Epstein–Barr virus LMP1 induces IL-8 production via regulation of the NF-κB pathway in human gingival epithelial cells

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Contents

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Abstract

Human chronic periodontitis is a major public health concern, the major etiology of which has recently been revealed to be neutrophil infiltration into the periodontium. It affects over half the adult population worldwide. Mounting evidence has indicated that chronic periodontitis is a risk factor for pre-term birth, heart disease, aspiration pneumonia, and diabetes. Although traditional microbiological research on periodontitis has focused on putative bacteria, emerging evidence has implicated an association between Epstein–Barr virus (EBV) and periodontitis. However, molecular mechanisms underlying the role of EBV in the pathogenesis of periodontitis are unknown. This study investigated the effects of EBV-encoded latent membrane protein 1 (LMP1) on the production of interleukin-8 (IL-8) in the human gingival epithelial cells. Real-time polymerase chain reaction, luciferase assay, enzyme-linked immunosorbent assay, and Western blotting were performed to determine *IL-8* mRNA expression, nuclear factor kappa B (NF-κB) transcription, IL-8 production, and the phosphorylation of NF-κB p65 and Inhibitor of kappa B alpha (IκBα), respectively, in Ca9-22 human gingival epithelial cells. Two *LMP1* mutants lacking *C*-terminal activating region (CATR) domains responsible for activating NF-κB were used. Overall, results suggest that extremely high IL-8 production was induced by *LMP*1 in time- and dose-dependent manner. In addition, *LMP1* stimulated simultaneous phosphorylation of IκBα and NF-κB p65 and the transcription of NF-κB. In contrast, IL-8 production as well as NF-κB activation was drastically inhibited by the dominant negative mutant of IκBα. Furthermore, the *LMP1* mutants failed to induce IL-8 production, indicating that owing to its CATR domains, *LMP1* activates NF-κB and subsequently leads to the induction of IL-8 production. These findings suggest that in human gingival epithelium, LMP1 contributes to the progression of human chronic periodontitis via IL-8 production attributable to NF-κB activation. Thus, EBV, as

well as periodontopathic bacteria, should be considered as a causative agent of the progression of human chronic periodontitis.

Keywords : Epstein–Barr virus ; Periodontitis ; Inflammatory cytokine
LMP1 ; NF-κB

Introduction

Chronic periodontitis, a chronic inflammatory and infectious disease causing the destruction of the periodontium including the alveolar bone, is prevalent worldwide (1, 2). Mounting evidence has indicated that chronic periodontitis is a risk factor for pre-term birth, heart disease, diabetes, and atherosclerosis (1, 2). Over the past decade, neutrophil infiltration in the periodontium has been revealed to be the major aetiology for periodontitis (2). Some oral endogenous bacteria are believed to trigger periodontitis *via* host–parasite interactions (2, 3). However, periodontopathic bacteria, such as *Porphyromonas gingivalis*, are not always detected in periodontal lesions (4–6); therefore, the conventional theory based on bacterial aetiology alone cannot fully explain the aetiology of periodontitis.

A positive association has been reported between chronic periodontitis and Epstein–Barr virus (EBV) infection (7–11). EBV, a member of the herpesvirus family, infects many adults. During primary EBV infection, the virus undergoes lytic replication in B-cells and epithelial cells of the upper aerodigestive tract, where it later establishes latency (12, 13). EBV can be reactivated and is commonly found in the saliva of infected people (9, 10, 14). Many reports have demonstrated that the amount of EBV DNA detected in periodontal pockets of patients with chronic periodontitis correlates with disease severity (7–11). Previous studies observed that EBV DNA was more frequently detected in patients with deeper periodontal pockets than in those with shallow ones or healthy controls (15, 16). Imai et al. observed that *P. gingivalis* was able to induce EBV reactivation through epigenetic regulation (17). Additionally, a large number of EBV-encoded small RNA (EBER)-positive B-cells were found in the gingival tissues of patients with chronic periodontitis (15). Thus, EBV is epidemiologically involved in the aetiology of chronic periodontitis. However, no causal relationship between EBV and chronic periodontitis has been delineated.

The level of gingival epithelial EBV infection is correlated with the severity of chronic periodontitis (18). EBV-infected cells reportedly express EBERs and EBV-encoded latent membrane protein (LMP1) (18). LMP1 is composed of 386 amino acids; it comprises a short *N*-terminal cytoplasmic domain, six transmembrane-spanning domains, and a *C*-terminal cytoplasmic tail (19–21), and mediates cell growth as an oncoprotein (19, 21). LMP1 expression is essential for EBV-mediated primary B-cell transformation *in vitro* and associated with a number of human malignancies (12, 13, 19, 21). In epithelial cells, LMP1 induces the expression of anti-apoptotic proteins and cell surface antigens (22–25). Additionally, it induces the production of matrix metallopeptidase-9, cyclo-oxygenase-2, and pro-inflammatory cytokines in immune cells (26–29). Taking these findings together, this viral protein activates inflammatory and immune-regulatory responses in EBV-infected cells.

Interleukin-8 (IL-8), a potent neutrophil chemoattractant and activator, is a mediator of human chronic periodontitis *via* the accumulation and degranulation of neutrophils, which causes the subsequent destruction of periodontium (30, 31). Moreover, it strikingly upregulates the release of elastase by neutrophils, which significantly amplifies periodontal inflammation (32). IL-8 thus plays a crucial role in the progression of chronic periodontitis.

This study demonstrates for the first time that LMP1 attributable to its *C*-terminal activator regions (CTAR) domains in a human gingival epithelial cell line induces IL-8 production *via* nuclear factor kappa B (NF-κB) activation. Moreover, the study discusses whether LMP1 in gingival epithelial cells possibly contributes to the progression of human chronic periodontitis.

Materials and Methods

Reagents and plasmids.

Antibodies against phospho-IκBα (Ser32) and phospho-p65 (Ser32) (Cell Signaling Technology, Danvers, MA, USA) and IκBα, p65, β-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were purchased. A reporter plasmid expressing firefly luciferase under the control of NF-κB (pGL3-5xκB-luc) and dominant negative mutant of IκBα (IκBαΔN) were used as previously described (33). The *LMP1* expression vector (pSG-LMP1), its mutants, and control vector (pSG) (20) were generous gifts from Dr Martin Rowe (School of Cancer Sciences, University of Birmingham, UK).

Cell culture and transfection.

The gingival epithelial cell line Ca9-22 was purchased from RIKEN BioResource Center (Tsukuba, Japan) and maintained at 37°C in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Rockford, IL, USA), penicillin, and streptomycin as previously described (34). Ca9-22 cells were transfected with pSG-LMP1 using Lipofectamine 2000 (Thermo Fisher Scientific), in accordance with the manufacturer's instructions.

mRNA preparation and real-time polymerase chain reactions (PCR).

Experimental procedures for RNA purification and real-time (RT)-PCR were performed as previously described (34). For cDNA synthesis, total RNA was reverse transcribed using an RNA PCR kit (PrimeScript; Takara Bio, Shiga, Japan). The resulting cDNA mixture was subjected to RT-PCR analysis using SYBR Premix Ex Taq solution

(Takara Bio) containing sense and antisense primers. The following primer sequences were used: *IL-8*, forward (5-CTT GTC ATT GCC AGC TGT GT-3) and reverse (5-TGA CTG TGG AGC TAT GGC TG-3); *Tumor necrosis factor-α (TNF-α)*, forward (5-ATG TCT TTG GCT GCT ATC C-3) and reverse (5-TGA CGG TGG AGA TAT GGC TG-3); *IL-6*, forward (5- TTC GGT CCA GTT GCC TTC TC -3) and reverse (5- GAG GTG AGT GGC TGT CTG TG -3); *GAPDH*, forward (5-TGC ACC ACC AAC TGC TAG C-3) and reverse (5-GGC ATG GAC TGT GGT CAT GAG-3). PCR assays were performed using a TP-800 Thermal Cycler Dice Real-Time System (Takara Bio) and analysed using software provided by the device manufacturer. Thermal cycling conditions were 40 cycles at 95°C for 5 s, 60°C for 30 s, and 72°C for 1 min. All RT-PCR experiments were performed in triplicate; the specificity of each product was verified by melting curve analysis. The calculated level of gene expression was normalised to that of *GAPDH* mRNA.

IL-8 measurements.

IL-8 in Ca9-22 cell culture supernatants was measured using a human enzyme-linked immunosorbent assay kit for IL-8 (R&D systems, Minneapolis, MN, USA), according to the manufacturer's instructions. All experiments were performed in triplicate, and data presented are representative of three independent experiments.

Preparation of cytoplasmic and nuclear extracts.

Ca9-22 cells (2×10^5 cells/ml) were transfected with or without pSG-LMP1 for 24 h. Cells were washed with cold phosphate-buffered saline, resuspended in lysis buffer, and centrifuged. The supernatant was collected (whole-cell extract) and stored at −80°C until use. Precipitated cells were resuspended in cytoplasmic lysis buffer (Chemicon International, Temecula, CA, USA) and incubated for 15 min on ice. The cells were vortexed and

centrifuged (10 min, 20,000×*g*), and the supernatant (cytoplasmic extract) was removed. Pelleted cells were washed twice with cytoplasmic buffer to remove any trace of proteins, resuspended in nuclear lysis buffer (Chemicon International), and incubated for 15 min on ice. Lysed nuclei were sonicated for 10 s and centrifuged (15 min, 20,000×*g*); the supernatant (nuclear extract) was stored at −80°C. The Pierce BCA Protein Assay–Reducing Agent Compatible kit (Thermo Fisher Scientific) was used for standardizing protein concentration.

Western blotting.

Experimental procedures for Western blotting were performed as previously described (34, 35). Briefly, equal amounts of protein (15 µg) were separated by sodium dodecyl sulfate - poly acrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (EMD Millipore Corporation, Billerica, MA, USA). The membrane was probed and visualised using a SuperSignal West Pico enhanced chemiluminescence kit (Thermo Fisher Scientific).

Transient luciferase assay.

Ca9-22 cells (4×10^5 cells/ml) were transfected with 200 ng of reporter plasmids (pGL3-5xκB-luc) and 10 ng of the internal control plasmid (pRL-TK) expressing Renilla luciferase (Promega, Madison, WI, USA), with or without LMP1 or IκBαΔN for 24 h, using Lipofectamine 2000. These cells were harvested using Passive Lysis Buffer (Promega); the extracts were assessed for luciferase activity using Dual-Luciferase Assay System (Promega) as previously described (17, 35).

Statistical analysis.

Mean values ± standard deviation (SD) were calculated. Statistical analysis was

performed using one-way analysis of variance with Tukey's multiple comparisons test; $p<0.05$ was considered to be statistically significant.

Results

LMP1 expression induces IL-8 production in a human gingival epithelial cell line.

RT-PCR analysis was used to investigate the effects of LMP1 on IL-8 expression in Ca9-22 cells. *LMP1* transfection induced significant IL-8 expression. The *IL-8* mRNA level was up-regulated time-dependently (Figure 1A) and dose-dependently (Figure 1B) in response to *LMP1* transfection, but was not in cells transfected with the control vector. Next, the effects of *LMP1* on IL-8 protein production were investigated. As shown in Figure 1C and D, extremely high concentrations of IL-8 were produced due to *LMP1* in time- and dose-dependent manners. These results indicate that LMP1 in human gingival cells may be a potent inducer of IL-8 production. In addition, *TNF-α* and *IL-6* mRNA level was up-regulated by *LMP1* (Figure 1G and H). These results indicate that LMP1 in human gingival cells may be a potent inducer of inflammatory cytokines production.

LMP1 activates NF-κB *transcription in Ca9-22 cells.*

NF-κB is an inducible cellular transcription factor that regulates a variety of cellular genes involved in controlling inflammatory and immune responses (36). NF-κB normally binds to its inhibitor IκB present in the cytoplasm. Upon stimulation, intracellular signalling activates the IκB kinase complex, which sequentially phosphorylates two serine residues (Ser32/36) in IκBα (36). This results in the degradation of IκB by the 26S proteasome and consequent nuclear translocation of NF-κB.

NF-κB activity is important in mediating IL-8 production (36, 37). In addition, whether *LMP1* activated NF-κB in Ca9-22 cells was examined using Western blotting. As shown in Figure 2A, the level of phosphorylated IκBα increased, and that of IκBα conversely decreased due to LMP1. In parallel with this, phosphorylation increased at Ser536 of the NF-κB p65 subunit, which plays a key role in the transcriptional competence of NF-κB and the

nuclear translocation of p65 (36). To further examine whether NF-κB activation by *LMP1* in Ca9-22 cells occurs at the transcriptional level, luciferase assays were performed using a reporter plasmid whose expression is proportional to NF-κB activity. As shown in Figure 2B, *LMP1* up-regulated NF-κB transcription in a dose-dependent manner. These results indicate that *LMP1* activates NF-κB in human gingival epithelial cells and that it may induce IL-8 production.

NF-κB *is involved in LMP1-induced IL-8 production from Ca9-22 cells*.

Next, whether NF-κB was involved in *LMP1*-induced IL-8 production in Ca9-22 cells was examined. A dominant negative mutant of IκBα (IκBαΔN) was used to interfere with NF-κB activity (38). As shown in Figure 3A, *LMP1*-induced NF-κB activity was inhibited by IκBαΔN in a dose-dependent manner. In parallel with this, *LMP1*-induced IL-8 production was also inhibited (Figure 3B). These results suggest that NF-κB is a potent regulator of *LMP1*-induced IL-8 production from human gingival epithelial cells.

The CTAR1 and CTAR2 domains of LMP1 contribute to LMP1-induced IL-8 production from Ca9-22 cells.

Previous studies have identified two functional domains in the *LMP1 C*-terminal cytoplasmic tail, CTAR1 (amino acids 187–231) and 2 (amino acids 351–386), which are important for NF-κB activation (20, 25, 39). Proximal CTAR1 interacts with several tumor necrosis factor (TNF) receptor-associated factors (TRAFs) and induces NF-κB signalling (25, 39). Distal CTAR2 binds to the TNF receptor-associated death domain (TRADD) protein and mediates NF-κB signalling (20). Thus, two *LMP1* mutants, LMP1Δ(187–351) lacking CTAR1 and LMP1Δ(349–386) lacking CTAR2 were employed to analyse the effects of such mutations on NF-κB activation. The expression of LMP1Δ(187–351) or LMP1Δ(349–386)

significantly reduced NF-κB activity (Figure 4A) and reduced IL-8 production by approximately 80-90% compared with expression of wild-type *LMP1* (Figure 4B). Therefore, both TRAF-interacting CTAR1 and TRADD-interacting CTAR2 should be necessary for *LMP1*-induced IL-8 production from human gingival epithelial cells.

Discussion

Although emerging evidence suggests an association between EBV and human chronic periodontitis, whose major aetiology has recently been revealed to be neutrophil infiltration into the periodontium, how EBV relates to the progression of periodontitis is not understood. To clarify this issue, it would be important to identify and characterise any complex pro-inflammatory cytokine network involved in periodontal pathogenesis and its link to EBV. Therefore, whether EBV *LMP1* in a human gingival cell line, Ca9-22, induced IL-8 production was first examined. As expected, *LMP1* induced IL-8 production. In addition, for IL-8, the activation of NF-κB was necessary.

Like other infectious diseases, adhesion to and subsequent invasion into the epithelia by pathogens are critical steps in the initiation of periodontitis. Bacterial adhesion to gingival epithelium reportedly induces the production of various immune-response mediators (1, 40). Among these, IL-8, a potent neutrophil chemoattractant and activator, is such a mediator of human chronic periodontitis that acts *via* its involvement in the accumulation and degranulation of neutrophils, which causes subsequent destruction of the periodontium (30, 31). Many investigators have demonstrated the presence of IL-8 in gingival crevicular fluid and pro-inflammatory cytokine levels in gingival crevicular fluid being closely associated with the severity of inflammation and periodontal destruction (41–44). Moreover, primed by IL1β, IL-8 strikingly upregulates neutrophils for elastase release, giving rise to the significant increase of periodontal inflammation (32). IL-8 is produced excessively or continuously in response to accumulated periodontopathic bacteria and their products in human gingival crevices or periodontal pockets and is an important determinant of the progression of chronic periodontitis (2). In the present study, extremely high levels of IL-8 were induced due to

LMP1 in Ca9-22 cells. This suggests that LMP1 contributes to the progression of chronic periodontitis by promoting the production of pro-inflammatory cytokines, IL-8 in particular. Genes of several pro-inflammatory cytokines, including chemokines, contain NF-κB-binding sites in their proximal promoters (36). Hence, NF-κB activation possibly also plays a key role in virus-dependent cytokine expression and pathogenesis with inflammation. In the present study, *LMP1* stimulated simultaneous phosphorylation of IκBα and NF-κB p65 and the transcription of NF-κB. Additionally, IκBαΔN inhibited *LMP1*-induced NF-κB activation and subsequent IL-8 production in a dose-dependent manner. This suggests that NF-κB is a potent regulator of *LMP1*-induced IL-8 production from human gingival epithelial cells. In this connection, this NF-κB-dependent IL-8 production has been reported to be associated with distinct human cells of the periodontium, such as a cervical squamous cell carcinoma cell line and umbilical vein endothelial cells (45, 46).

Regarding *LMP1*-related NF-κB activation, the mutation analysis of *LMP1* has identified CTAR1 and CTAR2 as independent effectors of NF-κB activation (20, 25, 39). CTAR1 binds to TRAF1, -2, -3, and -5 through a consensus TRAF-binding motif, while CTAR2 binds to the TRADD (20, 25, 39). In the present study, we found for the first time that LMP1Δ(187–351) and LMP1Δ(349–386), lacking CTAR1 and CTAR2, respectively, significantly reduced *LMP1*-induced IL-8 production. Therefore, *LMP1*, particularly its CTAR domains, may be responsible for IL-8 production from EBV-infected human gingival epithelial cells.

This study confirmed that NF-κB activation was responsible for *LMP1*-induced IL-8 production by the gingival epithelial cell line. However, this was only obtained from a single cell line. Although Ca9-22 has been employed in many periodontal studies (47–49), it is important to confirm the results obtained in this study in other gingival epithelial cells to clarify the physiological relevance of these findings.

Chronic periodontitis and EBV infection in the periodontium quite often coincide, where high numbers of EBER-positive B-cells and EBER-positive gingival epithelial cells are observed in the gingival tissues of patients with periodontal disease (15, 18). Moreover, the involvement of EBV in the aetiology of human chronic periodontitis is supported by several observations: Anti-herpesvirus drug treatment reportedly resulted in an undetectable level of EBV with simultaneous periodontal improvement (10), and increased concentrations of pro-inflammatory cytokines in sera were consistently observed in EBV-infected patients (21, 40). In addition, as described in a previous report, butyric acid produced by periodontopathic bacteria may cause EBV reactivation in the periodontium of EBV-infected individuals (17). Furthermore, the present study showed that *LMP1* in the human gingival epithelial cell line Ca9-22 markedly induced IL-8 production attributable to NF-κB activation.

Considering the observations mentioned above and the involvement of infiltrated neutrophils in the aetiology of human chronic periodontitis, it is suggested that inducible IL-8 production by EBV *LMP1* in the human gingival epithelium contributes to human chronic periodontitis. Therefore, EBV, as well as periodontopathic bacteria, should be considered as therapeutic targets when treating human chronic periodontitis. Further studies are needed to determine whether EBV is as an independent aetiological agent of human chronic periodontitis or whether it acts in combination with periodontopathic bacteria. The development of new treatments and superior preventative methods should be supported by a better understanding of the role of EBV in the aetiology of human chronic periodontitis.

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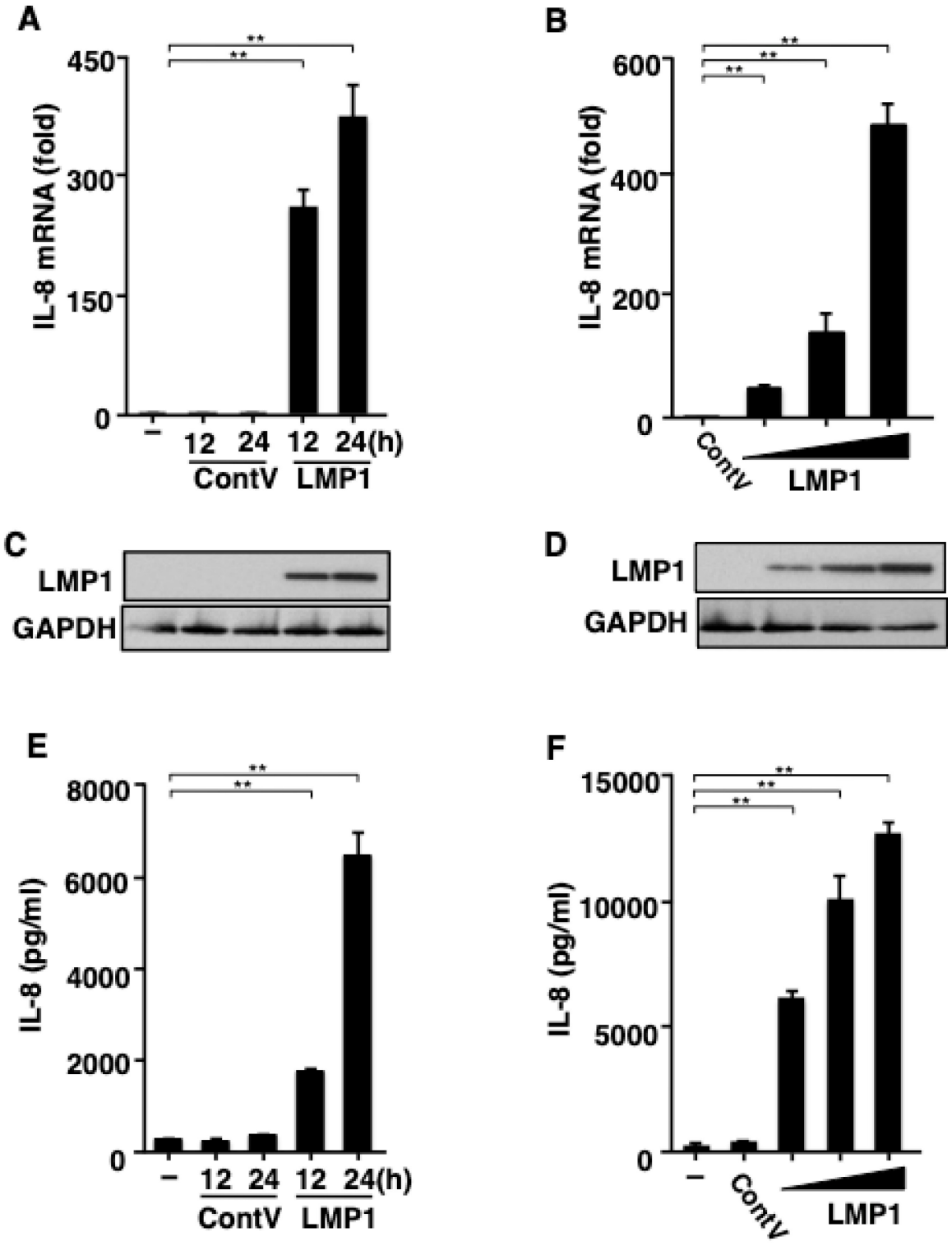
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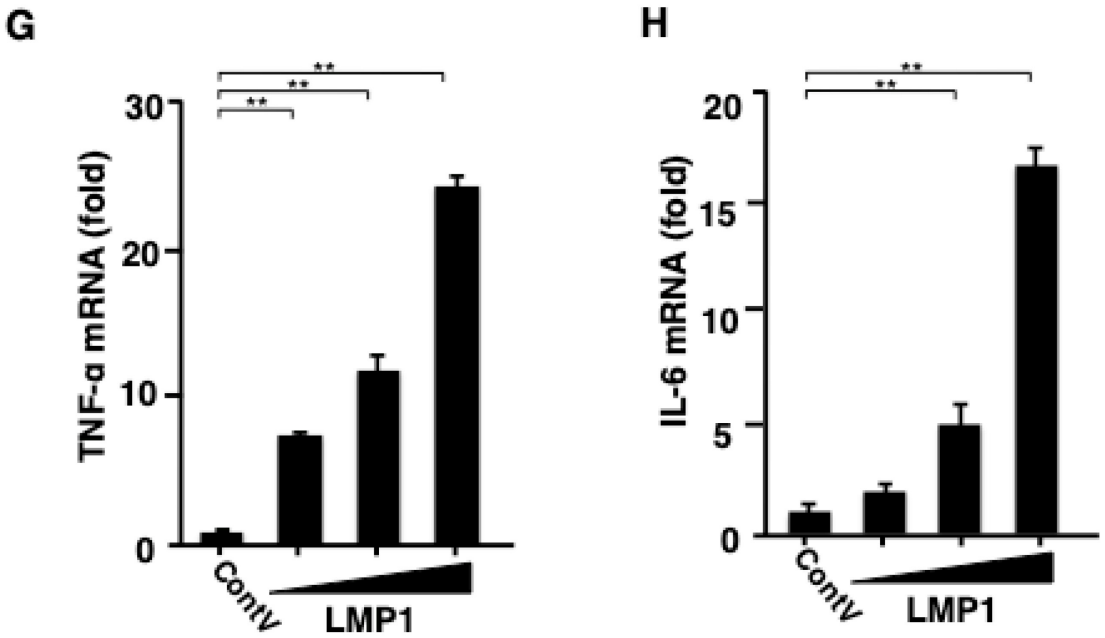

Figure 1. Latent membrane protein 1 (*LMP1*) promotes interleukin-8 (IL-8) production in a human gingival epithelial cell line. Ca9-22 cells were transfected with pSG-LMP1 (0.2 μg) or pSG (0.2 μg) as control vector (ContV) for different times (A, C, E) and with pSG-LMP1 at different concentrations (0.05, 0.1, or 0.2 μg) for 24 h (B, D, F, G, H). A, B, G, H: The cells were harvested and the level of *IL-8, TNF-a,* and *IL-6* mRNA were determined using reverse transcription polymerase chain reaction-analysis with specific primers. C, D: The cell lysates were prepared, and the LMP1 level was determined by Western blotting. E, F: IL-8 released into the culture supernatants was determined using enzyme-linked immunosorbent assay. The values are presented as mean ± SD; n=3. **Significantly different at p<0.0001).

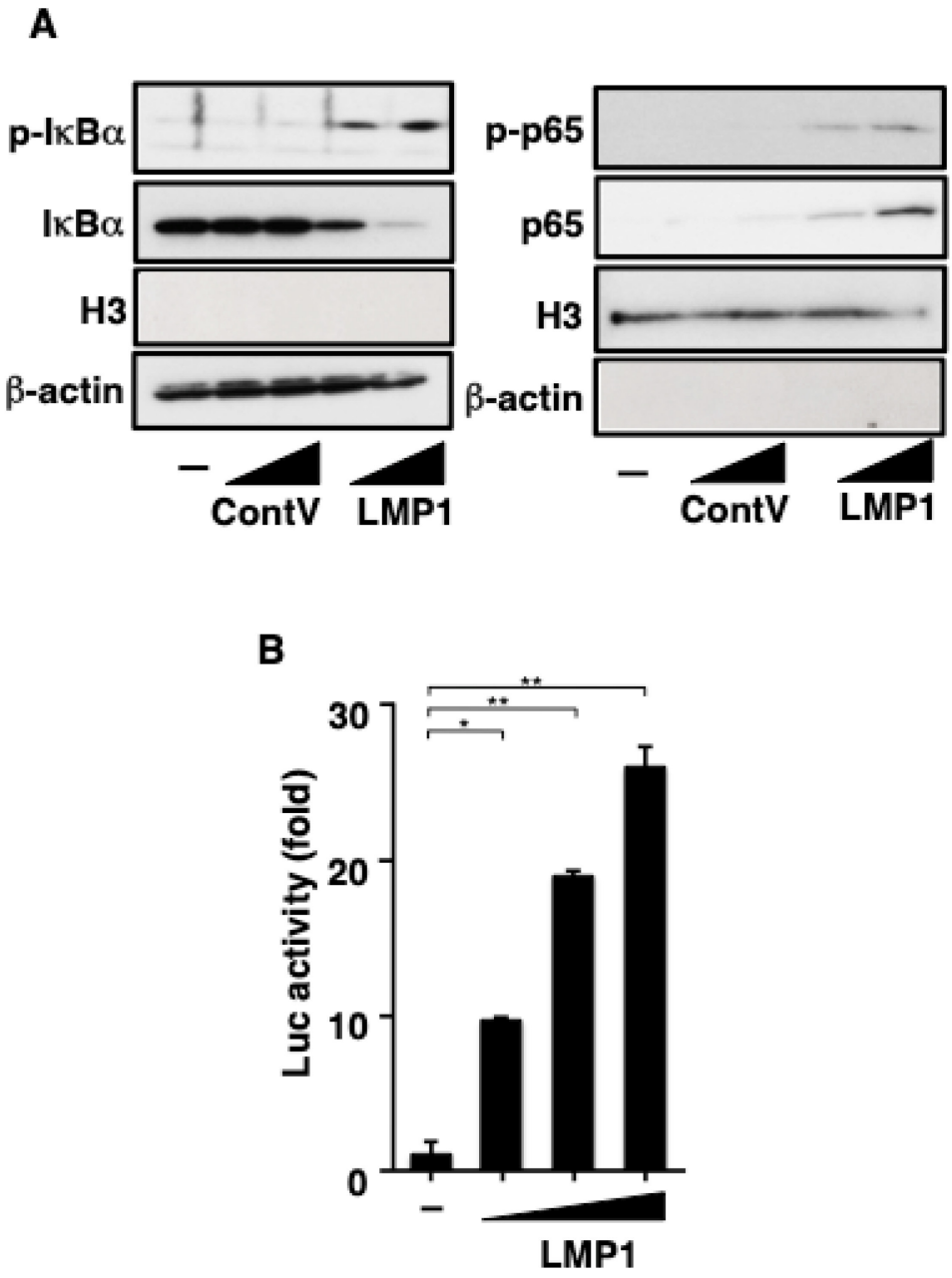

Figure 2. Latent membrane protein 1 (*LMP1*) induces nuclear factor kappa B (NF-κB) activation. Ca9-22 cells were transfected with pSG-LMP1 or pSG (0.05 or 0.2 μg) as control vector (ContV). A: Proteins in the cytoplasmic extract (left panel) and the nuclear extract (right panel) were separated by polyacrylamide gel electrophoresis and then immunoblotted. Purity of the cytoplasmic and nuclear extracts was confirmed using antibodies specific for histone H3 as a representative of nuclear proteins and β-actin as a representative of cytoplasmic proteins. B: Ca9-22 cells were transfected with pSG-LMP1 (0.05, 0.1, or 0.2 μg) and 20 ng of the pGL3-5xκB-luc reporter plasmid. The cells were harvested and luciferase (Luc) activity was measured 24 hours after transfection. The values are presented as mean ± SD; n=3. Significantly different at *p<0.05 and **p<0.0001.

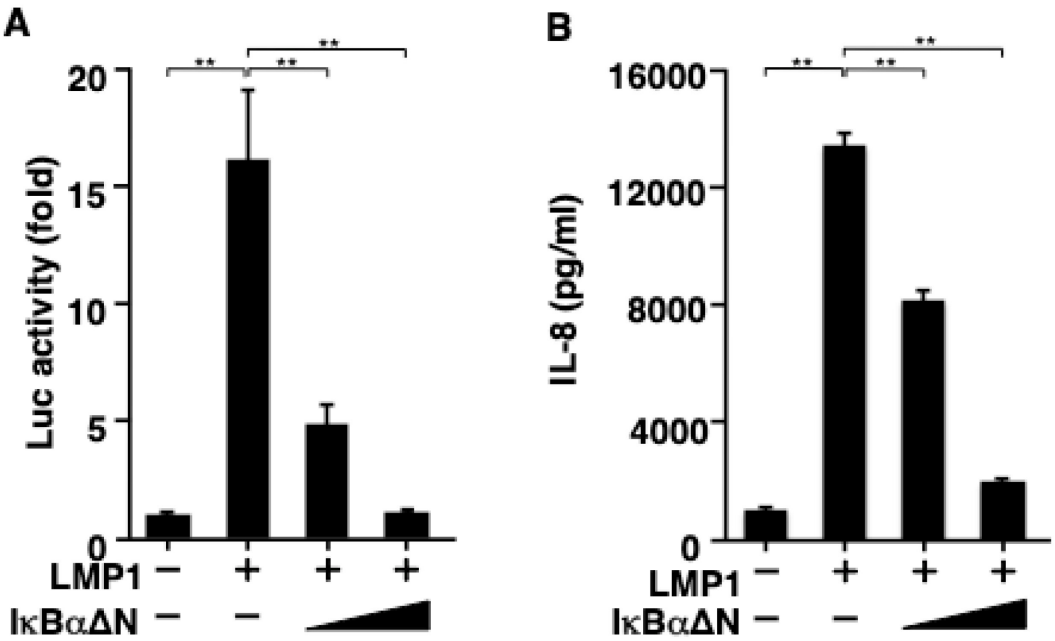

Figure 3. The inhibitor of kappa B alpha (IκBα) mutant inhibits EBV-encoded latent membrane protein 1 latent membrane protein 1 *(LMP1)*-induced nuclear factor kappa B (NF-κB) activation and interleukin-8 (IL-8) production. Ca9-22 cells were transfected with IκBαΔN-expressing plasmid (0.05 or 0.2 μg), 0.2 μg of pSG-LMP1, and 20 ng of pGL3-5xκB-luc reporter plasmid for 24 h. A: Luciferase (Luc) assay was then performed. B: Ca9-22 cells were transfected with IκBαΔN (0.05 or 0.2 μg) and 0.2 μg of pSG-LMP1 for 24 h. Enzyme-linked immunosorbent assay test for IL-8 was then performed. The values are presented as mean ± SD; n=3. **Significantly different at p<0.0001.

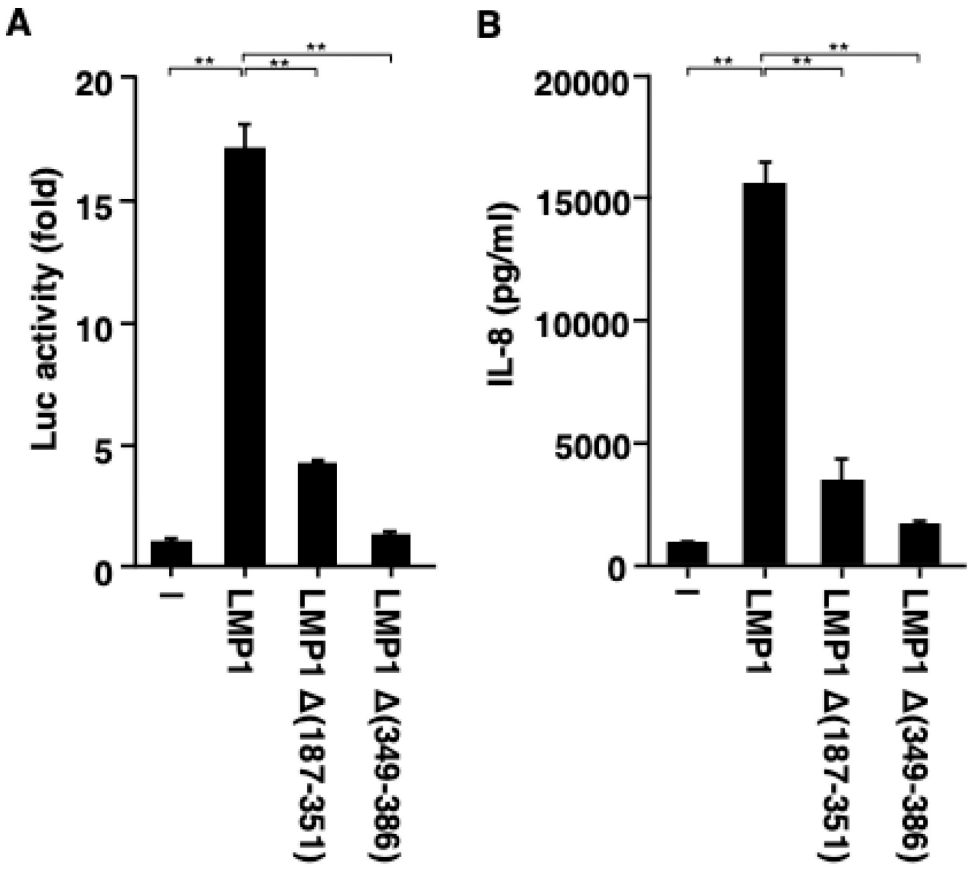

Figure 4. *C*-terminal activator region 1 (CTAR1) and CTAR2 domains of latent membrane protein 1 (LMP1) are involved in *LMP1*-induced nuclear factor kappa B (NF-κB) activation and interleukin-8 (IL-8) production. Ca9-22 cells were transfected with 0.2 μg of the wild-type or the indicated mutant *LMP1* plasmids together with 20 ng of the pGL3-5xκB-luc reporter plasmid for 24 h. A: Luciferase (Luc) assay was then performed as shown. B: Ca9-22 cells were transfected with wild-type or mutant (Δ) *LMP1* plasmids for 24 h. Cell culture supernatants were prepared and assayed for IL-8 protein. The values are presented as mean ± SD; n=3. **Significantly different at p<0.0001.