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

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ABSTRACT

Porphyromonas gingivalis produces different lipopolysaccharide isoforms with significant structural variations of their lipid A and O-antigen moieties that can affect its pro-inflammatory and bone resorbing potential.

We show here, for the first time, that *P.gingivalis* LPS isolated from W83 strain is highly sialylated and possesses significantly reduced inflammatory potential compared to less sialylated ATCC 33277 strain LPS. Nevertheless, the reduction in the endotoxin activity is not mediated by the presence of sialic acid LPS moieties since the sialic acid-free LPS produced by the mutant W83 strain exhibits a similar inflammatory potential as the wild-type strain. Furthermore, our findings suggest that 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 cells that cannot be out-competed by sialic acid containing *P.gingivalis* LPS. The present study also highlights the importance of endogenous sialic acid as a "self-associated molecular pattern" and CD33 receptors in modulation of innate immune response as human gingival fibroblasts, which do not express CD33 receptors, and desialylated THP-1 cells have both been found to have much higher spontaneous IL-8 production compared to naïve THP-1 cells.

INTRODUCTION

The ability to synthesize, take up, use and catabolize sialic acid is prevalent among both commensals and pathogens that colonize the oral cavity and the intestinal, respiratory and urogenital tracts (McDonald, Lubin et al. 2016). 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 (Socransky, Haffajee et al. 1998). Recent findings suggest that the host immune response is initially subverted by these keystone pathogens and is subsequently over-activated by pathobions leading to polymicrobial synergy and formation of dysbiotic subgingival microflora (Hajishengallis 2015). One of the mechanisms by which periodontopathogens can manipulate the immune responses of macrophages and monocytes is through their surface glycosylation (Amano, Chen et al. 2014). It has been shown 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 et al. 2015). *Porphyromonas gingivalis*, the keystone periodontal pathogen, 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. Whilst LPS recognition by the host is essential for clearing infections of this invading periodontal pathogen, at the same time it triggers a robust inflammatory reaction and is responsible for alveolar bone resorption. The 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 et al. 2006).

Sialic acids are a large family of sugars containing 9C atoms which predominantly derive from N-acetylneuraminic acid (NANA) (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. 2008). The mammalian glycome contains numerous sialylated glycans that can be recognized as ligands by special receptors present on the cell surface, called Siglecs (Sialic acid-binding 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 2010). The local concentration of sialic acid on the surface of immune cells is very high and as a consequence of this, Siglecs binding sites are usually masked by low-affinity *cis* interactions with endogenous glycans on the same cell (Varki, Gagneux 2012). However, high-affinity 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 of *cis* ligands (Collins, Blixt et al. 2006).

Siglecs are a family of ITIM-containing receptors (Immunoreceptor Tyrosine-based Inhibition Motif) that can recognise sialylated glycans and function as regulators of immune cells. CD33-related Siglecs are mainly expressed by cells of the innate immune systems such 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 cells of the nervous system (Siglec-4), placental trophoblasts (Siglec-6) and epithelial cells (Siglec-12) but not on fibroblasts (Poe, Fujimoto et al. 2004, Pilling, Fan et al. 2009). Ligand engagement of these inhibitory receptors causes phosphorylation of the ITIMs. 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).

Sialic acid is an important molecule for a number of bacteria too, serving as a substrate for capsule synthesis, LPS sialylation and as a potential growth factor (Steenbergen, Lee et al. 2006). A 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 receptors which mediate cell adhesion, protein-protein interaction and immune response (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. Sialylated LPS glycoforms play a key role in pathogenicity of *H.influenzae* and *N.meningitidis* (Bouchet, Hood et al. 2003).

There is a growing body of evidence that sialic acid and sialidases play a key role in the life, host mimicry and consequences of periodontal pathogen colonisation (Stafford, Roy et al. 2012). *P.gingivalis*, as a member of the "red complex bacteria", primarily inhabits the gingival sulci and periodontal pockets. Sialic acid is abundantly present in human serum which is the major component of the gingival crevicular fluid while salivary mucins and gingival connective tissue fibronectins also contain sialic acid. *P.gingivalis* is able to acquire sialic acid from the host and use it as an important factor that contributes to biofilm formation, capsule biosynthesis and pathogenicity of *P.gingivalis* (Li, Kurniyati et al. 2012). However, the role of sialyation of *P.gingivalis* LPS in the interaction with the host cells is still unknown. 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 determine the influence of LPS sialylilation on its inflammatory potential. Our results show for the first time that *P.gingivalis* W83 LPS is highly sialylated but the presence of sialic acid as an LPS carbohydrate moiety does not play a significant role in modulating the inflammatory potential of this periodontal pathogen's LPS.

MATERIALS AND METHODS

Porphyromonas gingivalis ATCC 33277 LPS was obtained from InvivoGen (San Diego, USA). *Porphyromonas gingivalis* W83 LPS was extracted using the method reported by Darveau and Hancock (Darveau, Hancock 1983). Sialic-acid free LPS extracted from *P.gingivalis* W83 ΔPG0083 (ΔW83), sialyltransferase defective mutants, was a kind gift from Dr Charles Shelburne, University of Michigan, Ann Arbor, US. This strain is not able to incorporate sialic acid into their LPS and was constructed and validated in the

same way as described by Sweier *et al*. (Sweier, Combs et al. 2003). Briefly, to replace the PG0083 gene an *erm*F cassette was constructed using the following primers (5'-3'): ACTGAATCCGATGGTGCAACTG and TAAGGCGAGTCCGACACGATAG and was introduced into *P.gingivalis* W83 by electroporation. Similar to the wild type strain, W83 ΔPG0083 mutants were able to adhere and invade a cultured human cell line, but they were not pigmented and the disruption of the PG0083 gene had a significant effect on their growth.

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 μg/ml) (Invitrogen). The cells were cultured between 10^3 to 10^6 cells/ml at 5% CO_2 atmosphere at 37^o C.

Human gingival fibroblasts (HGF) were established from explants of healthy gingival tissues obtained during routine clinical procedures (Lappin, Brown et al. 2016). The study was approved by the Research Ethics Committee of Northern Ireland, participant information sheets were provided, and written informed consent was obtained from patients wishing to participate in the study. 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 μg/ml of streptomycin in a humidified atmosphere of 5% CO_2 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.

SDS-PAGE Electrophoresis

Whole cell lysates were treated with sample loading buffer and fractionated on a 10% SDS polyacrylamide gel at 35mA. Proteins were transferred from the 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.

Immunoblotting

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

Immunoprecipation

200 µl of Protein G magnetic beads (Millipore) were pre-associated with 5µl of phospho-tyrosine- 4G10 (Millipore) antibodies on a rotor for 90 min at 4^{o}C. The antibody bound beads were pelleted at 2500g 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^{o}C for 2 hours. Afterwards

the beads were pelleted and washed 3 times in 0.5% TBST before being resuspended in 5µl of 5X Western Loading Buffer with 10% β-mercaptoethanol and 15µl of PBS. The samples were denatured at 95ºC for 5 min and placed on ice prior to loading onto a gel.

Lectin Blot analysis

Sialic acid content in LPSs was assessed using lectin blot analysis as described by Cao *et al*. (Cao, Guo et al. 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, ddH_2O was used as a negative control.

1µl of 1mg/ml of different LPS preparations (1µg of LPS, corresponding to 10^8 bacterial cells) with positive and negative controls were added directly onto the nitrocellulose membrane and allowed to air dry. The membrane was then incubated in 0.1% PBST for 1h at room temperature. Lectins were dissolved in 0.1% PBST at a final concentration of 1µl/ml and incubated with the membrane over night at $+4^0$C. The membrane was washed with PBST and incubated with Streptavidine HRP in PBST (1:5000). Having been washed, the membranes were developed using West-Femto chemiluminescence substrate and visualised in the Syngene G box.

ELISA

THP-1 cells were resuspended at $5x10^5$ cells/ml and human gingival fibroblast at $8x10^4$ cells/ml in corresponding media and treated with LPS for 4 hours. Cell free supernatants were removed and stored at -80°C until assessed for TNF-α and IL-8 (Peprotech, R&D Systems respectively). Absorbance was read at 405 nm (Genios Tecan spectrofluorimeter). Cytokines concentrations were extrapolated from the standard curve.

Flow cytometry

THP-1 cells and human gingival fibroblasts ($1x10^6$ cells/ml and 80000 cells/ml respectively) were washed in PBS, spun at 500g for 5 min and resuspended in a 100 µl of PBS supplemented with 2% FCS. Cells were then stained with 20µl of FITC conjugated anti-human CD33 antibody (eBioscience) or respective isotype control for 30 min at 20^{o}C 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.

Cell viability

Cell viability was determined with the Cell Proliferation Kit (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-MTT) (Promega, UK). THP-1 cells ($5x10^5$ cells/well) were treated with 1µg/ml of different LPS preparations for 4 h together with untreated control samples. Cells were subsequently incubated in a 96-well plate with MTT solution for a further 4 h. The absorbance of formazan dye was measured using a Genios Tecan plate reader, at 570 nm, with a reference wavelength of 690 nm.

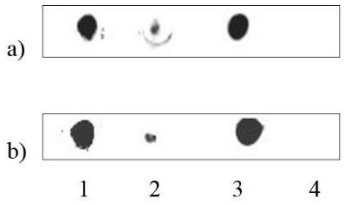
Statistical analysis

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 multiple treatments were compared by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as the mean ± 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 $P<0.01$ and *** means $P<0.001$).

RESULTS

Sialic acid detection

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 W83), the lectin-blot method was used with two sialic acid binding lectins as antibodies (SNA and MAA specific for α-2,6 and α-2,3 bond between the sialic acid and the terminal N-acetyl galactosamine respectively). Human serum was used as a positive control because of the high concentration of sialylated proteins present in it, while LPS extracted from sialyltransferase defective *P.gingivalis* W83 mutants (ΔW83) was used as a 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-2,3 linked sialic acids were abundantly present within the W83 LPS. Sialic acid was not detected in LPS extracted from *P.gingivalis* ΔW83 mutants (Figure 1.).



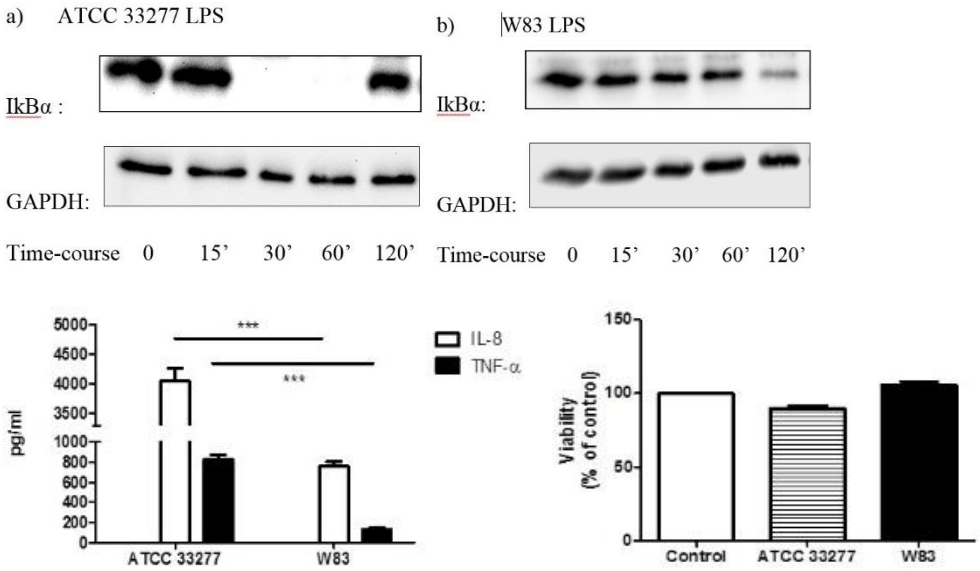
(Insert FIGURE 1.)

Figure 1: Lectin-blot detection of sialic acid LPS content from different *P.gingivalis* strains. Panel a) SNA lectin, panel b) MAA lectin. 1- human serum, 2- *P.gingivalis* ATCC 33277, 3- *P.gingivalis* W83 and 4- *P.gingivalis* ΔW83. The results are representative of three different experiments.

Inflammatory potential of LPSs

Inflammatory potential of the two types of *P.gingivalis* LPS was examined in a THP-1 cell model by monitoring IκBα degradation (Western-blotting) after 15, 30, 60 and 120

minutes of LPS challenge and by measuring TNF-α and IL-8 production in cells supernatants (ELISA) after 4h. Complete IκBα degradation occurred as early as after 30 minutes in the cells treated by *P.gingivalis* ATCC 33277 LPS while in the cells treated with *P.gingivalis* W83 LPS only partial degradation was observed after 2h of treatment (Figure 2a and 2b). Similarly, *P.gingivalis* ATCC 33277 LPS triggered much higher production of both TNF-α and IL-8 compared to W83 strain LPS (P<0.001) (Figure 2c). Cell viability was not affected by either of these two preparations (Figure 2d.)

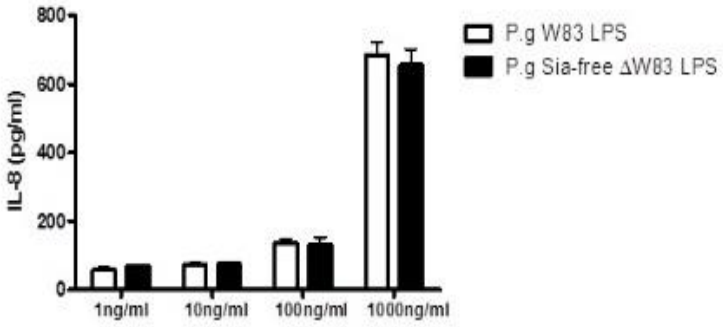


(Insert FIGURE 2a, FIGURE 2b, FIGURE 2c and FIGURE 2d)

Figure 2: Inflammatory potential of LPSs extracted from *P.gingivalis* ATCC 33277, and *P.gingivalis* W83. a) and b) THP-1 cells were challenged with 1μg/ml of ATCC 33277 LPS (a) or W83 LPS (b) for 15, 30, 60 and 120 minutes. GAPDH was used as a loading control. c) Production of TNF-α and IL-8 by THP-1 cells challenged with the two *P.gingivalis* LPS isoforms for 4h. d) Cell viability measured by the MTT assay. Values represent means ± SEM (n=3).

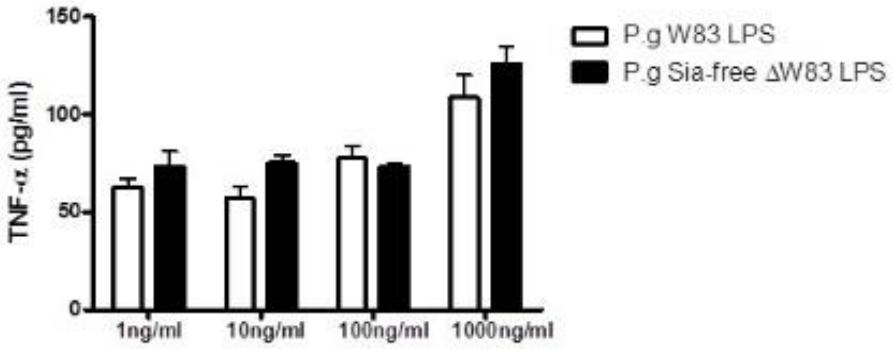
The influence of LPS sialylation on its inflammatory potential

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 for 4h with different concentrations of *P.gingivalis* W83 LPS, which was shown to be highly sialylated, and LPS isolated from sialyltransferase gene (PG00083) knock-out *P.gingivalis* W83 strain (ΔW83) and the production of TNF-α and IL-8 was measured in the supernatants (Figures 3. and 4.).



(Insert Figure 3.)

Figure 3: IL-8 production by THP-1 cells treated with different concentrations of *P.gingivalis* W83 LPS and *P.gingivalis* Sia-free ΔW83 LPS. Values represent the mean ± SEM (n=3).



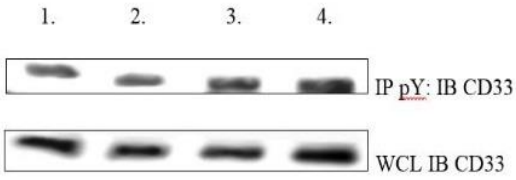
(Insert Figure 4).

Figure 4: TNF-α production by THP-1 cells treated with different concentrations of *P.gingivalis* W83 LPS and *P.gingivalis* Sia-free ΔW83 LPS. (n=3)

There was no difference in IL-8 and TNF-α levels produced by THP-1 cells treated with sialic acid free *P.gingivalis* ΔW83 LPS (Sia-free LPS) compared to sialic acid containing *P.gingivalis* W83 LPS in all concentrations examined.

CD33 activation

Upon activation of CD33, the 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, *P.gingivalis* W83 LPS and *P.gingivalis* ΔW83 LPS for 4 hours and phosphorylation of tyrosine residues within CD33 molecules was examined. Immunoprecipitation of THP-1 whole cell lysates with anti-phoshotyrosine antibodies was carried out followed by immunoblotting for CD33 (Figure 5).



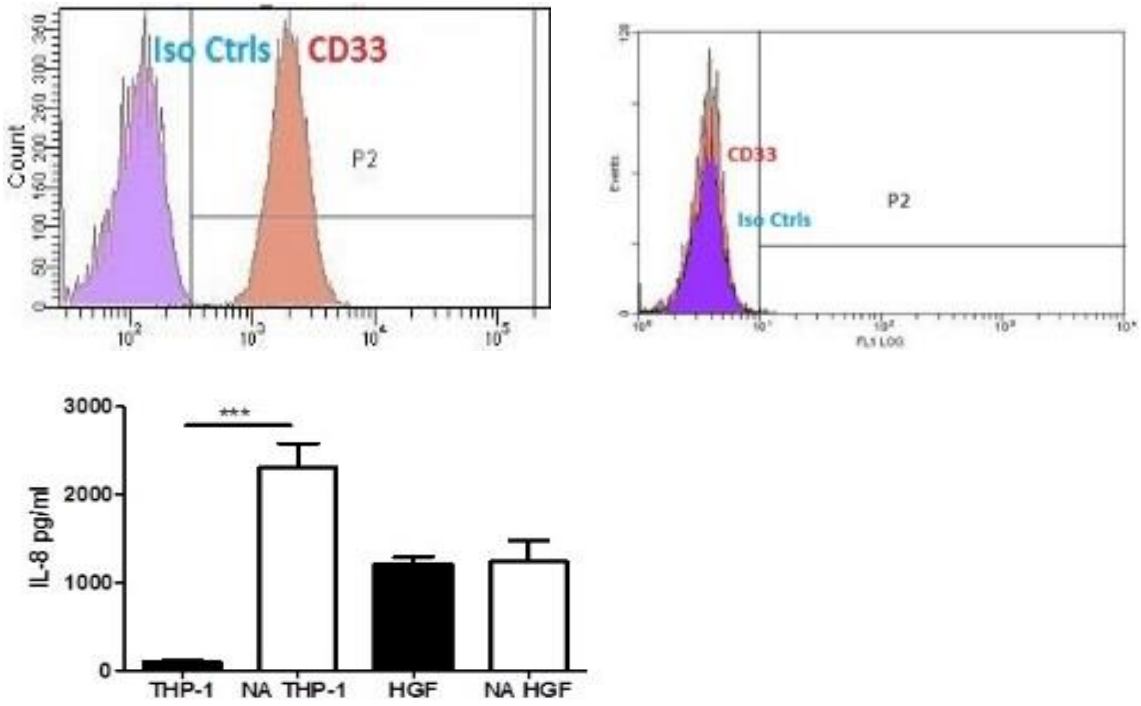
(Insert Figure 5)

Figure 5: CD33 phosphorylation. THP-1 cells were treated with: 1) medium alone; 2) 1μg/ml *P.gingivalis* ATCC 33277 LPS; 3) 1μg/ml *P.gingivalis* W83 LPS; and 4) 1μg/ml *P.gingivalis* 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. The results are representative of three different experiments.

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 alone. 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.

CD33 inhibits spontaneous secretion of IL-8

Since human gingival fibroblasts (HGF) do not express CD33 (Figure 6b), the spontaneous secretion of IL-8 by THP-1 cells, HGFs and neuraminidase (NA) treated THP-1 cells and HGFs over 4 hours without any treatment was investigated. Half a million of THP-1 cells, NA treated THP-1 cells and $8x10^4$ HGFs and NA treated HGFs per 1ml of corresponding media were incubated for 4 hours and IL-8 levels in cell supernatants were determined by ELISA. The concentration of IL-8 was adjusted according to the cell number (Figure 6c). Spontaneous production of IL-8 was significantly higher in NA treated THP-1 cells and human gingival fibroblasts compared to THP-1 cells, suggesting that endogenous sialic acid - CD33 interaction is likely to play an important role in inhibiting basal secretion of pro-inflammatory cytokines. NA treatment of HGFs did not affect the level of spontaneous IL-8 production by these cells.



(Insert Figure 6a, Figure 6b and Figure 6c)

Figure 6: CD33 expression in a) THP-1 cells, b) human gingival fibroblasts. c) Spontaneous production (over 4h) of IL-8 by THP-1 cells, neuraminidase (NA, Sigma) treated THP-1 cells, human gingival fibroblasts (HGF) and NA treated HGFs, adjusted according to cell number used in the experiments. (n=3)

DISCUSSION

Sialic acid residues are highly expressed in eukaryotic cells and act as a marker for self in the immune system (Chen, Brown et al. 2014). Pathogenic bacteria also use this molecule for biological mimicry, to coat themselves in sialic acid and increase resistance to components of the host's innate immune response, or they can use it as nutrient (Severi, Hood et al. 2007). Structural variations of bacterial LPS can have a major impact on the host immune response and clinical disease outcome. Sialylation of 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 et al. 2005) while the inflammatory response against *Campylobacter jejuni* is in direct correlation with the

level of its LPS sialylation (Stephenson, John et al. 2013). In addition, it has been shown that the 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 bacteria to evade recognition by LPS-specific bactericidal IgG and complement-mediated lysis (John, Phillips et al. 2016). Here, we show for 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 a much lower level of sialylation. Many previous studies have compared virulence-associated activities and 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 addition, a cluster of genes involved in the synthesis of highly sialylated capsular polysaccharides, transposase, helicase and fibronectin type 3 are present in W83 while it was not found in strain ATCC 33277 (Chen, Hosogi et al. 2004).

We compared the inflammatory potential of these two types of *P.gingivalis* LPS and found a rapid IκBα degradation and much higher production of TNF-α and IL-8 in human monocytes challenged by LPS extracted from the ATCC 33277 strain compared to W83 LPS. Whilst the measurement of TNF-α and IL-8 is not a comprehensive assessment of inflammatory potential, they have been shown to be good indicators of periodontal tissues damage (Zaric, Shelburne et al. 2010). Detection of LPS by Toll-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 chemical composition of the lipid A core of the LPS molecule (level of phosphorylation and acylation) and can be modulated by the O-antigen polysaccharide tail (Maldonado, Sa-Correia et al. 2016). In our case, highly sialylated W83 LPS triggered a significantly lower inflammatory response in THP-1 cells compared to less sialylated ATCC 33277 LPS. Similarly, sialylation of *Helicobacter bizzozeronii LPS* has been shown to play a crucial role in attenuation of the host's inflammatory response to this prominent animal pathogen (Kondadi, Revez et al. 2015).

P.gingivalis is capable of synthesizing heterogeneous isoforms of the lipid A molecule, which are subtly different from each other in structure. These subtle changes in the lipid A chemical composition have been shown to greatly influence the host's immune response to *P.gingivalis* LPS (Jain, Darveau 2010). In order to examine the influence of sialic acid O-antigen moieties of LPS on the host's inflammatory response and exclude lipid A variations, sialyltransferase deficient W83 mutants were grown under the same conditions as the wild type W83 and their LPS was extracted using the same method. These mutants produced sialic 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 inflammatory potency.

When CD33 receptor activation by LPS extracted from ATCC 33277, W83 and ΔW83 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 sialic acid on the surface of THP-1 cells from *cis* mediated interaction with CD33 receptors. Ishida *et al*. (Ishida, Akita et al. 2014) have found that CD33 activation by endogenous ligands negatively regulate TLR intracellular signalling and production of IL-12. Similarly, pre-treatment of sialic acid has been shown to efficiently prevent LPS-induced acute renal failure and TLR- mediated apoptotic signalling in a rat model (Hsu, Chen et al. 2016). On the contrary, desialylation of dendritic cells significantly improves their phagocytic activity and immunological potency through an actin-independent mechanism (Cabral, Silva et al. 2013). Interestingly, hyperglycaemia down-regulates CD33 expression in human monocytes and higher levels of TNF-α, IL-8 and IL12p70 were detected in the plasma of patients with type-2 diabetes compared to healthy individuals (Gonzalez, Herrera et al. 2012). In addition, an increase of α-2,6 and α-2,3-sialylation has been observed on the surface of endotoxin tolerant macrophages which responded to a repeated LPS challenge with significantly reduced production of TNF-α (Wu, Lan et al. 2016).

Endotoxin tolerance is not possible to be induced in human gingival fibroblasts (Zaric, Coulter et al. 2011) and they have been shown to play a crucial role in maintaining chronic inflammation (Buckley 2011). We showed here that human gingival fibroblasts do not 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 THP-1 cells treated with neuraminidase suggesting an important role of endogenous sialic acid – CD33 interaction in preventing production of pro-inflammatory cytokines during the resting state of the immune system.

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 attenuation of the host inflammatory response might reflect immune evasion by pathogens it may also represent a protective response that serves to prevent excessive inflammation in the host. Interactions between oral pathogens and Siglecs is a field of investigation that is likely to continue expanding in scope and dental clinical practice importance.

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The Authors declare that there is no conflict of interest.

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