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NF- κ B p50 subunits predominate in Chronic Periodontitis and in *P. gingivalis* LPS- pulsed Dendritic cells

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Abstract

The pathogenesis of a number of chronic inflammatory diseases has been linked to dysregulated functioning of nuclear transcription factor NF- κ B. In the present study, we examine NF- κ B activation in human oral mucosal tissues of chronic periodontitis (CP) patients. We find through EMSA and DNA binding ELISAs that there are elevated levels of transcriptionally repressive p50 subunits and increased p50:p65 ratio in CP tissues compared to healthy controls. Because *P. gingivalis* has been recognized to be a causal factor in CP, we used *P. gingivalis* LPS for in vitro studies with monocyte-derived dendritic cells (MoDCs). We find that *P. gingivalis* LPS, unlike *E. coli* LPS, induces an increased p50:p65 ratio in MoDCs. Using blocking antibodies, we observed that while both TLR2 and TLR4 are required for MoDC maturation by *P. gingivalis* LPS, only TLR4 signaling is sufficient to induce cytokine secretion. Our results suggest that increased levels of transcriptionally repressive p50 may be characteristic of CP and might be a result of suboptimal NF- κ B activation and dendritic cell maturation by *P. gingivalis*, a bacterium implicated in CP.

Keywords

NF-kappaB; p50- p65; periodontitis; Dendritic cells; *Porphyromonas gingivalis*; LPS

Introduction

In chronic periodontitis (CP) there is a heavy microbial load in oral mucosa; despite this load, there are few overt symptoms of inflammation and CP often goes unnoticed by the patients.¹ Prior studies have shown that the lack of inflammation may be related to endotoxin tolerance in the oral mucosa.² During endotoxin tolerance, repeated or prolonged activation of Toll-like Receptors (TLR) by lipopolysaccharide (LPS) renders the mucosa insensitive to subsequent stimulation.³ This hyposensitivity is evident by decreased production of inflammatory cytokines.

The microbial etiology of CP is complex; however, *Porphyromonas gingivalis*, (*P. gingivalis*), a Gram-negative bacterium, is considered a causal agent.^{1, 4} The LPS of *P. gingivalis* is structurally unique and biologically weak compared to the LPS of *Escherichia coli* (*E. coli*);⁴ however, the underlying reason for these LPS differences and their resulting significance remains unclear. One possibility is that whereas the LPS isolated from *E. coli*

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Conflicts of interest

The authors declare no conflicts of interest.

stimulates TLR4 of inflammatory cells, that of *P. gingivalis* utilizes TLR2 to activate cells.^{1,5}

Different TLRs have been shown to induce distinctive cytokine and maturation profiles in DCs; which can in turn elicit distinct effector responses in T lymphocytes. Studies have shown that NF- κ B transcription factors are important in DC maturation and cytokine production.^{6,7} NF- κ B is a family of dimeric transcription factors which consists of five members: p105 (processed to p50), p100 (processed to p52), p65 also known as Rel-A, Rel-B, and c-Rel.⁷ These subunits form homodimers and heterodimers to produce NF- κ B transcription factors. Interestingly, only three members of the NF- κ B family—p65, RelB and c-Rel—contain a transactivation domain (TAD), which is required for activation and transcription of NF- κ B-dependent genes; in contrast other NF- κ B members—p50 and p52—lack a TAD and thus can act as transcriptional repressors if paired as homodimers. These homodimer complexes have been shown to limit the strength of TLR responses, thereby inhibiting inflammatory cytokine production. In line with this, increased production of p50 homodimers occurs during endotoxin tolerance.³

In the present study, we sought to examine if the LPS of *P. gingivalis* preferentially signals DCs through unique TLRs to trigger immune suppressing p50 NF- κ B homodimers. These findings are correlated with the prevalence of NF- κ B p50 homodimers in gingival tissue from CP patients.

Materials and Methods

Gingival tissue collection and the preparation of cellular, nuclear, and cytoplasmic extracts

The Institutional Review Board at SUNY-Stony Brook approved all protocols involving human subjects. Informed consent was obtained from all subjects before commencement of the study. A total of 28 gingival tissues (14 healthy and 14 inflamed) were included in the present study. Tissues were classified according to the clinical criteria previously defined.⁸ Gingival tissues were flash frozen and stored at -80°C . Whole cell extracts from healthy and diseased (CP) gingival tissues were prepared using TransAM Nuclear extract kit according to manufacturer recommendations.

Functionally active NF- κ B p65 and p50 were measured using ELISA based transcription factor assay kit and following manufacturer's instructions (Active Motif, CA). Functionally active purified recombinant NF- κ B p65 and p50 proteins were used as standards as recommended by manufacturer (Active Motif, CA). The total protein concentration of extracts was measured by Bradford assay (Sigma-Aldrich). For protein assays, bovine gamma globulin IgG was used as standard. The protein extracts were aliquoted and stored at -80°C .

In vitro culture of MoDCs, monocytes, multiparameter flow cytometry analysis

MoDCs and their progenitors monocytes were generated as described.⁸ Briefly, monocytes were isolated from mononuclear fractions of peripheral blood of healthy donors and seeded in the presence of GM-CSF (100 ng/mL) and IL-4 (25ng/mL) at a concentration of $1-2 \times 10^5$ cells/mL for 6–8 days. Cell surface markers of MoDCs were evaluated by four-color immunofluorescence staining with the following mAbs: CD1a- FITC (BioSource International, Camarillo, CA); CD80-PE (BD Biosciences, Mountain View, CA); CD83-PE (Immunotech); CD86-PE (BD PharMingen, San Diego, CA); HLA-DR-PerCP (BD Biosciences); and CD14-APC (Caltag Laboratories, Burlingame, CA). Analysis was performed on a FACSCalibur (BD Biosciences). Marker expression was analyzed as the percentage of positive cells in the relevant population defined by forward and side scatter characteristics.

LPS purification and stimulation of human monocytes and dendritic cells

The methodology for isolation and purification of LPS from *P. gingivalis* 381 and *E. coli* American Type Culture Collection type strain 25922 (ATCC, Manassas, VA) is previously described.⁹ Briefly, whole cell pellets were subjected to hot-phenol water extraction; the aqueous phase was extensively dialysed against distilled water, followed by lyophilization and isopycnic density gradient centrifugation.⁹ In addition, some of the LPS preparations were provided courtesy of T.E. Van Dyke (Boston University, Goldman School of Dental Medicine). For stimulation, human monocytes and MoDCs were incubated with 100 ng/mL preparation of LPS for 1 hr. For blocking studies, cells were incubated with TLR2/TLR4 neutralizing mAb (eBiosciences), or with the specific NF- κ B specific inhibitor PDTC for 30 minutes before stimulation with either LPS.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were electrophoretically separated by SDS-PAGE (8% gel), transferred to a PVDF membrane, probed with anti-human p65/p50 (SantaCruz Biotechnology, CA) primary antibodies. Blots were developed using the ECL advance system (Amersham Biosciences). EMSA was performed using a non-radioactive (biotin-label) gel shift assay kit (Panomics Inc., CA).

For Western blots nuclear and cytoplasmic proteins was electrophoretically separated by SDS-PAGE (8% gel) and transferred to PVDF membrane. This was followed by blocking with 5% blocking agent (Amersham Bioscience –ECL blocking agent) in PBS. For probing, anti human -p65 (sc372) and p50 (sc114) antibodies were used at 1:20,000 ratio, followed by incubation for 1 hour with HRP conjugated rabbit anti- human IgG antibody (Amersham Bioscience) at ~1:20,000 dilution. The proteins were detected using an enhanced chemiluminescence detection system (Amersham Bioscience).

Cytometric Bead Assay for detection of cytokines

MoDC culture supernatants were collected after stimulation with either *P. gingivalis* or *E. coli* LPS for 18 hours. Culture supernatants were analyzed for inflammatory cytokines IL-1 β , TNF- α , and IL-6 by flow cytometry using cytometric bead array (CBA Kit, BD Biosciences). Concentrations of cytokines were calculated based on a standard curve for each cytokine. The CBA software calculates levels in pg/mL.

Results

It was hypothesized that the state of immunotolerance to bacteria present in CP patients is related to differences in the composition of NF- κ B dimers. To compare the NF- κ B dimer composition between CP patients and healthy controls, whole cell lysates of gingival tissues were analyzed by Western blotting for p65 and p50 subunits. Figure 1A demonstrates a semi-quantitative densitometric analysis from CP patients and healthy controls, showing that there was an increase in the level of NF- κ B p65 and to a lesser extent p50 present in tissue from CP patients.

Nuclear extracts were subsequently prepared and the activity of NF- κ B subunits was examined by an ELISA-based method that allows for quantification of transcription factor binding (TransAmTM, Active Motif). Results of this assay showed significant increases in activated nuclear p65 and p50 in diseased tissues, relative to health ($p < 0.05$, Student's t-test) (Figure 1B). While both p65 and p50 are increased in disease compared to health, the results showed that nuclear p50 displayed a greater increase despite relatively small changes in total cellular expression on Western blot analysis (Figure 1A). Therefore, there was a larger rise in the level

of nuclear p50 present in CP tissues compared to healthy controls, and this translated into a two-fold increase in the ratio of activated nuclear p50/p65 in CP tissue (Figure 1C).

One possibility to explain the presence of greater levels of nuclear p50 in CP tissue might be that the formation of p50 homodimers is enhanced. To test if this was the case, EMSAs were performed on nuclear extracts from CP and healthy tissue. Figure 1D demonstrates that while there was an increase in both p65/p50 and p50/p50 complexes, the most dramatic increase occurred for p50 homodimer complexes in CP tissue when compared to healthy controls. Overall, the results from Figure 1 suggest that in CP there is increased p50 homodimer production.

In light of the known role of p50 homodimers as repressors of transcription and mediators of endotoxin tolerance,³ we next sought to determine the influence of increased p50 homodimer complexes on inflammatory cytokine production by MoDCs. It has previously been shown that seropositivity to LPS from *P. gingivalis*,⁹ and infiltration of lamina propria with myeloid DCs,⁸ both correlate with periodontal disease activity. It was therefore hypothesized that *P. gingivalis* LPS may induce attenuated immune responses in DCs and macrophages relative to the LPS of *E. coli*. To test if this was the case, the ELISA-based DNA quantization assays employed in Figure 1 were used to calculate the ratios of nuclear p50/p65 after stimulation with either *P. gingivalis* LPS (10 ng/ml) or *E. coli* LPS (10 ng/ml). The p50/p65 ratio for *P. gingivalis* LPS increased after stimulation; while in contrast the ratio for *E. coli* LPS was significantly lower than controls (Figure 2A). Thus, the p50/p65 ratio resulting from stimulation with *P. gingivalis* LPS was significantly greater than that induced by *E. coli*, suggesting that *P. gingivalis* LPS is a much greater stimulator of nuclear p50-containing NF- κ B complexes.

To determine if these contrasting changes in nuclear p50/p65 ratios translated into alterations in immune function, MoDC cultures were assayed for the production of inflammatory cytokines after *P. gingivalis* or *E. coli* LPS stimulation. Figure 2B shows that supernatants collected after 18 hours of LPS stimulation demonstrate relatively low levels of production of the inflammatory cytokines IL-1 β , TNF- α , and IL-6 following *P. gingivalis* LPS (100ng/ml) compared to *E. coli* LPS (analyzed by cytometric bead array kit; BD Biosciences). Overall, Figure 2 demonstrates that cells stimulated with *P. gingivalis* LPS, which causes higher p50-65 ratios relative to *E. coli*, secrete lower levels of TNF- α , IL-1 β , and IL-6.

A mechanism was next sought to explain how cells stimulated with *P. gingivalis* LPS induce less p65 activation and produce less pro-inflammatory cytokines compared to *E. coli*. As mentioned, LPS isolated from *E. coli* stimulates TLR4 whereas that of *P. gingivalis* utilizes TLR2 and/or TLR4 to activate cells.^{1, 5} The role of TLRs in the observed differences in NF- κ B activation and inflammatory cytokine secretion was examined using TLR2 and TLR4 neutralizing antibodies. Surprisingly, secretion of the inflammatory cytokines TNF- α , IL-1 β , and IL-6 was inhibited only by anti-TLR4 antibodies in response to *P. gingivalis* LPS and not by anti-TLR2 antibodies (Figure 3A). However, in contrast to cytokine secretion, examination of TLR2 and TLR4 neutralizing antibodies on DC maturation demonstrated that either antibody was able to inhibit expression of the maturation marker CD83 (Figure 3B). Overall these results suggest that TLR4 is the main receptor mediating inflammatory cytokine production, but that dendritic cell maturation induced by *P. gingivalis* LPS requires both TLR4 and TLR2.

A link between NF- κ B activation and TLR signaling after *P. gingivalis* LPS stimulation was subsequently examined. MoDCs were pretreated with or without the NF- κ B inhibitor pyrrolidine dithiocarbamate (PDTC), and the ability of *P. gingivalis* or *E. coli* LPS to induce DC maturation was then analyzed. The results demonstrated that *P. gingivalis* LPS is a suboptimal inducer of DC maturation, as evidenced by smaller increases in maturation marker

CD83 (~17% CD83⁺ cells) induced by *P. gingivalis* LPS compared to (~36% CD83⁺ cells) for *E. coli* (Figure 4). However, DC maturation induced by either *P. gingivalis* or *E. coli* LPS required NF- κ B, as both were inhibited by PDTTC. Overall the results from Figure 4 confirm that NF- κ B was critical in DC maturation induced by LPS, but suggest that *P. gingivalis* LPS is a relatively weak stimulator of immune activation.

Discussion

The present study was conducted to examine the underlying mechanisms responsible for the state of immunotolerance in chronic periodontal disease (CP) patients. Our results demonstrate a predominance of p50 NF- κ B homodimers in the human gingiva from CP patients. Because p50 homodimers are considered immunosuppressive, these observations provide a mechanistic link to the previous observations of greater immunotolerance in CP patients. Prior work on NF- κ B has shown that increased production of p50 subunits drives increased formation of p50 homodimers.³ However, the current results indicate that even with mild changes in total p50 levels, there are dramatic differences in the amount of nuclear p50 homodimer complexes present in gingival tissues from CP patients.

A definitive explanation for the observations regarding preferential p50 activation in periodontal disease tissue is still lacking. However, it is hypothesized that an overabundance of the weakly immunogenic bacteria *P. gingivalis* may play a causal role. Here, it was demonstrated that activation of DCs by *P. gingivalis* LPS results in increases in p50/p65 ratios, with dramatically higher ratios compared to *E. coli* LPS. The increased p50 activation induced by *P. gingivalis* compared to *E. coli* translated into relatively decreased amounts of inflammatory cytokine production.

Interestingly, despite prior reports demonstrating that *P. gingivalis* LPS utilizes TLR2 to mediate inflammatory cytokine production, it was found that TLR4 was the receptor responsible for cytokine production. However, both TLR4 and TLR2 were found to be required for DC maturation induced by *P. gingivalis* LPS. The current findings are consistent with prior studies that indicated *P. gingivalis* LPS was 10-fold less potent in inducing an inflammatory reaction compared to *E. coli* LPS.¹⁰ Overall, the data suggest that there is suboptimal maturation of MoDCs and weak inflammatory cytokine production when *P. gingivalis* LPS is the primary inflammatory inducer in gingival tissue. Hindered maturation and cytokine production have previously been shown to cause weak adaptive immune responses and induce immune tolerance.¹¹

Tolerance has been demonstrated in monocytes, macrophages, T cells and tumor cells and has been reported to involve the upregulation of p50 mRNA and increased translocation of p50-homodimers into the nucleus.¹² It has previously been shown that the oral mucosa is in a state of endotoxin tolerance, likely due the repeated exposure to the oral biofilm.² Therefore, the current findings regarding upregulated p50 homodimer production in CP mesh well with existing literature on p50 homodimers in endotoxin tolerance.^{2, 3}

In summary, an imbalance in transcriptionally repressive and active subunits of NF- κ B, leading to ineffective dendritic maturation and inflammatory cytokine production, may be major contributing factors in the pathogenesis of CP. Furthermore, these findings suggest the possibility of selectively targeting the NF- κ B p50 to break immunosuppression and resolve chronic inflammation in periodontal disease.

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Figure 1. Increased nuclear NF-κB p50 homodimers in chronic periodontal disease tissue
(A) Densitometric analysis (in arbitrary units) of levels of NF-κB subunits p50 and p65 in whole cell extracts of gingival tissues from healthy and diseased (CP) patients; samples were analyzed by Western blotting and scanned for semiquantization. **(B)** ELISA experiments demonstrating the amounts of nuclear p50 or p65 bound (in ng/mL) to DNA in CP and healthy tissue (TransAM ELISA transcription factor assay). **(C)** Calculated ratio of p50/p65 levels in cell extracts of healthy and diseased tissues; ratios are calculated from values in 1B and expressed in arbitrary units. **(D)** Results of EMSA experiments showing p50-p65 heterodimers and p50 homodimers. Shown are the chemiluminescent bands of representative healthy and inflamed gingival tissue detected by Panaomics gel shift EMSA kit. Lane4: Healthy gingival tissue. Lane5: Inflamed (CP) gingival tissue. * Significant differences in relative levels in healthy versus diseased assessed by Students' T-test at $p < 0.05$.

Figure 2. Induction of nuclear p65 and resulting inflammatory cytokine production by *P. gingivalis* LPS is attenuated relative to *E. coli* LPS in cultured MoDCs

(A) An ELISA-based quantization of activated nuclear p65 and p50 NF-κB subunits was performed (as in Figure 1B) after stimulation with either *P. gingivalis* LPS (10 ng/mL) or *E. coli* LPS (10 ng/mL) for 1 hour. The p50/p65 ratio in nuclear extracts of MoDC is shown in arbitrary units. (B) *P. gingivalis* LPS is a weaker stimulator of inflammatory cytokines (TNF-α, IL-1β, and IL-6) in dendritic cells compared to *E. coli* LPS. MoDC were treated with 100 ng/mL of LPS and culture supernatants were collected after 18 hrs of incubation. Cytokines were quantified by cytometric bead assay (CBA Kit, BD Biosciences, San Diego, CA). Based on a standard curve for each cytokine, BD software calculates levels in pg/ml. * p<0.05; ** p<0.01.

Figure 3. *P. gingivalis* LPS signals through TLR4 to induce inflammatory cytokine production and through both TLR2 and TLR4 to induce dendritic cell maturation

(A) Inhibition of pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 by MoDC after blocking with TLR2 and TLR4 functional antibodies. Supernatants from dendritic cells stimulated with either control (C) or *P. gingivalis* LPS (100ng/mL) (labeled Pg) were assayed as in Figure 2B. (B) Cell surface markers of DC were evaluated by four-color immunofluorescence staining with the following mAb: CD80-PE (Becton Dickinson), CD83-PE (shown) (Immunotech), CD86PE (Pharmingen), HLA-DR-PerCP. Flow cytometric analysis demonstrates decreased MoDC maturation following stimulation with *P. gingivalis* LPS if either neutralizing antibodies to either TLR4 (T4) or TLR2 (T2) are added. * $p < 0.05$.

Figure 4. Prior treatment of MoDC with PDTC (1mM) inhibits upregulation of HLADR, CD80, CD83 and CD86 in *P. gingivalis* and *E. coli* LPS treated cells

MoDC were pretreated with 1 mM PDTC for 30 min prior to treatment with either *P. gingivalis* LPS 100 ng/mL (A) or *E. coli* LPS 100 ng/mL (B). Untreated cells served as controls. Cell surface markers of MoDC were evaluated by four-color immunofluorescence staining as described in Figure 3B.