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Abstract

Primary gingival epithelial cells were cultured in multilayers as a model for the study of interactions with oral bacteria associated with health and periodontal disease. Multilayers maintained at an air-liquid interface in low calcium medium displayed differentiation and cytokeratin properties characteristic of junctional epithelium. Multilayers were infected with fluorescently labeled *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* or *Streptococcus gordonii*, and bacterial association was determined by confocal microscopy and quantitative image analysis. *P. gingivalis* invaded intracellularly and spread cell to cell. *A. actinomycetemcomitans* and *F. nucleatum* remained extracellular and showed intercellular movement through the multilayer. *S. gordonii* remained extracellular and predominantly associated with the superficial cell layer. None of the bacterial species disrupted barrier function as measured by transepithelial electrical resistance. *P. gingivalis* did not elicit secretion of proinflammatory cytokines. However, *A. actinomycetemcomitans* and *S. gordonii* induced IL-1 β , TNF- α , IL-6 and IL-8 secretion; and *F. nucleatum* stimulated production of IL-1 β and TNF- α . *A. actinomycetemcomitans*, *F. nucleatum* and *S. gordonii*, but not *P. gingivalis*, increased levels of apoptosis after 24 h infection. The results indicate that the organisms with pathogenic potential were able to traverse the epithelium, while the commensal bacteria did not. In addition, distinct host responses characterized the interaction between the junctional epithelium and oral bacteria.

Keywords

oral pathogens, oral commensals, virulence, periodontal disease

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Interaction of oral bacteria with gingival epithelial cell multilayers

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Abstract

Primary gingival epithelial cells were cultured in multilayers as a model for the study of interactions with oral bacteria associated with health and periodontal disease. Multilayers maintained at an air-liquid interface in low calcium medium displayed differentiation and cytokeratin properties characteristic of junctional epithelium. Multilayers were infected with fluorescently labeled *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* or *Streptococcus gordonii*, and bacterial association was determined by confocal microscopy and quantitative image analysis. *P. gingivalis* invaded intracellularly and spread cell to cell. *A. actinomycetemcomitans* and *F. nucleatum* remained extracellular and showed intercellular movement through the multilayer. *S. gordonii* remained extracellular and predominantly associated with the superficial cell layer. None of the bacterial species disrupted barrier function as measured by transepithelial electrical resistance. *P. gingivalis* did not elicit secretion of proinflammatory cytokines. However, *A. actinomycetemcomitans* and *S. gordonii* induced IL-1 β , TNF- α , IL-6 and IL-8 secretion; and *F. nucleatum* stimulated production of IL-1 β and TNF- α . *A. actinomycetemcomitans*, *F. nucleatum* and *S. gordonii*, but not *P. gingivalis*, increased levels of apoptosis after 24 h infection. The results indicate that the organisms with pathogenic potential were able to traverse the epithelium, while the commensal bacteria did not. In addition, distinct host responses characterized the interaction between the junctional epithelium and oral bacteria.

Keywords

oral pathogens; oral commensals; virulence; periodontal disease

Introduction

The human oral cavity is a complex ecosystem that contains a diverse assemblage of microorganisms with differing pathogenic potential. Streptococci such as *S. gordonii* are early colonizers of the dental plaque biofilm and generally are commensals in the oral cavity, although capable of causing disease at systemic sites such as on defective heart valves (Nobbs *et al.*, 2009). A predominant anaerobic species in the subgingival biofilm is

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F. nucleatum that is prevalent in mature plaque in both health and disease (Dzink *et al.*, 1988, Moore *et al.*, 1994, Tanner *et al.*, 1989), and thus is considered an opportunistic pathogen. The presence of *S. gordonii* and *F. nucleatum* favors subsequent colonization by more pathogenic organisms, such as *P. gingivalis*, which play a role in the initiation and progression of chronic periodontitis (Lamont *et al.*, 1998, Kuboniwa *et al.*, 2010). Another pathogen is *A. actinomycetemcomitans*, a causal agent of the clinically distinct localized aggressive periodontitis (LAP) (Kachlany, 2010). Nevertheless, many recognized pathogens are frequently present in healthy individuals, and disease ensues when there is a disruption of the normally balanced host-microbe interaction (Marsh, 2003, Handfield *et al.*, 2008).

The epithelial cells that line the gingival compartment are amongst the first host cells encountered by subgingival bacteria. In addition to providing a mechanical barrier to microbial intrusion, gingival epithelial cells also produce effectors of innate immunity, such as cytokines, and act as sensors of infection by signaling to immune cells in the underlying periodontal tissues (Tribble *et al.*, 2010, Kagnoff *et al.*, 1997). The interactions between bacteria and gingival epithelial cells have been studied extensively using epithelial cell monolayers maintained in culture. *P. gingivalis* is highly invasive yet demonstrates stealth-like properties in primary cultures of gingival epithelial cells, suppressing production of cytokines such as IL-8 and preventing epithelial cell apoptosis (Lamont *et al.*, 1995, Mao *et al.*, 2007, Tribble *et al.*, 2010, Darveau *et al.*, 1998). *A. actinomycetemcomitans*, in contrast, invades poorly but stimulates expression of cytokines and induces apoptotic cell death (Handfield *et al.*, 2005, Stathopoulou *et al.*, 2010, Guentsch *et al.*, Huang *et al.*, 1998). *F. nucleatum* can invade epithelial cells and stimulate a proinflammatory response (Darveau *et al.*, 1998, Stathopoulou *et al.*, 2010, Han *et al.*, 2000, Huang *et al.*, 2004), whereas *S. gordonii* is essentially extracellular although capable of inducing inflammatory cytokines (Lamont *et al.*, 1995, Hasegawa *et al.*, 2007, Stathopoulou *et al.*, 2010).

While monolayers of cells are a convenient and reproducible model for the study of host-bacteria interactions, a cell multilayer more closely resembles the *in vivo* situation. Gingival epithelial cells can be grown in organotypic culture, and express differentiation and cytokeratin markers similar to those of junctional epithelial cells (Pan *et al.*, 1995, Papaioannou *et al.*, 1999, Rouabhia *et al.*, 2002). *P. gingivalis* has been shown to adhere to, invade and penetrate through multilayers of gingival epithelial cells (Andrian *et al.*, 2004, Sandros *et al.*, 1994). However, the responses of epithelial cell multilayers to challenge with *P. gingivalis* or with other oral organisms have not been investigated. In this study we generated multilayers of gingival epithelial cells and characterized their responses to *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum* and *S. gordonii*. The results show that distinct profiles of multilayer interactions are exhibited by the bacteria which may contribute to the pathogenic personalities that characterize each organism.

Materials and Methods

Primary Cell Culture

Primary cultures of gingival epithelial cells (GECs) were generated as described previously (Oda *et al.*, 1990). Briefly, healthy gingival tissue was collected from patients undergoing surgery for removal of impacted third molars and following Institutional Review Board Guidelines. Basal epithelial cells were separated and cultured in flasks in keratinocyte growth medium (KGM; Cambrix) containing 0.06 mM calcium and in the absence of antibiotics at 37 °C in 5% CO₂. At 80% confluence cells were removed by trypsinization for multilayer culture. Polyester (PET) Membrane Transwell Clear Inserts (12 mm diameter, 0.4 µm pore size; Tissue Culture Treated, Corning 3450) were placed in 12-well culture dishes (Corning), and GECs were seeded onto the membranes at 2×10^5 cells per well. KGM was added to the membrane and in the well under the membrane insert, and the cells cultured

until a confluent monolayer was attained (approximately 1 week). Medium was then removed from the insert to produce an air-liquid interface and the cells cultured to a 3 layer multilayer (approximately 3 weeks) that contained approximately 9×10^5 cells (Fig S1).

Bacteria and culture conditions

Bacteria were maintained as frozen stock cultures. *P. gingivalis* ATCC 33277 and *F. nucleatum* ATCC 25586 were cultured anaerobically at 37°C in trypticase soy broth (TSB) supplemented with yeast extract (1 mg ml⁻¹), hemin (5 µg ml⁻¹), and menadione (1 µg ml⁻¹). *A. actinomycetemcomitans* VT1169 was grown in TSB with yeast extract (0.6 mg ml⁻¹) in 10% CO₂ at 37°C. *S. gordonii* DL-1 was cultured anaerobically at 37°C in TSB with yeast extract (5 mg ml⁻¹) and glucose (5 mg ml⁻¹).

Bacterial challenge

Bacteria were stained with BacLight Green (Invitrogen) anaerobically at 37°C for 30 min, suspended KGM and added to the top of the multilayers at a MOI of 100. After incubation at 37°C in 5% CO₂, the multilayers were fixed with 4% paraformaldehyde for 20 min, washed with PBS, and blocked overnight at 4°C in 10% normal goat serum in PBS. The cells were permeabilized for 20 min at room temperature (RT) with 0.2% saponin in PBS with 10% normal goat serum. Actin microfilaments were stained Alexa 635 Phalloidin (Invitrogen) 1:200 for 30 min at RT. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (1 µg/ml, Sigma-Aldrich). Membranes were excised from the insert and mounted on a glass slide with Vectashield (Vector Laboratories). Images were acquired using a Leica DM IRM confocal microscope with Micro-Manager software (Applied Scientific Instrumentation). Three optical slices at 10 µm intervals were collected and Imaris software (V6, Bitplane) was used to compile the optical slices into a 3-dimensional volume view. The Ortho-Slicer function was used to “slice” the volume compilation of images into three sections (representing the three cell layers). The Spot-Counter function was then used to detect and count the number of fluorescent signals in a specified channel (corresponding to individual bacterial cells) and also within a segmented area of the volume. Counting was performed in 20 random fields.

Fluorescent antibody and annexin-V staining

The cells were fixed and permeabilized as above and reacted with primary antibodies for 1 h at RT. Antibodies used were: mouse anti-human Cytokeratins 1/10, 5/6, 13 and 19, or mouse anti-human TLR 2 and 4 (Invitrogen), 1:1000. Antigen-antibody binding was detected with goat anti-mouse Alexa 555 secondary antibody (Invitrogen). For quantitation of apoptosis, cells were stained with FITC-Annexin-V and propidium iodide (PI) using the ApoTarget kit (Invitrogen). Membranes were washed with PBS, excised from the insert, mounted on glass slides and images were acquired on a Leica DM IRM confocal microscope with Micro-Manager software as described above.

Transepithelial electrical resistance

Transepithelial electrical resistance (TER) was measured using the Millicell-ERS (Millipore) system. Membranes were submerged in PBS, electrodes inserted above and below the membrane, and electrical resistance measured. Background resistance from cell-free membranes was subtracted from initial resistance values.

Cytokine measurements

Supernatants from the upper chambers of cell membranes were collected, centrifuged to remove any bacteria, and stored at -20 °C until use. The supernatants were analyzed in the Millipore MILLIPLEX Map Kit Human Cytokine/Chemokine custom 7-Plex Multi-

Cytokine Detection System for IL-1 β , IL-10, IL-12(p40), IL-6, IL-8, MCP1, and TNF- α . The Luminex 100 System was used to acquire the results and MILLIPLEX Analyst Software (VigeneTech) was used to analyze the results.

Statistical analyses

One-way Analysis of Variance (ANOVA) multiple pairwise comparisons test with the Tukey or Bonferroni post tests were used to determine statistical significance.

Results

Keratin expression by GEC multilayers

GEC multilayers were probed with keratin antibodies and fluorescently labeled secondary antibodies. Keratin expression was examined by confocal microscopy. Cells in multilayers exhibited uniform strong expression of cytokeratins 13 and 19 (Figs S2 and S3), markers of junctional epithelium (Papaioannou *et al.*, 1999). In addition, there was moderate expression of cytokeratin 5/6, a marker of basal epidermal cells. Expression of cytokeratins 1/10, markers of terminal differentiation, was not detected. These results indicate that the GEC multilayers provide a model that reflects important aspects of the basal layer of the junctional epithelium.

Bacterial interactions with GEC multilayers

Multilayers were challenged with fluorescently labeled bacteria and the physical association examined by confocal microscopy. *P. gingivalis* penetrated the multilayers both inter- and intra- cellularly (Fig 1). After 2 h, approximately 80% of the total bacteria associated with the layers were present in the top layer. Over time, the organisms moved between the layers and after 24 h approximately 40% of the bacteria had located in the middle layer (Fig 1B). The majority of the *P. gingivalis* organisms associated with the multilayers were intracellular as determined by co-localization with actin (Fig 1C). In contrast, *A. actinomycetemcomitans*, *F. nucleatum* and *S. gordonii* penetrated to the lower epithelial layers but were not co-localized with actin indicating that they were not found intracellularly. *A. actinomycetemcomitans* penetrated the multilayers rapidly (Fig 2A), and after 2 h almost 40% of the bacteria had reached the middle layer (Fig 2B). At 24 h, almost 30% of the bacteria had reached the bottom layer and the organisms were more evenly dispersed through the 3 layers (Fig 2B). This contrasts with *P. gingivalis*, which did not penetrate to basal layer in the time period of the experiment. *F. nucleatum* spread through the three layers by 24 h (Fig 3A), although at the 2 h time point only 20% of the bacteria had reached the middle layer (Fig 3B). *S. gordonii*, which also traversed the epithelial layers through an extracellular route, penetrated them slowly, and after 24 h 70% of the organisms remained in the superficial layer (Fig 4).

Transepithelial resistance

GEC multilayers exhibited a stable transepithelial electrical resistance of $150 \Omega \times \text{cm}^2$. There was no significant change in transepithelial resistance following challenge with any of the test species of bacteria over 24 h (Fig 5). Thus, several of the bacteria penetrated the epithelial layers through an extracellular route but did this without disrupting the integrity of the epithelial barrier.

Cytokine secretion

Following 2 h and 24 h bacterial challenge, culture supernatants were analyzed for cytokine levels using Luminex technology. There was an increase in the secretion of IL-1 β and TNF- α following 24 h challenge with *S. gordonii*, *F. nucleatum*, and *A. actinomycetemcomitans*

(Figs 6 and 7). Levels of IL-6 and IL-8 increased following challenge with *S. gordonii*, and to a lesser degree *A. actinomycetemcomitans* (Fig 8 and 9). In contrast *P. gingivalis* did not increase the extracellular levels of IL-1 β , TNF- α , IL-6, or IL-8 (not shown). There were no statistically significant increases in the amounts of IL-10, IL-12, or MCP-1 following challenge with any of the bacterial species (not shown).

Apoptosis

The ability of the test bacteria to induce apoptosis in the GEC multilayers was tested with Annexin-V staining (Fig 10). *S. gordonii*, *F. nucleatum*, and *A. actinomycetemcomitans* stimulated the induction of apoptosis after 2 h, and apoptosis was sustained through 24 h. *P. gingivalis* caused a transient increase in Annexin-V staining after 2 h; however, at 24 h apoptosis levels had returned to the control value.

Discussion

The junctional epithelium is characterized by a relatively permeable structure, lack of keratinization and limited differentiation. Primary cultures of gingival epithelial cells maintained in monolayers exhibit these properties (Oda *et al.*, 1990), but they lack the stratified cell layers of tissue. A number of investigators have established multilayers of oral epithelial cells, although often the cells terminally differentiate when in an air-liquid interface and/or when cultured with serum or high calcium concentrations (Pan *et al.*, 1995, Papaioannou *et al.*, 1999, Rouabhia *et al.*, 2002). We have established multilayers of primary gingival epithelial cells in an air-liquid interface that retain limited differentiation status (K1/10 negative) by culture in medium lacking serum and with a low calcium concentration. This model provides an organotypic culture of phenotypically relevant cells that facilitates study of bacterial interactions with the junctional epithelium.

P. gingivalis rapidly invades monolayers of primary GECs in high numbers (Belton *et al.*, 1999, Lamont *et al.*, 1995), and can also adhere to and invade in gingival epithelial cells in multilayers (Papaioannou *et al.*, 2003, Andrian *et al.*, 2004, Sandros *et al.*, 1994). In our organotypic model, the majority of *P. gingivalis* organisms traverse the multilayers of GECs intracellularly, thus corroborating the importance of the intracellular lifestyle for *P. gingivalis*. While *A. actinomycetemcomitans* and *F. nucleatum* can invade monolayers of transformed epithelial cells (Han *et al.*, 2000, Edwards *et al.*, 2006, Meyer *et al.*, 1997), and *S. gordonii* can invade HeLa cells and endothelial cells (Stinson *et al.*, 2003, Nobbs *et al.*, 2007), in GEC multilayers any intracellular invasion was below the limit of detection. However, *A. actinomycetemcomitans* and *F. nucleatum* did spread through the multilayers intercellularly whereas *S. gordonii* was more confined to the upper cell layer. While *P. gingivalis* can effectively penetrate tissue by the paracellular route (Katz *et al.*, 2002, Balkovetz *et al.*, 2003, Hintermann *et al.*, 2002), the slower spread of the organism through the multilayers in this model may be a reflection of the predominantly intracellular location and the need for specialized cell-to-cell transmission systems (Yilmaz *et al.*, 2006). Indeed, *P. gingivalis* did not disrupt the barrier function of the multilayers as measured by TER, which may be related to the down regulation of protease production that occurs when *P. gingivalis* is in contact with GECs (Xia *et al.*, 2007). With monolayers of MDK cells, a critical threshold concentration of *P. gingivalis* is required to disrupt the barrier function (Katz *et al.*, 2000) and thus higher numbers of *P. gingivalis* may be capable of inducing disruption of the integrity of the multilayers. *A. actinomycetemcomitans* and *F. nucleatum* also did not disrupt the barrier function, indicating that the permeable structure of the multilayers is sufficient to allow intercellular bacterial penetration. The basis for the lack of *S. gordonii* spread through the multilayers is unknown; however, *S. gordonii* possess multiple adhesions for epithelial cells including Hsa and Ag I/II proteins (Nobbs *et al.*, 2009), and thus may remain attached to the first cells that are encountered.

Epithelial cell monolayers display a limited innate immune response following challenge with *P. gingivalis* (Darveau *et al.*, 1998, Hajishengallis, 2009). *P. gingivalis* exhibited even greater “stealth-like” characteristics with the GEC multilayers, and none of the tested cytokines were stimulated by the organism. As *P. gingivalis* can induce the secretion of IL-1 β , TNF- α and IL-6 from monolayers of GECs (La *et al.*, 2010; Stathopoulou *et al.*, 2010), this prompted us to question whether multilayers of GECs have diminished expression of TLRs. However, staining with fluorescent antibodies to TLR2 and TLR4 and quantitative image analysis revealed no significant differences in expression of TLR2 and TLR4 between mono- and multi layers (not shown). *F. nucleatum* and *S. gordonii* tended to be more proinflammatory, and induced secretion of IL-1 β and TNF- α . Moreover, IL-6 and IL-8 levels were also increased in response to *S. gordonii*. These data support the contention that a degree of inflammation, induced by commensal organisms, contributes to the control of potential pathogens, and to the maintenance of gingival health (Dixon *et al.*, 2004). *A. actinomycetemcomitans* stimulated the secretion of IL-1 β , TNF- α , IL-6 and IL-8 although levels of the latter three cytokines were significantly lower than those induced by *F. nucleatum* or *S. gordonii*. In combination with the *P. gingivalis* data, these findings suggest that there may be threshold concentrations of cytokine production that are associated with health and with the type and severity of disease.

Epithelial cell responses to bacterial challenge often include modulation of apoptotic pathways. Apoptosis in the GEC multilayers was increased following challenge with *A. actinomycetemcomitans*, *F. nucleatum* or *S. gordonii*, indicating that the gingival epithelial response to infection with different types of bacteria, ultimately includes increased cell death which may facilitate removal of excess bacteria. Interestingly, these three organisms also induced cytokine expression, which may contribute to the apoptotic process. *P. gingivalis*, in contrast, induced a transient increase in early apoptotic markers which returned to control levels after 24 h. This mimics the situation found in monolayers of GECs and is related to the ability of *P. gingivalis* to upregulate anti-apoptotic pathways in GECs in a time dependent manner (Nakhjiri *et al.*, 2001, Mao *et al.*, 2007). As *P. gingivalis* is predominantly located intracellularly, this is thought to represent a strategy of the bacteria to prolong the life of its eukaryotic host cell.

The bacterial inhabitants of the gingival compartment are diverse and exhibit a range of context-dependent pathogenic potentials. These pathogenic personalities may be established to a degree by the nature of the interaction with the junctional epithelium. *P. gingivalis*, a pathogen in severe and chronic manifestations of periodontitis, resides intracellularly and in gingival epithelial cells does not stimulate the production of proinflammatory cytokines. Persistence of the organism will be facilitated by the inhibition of apoptotic cell death. *A. actinomycetemcomitans*, a pathogen in more acute aggressive forms of periodontitis can penetrate multilayers and induce a degree of inflammation, but this may be insufficient to control the organism. *F. nucleatum*, which also has pathogenic potential, penetrates the multilayers; however, the induction of proinflammatory cytokines may be sufficient to control the organism in the absence of other pathogenic bacteria or host factors that predispose to disease. *S. gordonii* resides predominantly on the surface of the multilayers and over time induces the secretion of proinflammatory cytokines which may help control overgrowth of other bacterial species. An additional host strategy to control bacterial overgrowth may be to initiate apoptotic cell death that will contribute to the removal of cell-associated bacteria. Interestingly, the three organisms with pathogenic potential all had the capacity to traverse the epithelium, while the commensal organism did not. These studies provide insight into a mechanism that has been underappreciated, that strategies of traversing the epithelium represent an important component of bacterial pathogenicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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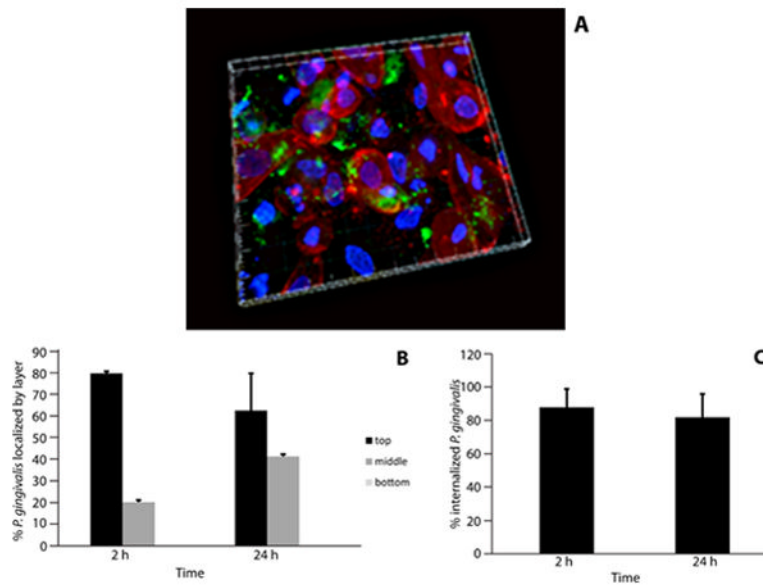


Figure 1. Physical association between *P. gingivalis* and GEC multilayers. A. Confocal microscopy of GECs stained with Alexa 635-Phalloidin (red) after infection for 24 h with *P. gingivalis* stained with BacLight Green. Nuclei are stained with DAPI (blue). A series of fluorescent optical $0.2 \mu\text{m}$ x - y sections were collected to create digitally reconstructed images with Imaris software. B. Percentage of *P. gingivalis* cells in the upper, middle or lower layers of the GEC multilayer. Layers were digitally separated using the Ortho-Slicer function of Imaris and the Spot-Counter function was used to detect and count the number of bacterial fluorescent signals. C. Percentage of total *P. gingivalis* that were internalized within all layers of the GECs at 2 and 24 h. Images are representative of three independent experiments. Data are means of three independent experiments in duplicate with standard deviation.

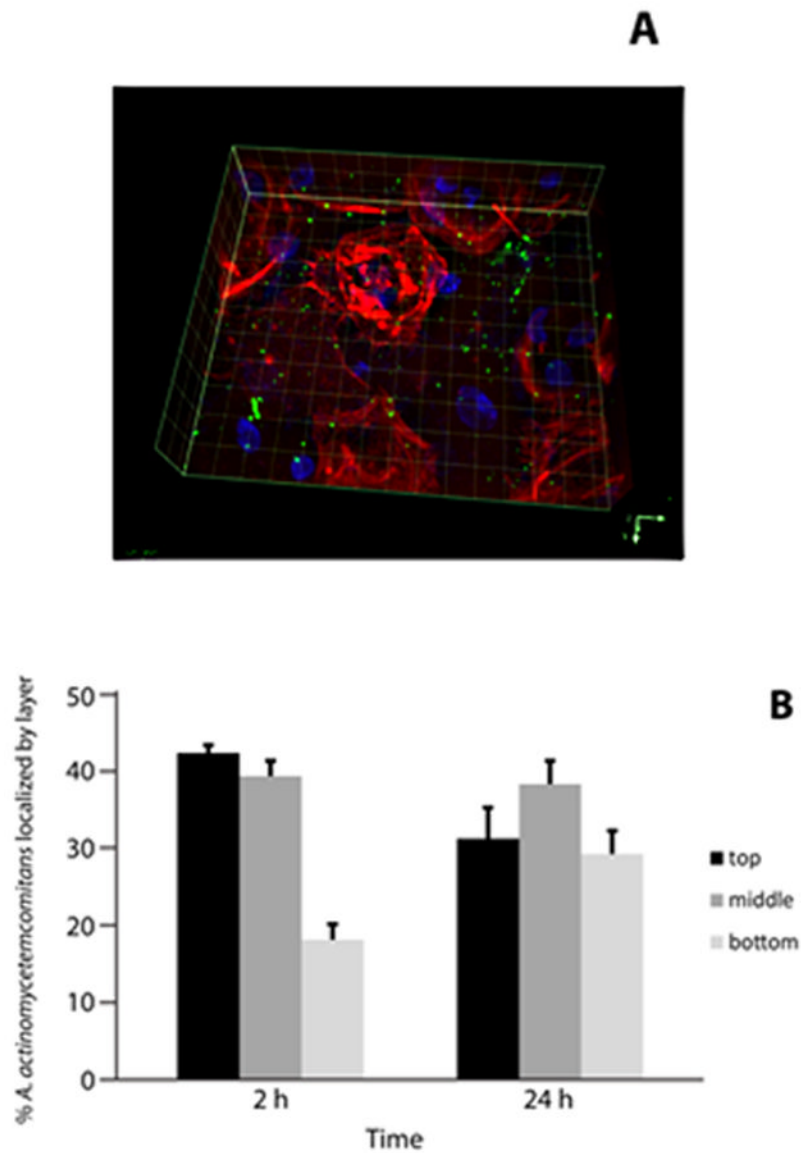
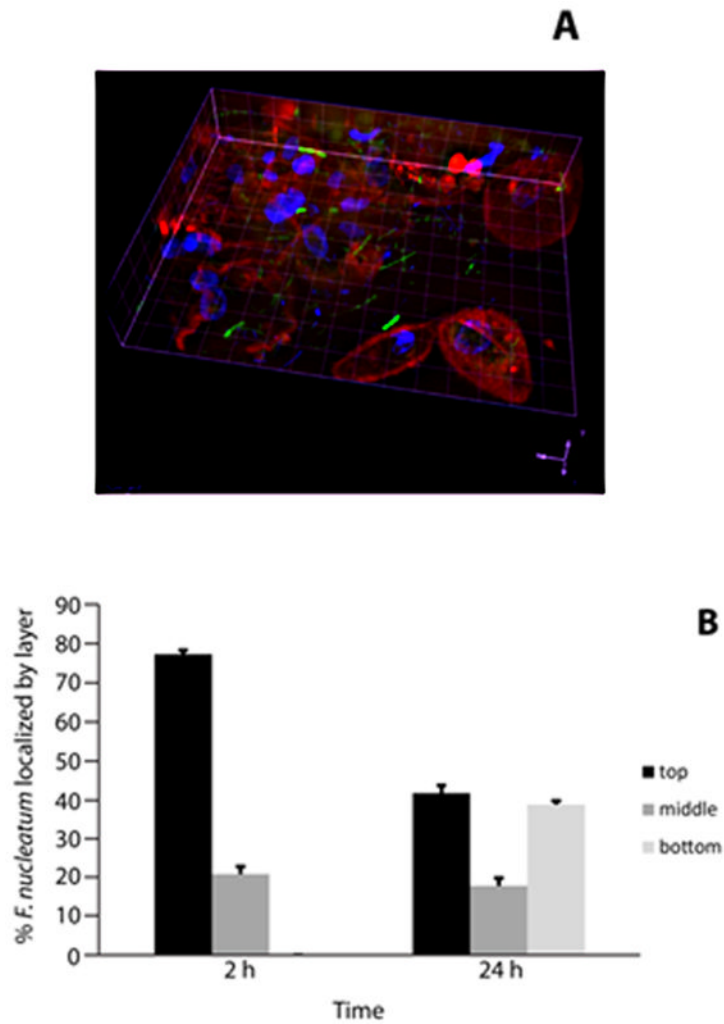


Figure 2. Physical association between *A. actinomycetemcomitans* and GEC multilayers. A. Confocal microscopy of GECs stained with Alexa 635-Phalloidin (red) after infection for 24 h with *A. actinomycetemcomitans* stained with Baclight Green. Nuclei are stained with DAPI (blue). A series of fluorescent optical $0.2 \mu\text{m}$ x - y sections were collected to create digitally reconstructed images with Imaris software. B. Percentage of *A. actinomycetemcomitans* cells in the upper, middle or lower layers of the GEC multilayer. Layers were digitally separated using the Ortho-Slicer function of Imaris and the Spot-Counter function was used to detect and count the number of bacterial fluorescent signals. Images are representative of three independent experiments. Data are means of three independent experiments in duplicate with standard deviation.

**Figure 3.**

Physical association between *F. nucleatum* and GEC multilayers. A. Confocal microscopy of GECs stained with Alexa 635-Phalloidin (red) after infection for 24 h with *F. nucleatum* stained with Baclight Green. Nuclei are stained with DAPI (blue). A series of fluorescent optical $0.2 \mu\text{m}$ x - y sections were collected to create digitally reconstructed images with Imaris software. B. Percentage of *F. nucleatum* cells in the upper, middle or lower layers of the GEC multilayer. Layers were digitally separated using the Ortho-Slicer function of Imaris and the Spot-Counter function was used to detect and count the number of bacterial fluorescent signals. Images are representative of three independent experiments. Data are means of three independent experiments in duplicate with standard deviation.

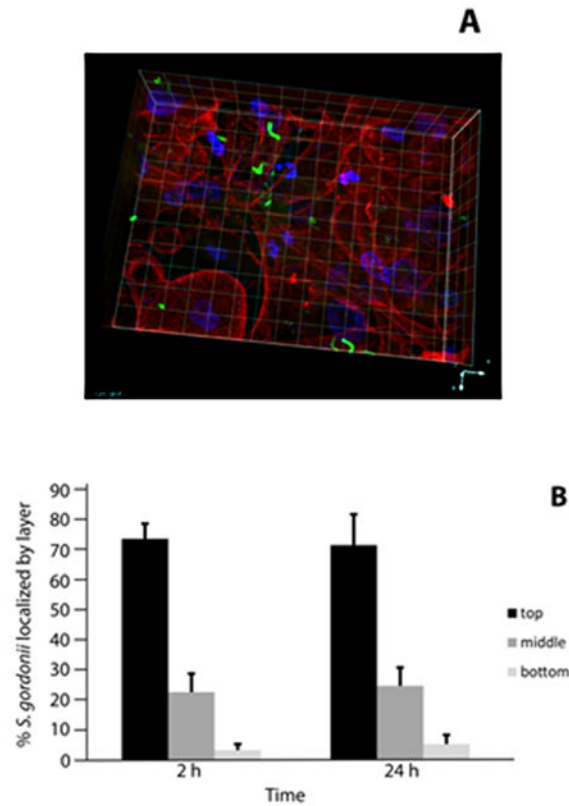


Figure 4. Physical association between *S. gordonii* and GEC multilayers. A. Confocal microscopy of GECs stained with Alexa 635-Phalloidin (red) after infection for 24 h with *S. gordonii* stained with BacLight Green. Nuclei are stained with DAPI (blue). A series of fluorescent optical $0.2 \mu\text{m}$ x - y sections were collected to create digitally reconstructed images with Imaris software. B. Percentage of *S. gordonii* cells in the upper, middle or lower layers of the GEC multilayer. Layers were digitally separated using the Ortho-Slicer function of Imaris and the Spot-Counter function was used to detect and count the number of bacterial fluorescent signals. Images are representative of three independent experiments. Data are means of three independent experiments in duplicate with standard deviation.

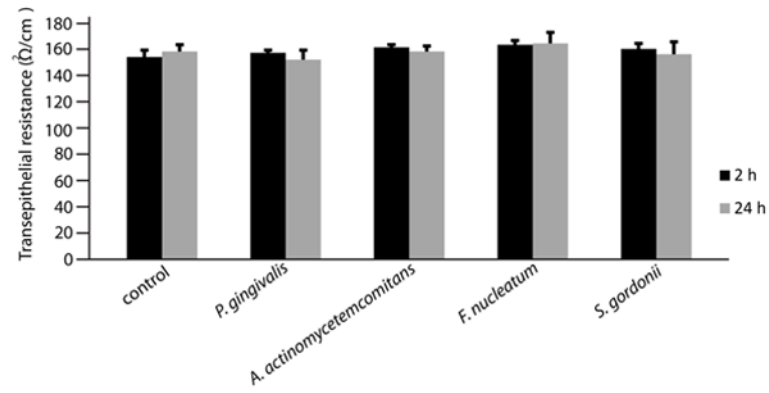


Figure 5. Transepithelial electrical resistance of GEC multilayers infected with *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum*, or *S. gordonii* for 2 and 24 h. Control is uninfected cells. Data are means of three independent experiments in duplicate with standard deviation.

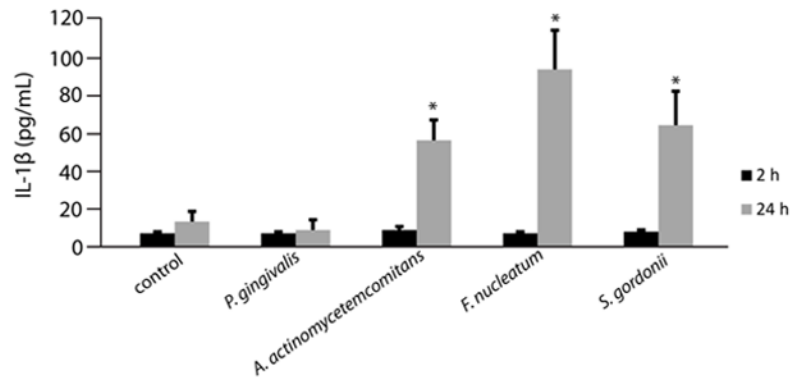


Figure 6.

IL-1 β accumulation in supernatants of GEC multilayers following infection with *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum* or *S. gordonii* for 2 h or 24 h as determined by a Luminex cytokine array. Control is uninfected cells. The error bars indicate standard deviations ($n = 3$). Asterisk denotes $P < 0.05$ compared to control.

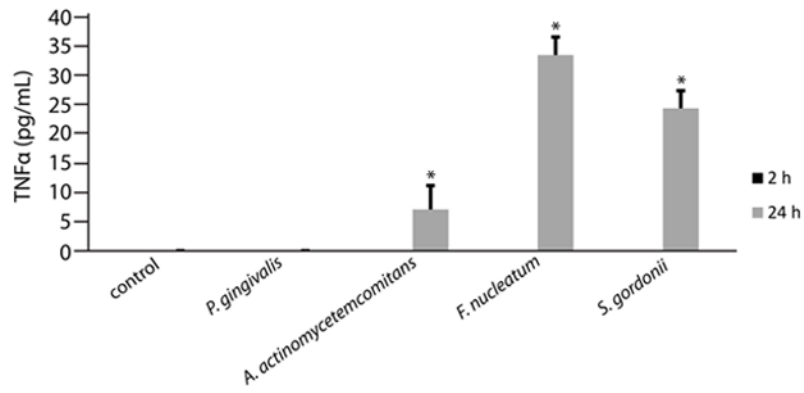


Figure 7. TNF α accumulation in supernatants of GEC multilayers following infection with *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum* or *S. gordonii* for 2 h or 24 h as determined by a Luminex cytokine array. Control is uninfected cells. The error bars indicate standard deviations ($n = 3$). Asterisk denotes $P < 0.05$ compared to control.

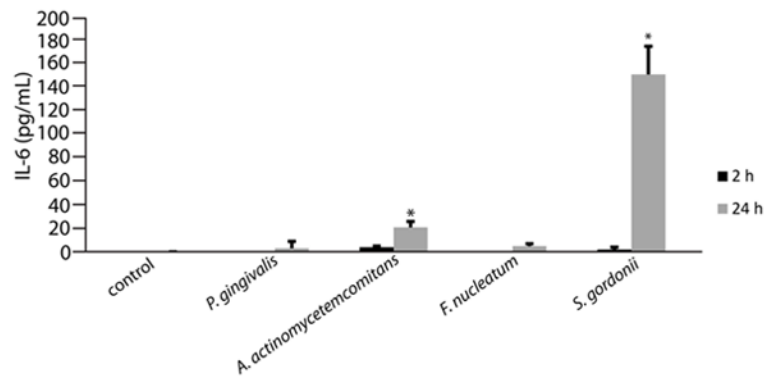


Figure 8.

IL-6 accumulation in supernatants of GEC multilayers following infection with *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum* or *S. gordonii* for 2 h or 24 h as determined by a Luminex cytokine array. Control is uninfected cells. The error bars indicate standard deviations ($n = 3$). Asterisk denotes $P < 0.05$ compared to control.

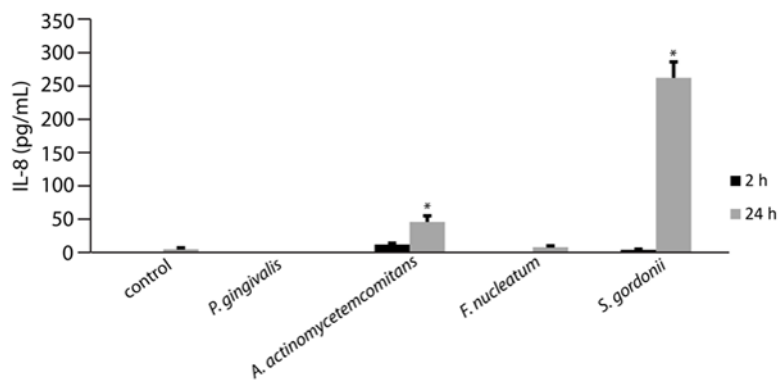


Figure 9.

IL-8 accumulation in supernatants of GEC multilayers following infection with *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum* or *S. gordonii* for 2 h or 24 h as determined by a Luminex cytokine array. Control is uninfected cells. The error bars indicate standard deviations ($n = 3$). Asterisk denotes $P < 0.05$ compared to control.

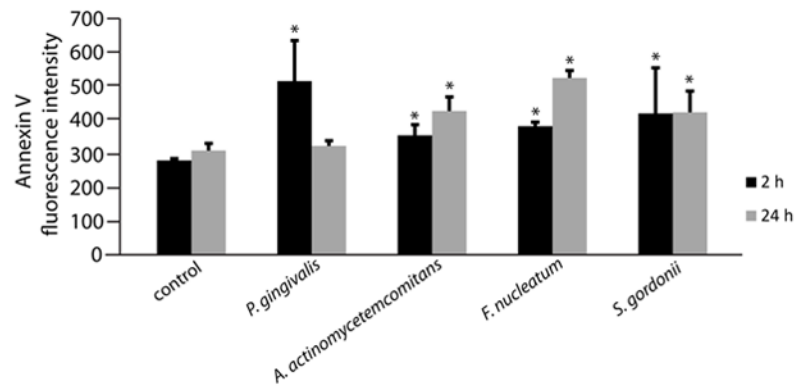


Figure 10.

Annexin V staining in GEC multilayers infected with *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum* or *S. gordonii* for 2 h or 24 h. Control is uninfected cells. FITC-Annexin V signals were collected by confocal microscopy. Mean intensity was calculated using Slidebook software. Error bars indicate standard deviations ($n = 3$). Asterisk denotes $P < 0.05$ compared to control.