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The *Porphyromonas gingivalis* O antigen is required for inhibition of apoptosis in gingival epithelial cells following bacterial infection

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Background and Objective: *Porphyromonas gingivalis* infection induces apoptosis inhibition in gingival epithelial cells; however, it is not fully understood which bacterial effectors are involved in this process. The aim of this study is to evaluate whether the *P. gingivalis* lipopolysaccharide (LPS), specifically the O-antigen region, affects adherence, invasion, viability and apoptosis of gingival epithelial cells.

Material and Methods: Gingival epithelial cells (OKF6/TERT2 line) were infected by different freshly prepared *P. gingivalis* clinical isolates, obtained from subjects with chronic periodontitis (CP3 and CP4) and healthy individuals (H1 and H3). Periodontitis and healthy isolates show differences in O-antigen production, as healthy isolates lack the O-antigen region. In addition, cells were infected by a site-specific mutant lacking the O-antigen portion. After 24 h postinfection, cell proliferation, viability and apoptosis were evaluated by Trypan blue, MTS and annexin V assays, respectively. Bacterial invasion, adhesion and proliferation were measured by gentamicin/metronidazole protection assays. Finally, toll-like receptor (TLR)2 and TLR4 mRNA expression was evaluated by quantitative reverse transcription–polymerase chain reaction. Statistical analysis was performed using ANOVA, Tukey's or Dunnett's tests ($p < 0.05$).

Results: At 24 h postinfection, strains lacking the O-antigen region (healthy isolates and O-antigen ligase-deficient strain) were unable to increase proliferation and viability, or decrease apoptosis as compared with strains producing intact LPS (periodontitis isolates and reference strain). However, the presence of the O-antigen neither contributed to changes in the ability of the bacteria to adhere to or invade cells, nor to intracellular survival. The presence of O-antigen also increased the expression of TLR4 (nearly sixfold), which correlated with inhibition of apoptosis.

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Conclusion: The O-antigen region of *P. gingivalis* LPS is required to increase gingival epithelial cell viability upon infection by bacteria and this increase is attributable to a reduction in apoptosis. Moreover, although bacterial internalization is required, the effects observed are not due to alterations in *P. gingivalis* adherence, invasion or intracellular survival. Interestingly, inhibition of apoptosis correlates with increased TLR4 expression, suggesting a role for TLR4 in this process.

Porphyromonas gingivalis is a gram-negative anaerobic bacteria that plays a key role in the etiology of periodontitis, an infectious disease affecting over 30–50% of the adult population worldwide (1). This disease is characterized by accumulation of bacterial plaque at the gingival margin, which induces an inflammatory response that leads to destruction of connective tissue attachment to teeth, alveolar bone resorption and tooth loss (2). Periodontitis has also been linked to an increased risk in developing atherosclerosis, diabetes and rheumatoid arthritis (1,3,4). Moreover, periodontitis has recently been suggested to increase the risk of development of different types of cancer (5,6).

Host colonization by *P. gingivalis* has been attributed to various processes, such as the formation of a biofilm with other oral species, epithelial cell invasion, intracellular bacterial growth, evasion of the immune response and inhibition of apoptosis in immune and gingival epithelial cells (GECs) (7–16).

In the subgingival compartment, the epithelial cells represent the largest area of contact and the initial sites of bacterial–host invasion. Hence, the interaction between GECs and periodontal bacteria determines whether the colonization process will be successful. *P. gingivalis* internalization requires bacterial virulence factors, such as the FimA fimbrae (17), SerB serine phosphatase and several proteases (18,19). Once internalized, the bacteria are able to survive and replicate in the intracellular milieu, without activation of an acute immune response (20). Upon infection by *P. gingivalis*, the epithelial cells express effector molecules that increase cell survival and decrease apoptosis (21,22). In this respect, it is interesting to note that in immortalized human gingival keratinocytes several genes related to increased cell survival and proliferation are altered upon *P. gingivalis* infection (23). The bacteria were further shown to promote survival of primary GECs by activating the phosphatidylinositol 3-kinase/Akt pathway and preventing cytochrome *c* release (9). In addition, *P. gingivalis* blocks apoptosis in primary GECs through the JAK/Stat pathway, which modulates the intrinsic cell death pathway and regulates the expression of several anti-apoptotic proteins (24,25).

To elicit these cellular responses, direct interaction between the bacterium and host cells is required (26). To understand better how these responses are triggered, components of the bacterial envelope, which mediate initial interactions with host cells and other microorganisms composing the oral biofilm, constitute important molecular targets that merit characterization in more detail. Among these surface virulence factors, the lipopolysaccharide (LPS) is the major constituent of the outer leaflet of the bacterial outer membrane. This complex glycolipid is composed of three covalently linked domains, i.e. lipid A (which is embedded in the outer membrane), central oligosaccharide core and the O antigen (OAg), which is the most external and variable LPS segment (27). The virulent *P. gingivalis* strain W50 synthesizes two different LPS molecules, which only differ in the OAg moiety. O-LPS consists of a repetitive tetrasaccharide unit of OAg $(\text{Glc-Rha-GalNAc-Gal})_x$ that is modified by phosphoethanolamine (28), while anionic LPS consists of a phosphorylated mannan-containing anionic polysaccharide (29).

It has been shown that *P. gingivalis* LPS can induce an inflammatory response in several cell types, such as macrophages (30), fibroblasts (31), endothelial cells (32) and GECs (33), via activation of either toll-like receptor (TLR)2 or TLR4. However, cytokine secretion induced by LPS in GECs is mainly mediated by TLR2. In addition, it has been demonstrated that *P. gingivalis* LPS favors inhibition of apoptosis in monocytes, neutrophils and polymorphonuclear leukocytes (12–14); however, it remains to be determined whether the LPS is involved in inhibition of apoptosis in GECs or whether TLR2/TLR4 participate in this process.

Importantly, specific modifications in the lipid A region of the *P. gingivalis* LPS are known to contribute to the ability of the pathogen to evade and even prevent an immune response (34). In addition, lipid A dephosphorylation at position 4′ decreases the negative charge of the bacterial surface, which favors the development of resistance to cationic antimicrobial peptides, such as polymyxin B (34). Despite the availability of such evidence, little is known about the relevance of other *P. gingivalis* LPS components (such as OAg, core or anionic LPS) to bacterial virulence.

Recent results from our group showed that isolates obtained from individuals suffering from periodontitis, but not those isolated from healthy subjects, possess complete LPS with high molecular mass OAg molecules (35). Interestingly, the presence of a complete OAg is associated with higher resistance to polymyxin B. However, it remains to be determined whether these differences in LPS composition are relevant to other steps of *P. gingivalis* colonization.

With these aspects in mind, the aim of our current study was to evaluate the role of *P. gingivalis* LPS, and specifically the OAg region, in adherence, invasion, viability and inhibition of apoptosis in GECs. Using freshly prepared clinical isolates and a site-specific mutant lacking the OAg portion, we found that the presence of the LPS OAg is necessary to increase viability and decrease apoptosis in GECs infected by *P. gingivalis*. However, this effect was neither due to changes in the bacterial ability to adhere to or invade cells, nor to favor intracellular survival. Rather, our observations indicate that the OAg presence increases the expression of TLR4, which correlates with inhibition of apoptosis, suggesting that signaling via this receptor may be involved in enhancing the viability of infected epithelial cells.

Material and methods

Bacterial strains and culture conditions

P. gingivalis reference strains ATCC 33277 and W50 (ATCC 53978), and clinical isolates from both patients with chronic periodontitis (CP3 and CP4) and healthy individuals (H1 and H3) (35), were grown anaerobically at 37°C in enriched brain–heart infusion liquid medium supplemented with hemin 1% (Calbiochem, San Diego, CA, USA) and menadione 1% (Sigma-Aldrich, St. Louis, MO, USA), or on blood agar plates supplemented with hemin 1% and menadione 1%. CP3, CP4, H1 and H3 isolates were chosen based on clinical parameters, such as probing depth and clinical attachment level exhibited by the corresponding individuals from whom the samples were obtained. CP3 and CP4 were the clinical isolates with the highest probing depth and clinical attachment level (CP3 patient: probing depth = 4.2 ± 0.7 mm and clinical attachment level = 4.8 ± 0.5 mm; CP4 patient: probing depth = 4.2 ± 0.7 mm and clinical attachment level = 4.6 ± 0.9 mm). Similarly, healthy isolates H1 and H3 were chosen because the individuals from whom they were isolated exhibit the lowest values for probing depth and clinical attachment level (H1 subject: probing depth = 2.5 mm ± 0.5 and clinical attachment level = 1.5 mm ± 0.2; H3 subject: probing depth = 2.5 mm ± 0.5 and clinical attachment level = 1.3 ± 0.3 mm).

ΔPG1051 and *CΔPG1051* strains were grown in the presence of erythromycin (5 μg/mL). The *ΔPG1051* strain corresponds to an isogenic mutant in the *PG1051* gene, which codes for the OAg ligase (WaaL) enzyme that catalyzes the final step in LPS assembly (36). WaaL transfers preformed OAg from an undecaprenyl lipid carrier to the lipid A-core moiety in the periplasmic face of the cytoplasmic membrane. In the complemented *CΔPG1051* strain, the *PG1051* gene was reincorporated in *cis* to allow the bacteria to produce a complete LPS molecule (Fig. S1).

Cell lines and culture conditions

The immortalized human oral keratinocyte cell line OKF6/TERT2 was kindly provided by Dr. Anna Dongari-Bagtzoglou, University of Connecticut Health Center, USA (37). OKF6/TERT2 cells were incubated in keratinocyte serum-free medium (Gibco, Carlsbad, CA, USA) supplemented with bovine pituitary (Gibco), epidermal growth factor (Gibco), calcium chloride solution 0.3 M (Merck, Darmstadt, Germany) and penicillin/streptomycin (Biological Industries, Kibbutz Beit-Haemek, Israel). Cells were incubated at 37°C in 5% CO_2 atmosphere.

MTS viability assay

OKF6/TERT2 monolayers were prepared by seeding 25,000 cells in each well of a 96-multiwell plate and incubating for 24 h at 37°C in 5% CO_2. Bacterial cultures were grown anaerobically to an optical density at 490 nm of 0.7, washed once with phosphate-buffered saline (PBS), suspended in PBS and added to OKF6/TERT2 cells at a multiplicity of infection (MOI) of approximately 100. After infection for 90 min, cell monolayers were washed and incubated with fresh media supplemented with gentamicin (300 μg/mL) and metronidazole (200 μg/mL) for an additional 2 or 24 h. Then, the viability of OKF6/TERT2 cells was measured using the CellTiter 96 cytotoxicity assay (Promega, Madison, WI, USA). The percentage of viability was calculated as follows: (OD_{560} of infected cells/OD_{560} of control cells) × 100.

Trypan blue exclusion assay

Cell proliferation was measured by Trypan blue dye exclusion after *P. gingivalis* invasion. Briefly, 80,000 cells/well were plated in 48-well plates and infected with *P. gingivalis* as described above. After incubation with fresh media supplemented with gentamicin (300 μg/mL) and metronidazole (200 μg/mL) for 2 or 24 h, cells were washed three times with PBS and detached by trypsin–EDTA (Hyclone, Logan, UT, USA) incubation for 3 min at 37°C. Then, cells were suspended in Trypan blue solution and viable cells were counted in a Neubauer chamber using an inverted microscope (Axiovert 40 CFL; Zeiss, Oberkochen, Germany). The percentage of proliferation was calculated as follows: (number of live infected cells/number of live non-infected cells) × 100.

Apoptosis assay

Apoptosis was evaluated by the annexin V cell surface labeling apoptosis assay. Cell monolayers were formed by plating 100,000 cells in each well of a 24-multiwell plate (BD Falcon, Franklin Lakes, NJ, USA). Subsequently, the cells were infected with *P. gingivalis* as described above. Then, 2 or 24 h postinfection, the number of apoptotic cells was determined by flow cytometry (BD FACSCanto Instrument, San Jose, CA, USA) using the annexin V–phycoerythrin Apoptosis Detection Kit (BD Pharmingen, San Jose, CA, USA).

Adhesion and invasion assays

OKF6/TERT2 monolayers were prepared by seeding 20,000 cells in each well of a 96-multiwell plate. Adhesion assays were performed by allowing bac-

teria to adhere at 4°C for 90 min. After that, the cells were rinsed with cold PBS and lysed by incubation with 100 μL of saponin (1% w/v in distilled water) for 10 min. Adequate dilutions were inoculated on blood agar plates supplemented with hemin 1% and menadione 1%, and colony forming units (CFU) were counted after 5 d incubation in anaerobiosis. Adhesion was calculated as follows: number of CFU in infected cells/number of CFU in control cells. Invasion was performed as described above, except that cells were infected at 37°C. Invasion was calculated as follows: 100 × (number of viable CFU in infected cells/number of CFU of initial bacteria inoculum).

Fluorescence invasion assays

OKF6/TERT2 monolayers were prepared by seeding 20,000 cells in each well of a 96-multiwell plate containing a circular coverslip. Bacterial cells used for the infection were stained previously with carboxyfluorescein succinimidyl ester by incubating for 30 min at room temperature. After 90 min, the coverslip was washed with PBS, treated 30 min with 1% rhodamine phalloidin, then incubated with 4% paraformaldehyde and finally fixed with Mowiol 4-88. Resulting coverslips were observed by confocal microscopy (LSM700; Zeiss).

Intracellular survival assay

OKF6/TERT2 cell monolayers were prepared by seeding 20,000 cells in each well of a 96-multiwell plate. After infection for 90 min, extracellular adherent bacteria were killed by incubating the monolayers with gentamicin (300 μg/mL) and metronidazole (200 μg/mL) for 2 h. Then, OKF6/TERT2 cells were lysed to release intracellular bacteria by addition of saponin (1%). Serial dilutions of the lysates were plated on blood agar plates supplemented with hemin 1% and menadione 1% and cultured anaerobically at 37°C for 5 d. CFU were counted at the appropriate dilutions. 100 × (number of viable CFU in infected cells/number of CFU of initial bacteria inoculum).

Gene expression analysis by quantitative reverse transcription–polymerase chain reaction

Total cytoplasmic RNA was isolated from stimulated GECs using the Igepal® lysis buffer method as described previously (38). Reverse transcription was performed using a First-Strand cDNA Synthesis SuperMix kit according to the manufacturer's instructions (SuperScrip™ III; Invitrogen, Grand Island, NY, USA). To quantify the mRNA expression for receptors TLR2 and TLR4, 50 ng of cDNA were amplified by quantitative real-time polymerase chain reaction (PCR) using appropriate primers (TLR2F: CTC TCGGTGTCGGAATGTC; TLR2R: AGGATCAGCAGGAACAGAGC; TLR4F CCCTCCCCTGTACCTTCT; TLR4R: TCCCTGCCTTGAATACC TTC) and the KAPA™ SYBR® Fast quantitative PCR reagent (KAPA Biosystems, Woburn, MA, USA) in a StepOnePlus® real-time PCR system (Applied Biosystems, Grand Island, NY, USA), as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Finally, a melting curve was obtained by incubating at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s, to detect non-specific product formation and false positive amplification. As an endogenous control, 18S rRNA expression levels were determined (39).

Data analysis and statistical considerations

The quantitative reverse transcription-PCR data were analyzed using the STEPONE Software v2.2.2 (Applied Biosystems) and the relative quantification was obtained by normalizing the TLR mRNA expression to 18S rRNA expression using the $2^{-\Delta\Delta C_t}$ method. Data were statistically analyzed using PRISM software (version 6.0; GraphPad, La Jolla, CA, USA). The normality of data distribution was determined using the Kolmogorov–Smirnov test. Results were compared pairwise by applying the ANOVA test. Tukey's or Dunnett's multiple comparison *post-hoc* tests were used to analyze statistical differences. All groups were from three or more independent experiments. Differences with $p < 0.05$ were considered statistically significant.

Results

Clinical isolates of *P. gingivalis* differentially modulate cell viability and apoptosis

In a previous study, we noted that a major difference between isolates from healthy and periodontitis subjects was that in isolates obtained from chronic periodontitis individuals (CP3, CP4) a complete LPS was detected and that this phenotype correlated with increased resistance to cationic peptides. The isolates from healthy subjects (H1, H3) in contrast, lacked the OAg region and were more susceptible to these molecules (35). In the current study, using the same isolates obtained from healthy and periodontitis subjects, we determine whether *P. gingivalis* OAg participates in the modulation of viability and proliferation of GECs.

Proliferation and viability of GECs infected by clinical isolates were evaluated by Trypan blue exclusion and MTS assays, respectively. Proliferation was measured 2 or 24 h postinfection and non-infected cells were considered as controls. After 2 h, no differences in proliferation were detected for GECs infected with either the two chronic periodontitis isolates (CP3 and CP4) or healthy isolates (H1 and H3), as compared to the non-infected cells (arbitrarily assigned a value of 100%). However, after 24 h, chronic periodontitis isolates (CP3 and CP4) increased GEC proliferation over twofold (324% and 362%, respectively) compared to the non-infected cells (148%). In contrast, GEC cells infected with the healthy isolates H1 and H3 did not show significant differences in proliferation at any time point postinfection when compared with non-infected cells (Fig. 1A).

Additionally, viability was evaluated by the MTS assay at early (2 h) or late (24 h) time points postinfection and the percentage of viability was calculated considering non-infected cells as controls (100%). No differences in viability

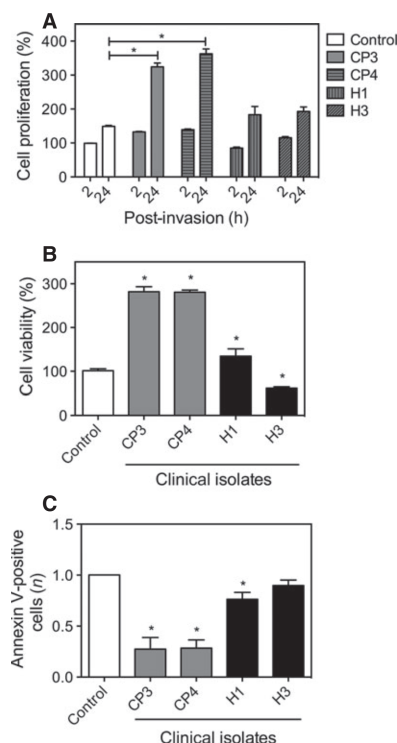

Fig. 1. (A) The percentage of proliferation of OKF6/TERT2 cells after 2 and 24 h post-invasion by *P. gingivalis* isolates was measured using Trypan blue exclusion assays. (B) The percentage of viability of OKF6/TERT2 cells after 24 h post-invasion by *P. gingivalis* isolates was measured using MTS assays. Viability was compared in each case to the viability of non-treated cells after the same periods of incubation (100%). (C) The dead cell populations (annexin V-positive/propidium iodide-negative) after 24 h infection by *P. gingivalis* isolates were compared to the values obtained for the control cells. CP3 and CP4 are isolates from subjects with chronic periodontitis and H1 and H3 from healthy individuals. The controls were non-treated cells. Values that differ significantly from controls are indicated (means ± SEM; $n = 3$; $*p \leq 0.001$).

were observed in the infected cells when compared to the non-infected cells after 2 h (not shown). However, the viability of GECs 24 h postinfection with CP3 and CP4 isolates increased nearly threefold as compared to the control (282% and 280%, respectively) (Fig. 1B). Interestingly, the isolate H1 also increased cell viability, but to a lesser extent (134%). By contrast, the isolate H3 also obtained from a healthy individual, significantly decreased cell viability by over a third as compared with non-infected cells (Fig. 1B).

To evaluate whether the changes in viability and proliferation induced by the clinical isolates were due to differential modulation of apoptosis, cell death was quantified postinfection by measuring phosphatidylserine exposure using Alexa Fluor 488-conjugated annexin V. Flow cytometry results revealed that infection by CP3 and CP4 isolates decreased apoptosis levels to 27% and 28% of the basal apoptosis levels detected in non-infected cells (1) (Fig. 1C). H1 also decreased apoptosis, but to a lesser extent than CP3 and CP4 isolates (76% of the values for non-infected cells). H3 infection did not yield statistically significant differences when compared to non-infected cells (Fig. 1C).

The O antigen of *P. gingivalis* lipopolysaccharide participates in increasing cell viability and proliferation in infected gingival epithelial cells

To confirm that the LPS OAg may contribute to the alterations observed, we used an isogenic mutant of W50 strain in the *PG1051* gene, which completely lacks the OAg moiety. In addition, a complemented strain *CΔPG1051* was used (Fig. S1).

Trypan blue assays revealed that no significant differences in viability/proliferation were observed in cells 2 h postinfection by W50, the *ΔPG1051* mutant or the *CΔPG1051* strain. In contrast, 24 h postinfection with either W50 or *CΔPG1051* strains, cell proliferation significantly increased (220% and 192%, respectively) when compared to non-infected cells. However, in GECs infected by *ΔPG1051* proliferation increased to a similar extent as observed for non-infected cells (Fig. 2A).

Likewise, no changes in viability were observed 2 h postinfection (not shown). However, 24 h postinfection, the parental strain W50 increased viability of infected GECs by over 20% compared to the non-infected cells (Fig. 2B). Importantly, this effect was lost upon infection of cells with the mutant *ΔPG1051*, but recovered when GECs were infected with the complemented C*ΔPG1051* strain.

The O antigen of *P. gingivalis* lipopolysaccharide participates in reducing apoptosis in infected gingival epithelial cells

To evaluate whether the observed changes in viability and proliferation result from modulation of apoptosis by the OAg region, annexin V staining was evaluated 24 h postinfection. Flow cytometry revealed that infection by the parental strain W50 decreased apoptosis by approximately 50% when compared to the non-infected cells. As expected, infection with the *ΔPG1051* mutant strain resulted in apoptosis levels similar to the basal levels observed in non-infected cells. In contrast, the complemented strain *CΔPG1051* behaved in a manner similar to the parental strain W50 (Fig. 2C).

Purified lipopolysaccharide and heat-inactivated *P. gingivalis* do not affect the viability of infected gingival epithelial cells

Previous studies indicated that incubating GECs with purified LPS is sufficient to induce the production of cytokines (33). In addition, the treat-

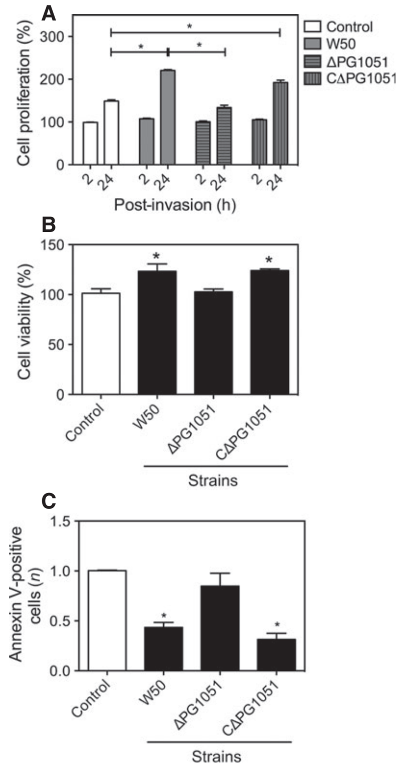

Fig. 2. The proliferation (A) and viability (B) of OKF6/TERT2 cells after 24 h infection by *P. gingivalis* O-antigen mutant (*ΔPG1051*) and complemented strain (*CΔPG1051*) was measured using the Trypan blue exclusion or MTS assays, respectively. Viability and proliferation were compared in each case to that of non-treated cells (100%). (C) Apoptotic cells (annexin V-positive/propidium iodide-negative) after 24 h infection by *P. gingivalis* were measured and compared to the values obtained for the control cells. Values that differ significantly from controls are indicated (means ± SEM; $n = 3$; $*p \leq 0.001$).

ment of monocytes and neutrophils with purified LPS was shown sufficient to inhibit apoptosis (13,14). To determine whether the LPS molecule is sufficient to increase the viability of GECs, we incubated the cells with different amounts of LPS purified from the W50, *ΔPG1051* mutant or C*ΔPG1051* strains (5, 10, 25, 50, 100, 200 and 500 μg/mL). Our results indicate that no changes were observed in GEC viability either at low or high concentrations of LPS (Fig. S2A). Similar results were observed when GECs were incubated with heat-inactivated W50, *ΔPG1051* mutant or C*ΔPG1051* strains (Fig. S2B).

The absence of O antigen does not affect either bacterial adhesion or invasion of gingival epithelial cells

To evaluate whether the modulation of GEC apoptosis was due to possible differences in bacterial internalization between the parental strain and the mutant, we assayed the ability of W50, *ΔPG1051* and *CΔPG1051* to adhere to and invade GECs. Our results revealed no changes in the number of viable adherent or intracellular bacteria when GECs were infected with the reference strain, the *ΔPG1051* mutant or the complemented *CΔPG105* strain (Fig. 3A,B).

In addition, visualization by confocal microscopy of intracellular *P. gingivalis* (stained with carboxyfluorescein succinimidyl ester) in GECs revealed the presence of similar levels of intracellular bacteria for *ΔPG1051*, C*ΔPG1051* and the parental strain (Fig. 3C).

The absence of O antigen does not affect bacterial intracellular survival

We also evaluated whether the absence of the OAg portion of the LPS affected bacterial survival once they were internalized. Quantification of viable intracellular bacteria recovered 2, 8, 16 and 24 h postinfection, revealed similar numbers of CFU when GECs where infected by the wild-type, *ΔPG1051* or C*ΔPG1051* strains (Fig. 4). Eight hours after infection, 25% of the initial intracellular bacteria remain alive. After 16 h, a small number of viable bacteria were still detected; however, 24 h after infection no viable intracellular bacteria were observed, independent of the bacterial strain employed.

O antigen-mediated inhibition of apoptosis correlates with increased toll-like receptor 4 expression in infected gingival epithelial cells

To evaluate if the effect of *P. gingivalis* OAg could be mediated by TLRs, we analyzed the expression of TLR4 and TLR2. Interestingly, our results showed that only the strains that possess a complete OAg molecule (W50 and C*ΔPG1051*) induced a nearly sixfold increase in TLR4 mRNA expression. For the cells infected with the *ΔPG1051* mutant, TLR4 mRNA expression levels were similar to those detected in non-infected GECs (Fig. 5A). These results suggest that OAg-mediated phenotypes may be due to alterations in TLR4 expression in GECs. In contrast, the strain W50 and the mutant *ΔPG1051* strains both increased the expression of TLR2 more than threefold (Fig. 5B), indicating that the OAg-associated phenotype is not likely linked to changes in TLR2.

Discussion

Epithelial cells represent the largest area of contact and the initial sites of host invasion by bacteria present in the subgingival compartment. Hence, the interaction between GECs and periodontal bacteria determines whether the colonization process is successful. On the other hand, LPS is the major constituent of the outer leaflet of the bacterial outer membrane and the first line of interaction with host cells. For these reasons, it

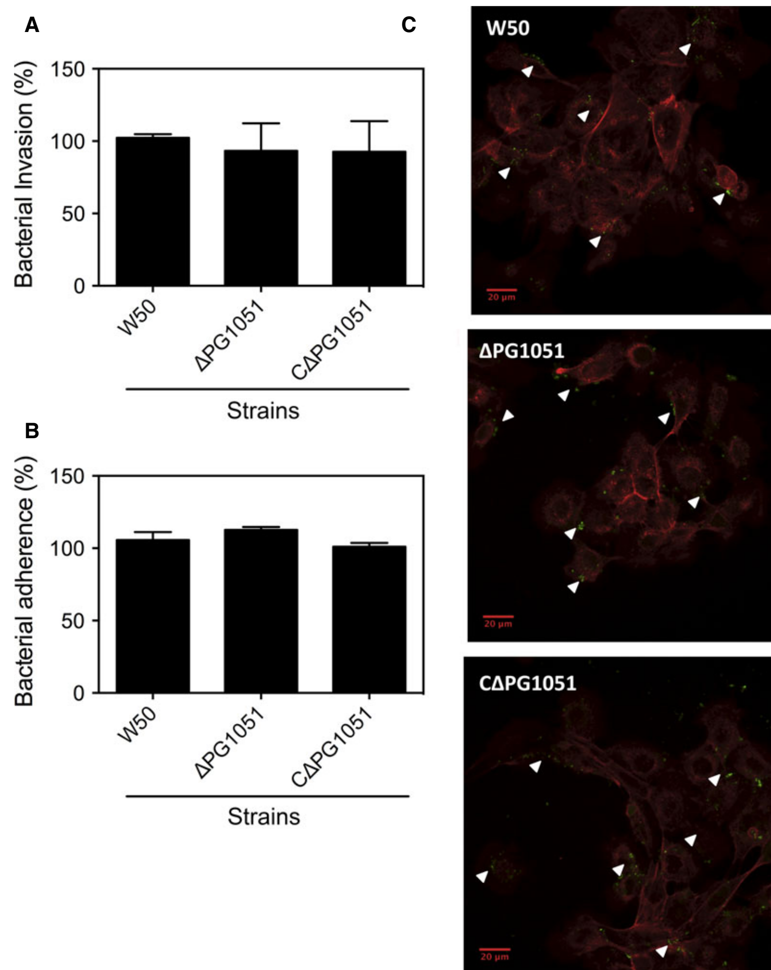

Fig. 3. (A) Invasion percentage of, or (B) adherence percentage to OKF6/TERT2 cells infected either with *P. gingivalis* wild-type or the mutant strain lacking O antigen. Invasion or adhesion were calculated as: (the number of viable CFU in infected cells/the number of CFU of initial bacteria inoculum) $\times$ 100. Values represent invasion or adherence of each strain relative to invasion or adherence of the wild-type strain (W50). Invasion and adherence assays were performed in triplicate on at least three independent occasions. Averages $\pm$ standard errors (error bars) are shown. (C) Confocal microscopy of OKF6/TERT2 cells 2 h postinfection by *P. gingivalis, P. gingivalis* O-antigen mutant (*ΔPG1051*) or the complemented strain (*CΔPG1051*). White arrowheads show the intracellular carboxyfluorescein succinimidyl ester-stained bacteria (green) and gingival epithelial cells were stained with phalloidin (red).

is important to identify factors and mechanisms involved in the interaction between bacterial LPS and epithelial cells, because these will determine the success rate of bacterial colonization.

In a recent study by our group, we compared *P. gingivalis* isolates obtained from patients with chronic periodontitis and from healthy individuals in terms of macro morphological characteristics and the presence of certain virulence factors. Important differences in the LPS profile of bacterial isolates were detected, whereby isolates from healthy subjects were shown to produce the LPS lacking the OAg polymeric region (35), raising the possibility that the OAg may contribute significantly to *P. gingivalis* virulence. Because the clinical isolates H1, H3, CP3 and CP4 were not genetically characterized in the previous study, we sought here to discard the possibility that differences observed in GEC proliferation and viability following infection with clinical isolates were due to other virulence factors. To this end, we used an isogenic mutant of the W50 strain with a deletion in the *PG1051* gene (*ΔPG1051*) and the corresponding complemented strain in which the gene was reconstituted (*CΔPG1051*).

In the present study, we demonstrate specifically that the presence of the OAg region of the LPS is essential for *P. gingivalis* to increase viability and proliferation, as well as inhibit apoptosis of infected GECs. These results are in agreement with previous reports showing that *P. gingivalis* LPS decreases apoptosis in polymorphonuclear leukocytes (12), monocytes (14) and neutrophils via a TLR2-mediated response (13) involving IL-10 modulation via the phosphatidylinositol 3-kinase pathway (40). Here it should be noted that these studies did not evaluate the importance of the OAg in this context. Importantly, our analysis of

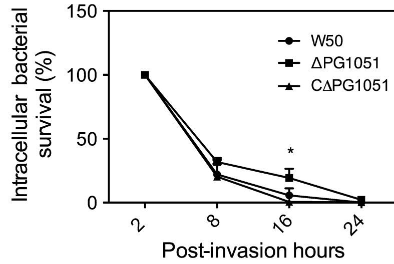

Fig. 4. Survival percentage of intracellular *P. gingivalis* W50, *P. gingivalis* O-antigen mutant (*ΔPG1051*) or the complemented strain (*CΔPG1051*) 2, 8, 16 and 24 h postinfection. Values were calculated as: (the number of viable CFU in infected cells/the number of CFU of initial bacteria inoculum) × 100. The CFU recovered after 8, 16 and 24 h were normalized with respect to the CFU recovered 2 h after invasion (100%). Assays were performed in OKF6/TERT2 cells, in triplicate on at least three independent occasions. Values that differ significantly from those for non-treated controls are indicated (means ± SEM; $n = 3$; $*p \leq 0.001$).

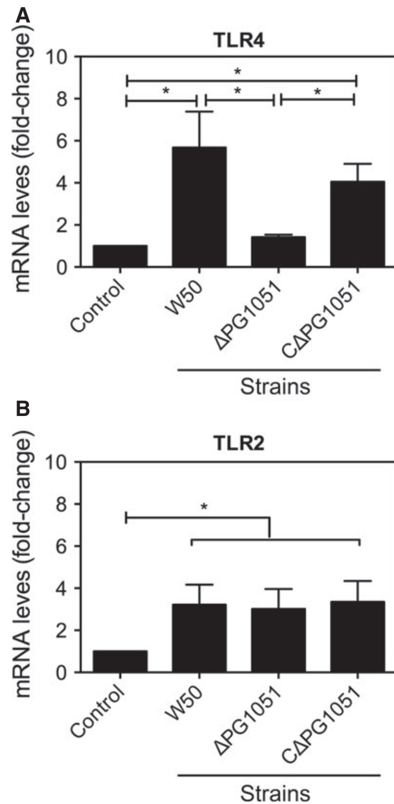

Fig. 5. TLR2 and TLR4 mRNA expression in gingival epithelial cells infected by *P. gingivalis* W50, *P. gingivalis* O-antigen mutant (*ΔPG1051*) or the complemented strain (*CΔPG1051*) 24 h postinfection. Data are represented as fold-change with respect to non-infected cells (control), which was arbitrarily assigned the value of 1. Data were obtained from four independent experiments, performed in duplicate. Values that differ significantly from controls are indicated (means ± SEM; $*p \leq 0.001$). TLR, toll-like receptor.

TLR expression in response to bacterial infection would suggest that TLR4, rather than TLR2, is critical for the responses in GECs given that we only observed differences in the expression of this receptor following infection by wild-type and mutant bacterial strains.

Interestingly, in a recent report, LPS was shown to be released by gram-negative bacteria into the cytosol of host cells where it binds directly to caspases and induces caspase-4/caspase-5/caspase-11 oligomerization and activation, suggesting a non-canonical mechanism of proinflammatory caspase activation by LPS (39). The recognition of intracellular LPS by caspases triggers bacterial escape from the vacuole through lysis of the bacteria containing vacuole. Our results suggest that the apoptosis inhibition mediated by OAg requires that the bacteria must be associated with or internalized by the epithelial cells, because incubation of GECs with different concentrations of purified LPS (5–500 μg/mL) or heat-inactivated bacteria did not have any effects on cell viability. These observations are in agreement with a previous study by Kusumoto *et al.* (41) showing that the stimulation with *P. gingivalis* LPS neither activated human GECs nor induced cytokine production or NF-κB activation.

The mechanism we propose explains how *P. gingivalis* CP3 and CP4 isolates can promote cell proliferation and links these observations to the formation of a complex between gingipains and the OAg moiety of the LPS. It has been previously reported that the OAg region of *P. gingivalis* interacts with an arginine gingipain (Rgp), a cysteine protease that plays an important role in bacterial infection and evasion from host defense mechanisms (36). In addition, it has also been shown that *P. gingivalis* LPS interacts with arginine and lysine gingipains (Rgp and Kgp) modifying specific domains that are essential for bacterial protease activity and the ability to promote cell survival (42,43). Specifically, it has been demonstrated that Hgp44, a peptide generated by proteolytic RgpA processing, is internalized by epithelial cells and induces a protective effect in these cells by increasing the expression of anti-apoptotic proteins and inhibiting apoptosis by a mitochondria-mediated mechanism (44). In the absence of OAg molecules (as is the case for H1 and H3 isolates), gingipains presumably do not bind LPS and are not processed, explaining why

purified LPS, heat-inactivated bacteria or the mutant lacking OAg have no effect on cellular survival.

Additionally, because the ability of the OAg mutant to invade or adhere to GECs was not affected, our results suggest that OAg-mediated apoptosis inhibition is not associated with changes in bacterial adherence or invasion. These results are in agreement with previous studies reporting that in other gram-negative intracellular bacteria, the OAg region is not required for adhesion to or invasion of epithelial cells, unlike the core region, which is essential for these processes (45,46). It remains to be determined whether other regions of *P. gingivalis* LPS, such as the core or lipid A, are involved in *P. gingivalis* adhesion or invasion.

Previously, it has been demonstrated that LPS stimulates the secretion of anti-inflammatory cytokines in OKF6/TERT2 GECs by increasing TLR2 mRNA expression (33), although in other studies it is shown that chemokine production by gingival epithelia infected with *P. gingivalis* is mediated by TLR2 in an LPS-independent manner (41). In our study, we observed that GECs infected by *P. gingivalis* W50 increased TLR2 mRNA levels, but did so to the same extent as the *ΔPG1051* mutant and the complemented strains, indicating that apoptosis inhibition mediated by LPS was not likely due to increases in TLR2 expression. This is not surprising given that the lipid-A portion of *P. gingivalis* LPS is a ligand for TLR2 (30) and this region was not altered in the *ΔPG1051* mutant.

On the other hand, it was previously reported that TLR4 is involved in cytokine secretion mediated by *P. gingivalis* (33) and that the variability in cytokine secretion and chemokine expression in dendritic cells stimulated with different serotypes of *P. gingivalis* is TLR4 dependent (38); however, it remains unknown whether TLR4 participates in modulation of GEC apoptosis. Interestingly, in this study, we observed an increase in TLR4 expression in GECs infected by the W50 strain, but not when cells were infected by the OAg mutant strain. These observations suggest for the first time a possible role for TLR4 in the inhibition of GEC apoptosis and that this mechanism is triggered by the OAg region. In agreement with these results, TLR4 activation by LPS was recently shown to enhance survival in prostate cancer cells (47), suggesting that LPS-mediated TLR4 activation is important for cellular survival responses following infection.

Here it is important to mention that the concept of apoptosis modulation in GECs is still controversial. Whereas some studies indicated that apoptosis is induced in GECs after infection by *P. gingivalis* (48,49), others showed that apoptosis is inhibited (9,21,24). The variability of these studies could be explained in part by the use of different MOIs. For instance, Pan *et al.* (50) showed that for MOIs of 50 or 100 the bacteria inhibits apoptosis; however, at higher MOIs (200 or 500) apoptosis is increased. In addition, for the infection protocols used in these studies several important variations were apparent. Some protocols indicated that antibiotics were added after infection to eliminate extracellular bacteria that were not internalized; however, other procedures indicated that extracellular bacteria were maintained over the entire period (24, 48 h or even more). This last procedure is likely to permit repetitive infection cycles, due to increases in the MOI following bacterial proliferation. Such reinfection events are likely to affect the expression of cytokines and other signaling molecules inside the cell (51). It is also important to note that most of the studies use reference strains of *P. gingivalis*. In our study, we observed that the virulent reference strain W50 inhibited apoptosis in GECs, and the effect was greater when GECs where infected by clinical isolates obtained from chronic periodontitis individuals. In contrast, isolates from healthy subjects did not inhibit apoptosis to the same extent as chronic periodontitis strains. Moreover, for one of the healthy isolates, no changes in apoptosis were detected. These strain-specific differences in behavior are currently being studied in our laboratory, using sequencing approaches and by characterizing lipid A.

In addition, for some studies showing apoptosis inhibition by *P. gingivalis* infection, primary culture GECs were employed (9,24). This may be considered a more physiological model than the use of an immortalized cell line (such as OKF6/TERT2). However, as Silva *et al.* (52) demonstrated, substantial differences in the cytokine profile produced by gingival tissue samples are detected for the different subjects, even when the same inclusion and exclusion criteria are employed. These results tend to suggest that results obtained with primary cultures are subject to considerable variability between samples. Alternatively, with an immortalized cell line more reproducible results are obtained because of the more homogeneous nature of the cells under study.

In summary, we demonstrate here that the OAg region of *P. gingivalis* LPS is essential for the increase in GEC viability induced by the bacteria and that this increase is attributable to a reduction in apoptosis. Moreover, although bacterial internalization is required, our results reveal that the effects observed are not due to alterations in *P. gingivalis* adherence and invasion, or intracellular survival of the bacteria. In addition, apoptosis inhibition by wild-type and OAg complemented mutant strains correlates with increased TLR4 expression, suggesting that TLR4 may be important for the observed differential responses in GECs.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 LPS electrophoretic profiles of *P. gingivalis* W50 wild-type, O-antigen ligase mutant *ΔPG1051* and complemented strain *CΔPG1051*. The LPS was obtained in stationary phase and each row was standardized to an OD_{600} = 2.0. The LPS was analyzed by SDS–tricine–polyacrylamide electrophoresis followed by silver staining.

Figure S2 The percentage of viability of OKF6/TERT2 cells after 24 h of infection with: (A) *P. gingivalis* LPS or (B) heat inactivated (HI), *P. gingivalis ΔPG1051*, or the complemented strain (*CΔPG1051*). Viability was quantified using the MTS assay. Controls correspond to non-treated cells (100%).

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