Oral Epithelial Cell Sensing of LPS:

A Novel Protection Mechanism of Oral Epithelium

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Abstract

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The oral epithelium plays a pivotal role in protecting the underlying periodontal tissues from the microbial community found in the periodontal pockets. Having an appropriate phenotype displayed by oral epithelial cells (OECs) is a critical host component required for protection against bacterial invasion into gingival tissues. In the present study, oral epithelial homeostasis associated with the CXCL-8/IL-8 chemokine response was investigated *in vitro* to determine the mechanisms which OECs utilize for sensing gram-positive and gram-negative microorganisms. The findings of this study have demonstrated a heterogeneous responsiveness of OECs by Toll-like receptor (TLR) 2, a lipoprotein sensor. Notably, however, lipopolysaccharide (LPS), a major virulence factor of gram-negative bacteria, is not recognized by OECs unless the LPS is internalized into OECs. Activation of TLR4 in OECs occurs in the endosome, an intracellular event that requires an unidentified mechanism involving protein transport from the Golgi apparatus to the endosome. Although OECs are not able to detect

LPS present in the microenvironment, cell wall derived from an oral commensal-turned pathogen *Fusobacterium nucleatum* promotes LPS internalization that partially involves FadA adhesion function. Cell wall proteins obtained from other pathogenic microbes that induce CXCL-8 fail to interact with intracellular TLR4 in OECs. Collectively, TLR4 only plays a role when microbial invasion or cell wall uptake occurs in the oral epithelium and thus partially contributes to CXCL-8–mediated periodontal homeostasis. This study has identified a unique LPS sensing mechanism of the oral epithelium to overcome a periodontal infection associated with LPS derived from gram-negative microbes that arises during dysbiosis.

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GLOSSARY

Aa	*Aggregatibacter Actinomycetemcomitans*
Aae	*Aggregatibacter Actinomycetemcomitans* epithelial cell binding
ATCC	American Type Culture Collection
CD14	Cluster domain 14
CFU	Colony forming unit
CXCL	Chemokine ligand, the two N-ternimal cysteines of CXC chemokines are separated by one amino acid, represented in this name with an “X”
CW	Cell wall
ER	Endoplasmic reticulum
Ec	*Escherichia coli*
ELISA	Enzyme-linked Immunosorbent assay
Fn	*Fusobacterium nucleatum*
FnCW	*F. nucleatum* cell wall
FadA	*Fusobacterium* adhesin A
ΔfadA	*F. nucleatum* mutant lacking *fadA*
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GECs	Gingival epithelial cells
hBD	Human beta-defensin
HEK293	Human embryonic kidney 293 cells
hTIGK	Normal human telomerase-immortalized gingival keratinocyte
hTLR	Human Toll-like receptor
IECs	Intestinal epithelial cells
IFN-γ	Interferon-gamma
JE	Junctional epithelium
LPS	Lipopolysaccharide (TLR4 agonist)

LAL	Limulus ameobocyte lysate
MD2	Myeloid differentiation protein-2
MOI	Multiplicity of Infection
nGEC	Normal gingival epithelial cell; primary gingival epithelial cell
NF-κB	Nuclear factor – kappa beta
OECs	Oral epithelial cells
OKF6/TERT2	Normal human telomerase-immortalized oral keratinocyte derived from the floor of mouth
Pam_3CSK_4	Pam3CysSerLys4, a synthetic tripamitoylated lipopeptide that mimicks the acylated amino terminus of bacterial lipoproteins (TLR2/1 agonist)
PAMPs	Pathogen-associated molecular patterns
PAR	Protease-activated receptor
PMB	Polymyxin B
Pg	*Porphyromonas gingivalis*
Pi	*Prevotella intermedia*
PRAT4A	Protein receptor associated with Toll-like receptor 4
qPCR	Quantitative polymerase chain reaction
Sg	*Steptococcus gordonii*
siRNA	Small interfering RNA; short interfering RNA
TLR	Toll-like receptor

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DEDICATION

To the Chakri dynasty of Thailand,

the University of Washington,

My parents and family

CHAPTER I

INTRODUCTION

Oral/gingival epithelium is a specialized oral tissue responsible for defending against oral microbial communities in the periodontal pockets. Once activated by an interaction between pathogen-associated molecular patterns (PAMPs) and Toll-like receptors (TLRs) on the host cells, oral epithelial cells (OECs) and gingival epithelial cells (GECs) are capable of producing inflammatory and chemotactic cytokines and anti-microbial peptides in response to bacterial challenge. Theoretically, both gram-positive and gram-negative bacteria are capable of activating host cells through the recognition of lipoproteins and lipopolysaccharides (LPS) by TLR2 and TLR4, respectively. Regardless of the presence of TLR2 and TLR4 transcripts in OECs, previous studies have revealed an unresponsiveness of OECs to synthetic lipoprotein and purified *Escherichia coli* LPS (EcLPS). This suggests a unique feature of TLR response to bacterial cell wall components in OECs and a possible protective strategy mechanistically utilized by oral epithelium to mediate a proper innate immune response, but yet beneficial for circumventing a microbial milieu in the periodontium where LPS is found.

BACKGROUND, KNOWLEDGE GAP AND SIGNIFICANCE

A. Oral/gingival epithelial cells (OECs, GECs) act as a frontline defense against oral microbes, and host-microbial interplays are essential for gingival health homeostasis via human beta-defensin (hBD) and chemokine ligand (CXCL) induction.

Periodontitis is an infectious disease of the periodontal tissues in which multi-species of oral microorganisms colonize and further populate in the gingival sulcus, resulting in a chronic host-bacterial inflammatory reaction. Drastic shifts of the microbial population from healthy to diseased periodontium have been reported in numerous studies asserting that gram-negative asaccharolytic anaerobic bacteria are more prevalent in the diseased sites, while gram-positive bacteria are predominant in the healthy periodontium (Marsh 1994). However, constant changes of the relative microbial composition in dental biofilms can dynamically affect host cellular responses. In other words, an imbalance of the relative abundance of oral commensals and pathogens alters the magnitude of the host inflammatory response, potentially leading to excessive tissue inflammation and eventual periodontal tissue destruction (Kumar et al. 2006, Curtis, Zenobia, and Darveau 2011). Resistance to the intrusion of microbial components into deeper periodontal tissues by gingival epithelium is likely a crucial innate mechanism for maintaining healthy underlying connective tissues.

With a continual transitioning of microenvironments in the gingival sulcus, oral/gingival epithelium unavoidably confronts the diversity of oral microbial species found in dental plaque, and the inflammatory response by oral/gingival epithelium is thus modulated by the variable microbial consortium. A dysbiotic oral microbial community has a stronger ability to potentiate and alter host innate immune responses than does a symbiotic community (Hajishengallis et al. 2011, Maekawa et al. 2014, Kolenbrander et al. 2006). In addition, subgingival plaque collected from healthy sites of periodontium differentially modulate host innate immune surveillance systems at both human TLR2 and TLR4 as compared to the plaque collected from diseased sites (To et al. 2016), suggesting that TLR2 and TLR4 of OECs are constantly challenged with an oral microbial community and possess a wide range of immunomodulatory activities.

Numbers of studies have investigated the expression profiles of TLRs in the eukaryotic cells and their roles in response to the specific ligands. Although there are thirteen mouse TLRs that have been identified to date, only ten TLRs are reported to be functional in human cells (Akira, Takeda, and Kaisho 2001) due to the presence of the stop codon in open reading frame of human TLR11 genomic sequence (Zhang et al. 2004), and the human homologs of TLR 12 and TLR13 have yet to be identified (Sasai and Yamamoto 2013, Tabeta et al. 2004).

Most of TLRs, i.e., TLR1, TLR2, TLR4, TLR5, and TLR6, are located exclusively on the cell surface and sense components on bacterial cell walls, while TLR3, TLR7, TLR8, and TLR9 are localized in cytosolic vesicles and detect bacterial nucleic acids (Kawai and Akira 2011), as shown in Figure 1. When TLRs recognize microbial patterns, myeloid differentiation factor 88 (MyD88) is a major adaptor protein recruited to interact with Toll/interleukin-1 receptor (TIR) domains in the intracellular compartment of TLRs at which signal transduction downstream of TLRs begins and results in NF-κB activation (Kawai and Akira 2010, Akira, Takeda, and Kaisho 2001). However, TLR3 and TLR4 also utilize TIR-domain-containing adaptor-inducing interferon-β (TRIF) that leads to the activation of NF-κB as well as interferon regulatory factor 3 (IRF3), as illustrated in Figure 1 (O'Neill, Golenbock, and Bowie 2013). In accordance with this notion, oral epithelial innate immunity is thus postulated to exploit numerous TLRs for a broad recognition of bacterial components, including both cell wall-derived and nucleic acid-associated molecular structures.

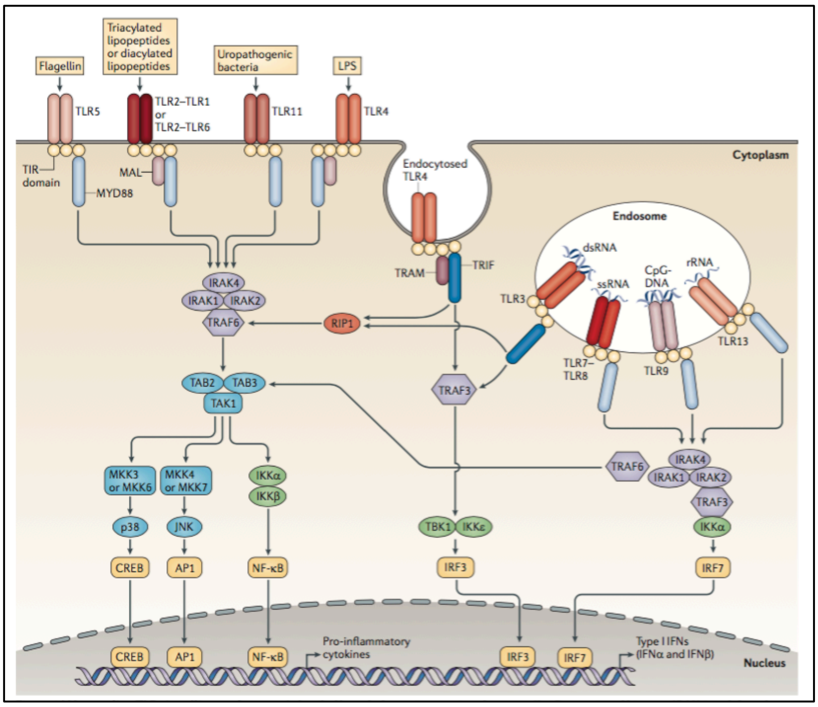

Figure 1. TLR signaling pathways in human cells (O'Neill, Golenbock, and Bowie 2013).

Activation of NF-κB by TLR-mediated signals leads to the production of inflammatory cytokines and/or type1-interferons. The process, where specificity of microbial components is crucial for activation, leads to the production of select inflammatory mediators.

The role of human OECs in the protection of underlying periodontal tissues from the harmful effects of oral bacteria has been studied extensively *in vitro,* as they are the first line of defense against oral pathogens. An intact epithelial cell layer provides a physical barrier that prohibits microbial intrusion into the underlying soft tissues and thus prevents the dissemination of microorganisms throughout the gingival tissue. Upon direct binding of bacteria to the OEC surface, the OECs are able to distinguish between gram-positive and gram-negative bacterial components using TLRs. Despite the abundance of TLRs in OECs (Sugawara et al. 2006, McClure and Massari 2014), their functions for recognizing bacterial components are seemingly distinct from those reported in sentinel cells.

1) Current understandings of oral epithelial cell biology and localization of TLRs in oral epithelial cells are still limited.

Although the induction of inflammatory signals via TLRs has been studied extensively in intestinal epithelial cells (IECs) (Abreu 2010, Abreu et al. 2002, Abreu, Fukata, and Arditi 2005, Abreu et al. 2001, Melmed et al. 2003), it is still unclear if the expression of major TLRs particularly TLR2 and TLR4 in OECs mirrors the presence of TLRs in IECs. However, a limited number of studies have shown findings similar to those reported from IEC studies showing an unresponsiveness of OECs to TLR agonists (Kim et al. 2012, Sugiyama et al. 2002, Uehara et al. 2007, Uehara, Sugawara, and Takada 2002, Uehara et al. 2005). Furthermore, it has not yet been elucidated where TLRs are located in OECs, unlike IECs in which the spatial distribution of TLRs has been more clearly defined.

While myeloid cells express a repertoire of TLRs on their surfaces and intracellularly (Sabroe et al. 2002, Akashi et al. 2000, Akira, Takeda, and Kaisho 2001, Akira and Takeda 2004), TLRs are sparsely present on IEC surfaces. This typical TLR expression thus contributes to the unresponsiveness of IECs to bacterial ligands. One clear example is the expression of TLR4 and TLR5 in the Golgi apparatus and at the basolateral surface of IECs, respectively (as depicted in Figure 2) (Hershberg 2002, Reed et al. 2002), thereby providing a protective strategy for epithelial cells from an overwhelming course of bacterial activation

(Abreu, Fukata, and Arditi 2005). Apparent redistribution of TLR2 and TLR4 from the apical surface of IECs to locations near basolateral membranes was shown when the cells were stimulated with peptidoglycan or LPS, respectively (Cario et al. 2002). However, whether TLR2 and TLR4 are polarized and separated from the apical surfaces in OECs is still unclear.

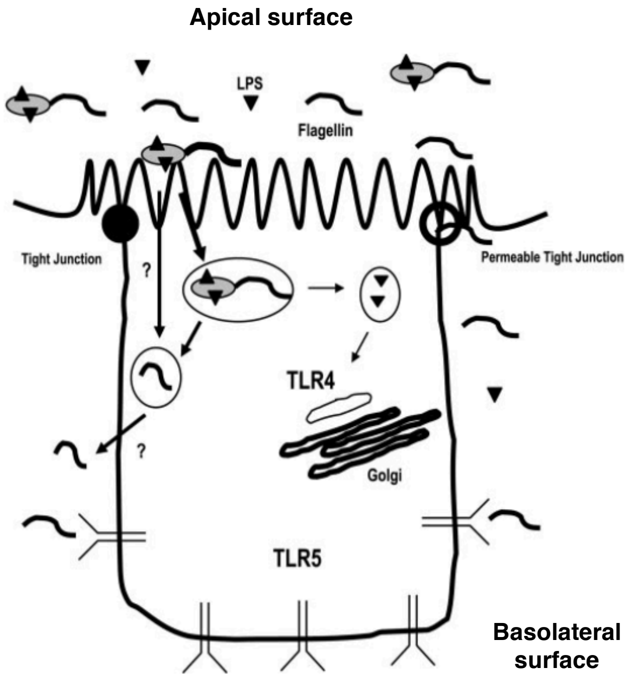

Figure 2. Polarization of TLRs in IECs (Hershberg 2002).

Distribution of TLRs, including TLR2, TLR4, and TLR5, has been shown from previous studies. The absence of TLR4 on the apical surface is believed to contribute to LPS hyporesponsiveness in IECs.

Since the roles of TLR2 and TLR4 in differential recognition of gram-positive and gram-negative bacterial cell wall components were discovered (Takeuchi et al. 1999), thorough investigations of TLRs and other surface receptors in OECs have been made. Unlike myelo-monocytic cells whose TLRs are strongly expressed (Muzio, Bosisio, et al. 2000, Applequist, Wallin, and Ljunggren 2002, Sabroe et al. 2002, Muzio, Polentarutti, et al. 2000), especially TLR2 and TLR4 that are found both extracellularly and intracellularly (Uronen-Hansson et al. 2004), epithelial cells express these receptors to an extent that was detectable at mRNA levels (Pivarcsi et al. 2003, Kusumoto et al. 2004, Kollisch et al. 2005, Uehara et al. 2001, Lebre et al. 2007) but location and abundance of these receptors present in epithelial cells, in particular OECs, are still controversial. Relative expression profiles of TLR transcripts in OECs were compared to those in human gingival fibroblasts (HGFs) and peripheral blood mononuclear cells (PBMCs), and distinct profiles between those cells were revealed. While PBMCs strongly express TLR1-TLR10 mRNAs, OECs and HGFs do not express TLR1, TLR7, TLR8, and TLR10 (Kusumoto et al. 2004). These findings thus indicate possible differential regulations of innate immunity by select TLRs in mucosal epithelia where the microorganisms predominantly found are bacteria. More importantly, TLR2 and TLR4 in OECs are most likely expressed intracellularly.

2) Bacterial infection of oral epithelium leads to the production of anti-microbial peptides and inflammatory cytokines by GECs.

Oral epithelium is a physical barrier that first interact with oral microbial community. Once the microbial community is found in close proximity to gingiva at the **junctional epithelium (JE)**, epithelial lining cells are constantly responding to external stimuli by mounting an inflammatory immune response. Ligation of TLRs for the recognition of pathogen-associated molecular patterns (PAMPs) triggers downstream signaling cascades in OECs and consequently leads to the induction of pro-inflammatory and chemotactic cytokines (chemokines). In addition, anti-microbial peptides, e.g., human defensins (Dale and Krisanaprakornkit 2001) and cathelecidins LL-37 (Dale and Fredericks 2005, Hosokawa et al. 2006), which directly affect bacterial cell viability, are also produced by OECs. Constitutive expression of human β-defensin (hBD) 1 and 2 is found in gingival epithelium (Krisanaprakornkit et al. 1998, Krisanaprakornkit et al. 2000). Interestingly, hBD-2, but not hBD-1, is inducible by the oral bridging organism *Fusobacterium nucleatum* (Fn), but not the periodontal pathogen *Porphyromonas gingivalis* (Pg) (Krisanaprakornkit et al. 2000), and this induction is mediated through p38 mitogen-activated protein kinase (MAPK) and c-Jun NH_2-terminal kinase (JNK) (Krisanaprakornkit, Kimball, and Dale 2002). Furthermore, an oral commensal *Streptococcus gordonii* (Sg) also utilizes MAPK, while oral pathogens *P. gingivalis* and *Aggregatibactor Actinomycetemcomitans* (Aa) share MAPK and nuclear factor-kappa B

(NF-κB) pathways for hBD-2 induction (Chung and Dale 2004). Clearly, hBD-2 induction in OECs involves several signaling mechanisms and each bacterium triggers intracellular signaling cascades differently (Chung and Dale 2008, 2004). This finding suggests that oral/gingival epithelium has a special sensing mechanism for differentiating oral commensals from pathogenic microbes.

Although oral epithelium is continuously exposed to a significant microbial load, a healthy state of periodontal tissues is usually maintained. There are several mechanisms utilized to protect underlying tissues from overwhelming inflammation caused by microbial burden. Constant transit of neutrophils into the gingival tissue, in particular the JE, is a key mechanism for controlling the number of bacteria (Hart, Shapira, and Van Dyke 1994) and thereby preventing epithelial cells from promoting deleterious inflammatory responses to bacterial stimulation. More importantly, both defective and excessive recruitment of neutrophils to the gingiva are conditions that lead to periodontal tissue breakdown (Roberts et al. 2015, Liu et al. 2001), indicating a pivotal role of neutrophil homing in periodontal health. Homing of neutrophil to gingival tissues is a biological process dependent on several key mediators including E-selectin (Darveau et al. 1995), intercellular adhesion molecule-1 (ICAM-1), and chemokine ligands (CXCLs) such as CXCL-8/IL-8 expressed in gingival tissues (Tonetti, Imboden, and Lang 1998, Tonetti 1997, Gemmell et al. 1994). A recent *in vivo* study reported that CXCL1 and CXCL2 are constitutively expressed in the JE (Zenobia et al. 2013). Remarkably, CXCL2, but not CXCL1, is induced by oral commensal bacteria in a MyD88-dependent manner, suggesting that direct sensing of oral commensal bacteria by OECs is crucial for the induction of select chemokine responses (Zenobia et al. 2013). Therefore, the presence of oral commensal bacteria in the oral microenvironment, leading to the expression of select neutrophil chemoattractants, might contribute to the periodontal health.

Expression of CXCL-8/IL-8, a human homologue of mouse CXCL1 and 2, by epithelial cells from different sites of the human body in response to microbial stimuli has been substantially investigated. Similar to other epithelial cell lineages, OECs are also capable of sensing oral bacteria and inducing CXCL-8 secretion in response to oral bacterial species found in dental plaque (Stathopoulou et al. 2010). Despite the absence of clinical gingival inflammation, CXCL-8 is constitutively expressed in normal gingival tissues, and its basal expression is found predominantly in epithelial layers (Sfakianakis, Barr, and Kreutzer 2002, Fitzgerald and Kreutzer 1995). Quantitative analysis of messenger RNA expression also showed that CXCL-8 is significantly up-regulated in chronically inflamed gingival tissues (Fitzgerald and Kreutzer 1995). Presumably due to the presence of diverse oral pathogens at the diseased sites, innate immune receptors on OECs are constantly stimulated, therefore, leading to a substantial CXCL-8 production. Notably, even in a germ-free condition, a significant number of neutrophils are detected in JE where both CXCL1 and CXCL2 are constitutively expressed (Zenobia et al. 2013). These findings thus indicate that 1) CXCLs play a pivotal role in the protection of periodontium from excessive bacterial loads by recruiting neutrophils to JE where they become activated (Tonetti, Imboden, and Lang 1998) and that 2) gingival epithelium is responsible for CXCL induction.

3) TLR4 mediates NF-κB inflammatory signaling in eukaryotic cells.

Due to the differential utilization of TLRs, host cells are able to differentiate between gram-positive and gram-negative bacteria. While recognition of gram-positive bacterial components relies on TLR2, bacterial LPS so called ‘endotoxin’ is sensed by TLR4 (Takeuchi et al. 1999). Immunosurveillance by TLR4 protects host cells from devastating bacterial infection as TLR4-LPS ligation leads to NF-κB driven immune reaction in response to invading microbes. Reduced expression of surface TLR4 results in the decreased potential of host cells to mediate the production of inflammatory cytokines (Nomura et al. 2000). Several factors are needed for the TLR4 priming step in order to initiate the signaling response. Aside from LBP and CD14

(Schroder et al. 2000, Jiang et al. 2000), MD-2, Myeloid Differentiation-2, is another key factor that governs LPS response in human immune cells (Shimazu et al. 1999, Akashi et al. 2000). Similar to the juxtaposition of CD14 and TLR4 prior to NF-κB nuclear translocation (Jiang et al. 2000), TLR4-mediated NF-κB signaling essentially requires physical association of MD-2 to the extracellular domain of TLR4 (Shimazu et al. 1999, Akashi et al. 2000) as shown in Figure 3. Participation of MD-2 in TLR4 signaling induces significant p38-MAPK, JNK, and extracellular signal-regulated kinases (ERK) activities (Yang et al. 2000), underscoring the functional versatility of MD-2 as a shared domain that host cells utilize in order to defend against danger signals, e.g., bacterial toxin, various cytokines, oxidative stress, and morphogenic cues (Roux and Blenis 2004). Nevertheless, one study conducted *in vitro* using several intestinal epithelial cell lines showed that intestinal epithelial cells express decreased TLR4 and MD-2 transcripts, associated with reduced NF-κB activation and CXCL-8 induction (Abreu et al. 2001). It is thus postulated that reduced LPS-mediated inflammatory reaction by down-regulation of TLR4 and MD-2 provides the host a protection from deleterious host-bacterium interactions.

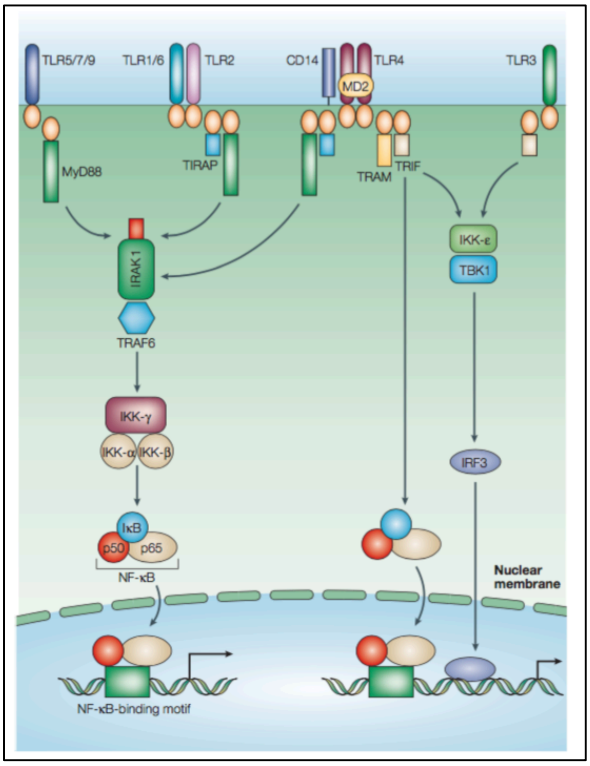

Figure 3. Utilization of co-receptors and TIR-domains for mediating TLR signaling to NF-κB (Akira and Takeda 2004).

Dimerization of TLR2 with TLR1 or TLR6 leads to the broader recognition of gram-positive bacteria in which lipopeptide heterogeneity is present. Sensing of gram-negative bacteria requires MD-2 and CD14 co-receptor for signaling transduction.

Taken together, despite TLR expression in epithelial cells, engagement of co-receptors is a requisite for initiating TLR2- and TLR4-mediated downstream signaling. Notably, epithelial cells are devoid of mCD14 and MD-2 on their surfaces, possibly rendering them hypo-responsive to bacterial LPS (Abreu et al. 2001, Zhang et al. 2009).

4) hBD-2 and CXCL-8 are differentially regulated in OECs and whether TLR2 and TLR4 are involved in these inductions is unclear.

Previous findings that TLR4-MD2 complexes are down regulated and TLR2 is unresponsive to gram-positive bacterial cell wall compartments in IECs (Melmed et al. 2003, Abreu et al. 2001) have addressed an undiscovered mechanism epithelial cells employ in order to tolerate the considerable numbers of gut microbiota. A similar phenomenon was also observed in OECs and GECs challenged with a variety of synthetic microbial patterns. Surprisingly, neither OECs nor GECs elicit inflammatory cytokines, i.e., CXCL-8 and interleukin-6 (IL-6), in response to ligand stimulation (Uehara et al. 2007, Uehara et al. 2005, Kusumoto et al. 2004, Kim et al. 2012). Even though these epithelial cells are derived from the different sites of human body, they coincidentally express common phenotypes in which their TLRs are inert to isolated microbial components.

The roles of TLRs, in particular TLR2 and TLR4, have been investigated extensively in OECs in defense against oral bacterial infection. Nevertheless, little is known how TLR2 and TLR4 on OECs recognize their putative ligands with respect to their structural requirement. In order to elucidate the functional roles of TLR in the induction of anti-microbial peptides and cytokines in OECs, purified bacterial agonists or synthetic analogs of microbial patterns are often used for *in vitro* stimulation to mimic the situations in which OECs encounter bacterial stimuli. Surprisingly, purified/synthetic TLR agonists are able to provoke hBD-2 production (Uehara et al. 2007) but not IL-6 and CXCL-8 (Uehara, Sugawara, and Takada 2002, Uehara et al. 2005, Uehara et al. 2007, Eskan, Hajishengallis, and Kinane 2007). In contrast, bacterial glycoproteins elicit an inflammatory immune response by OECs (Sugiyama et al. 2002). This indicates a discrepancy between microbial structures required for the induction of hBD-2 and CXCL-8 in OECs. It is most likely that an additional signaling in OECs is needed to trigger CXCL-8 response.

It has been so far inconclusive whether TLR2 and TLR4 play a pivotal role in oral epithelial cell sensing of bacteria. However, TLR2 has been reportedly implicated in the induction of hBD-2 and hBD-3 (Ji et al. 2009, Shin et al. 2013) as well as CXCL-8 (Onishi et al. 2008, Shin et al. 2013) in human OECs infected with live *F. nucleatum*. Contradicting results demonstrated that suppression of functional TLR2 in OECs by using small-interfering RNA (siRNA) prior to *F. nucleatum* challenge did not affect the levels of CXCL-8 (Ji et al. 2009), indicating a controversy as to which TLRs in OECs actively participate in bacterial recognition. Interaction between live bacterium and OEC at specific receptors triggers intracellular signaling that culminates in the production of both anti-microbial peptides and cytokines. More importantly, OECs are not capable of recognizing isolated bacterial ligands, but rather sense intact bacterial cell walls comprised of numerous microbial patterns.

5) CXCL-8 is induced by microbial invasion into epithelial cells by an unknown mechanism.

To date, oral/gingival epithelial phenotypes have been studied to a great extent, particularly their inherent ability to express CXCL-8. Conclusively from the large body of evidence discussed above, OECs challenged with TLR agonists do not produce IL-6, CXCL-8 but hBD-2, indicating a possibility that TLRs especially TLR2 and TLR4 do not participate in inflammatory signaling transmission triggered by gram-positive and gram-negative bacterial cell wall compartments at TLR2 and TLR4, respectively. However, this explanation is very unlikely since a recent study in which TLR1 or TLR6, required for TLR2 dimerization (Akira and Takeda 2004), was blocked by using antibodies to incapacitate TLR2-mediated signaling has demonstrated significantly reduced hBD-2 mRNA of GECs stimulated with FnCW-derived protein (Bhattacharyya et al. 2016). Decreased expression of functional TLR4 in OECs has been implicated in the reduced response of pro-inflammatory cytokines as well as chemokine CXCL-8 induced by live *P. gingivalis* (Eskan et al. 2008), suggesting the involvement of TLR4 for bacterial sensing by OECs. Interestingly, CXCL-8, strongly induced by *F. nucleatum*, is dampened when *F. nucleatum* loses its ability to invade OECs (Han et al. 2000). Taken together, these findings suggest that TLR4 might play a role in bacterial sensing in OECs; however, the TLR4-mediated signaling in OECs is not well understood.

The fact that OECs are unresponsive to the specific TLR2 and TLR4 agonists but not to invading bacteria has led us to speculate that intracellular recognition of bacterial components is essential for OEC response to microbial stimuli. It is most likely that there are TLR2 and TLR4 located intracellularly. Intracellular TLR4 was characterized for its location in mouse IECs, and the requirement of LPS internalization for the induction of inflammatory response by IECs has been revealed (Hornef et al. 2002). Trafficking of LPS to the Golgi apparatus where TLR4 is located initiates NF-κB-dependent CXCL2 production by murine IECs (Hornef et al. 2002, Hornef et al. 2003). Surprisingly, recently published studies have demonstrated the involvement of the endocytic pathway in TLR2 and TLR4 stimulation (Husebye et al. 2006, Marre et al. 2010, Motoi et al. 2014), suggesting the location of TLR2 and TLR4 in the endosomes. These findings thus help to better understand the nature of epithelial cells as to how they utilize novel TLR4 to sense microbial LPS. Most likely, TLR4 in OECs is presumably located intracellularly, specifically at the Golgi apparatus, and transported to the endosomes where LPS uptake or bacterial internalization occurs.

6) CXCL-8 response by epithelial cells involves multiple mechanisms of induction.

Bacterial recognition by epithelial cells is a highly organized process whereby the diversity of microbial components and foreign antigens is allowed to be sensed by a repertoire of receptors residing in a host cell in an antigen-receptor specific manner. As mentioned, various receptor-ligand interactions may converge toward one shared common pathway downstream of receptor components, thereby leading to the activation of one specific transcription factor.

Even though it is conceivable that CXCL-8 induction is mediated through NF-κB activation (Elewaut et al. 1999), recent studies have demonstrated the involvement of other signaling components and receptors that trigger CXCL-8 response by OECs. Activation of protease-activated receptor 2 (PAR-2) by the oral pathogen *A. actinomycetemcomitans* also results in CXCL-8 induction (Shimada et al. 2009). Robust CXCL-8 response to bacterial DNA and peptidoglycan by OECs, via TLR9 and their calcium channels, respectively (Kim et al. 2012, Son, Shin, and Hong 2015), indicates an orchestrated innate immune function of OECs in prompt response to the variety of bacterial stimuli found in an oral microenvironment. To summarize, induction of CXCL-8 expression by OECs is tightly regulated.

B. Knowledge gap and Significance

As a part of the innate immune system, oral/gingival epithelium provides the first line of defense and protection against periodontal tissue infection. Integrity of the gingival epithelium, especially the JE at which the epithelial surface is not keratinized, is therefore crucial for preventing the entry of oral microbes into the deeper connective tissues, thereby inhibiting bacterial colonization on the tooth surface in close proximity to the epithelium of periodontal pockets.

So far, current literature has provided insights regarding the innate immune mechanisms which gingival epithelium employs, e.g., secretion of the anti-microbial peptide hBD-2 and other pro-inflammatory cytokines, in order to overcome these microbial threats. The contribution of OECs to an oral innate immune defense has been extensively studied despite the unresponsive phenotype of oral epithelium to isolated microbial components. However, it remains unclear how inflammatory signals are initiated in OECs, i.e., which TLRs in OECs are involved in the sensing of oral bacteria. Of note, the role of TLR2 and TLR4 in the recognition of bacterial lipoproteins and LPS by OECs has not been well understood to date.

Understanding of the differential recognition of oral microbial structures by OECs at different TLRs, both extracellularly and intracellularly, is needed as it may provide a better fundamental basis of immunopathology of periodontal disease as well as the protective mechanism deliberately utilized by oral epithelium to overcome resident microbes found in the periodontium.

CHAPTER II

AIMS OF THE STUDY

Our hypotheses are that multiple TLRs, in particular TLR2 and TLR4, are engaged for the recognition of oral bacteria. The overall objectives of this project are to identify whether TLR2 and TLR4 are involved in the induction of chemokine expression in OECs. By using an *in vitro* cell culture system, we determined specifically which components of oral bacteria are capable of activating TLR and investigated how different bacterial species utilize intracellular machinery in OEC for inflammatory signaling.

According to the previous study, *F. nucleatum*, a co-evolved microorganism in the oral cavity, is a potent CXCL-8 inducer in OECs (Darveau et al. 1998, Han et al. 2000). Therefore, *F. nucleatum* was chosen as a study tool to elucidate the roles of TLR2 and TLR4 in CXCL-8 induction in OECs. Four different fractions of *F. nucleatum* that were prepared for an *in vitro* stimulation of cultured OECs included:

1. *F. nucleatum* whole cells, or live *F. nucleatum*
2. Cell wall proteins derived from *F. nucleatum* whole cells
3. Isolated LPS derived from *F. nucleatum* cell walls
4. *F. nucleatum* culture supernatants

Similar sample preparations were harvested from *E. coli* and used in all experimental setups as a positive control. Experimental protocols and procedures of OEC stimulation will be discussed in the details in the following chapters.

HYPOTHESES AND SPECIFIC AIMS

Specific Aim 1. Identification of microbial components that are differentially involved in the regulation of chemotactic cytokine CXCL-8 expression in response to direct host-bacterium engagement at TLR2 and TLR4 by human OECs.

Hypotheses:

1.1 CXCL-8 induction in human OECs is mediated by both TLR2 and TLR4. However, TLR4 in OECs is typically unresponsive and can thus only sense internalized LPS or LPS anchored to intact bacterial cell wall.

1.2 Bacterial internalization into OECs and its interaction with endosomal TLR4, shuttled from the ER, are required for this chemokine response.

Approaches:

1) To identify the structural components required for the induction of both hBD-2 and CXCL-8, oral bacterium *F. nucleatum* was tested in a series of experimental setups for their immune stimulatory potency in human OEC culture using ELISA.
2) To characterize the roles of TLR2 and TLR4 in hBD-2 and CXCL-8 induction, purified microbial patterns including LPS, and synthetic lipoprotein $\mathrm{Pam_3CSK_4}$ were used for stimulating OECs *in vitro*, comparing the responses triggered by *F. nucleatum* cell walls (FnCW). Functional roles of TLR2 and TLR4 were investigated using small interfering RNA (siRNA) to silence corresponding genes before challenging with *F. nucleatum* whole cells.
3) To elucidate the requirement of specific TLR ligands on bacterial cell walls for hBD-2 and CXCL-8 inductions, different treatments of FnCW were carried out prior to being used for *in vitro* OEC stimulation.
4) TLR2 and TLR4 activities of FnCW were investigated using the HEK293 NF-κB reporter system transfected with plasmid bearing human *tlr2* or *tlr4* sequences.

5) To elucidate the requirement of LPS uptake for TLR4-mediated OEC recognition, EcLPS or FnLPS, either isolated forms or shed forms in bacterial culture supernatants, were delivered into cultured OEC monolayer and subsequent CXCL-8 ELISA was performed. Knocking down of *TLR4* by using siRNA prior to LPS delivery was accomplished to confirm the presence of intracellular TLR4 in OECs.
6) Intracellular recognition of microbial patterns by TLR4 in OECs was examined using direct intracellular delivery of respective purified ligands with or without an inhibitor of endosomal maturation as well as an inhibitor of protein transportation from ER to the Golgi.

Specific Aim 2. Defining the role of bacterial entry into GECs and identifying bacterial requirement for LPS recognition by OECs.

Hypothesis:

2.1 Direct bacterial entry into OECs is crucial for LPS recognition.

2.2 *F. nucleatum* employs its cell wall proteins for mediating host response, due to the presence of unique adhesin proteins.

Approaches:

1) Bacterial invasion inhibition assays using inhibitors targeting membrane actin were performed to determine whether intracellular TLRs are responsible for partial CXCL-8 induction.
2) Similar to Chapter 1, *F. nucleatum* was also used as a bacterial model for the study. *F. nucleatum* clinical isolate strains 12230 wild-type and its counterpart lacking *FadA* adhisin gene were incorporated into this study aim to elucidate the requirement of bacterial invasion for LPS-mediated OEC response with respect to the ability of LPS to induce CXCL-8 protein.

3) To elucidate the uniqueness of *F. nucleatum* in its ability to mediate OEC response, FnCW proteins were digested with Proteinase K prior to OEC challenge. CXCL-8 response was compared between cell walls harvested from different bacterial species including *E. coli* and *A. actinomycetemcomitans*.
4) To determine the role of TLR2 and TLR4 in the recognition of bacterial cell wall proteins, untreated or Proteinase K-treated FnCW was tested for its ability to activate human TLR2 or TLR4 by using a well-established HEK293-TLR assay.

Detailed research methods and results are provided and discussed in the following chapters.

CHAPTER III

UTILIZATION OF AN INTRACELLULAR SENSING MECHANISM FOR LIPOPOLYSACCHARIDE (LPS) – INDUCED INFLAMMATORY RESPONSE BY ORAL EPITHELIAL CELLS

SYNOPSIS

Toll-like receptors (TLR) 2 and TLR4 are known to be involved in host innate immune defense against invading pathogens. However, mechanisms which oral epithelial cells (OECs) utilize for sensing bacterial cell wall components, particularly TLR2 and TLR4, still remain unclear. In this *in vitro* study, oral epithelial cells (OECs) were challenged with *Fusobacterium nucleatum* cell wall (FnCW) as well as agonists targeting TLR2 and TLR4. Unlike TLR2, none of OEC lines elicited CXCL-8/IL-8 in response to LPS-mediated TLR4 stimulation unless LPS was delivered into the cells. These findings demonstrate that oral epithelium utilizes a previously undiscovered intracellular mechanism mediated by TLR4 to recognize oral bacterial LPS in order to circumvent a constant exposure of OECs to oral microbes and to enable them to maintain periodontal tissue health.

INTRODUCTION

Oral or gingival epithelium is a specialized lining that protects underlying connective tissues and alveolar bone from potentially harmful burdens derived from oral microbial communities that colonize periodontal pockets. The roles of OECs in innate immune defense, therefore, have been investigated to a great extent since they are the main component of the sulcular epithelium, the cell layer that first encounters periodontal bacteria and forms a physical barrier to resist bacterial intrusion. Their close proximity to dental plaque, where the diverse oral microbial species are found, potentially contributes to a perpetual activation of immunological activities in OECs.

Induction of human anti-microbial peptide defensins (Dale and Krisanaprakornkit 2001) and cathelecidins LL-37 (Dale and Fredericks 2005, Hosokawa et al. 2006) in epithelial barriers is known to be a crucial mechanism for defending against microbial burdens as these peptides are known to possess broad anti-microbial activities (Dale and Krisanaprakornkit 2001, Greer, Zenobia, and Darveau 2013). It has been reported that human β-defensin (hBD) 1 and 2 are constitutively expressed in gingival epithelium (Krisanaprakornkit et al. 1998, Krisanaprakornkit et al. 2000), and the expression of hBD-2 is inducible by the oral bridging organism *Fusobacterium nucleatum* (Krisanaprakornkit et al. 2000). Interestingly, hBD-2 induction is differentially mediated via several signaling pathways depending on the interacting bacterial species (Krisanaprakornkit, Kimball, and Dale 2002, Chung and Dale 2004).

Expression of CXCL-8/IL-8 by epithelial cells from different sites of human body in response to microbial stimuli has been substantially investigated since the expression of this protein is required for neutrophil transit to JE (Tonetti, Imboden, and Lang 1998, Tonetti 1997, Gemmell et al. 1994). CXCL-8 is constitutively expressed in healthy gingival tissues and its basal expression is found predominantly in the epithelial layer (Sfakianakis, Barr, and Kreutzer 2002, Fitzgerald and Kreutzer 1995). However, a significant increase of CXCL-8 transcripts in chronically inflamed gingival tissues (Fitzgerald and Kreutzer 1995) and a distinct expression profile of pro-inflammatory cytokines in OECs challenged with different oral bacterial species found in dental plaque (Stathopoulou et al. 2010) have suggested that oral microbes are able to differentially modulate CXCL-8 response *in vivo* and that periodontal tissue health is tightly regulated by dynamic host–bacterium interactions.

Bacterial cell wall components are able to induce host innate immune response differently. Previous studies have investigated the ability of crude *F. nucleatum* cell wall (FnCW) to induce hBD-2 in human OECs. FnCW was clearly able to induce transcript expression of hBD-2 and CXCL-8 (Krisanaprakornkit et al. 2000, Krisanaprakornkit et al. 1998). In contrast, LPS from both *Escherichia coli* (EcLPS) and *F. nucleatum* (FnLPS) did not induce significant expression levels of hBD-2 and CXCL-8. However, results from a quantitative analysis of secreted hBD-2 and CXCL-8 from OECs challenged with LPS and other TLR agonists have demonstrated distinct expression profiles of hBD-2 and CXCL-8 (Uehara et al. 2007), suggesting a differential structural requirement for hBD-2 and CXCL-8 response by OECs.

It has been unclear so far as to how OECs recognize oral microbes, with respect to TLR signaling. The relative contribution of TLR2 and TLR4 still needs to be explored. In this study, I hypothesized that hBD-2 and CXCL-8 are differentially regulated via distinct signaling pathways in OECs, and the induction of these 2 host defense proteins requires host-microbial interactions at both TLR2 and TLR4. An oral commensal bacterium, *F. nucleatum,* was used as a study model to elucidate the distinct mechanisms by which hBD-2 and CXCL-8 are induced, since *F. nucleatum* was is found in both healthy and disease sites (Han 2015, Han and Wang 2013) and this bacterium provokes inflammatory response via TLR4 (Liu, Redline, and Han 2007). In addition to its abilities to trigger a robust host immune response, the engagement of *F. nucleatum* in the formation of dental biofilm as a bridging organism (Kolenbrander et al. 2002, Kolenbrander et al. 2006, Kolenbrander et al. 1995) highlights its pivotal role in the stabilization of microbial communities in periodontal pockets. Notably, we show that oral/gingival epithelium elicits both hBD-2 and CXCL-8 in response to oral bacterial infection, and this response is partly mediated at TLR2 and TLR4. While hBD-2 is induced by the ligation of bacterial lipoproteins with surface TLR2, CXCL-8 response was provoked in OECs by bacterial lipoproteins in a cell line-dependent manner. In contrast, surface priming with LPS is able to mount hBD-2 response but not CXCL-8, unless bacterial LPS is trafficked to intracellular TLR4. This study underscores a unique feature of TLR4 in OECs, with respect to the mechanism to recognize bacterial LPS, in oral epithelial innate immunity.

MATERIALS AND METHODS

Human oral/gingival epithelial cell culture

Normal human OECs were obtained from the gingival tissue cell collections at the department of Oral Health Sciences, School of Dentistry, the University of Washington. Briefly, normal human OECs were isolated from gingival tissue specimens obtained from healthy adults who underwent the third-molar surgical procedures. Gingival tissue explant cultures were performed to isolate primary epithelial cells as previously described (Krisanaprakornkit et al. 1998) and then stored in our collection. Primary OECs were grown in Keratinocyte Serum-Free medium (Ker-SFM) (Life Technologies) supplemented with 25 µg/mL bovine pituitary extracts, 0.2 ng/mL human recombinant epidermal growth factor, 5 µg/mL insulin (Sigma-Aldrich, St. Louis, MO), 0.5 µg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO), 0.15 mM calcium chloride, and 10% Penicillin–Streptomycin (Sigma-Aldrich, St. Louis, MO). In some experiments where live bacteria were used for the stimulation, OECs were grown in antibiotic-free Ker-SFM. Cultured OECs were seeded in 12- or 96-well plates and maintained under a 37°C humidified atmosphere with 5% CO_2. Monolayer of OECs reached confluence prior to the day of bacterial stimulation.

This study incorporated other 2 lines of epithelial cells including OKF6/TERT2 and hTIGK, in addition to primary, normal GECs. Normal human telomerase immortalized oral epithelial cell OKF6/TERT2 line was generously provided by the Division of Dermatology, Harvard medical school. Human telomerase-immortalized gingival epithelial cell hTIGK line was kindly gifted by Dr. Richard J. Lamont, school of Dentistry, University of Louisville. Both OKF6/TERT2 and hTIGK lines were grown and maintained in Ker-SFM supplemented with 25 µg/mL bovine pituitary extracts, 0.2 ng/mL human recombinant epidermal growth factor, 0.4 mM calcium chloride, and 10% Penicillin–Streptomycin (Sigma-Aldrich, St. Louis, MO).

Bacterial culture and crude cell wall preparation

Bacteria tested in this study were obtained from the Darveau laboratory's frozen bacterial stocks. In brief, *F. nucleatum* ATCC 25586 was grown overnight at 37°C under an anaerobic gas condition (80% N_2, 10% CO_2, 10% H_2) in trypticase soy yeast broth (TYK) supplemented with 10 µg/mL hemin (H) and 1 mg/mL menadione. Crude cell wall was prepared as previously described (Krisanaprakornkit et al. 1998) by using French Pressure Cell Press at 15,000 lb/in^2 with the cocktails of protease inhibitors including Pefabloc® SC (Boehringer Mannheim GmbH, Mannheim, Germany), Benzamidine (Sigma-Aldrich, St. Louis, MO), Nα-Tosyl-L-Lysine Chloromethyl Ketone Hydrochloride (Sigma-Aldrich, St. Louis, MO) at a concentration of 20 µM each and DNase (Sigma-Aldrich, St. Louis, MO). Cell wall fractions were harvested by several centrifugations and suspended in sterile phosphate-buffered saline (PBS) prior to fluorometric protein quantitation using Qubit® protein quantitation kit (Thermo Fisher Scientific). Crude FnCW in PBS suspension was kept frozen prior to experimental setup. LPS from *E. coli* and *F. nucleatum* was isolated and purified as previously described (Darveau and Hancock 1983).

In some experiments, *Porphyromonas gingivalis (Pg)* ATCC 33277, *Prevotella intermedia (Pi)* ATCC 25611, *Aggregatibacter actinomycetemcomitans (Aa)* ATCC 43718, and *Streptococcus gordonii (Sg)* ATCC 51656 were included. Similar to *F. nucleatum,* these bacteria were grown in TYHK broth overnight at 37°C under anaerobic gas conditions, with the exception of *Aa* in which an anaerobic atmosphere with 5%CO_2 is preferable. In some experiments, *Escherichia coli (Ec)* JM83 strain was used as a positive control. *E. coli* was grown in Luria-Bertarni (LB) broth under aerobic conditions in a vigorous 37°C shaker.

Preparation of bacterial culture supernatant

Bacterial culture supernatants were prepared from *F. nucleatum* ATCC 25586 and *E. coli* JM 83 overnight cultures. Briefly, bacterial whole cells in an overnight 20-mL culture of each strain were removed by centrifugation at 6,500 g for 25 minutes. Clear culture supernatants were then filtered with Steriflip® filter units with 0.22 µM pore size (Millipore Corporation, Billerica, MA, USA) to ensure a complete removal of bacterial cells and debris from the resulting supernatants. An approximate 20-mL bacterial supernatant was then concentrated into a smaller volume (roughly 500 µL) by using Centricon Plus-20 Centrifugal Filter Devices (Millipore Corporation, Billerica, MA, USA). A concentration of total proteins from each preparation was determined by using Qubit® protein quantitation kit (Thermo Fisher Scientific). 100 µg/mL and 10 µg/mL working stocks of supernatants were prepared from original stocks using 1X PBS (Gibco by Life Technologies), and stored at -20°C until needed.

Detection of endotoxin (LPS) level in bacterial supernatants

In some experiments in which concentrated *F. nucleatum* or *E. coli* culture supernatants were used, the presence of LPS in the supernatants was quantified using ToxinSensor™ Endotoxin Detection System (GenScript, Piscataway, NJ, USA). Briefly, aliquots of either *F. nucleatum* or *E. coli* culture supernatants were incubated with Limulus Ameobocyte Lysate (LAL) reagents for 12 minutes at 37°C in 96-well plate. Chromogenic substrate solution was then added to each reaction and allowed 6 minutes for incubation. Stop solution and color stabilizer were finally added to each well. Optical density at 454 nm wavelength was determined by using a plate reader (VMax microplate reader from Molecular Devices, Sunnyvale, CA). A wide-range dose of EcLPS and FnLPS (100 pg/mL – 1 µg/mL) was used as standards for the readout and PBS served as a negative control.

HEK293 TLR activation assays

Analysis of TLR2- and TLR4-mediated NF-κB activities of bacterial samples and purified agonists was performed using human embryonic kidney 293 cells (HEK293) transfected with plasmid DNAs bearing Firefly luciferase-labeled NF-κB sequence, Renilla luciferase-labeled β-actin sequence, in combination with plasmids encoding human TLR2/1 or TLR4 as described elsewhere (Coats et al. 2007).

HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum, and seeded in 96-well plates one day prior to transfection. Transfection with plasmids encoding human TLR2/1 or TLR4 was performed by a standard calcium phosphate precipitation method. Briefly, pTLR2 and pTLR1 (0.002 µg each) were co-transfected with pCD14 (0.002 µg) for transient TLR2/1 expression. Similarly, pTLR4 (0.002 µg) and pMD-2 (0.002 µg) were co-transfected with pCD14 (0.002 µg) to induce TLR4 expression. Reporter plasmid pBArenLuc (0.0004 µg) and pNFκBLuc (0.02 µg) were transfected in all wells. Stimulation of transfected HEK293 cells was performed on the following day in triplicate under normal physiological conditions for 4 hours, after which the HEK293 cells were rinsed with PBS and lysed with passive lysis buffer (Promega, Madison, WI). Luciferase activity was then assayed by using the Dual Luciferase Assay Reporter System (Promega, Madison, WI). Luminescent signals generated from firefly luciferase activity were normalized to signals from renilla luciferase, thereby resulting in the fold activity of NF-κB relative to β-actin internal control.

Signaling pathway inhibition

To segregate signaling pathways involved in both hBD-2 and CXCL-8 induction, inhibitors (listed in table 1) were pre-incubated with cultured OECs in 96-well plates for 1 hour before bacterial stimulation. Treated-OECs were then challenged with test bacteria at multiplicity of infection (MOI) 1:50 (50 bacteria per one OEC) for 24 hours prior to hBD-2 or CXCL-8 protein quantitation. The concentrations used with OEC were pre-determined by using CellTiter-Glo Luminescent cell viability assays (Promega, Madison, WI), as well as Trypan blue (Thermo Fisher Scientific) exclusion test, and demonstrated no cytotoxicity to OECs.

Neutralization of FnCW lipoproteins and LPS

10 ng FnCW or 1 µg synthetic lipoprotein Pam_3CSK_4 (Invivogen, San Diego, CA) were neutralized with lipoprotein lipase derived from a *Burkholderia sp.* (Sigma-Aldrich, St. Louis, MO), or polymyxin B (PMB) at 37°C overnight. Doses of lipoprotein lipase and PMB used in the study were 50 and 100 ng total mass. After 16 hours of incubation, samples were made to a final concentration of 100 ng/mL for FnCW and at 1000 ng/mL for Pam_3CSK_4 using Ker-SFM growth medium containing 0.4 mM calcium chloride. 100 µL resuspended samples were added into cultured hTIGKs and allowed 24 hours for OEC stimulation.

TLR2 and TLR4 gene silencing

OECs were seeded into 12-well plates ($5x10^4$ cells/well) using supplemented Ker-SFM medium containing 1.2 mM calcium chloride. Shortly after seeding OECs, small-interfering RNA was transfected using Fast-forward protocol according to manufacturer's instructions. Specific siRNA sequences of *TLR2*, *TLR4*, or non-silencing (NS) scramble RNA sequences (Qiagen, Valencia, CA) were allowed to equilibrate at room temperature for 10 minutes in the Ker-SFM medium in the presence of HiPerfect transfection reagents (Qiagen, Valencia, CA). The siRNA complexes were subsequently added to the cultured OECs and incubated for 48 hours prior to bacterial stimulation. The final concentration of each siRNA used in this study was 50 nM.

RNA isolation and quantitative RT-PCR (RT-qPCR) analysis

Total RNA from OECs was extracted and purified using RNeasy mini kit (Qiagen, Valencia, CA) according to manufacturer's instruction. Genomic DNA contamination was removed by using In-column treatment with DNase I (Qiagen, Valencia, CA). Subsequently, reverse transcription reaction was performed using 500 ng of total mRNA. The reactions were set up by using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems by

Thermo Fisher Scientific, Carlsbad, CA) and its protocol. In addition, control samples without reverse transcriptase enzyme, as well as controls without RNA templates, were prepared similarly and served as negative controls.

Quantitative analysis of *TLR2* and *TLR4* mRNA levels was subsequently performed by using a Lightcycler 480 (Roche Applied Sciences, Indianapolis, IN) in combination with a Power SYBR^{TM} Green Master Mix (Applied Biosystems by Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations. Briefly, each PCR reaction was setup to a total volume of 20 µL, containing 50 ng cDNA and 500 nM primers (table 2), in 96-well plates. The conditions for product amplification included initial denaturation at 95^{o}C for 5 minutes and followed by 45 cycles of denaturation (95^{o}C for 15 seconds), and annealing (60^{o}C for 1 minute). After the end of PCR amplification, 0.8% agarose gel electrophoresis was performed to examine the primer specificity, as well as the analysis of melt curve to assure there was no spurious PCR products. Crossing-point values (Cp) generated by the software after each run were utilized for calculating the expression level of genes of interest where the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene from the same sample was used for normalization. Relative expression was calculated by using the ΔΔCp method, and shown in the figures as fold increase over unstimulated controls.

OEC stimulation and intracellular delivery of LPS

All test bacteria were grown overnight before the day of stimulation. Bacterial pellets were obtained by centrifugation of 750 µL bacterial cultures, washed once with culture broth, and determined for the number of bacteria correlating to the optical density (OD600) by using an Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany). Bacterial stock of multiplicity of infection (MOI) 1:50 was pre-determined to yield an optimal CXCL-8 response by OECs from our preliminary experiments. All test bacteria were prepared with Ker-SFM medium without antibiotics to achieve to the stocks of MOI 1:50, a ratio of one OEC to 50 bacteria. Stimulation of OECs that were cultured in 96-well plates lasted for 24 hours, unless otherwise indicated, before hBD-2 and/or CXCL-8 proteins were quantified.

For intracellular LPS delivery experiments, LPS-liposome complexes were prepared according to the methodology developed for LPS transfection (Hagar et al. 2013) with a slight modification. Briefly, 500 ng isolated purified EcLPS or FnLPS was pre-mixed with 2 µL Lipofectamine 2000 (Invitrogen, Carlsbad, CA), spun down, and sat at room temperature for 10 minutes. Ker-SMF without antibiotics was added and brought up to a final concentration of 1µg/mL LPS. Cultured OECs were then stimulated with 100 µL LPS-liposome complexes for up to 24 hours, while mock transfection (LPS+medium) and Lipofectamine alone served as controls. Positive control wells were infected with 100 ng/mL FnCW. Culture supernatants were assayed for CXCL-8 proteins at 4 and 24 hours after stimulation. For elucidating the role of endosomal maturation in TLR4 activation, 400 nM bafilomycin A1 (Sigma-Aldrich, St. Louis, MO) was pre-incubated with OECs for 1 hour prior to LPS or bacterial challenging. Brefeldin A (40µg/mL) was also pre-incubated with OECs similarly in parallel experiments to investigate the role of protein transport from endoplasmic reticulum to the Golgi apparatus.

Similar to an *in vitro* delivery of purified LPS, parallel experiments were also performed by using concentrated supernatants harvested from *E. coli* or *F. nucleatum* cultures. In brief, 5 µL from 10 µg/mL culture supernatant was mixed with 2 µL Lipofectamine 2000 (Invitrogen, Carlsbad, CA), spun down, and sat at room temperature for 10 minutes. 13 µL Ker-SFM without antibiotics was then added to the LPS-Liposome mixture and incubated statically at room temperature for 30 minutes. Ker-SFM without antibiotics was added and brought up with 480 µL to a final concentration of 100 ng/mL total protein in the supernatant. 100 µL prepared samples were added to OEC cultures and incubated for 24 hours prior to CXCL-8 protein assays.

Measurement of hBD-2 and CXCL-8 secreted proteins

Culture supernatants were collected for measuring CXCL-8 levels after 24 hours of incubation, unless otherwise indicated. CXCL-8 monoclonal capture antibody (M801) and detection antibody (M802B) (Thermo Fisher Scientific, Rockford, IL) were used. For some experiments when hBD-2 needed to be detected, hBD-2 enzyme-linked immunosorbent assay (ELISA) development kit (Peprotech, Rocky Hill, NJ) was used. Avidin-horseradish peroxidase enzyme (HRP) (eBioscience, San Diego, CA) and tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, MO) were used in the study. Standard sandwich ELISA protocol was performed according to manufacturer's recommendations. The reported concentrations were calculated from the standard curve derived from correlating the OD reading of each sample at 450/570 nm wavelengths (VMax microplate reader from Molecular Devices, Sunnyvale, CA) to the known concentrations of serially diluted recombinant protein standards.

Data analysis

All experiments were performed at least 3 times in triplicate using 3 different epithelial cell lines, unless otherwise indicated in the figures. Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software Inc, La Jolla, CA). One-way Analysis of Variances (ANOVA) with Tukey's post hoc test, unless otherwise stated in the figure text, was used to determine the significance level where p-value < 0.05 was considered statistically different.

RESULTS

The oral bacterium *F. nucleatum* strongly interacts with human TLR2 and TLR4 due to the presence of lipoprotein and LPS components.

Gram-negative bacterial cell walls are not only composed of LPS, but also lipoproteins, both of which are anchored to the outer membrane of gram- negative bacteria (Silhavy, Kahne, and Walker 2010). We thus speculated that lipoprotein contents and LPS in FnCW might be able to interact with OECs at TLR2 and TLR4, respectively. We first investigated the ability of live *F. nucleatum* and its cell wall to activate human TLR2 and TLR4 by utilizing the HEK-TLR activation assay. We found that *F. nucleatum* has strong abilities to activate both human TLR2 and TLR4 (Figure 4).

Neutralization of bacterial LPS by using polymyxin B was reported to be effective in the number of studies (Uehara, Sugawara, and Takada 2002, Coats et al. 2009, Slocum et al. 2014, Zhang, Mann, and Tsai 1999). Likewise, an addition of lipoprotein lipase into LPS samples has been shown to remove co-purified lipoproteins contaminated in LPS preparations (Jain et al. 2013). We hence employed these neutralization strategies to elucidate the presence of lipoproteins and LPS in FnCW. FnCW treatment with lipoprotein lipase and polymyxin B reduced the activation of NF-κB, specifically at TLR2 and TLR4, respectively, in HEK293 (Figure 5A, 5B) as well as CXCL-8 secreted by hTIGK (Figure 6). Taken together, it thus demonstrates the presence of lipoproteins and LPS in FnCW preparations and that the CXCL-8 response is presumably mediated by TLR2 and TLR4 in hTIGK. Furthermore, it is also important to note that host-bacterium interactions potentially engage both TLR2 and TLR4. However, the mechanisms which OECs utilize for sensing both bacterial lipoprotein and LPS are not well understood.

Variability of CXCL-8 response to lipoproteins, but not LPS, suggest heterogeneity of OECs.

Previous studies reporting the hypo-responsiveness or unresponsiveness of human intestinal and oral epithelial cells to synthetic lipoprotein and bacterial LPS (Uehara et al. 2007, Uehara et al. 2005) led us to investigate whether this hypo-responsive phenotype is also applicable to other OEC lineages. Four lines of OECs were therefore challenged with *F.*

nucleatum (whole cell or cell wall), Pam_3CSK_4 (synthetic triacylated lipoprotein; TLR2/TLR1 agonist), or LPS (from *E. coli* and *F. nucleatum*) at a wide dose range of 1-1,000 ng/mL for 24 hours. Remarkably, Pam_3CSK_4-induced CXCL-8 levels were increased in a dose-dependent manner in hTIGK, as well as in nGEC1 stimulated with a 1 µg/mL Pam_3CSK_4 (Figure 7A, 7C). However, no induction of CXCL-8 was detected in the culture supernatants obtained from OKF6 and nGEC2 (Figure 7B, 7D). This finding thus demonstrates heterogeneity of TLR2 expression in OEC populations. More importantly, none of the test OEC lines secreted CXCL-8 in response to EcLPS and FnLPS.

Expression profiles of TLR2 and TLR4 transcripts in epithelial cells were investigated in several studies, and detectable mRNA levels were reported (Kusumoto et al. 2004, Sugawara et al. 2006). We then hypothesized that TLR2 and TLR4 might contribute to CXCL-8 induction in OECs. After an infection of OECs with live *F. nucleatum* or FnCW, all test OECs elicited robust CXCL-8 and hBD-2 expressions (Figure 7, 8), significantly higher than that of OECs stimulated with Pam_3CSK_4 or LPS. Essentially, this finding indicates that the magnitude of oral epithelial response to oral bacteria is determined by unidentified bacterial cell wall components. Interestingly, a slight increase of hBD-2, but not CXCL-8, was observed after OECs were challenged with LPS and Pam_3CSK_4 (Figure 7, 8). This discrepancy in OEC response to stimuli further underlines the differential regulation of signaling initiation between different host immune defense markers. Consistent with this, NF-κB signaling is pivotal for the induction of both CXCL-8 and hBD-2, while hBD-2 induction is also dependent on JNK-, p38-MAP kinases, and AP-1 pathways (Figure 9).

OECs expressing TLR2 and TLR4 are functionally involved in epithelial recognition of oral microbes.

Next, we sought to determine whether CXCL-8 induction is mediated by TLR2 and TLR4. TLR2, TLR4, or both TLR2 and TLR4 genes in human OECs were silenced with specific siRNA sequences for 48 hours prior to live *F. nucleatum* or FnCW stimulation. The efficiency of siRNA knock-down was determined by RT-qPCR showing that approximately 50% reduction of mRNA expression levels was achieved in each experimental setup (Figure 10A). After 24 hours of stimulation with live *F. nucleatum*, CXCL-8 levels secreted by OECs silenced for TLR2 or TLR4 were significantly lower than those of non-silenced OECs (Figure 10B). However, a partial reduction of CXCL-8 in *TLR2-* and *TLR4*-silenced OECs suggests a relatively minor role of TLR2 and TLR4 in bacterial sensing. Essentially, both TLR2 and TLR4 are expressed and partially contribute for host-bacterium interactions in OECs.

Intracellular recognition of bacterial LPS by OECs triggers CXCL-8 response.

Our observation that OECs are not responsive to bacterial LPS despite the requirement of TLR4 for bacterial recognition has led us to hypothesize that OECs express TLR4 elsewhere, most likely on intracellular compartments. To test our hypothesis, we adopted a liposome-LPS formation technique reported elsewhere (Hagar et al. 2013) for delivering purified, isolated EcLPS or FnLPS into OECs. After 24-hour incubation of OECs with liposome-LPS complexes, a considerable amount of CXCL-8 was induced (Figure 11) in all test lines, indicating that LPS needs to ne internalized. However, normal gingival epithelial cell (nGEC) lines elicited a limited amount of CXCL-8, compared to immortalized epithelial cells, possibly due to the poor efficiency of LPS transfection. An increase of CXCL-8 response upon successful LPS bypassing epithelial cell membranes indicated that, in order to be recognized by OECs, LPS trafficking to intracellular sensor is required for LPS-mediated CXCL-8 induction in OECs.

To mimic the natural events that oral epithelium is constantly exposed to bacterial LPS shed from bacterial cell membranes and vesicles (Ellis and Kuehn 2010), concentrated culture supernatants of *E. coli* and *F. nucleatum* were tested for the abilities to provoke CXCL-8 expression in OEC monolayers. All 4 OEC lines were challenged with bacterial supernatants and no increase of CXCL-8 response was detected, with an exception of OECs stimulated with 1,000 ng/mL supernatant proteins (Figure 12).

In order to survive in the host tissues, bacteria employ several strategies, i.e., secretion of proteins and toxin into the microenvironment, to overcome host-mediated inflammatory reactions (Lee and Schneewind 2001). To rule out the effect of secreted proteins in the culture supernatants on the inhibition of LPS-mediated CXCL-8 induction in OECs, we utilized HEK-TLR4 activation assay to test whether *F. nucleatum* supernatant blocked LPS-induced TLR4 activity. Strikingly, *F. nucleatum* culture supernatant showed a potent activation at human TLR4, relative to the activation by FnLPS (Figure 13, solid lines). Activation of human TLR4 by bacterial culture supernatant from *F. nucleatum* was synergistically augmented when TLR4-overexpressing HEK293 cells were co-stimulated with its naked LPS counterpart, and vice versa (Figure 13, dashed lines). This thus demonstrates that proteins in the *F. nucleatum* culture supernatant did not block TLR4 activation. In addition, LAL assays confirmed the abundance of LPS in the culture supernatants harvested from *E. coli* (Figure 14A) and *F. nucleatum* (Figure 14B). Notably, substantial amount of FnLPS was detected even at a lower concentration of *F. nucleatum* supernatant proteins (0.1 and 1 ng/mL), relative to LPS standard controls (Figure 14B). Taken together, these findings demonstrate that shed LPS, similar to species-matched isolated LPS, has a strong stimulatory activity at human TLR4 and that oral mucosal tissue lining is inert to direct stimulation by LPS, both naked and naturally-found forms. Strikingly, OECs were able to recognize LPS in the supernatants only when Lipofectamine was coupled with supernatant proteins, indicating that LPS uptake is crucial for OEC recognition of gram-negative bacteria (Figure 15).

Since we successfully delivered isolated LPS into OECs, particularly hTIGK and OKF6, by utilizing an *in vitro* LPS-liposome complex to bypass cell membranes, we, therefore, sought to determine the fate of internalized LPS *in vitro* and its downstream consequence. We speculated that LPS sensor, TLR4, is responsible for LPS-mediated CXCL-8 induction in

OECs. Two immortalized OEC lines, hTIGK and OKF6, were silenced for *TLR2* or *TLR4* for 2 days prior to LPS delivery mediated by Lipofectamine. As expected, knocking down TLR4 resulted in a significant reduction of CXCL-8 expression in response to internalized LPS (Figure 16). This finding thus confirmed the presence of intracellular TLR4 in human OECs and its role in CXCL-8–mediated periodontal homeostasis. However, a negligible decrease of CXCL-8 level secreted by TLR2-knocked down OECs stimulated with liposome-LPS complexes was also observed. This reduction was likely contributed by the presence of lipoproteins, TLR2 ligands known to be co-purified with LPS preparations (Jain et al. 2013), that were co-delivered into OECs by Lipofectamine.

To date, it is unclear as to where non-canonical TLR4 is expressed in human epithelial cells. However, intracellular TLR4 was found to be present in murine intestinal epithelial cells, located specifically at the Golgi apparatus (Hornef et al. 2002). Trafficking of LPS to the Golgi apparatus, where TLR4 is accommodated, induced NF-κB–dependent CXCL2 induction. Several lines of evidence have also suggested the role of endosomal TLR4 in LPS-dependent cellular signaling in myeloid cells (Uronen-Hansson et al. 2004, Kawai and Akira 2010, 2011) and epithelial cells from other body sites (Ueta et al. 2004). We thus postulated that TLR4 is located at the endosomal compartments in OECs, and an inhibition of endosome-lysosome fusion, the process required for TLR4 activation (Husebye et al. 2006), might result in the reduction of LPS-mediated TLR4 signaling. Pre-incubation of OECs with 400 nM bafilomycin A, a known inhibitor of endosomal acidification used in numerous *in vitro* studies investigating intracellular TLR signaling (Johnson et al. 1993, Kim et al. 2012, Park et al. 2008), resulted in a remarkable decrease of CXCL-8 induced by LPS-liposome complexes (Figure 17). This finding strongly supports the notion that the presence of non-canonical TLR4 in OECs is pivotal for LPS recognition. Most likely, TLR4 is abundantly expressed on the endosomes in OECs and its activation requires cytosolic engulfment of bacterial LPS in order to deliver LPS to endosomes where non-canonical TLR4 resides.

Functional TLR4, as shown here from our study, is expressed intracellularly on the endosomal compartments in OECs. Whether TLR4 is synthesized from endoplasmic reticulum (ER) and consequently transported to the Golgi apparatus during the course of infection remains to be elucidated. We, therefore, utilized brefeldin A, a pharmacological blocker known to promote retrograde transport of protein from the Golgi body to the ER (Hornef et al. 2002, Latz et al. 2002), as we postulated that TLR4 is trafficked to the Golgi apparatus and subsequently fated to endosomes. Our results showed that incubation of OECs with brefeldin A (40 µg/mL) dramatically dampened the level of CXCL-8 induction in response to internalized LPS (Figure 18), although the reduction was not statistically significant in OECs stimulated with Lipofectamine-coupled FnLPS, indicating that protein synthesis machinery in OECs is required for LPS recognition and that dynamic TLR4 translocation from ER to endosomes is evolutionarily programmed to govern an immediate innate immune response when oral epithelium is breached by virulent microbes.

DISCUSSION

Oral epithelium, consisting of a layer of stratified squamous epithelial cells, is the first tissue that encounters a complex periodontal microbial community. In addition to being a natural physical barrier that prevents oral bacterial invasion into deeper tissues, OECs also act as programmed microbial sensors that allow the human immunosurveillance system to mount an initial, rapid, non-specific response to polymicrobial infections, i.e., an induction of antimicrobial peptides, pro-inflammatory and chemotactic cytokines (Dale et al. 2001, Darveau 2010, Darveau et al. 1998, Greer, Zenobia, and Darveau 2013, Krisanaprakornkit et al. 2000, Zenobia et al. 2013). In this study, we clearly elucidated the roles of human TLR2 and TLR4 in OECs in the recognition of oral bacteria by employing *F. nucleatum* as a tool to reveal the underlying mechanisms which OECs utilize for this recognition process.

To date, it is unclear how human oral/gingival epithelial cells employ TLR2 and TLR4 for bacterial recognition. While TLR2 and TLR4 expressions in myeloid cell lineages are predominantly found on the cell surfaces (Akashi et al. 2000, Latz et al. 2002, Sabroe et al. 2002), previous studies have reported that TLRs are not abundantly expressed on the surface of epithelial cells (Ueta et al. 2004). Our data suggest that, most likely, TLR2 and TLR4 are expressed internally in OECs (Figure 7) and that NF-κB–driven inflammatory gene transcription is a shared signaling mechanism for both antimicrobial peptide and chemokine induction (Figure 9). Interestingly, a slight increase of hBD-2, but not CXCL-8, induced by isolated lipoprotein Pam_3CSK_4 and bacterial LPS indicates that both TLR2 and TLR4 might be present on the cell surfaces even if in low abundance. Our findings also suggest that CXCL-8 induction in OECs requires an additional signal transduction, and that TLR signaling from surface priming to ligands is not sufficient for CXCL-8 induction.

The mechanism of oral bacterial sensing by TLR2 and TLR4 in oral keratinocytes is considered to be unique in that a vast variability of the expression levels of TLR2, TLR4, as well as co-receptors among different OEC lines was reported. Regardless of TLR2 and TLR4 transcripts, OECs are typically hypo-responsive to their specific ligands (Sugawara et al. 2006), most likely due to the lack of TLRs on the apical cell surface. Previous studies have shown an increase of either TLR2 or TLR4 responsiveness in intestinal epithelial cells when TLR2/TLR6 or TLR4/MD-2 was over-expressed (Abreu et al. 2001, Melmed et al. 2003), indicating the absence of functional TLR2 and TLR4 proteins in those test epithelial cell lines. However, silencing of *tlr2* or *tlr4* gene in OECs in our study resulted in a decrease of CXCL-8 secreted from OECs in response to *F. nucleatum* infection (Figure 10). This clearly suggests the involvement of functional TLR2 and TLR4 in OEC sensing of bacteria. Unlike myeloid cells, surface priming of OECs with lipoproteins or LPS is not sufficient for OECs to recognize and initiate signal transduction in response to isolated microbial cell wall components. Unresponsiveness of OECs to a broad spectrum of isolated microbial components is thus beneficial for gingival health, similar to an event found in intestines where IECs continuously encounter diverse species of luminal bacteria (Abreu 2010, Abreu et al. 2002, Abreu, Fukata, and Arditi 2005, Abreu et al. 2001, Melmed et al. 2003) since it provides a recalcitrant barrier preventing the periodontium from an overwhelming inflammatory immune response.

The absence of membrane CD14 and MD-2 co-receptors may render OECs unresponsive to lipoproteins and LPS stimulation. Although interferon-gamma (IFN-γ) priming of OECs has been shown to up-regulate MD-2, TLR2, TLR4, but not CD14, at mRNA levels (Suzuki, Hisamatsu, and Podolsky 2003, Uehara, Sugawara, and Takada 2002), it is still unclear as to whether IFN-γ–primed OECs increasingly express these signaling components on the surface. Moreover, the addition of soluble CD14 (sCD14)–containing human serum in cultured OECs does not enhance TLR2- and TLR4-mediated immune response to isolated microbial components (Uehara et al. 2001), as reported by an earlier study as well as from our observations (data not shown), suggesting that the presence of sCD14 and the interaction between TLRs and their corresponding agonists on the surface of OECs are not sufficient for OECs to mediate an immune response via surface TLRs. Despite the absence of human serum, live *F. nucleatum* and FnCW elicited robust hBD-2 and CXCL-8 induction (Figure 7 and 8). FnCW induced a remarkable amount of hBD-2 and CXCL-8, relative to those induced by live bacteria after 24 hours of infection, albeit it lacks the viability that inherently allows it to attach to and vigorously invade OECs. This hence indicates that FnCW-induced OEC response was presumably mediated by cell wall proteins. Although isolated LPS used in this study elicited a strong TLR4 activation, it failed to induce CXCL-8 expression in OECs. Distinct CXCL-8 expression profile of OEC in response to isolated agonists versus live *F. nucleatum*, or rather its crude cell wall, suggest that OEC likely utilizes intracellular machinery for eliciting chemokine response. Essentially, an intact bacterial cell wall structure is required for this activation process.

It has become clear that oral epithelial lining secretes CXCL-8 in response to bacterial challenge (Darveau et al. 1998, Sfakianakis, Barr, and Kreutzer 2002, Stathopoulou et al. 2010, Darveau 2010) and this CXCL-8–mediated epithelial cell homeostasis, resulting in neutrophil recruitment to gingival tissues, requires both TLR2 and TLR4 for bacterium–induced CXCL-8 expression. We showed, for the first time, direct *in vitro* evidence that OECs recognize both LPS shed from gram-negative microbes and LPS isolated forms by TLR4 when they were forcibly delivered into the cells (Figure 11, 15, and 16).

Our data from inhibition experiments (Figure 17, 18) strikingly elucidated intracellular events of TLR4 trafficking during LPS infection. Firstly, it is clear that endosomal TLR4 serves as a LPS sensor in OECs, consistent with a previous investigation in which the role of endosomal TLR4 has been reported (Husebye et al. 2006), and this LPS-TLR4 ligation induced CXCL-8 expression. LPS can be naturally taken into an endosomal structure, as suggested by FnCW-induced hTIGK and OKF6 responses, and be subsequently fated to phagosomal fusion (Figure 17). This recycling process creates a pH change in a fused endo-phagosome (Johnson et al. 1993) leading to the activation of TLR9 located on the endosomes (Park et al. 2008). Strikingly, blocking of endosomal acidification attenuated CXCL-8 secretion by OECs in response to FnCW and internalized LPS. Our findings thus suggested for the first time that, upon LPS uptake, endosomal acidification is a critical phenomenon where subsequent TLR4 activation occurs, resulting in the induction of downstream inflammatory cascades including CXCL-8. Secondly, functional TLR4 is presumably synthesized from the ER, and subsequently transported to the Golgi apparatus where TLR4 is stored in a steady state. We showed that retrograde protein transport mediated by brefeldin A dampened CXCL-8 levels in response to internalized LPS (Figure 18), indicating that TLR4, a specific sensor of microbial LPS, is trafficked to endosomes where it interacts with LPS. Recycling of TLR4 from the Golgi apparatus to plasma membranes, and vice versa, was reported (Latz et al. 2002) and this process is known to regulate by a protein associated with TLR4 (PRAT4A) (Takahashi et al. 2007). Taken together, our findings strongly suggest that TLR4 in OECs is distributed intracellularly at the Golgi apparatus and subsequently shuttled to endosomes when bacterial LPS breaches oral epithelial interface.

Interestingly, lipid raft, a cholesterol-enriched cell membrane microstructure, might play a role for TLR4 trafficking in response to LPS. A recent study demonstrated that TLR4 undergoes a mobilization process to lipid rafts in OECs in order to mediate cytokine production in response to *A. actinomycetemcomitans* infection (Imai et al. 2016). Lipid rafts have also been shown to functionally associate with autophagy as the respiratory pathogen *Legionella pneumophila* and the enteric pathogen *E. coli* utilize lipid rafts as portals to enter macrophages and are immediately transferred to autophagosomes (so called autophagy) where they evade host killing (Amer and Swanson 2005). More importantly, autophagy is another avenue which has been shown to induce the expression of CXCL-8 in IECs (Li et al. 2011). The association between autophagy and CXCL-8 homeostasis is not well understood, even though TLR4 is reportedly involved in autophagy induction (Xu, Jagannath, et al. 2007, Xu et al. 2008). With respect to the periodontal microenvironment, oral pathogens such as *P. gingivalis* exploit lipid rafts to enter host cells in the same fashion (Wang and Hajishengallis 2008) and possibly avoid host defense via autophagy for their own benefit (Dorn, Dunn, and Progulske-Fox 2001). Taken together with our findings, TLR4 location in OECs and its docking between intracellular compartments, for instance lipid rafts, autophagosomes, and endosomes, are thus a complex intracellular signaling event in OECs. Essentially, TLR4 signaling for CXCL-8 induction is tightly regulated by numerous mechanisms including canonical TLR4 and lipid raft structures as well as autophagic pathways. Although PRAT4A is in part known to be involved in the compartmentalization of TLRs, how Golgi-associated TLR4 cargo is mechanistically trafficked to endosomal compartments when epithelial cells are infected remains unclear and, therefore, warrants further investigation. Understanding of the mechanisms by which TLR4 is compartmentalized in OECs and the natural process by which LPS is transported through host cell membranes will shed more light on the significance of oral epithelial innate immunity regarding its tolerance to LPS for maintaining periodontal health.

Epithelial cell polarization reportedly contributes to an unresponsiveness of intestinal epithelial cells to bacterium-derived components, a major hallmark of epithelial cells in response to microbial infection (Hershberg 2002). Similarly, this possibly explains the phenomenon found in our study that OECs were not able to sense bacterial LPS unless LPS was transfected into OECs with liposomes. Although the polarization of TLR4 was not investigated in this study, TLR4 was presumably located near the basolateral surface of epithelial cells (Cario et al. 2002). Polarization of TLR4 in OECs might be associated with the lack of PRAT4A protein in OECs, known to promote TLR4 docking to plasma membranes (Takahashi et al. 2007). Whether PRAT4A modulates location of TLR2 and TLR4 in OECs warrants further elucidation. A robust TLR4-mediated CXCL-8 induction upon LPS delivery (Figure 11) clearly substantiated a novel role of intracellular TLR4 in epithelial homeostasis. However, our data clearly suggested, aside from TLR4, the involvement of multiple signaling pathways for CXCL-8 expression by oral epithelium. Despite the requirement of TLR2 and TLR4 for CXCL-8 response by OECs, the ligation of other innate immune receptors, e.g., TLR9 and PAR-2 with their corresponding ligands, also contributes to the expression of CXCL-8 in OECs (Kim et al. 2012, Shimada et al. 2009). Orchestration of various innate immune receptors in OECs, in spite of an inert feature of TLR4, stimulates homing of neutrophils to the periodontal tissue to defend against gram-negative microbes, while simultaneously keeping the oral epithelial barrier unharmed by free LPS in the microenvironment.

To summarize, we have elucidated a novel mechanism which OECs utilize for sensing oral bacteria, with respect to the roles of TLR2 and TLR4, by using oral commensal *F. nucleatum* as an experimental approach *in vitro*. The abilities of *F. nucleatum* to engage at both human TLR2 and TLR4 enabled us to investigate a role of intracellular TLR4 machinery in OEC defense against invading microbes. While OEC populations have demonstrated the heterogeneity of TLR2 responsiveness, TLR4 remains silent unless bacterial LPS is internalized into OECs by a specific machinery that delivers LPS to an endosomal compartment. However, signals upstream of TLR4 required for trafficking of Golgi-associated TLR4 to LPS-containing endosomes need to be elucidated. The distinction of signal transduction between TLR2 and TLR4 in OECs indicates an inherent mechanism which oral epithelium employs to discriminate gram-negative from gram-positive microbes in order to avoid a devastating effect of gram negative bacteria predominantly found in deeper periodontal pockets and to prevent an excessive host-bacterium interaction resulting from constant exposure of OECs to oral pathogens. This strategy, e.g., TLR4 polarization possibly due to the lack of PRAT4A, may, therefore, developmentally provide a protective measure for the periodontium to avert the destruction of the underlying connective tissues and bone as a consequence of a polymicrobial milieu dominated by gram-negative anaerobes found in early stages of periodontal disease.

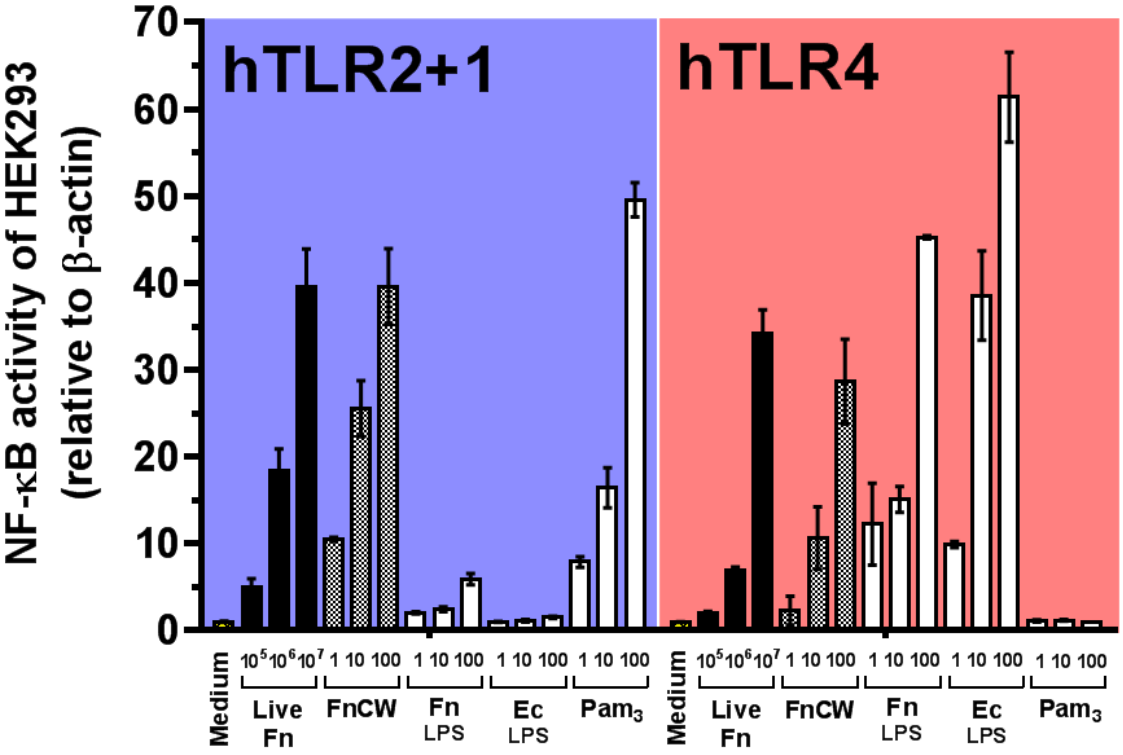

Figure 4. **TLR2 and TLR4 activation by live *F. nucleatum* or its cell walls.** Titration of live *Fn* (10^5-10^7 bacteria/mL) and FnCW (1-100 ng/mL) were tested for their abilities to activate NF-κB specifically via TLR2 or TLR4, relative to β-actin internal control. Synthetic lipoprotein Pam_3CSK_4 (1-100 ng/mL) served as a positive control for TLR2 assays. *E. coli* LPS (1-100 ng/mL) and *F. nucleatum* LPS (1-100 ng/mL) were used as positive controls of TLR4 stimulation assays. Experiments were performed in triplicate wells in 2 independent setups that yielded identical results. Data are shown as average fold TLR activity ± SEM, relative to unstimulated controls, from one independent experiment.

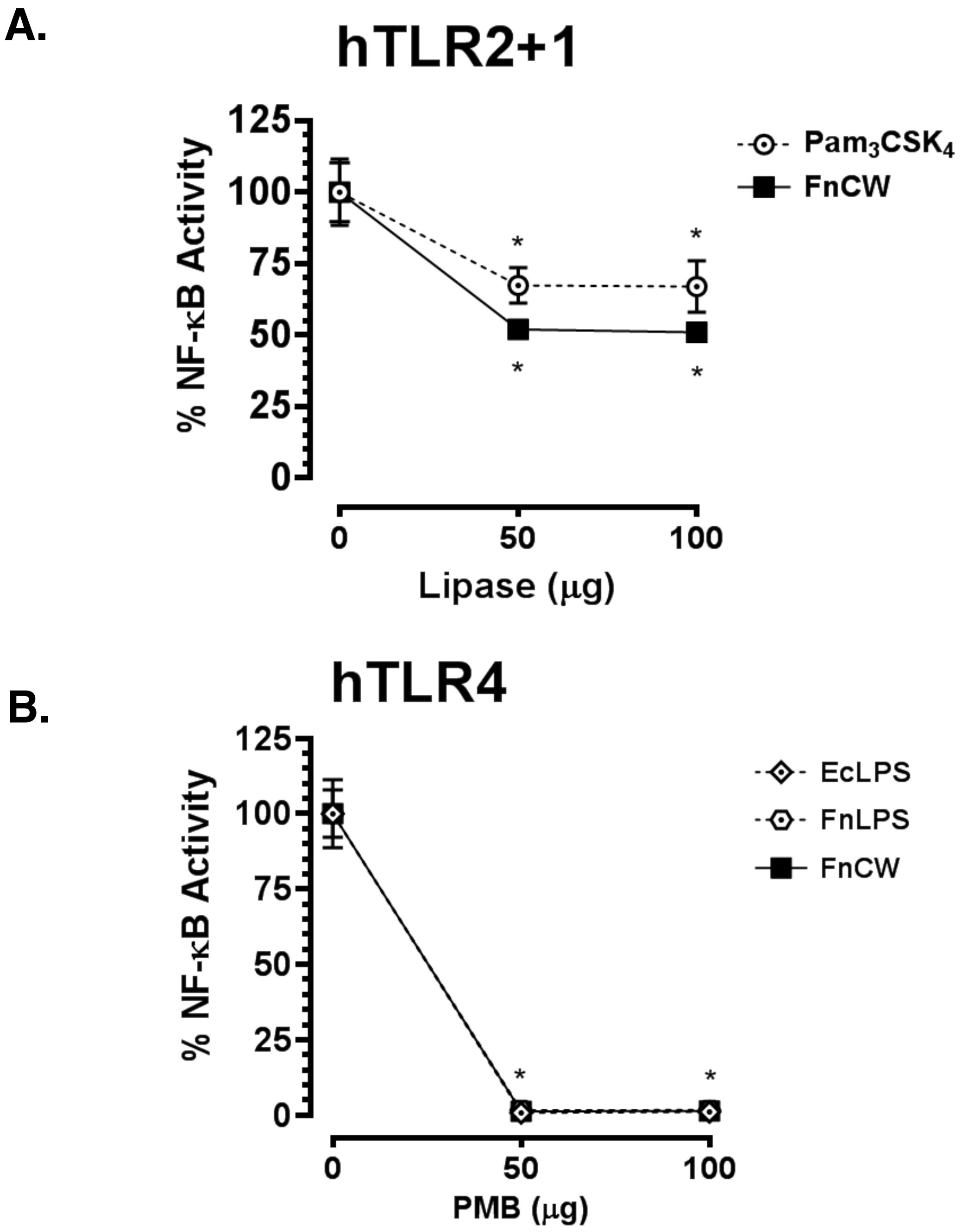

Figure 5. Reduction of TLR2+1 (A) and TLR4 (B) activities after FnCW neutralization. HEK293 overexpressed TLR2+1 or TLR4+MD2 was stimulated with native FnCW, or treated-FnCW for 4 hours in triplicate wells. Treatment of FnCW with lipoprotein lipase and polymyxin B resulted in a significant decrease of NF-κB activity. Data are shown as mean ± SEM and represented similar results from one of two independent assays. Asterisks indicate significant differences (*p-value<0.05), relative to corresponding controls.

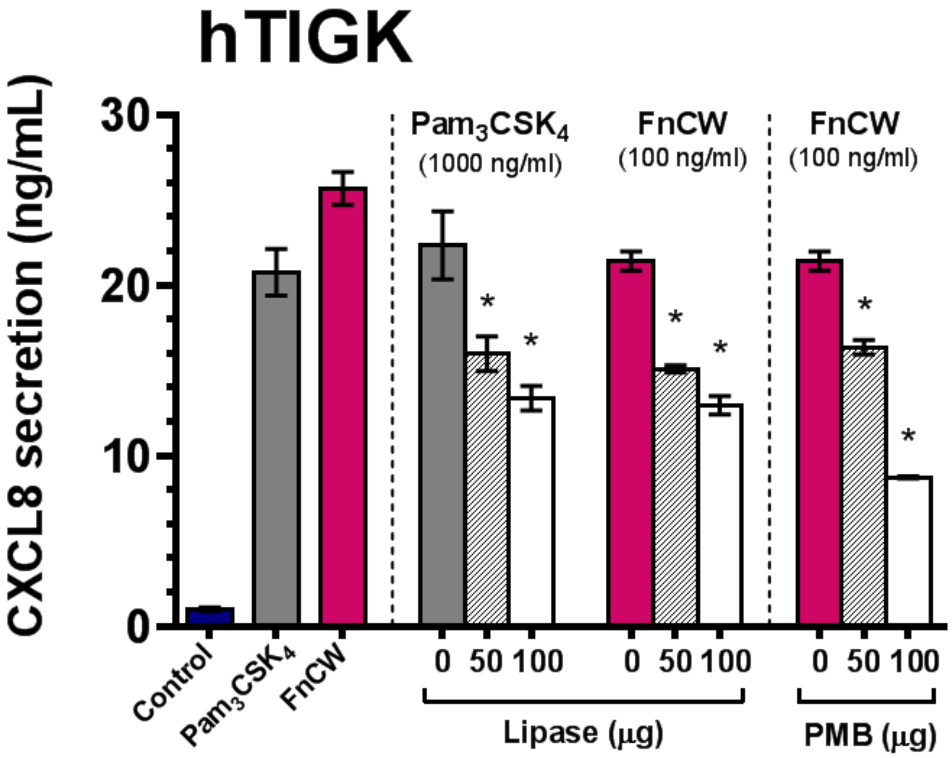

Figure 6. Reduction of CXCL-8 secretion by hTIGK after FnCW neutralization. hTIGK were stimulated with native FnCW, or treated-FnCW for 24 hours in triplicate wells. Treatment of FnCW with lipoprotein lipase and polymyxin B resulted in a significant decrease of CXCL-8 response. Data are shown as mean ± SEM and represented similar results from one of three independent assays. Asterisks indicate significant differences (*p-value<0.05), relative to corresponding mock controls

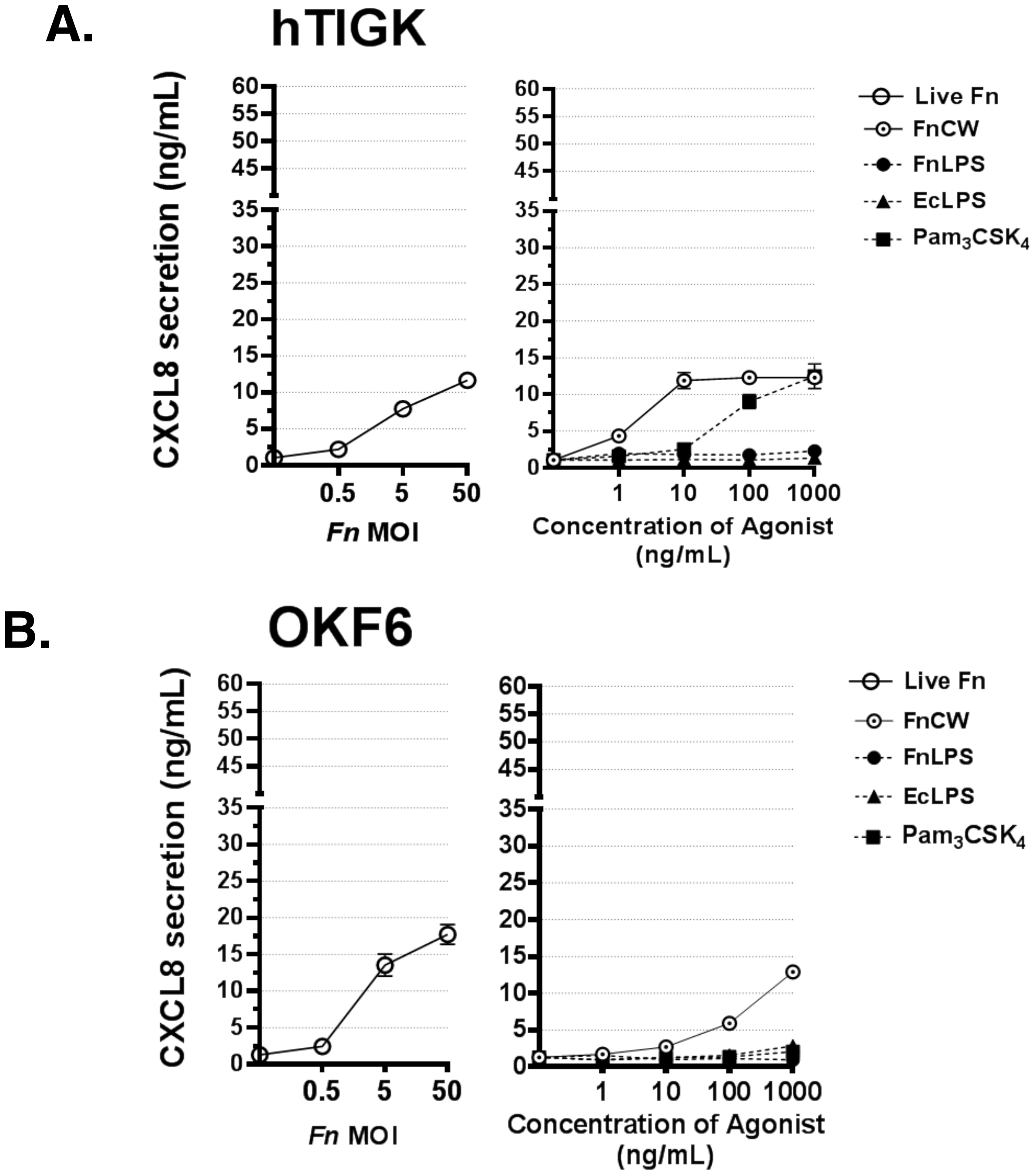

Figure 7. CXCL-8 response to *F. nucleatum* infection by different lines of OECs. Cultured hTIGK **(A)**, OKF6 **(B)**, normal GEC from donor 1 (nGEC1) **(C)** and from donor 2 (nGEC2) **(D)** were challenged with wide doses of live *Fn*, FnCW, EcLPS, FnLPS, or Pam_3CSK_4 for 24 hours prior to CXCL-8 protein determination by ELISA. Actual concentrations of secreted CXCL-8 are shown as mean ± SEM and represent similar results from one of two independent experiments in each line performed in triplicate.

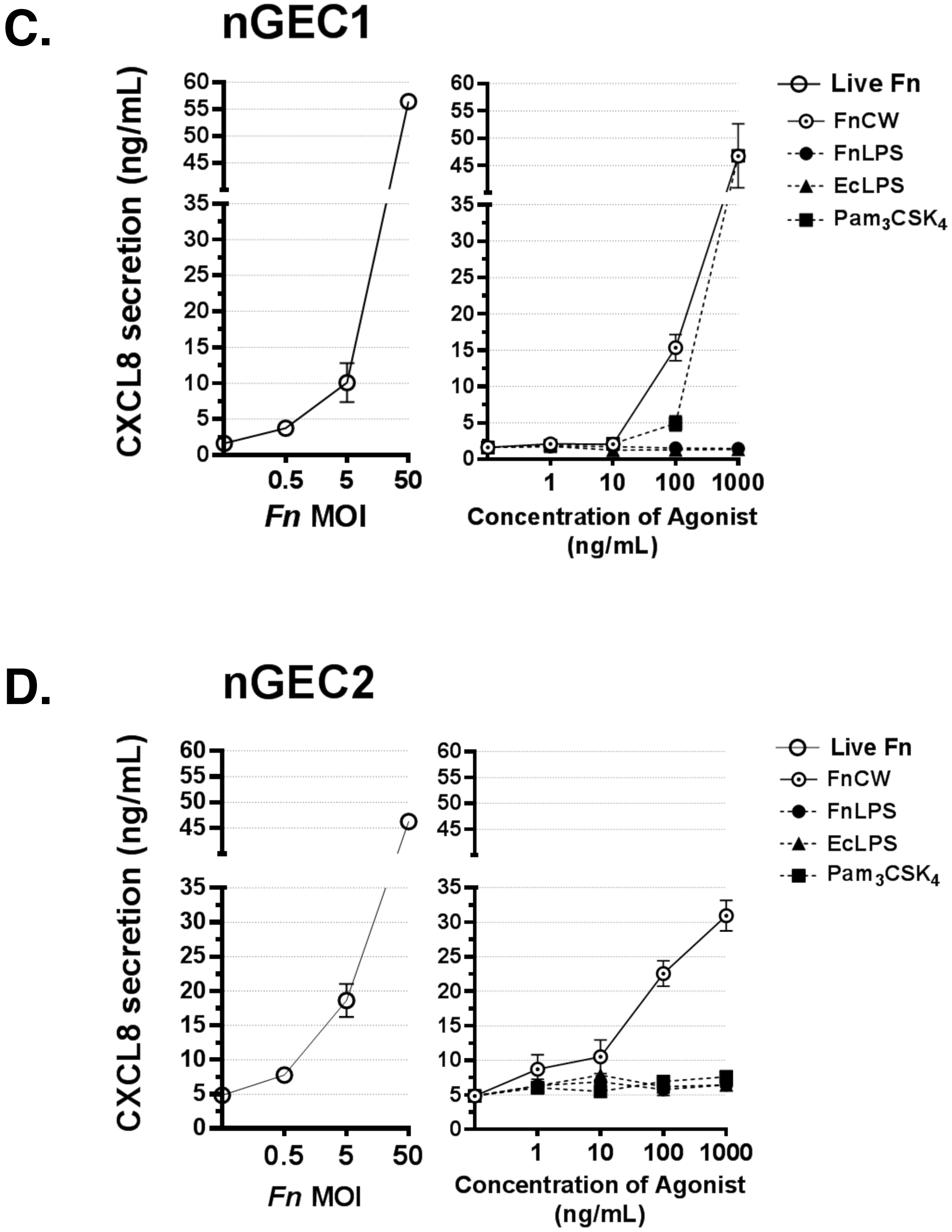

Figure 7. CXCL-8 response to *F. nucleatum* infection by different lines of OECs. (continued)

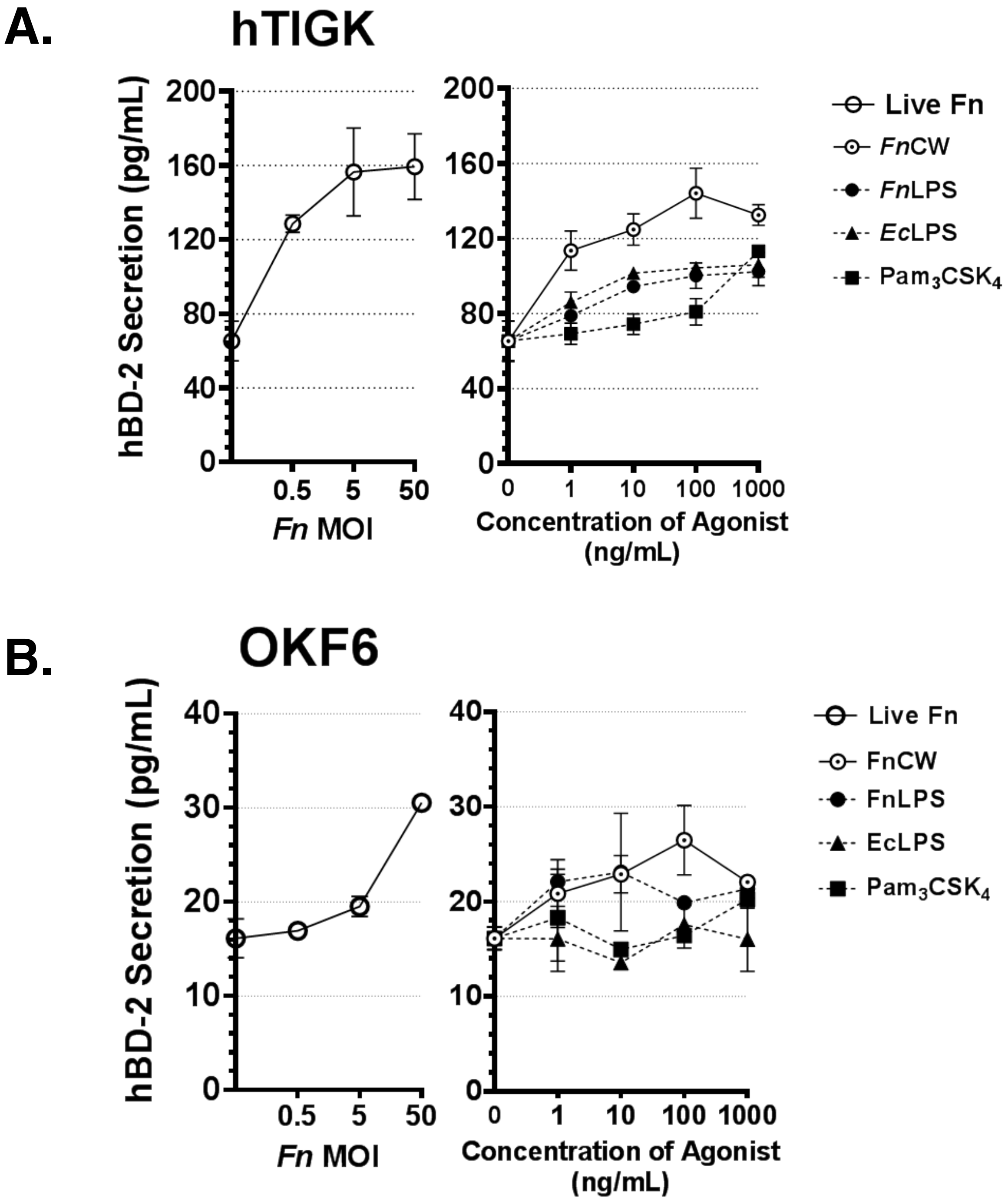

Figure 8. hBD-2 secretion in response to *F. nucleatum* infection by different lines of OECs. Cultured hTIGK **(A)**, OKF6 **(B)**, normal GEC from donor 1 (nGEC1) **(C)** and from donor 2 (nGEC2) **(D)** were challenged with wide doses of live *Fn*, FnCW, EcLPS, FnLPS, or Pam_3CSK_4 for 24 hours prior to hBD-2 protein quantitation by ELISA. Actual concentrations of secreted hBD-2 are shown as mean ± SEM and represented similar results obtained from one of two independent experiments in each line performed in triplicate.

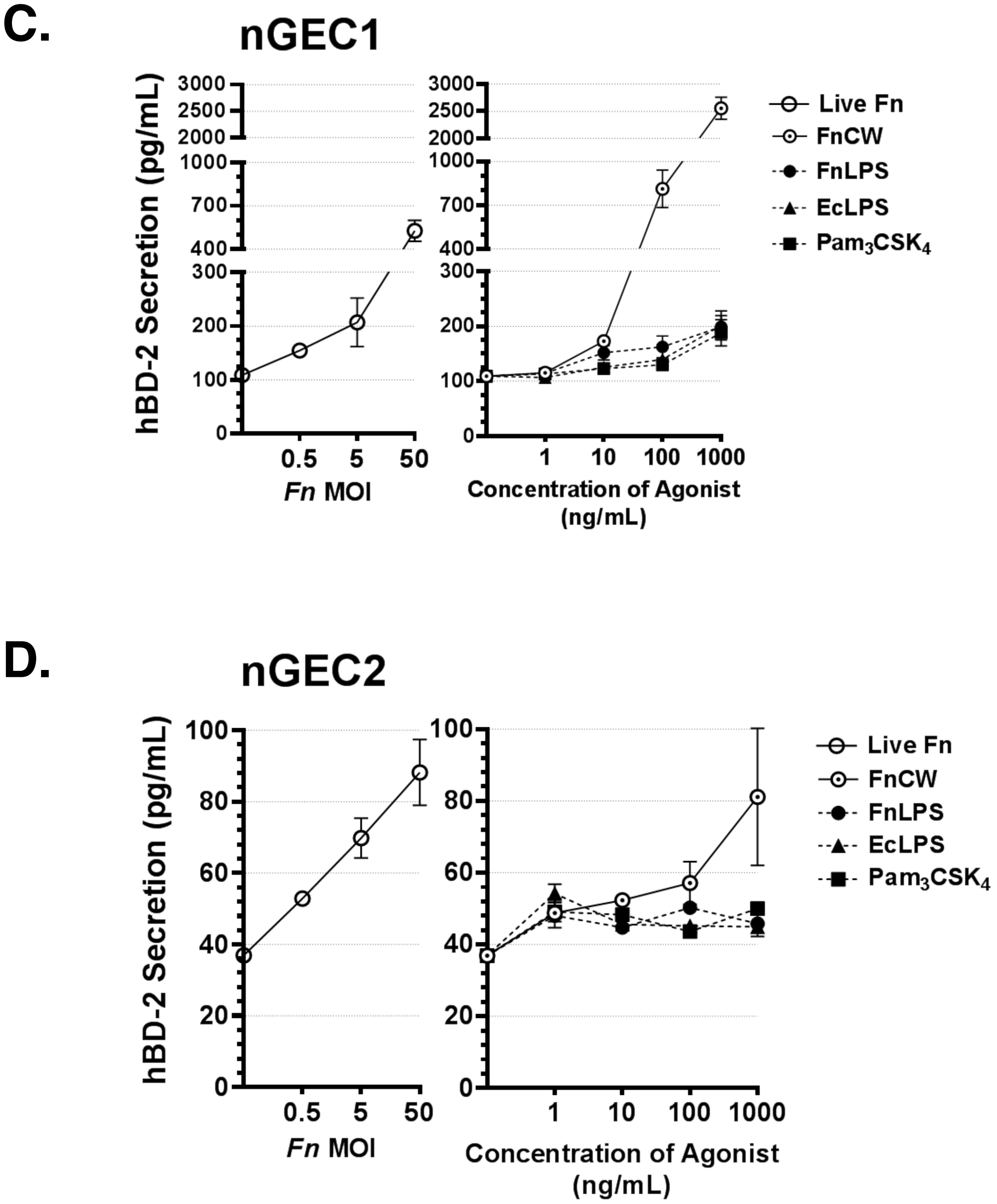

Figure 8. hBD-2 secretion in response to *F. nucleatum* infection by different lines of OECs. (continued)

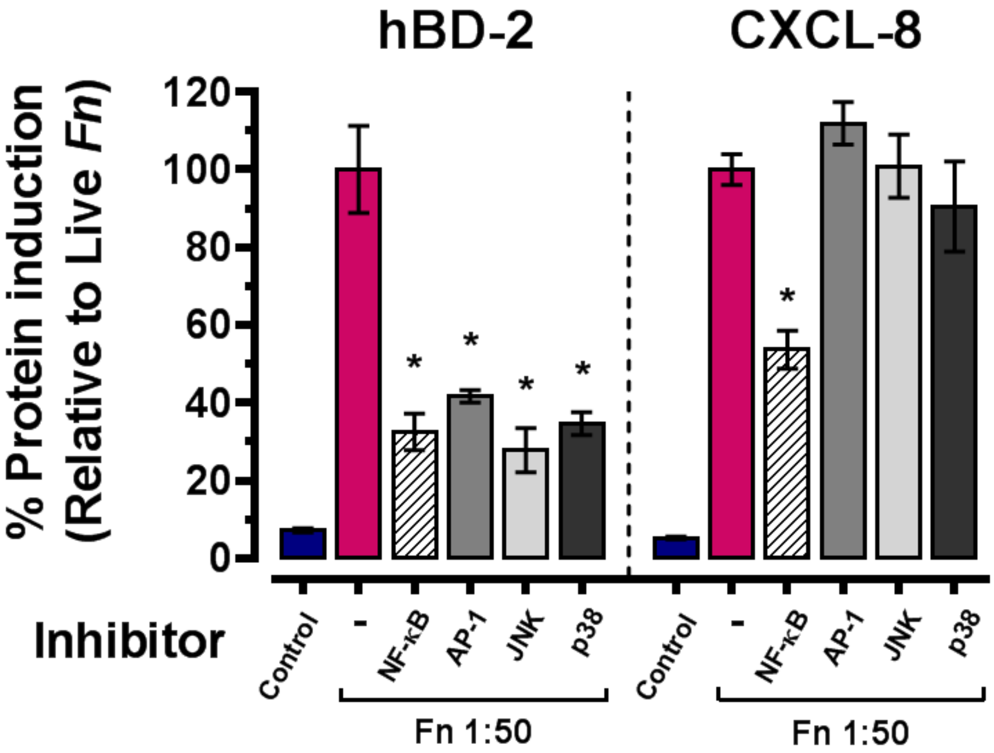

Figure 9. Differential regulation of hBD-2 and CXCL-8 induction in OECs in response to *F. nuclatum* infection. Confluent hTIGK monolayer was pre-incubated with a specific pathway inhibitor for 1 hour prior to *F. nucleatum* challenge. hBD-2 and CXCL-8 proteins were determined using ELISA. Data are derived from one of three independent experiments in triplicate, yielding identical results, and represented as mean ± SEM. Significant level was determined using One-way ANOVA with Tukey's multiple comparison test. Asterisks indicate statistical significance relative to *Fn*-stimulated groups (**p*-value<0.05).

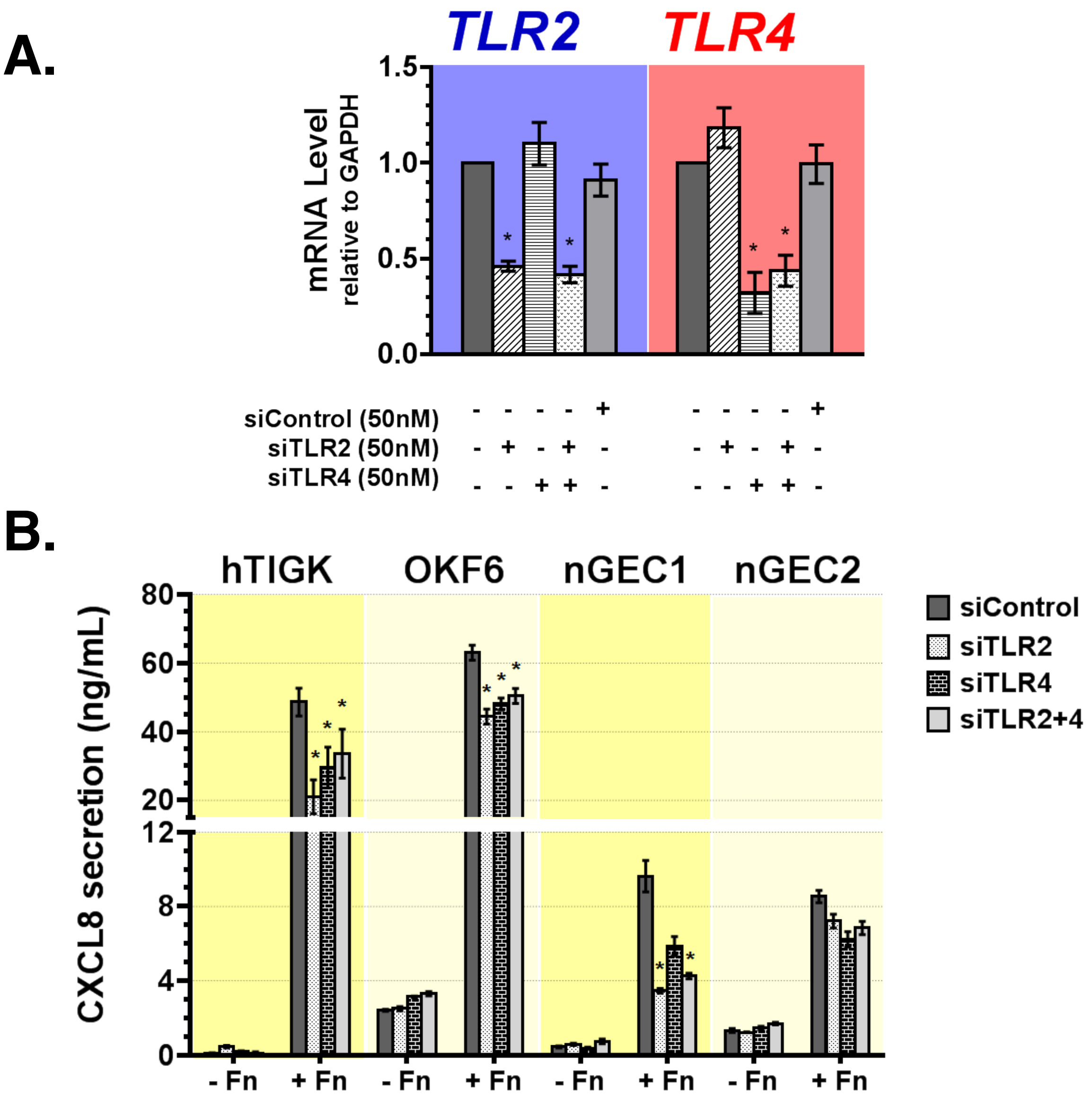

Figure 10. Silencing of TLR2 and TLR4 attenuated CXCL-8 response to *F. nucleatum* by OECs. Cultured cells were knocked-down for TLR2, TLR4 or both by siRNA for 48 hours before 24-hour stimulation with *F. nucleatum* whole cells. **(A)** Silencing efficiency was verified by RT-qPCR. Data represented mean of gene expression level ± SEM, derived from 4 independent experiments in all 4 OEC lines performed in duplicate. **(B)** CXCL-8 protein was quantified by ELISA. Data are shown as mean of actual CXCL-8 concentration ± SEM from 2 independent experiments in duplicate. Differences were tested using two-way ANOVA and Tukey's multiple comparison test. Asterisks indicate significant differences (*p-value<0.05), compared to corresponding non-silenced control cells.

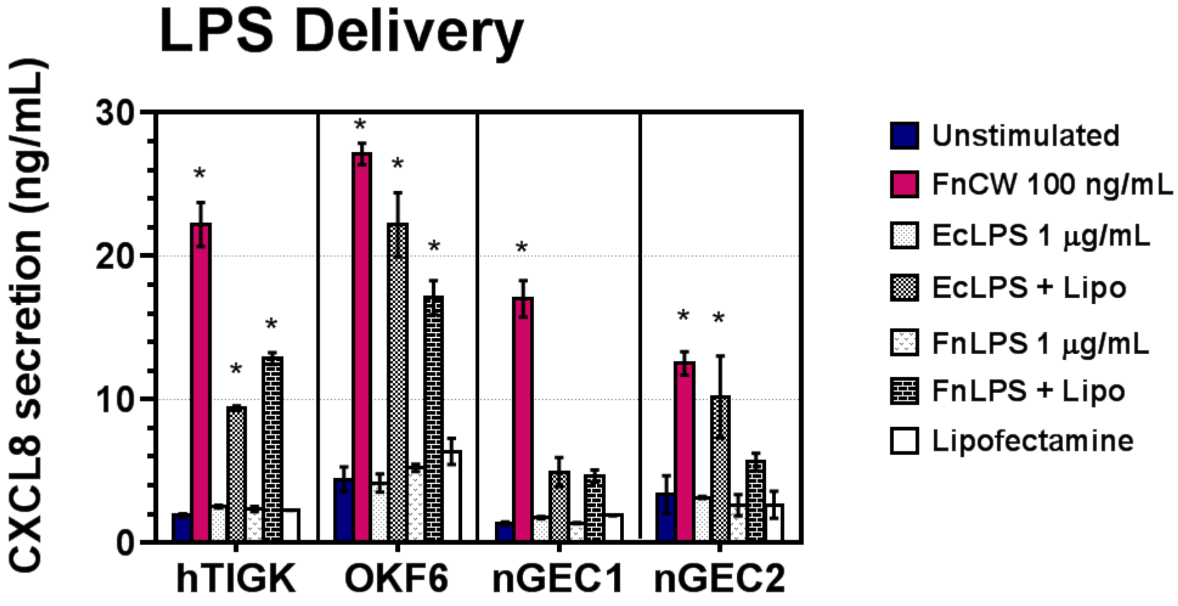

Figure 11. Liposome-coupled LPS induction of CXCL-8 expression in OECs. Cultured OECs were incubated for 24 hours with LPS pre-mixed with Lipofectamine. Culture supernatants were collected and assayed for CXCL-8 secreted protein. Data are shown as mean concentration ± SEM from one of two independent experimental setups performed in each line. Statistical significance was determined by using Two-way ANOVA with Tukey's post hoc test. Asterisks indicate significant difference (**p*-value<0.05), compared to unstimulated controls (for FnCW) or species matched LPS-stimulated controls (for LPS+Lipofectamine).

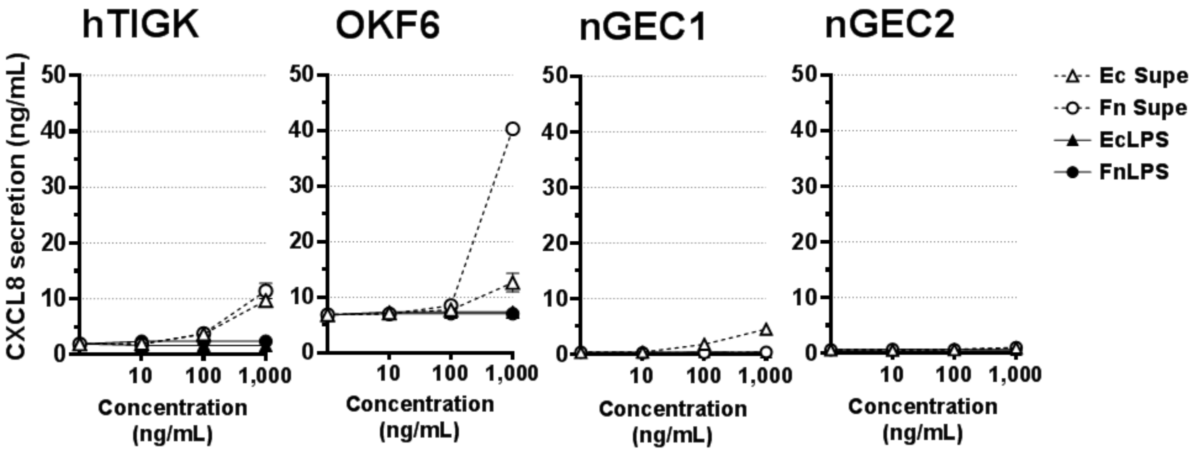

Figure 12. Induction of CXCL-8 expression by bacterial culture supernatants in OECs. LPS-containing bacterial culture supernatants were used for OEC stimulation assays. CXCL-8 protein levels were determined by ELISA assays. Data are representatives of similar results derived from one of two independent experiments, and shown as mean actual concentration ± SEM.

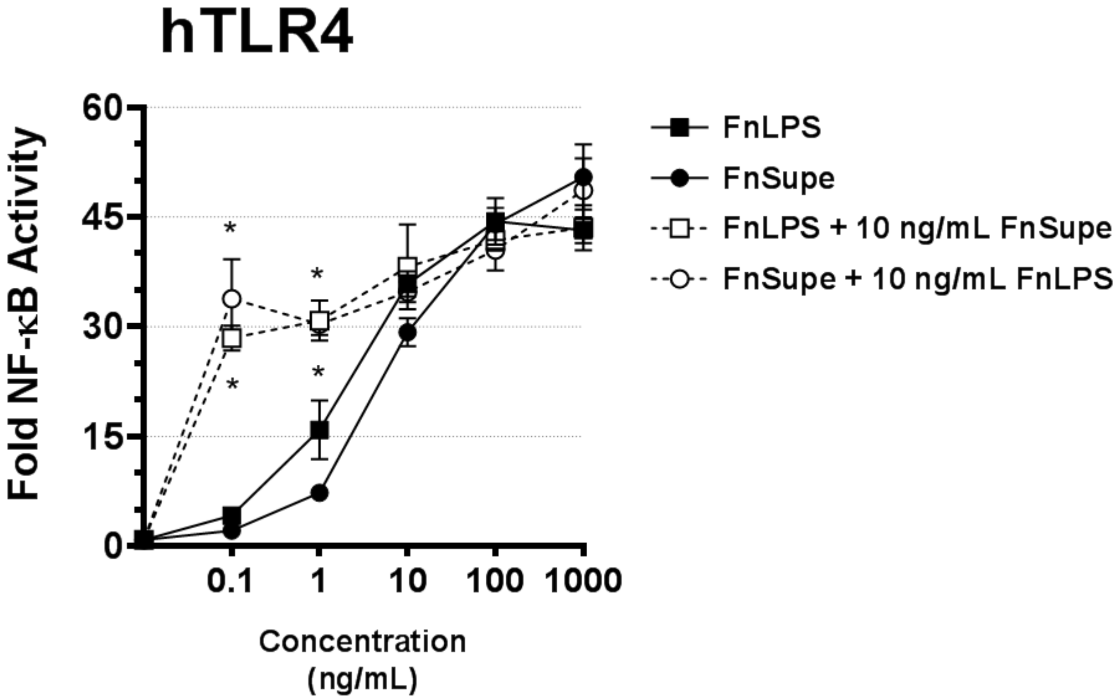

Figure 13. TLR4 activation by *F. nucleatum* culture supernatant (FnSupe). FnSupe (0.1-1000 ng/mL total protein) or purified FnLPS were tested with HEK293 overexpressing human TLR4 and MD2. Data are shown as fold NF-κB activity ± SEM, relative to unstimulated controls. Data are the results from one experiment representing 2 independent experiments that yielded identical results. Statistical difference was analyzed by using multiple unpaired t-test with Holm-Sidak's post hoc test. Asterisks indicate statistical significance (**p*-value<0.05), compared to FnSupe-stimulated controls at each concentration point.

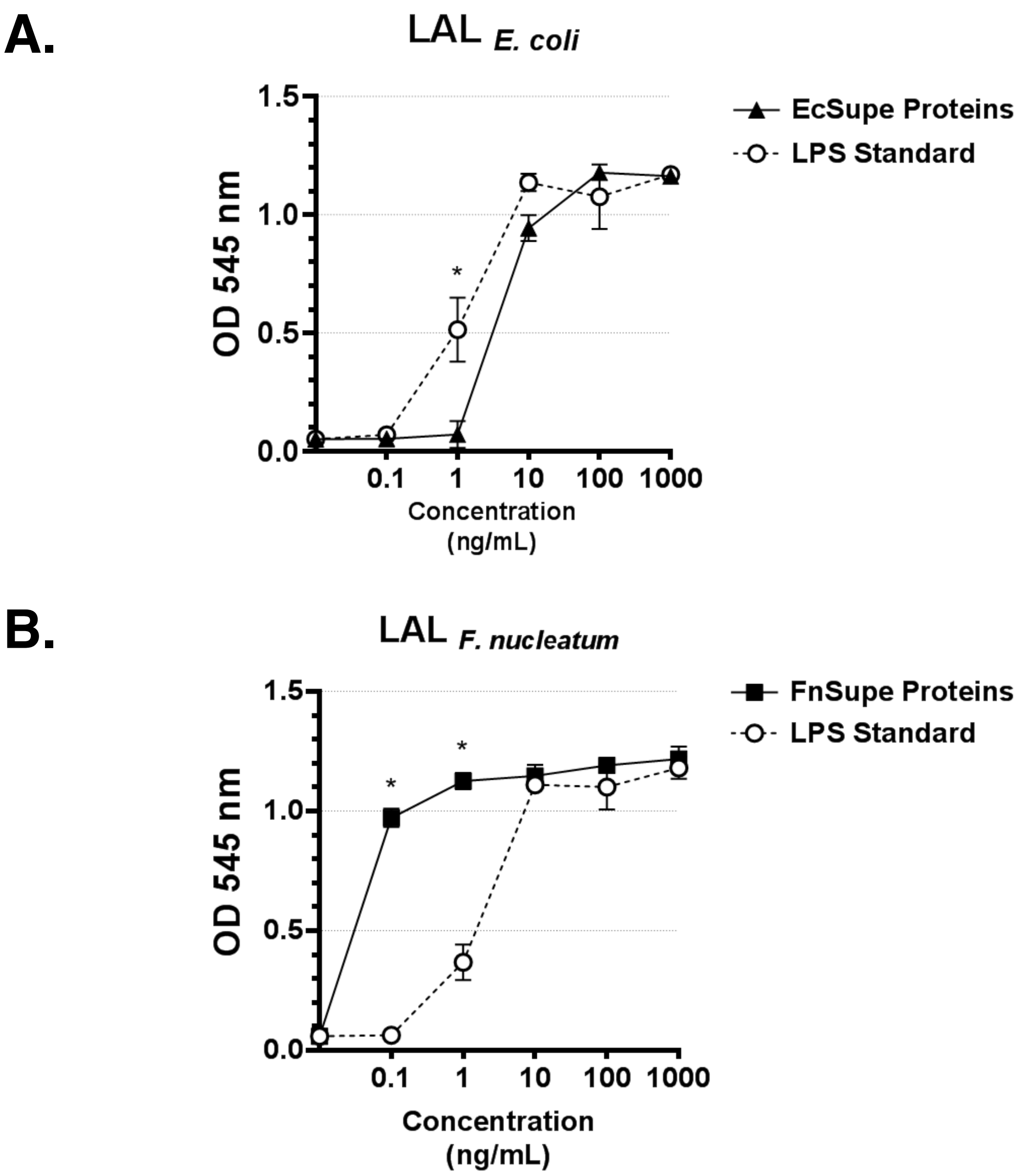

Figure 14. Relative quantity of LPS in bacterial culture supernatants. Supernatants from *E. coli* **(A)** and *F. nucleatum* **(B)** cultures were quantified for the amount of shed LPS by using LAL assays. Data represent mean OD545 ± SEM, obtained from one of two independent experiments in triplicate. EcLPS and FnLPS served as positive controls and showed similar OD values. Statistical difference was tested using two-way ANOVA with Bonferroni's multiple comparison test. Asterisks indicate statistical significance (**p*-value<0.05), compared to LPS standard.

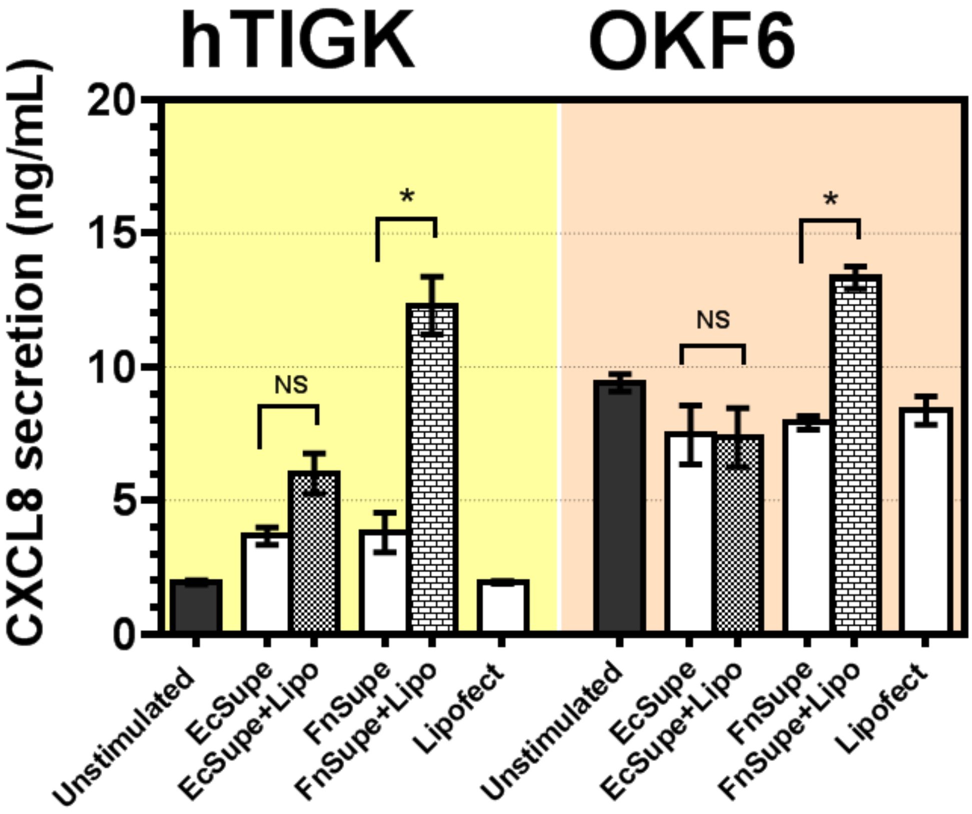

Figure 15. CXCL8 secretion by hTIGK and OKF6 in response to Lipofectamine-mediated internalization of shed LPS. Levels of CXCL-8 protein were determined by ELISA. Data are shown as mean actual concentration ± SEM, derived from one of two independent experiments in triplicate. Asterisks indicate statistical significance (*p-value<0.05, NS = not significant), compared to corresponding unstimulated controls.

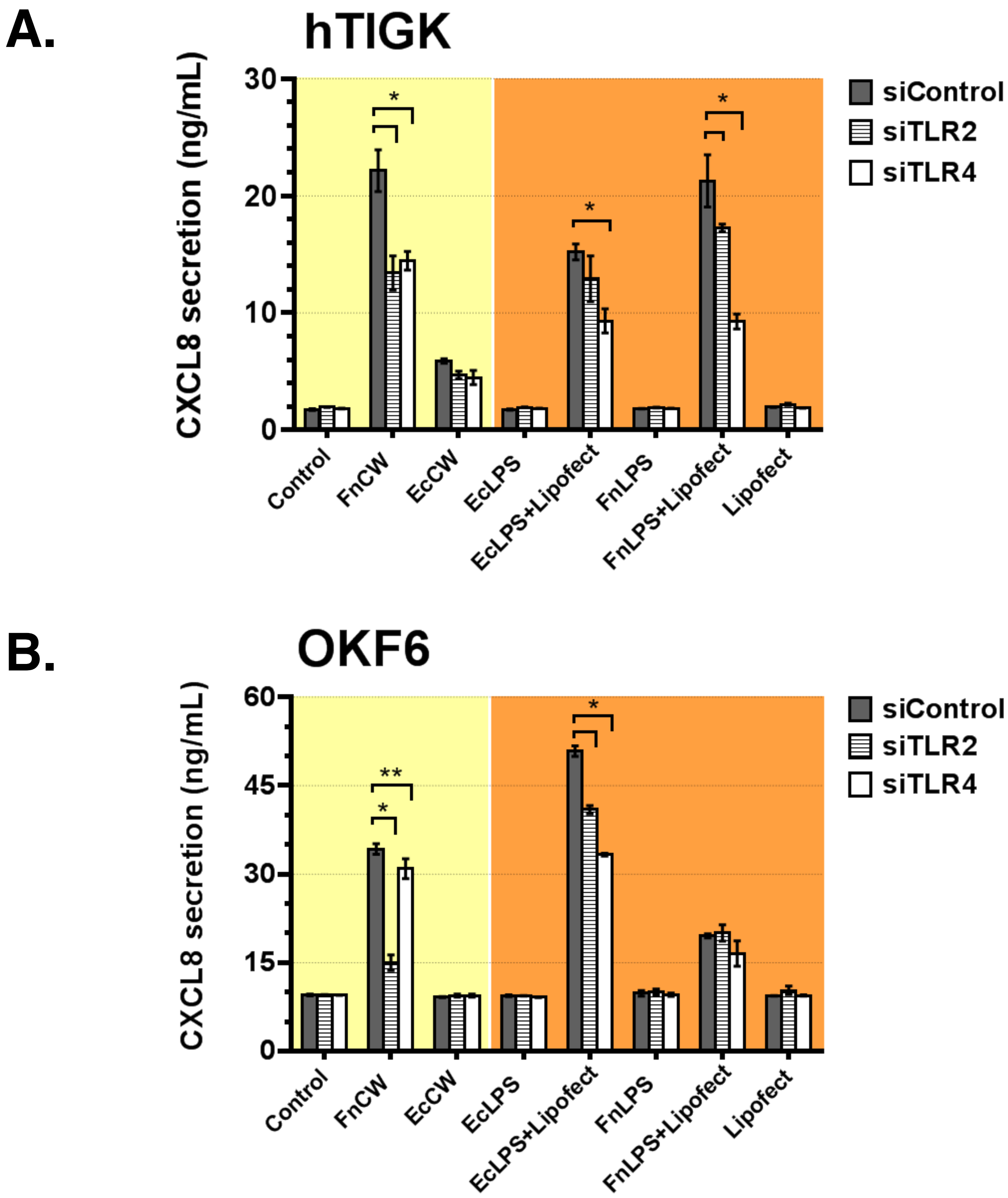

Figure 16. Trafficking of Internalized LPS to intracellular TLR4 in OECs. Cultured hTIGK **(A)** and OKF6 **(B)** were silenced for TLR2 or TLR4 by siRNA transfection for 48 hours prior to LPS delivery using Lipofectamine. CXCL-8 protein levels were determined after 24 hours of LPS challenge. Data represent mean actual CXCL-8 concentration ± SEM, derived from the experiments performed in triplicate wells. Asterisks indicate statistical significance (**p*-value<0.05, ***p-value*=0.0128), compared to corresponding controls.

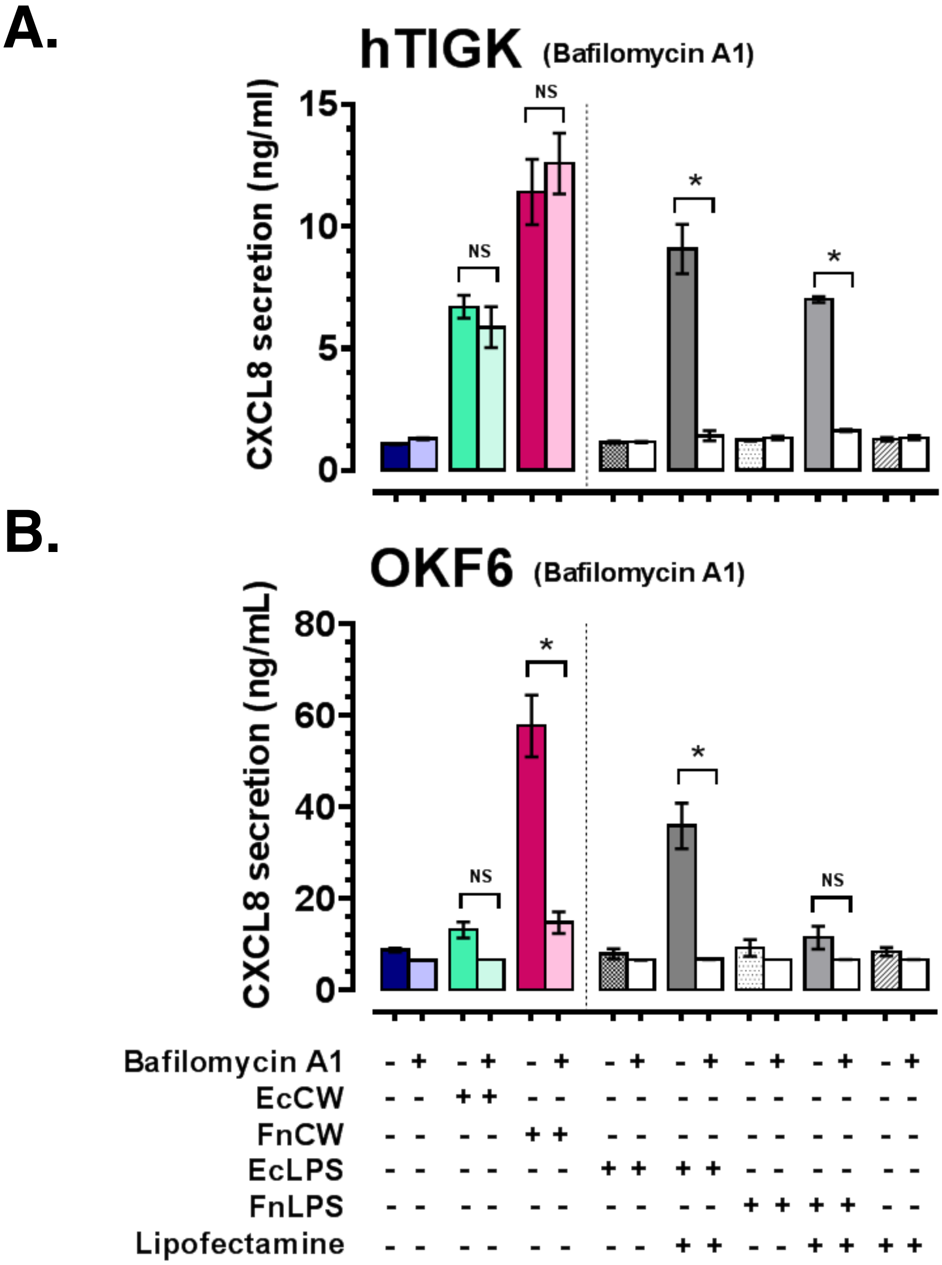

Figure 17. Inhibitory effects of bafilomycin A1 on CXCL-8 induction in OECs in response to internalized LPS. Cultured hTIGK **(A)** and OKF6 **(B)** were pre-incubated with bafilomycin A1 for 1 hour prior to LPS delivery. CXCL-8 secretion was assayed using ELISA. EcCW (100 ng/mL) and FnCW (100 ng/mL) served as positive controls. Data are shown as mean actual concentration ± SEM, derived from one of three independent experiments performed in triplicate. Asterisks indicate statistical significance (**p*-value<0.05, NS = not significant), compared to corresponding controls.

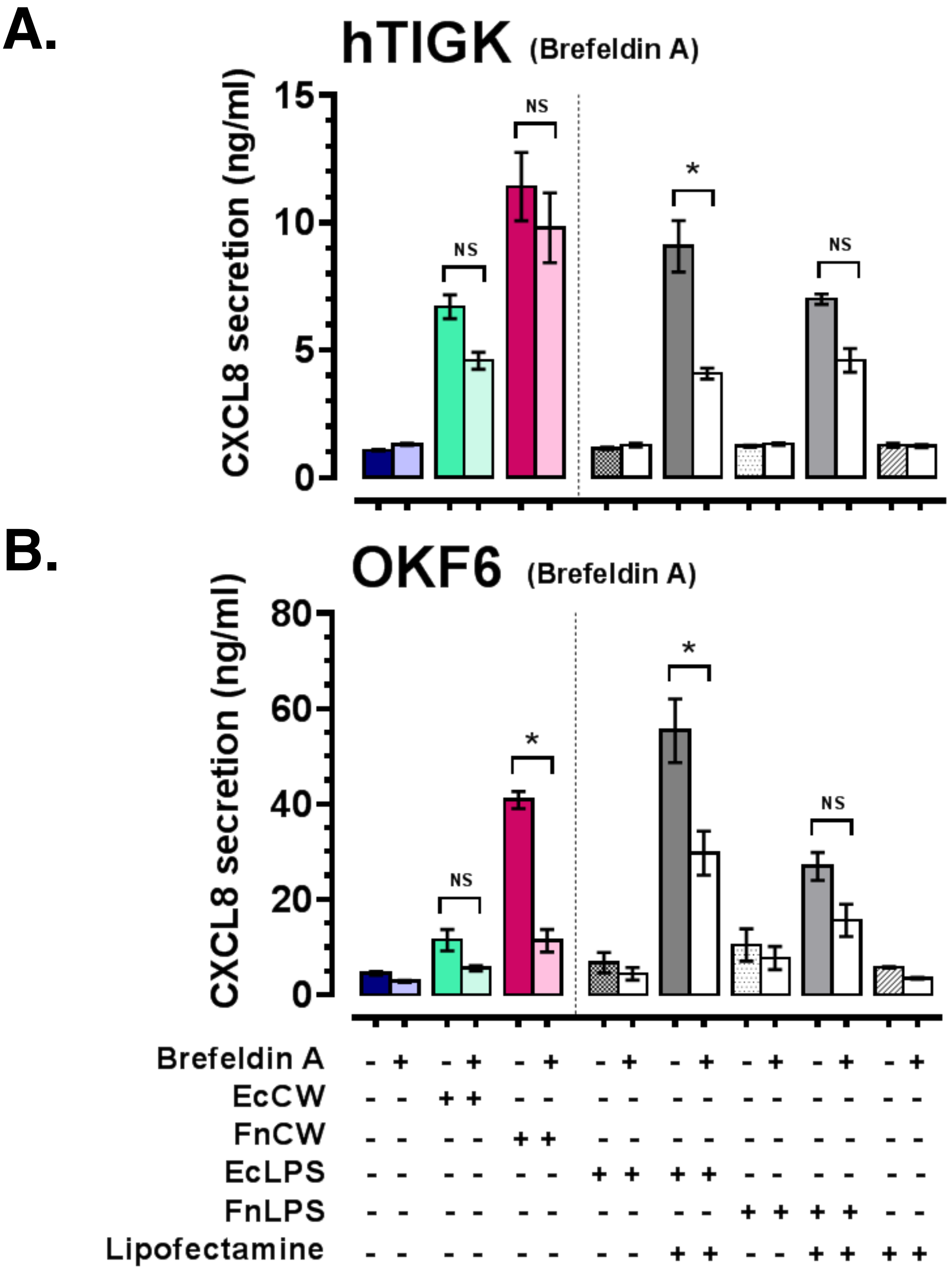

Figure 18. Inhibitory effects of brefeldin A on CXCL-8 induction in OECs in response to internalized LPS. Cultured hTIGK **(A)** and OKF6 **(B)** were pre-incubated with brefeldin A for 1 hour prior to LPS delivery. CXCL-8 secretion was assayed using ELISA. EcCW (100 ng/mL) and FnCW (100 ng/mL) served as positive controls. Data are shown as mean actual concentration ± SEM, derived from one of three independent experiments performed in triplicate. Asterisks indicate statistical significance (**p*-value<0.05, NS = not significant) relative to corresponding controls.

Table 1. Pathway inhibitors used in the study

Name of Inhibitor	Concentrations used [a]	Mechanism of Action	Reference
Bay11-7082 (InvivoGen)	20 µM	Inhibitor of IκB-α phosphorylation.	Mori *et al.* Blood J, 2002.
SR11302 (R&D Systems)	20 µM	Inhibitor of AP-1 transcription factor.	Huang C*et al.* PNAS, 1997.
SP600125 (InvivoGen)	20 µM	Inhibits JNK phosphorylation.	Bennett BL *et al.* PNAS, 2001.
SB203580 (InvivoGen)	20 µM	Inhibits phosphorylation of p38.	Krisanaprakornkit S *et al.* Infect Immun, 2002. Chung WO *et al.* Infect Immun, 2004.
Bafilomycin A1 (Sigma-Aldrich)	400 nM	Inhibit endosomal maturation / acidification of endolysosome.	Johnson LS *et al.* Mol Biol Cell, 1993. Kim Y *et al.* Immunol Cell Biol, 2012.
Brefeldin A (Sigma-Aldrich)	40 µg/mL	Inhibits GTPase involved in protein transport from ER to Golgi complex.	Latz E *et al.* J Biol Chem, 2002. Hornef MW *et al.* J Exp Med, 2003.

a. Optimal concentrations are non-toxic to hTIGKs as determined by Cell Titer-Glo® assays.

Table 2. Primer sequences used for quantitative PCR analysis

Target gene (Human)	Sequence (5'→3')	Amplicon size (bp)	Reference
GAPDH Forward	AAC GGA TTT GGT CGT ATT GGG C	160	Yilmaz Ö *et al.* Cell Microbiol, 2010.
GAPDH Reverse	CTT GAC GGT GCC ATG GAA TTT G		
TLR2 Forward	GGC CAG CAA ATT ACC TGT GTG	66	Horst OV *et al.* J Dent Res, 2009.
TLR2 Reverse	AGG CGG ACA TCC TGA ACC		
TLR4 Forward	CGG AGG CCA TTA TGC TAT GT	140	Horst OV *et al.* J Dent Res, 2009.
TLR4 Reverse	TCC CTT CCT CCT TTT CCC TA		

CHAPTER IV

INVASION OF *FUSOBACTERIUM NUCLEATUM* AND ITS CELL WALL PROTEINS IS REQUIRED FOR LPS RECOGNITION BY ORAL EPITHELIAL CELLS

SYNOPSIS

Extensive studies have emphasized how sentinel cells, i.e., monocytes, macrophages, and dendritic cells, recognize microbial patterns with respect to the innate mechanisms that they utilize to mount a proper innate immune defense, such as TLR-dependent signaling, in response to bacterial infection. Fundamentally, the basis of bacterial recognition by the myeloid cell lineage has been clearly demonstrated by a robust immune response when these cells encounter a milieu full of danger signals derived from microbial compartments including cell wall fragments as well as genetic materials, e.g., bacterial DNA and viral RNA. However, this is not the case for epithelial cells in which the unresponsiveness/hypo-responsiveness to bacterial components has been massively reported. Here we extended our study and showed that, although OECs are not responsive to isolated bacterial LPS, uptake of bacterial cell walls by OECs, owing to an active engagement of viable microbes with host cells, leads to the induction of inflammatory signals, thereby suggesting the natural mechanisms which OECs utilize for oral bacterial sensing and that LPS-impregnated bacterial cell wall proteins act as couriers delivering LPS to an intracellular TLR4. We thus concluded that bacterial invasion and native bacterial cell wall proteins such as FadA for *Fusobacterium nucleatum* are required for the recognition of bacterial LPS by OECs, and dissociation of LPS from its bound proteins present in native cell walls disarms OEC responsiveness to invading pathogens in that an unbound LPS loses its ability to enter and be trafficked to TLR4. This unique sensing of LPS

by OECs hence contributes to its immunosurveillance function for maintaining periodontal health.

INTRODUCTION

We previously demonstrated that OECs are capable of sensing bacterial LPS. However, in order for LPS to be recognized, LPS has to be trafficked to a specific intracellular compartment where TLR4 is located, presumably at the Golgi bodies. Consistent with several lines of evidence in which the responsiveness of epithelial cells to a variety of receptor agonists was investigated, regardless of where the tested cell lines were harvested from, OECs are inert to purified agonists, i.e., Pam_3CSK_4 or isolated LPS either from *E. coli* or *F. nucleatum.* Interestingly, FnCW is able to trigger a robust CXCL-8 response by OECs, suggesting an essential role of intact bacterial cell wall proteins in mediating a key chemokine response as well as in naturally transporting bacterial LPS into epithelial cells–a biological process required for LPS recognition.

In both healthy and inflamed gingiva, *F. nucleatum* is known to be a key pathogen that plays a role in promoting co-aggregation of the bacterial community (Kolenbrander et al. 2002, Kolenbrander et al. 1995) in the areas adjacent to junctional epithelium (JE). Its prevalence in the periodontium, as well as in distant sites associated with pathogenesis of several diseases (Han 2015, Han and Wang 2013), and its ability to provoke a strong immune response by OECs (Stathopoulou et al. 2010) have rendered it an oral commensal-turned pathogen. *F. nucleatum* is capable of invading OECs, and the level of CXCL-8 elicited by OECs is positively correlated with the number of *F. nucleatum* invading OEC monolayers (Han et al. 2000). More recently, an *F. nucleatum* mutant lacking FadA (ΔfadA), a virulence factor adhesin pivotal for *F. nucleatum* to attach to and invade numerous human cell types, has been constructed (Rubinstein et al. 2013, Xu, Yamada, et al. 2007). We, therefore, utilized the *F. nucleatum* ΔfadA as an *in vitro* research tool to study the role of bacterial invasion in the TLR-4dependent

OEC response. In addition, we examined the role of FnCW proteins in the induction of CXCL-8 response by OECs by employing an enzymatic digestion strategy to remove cell wall proteins as well as comparing FnCW to cell walls harvested from other invasive enteric and oral microbes. Our results demonstrated that the activity of digested-FnCW to stimulate human TLR2 was reduced, whereas TLR4 activity remained unchanged. Therefore, it suggests that FnCW proteins modulate human TLR2, and it plays a role in LPS uptake.

MATERIALS AND METHODS

Human oral/gingival epithelial cell culture

Normal human OECs were obtained from the gingival tissue cell collections at the department of Oral Health Sciences, School of Dentistry, the University of Washington. Briefly, normal human OECs were isolated from gingival tissue specimens obtained from healthy adults who underwent third-molar surgical procedures. Gingival tissue explant cultures were performed to isolate primary epithelial cells and then stored as previously described (Krisanaprakornkit et al. 1998). Primary OECs were grown in Keratinocyte Serum-Free medium (Ker-SFM) (Life Technologies) supplemented with 25 µg/mL bovine pituitary extract, 0.2 ng/mL human recombinant epidermal growth factor, 5 µg/mL insulin (Sigma-Aldrich, St. Louis, MO), 0.5 µg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO), 0.15 mM calcium chloride, and 10% Penicillin–Streptomycin (Sigma-Aldrich, St. Louis, MO). In some experiments where live bacteria were used for the stimulation, OECs were grown in antibiotic-free Ker-SFM. Cultured OECs were seeded in 12- or 96-well plates and maintained under a 37°C humidified atmosphere with 5% CO_2. Monolayers of OECs reached confluence prior to the day of bacterial stimulation.

This study incorporated 2 lines of epithelial cells, OKF6/TERT2 and hTIGK, in addition to normal OECs. Human telomerase immortalized normal oral epithelial cell OKF6/TERT2 line was generously provided by the Division of Dermatology, Harvard Medical School. Human telomerase-immortalized gingival epithelial cell hTIGK line was kindly gifted by Dr. Richard J. Lamont, School of Dentistry, University of Louisville. Both OKF6/TERT2 and hTIGK lines were grown and maintained in Keratinocyte Serum-Free medium (Ker-SFM) (Life Technologies) supplemented with 25 µg/mL bovine pituitary extract, 0.2 ng/mL human recombinant epidermal growth factor, 0.4 mM calcium chloride, and 10% Penicillin–Streptomycin (Sigma-Aldrich, St. Louis, MO)

Bacterial culture and crude cell wall preparation

Bacteria tested in this study were obtained from the collection of frozen bacterial stocks. Parental *F. nucleatum* clinical isolate strain 12230, and *Fn* 12230 Δ*fadA* counterpart were kindly provided by Dr. Yiping W. Han, Department of Microbiology and Immunology, School of Dental Medicine, Columbia University. In brief, *F. nucleatum* ATCC 25586, *F. nucleatum* clinical isolate 12230 or its ΔfadA mutant was grown overnight at 37^{o}C under anaerobic gas conditions (80% N_2, 10% CO_2, 10% H_2) in trypticase soy yeast broth (TYK) supplemented with 10 µg/mL hemin (H) and 1 mg/mL menadione. Crude cell walls were prepared as previously described (Krisanaprakornkit et al. 2000) by using French Pressure Cell Press at 15,000 lb/in^2 with the cocktails of protease inhibitors including Pefabloc® SC (Boehringer Mannheim GmbH, Mannheim, Germany), Benzamidine (Sigma-Aldrich, St. Louis, MO), Nα-Tosyl-L-Lysine Chloromethyl Ketone Hydrochloride (Sigma-Aldrich, St. Louis, MO) at a concentration of 20 µM each and DNase (Sigma-Aldrich, St. Louis, MO). Cell wall fractions were harvested by several centrifugations and suspended in sterile phosphate-buffered saline (PBS) prior to fluorometric protein quantitation using Qubit® protein quantitation kit (Thermo Fisher Scientific). Crude FnCW in PBS suspension was stored in -80^{o}C prior to experimental setup. LPS from *E. coli* and *F. nucleatum* were isolated and purified as previously described (Darveau and Hancock 1983).

In some experiments, *Porphyromonas gingivalis (Pg)* ATCC 33277, *Prevotella intermedia (Pi)* ATCC 25611, *Aggregatibacter actinomycetemcomitans (Aa)* ATCC 43718, and *Streptococcus gordonii (Sg)* ATCC 51656 were included. Similar to *F. nucleatum,* these bacteria were grown in TYHK broth overnight at 37°C under anaerobic gas conditions, with the exception of *Aa* in which an anaerobic atmosphere with 5%CO_2 is preferable. In some experiments, *Escherichia coli (Ec)* JM83 strain was used as a positive control. *E. coli* was grown in Luria-Bertarni (LB) broth under aerobic conditions in a vigorous 37°C shaker.

PCR screening of *FadA* gene in *F. nucleatum* genomic DNA

Fusobacterium nucleatum clinical isolate strain 12230 genetically deleted for *FadA* gene was a kind gift from Dr. Yiping W. Han. *F. nucleatum* 12230 WT and its FadA mutant were inoculated into TYHK broth and grown overnight at 37°C under anaerobic conditions. Bacterial cultures were harvested the next day and extracted for *F. nucleatum* genomic DNA by heating the pellets at 100°C for 10 minutes. Crude genomic DNA was then obtained from the supernatants after bacterial pellets were heated and spun down at 13K for 1 minute.

PCR amplification was setup afterwards to confirm the genotypes of tested *F. nucleatum* strains including *F. nucleatum* ATCC 25586, *F. nucleatum* 12230, and *F. nucleatum 12230* ΔfadA. A total 20 µL per reaction containing 1.5 µL crude genomic DNA, 1X Thermopol buffer, 200 nM dNTP, 500 nM each forward and reverse primers, and 0.5 U of Taq DNA polymerase was carried out under the following conditions: Initial denaturation of double strands DNA at 95°C for 5 minutes, Amplification for 30 cycles (consisting of denaturation at 95°C for 10 seconds, annealing at 50°C for 30 seconds, and elongation at 68°C for 1 minute), and final elongation at 68°C for 7 minutes. PCR was performed by using Bio-Rad iCycler iQ^{TM} (Hercules, California, USA).

A housekeeping gene, 16S rRNA, was used to control an equal load of DNA samples for each screen. PCR products were run in 1.2% agarose gel and visualized under UV light.

HEK293 TLR activation assays

Analysis of TLR2/1- and TLR4-mediated NF-κB activities of bacterial samples and purified agonists was performed using human embryonic kidney cells (HEK293) transfected with plasmid DNAs bearing firefly luciferase-labeled NF-κB sequence and renilla luciferase-labeled β-actin sequence, in combination with plasmids encoding human TLR2/1 or TLR4 as described elsewhere (Coats et al. 2007).

HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum, and seeded in 96-well plates one day prior to transfection. Transfection with plasmids encoding human TLR2/1 or TLR4 was performed by a standard calcium phosphate precipitation method. Briefly, pTLR2 and pTLR1 (0.002 µg each) were co-transfected with pCD14 (0.002 µg) for transient TLR2/1 expression. Similarly, pTLR4 (0.002 µg) and pMD-2 (0.002 µg) were co-transfected with pCD14 (0.002 µg) to induce TLR4 expression. Reporter plasmids pBArenLuc (0.0004 µg) and pNFκBLuc (0.02 µg) were transfected in all wells. Stimulation of transfected HEK293 cells was performed on the following day in triplicate under normal physiological conditions for 4 hours, after which the HEK293 cells were rinsed with PBS and lysed with passive lysis buffer (Promega, Madison, WI). Luciferase activity was then assayed by using the Dual Luciferase Assay Reporter System (Promega, Madison, WI). Luminescent signals generated from firefly luciferase activity were normalized to signals from renilla luciferase, thereby resulting in the fold activity of NF-κB relative to β-actin internal control.

Actin inhibition experiments

Although inhibitors derived from the cytochalasin group, i.e. cytochalasin B and D, are commonly used in a number of studies for blocking bacterial invasion into OECs (Han et al. 2000, Lamont et al. 1995), our preliminary experiments showed that both cytochalasin B and D (Sigma-Aldrich, St. Louis, MO) strongly induced CXCL-8 protein expression in hTIGK and

OKF6 by using ELISA (data not shown), consistent with an earlier study demonstrating that disruption of actin by cytochalasin D in human IECs induces CXCL-8 expression via NF-κB (Nemeth et al. 2004). Wiskostatin, a known actin blocker via the inhibition of Neuronal-Wiskott Aldrich Syndrome Protein (N-WASP) required for actin-related protein (Arp) 2/3 complex – mediated actin polymerization, was therefore chosen instead for our inhibition experiments as it showed an efficient blocking of endocytosis previously (Shintaku et al. 2013) as well as in our preliminary trials.

The concentration of wiskostatin (Sigma-Aldrich, St. Louis, MO) used with OECs (10 µM) was tested by using CellTiter-Glo Luminescent cell viability assays (Promega, Madison, WI), as well as Trypan blue (Thermo Fisher Scientific) exclusion tests after each experimental setup, and showed no cytotoxic effect on tested OECs and no increase of CXCL-8 basal expression (data not shown). To verify the efficacy of wiskostatin in the inhibition of bacterial uptake, I performed an *in vitro* infection of OKF6 with live *F. nucleatum*. Briefly, 10^5 OKF6 cells/well were seeded into 12 well-plates and grown for 2 days prior to *F. nucleatum* infection. An overnight culture of *F. nucleatum* was freshly prepared for OKF6 infection at MOI 1:1 using stimulation medium (Ker-SFM growth medium without antibiotics). Wiskostatin was pre-incubated with OKF6 for 1 hour before bacterial infection. Mock inhibition was performed in parallel using growth medium. To avoid an effect of oxygen on the viability of *F. nucleatum*, confluent OKF6 monolayers were infected with live *F. nucleatum* (total $2x10^5$ bacteria / 500 µL) for 4 hours, according to a previous study (Han et al. 2000). Un-infected OKF6 served as a negative control. After 4-hour incubation, infected OKF6 was washed with 1XPBS twice, and incubated with Ker-SFM growth medium containing gentamycin (300 µg/mL) and metronidazole (200 µg/mL) for 1 hour to kill extracellular bacteria attaching to OKF6. Cells were washed with 1XPBS twice and lysed with 500 µL ultrapure water before spotting 10 µL OKF6 lysates containing live *F. nucleatum* on blood agar plates in triplicate and incubating under anaerobic conditions until *F. nucleatum* colony forming units (CFUs) were visible for counting.

To elucidate the requirement of intact cellular actin function for OEC response to bacterial stimuli, wiskostatin (Sigma-Aldrich, St. Louis, MO) was pre-incubated with cultured OECs in 96-well plates for 1 hour before stimulation with FnCW prepared from *F. nucleatum* 25586. Wiskostatin-treated OECs were then challenged with 100 ng/mL FnCW for 24 hours prior to CXCL-8 protein measurement.

Digestion of bacterial cell wall proteins

To remove bacterial cell wall proteins, Proteinase K from *Engyodontium album* (Sigma-Aldrich, St. Louis, MO) was diluted in ultrapure water (Invitrogen by Life Technologies) to make a working stock containing 10 mg/mL Proteinase K. 10 ng bacterial cell walls were incubated at 37°C for 2 hours with 100 µg Proteinase K or water serving as a mock treatment. Proteinase enzyme was subsequently inactivated at 80°C for 20 minutes.

Shortly before stimulation, digested CW was resuspended in stimulation media of either HEK cells or OECs to achieve a 100 ng/mL final concentration, while treated live bacteria were diluted at 1:10 ratio with stimulation media prior to HEK or OEC stimulation. Cultured HEK cells were stimulated in triplicate for 4 hours with 100 µL prepared samples per well before cell lysis. In contrast, cultured OECs were stimulated for 24 hours, a time point at which OEC supernatants were collected and measured for CXCL-8 protein levels.

Detection of endotoxin (LPS) level in bacterial supernatants

In some experiments in which concentrated *F. nucleatum* or *E. coli* culture supernatants was used, the presence of LPS in the supernatants was quantified using ToxinSensor™ Endotoxin Detection System (GenScript, Piscataway, NJ, USA). Briefly, aliquots of either *F. nucleatum* or *E. coli* culture supernatants were incubated with Limulus

Amebocyte Lysate (LAL) reagents for 12 minutes at 37°C in 96-well plate. Chromogenic substrate solution was then added to each reaction and allowed 6 minutes for incubation. Stop solution and color stabilizer were finally added to each well. Optical density at 454 nm wavelength was determined by using a plate reader (VMax microplate reader from Molecular Devices, Sunnyvale, CA). A wide-range dose of EcLPS and FnLPS (100 pg/mL – 1 µg/mL) diluted with PBS was used as standards for the readout in each experimental setup and PBS blank served as a negative control.

RNA isolation, semi-quantitative RT-PCR (RT-PCR) analysis

Total RNA from OECs was extracted and purified using RNeasy mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Genomic DNA contamination was removed by using In-column treatment with DNase I (Qiagen, Valencia, CA). Total RNA quantitation and purity were determined using an Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany). A total of 5mg RNA in 50 µL volume was mixed with 1 µM oligo(dT), incubated at 65°C for 5 minutes, and stored at -80°C for subsequent analysis.

Reverse transcription and polymerase-chain reaction (RT-PCR) were performed by using one step Access RT-PCR System Kit (Promega, Madison, WI) in a total volume of 25 µL containing 300 ng total mRNA, AMV/*Tfl* 1X Reaction buffer, *400* nM forward and reverse primers (table 3), 0.2 mM dNTP mix, 1 mM $MgSO_4$, 0.2 U AMV Reverse Transcriptase, 0.2 U T*fl* DNA Polymerase. Control samples without reverse transcriptase enzyme, as well as controls without RNA templates, were prepared similarly and served as negative controls. First strand cDNA synthesis was conducted at 48°C for 50 minutes, followed by AMV RT inactivation at 95°C for 2 minutes. Second strand synthesis and PCR amplification were subsequently carried out for 30 or 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 68°C for 2 minutes. After each run, gel electrophoresis was performed with 20 µL PCR products combined with 1X loading dye in either 0.5% or 1.2% agarose gel, and visualized under UV light to examine the specificity of PCR products.

Bacterial stimulation of OECs

All test bacteria were grown overnight before the day of stimulation. Bacterial pellets were obtained by centrifugation of 750 µL bacterial cultures, washed once with corresponding culture broth, and determined for the number of bacteria correlating to the optical density (OD600) using an Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany). Bacterial stock of multiplicity of infection (MOI) 1:50 was pre-determined to yield an optimal CXCL-8 response by OECs from preliminary experiments. All test bacteria were prepared with Ker-SFM medium without antibiotics to achieve MOI 1:50, a ratio of one OEC to 50 bacteria. Stimulation of OECs that were cultured in 96-well plates lasted for 24 hours, unless otherwise indicated, before CXCL-8 proteins were quantified.

For elucidating the role of endosomal acidification in OEC sensing of bacterial whole cells, 400 nM Bafilomycin A1 (Sigma-Aldrich, St. Louis, MO) was pre-incubated with OECs for 1 hour prior to LPS or bacterial challenge.

Measurement of secreted CXCL-8 protein

Culture supernatants were collected for measuring CXCL-8 levels after 24 hours of incubation, unless otherwise indicated. CXCL-8 monoclonal capture antibody (M801) and detection antibody (M802B) (Thermo Fisher Scientific, Rockford, IL) were used. Avidin-horseradish peroxidase (HRP) (eBioscience, San Diego, CA) and tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, MO) were used in the study. Standard sandwich ELISA protocol was performed according to manufacturer's recommendations. The reported concentrations were calculated from the standard curve derived from correlating the OD reading of each sample at 450/570 nm wavelengths (VMax microplate reader from Molecular

Devices, Sunnyvale, CA) to the known concentrations of serially diluted human recombinant protein standards.

Data analysis

All experiments were performed at least 3 times in triplicate using 3 different epithelial cell lines, unless otherwise indicated in the figures. Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software Inc, La Jolla, CA). One-way Analysis of Variance (ANOVA) with Tukey's multiple comparison test, unless otherwise stated in the figure text, was used to determine the significance level where p-value < 0.05 was considered statistically different.

RESULTS

TLR-induced CXCL-8 response by OECs is associated with an internalization of bacterial cell wall fragments.

We have demonstrated earlier that despite LPS unresponsiveness of OECs, increasingly secreted CXCL-8 protein was detected when either isolated LPS or naturally-shed LPS was delivered intracellularly, indicating that LPS-TLR4 interaction was initiated by LPS engulfment specifically termed 'bacterial endocytosis'. We therefore postulated that, in an *in vivo* context of subgingival plaque where polymicrobial species reside, engulfment of bacterial cells or bacterial cell wall fragments is required for LPS sensing by intracellular TLR4 in OECs.

To test this hypothesis, wiskostatin, an actin blocker, was pre-incubated with cultured OECs for 1 hour prior to FnCW stimulation. The efficacy of wiskostatin for inhibiting bacterial uptake was tested in OKF6 challenged with live *F. nucleatum* at MOI of 1:50, and these preliminary experiments clearly showed that the number of CFU recovered from OKF6 cell lysates was significantly less in wiskostatin-treated OKF6 than untreated OKF6 (Figure 19).

Next, we tested similarly with FnCW in all OEC lines including primary cells from gingival explants obtained from 2 dental patients. We found that CXCL-8 secretion was abrogated nearly to basal levels in all tested OEC lines but not hTIGK (Figure 20), suggesting that an uptake of bacterial cell walls into OECs is pivotal for sensing of bacterial LPS. In other words, FnCW transport through plasma membranes via actin is crucial for OEC sensing of bacteria, as blocking the activity of actin substantially inhibited CXCL-8 induction. Furthermore, these findings thus also address the variability of responsiveness found between different OEC / GEC populations.

The contribution of *F. nucleatum* to the pathogenesis of systemic- and oral-infectious diseases has been explored extensively, and FadA, an adhesin protein, has been identified for its role in modulating host immune response (Han et al. 2005). FadA adhesin is responsible for attachment and invasion of *F. nucleatum* in a vast number of cell lines, including OECs (Xu, Yamada, et al. 2007). Thus, *F. nucleatum* lacking *fadA* (US1 strain), kindly gifted by Dr. Yiping W. Han (Figure 21), was utilized in our study to investigate whether *F. nucleatum* – induced OEC signaling is altered, in accordance with our hypothesis that bacterial LPS is sensed via intracellular TLR4. *F. nucleatum* ΔfadA mutant was expected to induce less CXCL-8 in OECs than does the wild-type strain, due to reduced ability to invade and deliver LPS into host cells. A significant CXCL-8 reduction was observed in hTIGK stimulated with *Fn* 12230 ΔfadA mutant at an MOI 1:50, compared to *Fn* 12230 WT (Figure 22A). A similar decrease of CXCL-8 secreted by OKF6 was notably evident when the cells were challenged with *Fn* 12230 US1 (Figure 22B) relative to its parental strain at an MOI of 1:50. In nGEC, although *F. nucleatum* ΔfadA mutant induced CXCL-8 to a similar level to that induced by *F. nucleatum* WT, wiskostatin completely inhibited CXCL-8 expression (Figure 22C). These findings demonstrate that 1) invasion of *F. nucleatum* into epithelial cells is essential for LPS-induced CXCL-8 induction and 2) OECs display a remarkable heterogeneity of an CXCL-8 response, most likely owing to their differential response to TLR2 ligands. Interestingly, blocking of *F.*

nucleatum invasion by wiskostatin completely attenuated CXCL-8 secretion by OKF6 and nGEC. This suggests that invasion of OECs by *F. nucleatum* is in part mediated by FadA (Figure 22B, 22C). Similar to the finding from the wiskostatin-inhibition experiment (Figure 20), wiskostatin-treated and *F. nucleatum* ΔfadA-treated hTIGK did not secrete a significantly different amount of CXCL-8 from hTIGK infected with wild-type *F. nucleatum* (Figure 22A). I incorporated one nGEC line in our study. This line (nGEC1) is responsive to Pam_3CSK_4, as shown in Chapter 1. Interestingly, nGEC behave similarly to hTIGK as it responded to *Fn* 12230 US1 as robustly as wild-type *Fn* (Figure 22C). Since hTIGK responds to TLR2 agonists while OKF6 does not, heterogeneity of OEC response at TLR2 is therefore a likely explanation of this discrepancy.

Bacterial recognition via NF-κB-dependent CXCL-8 induction requires a cell wall protein-mediated intracellular uptake of *F. nucleatum*.

Bacterial cell wall is an intricate structure which consists of peptidoglycan, LPS, and outer membrane proteins (Silhavy, Kahne, and Walker 2010). LPS, so called bacterial endotoxins, is a potent immune stimulator present in most gram-negative bacterial cell walls as well as in outer membrane vesicles (OMVs) where shed LPS is present (Ellis and Kuehn 2010, Haurat et al. 2011, Kaparakis-Liaskos and Ferrero 2015). The fact that OECs are able to recognize native FnCW and, more strikingly, an internalized LPS, has led to a conjecture that LPS transport through epithelial cell membranes is a crucial process whereby cell wall proteins, particularly present in oral bacterium *F. nucleatum*, might play a significant role in mediating epithelial cell sensing at human TLR4. We therefore employed an *in vitro* strategy using the enzyme Proteinase K to remove proteins from FnCW. Removal of cell wall proteins by proteinase K enzymatic activity significantly decreased TLR2 activation in HEK293 cells; however, TLR4 activity of digested-FnCW was unaffected (Figure 23). This clearly indicates that FnCW proteins mediate human immune response at TLR2 and that removal of cell wall proteins does not affect the stimulatory activity of FnLPS in FnCW. Digestion of FnCW proteins significantly dampened the CXCL-8 response by all tested OEC lines (Figure 24), indicating that the presence of cell wall proteins on *F. nucleatum* facilitates LPS-induced TLR4 response in OECs.

It has been demonstrated earlier from our data that despite the variability of CXCL-8 response to Live *F. nucleatum* among OECs was shown possibly due to the responsiveness of OECs to TLR2 ligands, the invasion of *F. nucleatum*, partially contributed by FadA, is seemingly associated with CXCL-8 induction in OECs. Remarkably, cell walls obtained from the FadA mutant (*Fn* 12230 US1: ΔfadA) significantly reduced CXCL-8 expression in all epithelial cell lines examined (Figure 25). Here, it clearly indicates that FadA can significantly contribute to CXCL-8 expression by *F. nucleatum*-infected OECs and that LPS uptake, when presented to the cells in the cell wall form, is a pivotal mechanism by which OECs utilize for LPS sensing at an intracellular TLR4. Despite losing its viability, FnCW still remained active as a CXCL-8 agonist (Figure 25), suggesting that cell wall structure of *F. nucleatum* is likely composed of proteins, aside from FadA, that might play a distinct role in the attachment and invasion into host cells.

We next investigated whether other microbes, including the gut pathogen *E. coli*, utilize a similar strategy to trigger host-mediated innate immunity specifically by using their cell wall proteins, like *F. nucleatum* does. Our preliminary experiments have shown that live *E. coli*, *F. nucleatum*, and *A. actinomycetemcomitans* are potent CXCL-8 inducers in OECs (data not shown), these 3 bacterial species were thus selected to elucidate the role of the bacterial cell wall in LPS sensing. Cultures of test bacterial strains including *E. coli*, *F. nucleatum*, and *A. actinomycetemcomitans* were harvested for their cell wall proteins and used for hTIGK, OKF6, and nGEC stimulation. Surprisingly, the levels of CXCL-8 expression by OECs, particularly OKF6, in response to EcCW and AaCW were relatively low (Figure 26B), even though their cell wall proteins contain an enormous amount of LPS (Figure 27B, 27C). However, CXCL-8 was induced strongly in hTIGK by EcCW and AaCW at a dose of 100 and 1,000 ng/mL, relative to the levels expressed by OKF6 in which EcCW and AaCW were not able to induce CXCL-8 secretion (Figure 26A, 26B). Interestingly, AaCW, but not EcCW and FnCW failed to induce CXCL-8 in nGEC (Figure 26C). The distinction of CXCL-8 expression profiles among OECs can be partially explained by the differential response of OECs to TLR2 ligands.

In spite of the strong ability to activate both human TLR2 and TLR4 of live *E. coli* and *A. actinomycetemcomitans* (Figure 28), a weak CXCL-8 induction in OKF6 by EcCW and AaCW and in nGEC by AaCW has underscored an essential role of viable bacterium in its interaction with OECs to mediate an innate immune response in oral epithelium. OECs are not able to recognize bacterial LPS unless it is transferred to cytoplasmic sensors. Discrepancy of the CXCL-8 response suggests that firstly, although bacterial cell wall components are able to activate host immunity, a whole live bacterium can possibly gain access into OECs more actively and thus stimulates a more robust chemokine response as a result of being sensed by a repertoire of intracellular host receptors. Secondly, *F. nucleatum* stands out from other oral microorganisms in that it possesses a specialized adhesin protein on its cell wall, known as FadA adhesion, responsible for *F. nucleatum* attachment to and invasion into host cells, rendering it more adherent to OECs.

Shed or isolated bacterial LPS, when delivered into OECs, up-regulates the level of secreted CXCL-8 as shown earlier. It is thus likely that LPS from different bacterial species might be similarly recognized via the endosomal pathway in OECs. We tested our speculation by pre-incubating OECs with 400 nM bafilomycin A1 prior to live bacterial challenge. Six bacterial strains, *E. coli*, *F. nucleatum*, *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia,* and *S. gordonii,* were included in our experiments. Interestingly, *E. coli*, *F. nucleatum*, and *A. actinomycetemcomitans* induced CXCL-8 strongly (Figure 29), while low to moderate levels of CXCL-8 expression were induced by *P. intermedia* and *S. gordonii*. Blocking of endosomal acidification by bafilomycin A1 significantly dampened CXCL-8 induction by corresponding bacterial species (Figure 29), suggesting that endocytosis is a shared pathway in which OECs strategize to overcome microbial infections.

Semi-quantitative RT-PCR analysis showed the differential transcript expression profiles of TLR2 and TLR4, as well as their required signaling components (Figure 30). Notably, *TRL1*, *TLR2*, and *MD2* transcripts are strongly expressed in OECs, but not *TLR4*, and *CD14.* Collectively, it is thus not well understood whether OECs utilize MD2 for LPS sensing. However, we demonstrated that TLR4 plays a role when LPS is engulfed by OECs.

DISCUSSION

We earlier elucidated the mechanisms that OECs utilize for bacterial LPS recognition, particularly how TLR4 participates in sensing of naked LPS. Unlike colonic epithelial cells, OECs are not able to sense bacterial LPS unless it is shuttled to intracellular compartments where TLR4 is located (data shown in Chapter 1). The distinction of the LPS sensing mechanisms between epithelial cells derived from different sites of the body thus highlights the differentially constructed architectures that allow for specialization of epithelial functions in different infectious arenas. Oral epithelium is the first cell layer encountering a microbial burden consisting of a vast diversity of bacterial species present in the gingival sulcus (Paster et al. 2001). In accordance with this notion, a recent study showing a marked association of periodontal health to the shift of indigenous microbial population found in subgingival plaque (Kumar et al. 2006) has signified the impact of the polymicrobial community on gingival or periodontal health. Juxtaposition of oral or sulcular epithelium to alveolar bone with an intervening connective tissue might, therefore, developmentally contribute to an unresponsiveness of OECs in order to avoid excessive inflammation arising from OEC-bacterium interactions, thus resulting in the preservation of the connective tissue attachment.

Canonical LPS sensing by mammalian cells requires several co-receptors for signal transduction, including membrane CD14 (mCD14) (Jiang et al. 2000, Zanoni et al. 2011).

Interestingly, the lack of mCD14 on epithelial cell surfaces is thought to confer epithelial cell hyporesponsiveness to bacterial LPS (Suzuki, Hisamatsu, and Podolsky 2003, Ueta et al. 2004). In contrast to IECs in which sCD14 was found to potentiate CXCL-8 in response to LPS (Uehara et al. 2001), sCD14 present in OEC culture microenvironments did not enhance CXCL-8 induction (data not shown). It is indeed still controversial whether CD14 facilitates LPS sensing in epithelial cells (Bocker et al. 2003). Our data, showing that OECs failed to elicit CXCL-8 in response to isolated microbial patterns (Pam_3CSK_4 and LPS) even with the presence of sCD14 derived from human serum, yet responded robustly to FnCW in the absence of sCD14, clearly suggested that OEC sensing of microbial LPS is an inflammatory event independent of CD14 expression. Even though CD14 is known to mediate LPS-induced endocytosis of TLR4 (Zanoni et al. 2011), it is unclear as to how OECs respond to LPS in FnCW as well as to fractionated glycoproteins derived from the pigmented oral bacterium *Prevotella intermedia* (Sugiyama et al. 2002).

In addition to CD14, TLR4-dependent LPS signal transduction is associated with the MD2 co-receptor (Shimazu et al. 1999). MD2 plays a pivotal role in the distribution of TLR4 from the Golgi apparatus to cell surfaces where TLR4 signaling is mediated (Nagai et al. 2002). However, our semi-quantitative RT-PCR analysis showed that MD2 is strongly expressed in OECs (Figure 30, A-D), similar to the finding in IECs (Suzuki, Hisamatsu, and Podolsky 2003), yet OECs are not able to elicit CXCL-8 in response to LPS challenge. This indicates that, apart from low-abundance expression of surface TLR4 in OECs, relocation of functional TLR4 in OECs is not solely regulated by MD2 but possibly by PRAT4A, a protein known to promote TLR4 trafficking to the cell surface (Takahashi et al. 2007). Whether OECs lack PRAT4A and if PRAT4A as well as MD-2 are idle in OECs need to be elucidated. Collectively, our data suggest that OECs have a unique LPS sensing machinery and, in addition, the mechanism upstream of TLR4 is independent of CD14 and MD2. Interestingly, intracellular TLR4/MD2 plays a role in the MyD88-dependent LPS-mediated inflammatory response, despite the absence of surface TLR4 (Guillot et al. 2004, Shibata et al. 2011) and MD2 (Shibata et al. 2011). Of note, LPS sensing by OECs might involve an undiscovered, novel pathway of which the signaling cascades are not initiated from the surface of OECs. OECs are, therefore, unique in the sense that they cannot recognize bacterial LPS from exogenous sources, unlike IECs in which LPS is autonomously internalized and subsequently trafficked to intracellular TLR4 as reported in an in vitro study using murine IECs (Hornef et al. 2003). Perhaps, differential LPS response by oral and intestinal epithelium might, in part, contribute to distinct progression and severity of inflammatory diseases related to those sites, compared between periodontitis and colitis.

The lack of CD14 and MD2 components necessary for TLR4-mediated transduction is considered an attribute of LPS unresponsiveness of epithelial cells. Consistent with this notion, unprimed OECs do express low levels of CD14, MD-2, and are not responsive to LPS. In contrast, when OECs were incubated with live bacteria or FnCW, a significant induction of CXCL-8 was observed (Chapter 2), suggesting that LPS recognition by OECs is not dependent on CD14 and MD2, but in fact OECs primarily utilize the endocytosis pathway for mediating LPS-induced TLR4 activity.

Since TLR4 responsiveness to LPS in epithelial cells is not dependent on IFN-γ priming, as suggested by our findings, we then speculated that CXCL-8 secretion by OECs detected when FnCW was used for stimulation requires host actin–bacterial cell wall engagement whereby LPS can be naturally taken to an endosomal structure via clathrin-dependent endocytosis, leading to the activation of TLR4 located on the endosomes (Husebye et al. 2006). Notably, blocking of actin polymerization activity by wiskostatin completely abolished CXCL-8 induction by FnCW (Figure 20), suggesting that bacterial internalization is mandatory for TLR4 sensing of bacterial LPS by OECs. Our findings are consistent with several studies in which CXCL-8 and murine CXCL-2, a homolog of human CXCL-8, were shown to be associated with an uptake of their co-evolved pathogens or LPS by epithelial cells

(Eckmann, Kagnoff, and Fierer 1993, Han et al. 2000, Hornef et al. 2002, Hornef et al. 2003). Nonetheless, these studies did not elucidate how bacterial LPS is sensed by epithelial cells.

We further support our speculation that invasion of OECs by live bacterium is a prerequisite for LPS recognition by utilizing cell wall preparations obtained from *E. coli* and *A. actinomycetemcomitans* overnight cultures. According to a number of studies, these two pathogenic species are invasive and therefore associated with diseases in the gut and periodontal tissues, respectively. *E. coli* possess FimH, a type I fimbriae-associated adhesin, that facilitates its invasion into bladder epithelial cells, of which intracellular TLR4 is a target receptor for both LPS and FimH (Mossman et al. 2008, Schilling et al. 2001). However, mechanisms of *A. actinomycetemcomitans* invasion into host cells are not well understood although Aae autotransporter adhesin (Rose, Meyer, and Fives-Taylor 2003) and CD14 (Takayama et al. 2003) are required for CXCL-8 response by epithelial cells. Our HEK-TLR4 data showed that both *Ec* and *Aa* have strong abilities to activate human TLR4 (Figure 28) due to the presence of LPS in the preparations (Figure 27) shown by LAL assays. Nonetheless, *in vitro* OEC stimulation assays indicated that CXCL-8 levels secreted by hTIGK or OKF6 challenged with either EcCW or AaCW were not as high as when stimulated with live counterparts (Figure 26). These findings thus underscore the essential requirements of bacterial viability and its invasiveness for targeting host cells as well as an immuno-silent feature of OECs being used as a self-protective measure to circumvent overstimulation of innate immunity in response to bacterial LPS.

Strikingly, CXCL-8 induction by bacterial cell wall fragments derived from *F. nucleatum* is intriguing, particularly in OKF6 in which the response was robust relative to CXCL-8 levels induced by EcCW and AaCW (Figure 26B). This profound distinction of FnCW from EcCW and AaCW, with respect to its ability to mediate OEC response, is most likely attributable to the FadA adhesin/invasin. FadA found in *F. nucleatum* is known to be crucial for its ability to attach to and invade host cells (Han et al. 2005, Xu, Yamada, et al. 2007). Our findings have elucidated clearly that FadA contributes to CXCL-8 response since the amount of CXCL-8 protein was secreted to a lesser extent when *Fn* 12230 ΔfadA was used for stimulation relative to its parental strain (Figure 22, 25). More interestingly, digestion of cell wall proteins strikingly dampened CXCL-8 induction in all tested OEC lines (Figure 24). Taken together, our results strongly indicate that bacterial invasiveness potentiated by its vitality is a requisite requirement for LPS sensing by OECs and that *F. nucleatum* cell wall proteins facilitate epithelial cell recognition of LPS most likely by directly delivering its LPS to intracellular TLR4 via the endocytosis pathway, partly contributed by FadA adhesin.

Recognition of microbial patterns by human cells is a complex immunological event in that a myriad of receptors driven by shared downstream machineries enables the host to recognize diverse microbial contents in an orchestrated fashion. Protease enzymes are also able to activate OECs to secrete CXCL-8 in response to *A. actinomycetemcomitans* infection via the PAR-2 receptor (Shimada et al. 2009). TLR9 and intracellular calcium signaling were previously reported to play a role in CXCL-8 induction in OECs (Kim et al. 2012, Son, Shin, and Hong 2015) in response to bacterial DNA and peptidoglycan, respectively. In addition, marked increase of CXCL-8 induction was detected when OECs were challenged with LL-37 (Montreekachon et al. 2014), indicating that oral epithelial homeostasis is operated by a highly orchestrated mechanism. Here, we demonstrated for the first time that TLR2 and TLR4 are involved in oral bacterial sensing by OECs and that *F. nucleatum* cell wall fragments actively trigger a robust CXCL-8 response by OECs due to the presence of unique cell wall proteins interacting with TLR2, unlike other bacteria of which the viability is crucial for mediating an immune response in oral epithelium. In addition, it is most likely that OECs expressed intracellular TLR2, according to a strong expression of *TLR1* and *TLR2* transcripts, although some lines are hypo-responsive at TLR2.

Host mechanisms for periodontal health homeostasis, partially due to an induction of CXCL-8 expression in oral epithelium, are elaborately regulated by the numbers of signaling cascades including direct host–bacterium interactions (Darveau et al. 1998) and indirect autocrine/paracrine activities (Assuma et al. 1998, Delima et al. 2002, Graves and Cochran 2003, Eskan et al. 2008), although transduction mechanisms are not clearly understood. Herein, direct activation of human OECs by bacterial LPS naturally bound to bacterial cells was investigated. To date, it is unclear how epithelial cells recognize invading microbes and how CXCL-8 is induced in response to bacterial infections. Particularly, whether human TLR2 and TLR4 in OECs play roles in sensing oral bacterial components has become our interest, and we showed earlier that both TLR2 and TLR4 are involved in CXCL-8 induction in OECs. However, TLR4 ligation occurs only when LPS is endocytosed by OECs. OECs share a common endocytosis pathway for CXCL-8–associated epithelial homeostasis, although the underlying mechanisms involved in TLR4 trafficking in OECs are not clear.

Microbial invasion into intestinal epithelium and interactions with TLRs are crucial for homeostasis in the intestine (Rakoff-Nahoum et al. 2004). Oral epithelium might utilize a similar strategy as well for maintaining periodontal homeostasis. In both health and disease, oral epithelium is infiltrated with TLR2- and TLR4-positive cells (Muthukuru, Jotwani, and Cutler 2005), indicating that TLRs play a role in defense against microbial infection. Presumably, the presence of *F. nucleatum* in the oral polymicrobial communitys helps protect underlying connective tissues and alveolar bone by inducing oral/junctional epithelium to release chemokine homologs e.g. CXCL-1, CXCL-2 in order to attract neutrophils to JE where polymicrobial infection persists (Zenobia et al. 2013), in addition to its role in the induction of antimicrobial peptides (Krisanaprakornkit et al. 2000).

In conclusion, our findings in this chapter underscore the unique features of oral epithelium essentially designed for withstanding a perilous environment in the oral cavity. As this naturally constructed barrier serve as the first line of defense against oral microbial insults, the fact that OECs do not respond to dead bacterial cells and their LPS renders oral epithelial barriers secured from microbial threats. However, oral commensal-turned pathogen *F.*

nucleatum possesses special characteristics in that it has a FadA protein capable of mediating OEC immune response via its role in host cell attachment and invasion. Despite being non-vital, fragments from dead *F. nucleatum* cell walls remain active at human TLR2 and TLR4 in OECs. This might be, in part, beneficial for periodontium to maintain a state of equilibrium between released immune factors from the host and the numbers of bacteria populating the gingival sulcus, an orchestrated process crucial for periodontal tissue health and integrity.

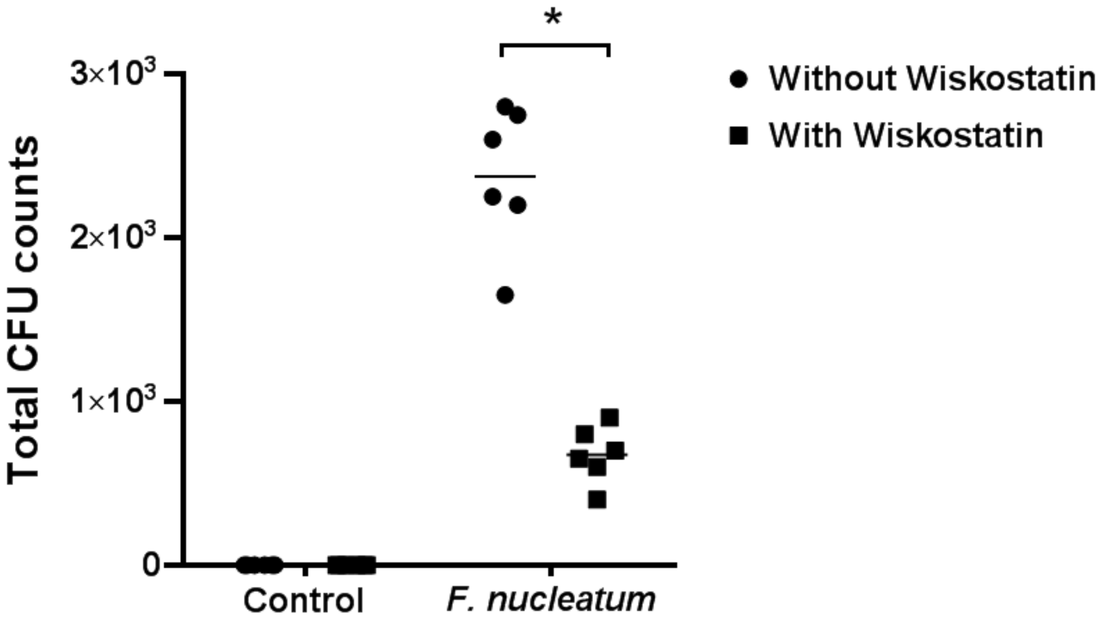

Figure 19. Wiskostatin inhibition of *F. nucleatum* uptake by OECs. Cultured OKF6 were pre-incubated with 10 µM wiskostatin for 1 hour before *F. nucleatum* infection (MOI 1:1). After infection and elimination of extracellular *F. nucleatum*, OKF6 lysates were plated on blood agar. CFUs were enumerated when visible colonies were formed. Data represent identical results from one of two experiments, showing the average, total number of CFUs. Statistical difference was tested using multiple t-tests with the Holm-Sidak method. Asterisk indicates statistical significance (**p*-value<0.05).

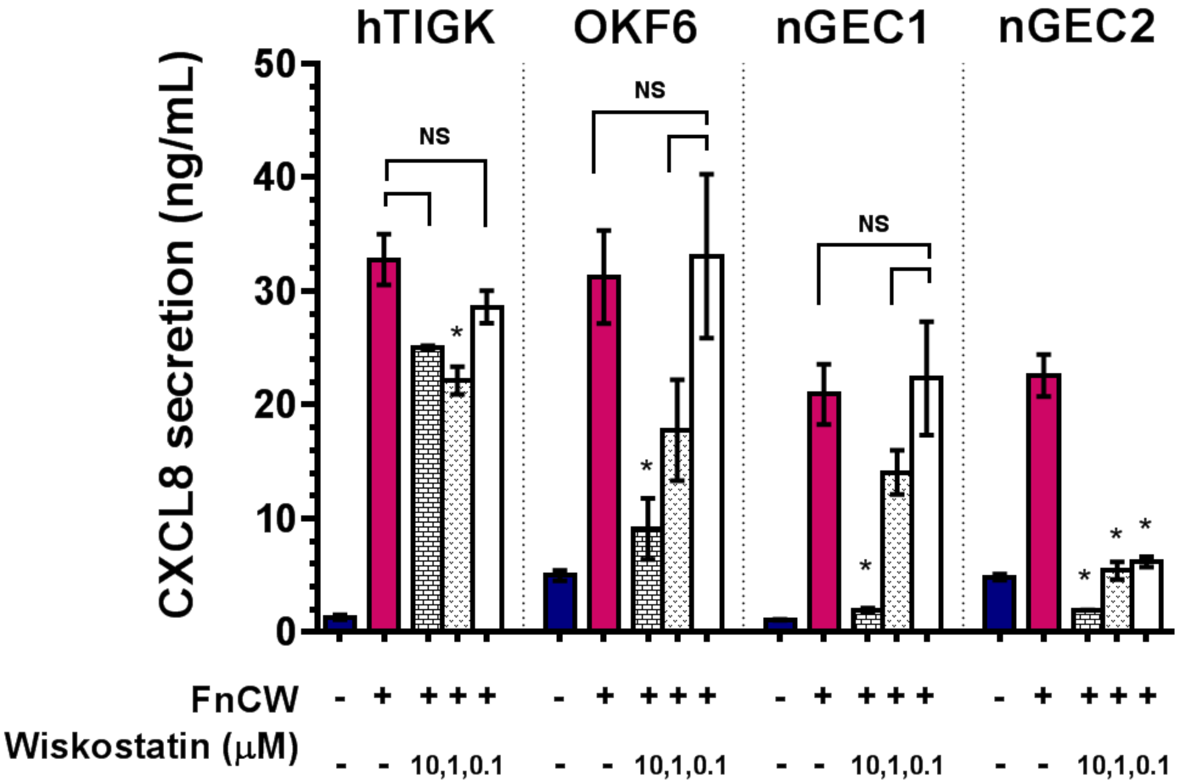

Figure 20. Inhibitory effect of Wiskostatin on CXCL-8 induction by FnCW in OECs. Cultured OECs were pre-incubated with wiskostatin for 1 hour before FnCW stimulation (100 ng/mL) for 24 hours. CXCL-8 was assayed by sandwich ELISA. Data are mean ± SEM, derived from one of two independent experiments of each cell line performed in triplicate. Asterisks indicate statistical significance between experimental groups and their corresponding FnCW-stimulated controls (**p*-value<0.05, NS = not significant).

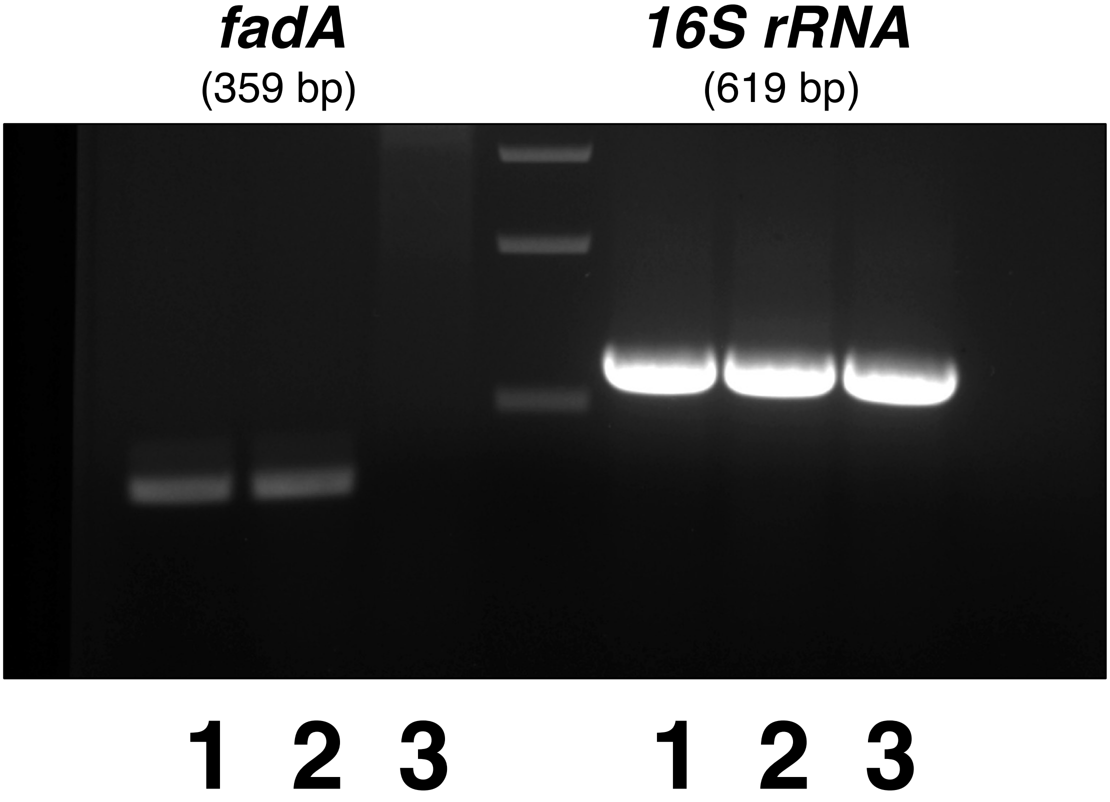

Figure 21. PCR screening of *fadA* gene expression in *F. nucleatum* strains used in the study. Lane 1: *Fn* ATCC 25586; Lane 2: *Fn* clinical isolates 12230; Lane 3: *Fn* clinical isolates 12230 US1 (ΔFadA). *16S rRNA* is a reference gene, used as an RNA loading control.

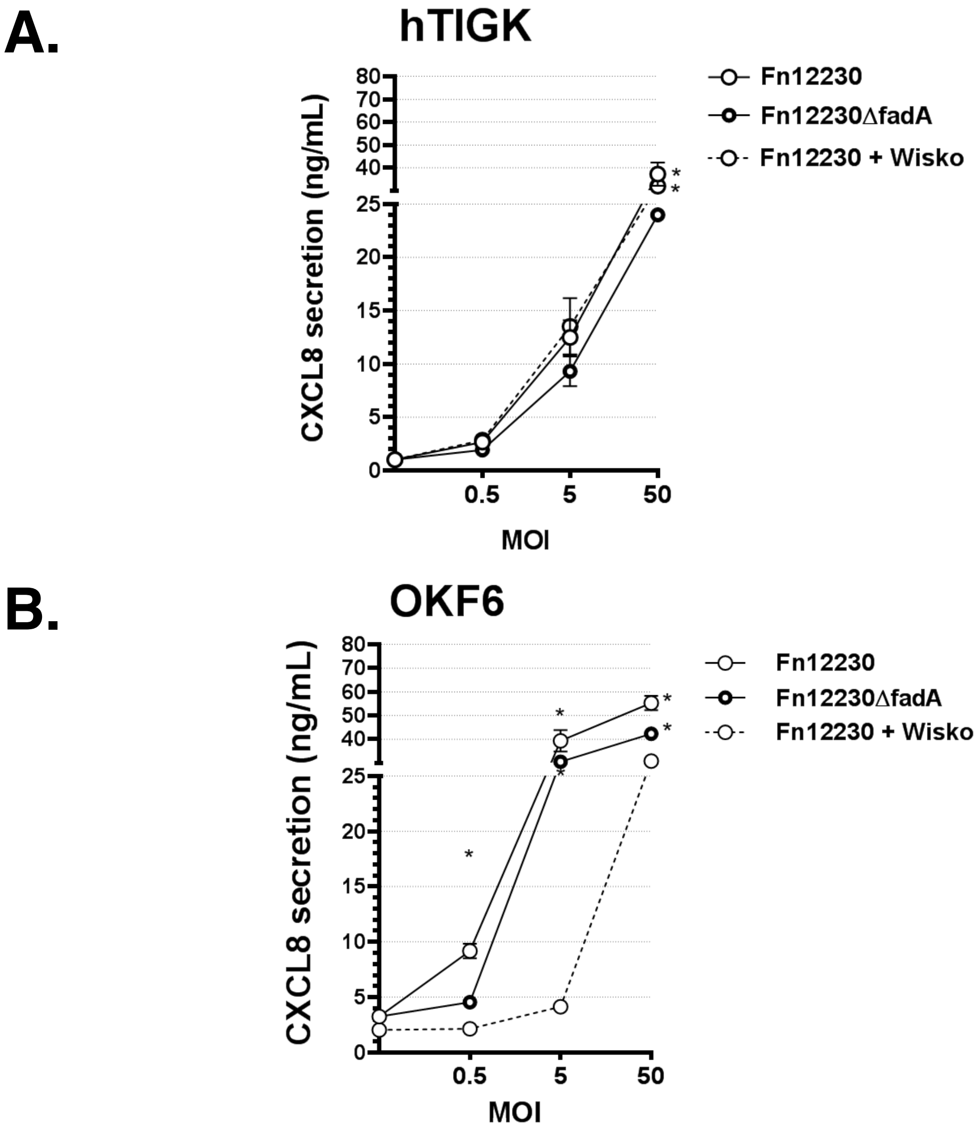

Figure 22. FadA contributes to CXCL-8 induction in OECs by *F. nucleatum* whole bacterial cells. hTIGK **(A)**, OKF6 **(B)**, and nGEC1 **(C)** were challenged with live (1) *Fn* 12230, (2) *Fn* 12230 ΔFadA, or (3) *Fn* 12230 after actin polymerization was blocked by wiskostatin. CXCL-8 protein was determined after 24-hour infection. Data are average of the actual concentration ± SEM, derived from one of two independent experiments of each cell line performed in triplicate. Two-way ANOVA with Tukey's post hoc was used to determine the significance levels. Asterisks indicate statistical significance between experimental groups and their corresponding wiskostatin-treated controls (*p-value<0.05).

C.

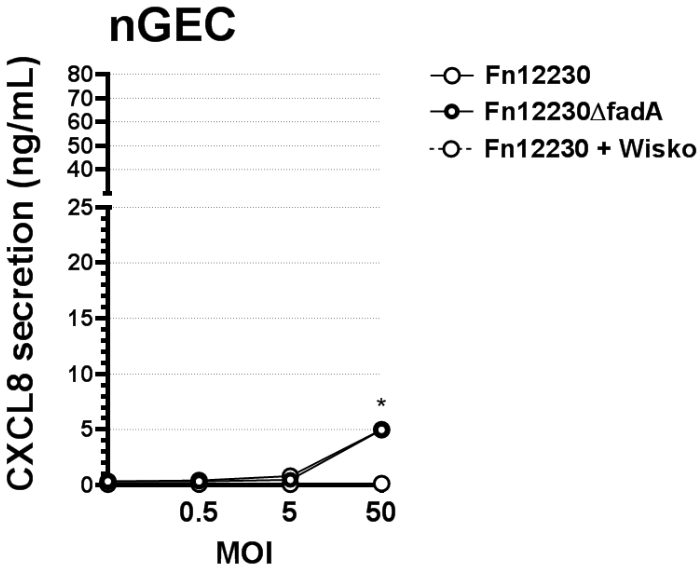

Figure 22. FadA contributes to CXCL-8 induction in OECs by *F. nucleatum* whole bacterial cells. (continued)

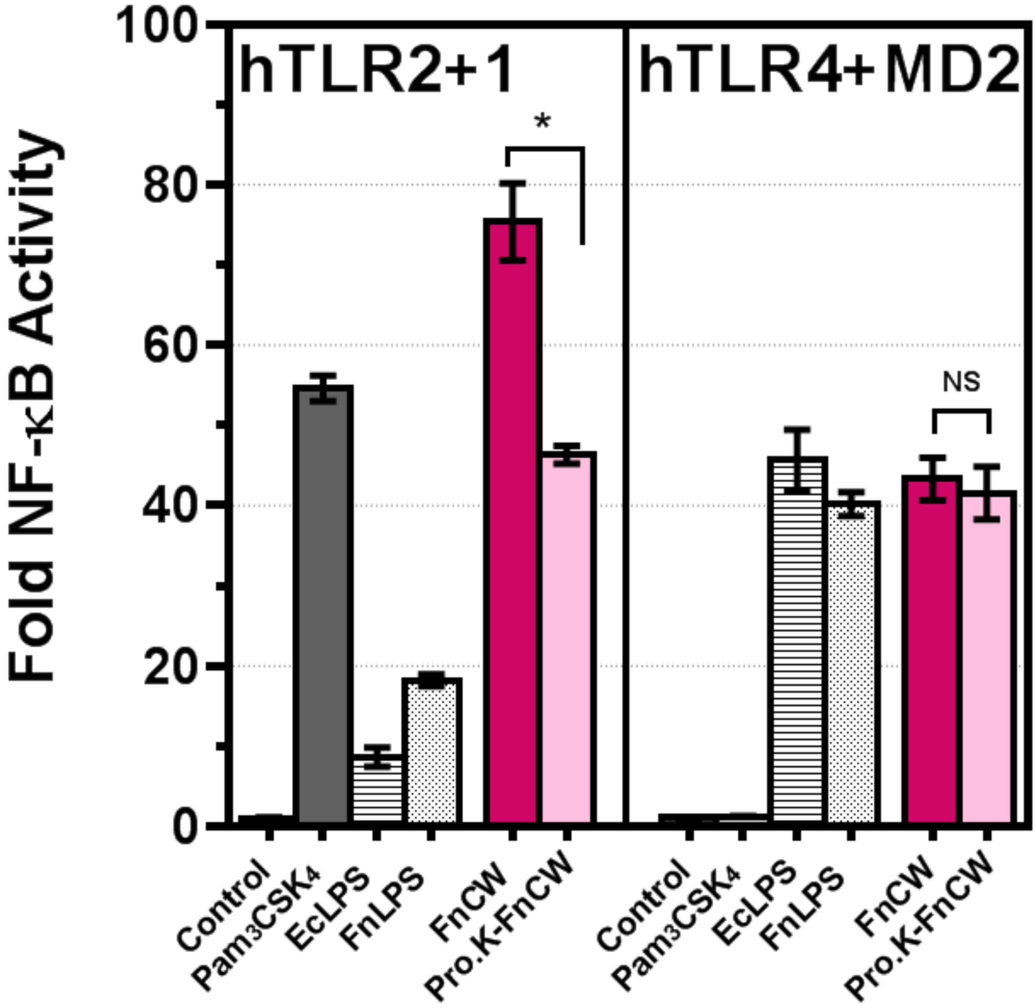

Figure 23. Modulation of human TLR2, but not TLR4, by FnCW proteins. Proteinase K-treated FnCW was tested for its ability to activate human TLR2 or TLR4 using HEK293 over-expressed TLR2+TLR1 or TLR4+MD-2. Data are shown as fold activity ± SEM, derived from one of three independent experiments in triplicate yielding similar results. Unpaired Student's t-tests were used to determine the significance level. Asterisk indicates statistical difference between experimental groups and their corresponding untreated-FnCW controls (**p*-value<0.05, NS = not significant).

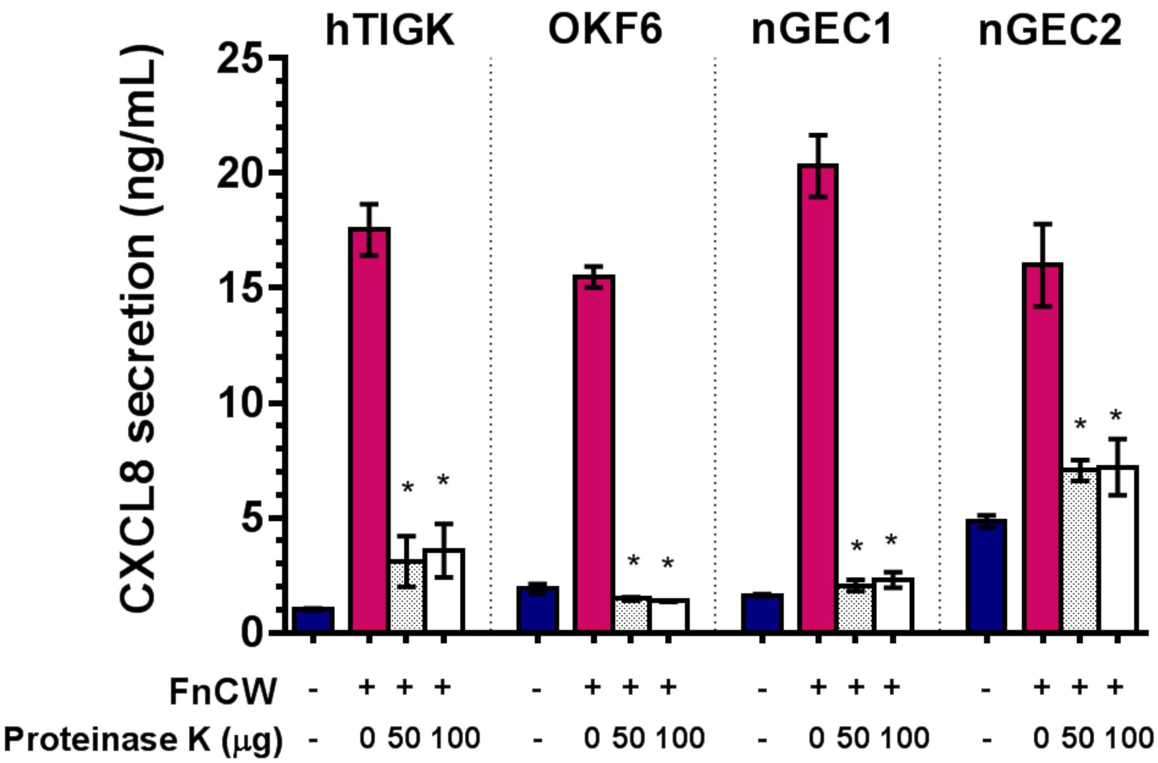

Figure 24. Reduced CXCL-8 response by OECs in response to digested FnCW. Native FnCW was enzymatically digested with 2 different doses of Proteinase K prior to OEC infection. Mock-digested FnCW (pink bars) was used as a negative control. Data are averages of the actual concentration ± SEM, derived from one of two independent experiments from each cell line performed in triplicate. Asterisks indicate statistical difference between experimental groups and their corresponding FnCW-infected controls (*p-value<0.05).

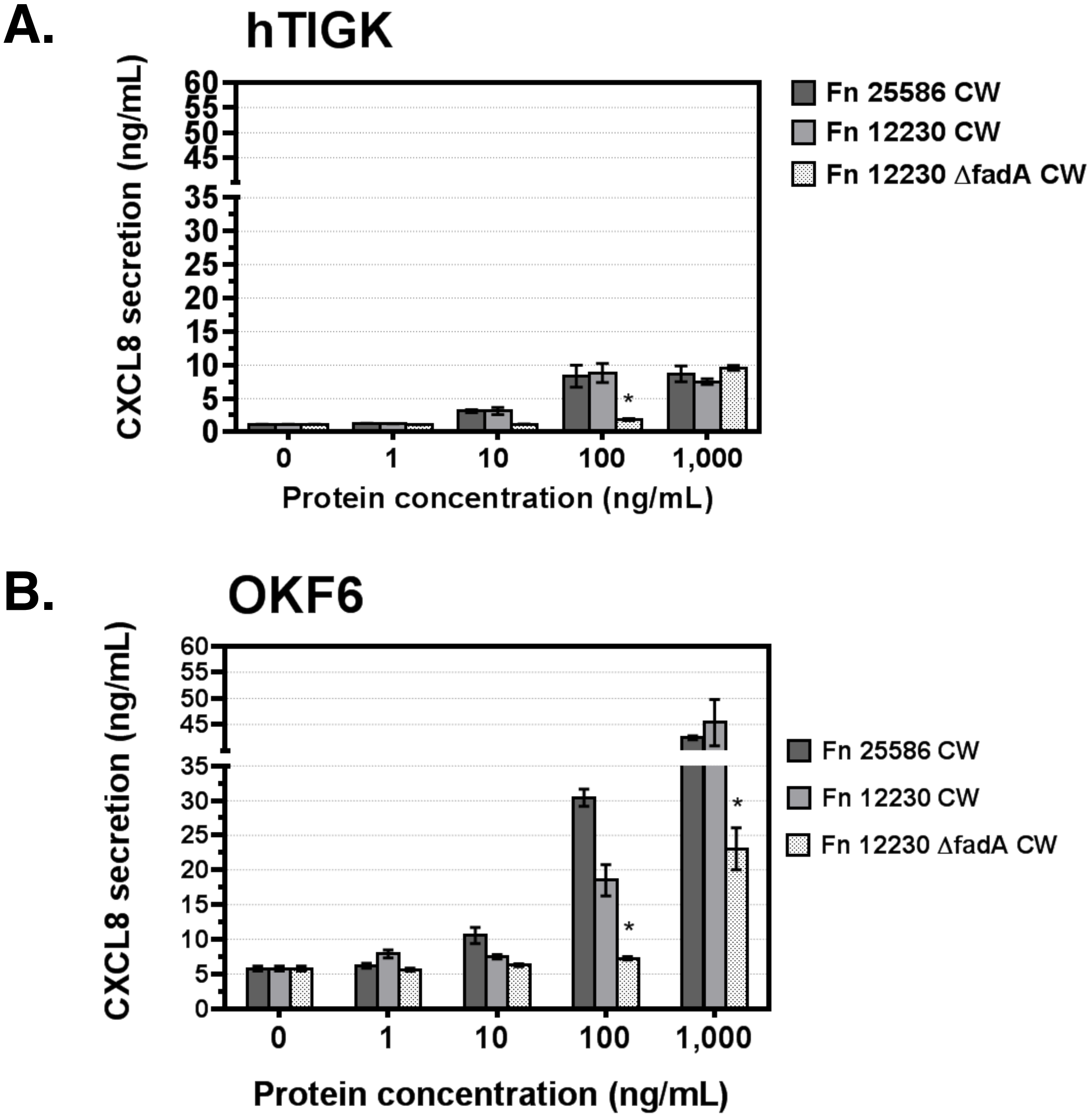

Figure 25. FadA-containing FnCW contributes to CXCL-8 induction in OECs. hTIGK **(A)**, OKF6 **(B)** and nGEC **(C)** were challenged with *Fn* 25586 CW, *Fn* 12230 CW, or *Fn* 12230 ΔFadA CW. CXCL-8 protein was assayed after 24-hour infection. Data are averages of the actual concentration ± SEM, derived from one of two independent experiments from each cell line performed in triplicate. Two-way ANOVA with Tukey's post hoc was used to determine the significance levels. Asterisks indicate statistical significance between experimental groupsand their corresponding *Fn* 12230 CW-infected controls (**p*-value<0.05).

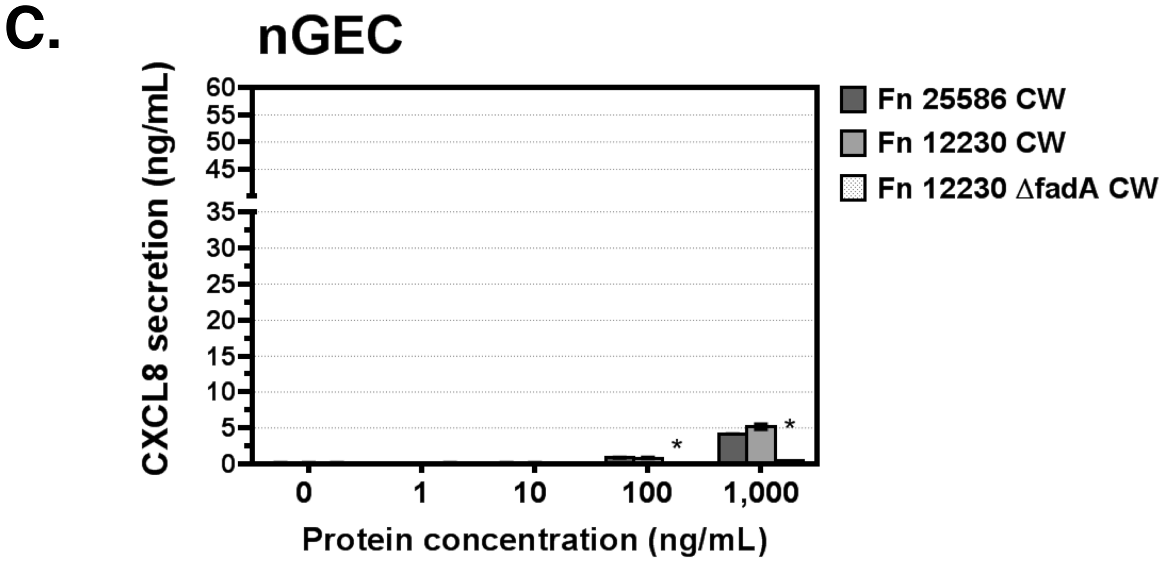

Figure 25. FadA-containing FnCW contributes to CXCL-8 induction in OECs.

(continued)

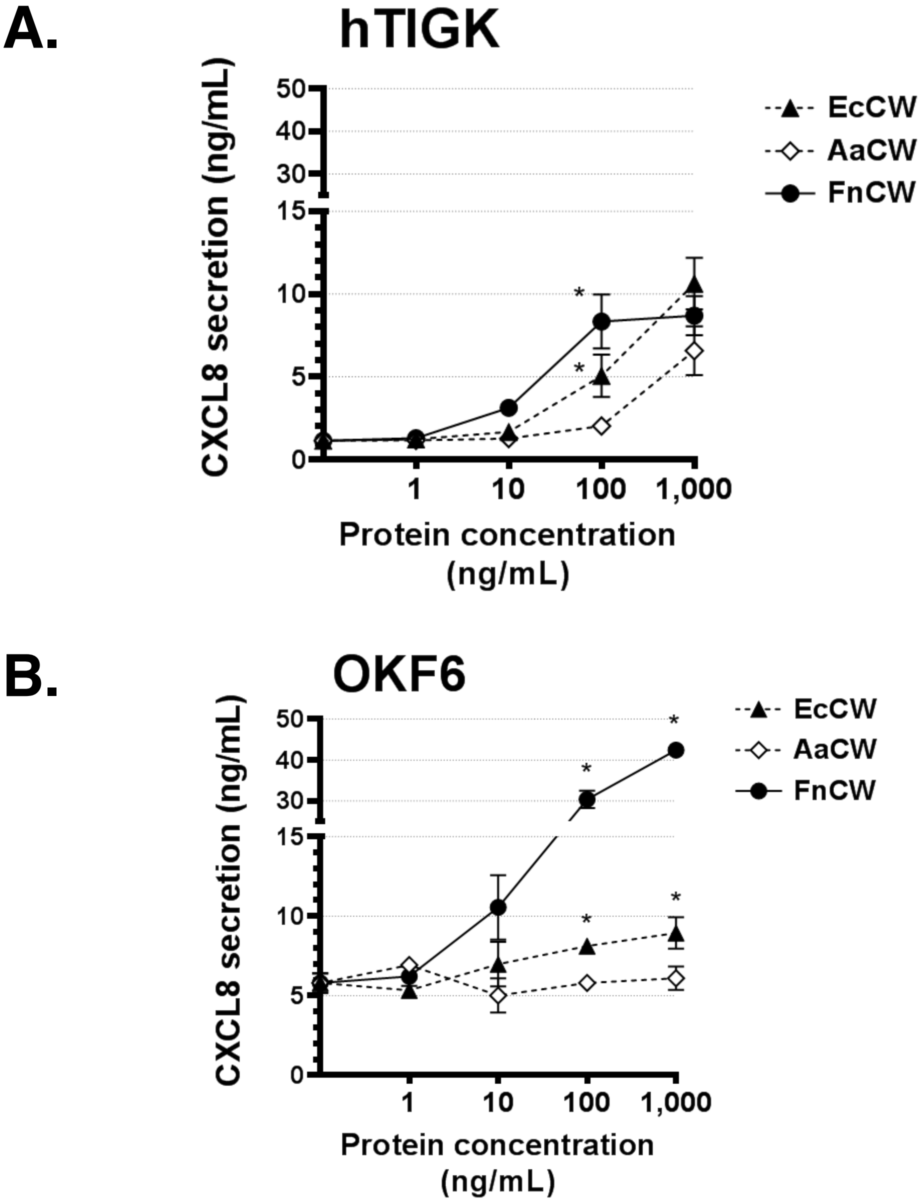

Figure 26. Differential OEC response to cell wall fractions of *E. coli*, *F. nucleatum*, and *A. actinomycetemcomitans*. Cultured hTIGK **(A)** and OKF6 **(B)** were challenged with various doses of EcCW, FnCW 25586 ATCC strain, or AaCW for 24 hours. CXCL-8 protein in the supernatants was assayed using ELISA. Data are averages of the actual concentration ± SEM, derived from one of two independent experiments in triplicate yielding similar results. Two-way ANOVA with Tukey's post hoc test was used to determine significant differences. Asterisks indicate significance between experimental groups and their corresponding AaCW-infected groups (*p-value<0.05).

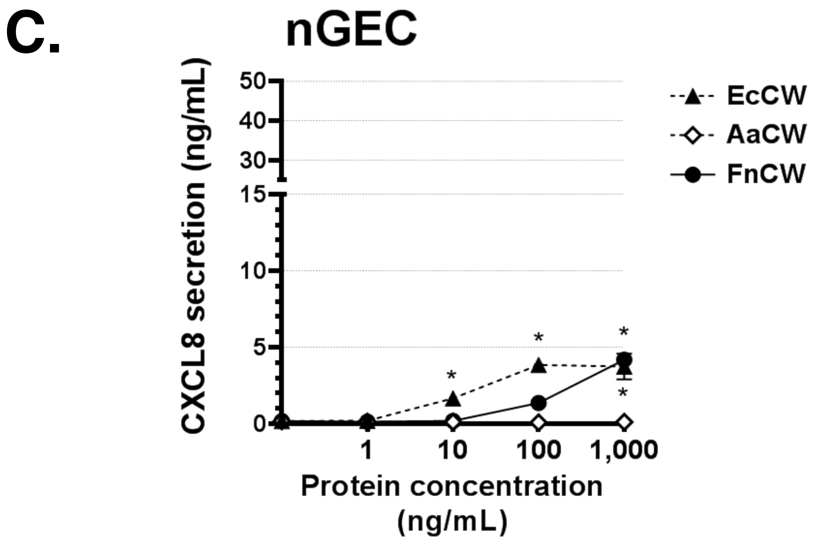

Figure 26. Differential OEC response to cell wall fractions of *E. coli*, *F. nucleatum*, and *A. actinomycetemcomitans*. (continued)

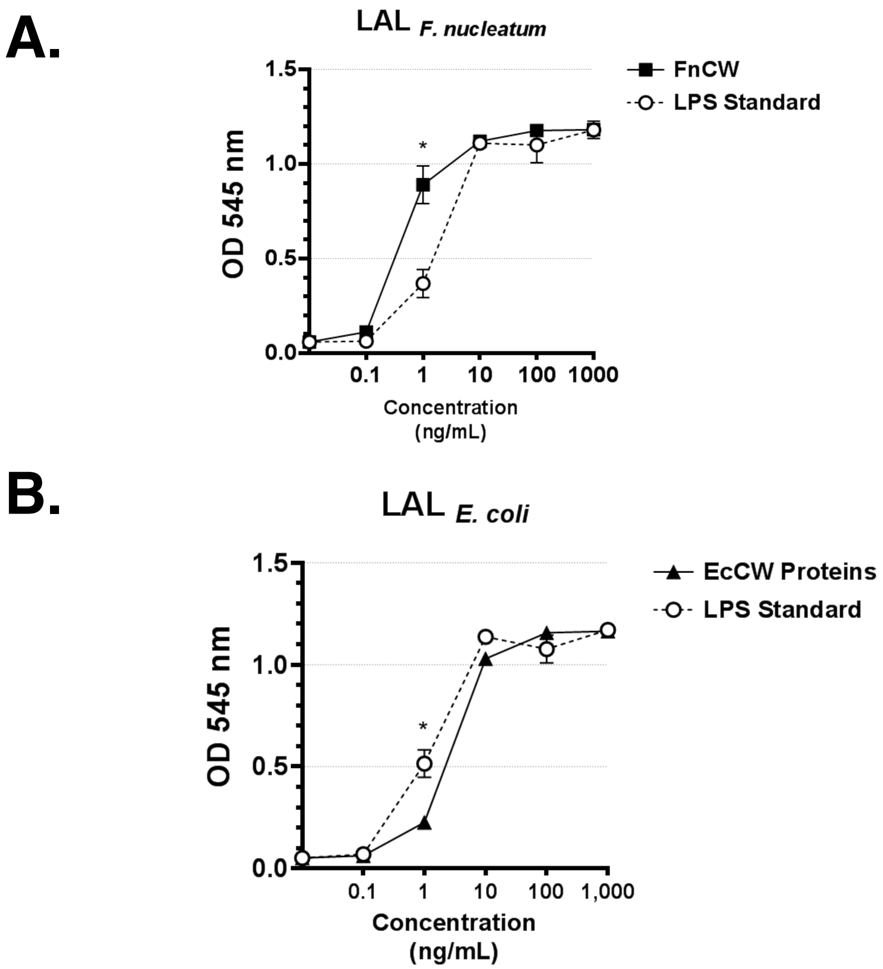

Figure 27. Relative quantity of LPS in cell wall preparations. Bacterial cell wall proteins harvested from *E. coli* **(A)**, *F. nucleatum* **(B)**, and *A. actinomycetemcomitans* **(C)** were quantified for the amount of LPS by using chromogenic LAL assay. Data are means ± SEM, representing of one of two independent experiments in triplicate yielding similar results. Statistical difference was tested using two-way ANOVA with Bonferroni's multiple comparison test. Asterisks indicate statistical significance (**p*-value<0.05), compared to LPS standard.

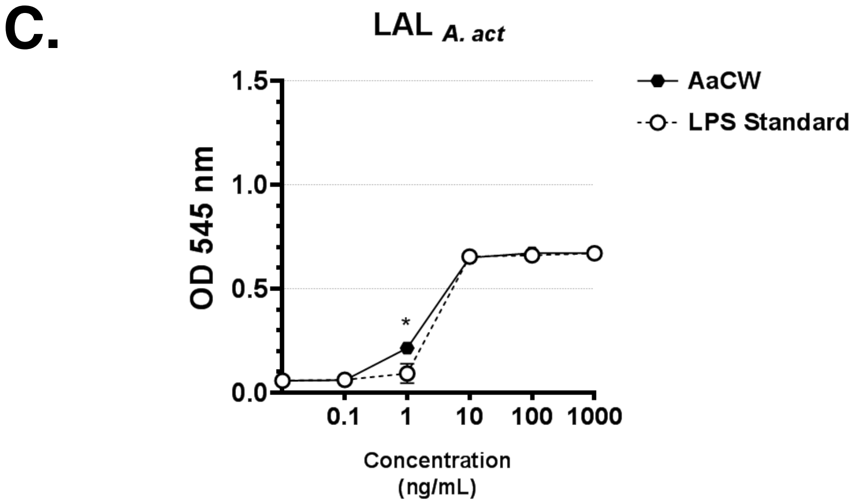

Figure 27. Relative quantity of LPS in cell wall preparations. (continued)

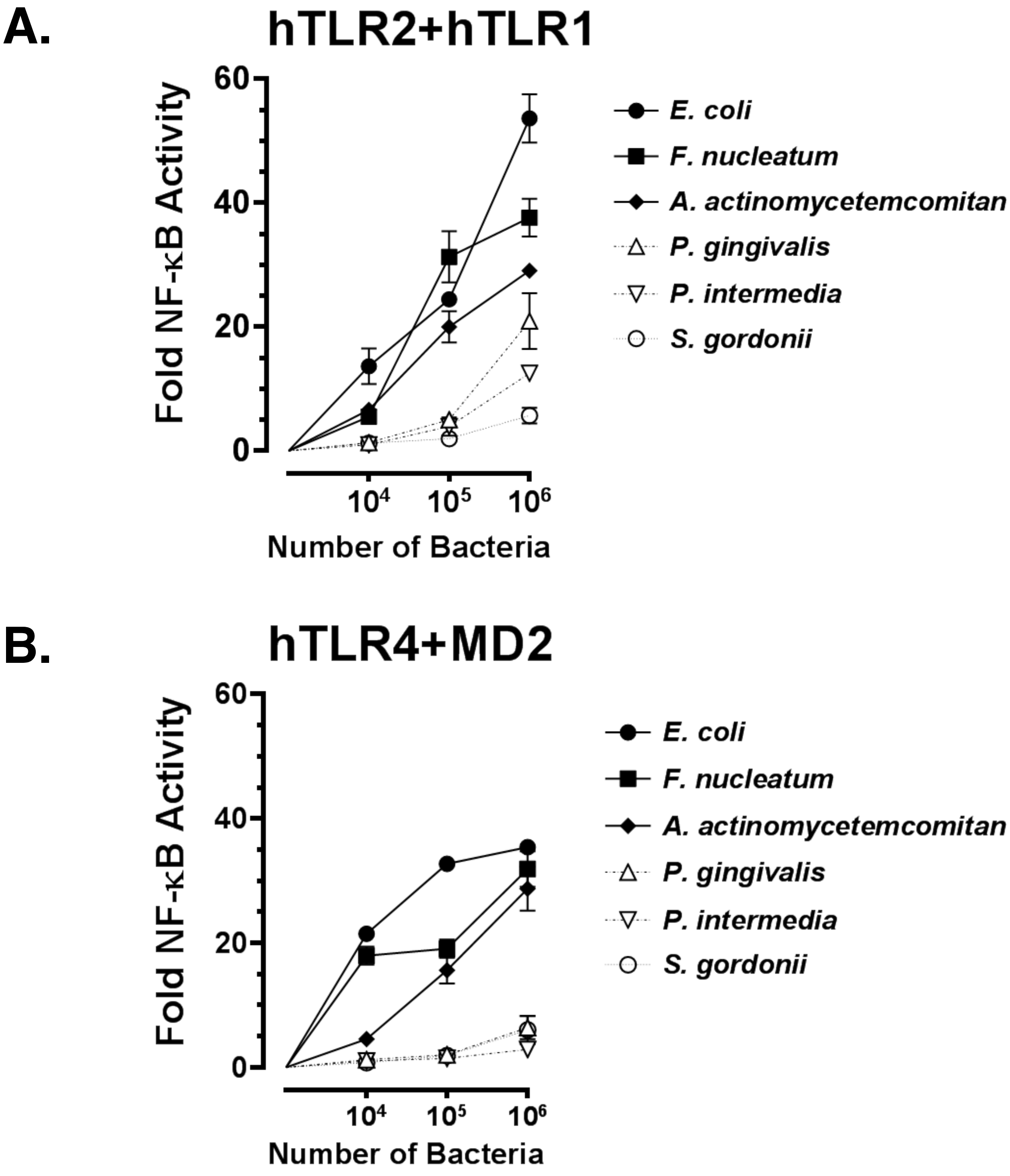

Figure 28. Differential activation of human TLR2 and TLR4 by various bacterial species. Six bacterial species included in our study (*Ec, Fn, Aa, Pg, Pi,* and *Sg*) were tested for their abilities to activate NF- κB signaling via human TLR2/1 **(A)** or TLR4/MD2 **(B)** using HEK293-TLR reporter system. Data represent the average of fold activity ± SEM, from experiments in triplicate.

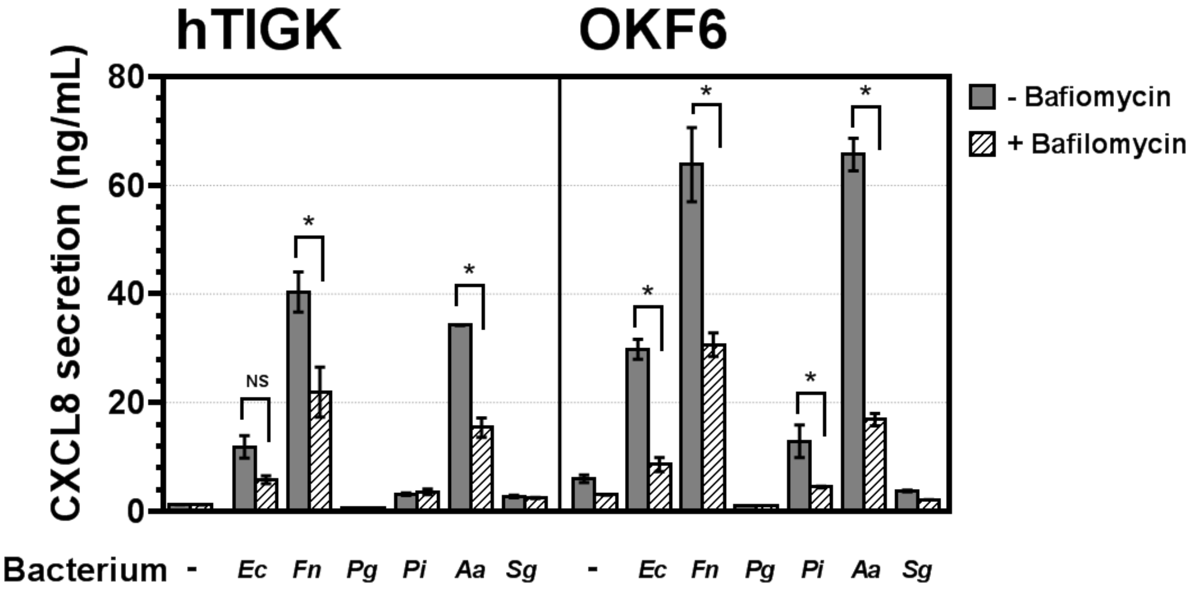

Figure 29. Requirement of endosomal acidification for CXCL-8 induction by various bacterial species. Bafilomycin A1 was pre-incubated with hTIGK and OKF6 for 1 hour prior to bacterial stimulation. Culture supernatants were assayed for CXCL-8 protein using ELISA after a 24-hour infection period. Data are means of the actual concentration ± SEM, representing similar results from one of two independent experiments of each cell line in triplicate. Asterisks indicate statistical difference between experimental groups and their corresponding live bacterium-infected controls (*p-value<0.05).

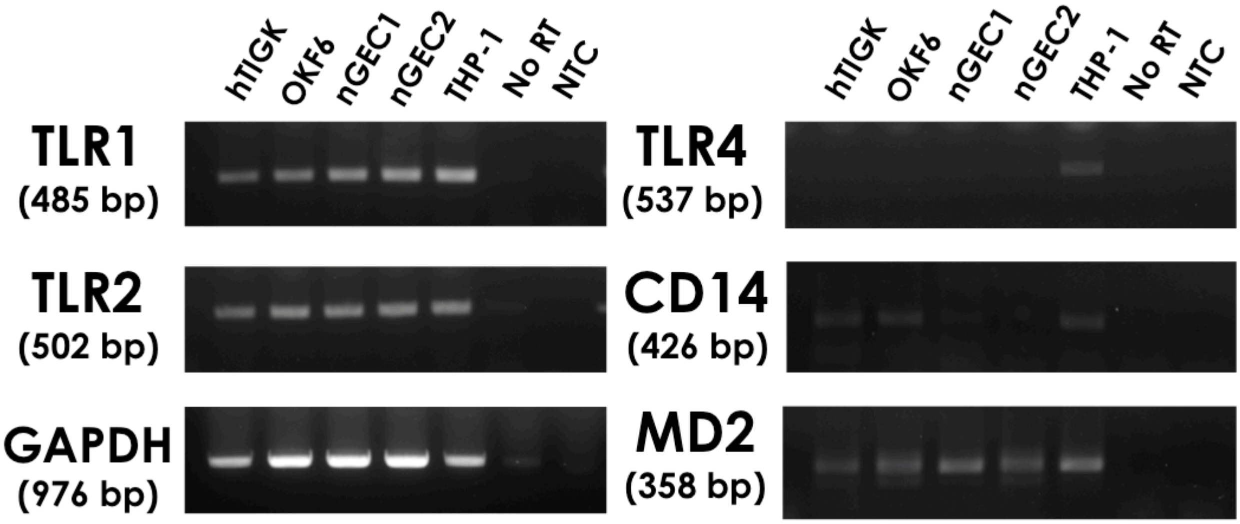

Figure 30. Semi-quantitative RT-PCR analysis of *TLR1*, *TLR2*, *TLR4*, *CD14*, and *MD2* gene expression. hTIGK **(A)**, OKF6 **(B)**, nGEC1 **(C)**, and nGEC2 **(D)** were grown in 6-well plates and subsequently extracted for total RNA and cleanup prior to RT-PCR amplification (40 cycles). A housekeeping gene *GAPDH* was used as a loading control. RNA of THP-1 – derived macrophage served as a positive control (No RT = PCR without reverse transcriptase, NTC = no template control).

Table 3. Primer sequences used in the study chapter II

Target gene (Human)	**Sequence (5'→3')**	**Amplicon size (bp)**	**Reference**
GAPDH Forward	TGA AGG TCG GAG TCA ACG GAT TTG GT	985	Abe K *et al.* PNAS, 1999.
GAPDH Reverse	CAT GTG GGC CAT GAG GTC CAC CAC		
TLR1 Forward	CTT TGG ACT TCT GAC ATC TTA TC	485	Kusumoto Y *et al.* J Periodontol, 2004.
TLR1 Reverse	GAT ATT AGA TAG TTC CAG ATT TG		
TLR2 Forward	TTA GCA ACA GTG ACC TAC AGA G	502	Kusumoto Y *et al.* J Periodontol, 2004.
TLR2 Reverse	CAA ATC AGT ATC TCG CAG TTC C		
TLR4 Forward	TTG CCC AGC TGG GTC CAA CAC TTG	537	Kusumoto Y *et al.* J Periodontol, 2004.
TLR4 Reverse	ATG GTT GTG AGC ATG TGT TAA TC		
CD14 Forward	CTC AAC CTA GAG CCG TTT AT	426	Uehara A *et al.* J Med Microbiol, 2002.
CD14 Reverse	CAG GAT TGT CAG ACA GGT CT		
MD2 Forward	TAT TGG GTC TGC AAC TCA T	358	Zhang J *et al.* Immunol Cell Biol, 2009.
MD2 Reverse	CTC CCA GAA ATA GCT TCA AC		

Target gene (*F. nucleatum*)	**Sequence (5'→3')**	**Amplicon size (bp)**	**Reference**
16S rRNA Forward	ATA CCC TGG TAG TCC ACG CC	619	Originally designed
16S rRNA Reverse	TGT GAC GGG CGG TGT GTA		
FadA Forward	TTA GCT GTT TCT GCT TCA GC	359	Han YW *et al.* J Bacteriol, 2005.
FadA Reverse	TTA CCA GCT CTT AAA GCT TG		

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

5.1 Summary of the findings

Epithelial barriers have indispensible roles in maintaining homeostasis in human tissues throughout the body. Essentially, integrity of epithelial tissues is a key element required for protecting the underlying connective tissues from being endangered by indigenous microbial populations. Pathogens that inhabit the external microenvironments or lumens are prohibited from disseminating into the deeper connective tissues by several strategies naturally occurring at mucosal surfaces, including chemokine ligand 8 or interleukin-8 (CXCL-8/IL-8) expression by the oral epithelial cell layer (Fitzgerald and Kreutzer 1995, Sfakianakis, Barr, and Kreutzer 2002). However, it is not well understood how oral bacterial components are recognized by oral epithelium as numbers of earlier studies have reported their unresponsive phenotypes in response to microbial components.

Herein, oral keratinocytes (oral epithelial cells, OEC) have demonstrated a similar pattern of response to exogenous stimuli. Lipopolysaccharides (LPS), a known TLR4 agonist derived from gram-negative bacterial species, failed to induce chemokine CXCL-8 expression by OECs. However, synthetic lipoprotein Pam_3CSK_4 was able to induce CXCL-8 response in some tested OEC lines in our study. We, therefore, hypothesized that TLR4 signaling in OECs is unique as it occurs only when LPS is internalized by OEC (Chapter 3). We utilized different approaches to elucidate the underlying mechanisms governing LPS responsiveness in OECs including the delivery of isolated LPS or LPS-containing bacterial supernatants from *E. coli* and *F. nucleatum.* Strikingly, although LPS either purified or shed from bacterial cells fails to induce CXCL-8, CXCL-8 is significantly induced when LPS is internalized into OECs. In addition, it is very important to note that perhaps TLR4 is not used by oral epithelia for an inflammatory defense against bacterial infection.

It has been reported that TLR4 is located in the Golgi apparatus (Latz et al. 2002), so we speculated that endocytosed LPS interacts with TLR4 shuttled from the Golgi apparatus to endosomes, leading to the activation of TLR4 signaling. Our data (Chapter 3) confirmed those speculations and established a novel basis of how TLR4 plays a role in LPS sensing in epithelial cells. Once LPS is internalized, TLR4 is trafficked from the Golgi body to endosomes where LPS is confined. Acidification within the endosome occurs afterwards and stimulates TLR4 signaling. The entire signaling process is initiated by LPS endocytosis by an as yet unknown mechanism. However, we summarized that most likely TLR4 is located on the Golgi apparatus, consistent with other studies, and that TLR4 trafficking to endosomes triggers an LPS-induced CXCL-8 response in oral epithelium (Figure 31).

In Chapter 4, we have shown that inhibition of actin function dampens CXCL-8 response by OECs. Our data suggest that bacterial endocytosis requires a direct engagement of live bacteria on the OEC surface or cell wall protein-mediated uptake. To recapitulate, based on the experimental strategies mentioned above, we found that LPS internalization is mandatory for TLR4 sensing of LPS by oral keratinocytes. In addition, a co-evolved bacterium, *F. nucleatum,* has a special characteristic due to the presence of protein on its cell wall that renders it stimulating for OECs. We have shown that FnCW proteins are not only crucial for CXCL-8 induction, but also required for inducing the LPS-mediated OEC response. Our overall findings thus clearly define OEC sensing of LPS and reveal a novel protection mechanism of oral epithelium: TLR4 unresponsiveness is used by epithelium to overcome the microbial burden that the oral epithelial interface encounters.

5.2 Future directions

Even though the roles of LPS sensors, including TLR4, in mammalian cells have been studied extensively in the field of immunology, most of the studies have emphasized how these innate immune receptors are utilized in sentinel cells, e.g., monocytes and macrophages. Current knowledge of epithelial cell innate immunity is thus still limited. According to the preceding experimental studies, a vast variability of phenotype between different epithelial cell lines has been clearly demonstrated, and that might contribute to inconclusive or controversial findings as to what the exact mechanisms mediated by TLRs are in epithelium. In addition, it is unclear whether epithelial cells possess intracellular machineries similar to those reported in myeloid cells. This discrepancy thus hinders the growing evidence of epithelial cell biology and renders it more challenging to researchers to gain a better understanding in the field of epithelial immunology. Several statements and questions, addressed here, need further clarification to enable us to firmly conclude as to how epithelial cells participate in maintaining homeostasis of mucosal surfaces.

It is still not well understood how TLR4 is shuffled from the Golgi apparatus to the endosome during infection.

We have provided the first evidence that, in OECs, endosomal TLR4 can mediate CXCL-8 induction. We demonstrated, by LPS delivery experiments in TLR4-silenced OECs, that TLR4 plays a role in LPS sensing when it is internalized into OECs. By using the pharmacological inhibitors, bafilomycin A1 and brefeldin A, we found that endosome-lysosome fusion was needed for TLR4 activation and that retraction of the Golgi membranes back to the ER attenuated TLR4-mediated CXCL-8 responses. This suggests that TLR4 is synthesized in ER, accumulates in the Golgi apparatus, and is rapidly shuttled to endosomes when OEC is breached by LPS. However, the molecular events of TLR4 utilization by OECs need to be confirmed by experimental studies using immunocytochemistry techniques to identify the specific location of TLR4 in OECs as well as the fate of TLR4 distribution during LPS infection. Further investigation on the signaling requirements enabling TLR4 to shuffle from the Golgi to the endosome will thus provide fundamental knowledge for the field of epithelial cell immunology.

Is another protein, caspase-4, involved in the homeostasis in oral / gingival epithelium?

In 2013-2014, the role of caspase-4, a human homolog of murine caspase-11, was first reported as a cytosolic LPS sensor in macrophages (Hagar et al. 2013, Kayagaki et al. 2013, Shi et al. 2014). Activation of caspase-4, or murine caspase-11, by internalized LPS leads to the production of the mature form of IL-1β, thereby enhancing the killing of bacteria by macrophages (Casson et al. 2015, Rathinam et al. 2012). Its activity also triggers host cell death, termed 'pyroptosis', which is in fact beneficial for the host as it promotes the shedding and the replacement of dead cells (Knodler et al. 2014). However, whether caspase-4 plays a role in LPS recognition in OECs remains to be elucidated.

Microbial components derived from invading microbes are able to interact with their corresponding sensors in the host cells at the same time. As mentioned, LPS shed from gram-negative bacteria might be concomitantly fated to both TLR4 and caspase-4, depending on the intracellular pathway to which the host-bacterium interaction leads. Our data clearly indicate the association of CXCL-8 induction with an endocytosis-mediated TLR4 signaling. Characterizing the role of caspase-4 in OECs by investigating IL-1β induction and cell death after LSP transfection might be an interesting avenue to explore since, according to studies in other cell types, it helps eliminate cytosolic bacteria. *P. gingivalis* and *F. nucleatum*, gram-negative periodontal pathogens that are able to invade OEC (Han et al. 2000, Lamont et al. 1995), might be the best representatives to use for this scenario.

Is TLR4 suppression in epithelial cells, particularly OECs, attributable to the lack of other protein responsible for TLR4 distribution?

In the early 2000s, emerging evidence had extensively characterized TLR4 functions as well as the signaling components required for its activation. The discovery of TLR4 and TLR2 captured researchers’ interest so that they strived to figure out the location of each component related to TLR4 signaling. The role of MD2 in LPS responsiveness was reported by Nagai *et al.* in 2002 (Nagai et al. 2002). They showed that MD2 mediates the distribution of TLR4 to cell membranes. However, our data suggest that OECs express *MD2* transcripts, even though OECs display an LPS unresponsiveness phenotype. It is possible that MD2, if expressed at the protein level in epithelial cells, might not be similarly functional. Takahashi *et al.* demonstrated in 2007 that PRAT4A, a protein associated with TLR4, is another candidate regulator of TLR4 trafficking to the surface (Takahashi et al. 2007). Whether OECs express PRAT4A warrants further studies. These findings might thus help explain the underlying, undiscovered mechanisms that oral keratinocytes exploit to prevent the epithelial layer from being harmed by an excessive TLR4 stimulation during periodontal health.

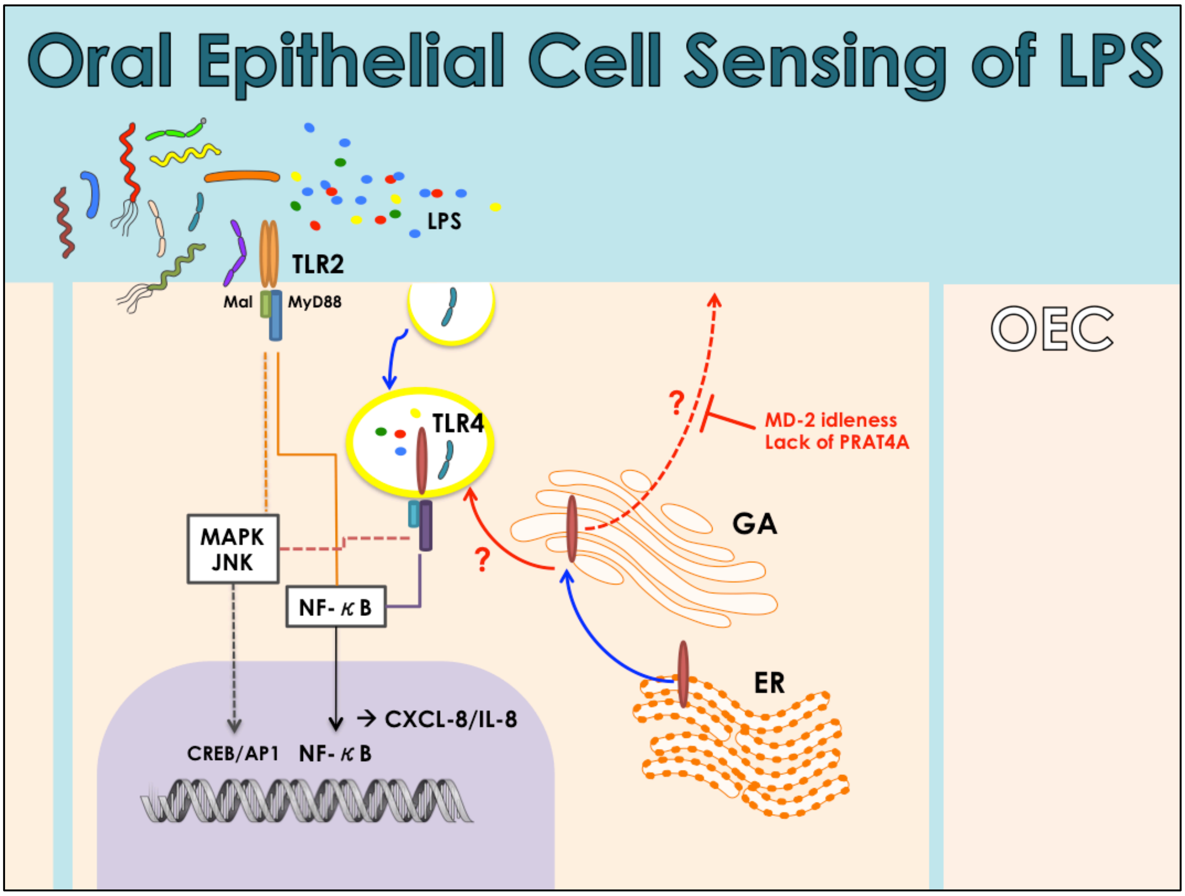

Figure 31. A diagram illustrates the proposed mechanism of LPS sensing by OECs. Upon infection / internalization of OECs with live gram-negative bacteria, LPS is directly ligated with endosomal TLR4 thereby activating CXCL-8/IL-8 induction. TLR4 is thought to be located on the Golgi apparatus (GA); however, it is not known how Golgi-associated TLR4 is trafficked to the endosome. The lack of PRAT4A or non-functional MD2 protein in OECs possibly contributes to LPS unresponsiveness (dashed line).

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