

Sialylation of Porphyromonas gingivalis LPS and its effect on bacterial-host interactions

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Porphyromonas gingivalis W83 LPS is highly sialylated but it does not affect its inflammatory potential

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1 SUMMARY

2 Inflammation and immune homeostasis are regulated by a tightly controlled network of 3 positive-negative regulatory mechanisms that guarantee proper defence and pathogen 4 clearance whilst avoiding hyperactivity and inflammatory diseases. Some pathogens have involved various means of evading the immune system and subverting normal host defences. 5 6 Porphyromonas gingivalis produces different lipopolysaccharide isoforms with significant structural variations of their lipid A and O-antigen moieties that can affect its pro-7 8 inflammatory and bone resorbing potential. In this study we examined the level of P.gingivalis LPS sialylation and the influence of sialic acid LPS content on the pro-9 10 inflammatory LPS potential in THP-1 cells. Our results indicate that *P.gingivalis* W83 LPS is 11 highly sialylated and possesses significantly reduced inflammatory potential compared to less 12 sialylated ATCC 33277 strain LPS. Nevertheless, the reduction in the endotoxin activity is 13 not mediated by the presence of sialic acid LPS moieties since the sialic acid - free LPS 14 produced by a mutant W83 strain exhibits a similar inflammatory potential as the wild-type 15 strain. In addition, the interaction between the sialic acid LPS moieties and the inhibitory CD33 receptor is prevented by endogenously expressed sialic acid on the surface of THP-1 16 cells that cannot be out-competed by sialic acid containing *P.gingivalis* LPS. Interestingly, 17 human gingival fibroblasts do not express CD33 receptors and have much higher spontaneous 18 19 IL-8 production compared to THP-1 cells. This basal IL-8 secretion is significantly increased 20 by THP-1 cells desialylation. Taken together, our results indicate that sialylation of 21 *P.gingivalis* LPS is not an important virulence factor of this prominent periodontal pathogen 22 and that the interaction between CD33 receptors and endogenously expressed sialic acid controls immune cell activation. 23

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26 INTRODUCTION

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Periodontal diseases are caused by dental plaque bacteria of so called "red complex" that can 28 29 use different molecular mechanisms and strategies to evade or overcome host defences (Ref, socransky). One of the mechanisms by which they can manipulate the cytokine responses of 30 31 macrophages and monocytes is through their surface glycosylation (Amano a, Chen C, Sharma A 2014). Recent studies have revealed that bacterial surface-expressed O-linked 32 glycans and especially terminally present sialic acid can help them evade the host innate 33 34 immune attacks and also modulate T-cell responses during periodontal inflammation (Megson, Koerdt, Messner, 2015). Porphyromonas gingivalis utilizes a panel of virulence 35 36 factors which can cause deregulation of the innate immune and inflammatory responses. 37 P.gingivalis lipopolysaccharide (LPS) is one of its most potent virulence factors that stimulates the host's innate immune system and bone resorption whilst LPS recognition by 38 39 the host is essential for clearing infections of this invading periodontal pathogen. Host's 40 inflammatory response can effectively be modulated by modifications of either lipid-A core of LPS or its oligo/polysaccharide domain (O-antigen) (Al-Qutub, Braham 2006). 41

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43 Sialic acids and Siglecs

Sialic acids are a large family of sugars containing 9C atoms which predominantly derive from N-acetylneuraminic acid (Angata 2006). N-acetylneuraminic acid (the most abundant and best-studied sialic acid) is usually a terminal sugar residue on the oligosaccharide chains of eukaryotic cells where it functions in recognition and anti-recognition phenomena (Vimr, Kalivoda et al. 2004). Sialic acids are transferred using α -2,3 or α -2,6 linkages to subterminal sugars by a family of sialyltransferases and are abundantly expressed on the periphery of mammalian immune cell surface glycans and serum proteins (Cao, Lakner et al. 51 2008). The mammalian glycome contains numerous sialylated glycans that can be recognized 52 as ligands by special receptors present on the cell surface, called Siglecs (Sialic acid-binding 53 Immunoglobulin Like Lectins). It is assumed that this recognition is important for modulating the function of Siglecs as regulators of adhesion, cell signalling and endocytosis (Chen, Varki 54 55 2010). The local concentration of sialic acid on the surface of immune cells is very high and 56 as a consequence of this, Siglecs binding sites are usually masked by low-affinity cis 57 interactions with endogenous glycans on the same cell (Varki 2012). This cis interaction does 58 not prevent the binding of exogenous ligands in the *trans* position. Moreover, high-affinity 59 exogenous ligands can out-compete *cis* ligands and more potently engage an inhibitory Siglec, proving that binding of Siglecs to *trans* ligands can occur dynamically in the presence 60 61 of cis ligands (Collins, Blixt et al. 2006).

62 Siglecs are a family of ITIM-containing receptors (Immunoreceptor Tyrosine-based 63 Inhibition Motif) that can recognise sialylated glycans and function as regulators of immune 64 cells. CD33-related Siglecs are mainly expressed by cells of the innate immune systems such 65 as monocytes (Siglec-3), macrophages, NK cells, neutrophils, eosinophils, DCs and mast cells (Crocker, Paulson et al. 2007). CD33-related Siglecs have also been found expressed on 66 cells of the nervous system (Siglec-4), placental trophoblasts (Siglec-6) and epithelial cells 67 (Siglec-12) but not on fibroblasts (Poe, Fujimoto et al. 2004) (Pilling, Fan Huang 2009 fibro). 68 69 In leukocytes, they regulate inhibition of cellular proliferation and activation, induction of 70 apoptosis and they can also function as endocytic receptors that may be important in the 71 clearance of sialylated antigens (Lock, Zhang et al. 2004). Currently, there are 11 functional 72 CD33-related Siglecs within the human genome (Crocker, Redelinghuys 2008) and all of 73 them have one or more Immunoreceptor Tyrosine-based Inhibition Motifs (ITIM) within the 74 cytoplasmic tail indicating their ability to mediate signalling functions (McMillan, Crocker 2008a). Ligand engagement of these inhibitory receptors causes phosphorylation of the 75

TIMs. This allows the recruitment of at least one of the SH2-domain-containing protein tyrosine phosphatases 1 and 2 (SHP1, SHP2) as well as the Inositol 5'-Phosphatase (SHIP). These phosphatases can subsequently dephosphorylate relevant intracellular substrates thus regulating cellular activation by attenuating or terminating tyrosine phosphorylation signal transduction (Barrow, Trowsdale 2006). Low membrane expression level of CD33 have been associated with higher levels of inflammatory cytokine production and increased spontaneous secretion of TNF-α, IL-8 and IL-6 by human monocytes (Gonzales, Herrera, Fabian 2012).

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84 Sialic acid and bacterial pathogens

85 Sialic acid is an important molecule for a number of bacteria too, serving as a substrate for 86 capsule synthesis, LPS sialylation and as a potential growth factor (Steenbergen 2005). A 87 variety of pathogens have been able to exploit sialic acid by expressing it on their surface, thus evading the immune system or binding to host cells via sialic acid - recognizing 88 89 receptors which mediate cell adhesion, protein-protein interaction and immune response 90 (Bukrinsky, St Hilaire et al. 2004). Some bacteria have the potential to incorporate sialic acid into their lipopolysaccharide as a terminal residue of the carbohydrate part of the molecule. 91 LPS is an important surface constituent of these bacteria and is involved in complex 92 93 interactions with the host immune system. Sialylated LPS glycoforms play a key role in 94 pathogenicity of H.influenzae and N.meningitidis (Bouchet, Hood et al. 2003). Sialylation of 95 LPS is an important mechanism used by the human pathogen H. influenzae to evade the innate immune response of the host and escape killing by human serum (Severi, Randle 96 2005) while the inflammatory response against *C.jejuni* is in direct correlation with the level 97 98 of its LPS sialylation (Stephenson, Jonh, Jarvis 2013). In addition, it has been shown that the 99 invasive strains of *N.meningitidis* have highly sialylated LPSs compared to carrier strains and that these structural features are likely to be important in the ability of the disease-causing 100

bacteria to evade recognition by LPS-specific bactericidal IgG and complement-mediated
lysis (John, Philips, Jarvis 2015).

103 There is a growing body of evidence that sialic acid and sialidases play a key role in 104 the life, host mimicry and consequences of periodontal pathogen colonisation (Stafford 2012). P.gingivalis, as a member of the "red complex bacteria", primarily inhabits the gingival sulci 105 106 and periodontal pockets. Sialic acid is abundantly present in human serum which is the major 107 component of the gingival crevicular fluid while salivary mucins and gingival connective 108 tissue fibronectins also contain sialic acid. *P.gingivalis* is able to acquire sialic acid from the 109 host and use it as an important factor that contributes to biofilm formation, capsule 110 biosynthesis and pathogenicity of *P.gingivalis* (Chen Li, Yaping Pan 2012). However, the 111 role of sialyation of *P.gingivalis* LPS in the interaction with the host cells is still unknown. 112 Thus, the aim of this study was to examine the sialic acid content in LPSs extracted from two different P.gingivalis strains (P.gingivalis ATCC 33277 and P.gingivalis W83) and to 113 114 determine the influence of LPS sialylilation on its inflammatory potential. Our results show 115 for the first time that *P.gingivalis* W83 LPS is highly sialylated but the presence of sialic acid 116 as an LPS carbohydrate moiety does not play a significant role in modulating the 117 proinflammatory potential of this periodontal pathogen's LPS.

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Porphyromonas gingivalis ATCC 33277 LPS was obtained from InvivoGen (San Diego, 128 129 USA). Porphyromonas gingivalis W83 LPS was extracted using the method reported by Darveau and Hancock (Darveau 1983). Briefly, P.gingivalis W83 was grown anaerobically in 130 131 anaerobic basal broth (Oxoid) containing 5µg/ml of haem for 7 days. Cells were harvested at 7,000 rpm for 15 minutes, lyophilized and resuspended in 15 ml of 10 mM Tris-HCl, pH 8.0, 132 133 2 mM MgCl₂. After sonication for 30s, DNase and RNase were added to the final 134 concentrations of 200 µg/ml and 50 µg/ml respectively. Suspension was incubated at 37°C 135 for 2 hours. 5 ml of 0.5 M EDTA (tetra sodium salt)/10 mM Tris, pH 8.0, 2.5 ml of 20% 136 SDS/10 mM Tris, pH 8.0 and 2.5 ml of 10 mM Tris-HCl, pH 8.0 were added, solution was 137 vortexed and centrifuged at 50,000 g for 30 minutes at 20°C to remove peptidoglycans. 138 Supernatant was saved and pronase was added to a final concentration of 200 µg/ml. Solution 139 was incubated at 37°C with constant shaking overnight. Next day two volumes of 0.375 M 140 MgCl₂/95% EtOH were added, mixed and cooled to 0°C in -20°C refrigerator. After the sample had cooled to 0°C, they were centrifuged at 12,000 g for 15 minutes at 0 - 4°C. Pellet 141 was resuspended in 25 ml of 0.1 M EDTA(tetra sodium salt), 2% SDS, 10 mM Tris-HCl, pH 142 143 8.0 and sonicated for 30s. The solution was incubated at 85°C for 30 minutes, cooled down to 144 room temperature and pH was brought to 9.5 by addition of 4M NaOH. Pronase was added 145 again to a final concentration of 25 µg/ml and incubated at 37°C overnight with constant shaking. The next day two volumes of 0.375 M MgCl₂/95% EtOH were added, the solution 146 147 cooled to 0°C as before, centrifuged at 12,000 g for 15 minutes at 0 - 4°C. The pellet was resuspended in 15 ml of 10 mM Tris-HCl, pH 8.0 and sonicated for 30s. Samples were 148 149 centrifuged at 1000 rpm for 5 minutes to remove insoluble Mg/EDTA complexes. MgCl₂ was added to give a final concentration of 25 mM and suspension was centrifuged at 200,000 g 150

151 for two hours. Pellet was resuspended in distilled water and the solution was lyophilized. Dry
152 LPS was measured and stock concentration of 1mg/ml was prepared by addition of
153 endotoxine free water.

154 *P.gingivalis* W83 Δ PG0083 (Δ W83) defective mutants were a kind gift from Dr 155 Charles Shelbourne, University of Michigan, Ann Arbor, US. This strain is not able to 156 incorporate sialic acid into their LPS.

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158 Cell culture

THP-1 cells (Human monocytic leukemia cell line) were purchased from ECACC (European Collection of Cell Cultures) and maintained in RPMI 1640 medium (Invitrogen) supplemented by 2 mM Glutamine, 10% Fetal Calf Serum (FCS), penicillin (100 units/ml) and streptomycin (100 μ g/ml) (Invitrogen). The cells were cultured between 10³ to 10⁶ cells/ml at 5% CO₂ atmosphere at 37° C.

Human gingival fibroblasts (HGF) were established from explants of healthy gingival tissues obtained during routine clinical procedures. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM-Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml of penicillin, and 100 μ g/ml of streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. Cells between the 5th and 13th passages were used for assays. 24 hours before treatment the medium is changed to 1% FCS DMEM supplemented with the same concentration of antibiotics and glutamine.

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172 Immunoprecipation

173 200 μl of Protein G magnetic beads (Millipore) were pre-associated with 5 μl of phospho174 tyrosine- 4G10 (Millipore) antibodies on a rotor for 90 min at 4°C. The antibody bound

beads were pelleted at 5000rpm for 1 min and washed in 0.5% TBST. The whole cell lysates were then added to the beads and incubated on rotation at 4°C for 2 hours. Afterwards the beads were pelleted and washed 3 times in 0.5% TBST before being resuspended in 5µl of SX Western Loading Buffer with 10% β-mercaptoethanol and 15µl of PBS. The samples were denatured at 95°C for 5 min and placed on ice prior to loading onto a gel.

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181 SDS-PAGE Electrophoresis

Samples were treated with sample loading buffer and fractionated on a 10% SDS polyacrylamide gel at 35mA. Each gel used a 4% stacking layer into which samples were loaded. Protein size was identified with the use of pre-stained molecular markers ranging from 4kDa to 200kDa (SeeBlue, Invitrogen). Proteins were transferred from gel to a nitrocellulose membrane using a Novex Mini Cell wet system apparatus from Invitrogen. The proteins were electrophoresed onto the membrane at 200 mA for 1.5 h.

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189 Immunoblotting

190 Following protein transfer, nitrocellulose membranes were rinsed in 1X PBS/0.1%Tween 20 191 and blocked with appropriate blocker (Table 2.1) for 1 hour. Membranes were probed using respective primary antibodies at the relevant dilutions in appropriate blotting solution. 192 193 Subsequently the membranes were washed six times 5 min each in 1X PBS/0.1% Tween 20. 194 Membranes were probed with an appropriate Horse-radish peroxidase (HRP) conjugated secondary antibody for 1h in 5% milk dissolved in 1X PBS/0.1%Tween 20 at 1:5000 195 196 concentration. The wash step was repeated as before and proteins were then detected using chemiluminescence method. Mouse monoclonal anti-CD33 antibodies were obtained from 197

Abcam, UK. To allow visualisation of proteins, membranes were covered for 5 min in
chemiluminescence detection reagent Super Signal West-Femto (Thermo Scientific). The
Syngene G Box imaging system was used to expose blots as per manufacturer's instructions.
Images were analysed using GeneSnap software.

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203 Lectin Blot analysis

Sialic acid content in LPSs was assessed using lectin blot analysis as described by Cao et al. (Cao, Guo, Ning 2013). Two types of biotinylated lectins (Vector Laboratories, USA) were used: *Sambucus nigra* lectin (SNA), that binds to α -2,6-sialic acid residues, and *Maackia amurensis* lectin (MAA) which interacts with α -2,3-linked sialic acid. Human serum was used as a positive control, ddH₂O was used as a negative control.

209 1µl of samples (1mg/ml LPS), positive and negative control were added directly onto 210 the nitrocellulose membrane and allowed to air dry. The membrane was then incubated in 211 0.1% PBST for 1h at room temperature. Lectins were dissolved in 0.1% PBST at a final 212 concentration of 1µl/ml and incubated with the membrane over night at $+4^{\circ}$ C. The membrane 213 was washed with PBST and incubated with Streptavidine HRP in PBST (1:5000). Having 214 been washed, the membranes were developed using West-Femto chemiluminescence 215 substrate and visualised in the Syngene G box.

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217 ELISA

218 THP-1 cells were resuspended at 5×10^5 cells/ml and human gingival fibroblast at 8×10^4 219 cells/ml in corresponding medium and treated with LPS for 4 hours. Cell free supernatants 220 were removed and stored at -80°C until they were assessed for TNF- α , IL-8 (Peprotech, 221 R&D Systems respectively). 222 Briefly, a 96 well plate was coated in capture antibody diluted in phosphate buffered 223 saline (PBS) overnight. All steps were carried out at room temperature. The wells were 224 washed three times in PBS/0.1% Polyoxyethylene sorbitan monolaurate (Tween 20) before 225 being blocked for one hour with 1% BSA dissolved in PBS. The washing step was repeated 226 and 100µl of supernatants or standards ranging from 2000 pg/ml to 0 pg/ml were added to the 227 wells and left for 2 hours. Subsequently supernatant was aspirated out, wells were washed 3 times and 100 µl of detection antibody diluted in 1% BSA/PBS was added for 2 hours. Again, 228 229 the wells were washed three times and Streptavidin-HRP was added at 1 in 2000 dilution in 1% 230 BSA/PBS for 1 hour. At this stage the plate was covered in aluminium foil. Once more, wells 231 were washed and ABTS (Invitrogen) was added for 20 minutes. Absorbance was read on a 232 plate reader at 405 nm (Genios Tecan spectrofluorimeter). Cytokines concentrations were 233 extrapolated from the standard curve.

234

235 Flow cytometry

THP-1 cells and human gingival fibroblasts ($1x10^6$ cells/ml and 80000 cells/ml respectively) were washed in PBS, spun at 2000 rpm for 5 min and resuspended in a 100 µl of PBS supplemented with 2% FCS. Cells were then stained with 20µl of FITC conjugated antihuman CD33 antibody (eBioscience) or respective isotype control for 30 min at room temperature in the dark. Cells were centrifuged again and washed twice with 1 ml PBS and resuspended in 500 ml of fresh PBS. Flow cytometry analysis was carried out on FACS CANTO II. Results were analysed using FlowJo software.

243

244 Statistical analysis

245 Differences between the means of treatments were analyzed by the Student t-test using GraphPad Prism version 4 (GraphPad Software, San Diego, CA). Differences between 246 multiple treatments were compared by one-way ANOVA followed by Tukey's post hoc test. 247 248 Values are expressed as the mean \pm standard error of the mean (SEM). A value of P<0.05 was considered to represent a statistically significant difference. (* means P<0.05. ** denotes 249 P<0.01 and *** means P<0.001). 250 251 252 253 254 RESULTS 255 256 Sialic acid detection 257 Sialic acid appears as a terminal sugar in polysaccharide chains. In order to examine the content of sialic acid in LPSs from two different P.gingivalis strains (ATCC 33277 and 258 259 W83), the lectin-blot method was used with two sialic acid binding lectins as antibodies 260 (SNA and MAA specific for α -2,6 and α -2,3 bond between the sialic acid and the terminal 261 N-acetyl galactosamine respectively). Human serum was used as a positive control because of the high concentration of sialylated proteins present in it, while water was used as a 262

negative control. Lectin-blot analysis revealed a high concentration of sialic acid within the W83 LPS and a much more attenuated signal for the ATCC 33277 strain. Both α -2,6 and α -

265 2,3 linked sialic acids were abundantly present within the W83 LPS. (Figure 1).

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Figure 1: Lectin-blot detection of sialic acid LPS content from different *P.gingivalis*strains. Panel a) SNA lectin, panel b) MAA lectin. 1- water, 2-human serum, 3-*P.gingivalis* ATCC 33277, 4- *P.gingivalis* W83.

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8 Inflammatory potential of LPSs

Inflammatory potential of the two types of *P.gingivalis* LPS was examined in a THP-1 cell model. THP-1 cells were challenged with 1µg/ml of the LPSs for 4 hours and the production of TNF- α and IL-8 was measured in the cell supernatants. ATCC 33277 LPS triggered much higher production of both TNF- α and IL-8 compared to W83 strain LPS (P<0.001) (Figure 2.)



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Figure 2: Inflammatory potential of LPSs extracted from *P.gingivalis* ATCC 33277, and *P.gingivalis* W83. THP-1 cells were challenged with 1μ g/ml of LPS for 4 hours and TNF- α and IL-8 were measured by ELISA.

289 The influence of LPS sialylation on its inflammatory potential

In order to examine the influence of sialic acid LPS moieties on the pro-inflammatory response of innate immune cells, THP-1 cells were treated with *P.gingivalis* W83 LPS, which was shown to be highly sialylated, and LPS isolated by the method of Darveau and Hancock from sialyltransferase gene (PG00083) knock-out *P.gingivalis* W83 strain (Δ W83). The absence of sialic acid in LPS extracted from *P.gingivalis* Δ W83 was confirmed by the lectinblot analysis, as explained previously, using two types of sialic acid binding lectins and *P.gingivalis* W83 LPS as a positive control (Figure 3).

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301 Figure 3: Sialic acid lectin-blot analysis of *P.gingivalis* W83 LPS and *P.gingivalis* ΔW83
302 LPS. Panel a) SNA lectins; panel b) MAA lectin; 1-*P.gingivalis* W83 LPS ; 2-*P.gingivalis*303 ΔW83 LPS.

As expected, LPS obtained from sialyltransferase mutated *P.gingivalis* W83 strain did not contain sialic acid. Both types of lectins failed to detect sialic acid moieties within this mutant type of *P.gingivalis* LPS (Sia-free LPS).

- 308 Furthermore, THP-1 cells were treated with different concentrations of *P.gingivalis* W83 LPS
- and Sia-free *P.gingivalis* Δ W83 LPS for 4 hours and the production of IL-8 and TNF- α in cell
- 310 supernatants was measured (Figures 4 and 5.).



- 312 Figure 4: IL-8 production by THP-1 cells treated with different concentrations of *P.gingivalis*
- 313 W83 LPS and *P.gingivalis* Sia-free Δ W83 LPS.



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315 Figure 5: TNF- α production by THP-1 cells treated with different concentrations of 316 *P.gingivalis* W83 LPS and *P.gingivalis* Sia-free Δ W83 LPS.

318 There was no difference in IL-8 and TNF- α levels produced by THP-1 cells treated with 319 sialic acid free *P.gingivalis* Δ W83 LPS (Sia-free LPS) compared to sialic acid containing 320 *P.gingivalis* W83 LPS in all concentrations examined.

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322 CD33 activation

323 Next, we wanted to check if the CD33 receptor is activated in THP-1 cells challenged with three different types of LPSs (ATCC 33277, W83 and Δ W83). Upon activation of CD33, the 324 325 tyrosine in position 340 in its intracellular tail becomes phosphorylated and is able to recruit SHP-1, SHP-2 or SHIP. THP-1 cells were treated with P.gingivalis ATCC 33277 LPS, 326 327 P.gingivalis W83 LPS and P.gingivalis AW83 LPS for 4 hours and phosphorylation of 328 tyrosine residues within CD33 molecules was examined. Imunoprecipitation of THP-1 whole 329 cell lysates with anti-phoshotyrosine antibodies was carried out followed by immunobloting 330 for CD33 (Figure 6).



Figure 6: CD33 phosphorylation. THP-1 cells were treated with: 1) medium alone; 2) 1µg/ml *P.g* ATCC 33277 LPS; 3) 1µg/ml *P.g* W83 LPS; and 4) 1µg/ml *P.g* Δ W83 LPS and the cell lysate was immunoprecipitated with anti-phosphotyrosine antibodies and immunoblotted for CD33. Whole cell lysate immunoblotted for CD33 served as a loading control.

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The results revealed that CD33 was phosphorylated at tyrosine residues in the cells treated with all types of LPSs in addition to control cells incubated in the medium only. These results show that CD33 is endogenously expressed in THP-1 cells and activated regardless of the type of LPS treatment. This is possibly due to the presence of sialic acids at the surface of monocytes as a constituent of glycoproteins, which interact with CD33 in *cis* interaction and block *trans* interaction of sialic acid present in LPS and CD33. Constitutive repressor activity of self-expressed sialic acid with CD33 receptors may be important for maintaining low levels of pro-inflammatory cytokine production during the resting state of the immune system.

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349 CD33 inhibits spontaneous secretion of IL-8

350 Since human gingival fibroblasts (HGF) do not express CD33 (Figure 7), the spontaneous secretion of IL-8 by THP-1 cells, neuraminidase (NA) treated THP-1 cells and HGFs over 4 351 hours without any treatment was investigated. Half a million ($5x10^5$ cells) THP-1 cells, NA 352 treated THP-1 cells and 8x10⁴ HGFs per 1ml of corresponding medium were incubated for 4 353 354 hours and IL-8 levels in cell supernatants were determined by ELISA. The concentration of 355 IL-8 was adjusted according to the cell number (Figure 7). Spontaneous production of IL-8 356 was significantly higher in NA treated THP-1 cells and human gingival fibroblasts compared 357 to THP-1 cells, suggesting that endogenous sialic acid - CD33 interaction is likely to play an 358 important role in inhibiting basal secretion of pro-inflammatory cytokines.





Figure 7: CD33 expression on a) THP-1 cells, b) human gingival fibroblasts. C)Spontaneous production of IL-8 by THP-1 cells, neuraminidase (NA, Sigma) treated THP-1 cells and human gingival fibroblasts (HGF), adjusted according to cell number used in the experiments.

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368 DISCUSION
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370 Sialic acid residues are highly expressed in eukaryotic cells and act as a marker for self in the 371 immune system (Chen, Brown, Zhen 2014). Pathogenic bacteria also use this molecule for biological mimicry, to coat themselves in sialic acid and increase resistance to components of 372 373 the host's innate immune response, or they can use it as nutrient (SEVERI, Thomas 2007). Structural variations of bacterial LPS can have major impact on the host immune response 374 375 and clinical disease outcome. Studies have suggested that the sialic acid decoration of LPS is 376 an important determinant of C.jejuni caused gastroenteritis and Neisseria meningitidis 377 caused meningitis (John, Wren, Jarvis 2013 and Jarvis, stein, Din 2015). Here, we show for 378 the first time that *P.gingivalis* W83 LPS is highly sialylated, containing both α -2,6 and α -2,3 attached sialic acid moieties, while P.gingivalis ATCC 33277 LPS expresses much lower 379 380 level of sialylation. Many previous studies have compared virulence-associated activities and 381 disease-promoting characteristic between *P.gingivalis* strains and have found the ATCC 33277 strain being avirulent while the W83 strain is generally considered virulent. In 382 383 addition, a cluster of genes involved in the synthesis of capsular polysaccharides, which are 384 highly sialylated, was present in W83 while it was not found in strain ATCC 33277 (Chen, 385 Hosogi, Abbey 2004).

We compared the inflammatory potential of these two types of *P.gingivalis* LPS and 386 found much higher production of TNF- α and IL-8 in human monocytes challenged by LPS 387 388 extracted from the ATCC 33277 strain compared to W83 LPS. Detection of LPS by Toll-389 Like Receptors (TLR) expressed on innate immune cells triggers a robust and essential inflammatory reaction. The interaction between LPS and TLR components depends on the 390 391 chemical composition of the lipid A core of the LPS molecule (level of phosphorylation and 392 acylation) and can be modulated by the O-antigen polysaccharide tail (Nina Maeshima and 393 Rachel C. Fernandez* 2013). In our case, highly sialylated W83 LPS triggered significantly lower inflammatory response in THP-1 cells compared to less sialylated ATCC 33277 LPS. 394

Similarly, sialylation of *Helicobacter bizzozeronii LPS* has been shown to play a crucial role
in attenuation of inflammatory host's response to this prominent animal pathogen (Kondadi,
Ravez 2015).

398 P. gingivalis is capable of synthesizing a heterogeneous isoforms of the lipid A molecule, which are subtly different from each other in structure. These subtle changes in the 399 400 lipid A chemical composition have been shown to greatly influence host's immune response 401 to P.gingivalis LPS (Jain, Darveau 2010). In order to examine the influence of sialic acid O-402 antigen moieties of LPS on the host's inflammatory response and exclude lipid A variations, 403 sialyltransferase deficient W83 mutants were grown under the same conditions as the wild 404 type W83 and their LPS was extracted using the same method. These mutants produced sialic 405 acid free LPS that triggered a similar level of TNF- α and IL-8 in THP-1 cells as the wild type LPS, suggesting that sialic acid is not an important determinant of *P.gingivalis* LPS 406 407 inflammatory potency. Since the structure of both the lipid A and the polysaccharide 408 components of LPS can significantly alter TLR activation and subsequent responses, we 409 examined the lipid A chemical composition of the ATCC33277 LPS and W83 LPS by mass-410 spectrometry (data not shown). The analysis revealed predominant m/z1690 peaks (penta-411 acylated monophosphorylated isoform) in ATCC 33277 LPS and m/z1195 and m/z 1435 412 peaks in W83 LPS (tri-acylated monophosphorylated and tetra-acylated monophosphorylated 413 isoforms respectively). As Darveau et al. suggested, the observed differences in the 414 inflammatory potential of these two preparations of *P.gingivalis* LPS are likely to be caused by the modifications of their lipid A core (Darveau, Hajjar 2004). 415

When CD33 receptor activation by LPS extracted from ATCC 33277, W83 and Δ W83 strains was examined by immunoblotting, CD33 was found to be activated in cells treated with all three types of LPS in addition to the control, untreated cells. This is probably a result of the inability of sialylated *P.gingivalis* LPS to displace and outcompete endogenously expressed 420 sialic acid on the surface of THP-1 cells from *cis* mediated interaction with CD33 receptors. 421 Ishida et al. (Ishida, Nakada 2014) have found that CD33 activation by endogenous ligands 422 negatively regulate TLR intracellular signalling and production of IL-12. Similarly, pre-423 treatment of sialic acid has been shown to efficiently prevent LPS-induced acute renal failure 424 and TLR- mediated apoptotic signalling in a rat model (Hsu, Chen, Chien, 2016). On the 425 contrary, desialylation of dendritic cells significantly improves their phagocytic activity and 426 immunological potency through an actin-independent mechanism (Cabral, Silva, Crespo, 427 2012). Interestingly, hyperglycaemia down-regulates CD33 expression in human monocytes 428 and higher levels of TNF-α, IL-8 and IL12p70 were detected in the plasma of patients with 429 type 2 diabetes compared to healthy individuals (Gonzales, Herrera, Torres, 2012). In 430 addition, an increase of α -2,6 and α -2,3-sialylation has been observed on the surface of 431 endotoxin tolerant macrophages which responded to a repeated LPS challenge with significantly reduced production of TNF- α (Wu, Lan, Ren, Chen 2016). 432

433 Endotoxin tolerance is not possible to be induced in human gingival fibroblasts (Zaric 434 et al 2011) and they have been shown to play a crucial role in maintaining chronic 435 inflammation (Buckley 2011). We showed here that human gingival fibroblasts do not 436 express CD33 receptors and their basal (spontaneous) IL-8 production was much higher than in THP-1 cells. Interestingly, the spontaneous IL-8 production was significantly increased in 437 438 THP-1 cells treated with neuraminidase suggesting an important role of endogenous sialic 439 acid – CD33 interaction in preventing production of pro-inflammatory cytokines during the resting state of the immune system. 440

Our data, in combination with previous studies suggest that although sialylation of *P.gingivalis* cell surface components may provide additional benefits to this prominent periodontal pathogen in biofilm formation and escaping complement killing, it does not affect the inflammatory potential of its LPS. While sialylation of pathogens' surface ligands and

445	attenuation of the host inflammatory response might reflect immune evasion by pathogens it
446	may also represent a protective response that serves to prevent excessive inflammation in the
447	host.
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