Role of *Fusobacterium nucleatum* in Periodontal Health and Disease

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Abstract
The pathogenesis of periodontitis involves the interplay of microbiota present in the subgingival plaque and the host responses. Inflammation and destruction of periodontal tissues are considered to result from the response of a susceptible host to a microbial biofilm containing gram-negative pathogens. Antimicrobial peptides are important contributors to maintaining the balance between health and disease in this complex environment. These include several salivary antimicrobial peptides such as β- defensins expressed in the epithelium and LL-37 expressed in both epithelium and neutrophils. Among gram-negative bacteria implicated in periodontal diseases, *Fusobacterium nucleatum*, is one of the most interesting. This review will focus on expression, function, regulation and functional efficacy of antimicrobial peptides against *F. nucleatum*. We are looking for how the presence of *F. nucleatum* induces secretion of peptides which have an impact on host cells and modulate immune response.

Introduction
Mucosal epithelial cells play an important role in the innate immune defense system by sensing signals from their environment, generating numerous molecules which affect growth, development and function not only of themselves but of other cells too, and maintaining the balance between health and disease (Kagnoff *et al.*, 1997). Gingival epithelium is a stratified squamous epithelium surrounding the tooth and forming an attachment to the tooth surface. It functions as a protective barrier against pathogenic microorganisms in dental plaque. Epithelial cells are in constant contact with bacteria or bacterial products from supra- and sub-gingival biofilms on the tooth surface. Before recent studies, the oral epithelium was considered as a passive covering that becomes damaged during the disease. Recently, the view has changed and the gingival epithelium is seen as providing not only a physical barrier to infection but playing an active role in innate host defense.

Epithelial cells respond to bacteria in an interactive way: they produce antimicrobial peptides, as chemokines that attract monocytes and neutrophils, cytokines that activate the adaptive immune system. Antimicrobial peptides are important contributors to maintaining the balance between health and disease in this complex environment. These include several salivary antimicrobial peptides, the β-defensines expressed in the epithelium, α-defensines expressed in neutrophils, and the cathelicidine LL-37, expressed in both epithelium and neutrophils.

One of the most studied bacteria implicated in periodontal disease is *F. nucleatum*. It belongs to the Bacteroidaceae family and is a dominant micro-organism within the periodonticum. It is a gram-negative anaerobic species of the phylum *Fusobacteria*, numerically dominant in dental plaque biofilms, and important in biofilm ecology and human infectious diseases. Dental plaque is a complex and dynamic microbial community that forms a biofilm on teeth, and harbors more that 400 distinct species *in vivo*. *F. nucleatum* is a prominent component quantitatively and is one of the first Gram-negative species to become established in plaque biofilms. It is a central species in physical interactions between Gram-positive and Gram-negative species that are likely to be important in biofilm colonization, and contributes to the reducing conditions necessary for the emergence of oxygen-intolerant anaerobes: it is considered as an intermediate colonizer bridging the attachment of commensals that colonize the tooth and epithelial surface with true pathogens (Kolenbrander, 2000 and 2002). *F. nucleatum* is also one of a small number of oral species that is consistently associated with, and increased in number at, sites of periodontitis, one of the most common infections in humans. *F. nucleatum* is not responsible for destructive periodontal disease, which is a major cause of tooth loss.

It is one of the most common oral species isolated from extra-oral infections, including blood, brain, chest, lung, liver, joint, abdominal, obstetrical and gynecological infections and abscesses. Further, *F. nucleatum* is a common anaerobic isolate from intrauterine infections and has been associated with pregnancy complications including the delivery of premature low birth weight infants. Thus, *F. nucleatum* is a significant pathogen in human infections, including several infections with real societal impact.

Antimicrobial peptides implicated in host response
F.nucleatum reacts on inflammatory response during periodontal disease. Within the gingival epithelium exposed to the bacterial biofilm, which forms at the surface of the tooth, some antimicrobial peptides have a crucial role in the maintenance of periodontal health.

Among these peptides, defensins seem to be the most studied. It was the first antimicrobial peptide identified in oral epithelium, described in bovine tongue (Zasloff et *al.,* 1995).

Defensins constitute a family of antimicrobial peptides largely implicated in innate immunity. They possess a broad-spectrum activity and play not only a role in infectious diseases, but also modulate the inflammatory response. They are present in gingival epihelia, saliva, and gingival crevicular fluid, placing them as a first line of defense in the oral cavity.

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Defensins participate in the awakening of the acquired immune response through chemotaxis of immature dendritic cells and memory T cells (by interacting with chemokine receptor CCR6). The hβD-2 gene responds to nuclear transcription factor NF-kB, which in turn is activated in response to lipopolysaccharide and proinflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and interleukin 1β(IL-1β). β-defensins could be a link between innate and adaptive immune responses.

These peptides are constituted from 41 to 50 amino acids, with 3 disulfide bonds localized at cysteine residues 1-5, 2-4 and 3-6. β-defensins are found as four different types in human (hBD 1-4) and it is believed, based on genomic targeting, that 28 other human β-defensins may exist. Within the gingival epithelium, keratinocytes are able to secrete these peptides.

Therefore, β-defensins elicit intracellular Ca^{2+} mobilization, and increased keratinocyte migration and proliferation (Niyonsaba *et al*., 2006). These peptides induced phosphorylation of EGFR, signal transducer and activator of transcription (STAT1), and STAT3, which are intracellular signalling molecules involved in keranitocyte migration and proliferation.

In most epithelia, including healthy gingival epithelium, hβD-1 is constitutively expressed, whereas hβD-2 and hβD-3 appear to be highly inducible by cytokines or after exposure to microorganisms.

Activities of these peptides against oral bacteria

Activity of hβD-2 against periodontopathogenic bacteria seems to be very important because this peptide was found to be 10-fold more potent than hβD-1 and exhibited activity against *Pseudomonas aeruginosa* at physiological concentrations (100 ng/ml) (Joly *et al*., 2003). In contrast, hβD-3 has shown broad-spectrum activity against both gram-negative and gram-positive bacteria at concentrations much lower than those for other members of the β-defensin family. In addition, its activity appears to be less salt-sensitive than those of hβD-1, -2, and -4. Therefore, hβD-3 is considered as the most potent β-defensin peptide described thus far. This is because hβD-3 is the most basic and positively charged peptide among those tested.

Several studies have shown susceptibility of bacteria to β-defensins. Some results showed that early microbial colonizers, even if they are very sensitive to these peptides, don't upregulate them. For hβD-2 and hβD-3, *in vitro* studies show that aerobes (*Streptococcus sanguis*, *Streptococcus mutans*, *Actinomyces naeslundii*, *Actinomyces israelii*, and *Escherichia coli*) were more susceptible to hβD-2 and hβD-3 than anaerobes (*Actinobacillus actinomycetemcomitans*, *F. nucleatum*, *Porphyromonas gingivalis*, and *Peptostreptococcus micros*). Therefore, small quantities are sufficient to inhibit growth of these aerobic bacteria; this could allow the organism to limit colonization by early colonizers. Results for anaerobic bacteria, as *F. nucleatum*, are different; important concentrations are necessary to inhibit growth of some *F. nucleatum* strains: in the study by Joly *et al.*, concentrations of 250 µg/ml are not sufficient to inhibit growth of some strains, but other strains are very sensitive (with the same concentration of commensal aerobic bacteria). This could be explained by the fact that aerobic and anaerobic bacteria demonstrated strain rather than species specificity in their susceptibilities to hβD-2 and hβD-3. Both peptides were active against selected gram-positive and gram-negative bacteria. Therefore, hβD-3 demonstrated greater activity against a broader array of organisms than hβD-2 and greater antimicrobial activity than hβD-2: this difference could be due to the capacity of hβD-3 to interact with different targets, while hβD-2 seems to interact preferentially with LPS.

However, the activities of hβD-2 and hβD-3 still appeared to be associated. This suggests that while hβD-2 and -3 may share a similar target, they may also possess very specific mechanisms of action. This hypothesis is supported by the fact that the antimicrobial activity of hβD-3 is not affected by increased ionic strength (unlike that of hβD-2), suggesting that binding of hβD-3 to a negatively charged bacterial membrane may not be its only mechanism of action. Starner *et al.* demonstrated that unlike that of hβD-2, the activity of hβD-3 was not mediated by binding to the lipopoligosaccharide of *Haemophilus influenzae*, suggesting that hβD-3 interacts with different binding sites or possesses a different mechanism of action than hβD-2. It has been theorized that hβD-3 could be more active in part due to a higher net cationic charge than that observed for hβD-2 or that it has the ability to form dimers. To explain the difference of susceptibility of different *F.n* strains, some authors say that β-defensins targets may be absent or modified in anaerobes. The resistance to antimicrobial β-defensins and other peptides may also be due to altered outer membrane proteins or altered lipopolysaccharide (LPS) structures. Recently, Brissette and Lukehart demonstrated that *Treponema denticola*, which lacks a traditional LPS, was naturally resistant to hβD-2. The fact that LPS or lipooligosaccharide can be variable between strains of the same species could partially explain the variable susceptibility pattern observed within a species, where, for example, *F. nucleatum* 49256 was very susceptible to the defensins tested compared to *F. nucleatum* 1594. Modification of such molecules by bacteria may be part of their strategy to evade the activities of antimicrobial peptides. Starner *et al*. demonstrated that the susceptibility of *H. influenzae* to hβD-2 was influenced by lipooligosaccharide acylation of the membrane, which has been associated with *P. aeruginosa* resistance to cationic antimicrobial peptides. Interestingly, *P. gingivalis*, one of the most resistant species in this study, and to a lesser extent *F. nucleatum*, are notorious for their production of a wide variety of proteolytic enzymes, which have been implicated in the inactivation of several known antimicrobial peptides. In conclusion, susceptibility to β-defensins doesn't seem to be specific to the species. For *F. nucleatum,* there is, *in vitro,* a strain specific rather than species specific activity.

Susceptibilities of *Fusobacterium nucleatum* to antimicrobial peptides

Numerous results show a great susceptibility of *F.nucleatum* to hβD-3. Ouhara *et al.* showed in 2005 that, compared with Gram-positive bacteria, Gram-negative bacteria, except *F. nucleatum*, tended to show low susceptibility to antimicrobial peptides. The strain *F. nucleatum* 21 had a remarkable susceptibility to hβD-3 (and LL37), having 100% susceptibility in the presence of 1 mg/L of the peptides. The MICs of hβD3 and LL37 for almost all *P. gingivalis, P. intermedia and A. actinomycetemcomitans* strains were 100 or 200 mg/L, whereas the MICs of the peptides for *F. nucleatum*

showed lowvalues (12.5 or 25 mg/L). As for Gram-positive bacteria, the MICs of the peptides were relatively lower than those for Gram-negative bacteria except for *F. nucleatum*. Comparison among the strains of the antibacterial effect of growing (microdilution method) and non-growing (PB) conditions revealed that there was no difference in terms of antimicrobial activity. The susceptibility of all *F. nucleatum* strains to hβD-3 and LL37 was higher than those of other species ((*A. actinomycetemcomitans* (20 strains), *P. gingivalis* (6), *Prevotella intermedia* (7), *F. nucleatum* (7), *S. mutans* (5), *Streptococcus sobrinus* (5), *Streptococcus salivarius* (5), *S. sanguis* (4), *Streptococcus mitis* (2) and *Lactobacillus casei* (1)). Among periodontopathogenic bacteria, all *F. nucleatum* strains tested in this study showed the highest sensitivity to hβD-3 and LL37 when compared with those of other bacteria. Authors measured the Zeta-potential, representing the net charge of whole bacteria, to study the relationship between susceptibility to cationic peptide and the net charge of the bacteria. Although they found some correlation in *A. actinomycetemcomitans* strains, they did not find a definite correlation with all the bacterial species. However, the net charge (negative charge) of *F. nucleatum* was not so strong compared with those of other Gram-negative bacteria. Therefore, the high susceptibility of *F. nucleatum* is not only due to the net charge, but also involves other factors (maybe chemical composition of LPS and/or the membrane in *F. nucleatum*).

These results are confirmed by other studies, *F. nucleatum* being the most sensitive of all bacteria tested.

Impact of *F. nucleatum* on defensin production

It is interesting to observe differences in susceptibility to β-defensins, but knowing if *F. nucleatum* can induce production of these peptides is very important too.

Generally, β-defensins are found in greater quantities in healthy than in inflamed tissues. Interestingly, some studies show significantly higher levels of hβD-3 expression in the healthy tissues as compared to the diseased ones. There was also a suggestion of higher expression of HβD-2 in the healthy tissues. Levels of hβD-1, hβD-2 and hβD-3 mRNA expression were correlated with one another. No difference was observed between levels of hβD-1 mRNA expression in healthy and diseased tissue samples. Furthermore, a study showed that hβD-1 mRNA is constitutively expressed in keratinocyte cell cultures and is not up-regulated when exposed to inflammatory mediators. These data from clinical samples confirm the constitutive or basal nature of hβD-1 mRNA expression, as the majority of samples in both the healthy and diseased categories demonstrated a low level expression with semi-quantitative PCR.

High levels of hβD-3 mRNA expression in healthy tissues suggest a potentially important protective role for defensins in the host immune response to infection by periodontal pathogens.

Localization studies (Bissel *et al*., Lu *et al*.) have shown that mRNA for hβD-1 and hβD-2 is most strongly expressed in the spinous layers of normal gingiva, whereas the peptides are present in more superficial epithelial layers, placing them in optimal defense position against bacterial infection. Dale and Krisanaprakornkit reported that mRNA expression for hβD-1 and hβD-2 was strongest at the gingival margin, adjacent to plaque formation, and in inflamed sulcular epithelium. Lu *et al.* showed that both hβD-1 and -2 peptides were detected (immunohistochemistry and quantitative analyze) in all periodontally healthy subjects, while hβD-1 was detected in all patients (healthy and patients with unresolved chronic periodontitis) and hβD-2 was found in most of the patients. Their expression was mainly confined to the granular and spinous layers of gingival epithelium, in which hβD-1 was detected in both intercellular spaces and cytoplasm, whereas hβD-2 was mainly observed in the cytoplasm.

These results are unexpected because the induction of defensins by periodontopathogenic bacteria seem to increase levels of these peptides. Many reports have shown an induction of these defensins in cell culture models with various inflammatory mediators (IL-1β, TNF-α, IFN-γ) and LPS, as well as increased expression in inflamed tissues. Taggart in 2003 showed that hβD-2 and -3 were degraded by the cysteine proteases cathepsins B, L and S: this study suggested that during infection, enhanced expression of cathepsins may increase degradation of hβD-2 and -3, with resultant bacterial colonization and infection. This would support the finding of diminished expression in periodontally diseased tissues.

F. nucleatum could have the capacity to induce a down-regulation of hβD-1 and LL-37: this down-regulation of the host defense may be another bacteria- mediated virulence mechanism.

Levels of mRNA are not necessarily correlated with increased or diminished amounts of functional peptides. Previous studies indicated that hβD-1 -2 and -3 showed poor antimicrobial activity against anaerobic periodontopathogenic organisms. Their primary activity may be directed against the earlier colonizers or commensal flora.

Regarding protein expression, hβD-2 is known to be upregulated after exposure to 2 periodontal bacteria (*F. nucleatum* and *A. actinomycetemcomitans*) which can also upregulate hβD-3. In this state, oral epithelium could be partially stimulated, giving the host the capacity to limit bacterial growth.

Some studies show that *F. nucleatum* doesn't stimulate hβD-1 production but has a stimulating effect on hβD-2 production. For hβD-3, a low but significant effect was seen for this bacteria. We could hypothesize that commsensal bacteria activate or keep the innate immune response in a limited activated state without being affected by it. By not stimulating hβDs or only to a low extent, these oral commensals seem to evade the innate host defense system and are able to maintain the colonization. From the host's point of view, by not upregulating hβDs the epithelium seems to be able to maintain these presumed protective commensals. On the other hand, high induction of other peptides as IL-8 can serve as a continuous stimulus to attract polymorphonuclear cells to the periodontal area where they are needed to protect the environment for new colonizing pathogens or to prevent the outgrowth of pathogens.

These conclusions confirm the hypothesis that stratified epithelia are able to develop a steady state and control innate immune response in presence of commensal bacteria, and expression of hβD-2 is the first step. These results are confirmed by other studies showing a high induction of hβD-2 production. hβD-2 expression was induced by cell wall extract of *F. nucleatum* but not by those of periodontal pathogens as *P. gingivalis* (bacteria of the red complex) or *P. intermedia* (bacteria of the orange complex).

hβD-2 peptide was inducted by TNFα and Phorbol myristate acetate (PMA), an epithelial cell activator (Krisanaprakornkit *et al.*, 2000). Kinetic analysis indicates involvement of multiple distinct signaling pathways in the regulation of hβD-2 mRNA. TNF-α and *F. nucleatum* cell wall induced hβD-2 mRNA rapidly, while PMA stimulation was slower. However, the role of TNFα as intermediary in *F. nucleatum* signaling was ruled out by addition of anti-TNFα that did not inhibit hβD-2 induction. Indeed, inhibitor studies show that *F. nucleatum* stimulation of hβD-2 mRNA requires both new gene transcription and new protein synthesis.

These hypotheses are confirmed: according to Chung and Dale in 2004 and Krisanaprakornkit in 2002, hβD-2 up-regulation in response to oral commensal bacteria does not seem to utilize the NF-kB intracellular signaling cascade typically associated with recognition of bacterial products, but instead utilizes the JNK and p38 mitogen activated protein kinase (MAPK) pathways that are associated with cytokine and stress responses. Intracellular calcium signaling is also involved (Krisanaprakornkit *et al*., 2003). Utilization of this signaling pattern has now been extended to show that commensal organisms from both oral and skin sites do not utilize the NF-kB pathway for hβD-2 up-regulation, in contrast to pathogens which use an NF-kB pathway (Chung and Dale, 2004). This may be due to the presence of commensal bacteria or to hβD-2 itself, which has been shown to act in a cytokine-like manner toward dendritic cells and peripheral blood mononuclear cells (Boniotto *et al*. 2006, Durr and Peschel, 2002, Niyonsaba *et al*., 2005, Yang *et al*., 1999).

Induction of immune response due to *F. nucleatum*

F. nucleatum induces significant changes in the expression of genes associated with immune defence responses. Studies show that *F. nucleatum* wall extracts induce significant changes in the expression of genes associated with immune and defence responses. The 20 most highly up-regulated genes include CCL20, S100A7, SKALP, IL8, IL1F9, CXCL5, C3, IL32, SAA1, SPRR2C and CXCL1. Fourteen out of twenty were cytokines, innate immune or inflammatory markers, antimicrobials, or protease inhibitors, while two additional strongly up-regulated genes (SPRR2B, SPRR2C, small proline-rich proteins) are related to structural aspects of the epithelial layer. The most down-regulated genes included cell cycle regulatory genes (CDC20, SKP2, PCNA, POLE2) and ubiquitine-proteasome-associated genes (UBAP2L, PSMD11).

Genes up-regulated by *F. nucleatum* included those encoding antimicrobial peptides and proteins; additional genes of defence responses include chemokines IL8 and CXCL1, 3,5 and 10, which attract neutrophils, monocytes and macrophages, or lymphocytes and CSF2 and -3 that stimulate neutrophil development.

Neutrophils are part of the continuous surveillance of the gingival sulcus. However, proteases released by neutrophils contribute to inflammation and tissue damage. Multiple protease inhibitors were strongly up-regulated in response to *F. nucleatum* cell wall extracts but not to hβD-2. These inhibitors are expected to target proteases released by neutrophils and therefore control potential tissue damage (Magert *et al*., 2005), and represent a protective response in the presence of commensal bacteria. Therefore, these protease inhibitors may protect against bacteria proteases secreted by pathogens. Periodontal pathogens, *P. gingivalis*, *T. denticola* and *Tannerella forsythensis*, have serine or cysteine proteases that are important virulence factors (Curtis 2001, Fenno, 2001, Van der Reijden, 2006). Cysteine proteases (gingipains) of *P. gingivalis* stimulate protease-activated receptors (Chung, 2004, Uehara, 2002) and this family of receptors has been implicated in periodontal disease (Holzhausen *et al.* 2005, 2006). In conclusion, up-regulation of protease inhibitor genes by commensal bacteria may specifically block effects of pathogenic bacteria, such as *P. gingivalis*, as well as limiting inflammatory tissue damage caused by neutrophil proteases.

Moreover, multiple genes that reduce NF-kB function were up-regulated with *F. nucleatum* cell wall extracts. NF-kB is a critical transcription factor involved in inflammatory responses such as IL-8 up-regulation.

In summary, *F. nucleatum* not only induces hβD-2 peptide, but influences immune response through the induction of cytokines and chemokines and probably suppression of NF-kB function, consistent with its expression in uninflamed oral tissue. *F. nucleatum* contributes to the maintenance of a healthy mucosal surface by increasing transcription of many protease inhibitors whose translation as active inhibitors may block tissue damage by proteases from neutrophils, which are continually migrating into the oral cavity via the gingival sulcus.

Interaction between LL-37 and *Fusobacterium nucleatum*

In mammalian skin, the other major class of anti-microbial peptides identified are cathelicidins. The sole cathelicidin in humans is LL-37/hCAP18 (human cationic peptid 18 -18 kDa-), and is expressed in leukocytes and in a kind of epithelial surface. LL-37 was also detected directly in human skin keratinocytes, but only where inflammation was present, suggesting this class of anti-microbial peptides functions primarily in response to injury rather than in modulating the surface colonization of the skin (Frohm *et al*., 1997). *F. nucleatum* is highly susceptible to LL-37, contrary to *P. gingivalis*, *S. sanguinis* and *Candida* species.

LL-37 has the property to link to and to neutralize LPS of bacteria, preventing a strong stimulation of cells, like macrophages. They have affinity for LPS, and these peptides can suppress cytokine production in response to endotoxic LPS and to varying extents can prevent lethal endotoxemia. In addition to its bactericidal activity, LL-37 is a chemoattractant for immune and inflammation cells, including T cells, neutrophils, monocytes and mast cells. It appears that LL-37 may serve not only as a bactericidal substance but also as an alarm signal when pathogens invade. The cathelin-like domain of LL-37 has antiproteasic properties which inhibits bacterial growth and limits tissue damage. This cathelicidin can stimulate the liberation of inflammation mediators as IL-8, MCP-1, IL-1β and TNFα.

LL-37 also causes functional changes in mast cells. Mast cells in the skin are involved in the innate immune system response against microbial infections *via* Toll-like receptors, such as TLR4, which is known to recognize LPS. They observed that LL-37 increased the level of TLR4 mRNA and TLR4 protein, and that LL-37 induced the release of IL-4, IL-5 and IL-1β from mast cells. Studies show that, although the up-regulation of LL-37-inducible Th2 cytokines

was cancelled by LPS, the increase of pro-inflammatory cytokine production was still observed. Theses findings indicate that LL-37 co- existing with the bacterial component switches mast cell function and directs human mast cells toward innate immunity. LL-37 may be a candidate modifier of the host defense against bacterial entry by serving as an alarm for sentinels such as mast cells.

These peptides possess bactericidal activity against a broad spectrum of microbes, including Gram-positive and negative bacteria, fungi and certain viruses.

LL-37, as human β-defensins, stimulates keratinocytes to increase their gene expression and protein production of IL-6, IL-10, IP-10, monocyte chemoattractant protein-1, macrophage inflammatory protein-3α, and RANTES. This stimulatory effect was markedly suppressed by pertussis toxin and U-73122, inhibitors of G protein and phospholipase C, respectively.

A genetic form of periodontal disease in young people, Morbus Kostmann syndrome, was shown to have a deficiency in the α-defensins, and a near absence of LL-37 (Putsep *et al.* 2002). This led to the suggestion that LL-37 may be particularly important for its effects vs. Gram-negative bacteria, especially *A. actinomycetemcomitans*, an organism associated with rapidly progressive periodontal disease especially in young people.

Impact of *Fusobacterium nucleatum* on IL-8 secretion

Regarding IL-8, results are often contradictory. Gursoy *et al*. studied the capacity of different strains of F. *nucleatum* to stimulate IL-8: they show that *F.n* bacteria increase IL-8 secretion by epithelial cells, and stimulate IL-8 liberation via LL-37. Other studies confirm this hypothesis.

However, other studies seem to show that *F.n* doesn't induce IL-8 secretion by not activating NF-kB signaling pathways, but MAP kinase signaling. Studies showed that hβD-2 was induced by *F. nucleatum* cell wall extracts without the involvement of transcriptional factor NF-kB, typically associated with innate immunity and inflammation. They showed that cell wall preparations of *F.n* were particularly effective in up-regulating hβD-2 mRNA expression but this up-regulation is not coupled with general signaling of other aspects of the innate immune system, such as IL-8 expression. Furthermore, it does not utilize the NF-kB intracellular signaling cascade typically used by proinflammatory stimuli, but instead utilizes a mitogen activated protein (MAP) kinase signaling cascade (*F. nucleatum* involves MAP kinases JNK and p38 preferentially) that is associated with cytokine and stress responses.

***Fusobacterium nucleatum*: a protective or an aggressive bacterium?**

Theses results seem to classify *F. nucleatum* as a commensal bacteria: poor inducer of an immune response, important susceptibility to cytokines (β-defensins, LL-37) and to phagocytosis: the minimum inhibitory concentrations of LL-37 and human beta-defensin-3, and the susceptibility to phagocytosis, were converted to a susceptibility index with scores ranging from + to ++++, in a study of "Susceptibility of various oral bacteria". *F. nucleatum* was the most susceptible bacterium, having a total score of 12+; whereas *S. gordonii* and *T. forsythia* were the most resistant, having a total score of 4+.

However, *F. nucleatum* can induce an inflammatory response by upregulating pro-inflammatory cytokines as well as metalloproteases. All *F. nucleatum* strains induce secretion by activated epithelial cells of a number of proteolytic enzymes, including metalloproteases (MMPs) that modify inflammatory reactions and facilitates cell migration. *F. nucleatum* has been shown to be a potent inducer of collagenase 3 (MMP-13) production. Both MMP-13 and IL-8 production is at least partly regulated by p38 MAP kinase signaling in the epithelial cells infected by *F. nucleatum*. *F. simiae*, and to a lesser extent *F. nucleatum* and *F. necrophorum* increased epithelial MMP-2 secretion. MMP-2 (gelatinase A) has several functions in the control of inflammation. We can conclude that the pathogenic potential of fusobacteria may partly result from their ability to stimulate secretion of MMP-9, MMP-13 and IL-8 from epithelial cell.

Moreover, *F. nucleatum* increased levels of 12 protein kinases involved in cell migration, proliferation, and cell survival. Of the 13 anaerobic oral bacterial species, *F. nucleatum* and *necrophorum* were among the best inducers of collagenase 3 mRNA levels, a powerful matrix metalloproteinase. This suggests that *F. nucleatum* may be involved in the pathogenesis of periodontal diseases by activating multiple cell signaling systems that lead to stimulation of collagenase 3 expression and increased migration and survival of the infected epithelial cells (Uitto *et al*., 2005). A crucial part of the epithelial defense system is the efficient recruitment of professional defense cells, especially of neutrophils, to the infection site. IL-8 is a key cytokine in this process. Studies about the amount of IL-8 in a medium of fusobacteria-treated epithelial cells show that all *Fusobacterium* species were able to induce IL-8 in vitro. The best inducers were *F. necrophorum*, *F. nucleatum* AHN 9500 and *F. varium*.

F. nucleatum can also secrete serine proteases. Proteases are considered as virulence factors employed by several periodontal pathogens including *P. gingivalis* and *T. denticola*. While supplying the nutritional requirements of these oral microorganisms, proteases were found to degrade elements of the periodontal connective tissue and the host defense systems such as immunoglobulins and complement. Proteases can inactivate key components of the plasma proteinase cascade and blood clotting systems, and degrade serum protease inhibitors. The *F. nucleatum* protease was found to degrade the extracellular matrix proteins fibrinogen and fibronectin as well as collagen I and collagen IV. The 65 kDa protease is also able to digest the α-chains of immunoglobulin A but not immunoglobulin G. This protease, able to degrade native proteins, may play an important role in both the nutrition and pathogenicity of these periodontal microorganisms. The degradation of extracellular matrix proteins by bacterial enzymes may contribute to the damage of periodontal tissues, and degradation of IgA may help the evasion of the immune system of the host by the bacteria. In comparison with the proteolytic activity of other periodontal bacteria, the specific activity of this protease is very low. The purified *F. nucleatum* 65 kDa protease required overnight incubation to reach the same proteolytic activity obtained by the *Treponema denticola* phenylalanine protease after 2 hours. The fact that *F. nucleatum* coaggregates, and is most often found together with other highly proteolytic microorganisms, might reduce the necessity for *F. nucleatum* to possess a strong proteolytic activity.

One of the last properties showing a potential pathogen role for *F. nucleatum* is his immunosuppressive role, a property of periodontopathogenic bacteria of the red complex. Studies show that this role is largely due to the ability of this organism to induce apoptotic cell death in peripheral blood mononuclear cells (PBMCs) and in polymorphonuclear cells (PMNs). The ability of *F. nucleatum* to induce apoptosis was abolished by either heat treatment or proteinase digestion but was retained after formaldehyde treatment, suggesting that a heat-labile surface protein component is responsible for bacterium-mediated cell apoptosis. Data also indicated that *F. nucleatum*-induced cell apoptosis requires activation of caspases, or cysteine-aspartic acid proteases - caspases are a family of cysteine proteases, which play essential roles in apoptosis, necrosis and inflammation - and is protected by NF-kB.

The immunosuppressive nature of certain invasive pathogenic oral bacteria, like *F. nucleatum*, has been reported previously. Inhibition of both B- and T- cell functions have also been reported in the presence of *F. nucleatum*. However, the detailed mechanisms of *F. nucleatum*-mediated immunosuppression have yet to be established. NF-kB and interleukin- converting enzyme (ICE) pathways seem to be involved in *F. nucleatum*- mediated lymphocyte death.

Initial observations by Shenker and Dirienzo indicated significant inhibition of peripheral blood lymphocyte function by cytoplasmic extracts obtained from *F. nucleatum*. Since then, the authors have purified and characterized FIP (*F. nucleatum* inhibitory protein) as the putative protein responsible for the immunosuppressive effect mediated by *F. nucleatum*. FIP was shown to elicit arrest at the G0/G1 phase of the cell cycle.

Preceding the induction of apoptotic cell death by *F. nucleatum*, significant aggregation of PBMCs was observed within a few minutes of the addition of that oral bacterium and not the other oral bacterial species tested (*P. gingivalis*, *P.intermedia*, *T. denticola*). Only viable or formaldehyde-treated *F. nucleatum* cells were able to induce aggregation and the induction of death in PBMCs. The ability of *F. nucleatum* to induce aggregation and apoptotic cell death was lost when the bacterium was killed by heat treatment. A close relationship was observed between the ability of *F. nucleatum* to induce aggregation of the PBMCs and its ability to cause apoptotic cell death.

It is possible that aggregation is a necessary step for the induction of death in PBMCs. *F. nucleatum*-mediated aggregation of peripheral blood lymphocytes has also been observed by Kinder Haake and Lindemann. The aggregation of PBMCs was inhibited by L-arginine, L-lysine, and heat treatment. Phytohemagglutinin-stimulated DNA synthesis and interleukin 2Rα expression of PBMCs were also inhibited in the presence of *F. nucleatum*.
F. nucleatum directly delivers death signals to PBMCs through the binding of a putative surface protein. It is likely that *F. nucleatum* delivers a direct death signal through its surface component as well as aiding in the upregulation of cell death machinery (Fas- and TNF receptor-mediated signaling) in PBMCs.

One possible explanation for bacterium-induced apoptosis is that bacterial lipopolysaccharide mediates the induction of cell death by triggering TNF-α release by PBMCs. However, these preliminary data are inconsistent with this hypothesis because LPS is heat stable, while the putative bacterial apoptosis-inducing mocelule(s) is(are) heat labile. Moreover, other bacteria were tested and, while all were able to induce the production of TNF-α at similar levels, only *A. actinomycetemcomitans* and *F. nucleatum* were able to induce apoptosis of PBMCs. Therefore, the rate of apoptosis of lymphocytes induced by the addition of exogenous TNF-α was much less than that induced by the bacteria.

F. nucleatum induced significantly higher levels of death in PMNs than in PBMCs when they were cocultured in the presence of similar numbers of the oral bacteria. PMNs are important effector cells in first-line defense against bacterial pathogens. Indeed, induction of death in both PMNs and PBMCs by *F. nucleatum* indicates the ability of this organism to mediate a generalized paralysis of the immune system. Although any significant induction of death by *F. nucleatum* on Cal 27 and SCC4 oral keratinocyte cell lines, the effect of this bacterium on normal human keratinocytes remains to be elucidated. Sonicated extracts from *F. nucleatum* and *A. actinomycetemcomitans* have been shown to have cytotoxic effects on human gingival fibroblasts. The overall paralysis of immune function by *F. nucleatum* might play an important role in initiation and progression of periodontal disease. We could emit one hypothesis regarding the role of *F. nucleatum*: initial colonization and increase in the number of *F. nucleatum* cells can cause depletion of immune cells at the site of the infection due to the induction of apoptotic cell death. This immunosuppression will lead to the recruitment and the binding of other pathogenic microorganisms to the sites previously colonized by *F. nucleatum*.

Is this immunosuppression positive or negative for the evolution of the periodontal disease? By eliminating immune cells that are important for immune defense against oral bacteria, *F. nucleatum* can contribute to the recruitment of other pathogenic bacteria and subsequently to the initiation and the progression of periodontal disease.

Indeed, positive association between *F. nucleatum*, *P. gingivalis* and *P. intermedia* and *Bacteroides forsythus* in sub-gingival plaque samples have been reported previously.

More importantly, colonization by *P. intermedia* was found to be due to *F. nucleatum*, since *P. intermedia* was never detected in a site unless *F. nucleatum* was also present. Combinations of *F. nucleatum*, *B. forsythus*, and *Campylobacter rectus* were also found in periodontal sites that had the most attachment loss and the deepest pockets. Complexes formed by *F. nucleatum*, *B. forsythus* and *C. rectus* were also found in sites refractory to treatment.

Increase in the number of bacteria associated with *F. nucleatum* might later serve to recruit and activate local immune cells, resulting in tissue destruction and the progression of periodontal disease. Indeed, colonization by other oral bacteria can serve to either compete with or cover the sites on *F. nucleatum* which are responsible for the induction of death in PBMCs.

Conclusion

All these results show the complexity of the immune defense in the oral cavity in order to ensure a balanced state of oral health. It is very difficult to determine, considering *F. nucleatum's* properties, if this bacteria is commensal or pathogenic. *F. nucleatum* has periodontopathogenic

properties but the susceptibility of this bacteria seems to show that it is a very sensitive microorganism. However, *F. nucleatum* has coaggregration properties which allows it to transport periodontopathogenic bacteria.

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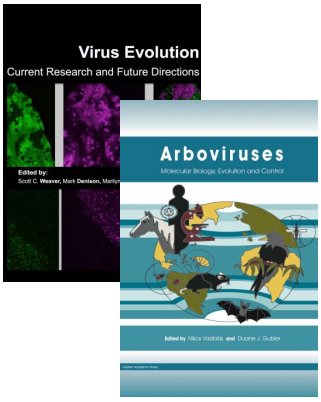

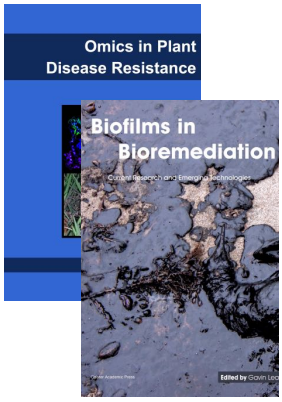

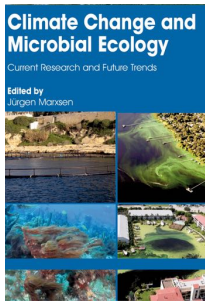

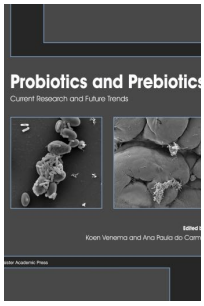

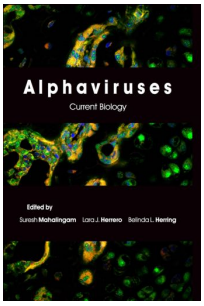

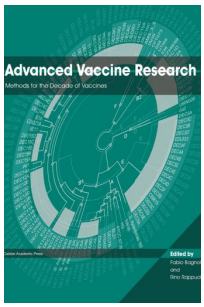

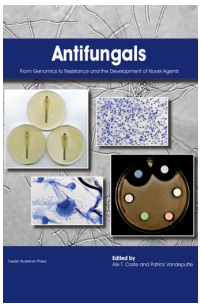

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