Porphyromonas gingivalis is an etiologic pathogen of periodontitis that is one of the most common inflammatory diseases. Recently, we found that P. gingivalis LPS activated the transcription factor nuclear factor-κB (NF-κB) through the IκB kinase complex (IKK). NF-κB is a transcription factor that controls inflammation and host responses. In this study, we examined the role of IKK/NF-κB in P. gingivalis LPS-induced gene expression on a genome-wide basis using a combination of microarray and biochemical approaches. A total of 88 early response genes were found to be induced by P. gingivalis LPS in a human THP.1 monocytic cell line. Interestingly, the induction of most of these genes was abolished or attenuated under the inactivation of IKK/NF-κB. Among those IKK/NF-κB-dependent genes, 20 genes were NF-κB-inducible genes reported previously, and 59 genes represented putative novel NF-κB target genes. Using transcription factor binding analysis, we found that most of these putative NF-κB target genes contained one or multiple NF-κB-binding sites. Also, some transcription factor-binding motifs were overrepresented in the promoter of both known and putative NF-κB-dependent genes, indicating that these genes may be regulated in a similar fashion. Furthermore, we found that several transcription factors associated with metabolic and inflammatory responses, including nuclear receptors, activator of protein-1, and early growth responses, were involved in the induction induced by P. gingivalis LPS through IKK/NF-κB, indicating that IKK/NF-κB may utilize these transcription factors to mediate secondary responses. Taken together, our results demonstrate that IKK/NF-κB signaling plays a dominant role in P. gingivalis LPS-induced early response gene expression, suggesting that IKK/NF-κB is a therapeutic target for periodontitis.

Periodontitis is a chronic Gram-negative anaerobic bacterial infection leading to inflammation and immune response of the gingivae and destruction of periodontal tissues (1, 2). Porphyromonas gingivalis is a Gram-negative bacterium that is recognized as one of the etiologic agents of periodontitis. Periodontitis impinges on a large proportion of the population and is characterized by alveolar bone resorption, ultimately resulting in tooth loss. LPS, a major component of the outer membrane of these bacteria, has been found in infected periodontal tissues and root surfaces (1, 2). LPS isolated from P. gingivalis has been shown to activate monocytes/macrophages and gingival fibroblasts (3, 4). P. gingivalis LPS induced monocytes/macrophages to produce bone-resorptive cytokines, including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and IL-6, chemokines, cell adhesion molecules, and matrix metalloproteinases (5–7). Experimental periodontitis could be induced by delivering a P. gingivalis LPS solution to the oral cavity in mice. The local injection of P. gingivalis LPS has been found to stimulate the production of inflammatory cytokines and bone resorption (6, 8, 9).

Toll-like receptors have been found to play a critical role in transducing LPS signaling and host responses (2, 9, 10–12). Interestingly, whereas LPS isolated from Gram-negative bacteria usually utilizes TLR4 to stimulate intracellular signaling pathways, P. gingivalis LPS has been shown to utilize TLR-4 and/or TLR-2 to facilitate cell activation (2, 9). It is well known that the LPS/TLR interaction activates the transcription factor nuclear factor κB (NF-κB), which turns on transcription of inflammatory mediators (2, 13–16). NF-κB was originally identified as a transcription activator that bound to a specific DNA motif (GGGAGCTCC) in the intronic enhancer of the immunoglobulin κ light chain gene in B lymphocytes. Subsequently, it was found that NF-κB was a ubiquitous cellular factor that was retained in the cytoplasm by inhibitory proteins IκBs. NF-κB consists of homo- and heterodimeric complexes of the Rel family proteins, including p50, p52, p65/RelA, c-Rel, and RelB. In mammalian cells, the most widely distributed κB-binding activity is a heterodimer of p50 and p65/RelA proteins, in which the p65/RelA subunit has potent transactivation activity (17–19). The IKK complex is mainly composed of two catalytic subunits, IKKα and IKKB, and IKKy (also known as NF-κB essential modulator), a scaffold molecule without catalytic activity (17–20).
Gene depletion studies demonstrate that IKKβ, but not IKKα, plays an essential role in NF-κB activation mediated by LPS and proinflammatory cytokines (18). Although IKKγ lacks catalytic function, it plays a critical role in assembling the IKK complex. Gene knock-out experiments found that IKKγ was essential for IKK activation induced by proinflammatory cytokines and LPS (18). IKKγ contains several predicted functional domains, including two coiled-coil regions that are separated by α helices, a leucine zipper motif, and a putative zinc finger domain at the C terminus. The C terminus of IKKγ has been found to play a regulatory role in IKK activation (3, 15, 21–25). Recently, we examined the zinc finger domain of IKKγ and found that C417R mutation in IKKγ zinc finger domain impaired LPS- or TNF-mediated IKK activation and NF-κB transcription. The overexpression of IKKγC417R inhibited the expression of IKK/NF-κB-dependent genes, such as IL-8, in a dominant negative fashion (15, 25). To further understand bacterial infection-mediated host response and inflammation, we examined P. gingivalis LPS-induced genes in human monocytes on a genomewide basis. Using the dominant-negative approach to block IKK/NF-κB signaling, we compare gene expression patterns induced by P. gingivalis LPS in monocytes. We found that most of the early response genes induced by P. gingivalis LPS were dependent on IKK/NF-κB, suggesting that IKK/NF-κB is a therapeutic target for periodontitis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Retroviral Infection**—Human THP.1 monocytes and murine RAW264.7 macrophages (ATCC) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin G, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. LPS from P. gingivalis 7346 was provided by Dr. Ronald Arnold (University of North Carolina, Chapel Hill, NC). A specific IKKβ inhibitor, IKKVI, was purchased from Calbiochem. For isolation and culture of primary mouse macrophages from bone marrow, C57BL/6j mice were sacrificed and sterilized with 70% alcohol. Under an aseptic condition, femurs were collected and cut with scissors. The bone marrow was flushed with medium using a syringe. Bone marrow cells were transferred to 100-mm tissue culture plates. Nonadherent cells were removed, and adherent cells were maintained for subsequent experiments.

To generate human THP.1 cells stably expressing the dominant negative mutant of IKKγ (IKKγ-DN), we utilized retrovirus-mediated transduction, as reported previously (14, 15, 26). Briefly, HEK293T cells were transfected with the retroviral vectors encoding IKKγ-DN or control empty vector using the calcium phosphate method. 24 h after transfection, cells were treated with 0.01 M sodium butyrate (Sigma) overnight to boost retrovirus production. Retrovirus-containing supernatants were collected 48 h later, filtered, and stored at −70 °C. Human THP.1 monocytes were infected with retroviruses in the presence of 6 μg/ml Polybrene (Sigma). 48 h after infection, cells were treated with G418 (600 μg/ml) for 2 weeks. The resistant cells were pooled, and cells expressing IKKγ-DN were confirmed by Western blot analysis.

**Western Blot Analysis**—Human THP.1 monocytes were treated with P. gingivalis LPS (500 ng/ml) for different time periods. Cells were harvested and washed once with cold PBS. Cells were pelleted and lysed with cell lysis buffer containing 1% Nonidet P-40, 5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 100 mM sodium orthovanadate, and 1:100 protease inhibitor mixtures (Sigma). The protein concentration was measured according to the manufacturer’s protocol (Bio-Rad). 50-μg aliquots of whole cell lysates were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad) using a semidry gel transfer cell. The blots were blocked with 5% nonfat milk overnight at 4 °C and incubated with the primary antibodies. The immunocomplexes were visualized with horseradish peroxidase-coupled goat anti-rabbit or anti-mouse IgG (Promega) using the SuperSignal reagents (Pierce), as described previously (26, 27). The primary antibodies were from the following sources: anti-IκBα, anti-ATF-3, anti-c-Fos, and anti-Fra-1 polyclonal antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phospho-IκBα (serine 32) antibodies from Cell Signaling; and anti-α-tubulin from Sigma.

**Affymetrix Human Microarray**—Cells were treated with P. gingivalis LPS for 1 h. After treatment, cells were harvested, and total RNAs were extracted with Trizol reagents in accordance with the manufacturer’s instruction (Invitrogen). To eliminate contaminated genomic DNA, total RNAs were cleaned with an RNeasy kit (Qiagen). 10-μg aliquots of total RNA from each sample were utilized for microarray analysis as described previously (28). Briefly, RNAs were transcribed to double-stranded cDNA using SuperScript II reverse transcriptase (Invitrogen) with an oligo(dT) primer that has a T7 RNA polymerase site on the 5'-end. Then, the cDNAs were used in an in vitro transcription reaction in the presence of biotin-modified ribonucleotides to generate single-stranded RNAs. The biotin-labeled RNAs were fragmented and hybridized with an Affymetrix (Santa Clara, CA) human U133A gene chip at 45 °C for 16 h in a mix that contained 10 μg of fragmented RNA, 6× SSPE, 0.005% Triton X-100, and 100 μg/ml herring sperm DNA in a total volume of 200 μl. Labeled bacterial RNAs were spiked into the hybridization mix for an internal standard and normalization. Chips were washed and stained with Streptavidin R-phycocerythrin (Molecular Probes, Inc., Eugene, OR). The arrays were scanned with the GeneArray scanner (Affymetrix). Signal intensity was calculated using the one-step Tukey’s biweight estimate (28). Affymetrix® Microarray Suite 5.0 was used for data analysis. Scaling was performed to facilitate the comparison of multiple arrays. We chose a target intensity of 500 in scaling. The Microarray Suite 5.0 detection algorithm generates a detection p value from probe pair intensities and assigns a present (p), marginal (M), or absent (A) call to each probe set. We applied the default cut-off value of 0.04 (i.e. a p value under 0.04 indicates a P call). The Microarray Suite change algorithm utilized Wilcoxon’s signed rank test to compare each probe set on the experiment array with its counterpart on the base-line array, and a change p value was calculated. We assigned increase (I) (p < 0.0025), decrease (D) (p > 0.9975), or no change call (NC) (p between 0.0025 and 0.9975) to each probe set. Significant increase was assigned to a probe set if it had a
detection call of P on experiment array, a change call of I, and a -fold change of at least 2. In the same way, significant decrease was assigned to a probe set if it had a detection of P on base-line array, a change call of D, and a -fold change of 0.5 or lower.

**Gene Function Annotation**—Gene Ontology (GO) terms for genes were obtained from the hgu74av2 and mgu74av2 package of bioconductor (29) (available on the World Wide Web at www.bioconductor.org/packages/data/annotation/stable/src/contrib/html/index.html). We used those packages to build an association file as an input for TermFinder (available on the World Wide Web at search.cpan.org/dist/GO TermFinder/) (30), which calculates a p value for the overexpression of specific GO terms in gene sets using a hypergeometric distribution, and output overrepresented GO terms.

Gene Set Enrichment Analysis (38) was performed using the stand alone javaGSEA package (available on the World Wide Web at www.broad.mit.edu/gsea/software/software_index.html) and the “s2.hgu133agmt” functional gene sets downloaded from the same site. Gene sets achieving a nominal p value of less than 0.05 are defined as "enriched."

Transcription factor binding site analysis was performed by obtaining sequences from the ENSEMBL data base (available on the World Wide Web at www.ensembl.org/index.html) (32) and scanning the genomic sequence regions spanning 3 kb around each transcription start site using the TRANSFAC® MATCH program (23, 34, 35). Default weight matrix score cut-offs were used.

**Quantitative Real Time PCR**—Total RNAs were isolated using Trizol reagent. 2-μg aliquots of total RNAs from each sample were subjected to reverse transcription using a Superscript first strand cDNA synthesis kit (Invitrogen) according to the manufacturer’s protocol. Quantitative real time PCR was performed on an iCycler (Bio-Rad) using SYBR Green PCR master mixture (Applied Biosystems) according to the manufacturer’s instructions. The human primers used for THP-1...
cells were as follows: egr-1, 5'-TTGGATCTTCTCTCCA-CTC-3' (forward) and 5'-GGTGCAGCGAGTTCTT-3' (reverse); egr-2, 5'-GAGTGGTGGTCTCCAGGTGTT-3' (forward) and 5'-CACCAGGTAGATGTTGTCAG-3' (reverse); egr-3, 5'-CCACCACTTCTCTTAC-3' (forward) and 5'-AAAGCCAACATTTCTCTGAATA-3' (reverse); pnrC1, 5'-TGCCACTAACCAGATCC-3' (forward) and 5'-GCTGGATGGTGGGA-3' (reverse); nr4a1, 5'-AGAAGAG-CTTGGAGTGGTGGA-3' (forward) and 5'-TGTTGAGGAGGGTAGGAAG-3' (reverse); nr4a2, 5'-GACCCAAGAT-ATTGCCTCT-3' (forward) and 5'-GCCTTGGCCTTCCTTCT-3' (reverse). The mouse primers used for RAW 264.7 were as follows: egr-1, 5'-AACCTATGAGGACCTGAC-3' (forward) and 5'-GTGCTGGTGCCTGAGATACT-3' (reverse); egr-2, 5'-CAGACTCAGCCTGACTGAG-3' (forward) and 5'-GAATGCTGAAGGATCTGGT-3' (reverse); egr-3, 5'-CGTCACTTCTAGACCCACA-3' (forward) and 5'-GGGTGGTTCAAAGGAATTACC-3' (reverse); pnrC1, 5'-CTCTCAAGGTGCAGACCTA-3' (forward) and 5'-CTCAC-TGCTGATCCCGTCC-3' (reverse); nr4a1, 5'-CAGCTTG-GGTGTTGATGTT-3' (forward) and 5'-TCAGTGATGAGGAGGACAGAGC-3' (reverse); nr4a2, 5'-ATCTCCTGACGGGCTCTATG-3' (forward) and 5'-TGGTGGAGCTCTAGTCT-3' (reverse). The cyclic parameters were as follows: 95 °C for 15 min and amplification at 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s for 40 cycles. Melting curves were analyzed to control for specificity of PCRs. The cumulative fluorescence for each amplicon was normalized to that seen with 18 S RNA control for specificity of PCRs. The cumulative fluorescence for each amplicon was normalized to that seen with 18 S RNA control for specificity of PCRs. The cumulative fluorescence for each amplicon was normalized to input.

**RESULTS**

**Inhibition of P. gingivalis LPS-induced IKK Activation by Overexpression of IKKγ Dominant Negative Mutant (IKKγ-DN)**

Previously, we have found that the overexpression of IKKγC417R could act as a dominant negative mutant to inhibit TNF- and LPS-mediated IKK activation and NF-κB transcripion in human THP.1 monocytic cells (15). However, human THP.1 cells expressing IKKγ-DN were not very stable. After prolonged passages, we found that the level of IKKγ-DN was significantly reduced and was not sufficient to inhibit the IKK/NF-κB signaling pathway. Therefore, to globally examine IKK/
NF-κB-dependent genes, we prepared fresh THP.1 cells expressing IKK−DN. The pooled clones from retrovirus-mediated infection were utilized in order to minimize artifacts due to clonal variation. As shown in Fig. 1A, Western blot analysis detected IKKγ-DN in THP.1 cells infected with retroviruses expressing HA-IKKγ-DN but not control empty vector. To confirm that IKKγ-DN functionally suppressed IKK activation, both THP.1 cells expressing IKKγ-DN (THP.1/IKKγ-DN) and control cells (THP.1/V) were treated with P. gingivalis LPS. As shown in Fig. 1, B and C, although P. gingivalis LPS rapidly induced the phosphorylation and degradation of IκBα by activating IKK activities in THP.1/V cells, the phosphorylation and degradation of IκBα was inhibited in THP.1/IKKγ-DN cells. Also, P. gingivalis LPS-induced NF-κB transcription was inhibited in THP.1/IKKγ-DN cells using a κB-dependent luciferase reporter assay (data not shown).

### TABLE 2

| Gene Entrez gene ID | Change (THP.1/V cells) |
|---------------------|------------------------|
| Aminolevulinate, δ-synthase 1 211 | 2 | 0.00002 |
| BCL2-related protein A1alpha 597 | 4 | 0.000068 |
| CD24 antigen 934 | 2.1 | 0.001336 |
| CD44 antigen 960 | 4.9 | 0.00002 |
| Kruppel-like factor 6 1316 | 2.6 | 0.000346 |
| Connective tissue growth factor 1490 | 9.2 | 0.000078 |
| Down syndrome critical region gene 1 1827 | 2.5 | 0.000232 |
| Dual specificity phosphatase 2 1844 | 19.7 | 0.00002 |
| Epstein-Barr virus-induced gene 2 (lymphocyte-specific G protein-coupled receptor) 1880 | 2 | 0.000241 |
| Early growth response 1 1958 | 11.3 | 0.00002 |
| Early growth response 2 1959 | 39.4 | 0.000167 |
| Early growth response 3 1960 | 24.3 | 0.000023 |
| Early growth response 4 1961 | 21.1 | 0.00002 |
| Coagulation factor III (thromboplastin, tissue factor) α 2152 | 2.1 | 0.000346 |
| c-fos 2353 | 2.1 | 0.00002 |
| GTP-binding protein 2669 | 2.5 | 0.000438 |
| Helicase, lymphoid-specific 3070 | 2.5 | 0.000035 |
| Nuclear receptor subfamily 4, group A, member 1 3164 | 6.1 | 0.00004 |
| Interleukin 23, α-subunit (CD123) 3383 | 3.2 | 0.00002 |
| Intercellular adhesion molecule 1 (CD54) α 3552 | 6.1 | 0.000249 |
| Interleukin-1, α 3593 | 2.8 | 0.000473 |
| Chemokine (CXC motif) ligand 10 α 3627 | 2.1 | 0.00006 |
| c-Jun 3725 | 16 | 0.00002 |
| JunB 3726 | 4.9 | 0.00002 |
| mL1 (BCL2-related) 4170 | 2 | 0.000078 |
| Membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3) 4356 | 2.6 | 0.000346 |
| Growth arrest and DNA damage-inducible, β 4616 | 2.3 | 0.000966 |
| Nuclear factor (erythroid-derived 2)-like 2 4780 | 2.1 | 0.00006 |
| Nuclear factor, interleukin-3-regulated 3627 | 2.1 | 0.00006 |
| Nuclear receptor subfamily 4, group A, member 2 4783 | 3.2 | 0.000147 |
| Pleiomorphic adenoma gene-like 2 4929 | 12.1 | 0.00027 |
| Pleckstrin 5326 | 2 | 0.00002 |
| Phospholipase A2, Group IIA 5341 | 3.7 | 0.00002 |
| Phospholipase A2, Group IVA 5366 | 2.8 | 0.00003 |
| Mitogen-activated protein kinase kinase 3 5606 | 2 | 0.000966 |
| Prostaglandin E receptor 4 5734 | 3.2 | 0.00002 |
| Pentraxin-related gene, rapidly induced by IL-1βα 5806 | 6.1 | 0.00002 |
| Regulator of G-protein signaling 16 6004 | 2.1 | 0.00002 |
| Syndecan-4 6385 | 2 | 0.000046 |
| Superoxide dismutase 2, mitochondrial β 6648 | 2.5 | 0.00002 |
| Tumor necrosis factor, α-induced protein 2 7127 | 3 | 0.00002 |
| Twist 7291 | 2.5 | 0.00002 |
| X-box-binding protein 1 7494 | 2.4 | 0.00003 |
| Zinc finger protein 36, C3H type 7538 | 8 | 0.000023 |
| CD83 antigen 9308 | 7 | 0.00002 |
| Immediate early response 2 9592 | 7 | 0.00002 |
| DEAD (Asp-Glu-Ala-Asp) box polypeptide 17 10,521 | 2.1 | 0.000101 |
| EH domain-containing 1 10,938 | 6.1 | 0.000774 |
| Proline-rich nuclear receptor coactivator 1 10,957 | 3.7 | 0.000046 |
| DnaJ (Hsp40) homolog, subfamily B, member 4 11,080 | 9.8 | 0.000232 |
| Dmx-like 2 23,312 | 2.8 | 0.00002 |
| Protein phosphatase 1, regulatory subunit 15A 23,645 | 9.8 | 0.00002 |
| Immediate early response 5 51,278 | 2.8 | 0.00002 |
| Interleukin 23, α subunit p19 51,561 | 2.5 | 0.00006 |
| Hypothetical protein FLJ10038 55,056 | 5.3 | 0.001486 |
| Zink finger and BTB domain-containing 10 65,986 | 4.6 | 0.000552 |
| Hypothetical protein MGC5618 79,099 | 6.1 | 0.00002 |
| Pleckstrin homology domain containing, family F (with FYVE domain) member 2 79,666 | 2.5 | 0.000552 |
| Ischemia/reperfusion-inducible protein 79,693 | 2 | 0.00006 |
| Hypothetical protein FLJ121986 79,974 | 2.1 | 0.000355 |
| KIAA1659 protein 85,373 | 2.1 | 0.000132 |
| Trophoblast-derived noncoding RNA 283,131 | 3.2 | 0.000241 |

*NF-κB target genes reported previously.*
**TABLE 3**

| GO term                              | p value  | Number of genes |
|--------------------------------------|----------|-----------------|
| Response to stress                   | 1.31E-17 | 31              |
| Immune response                      | 6.81E-15 | 27              |
| Response to biotic stimulus          | 1.30E-14 | 30              |
| Inflammatory response                | 2.96E-14 | 16              |
| Chemotaxis                           | 1.42E-08 | 10              |
| Antimicrobial humoral response       | 2.16E-04 | 7               |
| Apoptosis                            | 1.91E-03 | 12              |
| Programmed cell death                | 2.08E-03 | 11              |

* p values were calculated by GO::TermFinder based on hypergeometric distribution. Significantly overrepresented (p < 0.05) GO terms are listed.

**TABLE 4**

| Enriched gene sets                  | Description                                        | p value |
|-------------------------------------|----------------------------------------------------|---------|
| Cil14Pathway                        | Co-stimulatory signal during T-cell activation     | 0       |
| INSULIN_2F_DOWN                     | Down-regulation during insulin infusion             | 0.003   |
| MAP00500_Prostaglandin_and_leukotriene_metabolism | Prostaglandin and leukotriene metabolism           | 0.0004  |
| inflamPathway                       | Cytokines and inflammatory response                | 0.007   |
| MAP00100_Androgen_and_estrogen_metabolism | Androgen and estrogen metabolism                   | 0.009   |
| SA_MMP_Cytokine_CONNECTION          | Activation of matrix metalloproteinases by cytokines| 0.013   |
| Eicosanoid_Synthesis                | Eicosanoid synthesis                               | 0.018   |
| cell_surface_receptor_linked_signal_transduction | Signal transduction                              | 0.021   |
| MAP00230_Purine_metabolism          | Purine metabolism                                  | 0.027   |
| GPCRs_Class_C_Metabotropic_glutamate_pheromone | GPCR related to the metabotropic glutamate receptors| 0.03    |
| dcPathway                           | Dendritic cells in regulating TH1 and TH2 development | 0.031   |
| Tob1Pathway                         | Role of Tob in T-cell activation                   | 0.032   |
| ST_JNK_MAPK_Pathway                 | MAP kinases                                        | 0.033   |
| Cell_Cycle                          | Cell cycle                                         | 0.037   |
| erythPathway                        | Erythrocyte differentiation in bone marrow         | 0.039   |
| cytokinePathway                     | Cytokine network                                   | 0.043   |
| il17Pathway                         | Activated T cells secrete IL-17, which stimulates fibroblasts and other cells to secrete inflammatory and hematopoietic cytokines | 0.048   |

* p values were nominal and were calculated by gene set enrichment analysis software based on permutation of the genes. Significantly enriched gene sets (p < 0.05) are listed.
suggest that NF-κB may utilize these transcription factors to further modulate or amplify inflammatory responses.

To confirm these results, we first performed Western blot analysis to examine whether AP-1 family proteins were induced by LPS through IKK/NF-κB pathway. We utilized a specific IKKβ inhibitor, IKKVI. As shown in Fig. 3A, IKKVI potently inhibited the phosphorylation and degradation of 1xβA induced by P. gingivalis LPS in THP.1 cells. Consistent with our microarray data, we found that LPS induced the expression of c-Fos, Fra-1, and ATF-3 expression in THP.1 cells. The pretreatment of the IKKVI inhibitor abolished their induction (Fig. 3B). In RAW264.7 cells, we found that the induction of ATF-3 and Fra-1 by P. gingivalis LPS was also dependent on the IKK/NF-κB signaling pathway. Interestingly, c-Fos was constitutively expressed in RAW264.7 cells. Although P. gingivalis LPS could not induce its expression, the IKKVI inhibitor abolished its basal expression.

We utilized real-time PCR to confirm the expression of egr-1, -2, and -3, nr4a1, nr4a2, and pnrc1 induced by P. gingivalis LPS. As shown in Fig. 4, P. gingivalis LPS significantly induced their expression in THP.1 cells, and pretreatment of the IKKVI inhibitors suppressed their induction, validating our microarray results. Except for nr4a2, we also confirmed that P. gingivalis LPS induced their expression in RAW264.7 cells through IKK/NF-κB signaling (Fig. 5).

We also further examined whether P. gingivalis LPS induced these genes in primary macrophages through IKK/NF-κB signaling. Primary macrophages were prepared from mouse bone marrow. As shown in Fig. 6, real-time PCR revealed that the expression of egr-1, -2, and -3 and nr4a1 was also induced by P. gingivalis LPS and that the addition of IKKVI inhibitor suppressed their expression. However, probably due to cell type, we could not detect that P. gingivalis LPS induced nr4a2 and pnrc1 in primary bone marrow macrophages.

NF-κB Directly Binds to the egr-2 Promoter—It is known that NF-κB usually stimulates gene transcription by binding to a specific DNA motif. 22 known NF-κB target genes that we identified as induced by P. gingivalis LPS contain NF-κB-binding sites. Since 57 genes were putative novel NF-κB-dependent genes, we further analyzed the 3-kb promoter region upstream of the transcription start site of these genes using transcription factor binding site analysis. The sequences of
IKK in LPS-induced Gene Expression

Figure 6. Examination of P. gingivalis LPS-induced genes by real-time PCR in primary macrophages.

Figure 7. P. gingivalis LPS induces NF-κB bound to the egr-2 promoter.

The detection of putative NF-κB-binding sites in the egr-2 promoter by TRANSFAC® MATCH. A, the detection of putative NF-κB-binding sites in the egr-2 promoter by TRANSFAC® MATCH. B, NF-κB directly bound to the egr-2 promoter upon P. gingivalis LPS stimulation in THP-1 cells. C, the induction of egr-2 by P. gingivalis LPS was dependent on IKK. D, the induction of nr4a1 by P. gingivalis LPS was dependent on IKK.

DISCUSSION

Our microarray analysis demonstrated that P. gingivalis LPS induced a broad range of immune and inflammatory response genes in monocytes. Importantly, we found that IKK/NF-κB signaling played a dominant role in P. gingivalis LPS-induced early gene responses in monocytes by activating IKK, since the inhibition of IKK activities abolished or attenuated expression of most of the genes induced by P. gingivalis LPS. Our results suggest that IKK may be a critical target for inhibiting periodontal inflammation induced by oral pathogens, such as P. gingivalis LPS.

During the early stage of periodontal infection, it is known that bacteria or bacterial products induce monocytes/macrophages to secrete proinflammatory mediators and chemokines. Subsequently, these cytokines and chemokines recruit neutrophils and lymphocytes to infected sites (1, 4, 9, 41, 42). Consistently, we found that these proinflammatory cytokines and chemokines were induced by P. gingivalis LPS. Our results highlight the critical role of the IKK/NF-κB signaling pathway suppressed or attenuated expression of these genes in monocytes. Our results suggest that IKK may be a critical target for inhibiting periodontal inflammation induced by oral pathogens, such as P. gingivalis LPS.

The induction of antiapoptotic genes by P. gingivalis LPS was dependent on IKK, since the inhibition of IKK activities abolished or attenuated expression of these genes in monocytes/macrophages spontaneously undergo apoptosis in inflamed sites, the life span of monocytes/macrophages in periodontitis may be associated with the resolution of inflammation (43). The induction of antiapoptotic genes by P. gingivalis LPS may promote monocyte/macrophage survival, which contributes to the prolonged inflammation in chronic periodontitis (43).

Interestingly, we have identified a group of transcription factors, including AP-1 family proteins, nuclear receptor subfamily proteins, and EGR-1 family proteins, which were induced by P. gingivalis LPS through the IKK/NF-κB signaling pathway. Although functional roles of some transcription factors are unclear in the context of LPS-induced inflammation and immune responses, activation of AP-1 has been found to play
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an important role in the induction of inflammatory mediators and immune responses (18). For example, IL-6 and TNF have also been reported to be regulated by AP-1, and matrix metalloproteinase family proteins are induced by inflammatory mediators through activation of AP-1 (9, 19). In fact, several matrix metalloproteinases have been reported to be highly expressed in inflamed periodontal tissues and inhibition of their expression attenuated tissue destruction (44). Like AP-1, EGR family proteins are also involved in the transcriptional regulation of inflammatory mediators (45). Since the inhibition of IKK/NF-κB blocked expression of AP-1 and ERG family members, our results suggest that IKK/NF-κB activated by P. gingivalis LPS may control the activation of AP-1 and EGR during inflammatory responses. It is possible that P. gingivalis LPS may activate NF-κB as a primary response and subsequently induce AP-1 and ERG to further modulate inflammatory responses. Moreover, NF-κB and AP-1 have been reported to cooperatively induce gene transcription. Therefore, NF-κB and AP-1 may synergistically stimulate secondary gene responses mediated by LPS, which may lead to induction of a high level of inflammatory mediators (18, 23).

Additionally, we also found that P. gingivalis LPS induced nuclear receptor subfamily proteins NR4A1 and NR4A2 through the IKK/NF-κB signaling pathway. Currently, the role of these proteins in inflammation and periodontal diseases is unclear. Growing evidence has suggested that human periodontal infection is a risk factor for atherosclerosis. P. gingivalis has been identified in atheromatous plaques from patients suffering from atherosclerosis. Atherosclerosis has been considered as both a disorder of lipid metabolism and a chronic inflammatory disease (2, 46). Monocytes/macrophages play a key role in initiation and development of atherosclerosis from both metabolic and inflammatory aspects (2, 46). In general, expression of nuclear receptors in monocytes/macrophages regulates lipid-dependent gene expression and inflammation (47). Although we do not know the direct targets regulated by NR4A family proteins, our results raise the possibility that P. gingivalis LPS as well as other bacterial components from periodontal pathogens may affect metabolic and inflammatory responses in the artery wall by inducing NR4A family proteins.

LPS-stimulated TLRs can transduce several kinase signaling cascades, such as ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase), p38, and phosphatidylinositol 3-kinase/Akt in addition to IKK/NF-κB. These signaling pathways have also been reported to play a role in inflammation and immune response by modulating gene expression (9, 18, 48). Based on genome-wide analysis, our results suggest that IKK/NF-κB plays a dominant role in early gene responses induced by P. gingivalis LPS. Because NF-κB plays a critical role in inflammation and other chronic diseases, the IKK/NF-κB signaling pathway is under intensive investigation as a therapeutic target. Most studies have been focused on developing small molecule inhibitors to inhibit IKKβ because of its catalytic activities. May et al. (49) reported that IKKγ associated with a segment at the C terminus of both IKKβ and IKKα. A cell-permeable peptide spanning this segment disrupted the association of IKKγ with both IKKβ and IKKα in vitro, inhibited TNF-α-induced NF-κB activation in various cell types, and effectively ameliorated inflammatory responses in animal models (49, 50). Our results presented here suggest that the zinc finger domain of IKKγ may be an alternative target for screening small molecule inhibitors. In future studies, it will be interesting to test whether the inhibition of IKK/NF-κB inhibits periodontal inflammation and bone destruction.

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