gordonii
C. gingivalis
A.a. serotype a
C. sputigena
S. constellatus
C. rectus
C. showae
F. nucleatum nuc
F. nucleatum poly
P. gingivalis
F. nuc. subsp. nuc.
F. nuc. subsp. polym.

Figure 3

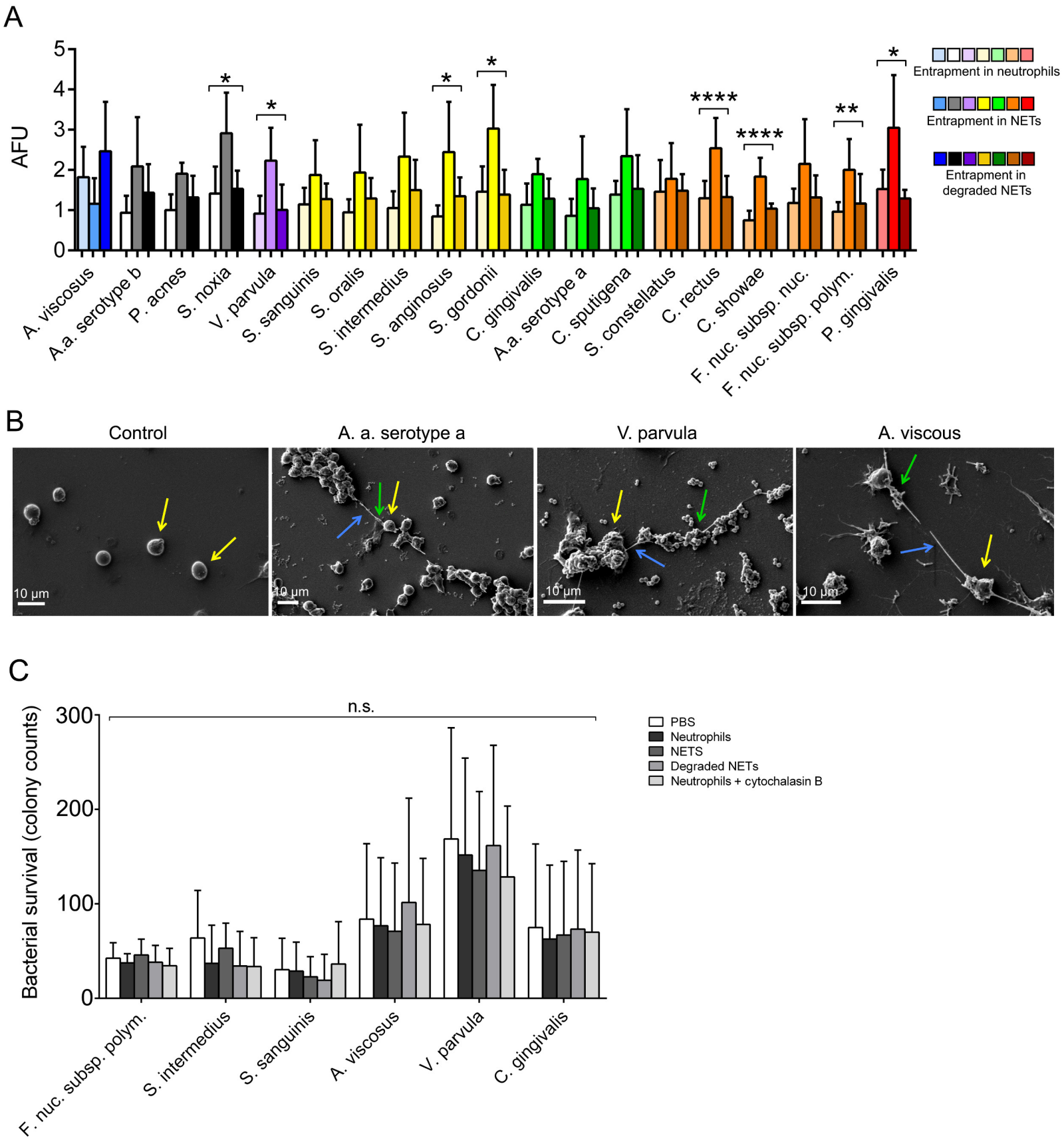

A
AFU
0
1
2
3
4
5
*
*
*
*
****
****
**
*
Entrapment in neutrophils
Entrapment in NETs
Entrapment in degraded NETs
A. viscosus
A.a. serotype b
P. acnes
S. noxia
V. parvula
S. sanguinis
S. oralis
S. intermedius
S. anginosus
S. gordonii
C. gingivalis
A.a. serotype a
C. sputigena
S. constellatus
C. rectus
C. showae
F. nuc. subsp. nuc.
F. nuc. subsp. polym.
P. gingivalis
B
Control
A. a. serotype a
V. parvula
A. viscous
10 µm
10 µm
10 µm
10 µm
C
Bacterial survival (colony counts)
0
100
200
300
n.s.
PBS
Neutrophils
NETS
Degraded NETs
Neutrophils + cytochalasin B
F. nuc. subsp. polym.
S. intermedius
S. sanguinis
A. viscosus
V. parvula
C. gingivalis

Figure 4

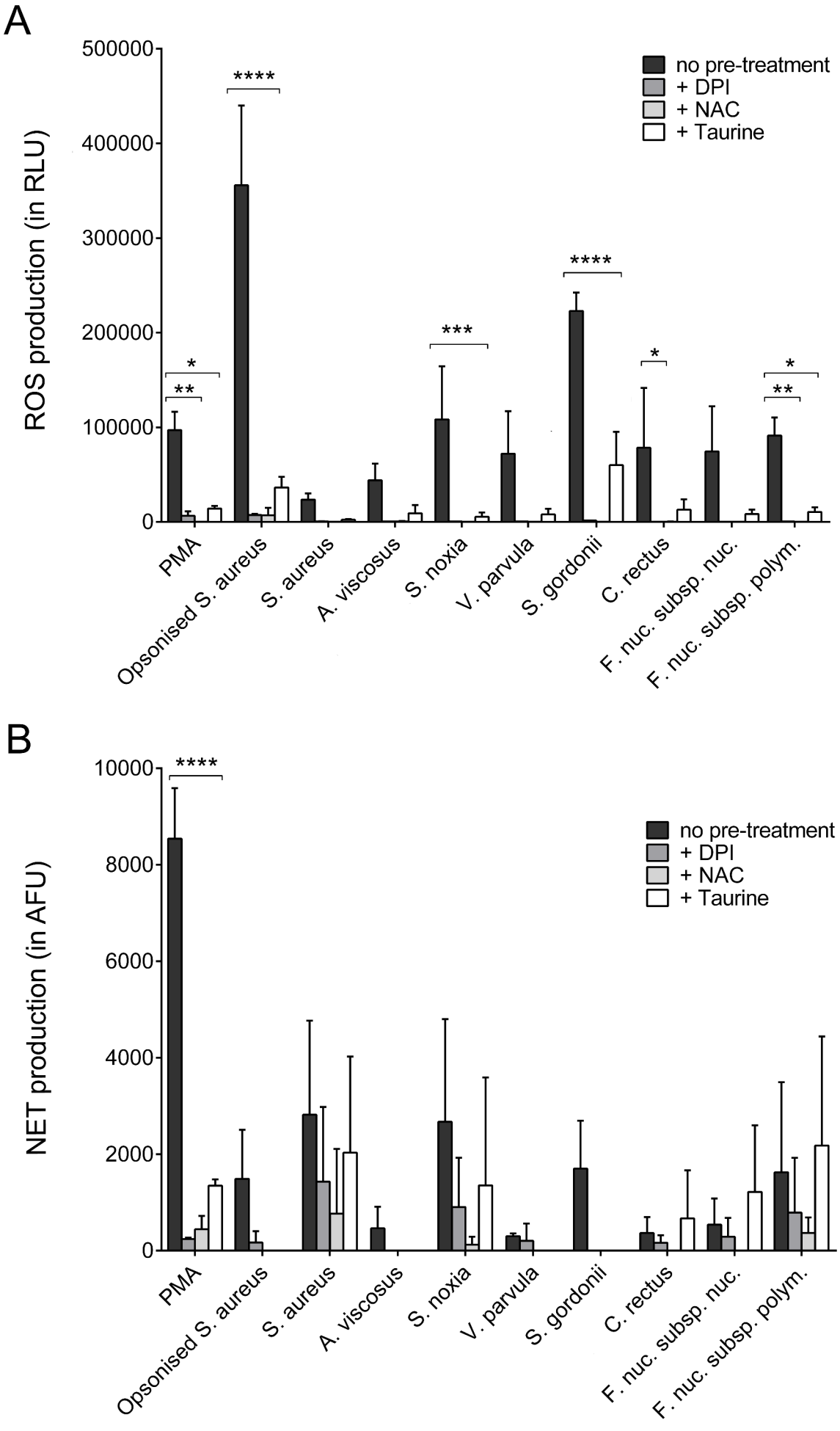
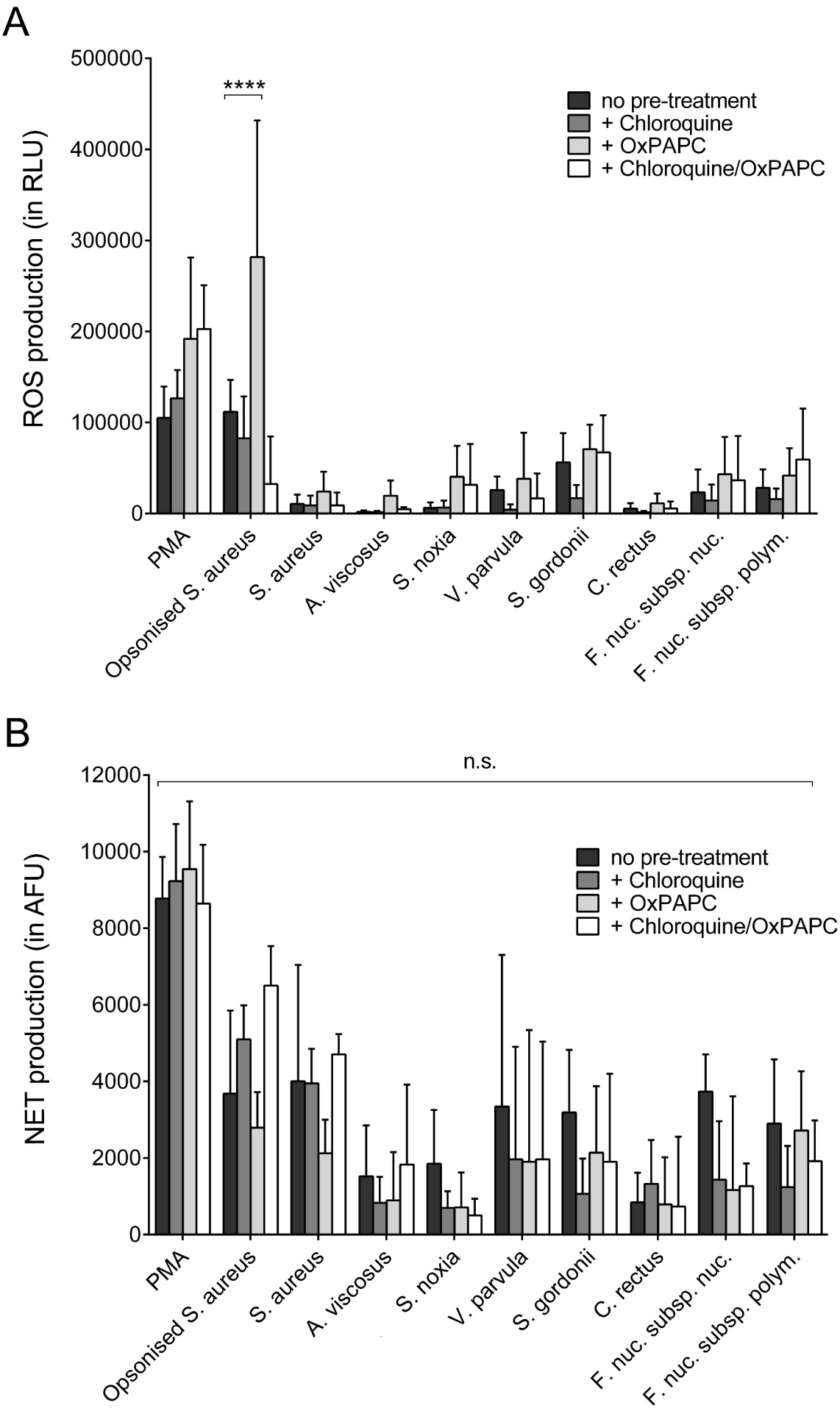

A
ROS production (in RLU)
500000
400000
300000
200000
100000
0
no pre-treatment
+ DPI
+ NAC
+ Taurine
****
*
**
***
****
*
*
**
PMA
Opsonised S. aureus
S. aureus
A. viscosus
S. noxia
V. parvula
S. gordonii
C. rectus
F. nuc. subsp. nuc.
F. nuc. subsp. polym.
B
NET production (in AFU)
10000
8000
6000
4000
2000
0
no pre-treatment
+ DPI
+ NAC
+ Taurine
****
PMA
Opsonised S. aureus
S. aureus
A. viscosus
S. noxia
V. parvula
S. gordonii
C. rectus
F. nuc. subsp. nuc.
F. nuc. subsp. polym.

Figure 5


A
ROS production (in RLU)
500000
400000
300000
200000
100000
0
****
no pre-treatment
+ Chloroquine
+ OxPAPC
+ Chloroquine/OxPAPC
PMA
Opsonised S. aureus
S. aureus
A. viscosus
S. noxia
V. parvula
S. gordonii
C. rectus
F. nuc. subsp. nuc.
F. nuc. subsp. polym.
B
NET production (in AFU)
12000
10000
8000
6000
4000
2000
0
n.s.
no pre-treatment
+ Chloroquine
+ OxPAPC
+ Chloroquine/OxPAPC
PMA
Opsonised S. aureus
S. aureus
A. viscosus
S. noxia
V. parvula
S. gordonii
C. rectus
F. nuc. subsp. nuc.
F. nuc. subsp. polym.