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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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Running head: Sialylation of *P.gingivalis* LPS

Keywords: *Porphyromonas gingivalis*, LPS, sialic acid, inflammation, periodontal disease

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- **Word count=**

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
5 involved various means of evading the immune system and subverting normal host defences.
6 *Porphyromonas gingivalis* produces different lipopolysaccharide isoforms with significant
7 structural variations of their lipid A and O-antigen moieties that can affect its pro-
8 inflammatory and bone resorbing potential. In this study we examined the level of
9 *P.gingivalis* LPS sialylation and the influence of sialic acid LPS content on the pro-
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
16 CD33 receptor is prevented by endogenously expressed sialic acid on the surface of THP-1
17 cells that cannot be out-competed by sialic acid containing *P.gingivalis* LPS. Interestingly,
18 human gingival fibroblasts do not express CD33 receptors and have much higher spontaneous
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
23 controls immune cell activation.

24

25

26 INTRODUCTION

27

28 Periodontal diseases are caused by dental plaque bacteria of so called “red complex” that can
29 use different molecular mechanisms and strategies to evade or overcome host defences (Ref,
30 socransky). One of the mechanisms by which they can manipulate the cytokine responses of
31 macrophages and monocytes is through their surface glycosylation (Amano a, Chen C,
32 Sharma A 2014). Recent studies have revealed that bacterial surface–expressed O-linked
33 glycans and especially terminally present sialic acid can help them evade the host innate
34 immune attacks and also modulate T-cell responses during periodontal inflammation
35 (Megson , Koerdt, Messner, 2015). *Porphyromonas gingivalis* utilizes a panel of virulence
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
38 stimulates the host’s innate immune system and bone resorption whilst LPS recognition by
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
41 of LPS or its oligo/polysaccharide domain (O-antigen) (Al-Qutub, Braham 2006).

42

43 Sialic acids and Siglecs

44 Sialic acids are a large family of sugars containing 9C atoms which predominantly derive
45 from N-acetylneuraminic acid (Angata 2006). N-acetylneuraminic acid (the most abundant
46 and best-studied sialic acid) is usually a terminal sugar residue on the oligosaccharide chains
47 of eukaryotic cells where it functions in recognition and anti-recognition phenomena (Vimr,
48 Kalivoda et al. 2004). Sialic acids are transferred using α -2,3 or α -2,6 linkages to sub-
49 terminal sugars by a family of sialyltransferases and are abundantly expressed on the
50 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
54 the function of Siglecs as regulators of adhesion, cell signalling and endocytosis (Chen, Varki
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
60 Siglec, proving that binding of Siglecs to *trans* ligands can occur dynamically in the presence
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
66 cells (Crocker, Paulson et al. 2007). CD33-related Siglecs have also been found expressed on
67 cells of the nervous system (Siglec-4), placental trophoblasts (Siglec-6) and epithelial cells
68 (Siglec-12) but not on fibroblasts (Poe, Fujimoto et al. 2004) (Pilling, Fan Huang 2009 fibro).
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
75 2008a). Ligand engagement of these inhibitory receptors causes phosphorylation of the

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

83

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,
88 thus evading the immune system or binding to host cells via sialic acid - recognizing
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
91 into their lipopolysaccharide as a terminal residue of the carbohydrate part of the molecule.
92 LPS is an important surface constituent of these bacteria and is involved in complex
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
96 innate immune response of the host and escape killing by human serum (Severi, Randle
97 2005) while the inflammatory response against *C.jejuni* is in direct correlation with the level
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
100 that these structural features are likely to be important in the ability of the disease-causing

101 bacteria to evade recognition by LPS-specific bactericidal IgG and complement-mediated
102 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).
105 *P.gingivalis*, as a member of the “red complex bacteria”, primarily inhabits the gingival sulci
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 sialylation 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
113 different *P.gingivalis* strains (*P.gingivalis* ATCC 33277 and *P.gingivalis* W83) and to
114 determine the influence of LPS sialylation 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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126 **METHODS**

127

128 *Porphyromonas gingivalis* ATCC 33277 LPS was obtained from InvivoGen (San Diego,
129 USA). *Porphyromonas gingivalis* W83 LPS was extracted using the method reported by
130 Darveau and Hancock (Darveau 1983). Briefly, *P.gingivalis* W83 was grown anaerobically in
131 anaerobic basal broth (Oxoid) containing 5µg/ml of haem for 7 days. Cells were harvested at
132 7,000 rpm for 15 minutes, lyophilized and resuspended in 15 ml of 10 mM Tris-HCl, pH 8.0,
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
141 sample had cooled to 0°C, they were centrifuged at 12,000 g for 15 minutes at 0 - 4°C. Pellet
142 was resuspended in 25 ml of 0.1 M EDTA(tetra sodium salt), 2% SDS, 10 mM Tris-HCl, pH
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
146 shaking. The next day two volumes of 0.375 M MgCl₂/95% EtOH were added, the solution
147 cooled to 0°C as before, centrifuged at 12,000 g for 15 minutes at 0 - 4°C. The pellet was
148 resuspended in 15 ml of 10 mM Tris-HCl, pH 8.0 and sonicated for 30s. Samples were
149 centrifuged at 1000 rpm for 5 minutes to remove insoluble Mg/EDTA complexes. MgCl₂ was
150 added to give a final concentration of 25 mM and suspension was centrifuged at 200,000 g

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 ΔPG0083 (ΔW83) 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.

157

158 **Cell culture**

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

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

171

172 **Immunoprecipitation**

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

175 beads were pelleted at 5000rpm for 1 min and washed in 0.5% TBST. The whole cell lysates
176 were then added to the beads and incubated on rotation at 4°C for 2 hours. Afterwards the
177 beads were pelleted and washed 3 times in 0.5% TBST before being resuspended in 5µl of
178 5X Western Loading Buffer with 10% β-mercaptoethanol and 15µl of PBS. The samples
179 were denatured at 95°C for 5 min and placed on ice prior to loading onto a gel.

180

181 **SDS-PAGE Electrophoresis**

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

188

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
192 respective primary antibodies at the relevant dilutions in appropriate blotting solution.
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
195 secondary antibody for 1h in 5% milk dissolved in 1X PBS/0.1%Tween 20 at 1:5000
196 concentration. The wash step was repeated as before and proteins were then detected using
197 chemiluminescence method. Mouse monoclonal anti-CD33 antibodies were obtained from

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

202

203 **Lectin Blot analysis**

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

209 1 μ l 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 1 μ l/ml and incubated with the membrane over night at +4⁰C. 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.

216

217 **ELISA**

218 THP-1 cells were resuspended at 5x10⁵ cells/ml and human gingival fibroblast at 8x10⁴
219 cells/ml in corresponding medium and treated with LPS for 4 hours. Cell free supernatants
220 were removed and stored at -80⁰C 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
228 times and 100 µl of detection antibody diluted in 1% BSA/PBS was added for 2 hours. Again,
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**

236 THP-1 cells and human gingival fibroblasts (1×10^6 cells/ml and 80000 cells/ml respectively)
237 were washed in PBS, spun at 2000 rpm for 5 min and resuspended in a 100 µl of PBS
238 supplemented with 2% FCS. Cells were then stained with 20µl of FITC conjugated anti-
239 human CD33 antibody (eBioscience) or respective isotype control for 30 min at room
240 temperature in the dark. Cells were centrifuged again and washed twice with 1 ml PBS and
241 resuspended in 500 ml of fresh PBS. Flow cytometry analysis was carried out on FACS
242 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
246 GraphPad Prism version 4 (GraphPad Software, San Diego, CA). Differences between
247 multiple treatments were compared by one-way ANOVA followed by Tukey's post hoc test.
248 Values are expressed as the mean \pm standard error of the mean (SEM). A value of $P < 0.05$
249 was considered to represent a statistically significant difference. (* means $P < 0.05$. ** denotes
250 $P < 0.01$ and *** means $P < 0.001$).

251

252

253

254 **RESULTS**

255

256 **Sialic acid detection**

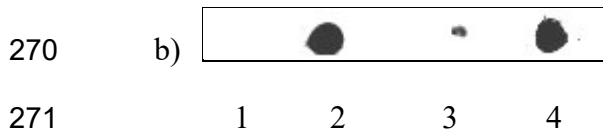
257 Sialic acid appears as a terminal sugar in polysaccharide chains. In order to examine the
258 content of sialic acid in LPSs from two different *P.gingivalis* strains (ATCC 33277 and
259 W83), the lectin-blot method was used with two sialic acid binding lectins as antibodies
260 (SNA and MAA specific 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
262 of the high concentration of sialylated proteins present in it, while water was used as a
263 negative control. Lectin-blot analysis revealed a high concentration of sialic acid within the
264 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).

266

267



269



272

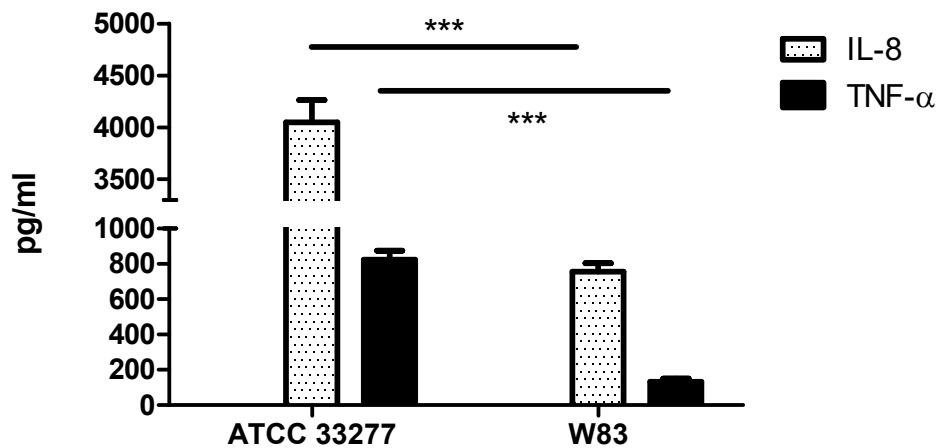
273

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

277

278 Inflammatory potential of LPSs

279 Inflammatory potential of the two types of *P.gingivalis* LPS was examined in a THP-
 280 1 cell model. THP-1 cells were challenged with 1µg/ml of the LPSs for 4 hours and
 281 the production of TNF-α and IL-8 was measured in the cell supernatants. ATCC
 282 33277 LPS triggered much higher production of both TNF-α and IL-8 compared to
 283 W83 strain LPS (P<0.001) (Figure 2.)



284

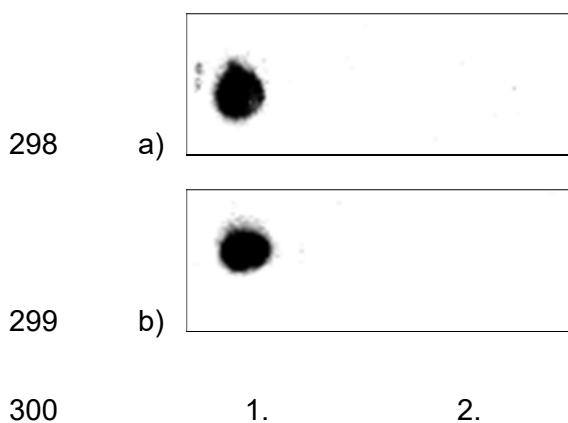
285 Figure 2: Inflammatory potential of LPSs extracted from *P.gingivalis* ATCC 33277,
 286 and *P.gingivalis* W83. THP-1 cells were challenged with 1µg/ml of LPS for 4 hours
 287 and TNF-α and IL-8 were measured by ELISA.

288

289 **The influence of LPS sialylation on its inflammatory potential**

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

297

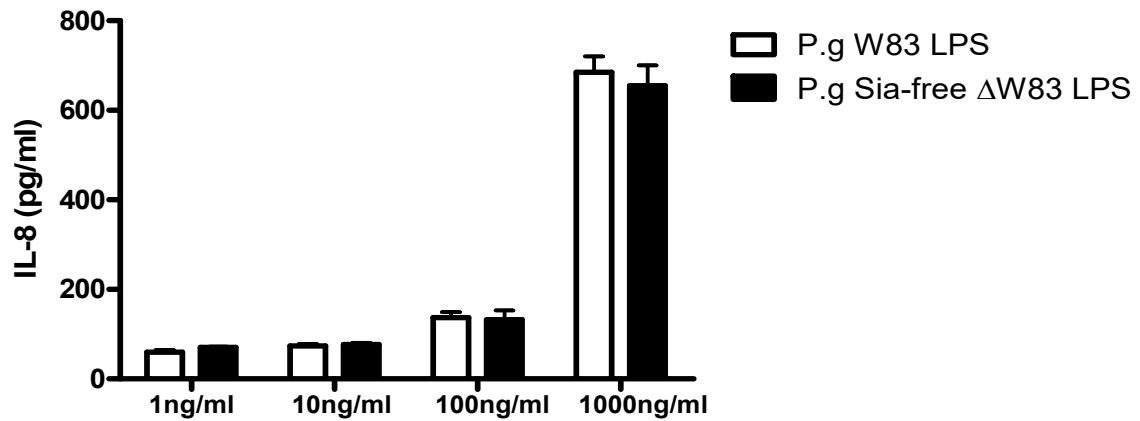


301 Figure 3: Sialic acid lectin-blot analysis of *P.gingivalis* W83 LPS and *P.gingivalis* Δ W83
302 LPS. Panel a) SNA lectins; panel b) MAA lectin; 1-*P.gingivalis* W83 LPS ; 2-*P.gingivalis*
303 Δ W83 LPS.

304

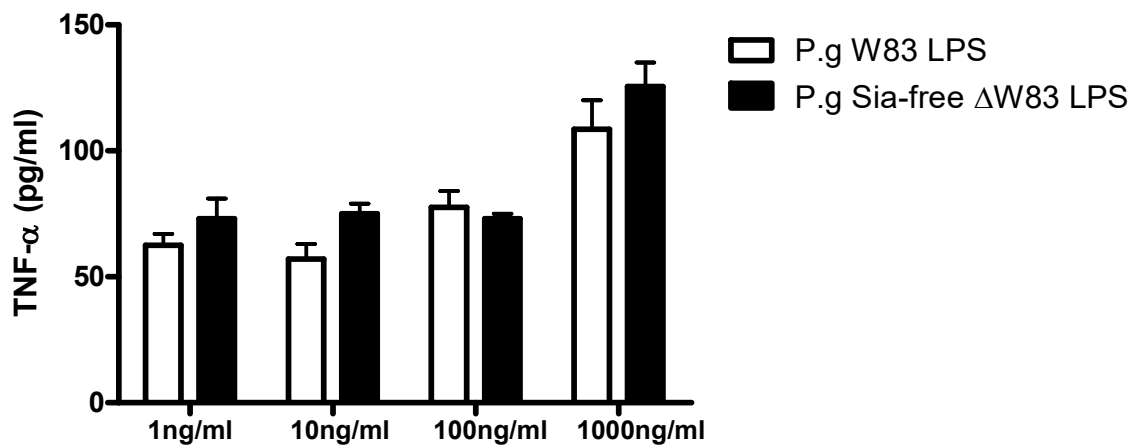
305 As expected, LPS obtained from sialyltransferase mutated *P.gingivalis* W83 strain did not
306 contain sialic acid. Both types of lectins failed to detect sialic acid moieties within this mutant
307 type of *P.gingivalis* LPS (Sia-free LPS).

308 Furthermore, THP-1 cells were treated with different concentrations of *P.gingivalis* W83 LPS
309 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).



311

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.



314

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.

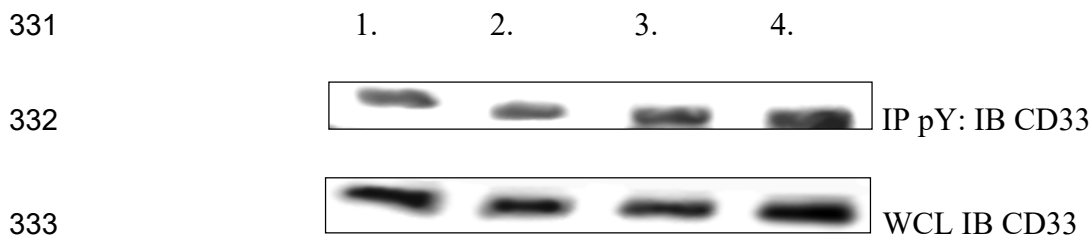
317

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.

321

322 **CD33 activation**

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



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

338

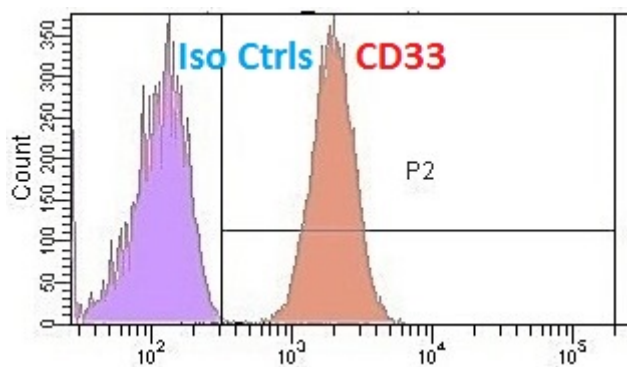
339 The results revealed that CD33 was phosphorylated at tyrosine residues in the cells
340 treated with all types of LPSs in addition to control cells incubated in the medium only.

341 These results show that CD33 is endogenously expressed in THP-1 cells and activated
342 regardless of the type of LPS treatment. This is possibly due to the presence of sialic acids at
343 the surface of monocytes as a constituent of glycoproteins, which interact with CD33 in *cis*
344 interaction and block *trans* interaction of sialic acid present in LPS and CD33. Constitutive
345 repressor activity of self-expressed sialic acid with CD33 receptors may be important for
346 maintaining low levels of pro-inflammatory cytokine production during the resting state of
347 the immune system.

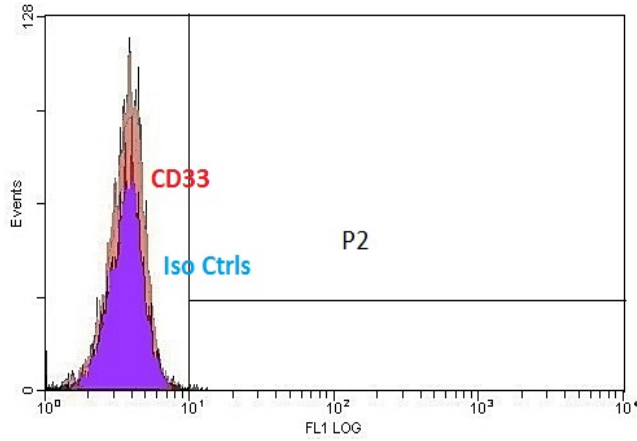
348

349 **CD33 inhibits spontaneous secretion of IL-8**

350 Since human gingival fibroblasts (HGF) do not express CD33 (Figure 7), the spontaneous
351 secretion of IL-8 by THP-1 cells, neuraminidase (NA) treated THP-1 cells and HGFs over 4
352 hours without any treatment was investigated. Half a million (5×10^5 cells) THP-1 cells, NA
353 treated THP-1 cells and 8×10^4 HGFs per 1ml of corresponding medium were incubated for 4
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.

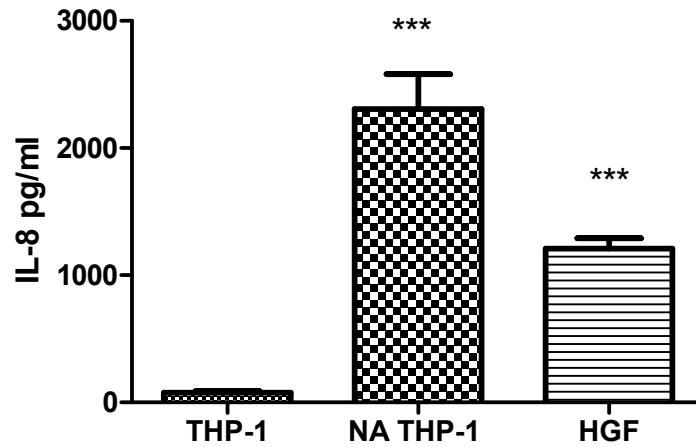


359 a)



360 b)

361



362 c)

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

367

368 **DISCUSSION**

369

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
372 biological mimicry, to coat themselves in sialic acid and increase resistance to components of
373 the host's innate immune response, or they can use it as nutrient (SEVERI, Thomas 2007).
374 Structural variations of bacterial LPS can have major impact on the host immune response
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
379 attached sialic acid moieties, while *P.gingivalis* ATCC 33277 LPS expresses much lower
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
382 33277 strain being avirulent while the W83 strain is generally considered virulent. In
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).

386 We compared the inflammatory potential of these two types of *P.gingivalis* LPS and
387 found much higher production of TNF- α and IL-8 in human monocytes challenged by LPS
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
390 inflammatory reaction. The interaction between LPS and TLR components depends on the
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
394 lower inflammatory response in THP-1 cells compared to less sialylated ATCC 33277 LPS.

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

398 *P. gingivalis* is capable of synthesizing a heterogeneous isoforms of the lipid A
399 molecule, which are subtly different from each other in structure. These subtle changes in the
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
406 LPS, suggesting that sialic acid is not an important determinant of *P.gingivalis* LPS
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
415 by the modifications of their lipid A core (Darveau, Hajjar 2004).

416 When CD33 receptor activation by LPS extracted from ATCC 33277, W83 and Δ W83 strains
417 was examined by immunoblotting, CD33 was found to be activated in cells treated with all
418 three types of LPS in addition to the control, untreated cells. This is probably a result of the
419 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
432 significantly reduced production of TNF- α (Wu, Lan, Ren, Chen 2016).

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
437 in THP-1 cells. Interestingly, the spontaneous IL-8 production was significantly increased in
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
440 resting state of the immune system.

441 Our data, in combination with previous studies suggest that although sialylation of
442 *P.gingivalis* cell surface components may provide additional benefits to this prominent
443 periodontal pathogen in biofilm formation and escaping complement killing, it does not affect
444 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.

448

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