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


Published in:
Innate Immunity

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

Publisher rights
© The Authors 2017. This work is made available online in accordance with the publisher's policies. Please refer to any applicable terms of use of the publisher.

General rights
Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person’s rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.
Porphyromonas gingivalis W83 LPS is highly sialylated but it does not affect its inflammatory potential

Svetislav S. Zaric1*, Mark J. Lappin2, Catherine R. Fulton3, Fionnuala T. Lundy3, Wilson A. Coulter4, Christopher R. Irwin2

1. Plymouth University, Peninsula Schools of Medicine and Dentistry, Plymouth, PL4 8AA, UK.
2. Queen’s University Belfast, Centre for Dental Education, Belfast, BT12 6BP, Northern Ireland.
3. Queen’s University Belfast, Centre for Experimental Medicine, Belfast, BT9 7BL, Northern Ireland.
4. University of Ulster, School of Biomedical Science, Coleraine, BT52 1SA, Northern Ireland.

Running head: Sialylation of P. gingivalis LPS

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

*Correspondence: Dr Svetislav Zaric, Peninsula Dental School, C406 Portland Square, Plymouth, PL4 8AA.

Word count=
**SUMMARY**

Inflammation and immune homeostasis are regulated by a tightly controlled network of positive-negative regulatory mechanisms that guarantee proper defence and pathogen clearance whilst avoiding hyperactivity and inflammatory diseases. Some pathogens have involved various means of evading the immune system and subverting normal host defences. *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. In this study we examined the level of *P. gingivalis* LPS sialylation and the influence of sialic acid LPS content on the pro-inflammatory LPS potential in THP-1 cells. Our results indicate that *P. gingivalis* W83 LPS 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 a mutant W83 strain exhibits a similar inflammatory potential as the wild-type strain. In addition, the interaction between the sialic acid LPS moieties and the inhibitory CD33 receptor is prevented by endogenously expressed sialic acid on the surface of THP-1 cells that cannot be out-competed by sialic acid containing *P. gingivalis* LPS. Interestingly, human gingival fibroblasts do not express CD33 receptors and have much higher spontaneous IL-8 production compared to THP-1 cells. This basal IL-8 secretion is significantly increased by THP-1 cells desialylation. Taken together, our results indicate that sialylation of *P. gingivalis* LPS is not an important virulence factor of this prominent periodontal pathogen and that the interaction between CD33 receptors and endogenously expressed sialic acid controls immune cell activation.
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.
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 2012). This cis interaction does not prevent the binding of exogenous ligands in the trans position. Moreover, 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 Huang 2009 fibro). In leukocytes, they regulate inhibition of cellular proliferation and activation, induction of apoptosis and they can also function as endocytic receptors that may be important in the clearance of sialylated antigens (Lock, Zhang et al. 2004). Currently, there are 11 functional CD33-related Siglecs within the human genome (Crocker, Redelinghuys 2008) and all of them have one or more Immunoreceptor Tyrosine-based Inhibition Motifs (ITIM) within the cytoplasmic tail indicating their ability to mediate signalling functions (McMillan, Crocker 2008a). 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). Low membrane expression level of CD33 have been associated with higher levels of inflammatory cytokine production and increased spontaneous secretion of TNF-α, IL-8 and IL-6 by human monocytes (Gonzales, Herrera, Fabian 2012).

**Sialic acid and bacterial pathogens**

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 2005). 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. LPS is an important surface constituent of these bacteria and is involved in complex interactions with the host immune system. Sialylated LPS glycoforms play a key role in pathogenicity of *H. influenzae* and *N. meningitidis* (Bouchet, Hood et al. 2003). 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 2005) while the inflammatory response against *C. jejuni* is in direct correlation with the level of its LPS sialylation (Stephenson, Jonh, Jarvis 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, Philips, Jarvis 2015).

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 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* (Chen Li, Yaping Pan 2012). However, the role of sialylation 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 sialylation 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 proinflammatory potential of this periodontal pathogen’s LPS.
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 1983). Briefly, *P. gingivalis* W83 was grown anaerobically in anaerobic basal broth (Oxoid) containing 5μg/ml of haem for 7 days. Cells were harvested at 7,000 rpm for 15 minutes, lyophilized and resuspended in 15 ml of 10 mM Tris-HCl, pH 8.0, 2 mM MgCl₂. After sonication for 30s, DNase and RNase were added to the final concentrations of 200 μg/ml and 50 μg/ml respectively. Suspension was incubated at 37°C for 2 hours. 5 ml of 0.5 M EDTA (tetra sodium salt)/10 mM Tris, pH 8.0, 2.5 ml of 20% SDS/10 mM Tris, pH 8.0 and 2.5 ml of 10 mM Tris-HCl, pH 8.0 were added, solution was vortexed and centrifuged at 50,000 g for 30 minutes at 20°C to remove peptidoglycans. Supernatant was saved and pronase was added to a final concentration of 200 μg/ml. Solution was incubated at 37°C with constant shaking overnight. Next day two volumes of 0.375 M MgCl₂/95% EtOH were added, mixed and cooled to 0°C in -20°C refrigerator. After the sample had cooled to 0°C, they were centrifuged at 12,000 g for 15 minutes at 0 - 4°C. Pellet was resuspended in 25 ml of 0.1 M EDTA(tetra sodium salt), 2% SDS, 10 mM Tris-HCl, pH 8.0 and sonicated for 30s. The solution was incubated at 85°C for 30 minutes, cooled down to room temperature and pH was brought to 9.5 by addition of 4M NaOH. Pronase was added again to a final concentration of 25 μg/ml and incubated at 37°C overnight with constant shaking. The next day two volumes of 0.375 M MgCl₂/95% EtOH were added, the solution cooled to 0°C as before, centrifuged at 12,000 g for 15 minutes at 0 - 4°C. The pellet was resuspended in 15 ml of 10 mM Tris-HCl, pH 8.0 and sonicated for 30s. Samples were centrifuged at 1000 rpm for 5 minutes to remove insoluble Mg/EDTA complexes. MgCl₂ was added to give a final concentration of 25 mM and suspension was centrifuged at 200,000 g
for two hours. Pellet was resuspended in distilled water and the solution was lyophilized. Dry LPS was measured and stock concentration of 1mg/ml was prepared by addition of endotoxine free water.

*P.gingivalis* W83 ΔPG0083 (ΔW83) defective mutants were a kind gift from Dr Charles Shelbourne, University of Michigan, Ann Arbor, US. This strain is not able to incorporate sialic acid into their LPS.

**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°C.

Human gingival fibroblasts (HGF) were established from explants of healthy gingival tissues obtained during routine clinical procedures. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM-Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml of penicillin, and 100 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.

**Immunoprecipitation**

200 μl of Protein G magnetic beads (Millipore) were pre-associated with 5 μl of phosphotyrosine- 4G10 (Millipore) antibodies on a rotor for 90 min at 4°C. The antibody bound
beads were pelleted at 5000rpm for 1 min and washed in 0.5% TBST. The whole cell lysates were then added to the beads and incubated on rotation at 4°C for 2 hours. Afterwards the beads were pelleted and washed 3 times in 0.5% TBST before being resuspended in 5μl of 5X Western Loading Buffer with 10% β-mercaptoethanol and 15μl of PBS. The samples were denatured at 95°C for 5 min and placed on ice prior to loading onto a gel.

**SDS-PAGE Electrophoresis**

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

**Immunoblotting**

Following protein transfer, nitrocellulose membranes were rinsed in 1X PBS/0.1%Tween 20 and blocked with appropriate blocker (Table 2.1) for 1 hour. Membranes were probed using respective primary antibodies at the relevant dilutions in appropriate blotting solution. Subsequently the membranes were washed six times 5 min each in 1X 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 1X 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. 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.

**Lectin Blot analysis**

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

1μl of samples (1mg/ml LPS), positive and negative control 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°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 5x10⁵ cells/ml and human gingival fibroblast at 8x10⁴ cells/ml in corresponding medium and treated with LPS for 4 hours. Cell free supernatants were removed and stored at -80°C until they were assessed for TNF-α, IL-8 (Peprotech, R&D Systems respectively).
Briefly, a 96 well plate was coated in capture antibody diluted in phosphate buffered saline (PBS) overnight. All steps were carried out at room temperature. The wells were washed three times in PBS/0.1% Polyoxyethylene sorbitan monolaurate (Tween 20) before being blocked for one hour with 1% BSA dissolved in PBS. The washing step was repeated and 100μl of supernatants or standards ranging from 2000 pg/ml to 0 pg/ml were added to the wells and left for 2 hours. Subsequently supernatant was aspirated out, wells were washed 3 times and 100 μl of detection antibody diluted in 1% BSA/PBS was added for 2 hours. Again, the wells were washed three times and Streptavidin-HRP was added at 1 in 2000 dilution in 1% BSA/PBS for 1 hour. At this stage the plate was covered in aluminium foil. Once more, wells were washed and ABTS (Invitrogen) was added for 20 minutes. Absorbance was read on a plate reader 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 2000 rpm for 5 min and resuspended in a 100 μl of PBS supplemented with 2% FCS. Cells were then stained with 20μl of FITC conjugated anti-human CD33 antibody (eBioscience) or respective isotype control for 30 min at room temperature in the dark. Cells were centrifuged again and washed twice with 1 ml PBS and resuspended in 500 ml of fresh PBS. Flow cytometry analysis was carried out on FACS CANTO II. Results were analysed using FlowJo software.

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 $\alpha$-2,6 and $\alpha$-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 water 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 $\alpha$-2,6 and $\alpha$-2,3 linked sialic acids were abundantly present within the W83 LPS. (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- water, 2-human serum, 3-*P. gingivalis* ATCC 33277, 4-*P. gingivalis* W83.

**Inflammatory potential of LPSs**

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

![Graph showing inflammatory potential of LPSs](image)

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

In order to examine the influence of sialic acid LPS moieties on the pro-inflammatory response of innate immune cells, THP-1 cells were treated with *P. gingivalis* W83 LPS, which was shown to be highly sialylated, and LPS isolated by the method of Darveau and Hancock from sialyltransferase gene (PG00083) knock-out *P. gingivalis* W83 strain (ΔW83). The absence of sialic acid in LPS extracted from *P. gingivalis* ΔW83 was confirmed by the lectin-blot analysis, as explained previously, using two types of sialic acid binding lectins and *P. gingivalis* W83 LPS as a positive control (Figure 3).

![Image](image-url)

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

As expected, LPS obtained from sialyltransferase mutated *P. gingivalis* W83 strain did not contain sialic acid. Both types of lectins failed to detect sialic acid moieties within this mutant type of *P. gingivalis* LPS (Sia-free LPS).
Furthermore, THP-1 cells were treated with different concentrations of *P. gingivalis* W83 LPS and Sia-free *P. gingivalis* ∆W83 LPS for 4 hours and the production of IL-8 and TNF-α in cell supernatants was measured (Figures 4 and 5.).

![IL-8 Production](image1)

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

![TNF-α Production](image2)

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

Next, we wanted to check if the CD33 receptor is activated in THP-1 cells challenged with three different types of LPSs (ATCC 33277, W83 and ΔW83). 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-phosphotyrosine antibodies was carried out followed by immunoblotting for CD33 (Figure 6).

![IP pY: IB CD33](image)

**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.

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

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

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

DISCUSSION
Sialic acid residues are highly expressed in eukaryotic cells and act as a marker for self in the immune system (Chen, Brown, Zhen 2014). Pathogenic bacteria also use this molecule for biological mimicry, to coat themselves in sialic acid and increase resistance to components of the host’s innate immune response, or they can use it as nutrient (SEVERI, Thomas 2007). Structural variations of bacterial LPS can have major impact on the host immune response and clinical disease outcome. Studies have suggested that the sialic acid decoration of LPS is an important determinant of _C.jejuni_ caused gastroenteritis and _Neisseria meningitidis_ caused meningitis (John, Wren, Jarvis 2013 and Jarvis, Stein, Din 2015). 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 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 capsular polysaccharides, which are highly sialylated, was present in W83 while it was not found in strain ATCC 33277 (Chen, Hosogi, Abbey 2004).

We compared the inflammatory potential of these two types of _P.gingivalis_ LPS and found 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. 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 (Nina Maeshima and Rachel C. Fernandez* 2013). In our case, highly sialylated W83 LPS triggered significantly lower inflammatory response in THP-1 cells compared to less sialylated ATCC 33277 LPS.
Similarly, sialylation of *Helicobacter bizzozeronii* LPS has been shown to play a crucial role in attenuation of inflammatory host’s response to this prominent animal pathogen (Kondadi, Ravez 2015).

*P. gingivalis* is capable of synthesizing a heterogeneous isoforms of the lipid A molecule, which are subtly different from each other in structure. These subtle changes in the lipid A chemical composition have been shown to greatly influence 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. Since the structure of both the lipid A and the polysaccharide components of LPS can significantly alter TLR activation and subsequent responses, we examined the lipid A chemical composition of the ATCC33277 LPS and W83 LPS by mass-spectrometry (data not shown). The analysis revealed predominant m/z1690 peaks (penta-acylated monophosphorylated isoform) in ATCC 33277 LPS and m/z1195 and m/z 1435 peaks in W83 LPS (tri-acylated monophosphorylated and tetra-acylated monophosphorylated isoforms respectively). As Darveau et al. suggested, the observed differences in the inflammatory potential of these two preparations of *P. gingivalis* LPS are likely to be caused by the modifications of their lipid A core (Darveau, Hajjar 2004).

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, Nakada 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, Chien, 2016). On the contrary, desialylation of dendritic cells significantly improves their phagocytic activity and immunological potency through an actin-independent mechanism (Cabral, Silva, Crespo, 2012). 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 (Gonzales, Herrera, Torres, 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, Ren, Chen 2016).

Endotoxin tolerance is not possible to be induced in human gingival fibroblasts (Zaric 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.

ACKNOWLEDGEMENTS

We are immensely grateful to Dr Charles Shelburne, University of Michigan, School of
Dentistry for sharing his sialic acid free *P. gingivalis* LPS with us.

REFERENCES