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Filifactor alocis interactions with gingival epithelial cells

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SUMMARY

An association between the gram-positive anaerobe *Filifactor alocis* and periodontal disease has recently emerged; however, possible pathogenic mechanisms have not been investigated. In this study we examined the responses of primary cultures of gingival epithelial cells (GECs) to infection with *F. alocis*. Secretion of the pro-inflammatory cytokines interleukin-1β, interleukin-6 and tumor necrosis factor-α from GECs was stimulated by *F. alocis* infection. *F. alocis* also induced apoptosis in GECs through pathways that involved caspase-3 but not caspase-9. Apoptosis was coincident with inhibition of mitogen-activated protein kinase kinase (MEK) activation. These results show that *F. alocis* has characteristics in common with established periodontal pathogens and has the potential to contribute to periodontal tissue destruction.

INTRODUCTION

Periodontitis, one of the most prevalent diseases worldwide (Brown *et al.*, 2002; Albandar, 2011), is a chronic bacterial inflammatory infection leading to destruction of the periodontal tissue, and culminating in alveolar bone loss and exfoliation of the teeth. Until recently, research into the etiology of periodontal disease has focused primarily on a small group of bacteria that can be recovered in high numbers from periodontal lesions. On the basis of association, Socransky *et al.,* (1998) proposed that *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, designated the red complex, were the primary pathogens, with orange-complex organisms, including *Fusobacterium nucleatum*, *Prevotella intermedia* and *Campylobacter rectus*, also contributing to disease to a lesser degree. A large number of studies have since revealed the pathogenic properties of these organisms, along with the nature of protective and destructive host responses (Lamont & Jenkinson, 1998; Holt & Ebersole, 2005; Feng & Weinberg, 2006; Darveau, 2010; Sharma, 2010; Frederick *et al.*, 2011).

Approximately 300 bacterial species from the oral cavity have been isolated in culture and formally named; however, it is estimated that less than half of the bacterial species present in the oral cavity can be readily cultivated (Wade, 2011). The development of culture-independent techniques such as 16S ribosomal RNA sequencing and high throughput sequencing, which allow for the identification of bacterial species directly from DNA, has led to a broader understanding of the diversity of bacterial species present in the oral environment (Dewhirst *et al.*, 2010; Griffen *et al.*, 2011; Wade, 2011). Indeed, a recent study using 16S rRNA sequencing found over 1,000 phylogenetically different taxa in the oral cavity,

around 400 of which were novel (Dewhirst *et al.*, 2010).

Molecular methods of bacterial identification have facilitated the ability to identify previously overlooked bacteria associated with disease. One such organism is *Filifactor alocis*, a gram-positive anaerobic rod. First isolated in 1985 from the gingival sulcus, the bacterium was taxonomically classified as *Fusobacterium alocis* (Cato *et al.*, 1985), with later phylogenetic analysis leading to its reassignment to *Filifactor* in 1999 (Jalava & Eerola, 1999). Although cultivable, this organism is slow-growing and difficult to detect by conventional culture-based methods. However, through molecular approaches it is becoming increasingly apparent that the presence of *F. alocis* is indicative of a number of oral diseases including caries, endodontic infections and periodontal disease. *Filifactor alocis* is weakly glycolytic, and children with caries have been shown to have elevated levels of *F. alocis* in plaque (Dahlen *et al.*, 2010). *Filifactor alocis* is among the most commonly detected taxa in sites of endodontic infection (Siqueira & Rocas, 2004; Sakamoto *et al.*, 2006), and it is present in the root canals of teeth with primary apical periodontitis (Siqueira *et al.*, 2009), and in periapical lesions of root-filled teeth (Gomes *et al.*, 2008). Several studies have found *F. alocis* at increased frequency and in higher numbers in periodontal disease sites compared with healthy sites, leading to the proposal that *F. alocis* should be included as a diagnostic indicator of disease (Kumar *et al.*, 2005, 2006; Dahlen & Leonhardt, 2006; Colombo *et al.*, 2009). Hence, there is a growing body of evidence supporting the notion that *F. alocis* may be a key causative agent in the development of oral diseases.

A recent study reported that *F. alocis* forms biofilms *in vivo*, preferentially colonizing the apical parts of the gingival pocket in close proximity to the soft tissues (Schlafer *et al.*, 2010). We hypothesized, therefore, that *F. alocis* would exert an influence on gingival epithelial cells that was consistent with the characteristics of a periodontal pathogen. Hence we investigated the ability of *F. alocis* to induce pro-inflammatory cytokine secretion and apoptotic cell death in gingival epithelial cells. Infection by *F. alocis* leads to the secretion of interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α) from gingival epithelial cells, and eventually causes apoptotic cell death. Our results begin to establish pathogenic credentials for *F. alocis* and support a role for the organism in the etiology of periodontal disease.

METHODS

Bacterial and eukaryotic cell culture

Filifactor alocis strain ATCC 38596 was routinely cultured anaerobically at 37°C on *Brucella* agar plates containing hemin and menadione (Sigma, St. Louis, MO) and supplemented with 5% sheep's blood. Primary cultures of gingival epithelial cells (GECs) were generated as described previously (Mao *et al.*, 2007). 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 keratinocyte growth medium (DermaLife Basal Medium; Lifeline Cell Technology, Walkersville, MD) in the absence of antibiotics. Eukaryotic cells were cultured at 37°C in 5% CO_2.

Confocal microscopy

For examination of *F. alocis*-GEC association, GECs were seeded at 1×10^5 cells on glass coverslips in 12-well plates and grown until ≈40% confluent. Cells were infected with Syto 17 (Invitrogen, Carlsbad, CA) –labelled *F. alocis* at a multiplicity of infection (MOI) 20 for 1 h. Coverslips were washed four times in phosphate-buffered saline (PBS) and fixed for 10 min in 4% paraformaldehyde. Following a 20-min block in 10% goat serum, actin was labelled using 1 : 100 fluorescein isothiocyanate–phalloidin (Sigma) for 40 min at room temperature. After four washes in PBS, coverslips were mounted using ProLong Gold with DAPI mounting medium (Invitrogen). Images were acquired on an Olympus DSU Spinning Disk Confocal Scanner mounted on an Olympus IX81 inverted microscope, using a 60× water immersion objective. *Z*-stacks were obtained (1 μm between layers, 20 layers/stack from base to top of cells) through the *z*-axis of cells (three *z*-stacks/coverslip), and numbers of associated *F. alocis*/cell were enumerated using means of bacteria associated with ≈50 cells/assay (three coverslips/group, three *z*-stacks/coverslip, average of six GECs/field).

For apoptosis assays, GECs were cultured on glass coverslips until ≈40% confluent and infected

with *F. alocis* at MOI 100 for 24 h. Coverslips were washed four times in PBS and fixed for 10 min in 4% paraformaldehyde. Permeabilization was with 0.2% Triton X-100 for 10 min at room temperature, before blocking in 10% goat serum for 20 min. Caspases were detected by reacting with primary active caspase-3 or caspase-9 antibodies (Sigma) at 1 : 100 dilution for 1 h, followed by Alexa-647-conjugated anti-rabbit secondary antibody (1 : 200) for 1 h in the dark. After four washes in PBS, coverslips were mounted using ProLong Gold with DAPI mounting medium (Invitrogen). Images were acquired on a Leica DM IRE2 inverted fluorescent microscope, with a Leica TCS SP2 AOBS spectral confocal scanner, using a 63× water immersion HCX PL APO WCORR objective. *Z*-stacks were obtained (10 layers/stack, 2 μm between layers) through the *z*-axis of cells (three *z*-stacks/coverslip), and maximum projections were obtained using Leica LCS software.

ELISA

GECs were cultured to 80% confluence and infected with *F. alocis* (MOI 100) for 6 h, 24 h or 48 h. Supernatants were collected and centrifuged at 4,000 ***g*** for 10 min to remove bacteria. Secretion of IL-1β, IL-6, IL-8 and TNF-α, was assessed using Quantikine kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Western immunoblotting

Filifactor alocis-infected GECs were lysed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) buffer, separated by SDS–PAGE, and transferred onto nitrocellulose membranes by electroblotting. Membranes were blocked in 10% skimmed dry milk in Tris-buffered saline overnight at 4°C. Primary antibody was rabbit anti-mitogen-activated protein kinase kinase (MEK) 1/2, rabbit anti-phospho-MEK1/2 or rabbit anti-GAPDH (Cell Signaling, Danvers MA), 1 : 1000 for 2 h at room temperature. Antigen–antibody binding was detected using horseradish peroxidase-conjugated species-specific secondary antibodies followed by ECL Western Blotting detection reagents (Thermo Pierce, Rockford, IL). Densitometric analysis was performed and phospho- (p-) MEK : MEK ratios were calculated following normalization to GAPDH.

Annexin V/Sytox Green flow cytometry assay

The GECs were infected with *F. alocis* (MOI 100) or treated with 10 μM campthothecin (apoptosis control) or 0.3% H_2O_2 (necrosis control). Cells were harvested by trypsinization, and a phycoerythrin Annexin V/Dead cell Apoptosis kit for flow cytometry (Invitrogen) was employed according to the manufacturer's instructions. Briefly, cell pellets were resuspended in Annexin-binding buffer for washing, then were centrifuged and stained with Annexin V and Sytox Green in the dark at 37°C in 5% CO_2 for 15 min before flow cytometry analysis.

Caspase-3 and caspase-9 luminosity assay

GECs were infected with *F. alocis* (MOI 100) for 24 h. Cells were then incubated with Caspase-Glo assay substrates for caspase-3 and caspase-9 (Promega, Madison, WI), at room temperature in the dark for 1 h. Luminosity was measured using a Wallac Victor^3 1420 Multilabel Counter Luminometer (Perkin-Elmer).

RESULTS

Filifactor alocis associates with GECs

Initially, we undertook fluorescent image analysis to investigate whether *F. alocis* associates with gingival cells, and *F. alocis* was observed to adhere to the surface of GECs (Fig. 1A, arrows). Examination of the *z*-stacks through layers indicated that *F. alocis* was also located within the cells, as bacteria were visible within the cytoplasmic region through central layers of the stack; however, this requires further investigation. Similarly, clinical isolates of *F. alocis* have been shown to invade epithelial cells (H. Fletcher, personal communication). The *F. alocis* associated with cells were enumerated (Fig. 1B), and there was ≈1 bacterium/cell at an MOI of 20.

Pro-inflammatory cytokine secretion is stimulated in *F. alocis*-infected GECs

Next, we sought to determine the cytokine responses of GECs to *F. alocis*. Levels of IL-1β, IL-6, IL-8 and TNF-α in GEC culture supernatants were quantified by ELISA. In response to *F. alocis,* IL-1β levels showed a slight increase 24 h after infection

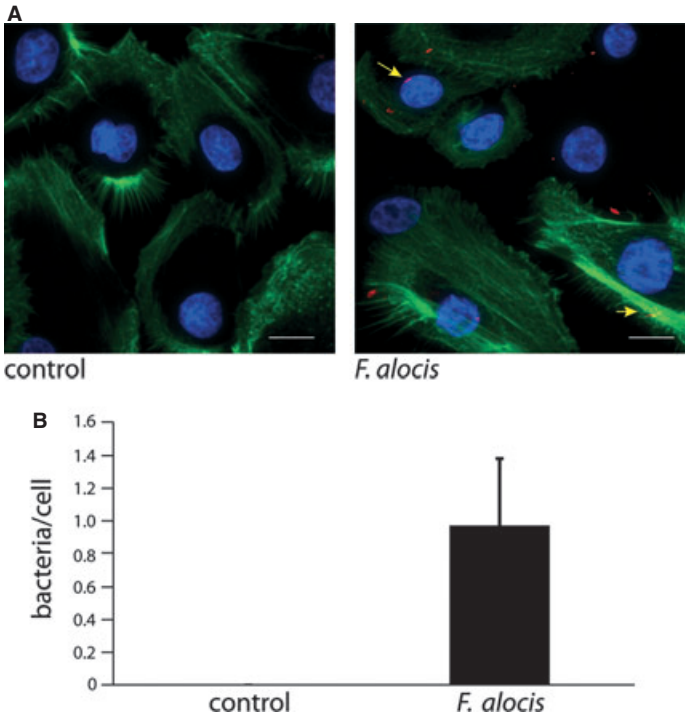

Figure 1 *Filifactor alocis* localizes to gingival epithelial cells (GECs). (A) The GECs were infected with *F. alocis* (multiplicity of infection 20) for 1 h and analyzed by confocal microscopy. Uninfected GECs acted as control. The *F. alocis* (red) was labeled with Syto 17 before infection, actin (green) was stained with fluorescein isothiocyanate–phalloidin, and nuclei (blue) were stained with DAPI. Results are representative of two independent assays. Data shown are maximum projections of *z*-stacks (20 slices/*z*-stack, three coverslips/group). (B) Levels of *F. alocis* associated with GECs. Numbers of bacteria co-localized with host cells were counted throughout *z*-stacks (20 slices/stack; three coverslips/group). Results are representative of two independent assays. Data are means of bacteria associated with ≈ 50 cells/assay (three coverslips/group, three *z*-stacks/coverslip, average of six GECs/field), and error bars indicate standard deviations. Scale bar 5 μm.

($P < 0.01$), and a more substantial increase following 48 h infection ($P < 0.001$) (Fig. 2A). The amount of IL-6 secreted from infected cells was comparable with that in uninfected controls at 6 h; however, after 24 h IL-6 levels were elevated more than four-fold in *F. alocis*-infected GECs ($P < 0.001$) (Fig. 2B). At 48 h, IL-6 levels were comparable between control and infected conditions. Infection with *F. alocis* caused a significant increase in TNF-α secretion (Fig. 2C) following a 24-h incubation ($P < 0.001$), and secretion levels continued to increase up to 48 h ($P < 0.001$). In contrast, IL-8 levels were unchanged following *F. alocis* infection at all time periods (not shown). These results indicate that *F. alocis* selectively induces a pro-inflammatory cytokine response from gingival epithelial cells.

Apoptosis is induced in *F. alocis*-infected GECs

To investigate whether *F. alocis* may affect cell viability, we examined the levels of apoptotic and necrotic cells following infection. Flow cytometry plots (Fig. 3A) revealed that after 4 h incubation, levels of apoptosis in infected cells and uninfected controls were comparable. However, after 24 h >50% of infected cells were apoptotic ($P < 0.001$), increasing to 88% apoptotic after 48 h of infection ($P < 0.001$) (Fig. 3B). Uninfected controls showed no higher than 4% apoptosis. No significant necrosis was detected in either group. This result provides the first evidence that *F. alocis* induces apoptosis in primary gingival epithelial cells.

Filifactor alocis activates an extrinsic apoptosis pathway in GECs

To begin to address whether apoptosis induction occurred through intrinsic or extrinsic pathways, activation of caspase-3 and caspase-9 in infected cells was determined. Following 24 h of infection, caspase-3 activity increased compared with uninfected controls ($P < 0.001$), (Fig. 4A). In contrast, no caspase-9 activation was detected (Fig. 4B). Caspase-3 and

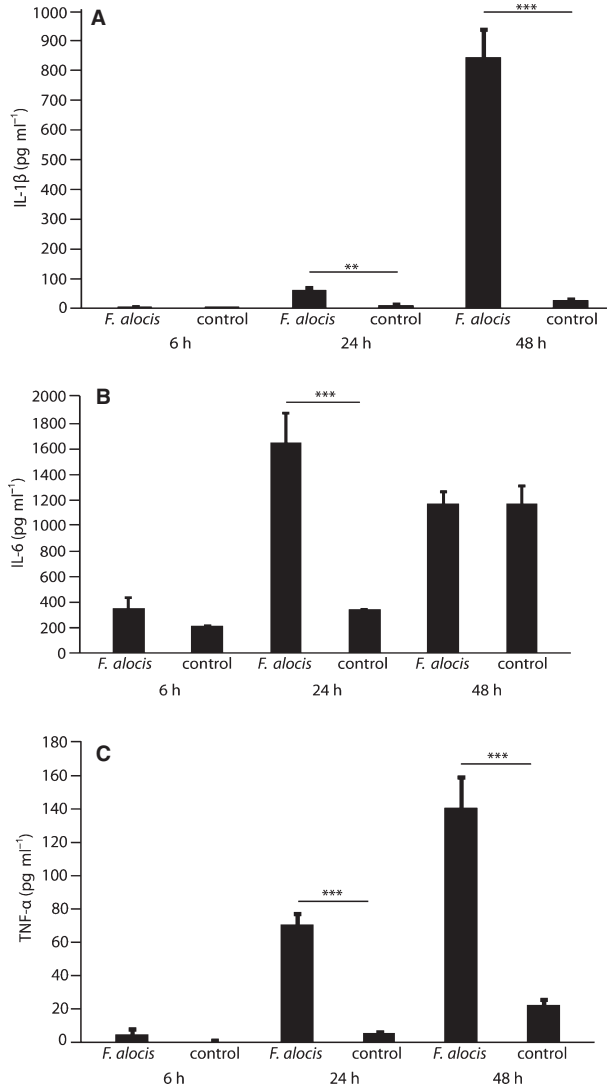

Figure 2 *Filifactor alocis* induces secretion of interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α) from gingival epithelial cells (GECs). Supernatants were obtained from *F. alocis*-infected GECs, or uninfected controls, and analyzed by ELISA. (A) IL-6, (B) IL-1β and (C) TNF-α. Data are means and error bars indicate standard deviation ($n = 3$). Data are representative of three independent experiments. $^{**}P < 0.01$ $^{***}P < 0.001$ by Tukey–Kramer multiple comparison test.

caspase-9 activation levels were also examined by confocal microscopy. As shown in Fig. 4C, caspase-3 activation was increased in cells infected with *F. alocis* compared with uninfected controls. Caspase-9 activation was not detectable in either infected or uninfected (Fig. 4D) cells. As activation of caspase-9 is indicative of intrinsic, mitochondrially induced apoptosis, these results support the concept that *F. alocis* activates an extrinsic apoptotic pathway in GECs.

Filifactor alocis modifies MEK signaling in GECs

Inhibition of MEK activity can induce apoptosis, and can impact both the intrinsic and extrinsic pathways (Dai *et al.*, 2003; Liu *et al.*, 2006; Wang *et al.*, 2007; Meng *et al.*, 2010). Therefore, the impact of *F. alocis* on MEK phosphorylation was investigated by Western blotting with specific MEK1/2 and phospho(p)-MEK1/2 antibodies (Fig. 5A). While *F. alocis* caused transient phosphorylation of MEK1/2 after 5 min of bacterial challenge, levels of phospho-MEK1/2 were reduced compared with uninfected controls after 30 min and for up to 5 h. Densitometric analysis of bands showed an approximately 70% reduction in the ratio of p-MEK to MEK after 45 min of *F. alocis* infection (Fig. 5B).

DISCUSSION

Periodontal diseases ensue from the disruption of the balance between the host and the complex polymicrobial community that colonizes the gingival crevice. As periodontal pathogens are also frequently present in the absence of disease, the identities of the organisms associated with the initiation and progression of disease are difficult to determine with certainty. Criteria that are used to impute pathogenic potential to periodontal bacteria include: an increase in number at disease sites; a reduction in number after treatment; pathogenicity in animal models; and display of appropriate virulence factors (Socransky & Haffajee, 1992). These criteria have been very successful in identifying key components of the pathogenic microbial communities in periodontal disease, and the virulence of organisms such as *P. gingivalis*, *Tannerella forsythia* and *Treponema denticola* is now well established. With the development and successful implementation of culture-independent identification technology it is now possible to more accurately catalogue the complete range of organisms present in health and disease. *Filifactor alocis* has emerged as an organism that increases in number in diseased periodontal sites in comparison to healthy sites (Kumar *et al.*, 2005, 2006; Dahlen & Leonhardt, 2006). In addition, cessation of smoking reduces the prevalence of *F. alocis*, along with other bacterial species associated with periodontal disease (Delima *et al.*, 2010). In terms of association with disease, therefore, *F. alocis* exhibits characteristics of a peri-

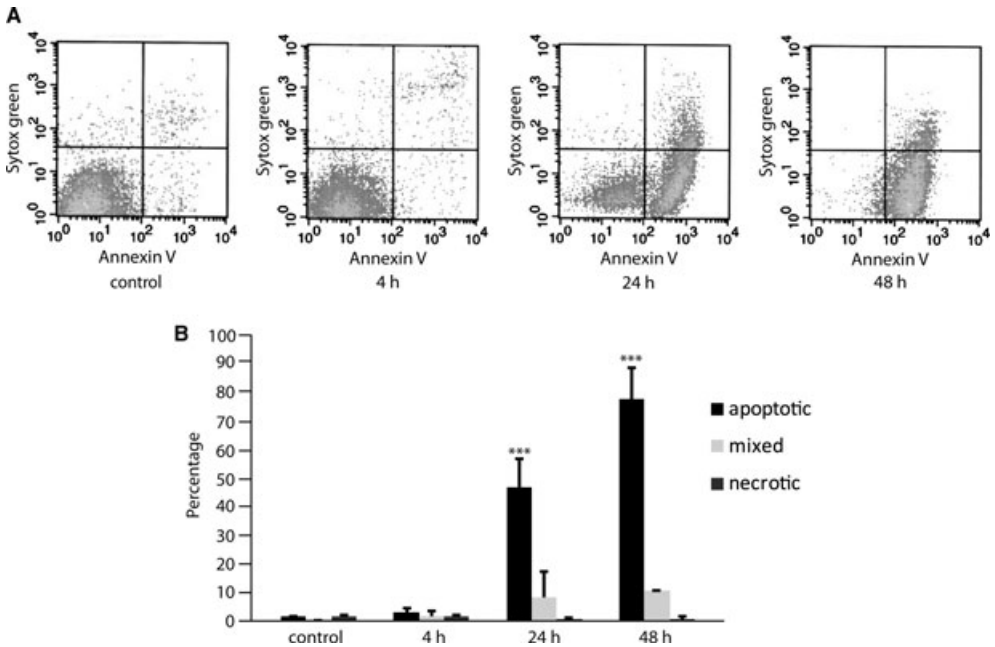

Figure 3 *Filifactor alocis* stimulates apoptosis in gingival epithelial cells (GECs). *F. alocis*-infected GECs were stained with Annexin V and Sytox Green. (A) FACS profiles showing apoptotic (lower right quadrants), necrotic (upper left) or mixed apoptotic and necrotic (upper right) cells. (B) Percentages of cells undergoing apoptosis/necrosis. Data are means and error bars indicate standard deviation. Results shown are the average from two independent assays. ***$P < 0.001$ compared with control by Tukey–Kramer multiple comparison test.

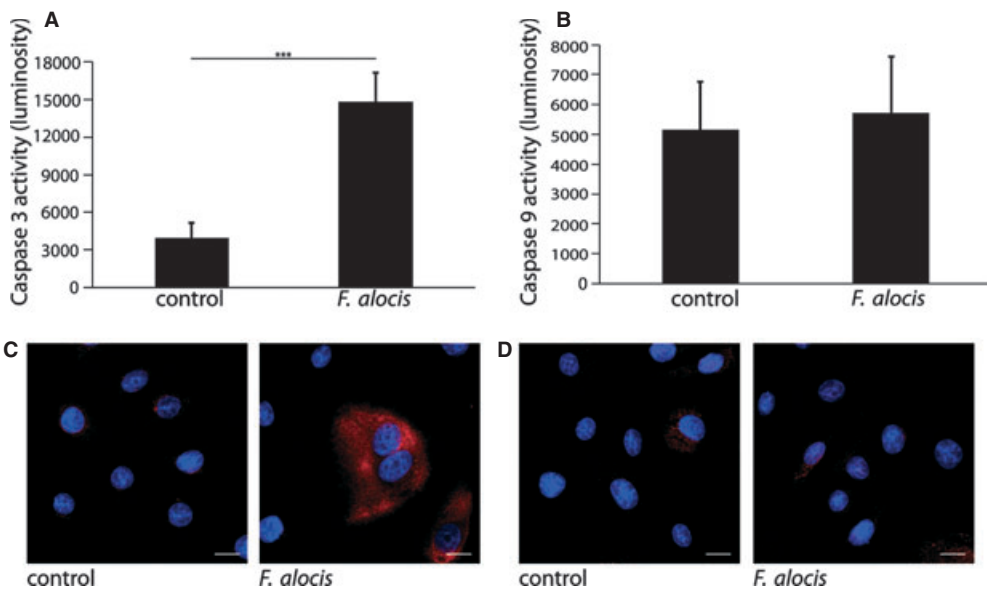

Figure 4 Caspase-3 is activated in *Filifactor alocis*-infected gingival epithelial cells (GECs). The GECs were infected with *F. alocis* (multiplicity of infection 100) for 24 h and reacted with proluminescent substrates for (A) caspase-3 and (B) caspase-9. Luminosity (arbitrary units) data are means and error bars indicate standard deviation ($n = 3$). Data are representative of three independent experiments. ***$P < 0.001$ by Student's *t*-test. (C, D) *Filifactor alocis*-infected cells or uninfected controls were labeled with (C) caspase-3 antibodies or (D) caspase-9 antibodies (red) and nuclei (blue) stained with DAPI. Cells were subsequently analyzed by confocal laser scanning microscopy. Results are representative of three independent assays. Data shown are maximum projections of *z*-stacks (10 slices/*z*-stack, three coverslips/group). Scale bar 5 μm.

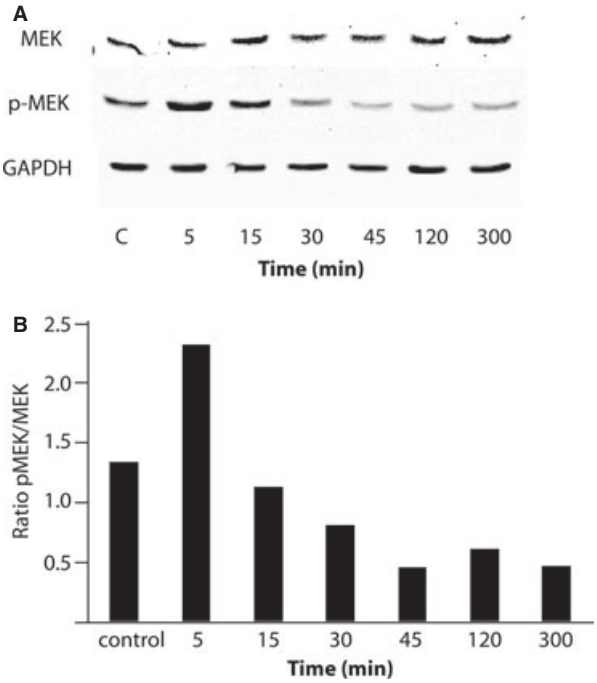

Figure 5 Mitogen-activated protein kinase kinase (MEK) 1/2 activity is modulated by *Filifactor alocis*. (A) Lysates of *F. alocis*-infected (inf) or uninfected control (C) gingival epithelial cells (GECs) were examined by Western blotting with antibodies to MEK1/2 or phospho(p)-MEK1/2. GAPDH was used as a loading control. (B) Scanning densitometry showing the ratio of p-MEK to MEK. Data are representative of three independent experiments.

odontal pathogen. We undertook this study to begin to investigate the pathogenic profile of *F. alocis*.

Epithelial cells that line the gingival crevice are among the first host cells encountered by periodontal 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 (Kagnoff & Eckmann, 1997; Tribble & Lamont, 2010). Successful periodontal pathogens can disrupt cytokine networks and also impact apoptotic cell death in gingival epithelial cells. We therefore examined the interaction between *F. alocis* and primary cultures of GECs in the context of cytokine responses and apoptosis.

Filifactor alocis induced the secretion of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, but not IL-8, from GECs. In terms of relevance to periodontal disease, IL-1β, IL-6 and TNF-α are capable of upregulation of pathways that stimulate osteoclasts and increase alveolar bone resorption (Preshaw & Taylor, 2011). Interleukin-1β, IL-6 and TNF-α can also contribute to tissue degradation through the induction of matrix metalloproteinases and other inflammatory mediators (Birkedal-Hansen, 1993; Graves & Cochran, 2003; Graves, 2008). Moreover, a number of studies have demonstrated increased IL-1β, IL-6 and TNF-α levels in periodontitis patients (Howells, 1995; Okada & Murakami, 1998), and the application of antagonists to IL-1 and TNF reduces the severity of experimental periodontitis (Graves & Cochran, 2003). Thus, much of the tissue destruction in periodontal disease is thought to result from disruption of cytokine homeostasis (Preshaw & Taylor, 2011). Interestingly, the GEC cytokine responses to *F. alocis* bear a remarkable resemblance to those of the consensus periodontal pathogen, *P. gingivalis*. In response to *P. gingivalis* infection, GECs produce IL-1β, TNF-α and IL-6, but not IL-8 (Darveau *et al.*, 1998; Stathopoulou *et al.*, 2010). In addition, *P. gingivalis* can antagonize production of IL-8 in response to stimulation with other oral bacteria (Darveau *et al.*, 1998). Suppression of the neutrophil chemokine IL-8 contributes localized immune suppression and may allow overgrowth of other destructive bacteria. The ability of *F. alocis* to antagonize IL-8 production remains to be investigated.

Epithelial cell apoptosis can be demonstrated in periodontal lesions (Tonetti *et al.*, 1998; Vitkov *et al.*, 2005), and apoptosis may be the direct result of bacterial action or the indirect result of pro-inflammatory cytokine secretion. *F. alocis* was capable of inducing apoptosis in GECs, and apoptosis was associated with the activation of caspase-3 but not caspase-9. The absence of caspase-9 activation would tend to suggest that *F. alocis*-induced apoptosis occurs through the extrinsic pathway. In contrast to the concordance between *P. gingivalis* and *F. alocis* in cytokine expression, *P. gingivalis* does not induce apoptosis in GECs (Mao *et al.*, 2007); however, other periodontal pathogens such as *Treponema denticola* can cause epithelial cell apoptosis (Leung *et al.*, 2002).

MEK1/2 is a member of the dual specificity protein kinase family, which lies upstream of the MAPKs (extracellular signal-regulated kinases or ERKs). MEK1/2 can activate MAPK pathways upon stimulation by a variety of extracellular and intracellular signals, and MAPK signaling can control cell proliferation and differentiation. *Filifactor alocis* caused a

transient activation of MEK1/2, and a longer-term inhibition of MEK activity. Apoptosis induction resulting from the inhibition of MEK1/2 has been reported in several cell types, and can impact both the intrinsic and extrinsic pathways (Dai *et al.*, 2003; Liu *et al.*, 2006; Wang *et al.*, 2007; Lunghi *et al.*, 2008; Meng *et al.*, 2010; Pellicano *et al.*, 2011). Hence, the pro-apoptotic effect of *F. alocis* may be related to its ability to suppress MEK activity. It is also possible that the pro-inflammatory cytokines induced by *F. alocis* may play a role in apoptosis, and the matter requires further investigation.

In conclusion, we have begun the characterization of the virulence properties of the recently recognized periodontal pathogen *F. alocis*. This organism can induce the secretion of pro-inflammatory cytokines from GECs. In addition, *F. alocis* causes apoptosis in GECs coincident with the suppression of MEK1/2 activation. The pro-inflammatory, pro-apoptotic phenotype of *F. alocis* may have relevance to the pathogenesis of periodontal disease.

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