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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- **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.

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INTRODUCTION

Periodontal diseases are caused by dental plaque bacteria of so called "red complex" that can 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 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 glycans and especially terminally present sialic acid can help them evade the host innate immune attacks and also modulate T-cell responses during periodontal inflammation (Megson , Koerdt, Messner, 2015). *Porphyromonas gingivalis* utilizes a panel of virulence factors which can cause deregulation of the innate immune and inflammatory responses. *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 the host is essential for clearing infections of this invading periodontal pathogen. Host's 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).

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 sub-terminal 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.

3

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 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

113   different *P.gingivalis* strains (*P.gingivalis* ATCC 33277 and *P.gingivalis* W83) and to

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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126 **METHODS**

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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 $MgCl_2$. 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 $MgCl_2$/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 $MgCl_2$/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. $MgCl_2$ 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 10^3 to 10^6

163 cells/ml at 5% CO_2 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% CO_2 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 **Immunoprecipation**

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, ddH$_2$O 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^{0}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.

216

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

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 ($1x10^6$ 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 ± 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 $P<0.001$).

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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

268 a)

269

270   b)

271   1   2   3   4
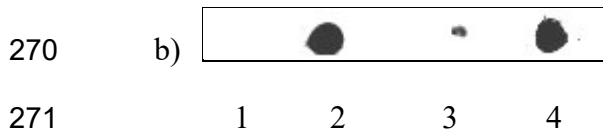
274   Figure 1: Lectin-blot detection of sialic acid LPS content from different *P.gingivalis*

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.

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.)
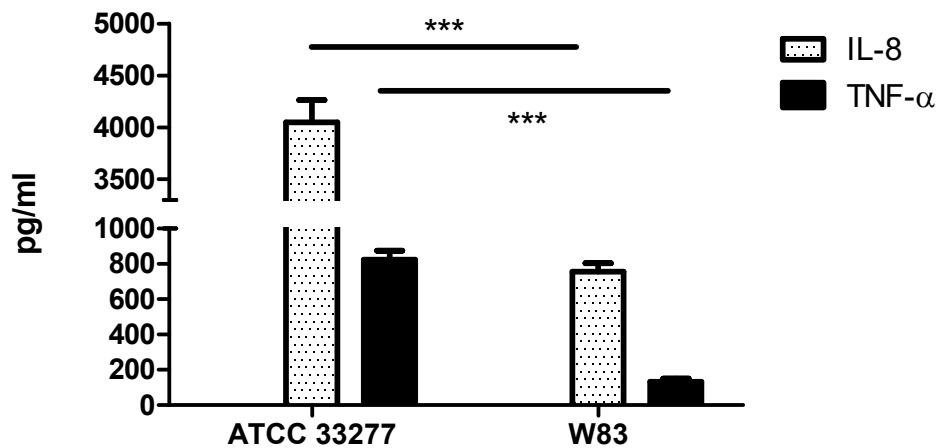
285   Figure 2: Inflammatory potential of LPSs extracted from *P.gingivalis* ATCC 33277,

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.

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 (ΔW83). The

294 absence of sialic acid in LPS extracted from *P.gingivalis* ΔW83 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

298 a)

299 b)

300   1.   2.
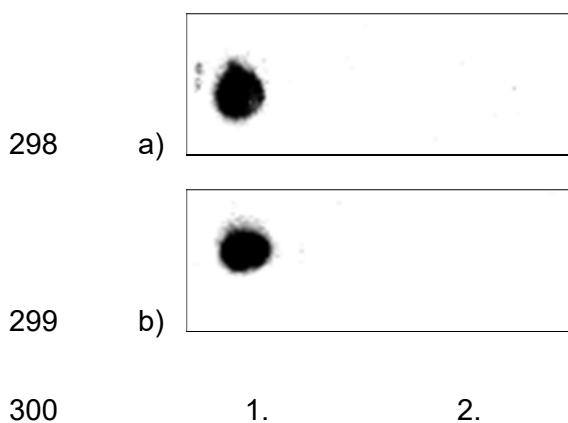
301 Figure 3: Sialic acid lectin-blot analysis of *P.gingivalis* W83 LPS and *P.gingivalis* ΔW83

302 LPS. Panel a) SNA lectins; panel b) MAA lectin; 1-*P.gingivalis* W83 LPS ; 2-*P.gingivalis*

303 ΔW83 LPS.

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* ΔW83 LPS for 4 hours and the production of IL-8 and TNF-α in cell

310 supernatants was measured (Figures 4 and 5.).
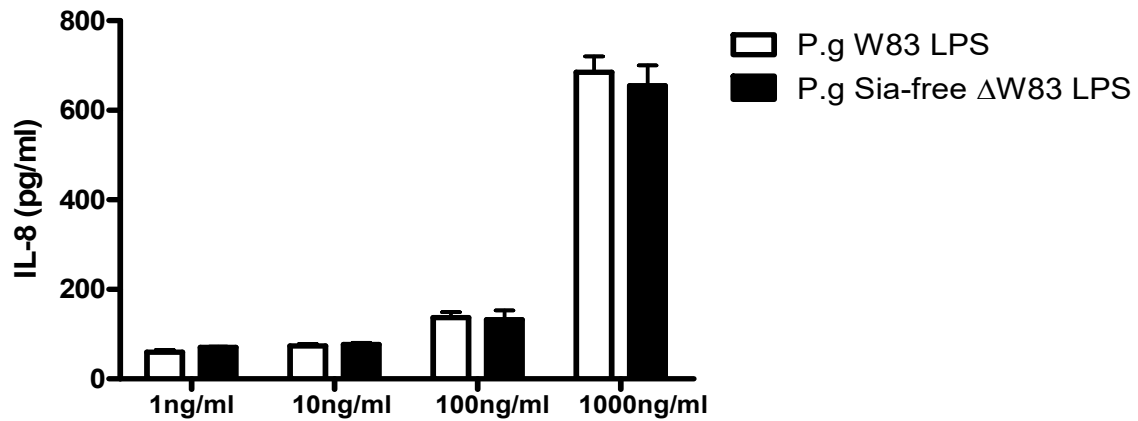
311

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 ΔW83 LPS.
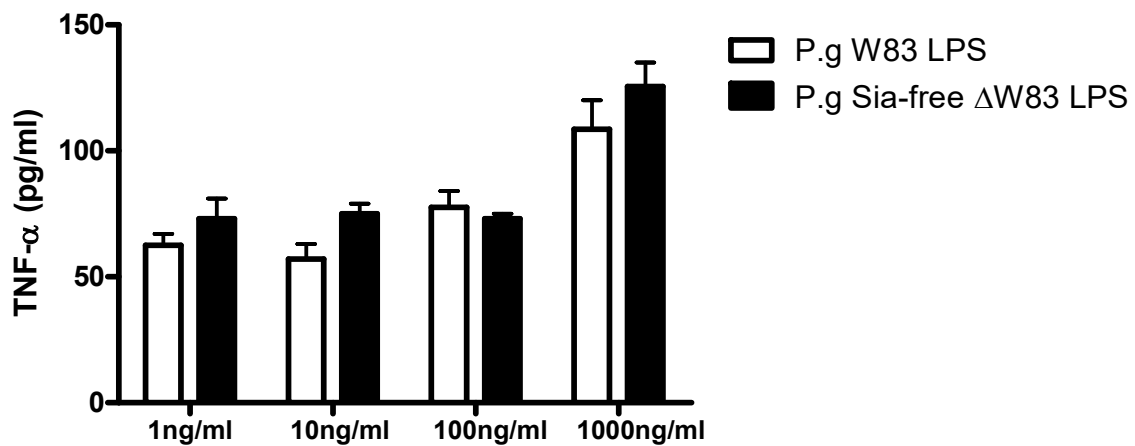
314

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 ΔW83 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* ΔW83 LPS (Sia-free LPS) compared to sialic acid containing

320 *P.gingivalis* W83 LPS in all concentrations examined.

321

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 ΔW83). 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* ΔW83 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).

331
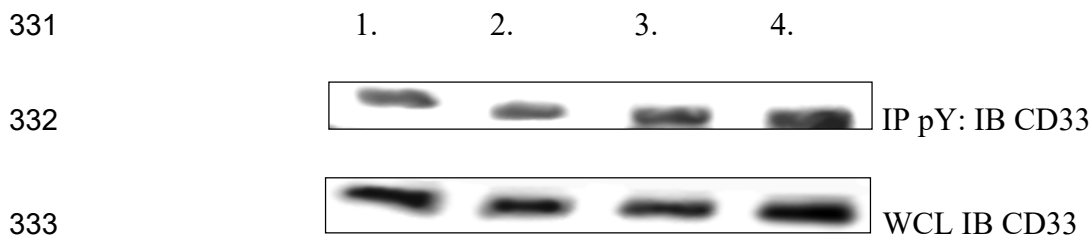
Figure 6: CD33 phosphorylation. THP-1 cells were treated with: 1) medium alone; 2) 1μg/ml *P.g* ATCC 33277 LPS; 3) 1μg/ml *P.g* W83 LPS; and 4) 1μg/ml *P.g* ΔW83 LPS and the cell lysate was immunoprecipitated with anti-phosphotyrosine antibodies and immunoblotted for CD33. Whole cell lysate immunoblotted for CD33 served as a loading control.

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 ($5x10^5$ cells) THP-1 cells, NA

353 treated THP-1 cells and $8x10^4$ 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.
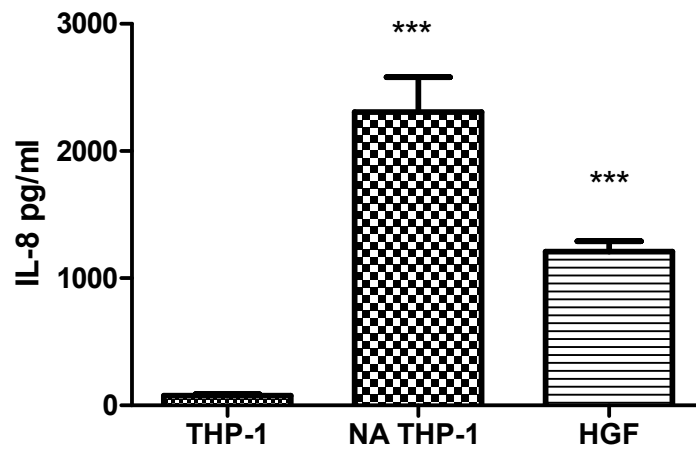

359 a)

17

360 b)

361
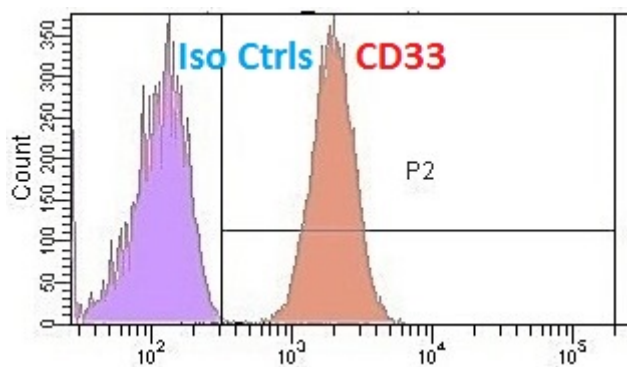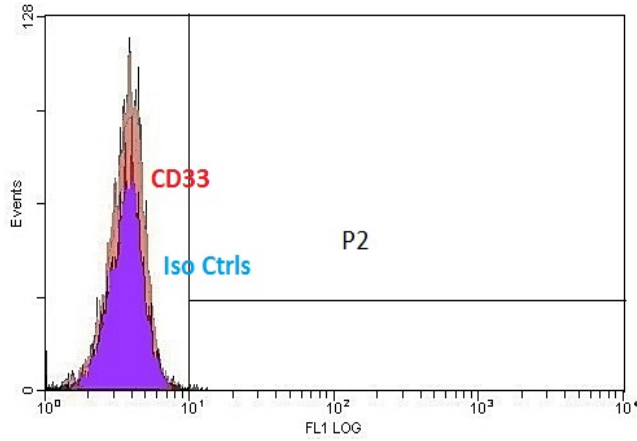
362   c)

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.

DISCUSION

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 ΔW83 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.

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453 **REFERENCES**

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