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Short communication

Differential regulation of innate immune response genes in gingival epithelial cells stimulated with *Aggregatibacter actinomycetemcomitans*

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Background and Objective: The gingival epithelium provides the first line of defense against colonization by periodontal pathogens, both as a physical barrier and by the production of inducible innate immune mediators such as β-defensins and pro-inflammatory cytokines. The gram-negative bacterium *Aggregatibacter actinomycetemcomitans* is implicated in the pathogenesis of localized aggressive periodontitis, although the bacterium is found widely in the healthy population. We hypothesized that gingival epithelial cell-derived innate immune mediators triggered in response to *A. actinomycetemcomitans* infection may play an important role in increased susceptibility to infection.

Material and Methods: Primary cultures of human gingival epithelial cells were cultured in the presence of *A. actinomycetemcomitans*. Total mRNA was examined for the presence of innate immune markers using RT-PCR.

Results: We show here that the mRNA levels of human β-defensin 2 and interleukin-8 are elevated by live cultures of a clinical isolate of *A. actinomycetemcomitans* in cultured gingival epithelial cells from healthy individuals, but not by *A. actinomycetemcomitans* lipopolysaccharide. Cells from a patient with localized aggressive periodontitis, however, did not respond to this bacterial stimulation. In contrast, the pro-inflammatory cytokine interleukin-19 was induced in cells from both localized aggressive periodontitis and healthy subjects. Examination of Toll-like receptors and associated adapter molecules indicated lower levels of Toll-like receptor 2 mRNA in the localized aggressive periodontitis patient-derived cells compared with cells from healthy subjects.

Conclusion: These results suggest that a differential expression of innate immune response genes to *A. actinomycetemcomitans* in the gingival epithelium could be an underlying factor of susceptibility to localized aggressive periodontitis.

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Periodontal infection with *Aggregatibacter actinomycetemcomitans* and the consequent inflammatory response lead to localized aggressive periodontitis. This cannot occur unless the pathogen is able to breach the initial line of host defense in the periodontium, largely represented by gingival epithelial cells. These cells represent the first line of oral innate immunity, producing antimicrobial agents and inflammatory mediators; however, little is known about how the gingival epithelium responds to the presence of bacteria in general, or to *A. actinomycetemcomitans* in particular. Furthermore, while localized aggressive periodontitis disease progression and epidemiology are well documented (1), as are differences in neutrophil function between patients and healthy individuals (2,3), deficiencies in the host defense capabilities of the gingival epithelium have not been examined.

The initial response of the mucosal epithelium occurs through the recognition of microbe-associated molecular patterns, such as lipopolysaccharide, by Toll-like receptors and other pattern recognition receptors. Following this receptor–ligand interaction, signal transduction is initiated, involving numerous adapter molecules and mediators, and activating a transcriptional response that includes the induction of host defense peptides such as the β-defensins and cytokines, including interleukin-8 (reviewed in ref. 4).

Recent studies have described the expression of β-defensins in the oral cavity, suggesting that they contribute to the innate host defense in this tissue (reviewed in ref. 5). It has been proposed that these antimicrobial peptides are secreted by the epithelial cells of the mouth and, by associating with salivary mucins, are distributed throughout the oral cavity (6). The concentrations of defensin in the saliva are believed to be sufficient for microbicidal activity against oral pathogens (7), including *A. actinomycetemcomitans* (8).

Based on our earlier findings that β-defensin genes are up-regulated by infectious agents and inflammatory mediators (9), it has been demonstrated that human β-defensin 2 expression is increased in the gingival epithelium by interleukin-1β, lipopolysaccharide, tumor necrosis factor-α and bacterial products (7,10,11), as well as by live cultures of *Porphyromonas gingivalis* (12). Challenge of cultured gingival epithelial cells with *A. actinomycetemcomitans* has resulted in variable expression of β-defensins, potentially because of *A. actinomycetemcomitans* strain variability (13,14), and this induction can be attributed, at least in part, to the outer membrane protein, OMP100, leading to the secretion of pro-inflammatory cytokines, which, in turn, can further induce human β-defensin 2 expression (15). However, because OMP100 was observed in all strains examined (15), other factors are probably also involved in the induction of innate immune gene expression.

However, *A. actinomycetemcomitans* is found commensally in 15% of the population (16) and it is unlikely that this constant interaction between bacteria and gingival epithelium would result in the continual induction of innate immunity. We have recently shown in a rat *A. actinomycetemcomitans* infection model that initial colonization by the bacterium results in an elevation in the levels of β-defensin mRNA, but that those levels return to precolonization levels after 7 d, even in the presence of bacteria (17). This suggests that a tolerance to bacterial colonization occurs in the oral epithelium, similar to that which may occur in the airway (18). Thus, because *A. actinomycetemcomitans* is found in healthy oral flora in addition to infected pockets, we sought to discover how this bacterium regulates β-defensin and cytokine gene regulation in health and disease.

To study this, we challenged primary epithelial cell cultures derived from both healthy individuals and those with localized aggressive periodontitis with the same strain of *A. actinomycetemcomitans* to determine whether innate immune response genes are differentially regulated in these cells. In addition to β-defensins, we analyzed these cultures for changes in the expression of the pro-inflammatory cytokine interleukin-8. Furthermore, as human β-defensin 2 was initially identified in psoriatic, but not in normal, skin (19), we also examined the gingival epithelium for the expression of interleukin-19. This is a novel member of the interleukin-10 family (20) whose action promotes the development of the T-helper 2 response in humans (21–23), and is also found in psoriatic lesions (24) and asthma (23). Several recent reports have demonstrated the importance of the T-helper 2 response in human periodontitis (25–27), suggesting that interleukin-19 may play a role in inflammatory diseases, such as localized aggressive periodontitis.

Material and methods

Primary cultures of human gingival epithelial cells were stimulated with live bacteria. Expression of innate immune mediators was evaluated by quantitative RT-PCR and ELISA.

Bacterial culture

A. actinomycetemcomitans strain CU1000 (28) was obtained from the laboratory of Dr Daniel Fine, UMDNJ. A single colony of *A. actinomycetemcomitans* was used to inoculate 30 mL of trypticase soy broth supplemented with 6 g/L of yeast extract, 0.8 g/L of dextrose, 0.4 g/L of sodium bicarbonate, 75 mg/L of bacitracin and 5 mg/L of vancomycin in a 100-mm polystyrene tissue culture dish (TPP, Trasadingen, Switzerland). The bacteria were grown at 37°C/10% CO_2 for 40 h, washed twice in sterile phosphate-buffered saline and scraped into 1 mL of phosphate-buffered saline. To obtain a single-cell suspension, the bacteria were vortexed for 1 min at top speed and allowed to settle for 5 min. The top 750 mL was removed and used in the experiments. Heat-killed bacteria were heated to 65°C for 30 min prior to challenge.

Isolation of *A. actinomycetemcomitans* lipopolysaccharide

Lipopolysaccharide was isolated by the Tri-reagent method, as described previously (29), and further purified by the method of Hirschfeld *et al.* (30). Concentrations of lipopolysaccharide were

determined by the limulus amebocyte lysate assay.

Cell culture of primary human gingival epithelial cells

Gingival tissue was obtained from third molar extractions (from healthy individuals) or from periodontal surgery (from patients) with informed consent under approval from the Institutional Review Board. The infection status of *A. actinomycetemcomitans* was not determined for these donors. The tissue was rinsed three times in HEPES-buffered saline solution supplemented with 1% Antibiotic/Antimycotic (Invitrogen, Carlsbad, CA, USA) and treated with 6 mg/mL of dispase (Gibco BRL, Carlsbad, CA, USA) in HEPES-buffered saline overnight at 4°C to detach the epithelial sheet from the basement membrane. The epithelium was removed, cut into small pieces, and incubated in 5 mL of trypsin-EDTA (0.5 g of porcine trypsin, 0.2 g of EDTA·4Na per litre of Hanks' balanced salt solution; Sigma-Aldrich, St Louis, MO, USA) at 37°C for 10 min. Trypsinization was stopped by adding 5 mL of Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (Invitrogen), and the cells were collected by centrifugation for 10 min at 200 ***g*** in a swing-bucket centrifuge (Jouan C312; Jouan, Waltham, MA, USA). The cells were resuspended in serum-free keratinocyte growth medium (Cambrex Bioscience, Walkersville, MD, USA) supplemented with bovine pituitary extract, human epidermal growth factor, bovine insulin, hydrocortisone, gentamicin, amphotericin B and 0.15 M $CaCl_2$, plated in a T-25 flask (Corning Glass Works, Corning, NY, USA) and cultured in a humid 37°C/5% CO_2 incubator. All cells used in these experiments were passaged once, into six-well plates (Corning Glass Works), and used when the cell count reached 1×10^5 cells/well.

Cell culture of THP-1 cells

The human peripheral blood cell line, THP-1 (ATCC, Manassas, VA, USA), was propagated according to the manufacturer's instructions. Briefly, cells were grown in modified RPMI-1640 (2 mM L-glutamine, 1.5 g/L of sodium bicarbonate, 4.5 g/L of glucose, 10 mM HEPES and 1.0 mM sodium pyruvate), supplemented with 0.05 mM β-mercaptoethanol and 10% fetal bovine serum (Invitrogen), to a maximum density of 1×10^6 cells/mL.

RNA isolation

Cells were lysed using 350 μL of Buffer RLT (Qiagen, Valencia, CA, USA) with 10 μL/mL of β-mercaptoethanol and collected using a cell scraper. Samples were homogenized with a QIAshredder (Qiagen) and RNA was isolated using the RNeasy mini kit with the additional on column DNAse (Qiagen), according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction

Total mRNA was subjected to reverse transcription-polymerase chain reaction using standard conditions and reagents (Invitrogen). Amplification was carried out at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, 50°C for 30 s and 68°C for 1 min, with a 10-min extension at 72°C. Samples were electrophoresed on a 1.5% agarose gel, and DNA was visualized and analyzed using the Typhoon 8600 imaging system (GE Healthcare, Waukesha, WI, USA). All PCR products were subjected to DNA sequence analysis to verify amplification of the proper gene. Primers were synthesized and sequencing was performed by the Molecular Resource Facility at UMDNJ. Primer sequences are shown in Table 1. Primer sequences of Toll-like receptors 1–8 were designed by C. Desanti, Case Western Reserve University School of Dental Medicine (Cleveland, OH, USA). Other primer sequences were designed based on published genomic sequences using MACVECTOR software (Accelrys, San Diego, CA, USA).

Reverse-transcriptase quantitative PCR

RNA was dissolved in polymerase chain reaction grade water (Invitrogen) to a concentration of 50 μg/mL. One microgram of this RNA was incubated at 70°C for 10 min and then reverse transcribed with 800 U of Murine leukaemia virus reverse transcriptase in 1 × Buffer II, 5 mM $MgCl_2$, 5 μM random hexamers, 20 U of RNAse inhibitor and 1 mM of each dNTP. All reverse-transcriptase quantitative PCR reagents were obtained from Applied Biosystems (Foster City, CA, USA). The sample was incubated at 25°C for 10 min, at 42°C for 45 min and then heat-inactivated at 95°C for 5 min. Five-hundred nanograms of cDNA was amplified using 1 × 'SYBR Green Master Mix' and primers in a final volume of 20 μL. For all products except interleukin-19, samples were incubated at 50°C for 2 min, at 95°C for 1 min and then for 50 cycles of 95°C for 15 s and 60°C for 1 min in a Stratagene MX4000 thermal cycler (Stratagene, La Jolla, CA, USA). Samples were then incubated for 41 cycles at 55°C for 30 s, increasing by 1°C per cycle to a final temperature of 95°C, for melting-point analysis. For interleukin-19, amplification was performed as described previously (22), using the MX4000 multiplex quantitative PCR system (Stratagene). PCR conditions consisted of an initial denaturation step of 95°C for 10 min, followed by 45 cycles at 95°C for 30 s and 60°C for 1 min. Primers were synthesized by the Molecular Resource Facility at UMDNJ. Primer sequences are shown in Table 1.

Enzyme-linked immunosorbent assay

The levels of interleukin-8 in culture supernatants were determined by enzyme-linked immunosorbent assay, as described previously (31). Experiments were performed in triplicate and data are presented as the mean ± standard deviation.

Results and Discussion

A number of laboratories have observed variability in β-defensin gene expression in normal gingival epithelium in response to live or heat-killed *A. actinomycetemcomitans*, using strains whose origins with respect to

Table 1. Primer sequences used for reverse transcription-polymerase chain reaction

Gene	Human PCR primers		Product size
	Forward primer (5′–3′)	Reverse primer (5′–3′)	
hBD-2	GGG TCT TGT ATC TCC TCT TCT CGT TC	TCC GCA TCA GCC ACA GCA G	223 bp
TLR1	TAG TGT GCT GCC AAT TGC TC	TCC AGC TGA CCC TGT AGC TT	402 bp
TLR2	GCC AAA GTC TTG ATT GAT TGG	TTG AAG TTC TTC AGC TCC TG	346 bp
TLR3	AAA TTG GGC AAG AAC TCA CAG G	GTG TTT CCA GAG CCG TGC TAA	320 bp
TLR4	TGG ATA CGT TTC CTT ATA AG	GAA ATG GAG GCA CCC CTT C	506 bp
TLR5	GCA ACC AGC TCC TAG CTC CT	GAT GGC ATC CTG GAT ATT GG	575 bp
TLR6	TGG ATC TGC CCT GGT ATC TC	GCT GTT CTG TGG AAT GGG TT	404 bp
TLR7	AAT GTC ACA GCC GTC CCT AC	GCA GAA ATT TAG CAT CCC CA	367 bp
TLR8	TTG GAG ATT TCC GAA GAT GG	TTG CTT TGG TTG ATG CTC TG	481 bp
TLR9	AGG TGT ACC CGC TAC TGG TG	ACA GCA GCT ACA GGG AAG GA	353 bp
TLR10	CAA GAG AAA TGT CCG ATT CCA CGC	CGA AGG TTT GCC CAG AAA AGC C	432 bp
TRIF	TTT GAC AGA GCA GGG GTT TTT G	CAA CTT GGA AAT CAG CCA GTC C	373 bp
TRAM	GAT GAT GCT GTA AAT GGG TCT GC	CCA CGA CTT TCT TCC TCT AAG GC	227 bp
SARM	AGC ACG AAG CCC TCA CAA ATC	GCA ATA GCC CAG CCT CAG AAT G	252 bp
MyD88	GGT GTA GTC GCA GAC AGT GAT GAA C	ACC CAG CAT TGA GGA GGA TTG	514 bp
MD2	TCT GCA ACT CCT CCG ATG CAA	TTC AGA GCT CTG CAA AAA GA	256 bp
TANK	GCG AGC AAC TCA ATA AAG CGT ATG	CTT CAA GCA GAG GAA CAC AGC C	225 bp
TOLLIP	GGA CAG AAA GAG AAT GCC CAC AAG	TGA CAA AAA CCA CCC CCA ATC G	163 bp
Mal	AGG CAA GAT GGC TGA CTG GTT C	TAG TGG GTT GTC CTG TGA GGT AGG	118 bp
Dectin-1	TTT GGG AAT CCT ATG CTT GG	ATC AGG TTG GGA AGA CAC TTG	244, 382 bp
G3PD	ACC ACT GAC ACG TTG GAC GT	ATC TTC CAG GAG CGA GAT CC	506 bp
interleukin-19	TCT GCG GCA ATG TCA GGA A	GGA CCT CCA GCT GAT CAT AGT TGT	97 bp
HPRT	CAG CCC TGG CGT CGT GAT TAG	GCA AGA CGT TCA GTC CTG TCC ATA A	113 bp

bp, base pairs; G3PD, glyceraldehyde-3-phosphate dehydrogenase; hBD-2, human β-defensin 2; HPRT, hypoxanthine-guanine phosphoribosyltransferase; MD2, myeloid differential protein-2; MyD88, myeloid differentiation factor 88; Mal, MyD88 Adapeter-like; PCR, polymerase chain reaction; SARM, sterile α- and armadillo-motif-containing protein; TANK, TRAF-associated NF-κB kinase; TOLLIP, Toll-interacting protein; TLR, Toll-like receptor; TRAM, TRIF, TOLL/1L-1R domain-containing adaptor-inducing IFN-beta; TRIF-related adaptor molecule.

localized aggressive periodontitis status or phenotype (i.e. rough vs. smooth) are not described (13–15,32). This variability may be the result of a variety of factors, including bacterial morphology and serotype, as recent studies using 16SRNA analysis have identified all serotypes in localized aggressive periodontitis patients (33). To confirm that induction by *A. actinomycetemcomitans* occurs in our system, we incubated primary cultures of normal human gingival epithelial cells with a clinical isolate of *A. actinomycetemcomitans* (CU1000, serotype f) exhibiting a rough phenotype (28). Cells were challenged either with live *A. actinomycetemcomitans* (at a multiplicity of infection of 200 : 1) or with purified *A. actinomycetemcomitans* lipopolysaccharide (100 ng/mL), as well as with interleukin-1β as a positive control, for 6 h at 37°C. The results, shown in Fig. 1A, demonstrate that this strain of *A. actinomycetemcomitans* results in a greater than 10-fold elevation of human β-defensin 2 mRNA levels. In contrast to epithelial cells from other tissues (34–37), purified lipopolysaccharide from this bacterium or from *P. aeruginosa* (data not shown) does not induce human β-defensin 2 gene expression. This pattern was also observed after induction for 24 h (data not shown), indicting that the induction is maintained at that level for at least 1 d, as also observed *in vivo* (17). Induction of human β-defensin 2 levels by live bacteria in these cells was reproducible, but varied from fourfold to greater than 30-fold between samples (Fig. 1B). Such patient-to-patient variability is consistent with a published report of the response to *P. gingivalis* (38).

As *A. actinomycetemcomitans* can be found as a commensal microorganism in healthy individuals, we sought to discover if the response to these bacteria differed in the gingival epithelium of patients suffering from localized aggressive periodontitis. We obtained epithelial tissue from an individual with localized aggressive periodontitis and examined the response of the cultured cells to *A. actinomycetemcomitans*. While the human β-defensin 2 levels in these cells increased in response to interleukin-1β, as with those from healthy individuals, there was no response to live *A. actinomycetemcomitans*, even at a multiplicity of infection of 1000 : 1 (Fig. 1A). This suggests that the pathway which leads to the interleukin-1β-mediated human β-defensin 2 induction is not impaired in the localized aggressive periodontitis sample. Although we were able to grow cells routinely from healthy individuals, because of the small number of available patients with localized aggressive periodontitis and the poor proliferative capacity of the cells we obtained from patient tissue, in *in vitro* culture we were only able to culture tissue from a single individual with this disorder.

Challenge of human gingival epithelial cells with *A. actinomycetemcomitans* has been shown to induce the secretion of several pro-inflammatory

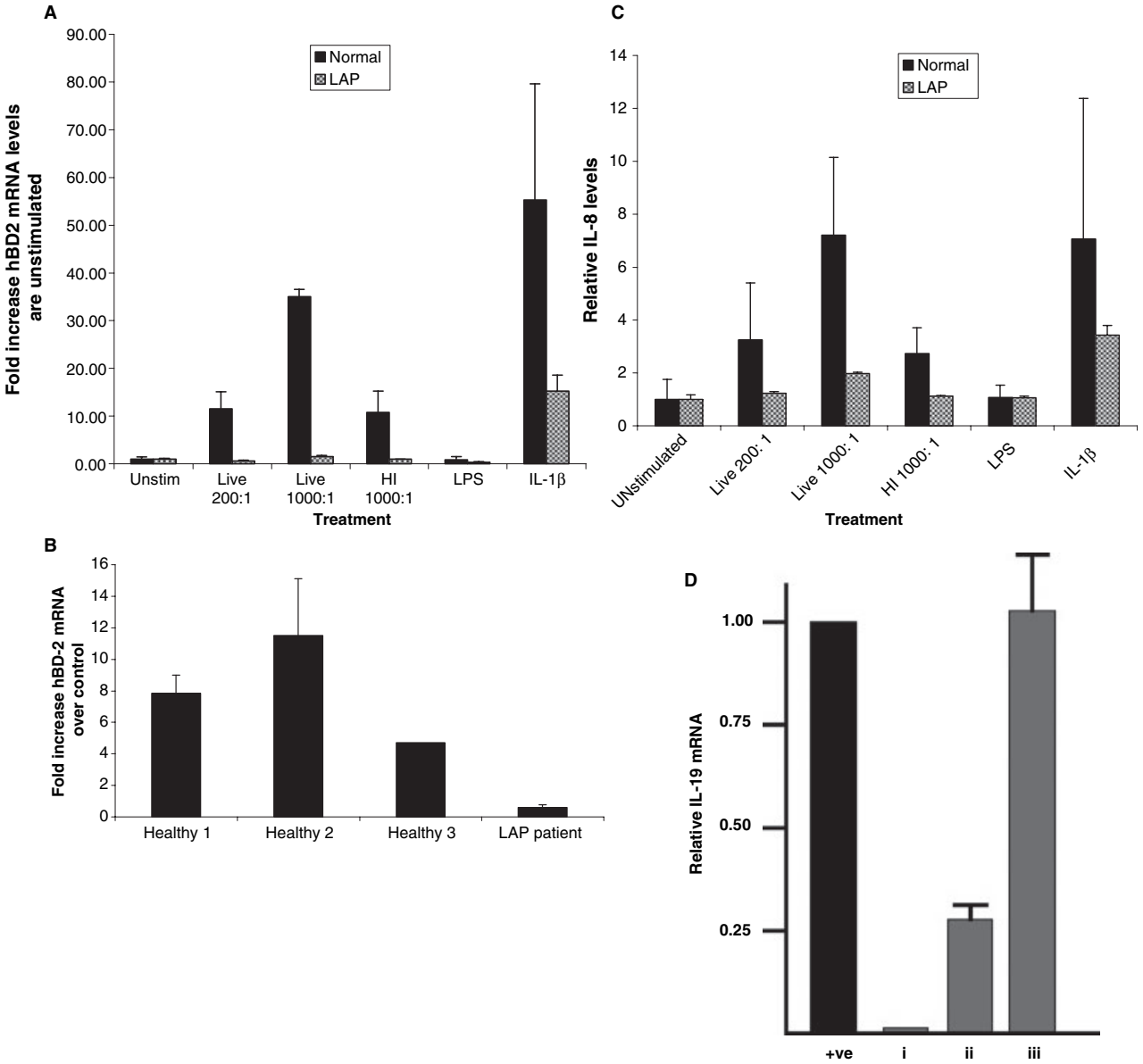

Fig. 1. Innate immune gene expression in human gingival epithelial cells in response to *Aggregatibacter actinomycetemcomitans*. (A) Human gingival epithelial cells from either normal individuals (representative of one of three, black bars) or an individual with localized aggressive periodontitis (grey bars) were cultured in the presence of live *A. actinomycetemcomitans* at a multiplicity of infection of 200 : 1 or 1000 : 1, with heat-inactivated *A. actinomycetemcomitans* (equivalent multiplicity of infection 1000 : 1), with purified *A. actinomycetemcomitans* lipopolysaccharide or with recombinant human interleukin-1β, for 6 h. Culture supernatants were collected, and total mRNA was isolated from the cell lysates. (B) Interindividual variability in response to live bacterial stimulation. Shown are the responses of cultured cells from three representative healthy individuals and the localized aggressive periodontitis patient also shown in (A). Relative levels of human β-defensin 2 (A,B) mRNA were quantified by real-time polymerase chain reaction; levels of interleukin-8 (C) were determined by enzyme-linked immunosorbent assay. (D) Expression of interleukin-19 in human gingival epithelial cells. Levels of interleukin-19 mRNA were determined by real-time polymerase chain reaction, compared with human peripheral blood mononuclear cells stimulated with 100 ng/mL of *Escherichia coli* lipopolysaccharide (positive control). Cells were stimulated with *A. actinomycetemcomitans* lipopolysaccharide (i), live *A. actinomycetemcomitans*, at a multiplicity of infection of 1000 : 1 for 6 h (ii), or with heat-inactivated *A. actinomycetemcomitans* (equivalent multiplicity of infection of 1000 : 1) (iii), and subjected to reverse transcription-polymerase chain reaction using primers for interleukin-19 normalized to β-actin. Error bars represent ± one standard deviation. hBD-2, human β-defensin 2; HI, heat inactivated; IL-1β, interleukin-1β; LAP, localized aggressive periodontitis; LPS, lipopolysaccharide; Unstim, unstimulated.

cytokines (31). To determine whether human β-defensin 2 was secreted in parallel with such cytokines, we also examined the interleukin-8 levels from culture supernatants of healthy and localized aggressive periodontitis patient-derived cells. The results shown in Fig. 1C demonstrate a similar response to the live *A. actinomycetemcomitans*

in normal individuals. The lack of induction by lipopolysaccharide is consistent with the report by Sfakianakis *et al*. (39) that polymyxin B failed to inhibit the *A. actinomycetemcomitans*-mediated induction of interleukin-8 in gingival epithelial cells. Consistent with the human β-defensin 2 findings, we observed a minimal response to live bacteria in cells from the localized aggressive periodontitis patient (at a multiplicity of infection of 1000 : 1 we observed a twofold increase in interleukin-8 levels). Given the interindividual variability in the responses discussed above, this suggests that human β-defensin 2 and interleukin-8 are induced through a common pathway, which may be deficient in localized aggressive periodontitis patients.

To determine whether this difference in response between subjects was prevalent among pro-inflammatory cytokines, we examined the cells for expression of interleukin-19. By reverse transcription-polymerase chain reaction we observed low basal levels of interleukin-19 mRNA in unstimulated cultures, which increased approximately fivefold upon stimulation with live bacteria (D. Laube, J. Eskdale, unpublished observations). Quantification by real-time polymerase chain reaction demonstrated that interleukin-19 expression was induced by both live *A. actinomycetemcomitans* and heat-killed *A. actinomycetemcomitans* (Fig. 1D). In contrast to human β-defensin 2 and interleukin-8, however, this stimulation was greater with heat-killed bacteria than with live bacteria. Stimulation was observed in both localized aggressive periodontitis and normal cells, as measured by quantitative PCR (data not shown), and there was no significant difference in the basal levels of interleukin-19 mRNA between the samples. This is the first demonstration of interleukin-19 expression in an epithelial tissue and suggests that interleukin-19 may participate in the inflammatory response observed in periodontal disease. However, as the response differs somewhat from interleukin-8 and human β-defensin 2, interleukin-19 may serve a different function, such as increasing the induction of interleukin-8 and interleukin-6 by local tissue macrophages, as a way to increase neutrophil infiltration (40). The differential response to heat-killed bacteria, for example, suggests that interleukin-19 may be regulated by heat-stable factors such as some lipoproteins (41) in addition to the heat-labile factors that stimulate human β-defensin 2 expression.

To begin to address the mechanism behind this observation, we examined the spectrum of Toll-like receptors and their related intracellular adapter molecules expressed in unstimulated primary cell cultures. As shown in Table 2, we observed the expression of most Toll-like receptors in the gingival epithelium, as well as all adapter molecules examined (Toll/1L-1R domain-containing adaptor-inducing IFN-beta (TRIF), TRIF-related adaptor molecule (TRAM), sterile α- and armadillo-motif-containing protein (SARM), myeloid differentiation factor 88 (MyD88), myeloid differential protein-2 (MD2), TRAF-associated NF-κB Kinase (TANK), Toll-interacting protein (TOLLIP) and MyD88 adapter-like (Mal)). Notably absent in our gingival epithelial cultures among the Toll-like receptors is the expression of Toll-like receptors 4, 5 and 7. Other laboratories have reported the presence of Toll-like receptor 4 in inflamed gingival epithelium, but under conditions used here for cell culture, we were repeatedly unable to observe any visible amplified product. Our conditions may thus reflect a healthy system and would explain the lack of response to purified lipopolysaccharide in our experiments. Also absent was mRNA encoding either isoform of the glucan-specific pattern recognition receptor, Dectin-1, consistent with findings in epithelial cells from the gastrointestinal and other mucosal sites (42,43). No significant change in the levels of any of the receptors or adapter molecules examined was observed after incubation with bacteria (data not shown).

As Toll-like receptor 2 can recognize patterns found on both gram-negative and gram-positive bacteria, it is reasonable to hypothesize that this receptor may recognize live *A. actinomycetemcomitans*, although there may be other factors found only in viable bacteria, as heat-killed bacteria elicited a greatly diminished response. To determine whether Toll-like receptor 2 might be involved, we examined the levels of mRNA encoding this receptor in the localized aggressive periodontitis cells. As seen in Fig. 2, there was a modest increase in Toll-like receptor 2 mRNA levels in normal cells incubated with live *A. actinomycetemcomitans* or interleukin-1β. However, in comparison with unstimulated cells from a normal individual, there was only ≈7% of Toll-like receptor 2 mRNA levels in unstimulated cells from the localized aggressive periodontitis patient. An examination of the coding sequence of the Toll-like receptor 2 cDNA from the localized aggressive periodontitis patient showed no sequence difference when compared with that of normal individuals (data not shown).

Table 2. Expression of Toll-like receptor and associated adapter molecules in gingival epithelial cells

Gene	HGE	THP-1
TLR1	+	+
TLR2	+	+
TLR3	+	+
TLR4	–	+
TLR5	–	+
TLR6	+	+
TLR7	–	+
TLR8	+	+
TLR9	+	+
TLR10	+	+
TRIF	+	+
TRAM	+	+
SARM	+	+
MyD88	+	+
MD2	+	+
TANK	+	+
TOLLIP	+	+
Mal	+	+

Positive expression was determined by visualization on agarose gel electrophoresis after 35 cycles of reverse transcription-polymerase chain reaction. mRNA from THP-1 cells was used as a positive control. MD2, myeloid differential protein-2; MyD88, myeloid differentiation factor 88; Mal, MyD88 adapter-like; SARM, sterile α- and armadillo-motif-containing protein; TANK, TRAF-associated NF-κB Kinase; TOLLIP, Toll-interacting protein; TLR, Toll-like receptor; TRAM, TRIF, TOLL/1L-1R domain-containing adaptor-inducing IFN-beta, TRIF-related adaptor molecule.

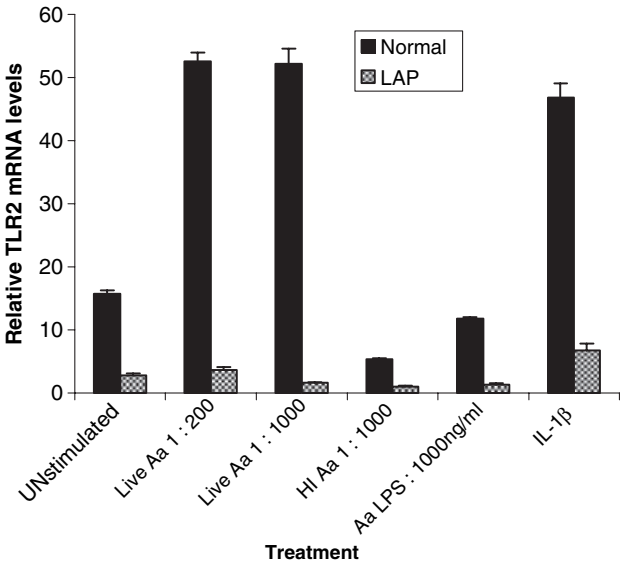

Fig. 2. Expression of Toll-like receptor 2 in human gingival epithelial cells. Human gingival epithelial cells from either normal individuals (representative of one of three, black bars, black bars) or an individual with localized aggressive periodontitis (grey bars) were cultured in the presence of stimuli as for Fig. 1, for 6 h. Culture supernatants were collected, and total mRNA was isolated. Relative levels of Toll-like receptor 2 mRNA were quantified by real-time polymerase chain reaction. Aa, *Aggregatibacter actinomycetemcomitans*; HI, heat inactivated; IL-1β, interleukin-1β; LAP, localized aggressive periodontitis; LPS, lipopolysaccharide.

The expression of Toll-like receptors 1, 2, 3, 6, 8, 9 and 10 suggests that the gingival epithelium is capable of recognizing a wide range of microbe-associated molecular patterns, including those associated with viruses (i.e. Toll-like receptors 3 and 8). However, the absence of Dectin-1 suggests that the gingival epithelium does not participate in glucan-mediated responses to fungal organisms. As all of the known downstream adapter proteins associated with Toll-like receptor-mediated induction of innate immunity are expressed in these cells, the machinery exists for the innate immune responses to these patterns via the Toll-like receptor pathways. This includes negative regulation, as SARM has just been identified as a negative regulator of Toll-like receptor signaling (44). Thus, it is interesting that bacterial challenge of cells from a patient with localized aggressive periodontitis results in little to no induction of human β-defensin 2 or interleukin-8 compared with cells from a healthy individual. This may be a result of the reduced basal levels of Toll-like receptor 2, whereas no apparent Toll-like receptor 2 sequence variation was observed in the Toll-like receptor 2 coding sequence of the localized aggressive periodontitis patient. The reduced mRNA levels may be controlled at the level of transcription by other mediators and may be specific to oral tissues. The reduced response of the localized aggressive periodontitis cells to interleukin-1β (Fig. 1A) supports this idea that other components which regulate the response may be involved. A reduction in the Toll-like receptor 2-mediated recognition of periodontal pathogens, such as *A. actinomycetemcomitans*, could lead to an increased susceptibility to colonization, as has been suggested for observed variability with Toll-like receptor 4 expression and recognition of *P. gingivalis* (38).

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