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DNA methyltransferase DNMT1 inhibits lipopolysaccharide-induced inflammatory response in human dental pulp cells involving the methylation changes of IL-6 and TRAF6

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Abstract. Dental pulp inflammation is a pathological process characterized by local lesions in dental pulp and the accumulation of inflammatory mediators. DNA methylation of cytosine residues is a key epigenetic modification that is essential for gene transcription, and plays pivotal roles in inflammatory reactions and immune responses. However, the function of cytosine DNA methylation in the innate immune defense against the inflammation of dental pulp is poorly understood. To investigate the effect of DNA methylation in inflamed dental pulp upon innate immune responses, expression levels of the DNA methyltransferases (DNMT1, DNMT3a and DNMT3b) in human dental pulp cells (hDPCs) after lipopolysaccharide (LPS) stimulation were evaluated by western blotting and reverse transcription-quantitative (RT-q) PCR. Only DNMT1 expression was decreased, while the transcription of inflammatory cytokines was increased. In the immune responses of LPS-induced hDPCs, the results of RT-qPCR and ELISA showed that DNMT1 knockdown promoted the production of the pro-inflammatory cytokines, interleukin (IL)-6 and IL-8. Western blotting demonstrated that DNMT1 knockdown increased the phosphorylation levels of IKKα/β and p38 in the NF-κB and MAPK signaling pathways, respectively. Furthermore, MeDIP and RT-qPCR analysis demonstrated that the 5-methylcytosine levels of the *IL-6* and TNF receptor-associated factor 6 (*TRAF6*) promoters were significantly decreased in DNMT1-deficient hDPCs. Taken together, these results indicated that the expression of DNMT1 was decreased after LPS stimulation in hDPCs. DNMT1 depletion increased LPS-induced cytokine secretion, and activated NF-κB and MAPK signaling; these mechanisms may involve the decreased methylation levels of the *IL-6* and *TRAF6* gene promoters. This study emphasized the role of DNMT1-dependent DNA methylation on the inflammation of LPS-infected dental pulp and provides a new rationale for the investigation of the molecular mechanisms of inflamed dental pulps.

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Introduction

Dental pulp inflammation is a pathological process characterized by various bacterial virulence factors that often elicits a dental emergency, and may develop into periapical disease or pulp necrosis (1). Lipopolysaccharide (LPS) is commonly released from gram-negative bacteria. When LPS enters the dental pulp, it can evoke an inflammatory response; LPS is also closely associated with pulpitis and periapical periodontitis (2). Previous studies have found that LPS can stimulate Toll-like receptor (TLR)4 in the cell membranes of human dental pulp cells (hDPCs), and activate the NF-κB, ERK1/2 and p38 pathways, thereby producing inflammation-related cytokines, including interleukin (IL)-6 and IL-8 (3,4). Although there are a number of mechanisms associated with the development of dental pulp infection, the specific molecular mechanism is still unclear (5,6). Recent studies have suggested that epigenetic alterations are crucial regulators in the occurrence and development of dental pulp infection (7-9).

DNA methylation that occurs at cytosine-phosphate-guanine (CpG) dinucleotide sites is the most common epigenetic modification event in the genome (10). The DNA methylation process involves placing a methyl group onto the 5-position of cytosines situated in CpG dinucleotides and turning the cytosine into 5-methylcytosine (5mC), which is catalyzed by members of the DNA methyltransferase (DNMT) family (11). DNMT1 can methylate hemimethylated CpGs and is a well-known maintenance methyltransferase that can preserve methylation patterns during DNA replication (12). DNMT3a and DNMT3b are *de novo* methyltransferases that can methylate unmethylated

and hemimethylated DNA, and establish DNA methylation patterns in embryo development (13). The roles and functions of DNA methylation patterns have attracted extensive attention, but there has been particular emphasis on their roles in the pathological processes of cancer (14). Only recently have studies begun to shed light on the contribution of DNMTs to the initiation and progression of inflammatory diseases (15). A study on inflamed peripheral blood mononuclear cells (PBMCs) showed that DNMT1 expression decreased after treatment with LPS; DNMT1 modulated the methylation level of gene promoters, thus mediating the transcription of pro-inflammatory cytokines, including IL-6, IL-8 and tumor necrosis factor-α (TNF-α) (16). In macrophages, DNMT1 contributes to the hypermethylation of suppressor of cytokine signaling 1, a negative regulator of cytokine signal transduction, thereby enhancing the secretion of pro-inflammatory cytokines induced by LPS indirectly (17). DNA methylation could also affect inflammatory reactions by modulating the activation levels of crucial proteins of the NF-κB and/or MAPK pathways (18,19). In addition, DNA methylation epigenetically regulates the transcription of TLRs and signal transduction molecules, including TNF receptor-associated factor 6 (TRAF6) and myeloid differentiation primary response 88 (MyD88). This suggests that DNA methylation is engaged in signaling pathways related to inflammation (20,21). These studies provide evidence indicating that DNA methylation can epigenetically regulate inflammatory reactions via several different mechanisms. However, whether DNA methylation is involved in the modification of dental pulp immunity remains unclear.

Preliminary experiments by our lab showed that in LPS-treated hDPCs, 5-aza-2'-deoxycytidine (5-Aza-CdR), a DNA methyltransferase inhibitor, increased the production of several inflammation-related cytokines (unpublished data). The present study aimed to investigate the effect of DNMT1 on the LPS-induced inflammatory response in hDPCs, thereby exploring the role of DNA methylation in dental pulp inflammation. The results demonstrated that *DNMT1* knockdown promoted the expression of pro-inflammatory cytokines and the phosphorylation of IKKα/β and p38 in LPS-treated hDPCs. Moreover, *DNMT1* depletion decreased the 5mC level in the *IL-6* and *TRAF6* promoters. These data suggested that DNMT1 may be involved in inhibiting the LPS-induced inflammatory response in hDPCs.

Materials and methods

Isolation and culture of hDPCs. Healthy permanent premolars and third molars were collected from donors aged 18 to 25 for orthodontic reasons from the Department of Oral and Maxillofacial Surgery, Guanghua School of Stomatology, Sun Yat-sen University for approximately one year between March 2018 and April 2019. Only healthy teeth without carious disease or hyperemic pulp tissue were selected. A total of 128 teeth from 58 donors (29 males and 29 females) were obtained for dental pulp tissue isolation and cell culture. hDPCs were isolated and cultivated using an enzymatic method as described by Gronthos *et al* (22). After extraction, the teeth were washed with 70% ethanol and PBS (pH 7.4) and then split open to expose the pulp chamber. The dental pulp tissue was gently isolated with forceps and minced into small pieces, which were then digested in 3 mg/ml collagenase type I (Gibco; Thermo Fisher Scientific, Inc.) for 20 min at 37˚C. Subsequently, the minced pulp tissue was cultured in DMEM containing 20% FBS, 100 mg/ml streptomycin and 100 U/ml penicillin (all purchased from Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. The medium was changed every 3 days. When the cells reached 80% confluence, they were detached using trypsin/EDTA (Gibco; Thermo Fisher Scientific, Inc.) and subcultured at a ratio of 1:2. Generally, 2-3 teeth from one donor were used for each primary culture. For each primary culture, ~10^6 cells at the zero passage were obtained. All experiments were performed with cells from passages two or three. To avoid inter-individual variation, the experiments were performed at least three times for each sample and each experiment, and average data were generated. For each parameter, experiments were replicated three times each using donor cells from three samples, and average data for the three different cell types were obtained.

Treatment with LPS. hDPCs were stimulated for the indicated times (0, 3, 6, 12 and 24 h) with 1 μg/ml purified *Escherichia coli* (*E. coli*) LPS (Sigma-Aldrich; Merck KGaA) at 37˚C with 5% CO_2 (4,23). The blank controls were cells without LPS stimulation.

DNMT1 small interfering RNA (siRNA) transfection. siRNA was used in hDPCs to knockdown *DNMT1*. A total of 3 siRNA sequences (Invitrogen; Thermo Fisher Scientific, Inc.) were designed to target the human *DNMT1* gene. Before transfection, hDPCs were seeded in 6-well plates in 2 ml of α-MEM at 4x10^5 cells/well containing 10% FBS for 24 h. After attachment overnight, hDPCs were then transfected with siRNA (50 nM) against *DNMT1* or a nontargeting siRNA control using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. After incubation for 24 h, the media was changed, and DMEM supplemented with 10% FBS was added with or without 1 μg/ml *E. coli* LPS. All siRNA sequences are listed in Table I. siRNA #1 with the best interference effect was selected as the DNMT1 target sequence for the subsequent experiments.

Reverse transcription quantitative (RT-q)PCR. Cells were lysed using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocols, and RNA was extracted and reverse transcribed into cDNA with a RevertAid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.). PCR was performed using the complementary DNA as a template. SYBR-Green I (Roche Diagnostics) RT-qPCR results were detected by a LightCycler® 480 thermal cycler. Thermal cycling conditions consisted of initial denaturation at 95˚C for 5 min, followed by 45 cycles of 95˚C for 10 sec, 65˚C for 20 sec and 72˚C for 30 sec. The relative results were normalized to the *GAPDH* mRNA levels (24). The primer sequences were designed using Primer Express Software v3.0.1 (Thermo Fisher Scientific, Inc.) and are listed in Table II.

Western blot analysis. Protein was extracted from hDPCs using RIPA buffer (Beyotime Institute of Biotechnology), and the concentrations were detected using a BCA Protein Assay kit

Table I. Sequences used for DNMT1 siRNA.

DNMT1 siRNA	Sequence (5'-3')
#1 siRNA	Sense: GGGACUGUGUCUCUGUUAUTT dTdT
	Antisense: dTdT AUAACAGAGACACAGUCCCTT
#2 siRNA	Sense: GCACCUCAUUUGCCGAAUATT dTdT
	Antisense: dTdT UAUUCGGCAAAUGAGGUGCTT
#3 siRNA	Sense: GAGGCCUAUAAUGCAAAGATT dTdT
	Antisense: dTdT UCUUUGCAUUAUAGGCCUCTT

DNMT1, DNA methyltransferases; siRNA, small interfering RNA.

Table II. Primers used for the analysis of mRNA levels by reverse transcription-quantitative PCR.

Gene	Primer sequences (5'-3')
DNMT1	F: GGCTGAGATGAGGCAAAAAG
	R: ACCAACTCGGTACAGGATGC
DNMT3 A	F: AGGGAAGACTCGATCCTCGTC
	R: GTGTGTAGCTTAGCAGACTGG
DNMT3 B	F: GCCTCAATGTTACCCTGGAA
	R: CAGCAGATGGTGCAGTAGGA
IL-6	F: TGCAATAACCACCCCTGACC
	R: AGCTGCGCAGAATGAGATGA
IL-8	F: GGTGCAGTTTTGCCAAGGAG
	R: TTCCTTGGGGTCCAGACAGA
GAPDH	F:TCTCCTCTGACTTCAACAGCGACA
	R: CCCTGTTGCTGTAGCCAAATTCGT

IL, interleukin; F, forward; R, reverse.

(Beyotime Institute of Biotechnology). Proteins (30 μg) were separated using electrophoresis on 8% SDS-polyacrylamide gels and transferred to PVDF membranes (EMD Millipore). Next, the membranes were blocked with TBS-Tween 20 (20 mmol Tris-HCl, 150 mmol NaCl, 0.05% Tween-20) containing 5% BSA (Biofroxx; neoFroxx GmbH) for 1 h at room temperature. Then, the membranes were incubated with primary antibodies against DNMT1 (1:2,000; Abcam), IκB kinase αβ (IKKαβ), phosphorylated (p)-IKKαβ, p65, p-p65, IκBα, p-IκBα (1:1,000; NF-κB Pathway Sampler kit, 9936, Cell Signaling Technology, Inc.), p38, ERK, JNK (1:1,000; MAPK Family Antibody Sampler kit, 9926, Cell Signaling Technology, Inc.), p-p38, p-ERK, p-JNK (1:1,000; phospho-MAPK Family Antibody Sampler kit, 9910, Cell Signaling Technology, Inc.) and GAPDH (1:1,000; Cell Signaling Technology, Inc.) overnight at 4˚C. After rinsing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000; AQ160P and AP307P, EMD Millipore) at room temperature for 1 h. An enhanced chemiluminescence system (EMD Millipore) was used to visualize the antibody binding. The relative protein expression levels were normalized to that of the GAPDH gene, and the protein band densities were determined by ImageJ v1.47 software (National Institutes of Health). ReBlot Plus (EMD Millipore) was used to strip and re-probe with the p-antibodies for IκBα, p38, ERK and JNK to distinguish different target proteins when they share similar molecular weights with the total IκBα, p38, ERK and JNK, respectively, on the same membrane.

ELISA. Human IL-6 ELISA kits (D6050, R&D Systems, Inc.) and Human IL-8 ELISA kits (D8000C, R&D Systems, Inc.) were used to analyze the culture supernatant protein concentrations of IL-6 and IL-8 collected after LPS stimulation for 6 h according to the manufacturer's protocols. A microplate reader (Tecan Safire microplate reader; Tecan Group, Ltd.) was used to evaluate the optical density (OD) values. Based on the standard solution concentration and corresponding OD value, sample concentrations were calculated.

Methylated DNA immunoprecipitation (MeDIP) and RT-qPCR. DNA was extracted from hDPCs and fragmented to 200-500-bp fragments with a Bioruptor Waterbath Sonicator (8 cycles, 15 sec on/15 sec off, at the highest output level while cooling the tube to 1˚C in a waterbath). Then, the DNA fragments were diluted to 700 μl with TE buffer (Invitrogen; Thermo Fisher Scientific, Inc.) with 60 μl Protein G Magnetic Beads (S1430S; New England BioLabs, Inc.) and denatured for 10 min at 94˚C. Following denaturation, DNA was immunoprecipitated at 4˚C overnight with an anti-5mC antibody (1:40, C02010031; Diagenode SA). Then it was incubated for 2 h with anti-IgG Magnetic Beads (S1430S; New England BioLabs, Inc.) at 4˚C with agitation. The beads were trapped on a magnetic rack, the supernatant discarded, and washed three times with 1 ml 1XIP buffer [2 mM EDTA, 20 mM Tris (pH=8.0), 1% Triton X-100, 0.1% SDS, 150 mM NaCl] for 10 min at 4˚C with agitation. Beads were then resuspended in 400 μl of Elution Buffer (50 mM Tris-HCl, pH=8.0; 10 mM EDTA, Ph=8.0; 1% SDS) with 10 μl of Proteinase K (Qiagen GmbH). IP with non-specific human IgG was measured as a negative control. After IP, the DNA samples were eluted using phenol-chloroform and precipitated using ethanol. After resuspending the precipitated samples in 10 μl Tris buffer, RT-qPCR was performed using 1 μl harvested DNA fragments. The primers designed for MeDIP-PCR are shown in Table III.

Statistical analysis. All experiments were carried out at least three times. The data were analyzed by the SPSS 20.0 software

Table III. Primers used for methylated DNA immunoprecipitation PCR.

Gene	Primer sequences (5'-3')
IL6	F: TGGCAGCACAAGGCAAACC
	R: GCTTCAGCCCACTTAGAGGAGG
IL8	F: TAGGAAGTGTGATGACTCAGGTT
	R: GTCAGAGGAAATTCCACGATT
TRAF6	F: GCTTACTGTAGCCTTGACTGCC
	R: GTGGTGCATATCTGTAGTCTCGG
MYD88	F: TTCGCTCACCGACACAGATG
	R: GGTCACTGCGGCTGCTCTT

IL, interleukin; TRAF6, TNF receptor-associated factor 6; MyD88, myeloid differentiation primary response 88; F, forward; R, reverse.

package (IBM Corp.) and are shown as the mean ± SD. Student's t-test was used to measure the differences between two groups. To evaluate the differences in multiple sets of data, one-way ANOVA or repeated-measures ANOVA with a post hoc Dunnett's test was performed. $P<0.05$ was considered statistically significant.

Results

DNMT1 expression in LPS-inflamed hDPCs. To detect the effect of LPS on the inflammatory reaction in hDPCs, hDPCs were stimulated with LPS at a concentration of 1 μg/ml for the indicated times. As illustrated in Fig. 1A and B, compared with the control group, *IL-6* and *IL-8* mRNA and protein expression was significantly increased by LPS. IL-6 and IL-8 expression was upregulated and peaked after 3 h, which was followed by a gradual decrease. The levels of *DNMT1* mRNA were significantly reduced within 24 h after treatment with LPS. DNMT1 protein expression also decreased, with the most significant change at 3 h (Fig. 1C and D). Moreover, the mRNA expression of *DNMT3a* and *DNMT3b* did not change significantly before or after LPS treatment (Fig. 1).

Effects of DNMT1 on inflammatory cytokine expression in LPS-induced hDPCs. Our preliminary study found that 5-Aza-CdR, a DNMT inhibitor, can increase the secretion of inflammatory cytokines in LPS-stimulated hDPCs, and among the upregulated cytokines, IL-6 and IL-8 experienced the greatest increase (unpublished data). To investigate the effect of DNMT1-dependent methylation on inflammatory cytokine production in hDPCs stimulated with LPS, the IL-6 and IL-8 expression levels after *DNMT1* knockdown in hDPCs transfected with siRNAs were measured. As shown in Fig. 2A and B, after *DNMT1* siRNA (#1, #2 and #3) interference, *DNMT1* mRNA expression levels were significantly reduced when compared to the negative control group. These data were further confirmed by western blotting, which showed a reduction in the protein expression. Among the *DNMT1* siRNAs, the siRNA #1 group showed the best interference effect at ~72% (Fig. 2B). Therefore, siRNA #1 was selected as the *DNMT1* target sequence for the subsequent experiments.

IL-6 and *IL-8* gene expression levels were then measured in cells stimulated by LPS after *DNMT1* depletion (Fig. 2C). The results showed that *IL-6* and *IL-8* mRNA expression within 24 h after LPS stimulation was notably higher in the *DNMT1* knockdown group compared with the control group. In LPS-inflamed hDPCs, the protein levels of IL-6 and IL-8 were also significantly increased after *DNMT1* knockdown (Fig. 2D).

Effects of DNMT1 on the NF-κB signaling pathway in LPS-induced hDPCs. One of the most important signaling pathways that influences inflammatory cytokine production in inflammation induced by LPS is the NF-κB signaling pathway (25). By means of western blotting, the phosphorylation levels of three crucial proteins of the NF-κB signaling pathway were examined (IKKα/β, p65 and IκBα) to determine whether DNMT1 is engaged in NF-κB pathway activation. As illustrated in Fig. 3A and B, *DNMT1* knockdown significantly increased the phosphorylation of IKKα/β at 15 and 30 min after LPS treatment in hDPCs. The p65 and IκBα phosphorylation levels also increased at several time points, but there was no significant difference.

Effects of DNMT1 on the MAPK signaling pathway in LPS-induced hDPCs. Another vital signaling transduction pathway involved in inflammation in the LPS-related inflammatory response is the MAPK signaling pathway (26). The phosphorylation levels of three key proteins in the MAPK signaling pathway were assessed (p38, ERK1/2 and JNK) to determine whether DNMT1 plays an important role in MAPK signaling pathway activation. As illustrated in Fig. 4A and B, after *DNMT1* knockdown in LPS-inflamed hDPCs, the p38 phosphorylation level was increased, while both p-ERK and p-JNK levels were not significantly altered.

Effects of DNMT1 on the dynamic methylation levels of the IL-6, IL-8, TRAF6 and MyD88 gene promoters in LPS-induced hDPC inflammation. DNA methylation can regulate the occurrence and progression of inflammatory responses by modulating the methylation levels of inflammation-related cytokines and signaling molecule promoters (27). TRAF6 and MyD88 are key intracellular signal transducers of LPS-induced signaling pathways (28). To identify whether the methylation of *IL-6, IL-8, TRAF6* and *MyD88* was regulated through DNMT1, the levels of 5mC present at their gene promoters were examined by means of MeDIP-PCR. The results illustrated that the levels of 5mC at the *IL-6* and *TRAF6* promoters decreased notably in LPS-stimulated hDPCs after *DNMT1* knockdown. However, no significant change was observed in the 5mC levels of the *IL-8* and *MyD88* promoters (Fig. 5). These experimental results indicated that DNMT1 can modulate the methylation of *IL-6* and *TRAF6* in hDPCs stimulated by LPS.

Discussion

As a major component of the outer membrane of gram-negative bacteria, LPS serves as the primary pathogenic factor leading to dental pulp inflammation (29). When healthy dental pulp cells are exposed to LPS, pro-inflammatory chemokines and cytokines, including IL-6 and IL-8, are released,

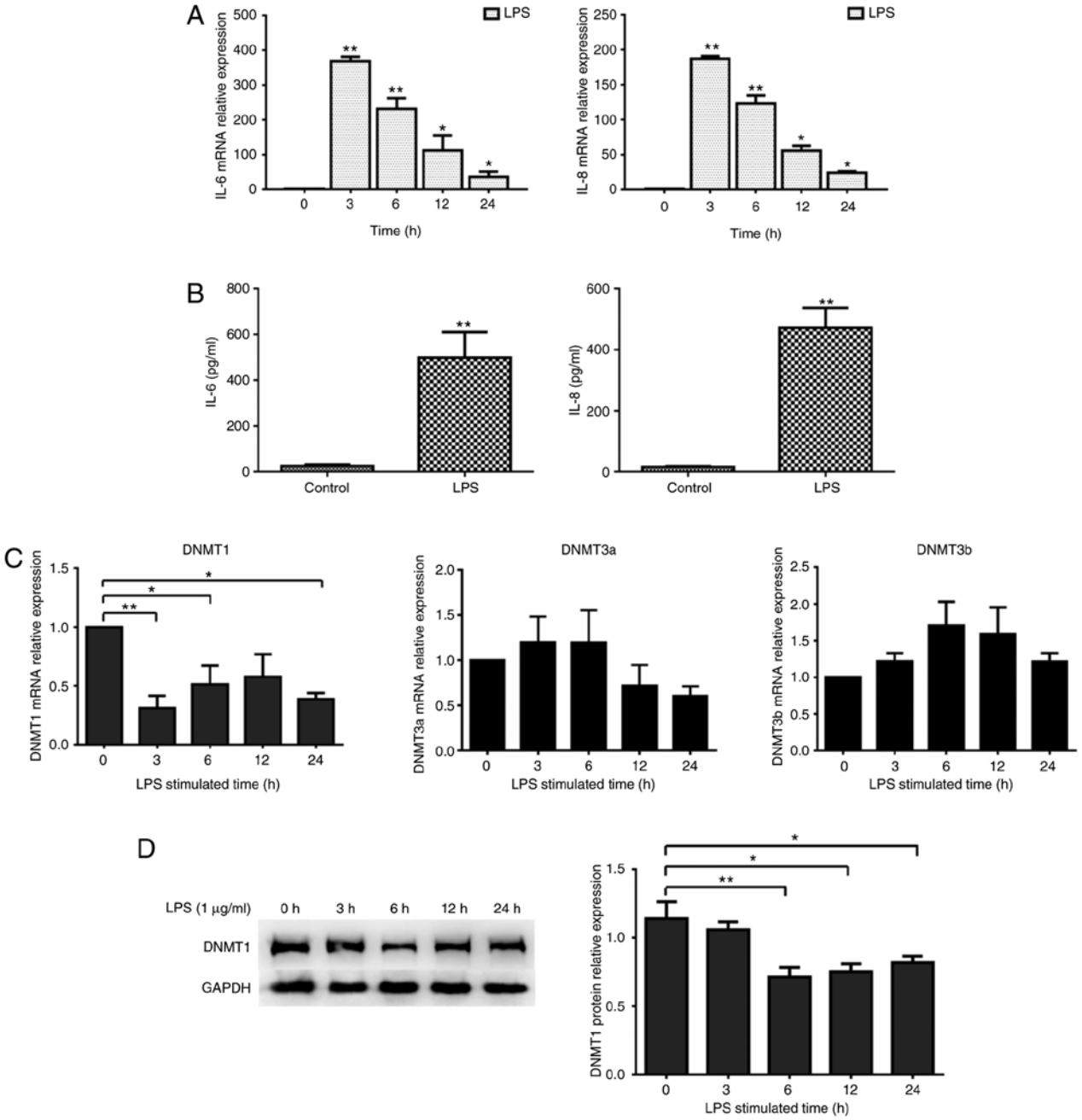

Figure 1. DNMT expression in hDPC inflammatory responses. (A) mRNA expression of *IL-6* and *IL-8* in hDPCs was measured by RT-qPCR after stimulation with LPS for 0, 3, 6, 12 and 24 h; *P<0.05; **P<0.01. (B) Protein expression levels of IL-6 and IL-8 from the supernatant were detected using ELISA; **P<0.01. (C) mRNA expression of *DNMT1*, *DNMT3a* and *DNMT3b* was examined using RT-qPCR in hDPCs after the stimulation with LPS for 3, 6, 12 and 24 h. (D) Protein levels of DNMT1 were determined by western blotting after relative quantitative analysis; GAPDH served as the control. Results were presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01. DNMT, DNA methyltransferase; hDPCs, human dental pulp cells; RT-qPCR, reverse transcription-quantitative PCR; IL, interleukin; LPS, lipopolysaccharide.

thus triggering subsequent inflammatory events (2). DNA methylation is a major epigenetic regulator that can influence the transcriptional expression of pro-inflammatory cytokines in the initiation and development of the inflammatory response (15-17). However, very little research has sought to define the function of DNA demethylation in the development of the LPS-inflamed dental pulp.

DNA methylation plays a pivotal role in a wide range of inflammatory diseases, and aberrant DNA methylation is often observed in some inflammation-related conditions (30). DNMT1 expression is increased in the rectal epithelium during ulcerative colitis progression in patients and may be a relatively early event in ulcerative colitis-associated tumorigenesis; consequently, this factor may be useful for predicting the risk of colorectal neoplasia in ulcerative colitis (31). In periodontitis, treating human oral keratinocytes with LPS downregulated DNMT1 expression (32). In Sjögren's syndrome, the global DNA methylation level in patient salivary gland epithelial cells was reduced, with a 7-fold decrease in DNMT1 but no significant difference in DNMT3a/b expression (33). To determine the relationship between DNMTs and LPS-inflamed dental pulp, the expression of three DNMTs

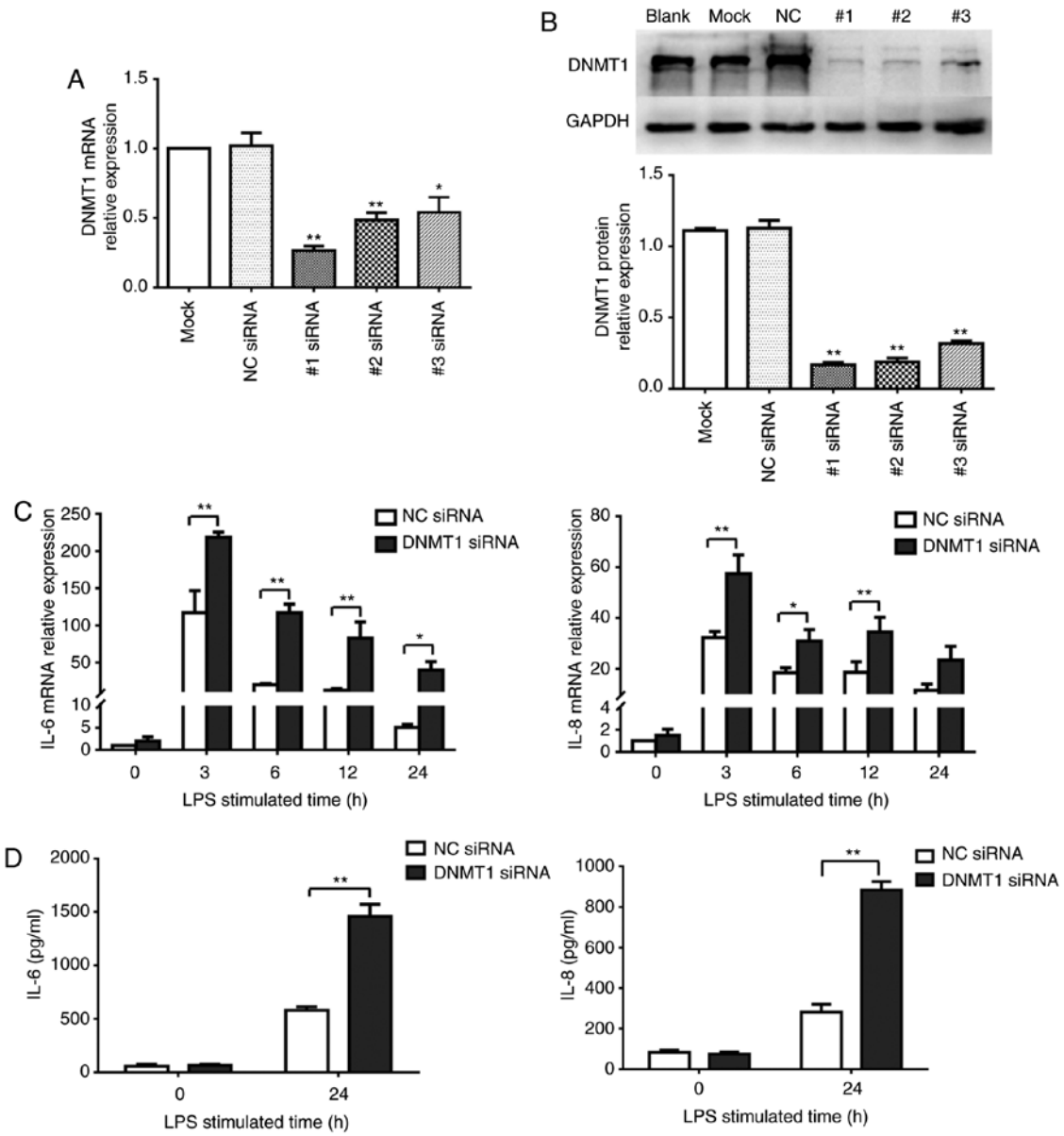

Figure 2. Effects of siRNA-mediated inhibition of DNMT1 expression in hDPCs on LPS-induced inflammatory cytokines. (A) *DNMT1* mRNA expression in hDPCs was assessed by RT-qPCR after interference by *DNMT1* siRNAs for 48 h (#1, #2 and #3). (B) Protein expression levels of DNMT1 were examined through western blotting and relative quantitative analysis was performed; GAPDH served as the control. (C) *IL-6* and *IL-8* mRNA expression levels were detected through RT-qPCR. (D) IL-6 and IL-8 protein levels were evaluated through ELISA. Results were presented as the mean ± SD of three independent experiments; *P<0.05; **P<0.01. siRNA, small interfering RNA; DNMT, DNA methyltransferase; hDPCs, human dental pulp cells; LPS, lipopolysaccharide; RT-qPCR, reverse transcription-quantitative PCR; IL, interleukin; NC, negative control.

in LPS-treated hDPCs were examined. After LPS stimulation, both mRNA and protein expression levels of DNMT1 decreased and reached their lowest level 3 h after stimulation. In addition, the mRNA expression levels of *DNMT3a* and *DNMT3b* fluctuated but did not differ significantly. These results suggested that DNMT1-dependent methylation may be involved in the inflammatory progression of dental pulp.

Researchers previously demonstrated that DNA methylation can function as a key epigenetic regulator in the pathogenesis of inflammation-related diseases (34,35). LPS stimulation can induce pro-inflammatory cytokine expression, and the methylation status of their gene promoters is involved in regulating the inflammatory response. In bovine endometrial cells, treatment with LPS can increase *IL-6* and *IL-8* mRNA expression and decrease the methylation levels of specific CpG sites at the *IL-6* promoter (at -366 and -660) and the *IL-8* promoter (at -120 and -48) (35). Treating PBMCs with LPS induces the expression of pro-inflammatory cytokines, including IL-6, TNF-α and IL-1β, while also demethylating the *IL-6* gene at the -302 and -264 CpG sites, as well as the *TNF-α* gene at the -371 CpG site (36). However, in human intestinal epithelial cells, the 5 CpG sites located near the *IL-8* transcription start site (-83, -7, +73, +119 and +191) were unmethylated on the lower and upper strands in both LPS treated and untreated groups (37). In our previous research, SEQUENOM MassARRAY was used to measure the methylation levels of the *IL-6* and *IL-8* promoters in hDPCs after LPS stimulation. The results showed that the methylation level at the -276 CpG

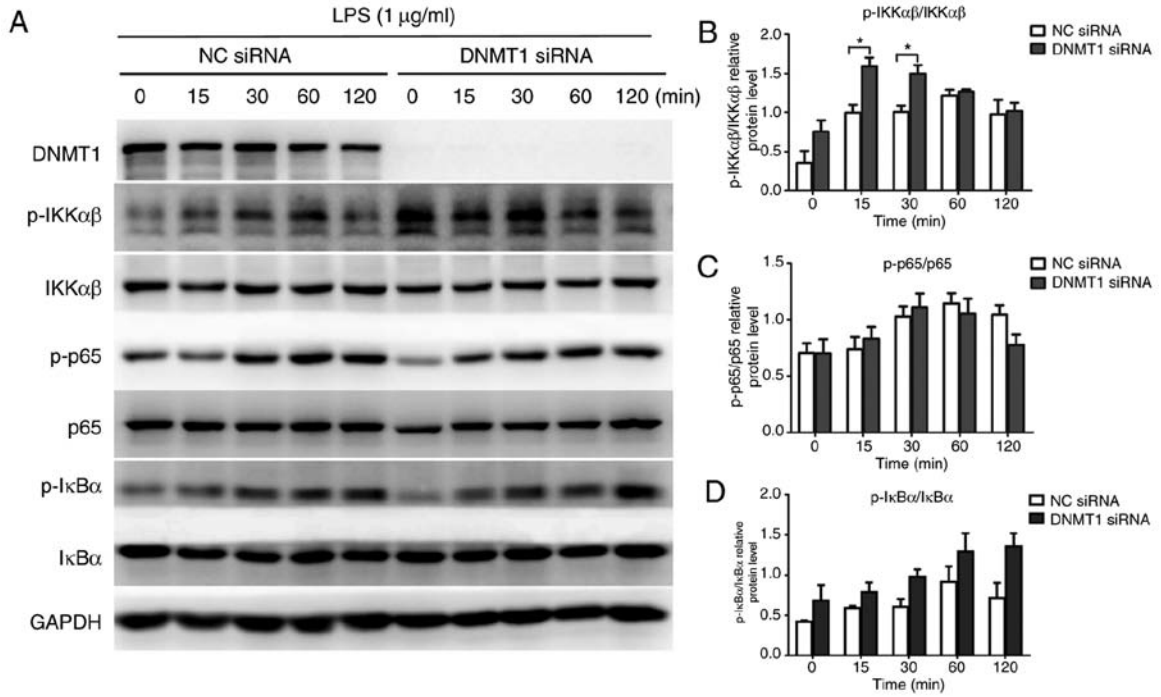

Figure 3. Effects of *DNMT1* knockdown on the activation of NF-κB signaling pathways in LPS-induced hDPCs. (A) Expression of key proteins involved in NF-κB signaling was evaluated using western blotting. The protein samples were collected after a 24 h siRNA transfection followed by LPS stimulation. GAPDH served as the internal reference. (B-D) Vertical histogram reveals the relative quantitative analysis of the phosphorylation levels of the key proteins in the NF-κB pathway in *DNMT1*-depleted hDPCs after stimulation with LPS. All results were represented as the mean ± SD of three independent experiments; *P<0.05. DNMT, DNA methyltransferase; hDPCs, human dental pulp cells; IKKαβ, IκB kinase αβ; LPS, lipopolysaccharide; siRNA, small interfering RNA; NC, negative control; p, phosphorylated.

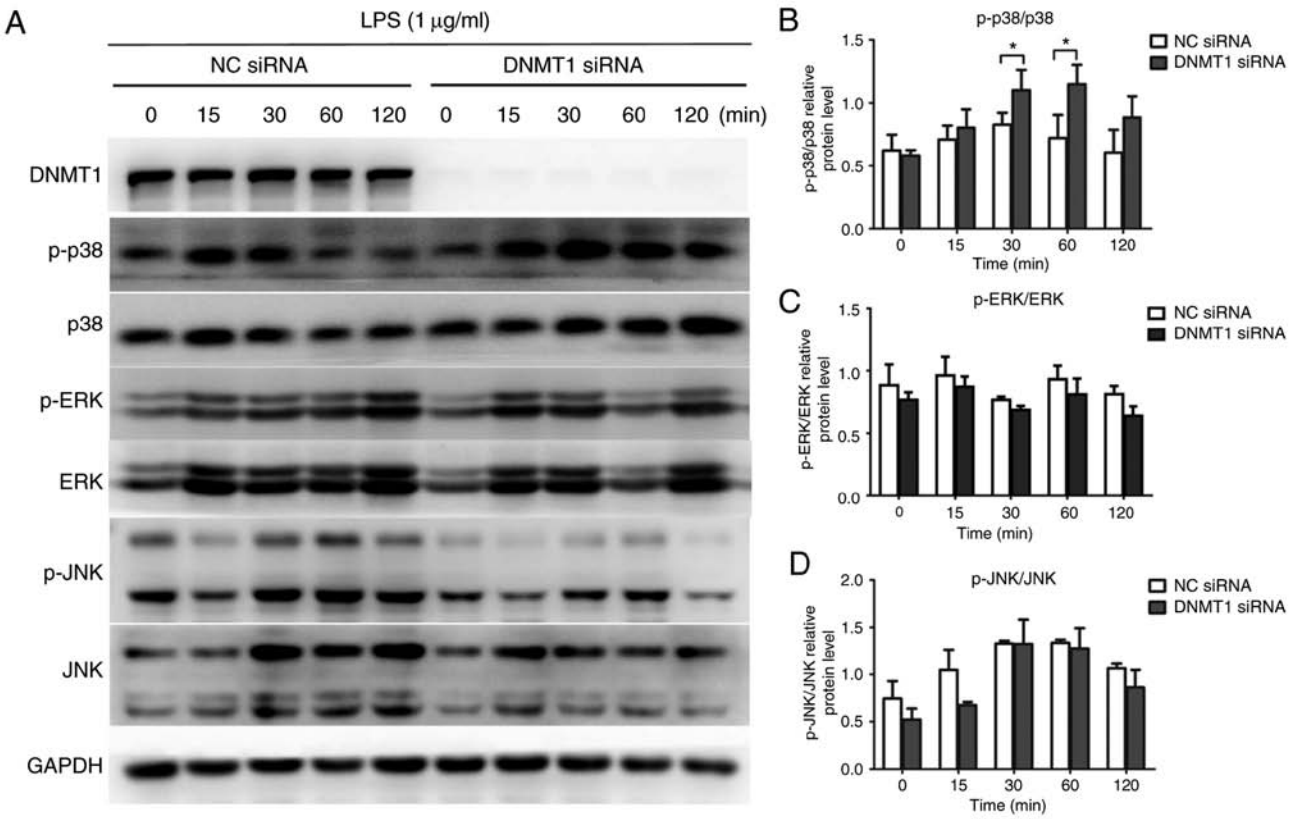

Figure 4. Effects of *DNMT1* knockdown on the activation of MAPK signaling pathways in LPS-induced hDPCs. (A) Expression of key proteins in the MAPK signaling pathway was evaluated using western blotting. The protein samples were collected after 24 h siRNA transfection followed by LPS stimulation. GAPDH served as the internal reference. (B-D) Vertical histogram reveals the relative quantitative analysis of the phosphorylation levels of p38, ERK and JNK in *DNMT1*-depleted hDPCs after stimulation with LPS. Results were represented as the mean ± SD of three independent experiments; *P<0.05. DNMT, DNA methyltransferase; hDPCs, human dental pulp cells; LPS, lipopolysaccharide; siRNA, small interfering RNA; NC, negative control; p, phosphorylated.

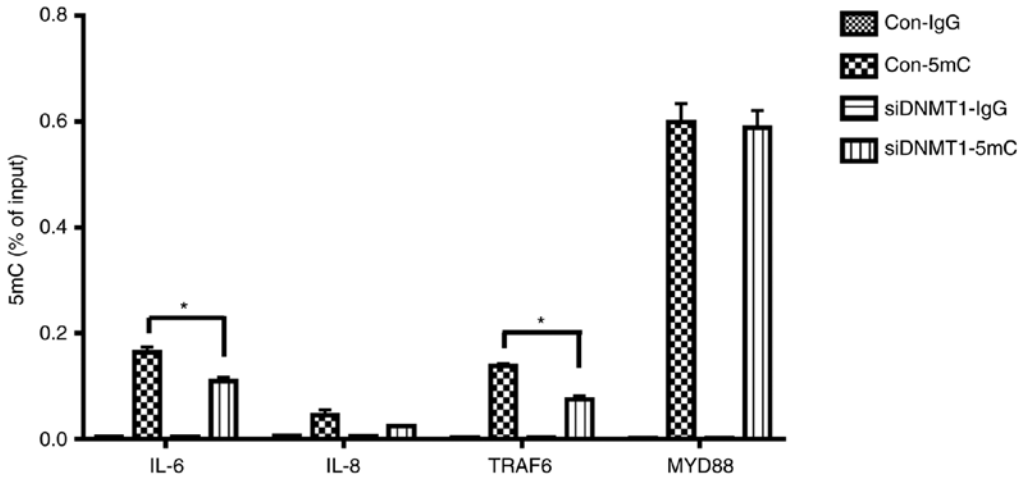

Figure 5. Effects of *DNMT1* knockdown on the 5mC levels of the *IL-6*, *IL-8*, *MyD88* and *TRAF6* gene promoters in LPS-induced human dental pulp cells. *IL-6*, *IL-8*, *MyD88* and *TRAF6* gene promoters' dynamic methylation levels were assessed using methylated DNA immunoprecipitation PCR after DNMT1 knockdown, and after 3 h of stimulation with LPS (control groups). The results were repeated at least three times and presented as the mean ± SD of three independent experiments; *P<0.05. DNMT, DNA methyltransferase; IL, interleukin; LPS, lipopolysaccharide; 5mC, 5-methylcytosine; TRAF6, TNF receptor-associated factor 6; MyD88, myeloid differentiation primary response 88; si, small interfering (RNA).

site in the *IL-6* promoter decreased after LPS stimulation. However, there was no difference in the methylation level of the *IL-8* promoter (unpublished data). In the present study, to investigate the function of DNMT1 in inflammatory cytokine production by hDPCs after LPS stimulation, DNMT1 knockdown in hDPCs was established through siRNA transfection. The expression of DNMT1 was significantly decreased following depletion of DNMT1, which is consistent with our previous research (20,38). Next, the LPS-stimulated cytokine expression after knocking down *DNMT1* was examined. *DNMT1* silencing prominently enhanced the production of the cytokines IL-6 and IL-8, thereby indicating that DNMT1 may be a regulator that negatively targets cytokine accumulation in hDPCs inflamed by LPS.

It is commonly known that the MAPK and NF-κB signaling pathways play critical roles in mediating inflammatory reactions and are likely regulated by DNA methylation (19,39). In aged mouse macrophages, phosphorylation of IκBα in the NF-κB signaling pathway was increased after treatment with the demethylation agent 5-Aza-CdR (40). 5-Aza-CdR also increased IκBα and IKKα/β phosphorylation levels to promote the activation of NF-κB signaling in gastric cancer cells (41). A study on lung tissue inflammation revealed that 5-Aza-CdR can markedly decrease p38, JNK and ERK phosphorylation levels, thereby inhibiting MAPK signaling pathway activation under LPS stimulation (19). The levels of DNA methylation were affected in 27 gene promoters of the MAPK pathway in PBMCs and plasma samples from children who were constantly exposed to air pollutants (42). To explore whether DNA methylation influences the signaling pathways in LPS-treated hDPCs, the phosphorylation levels of several important signaling molecules in the MAPK and NF-κB signaling pathways were examined. The data from the present study showed that compared to LPS exposure alone, *DNMT1* depletion upregulated the phosphorylation levels of IKKα/β in the NF-κB signaling pathway and the phosphorylation level of p38 in the MAPK signaling pathway. Therefore, DNMT1 suppressed both the MAPK and NF-κB signaling pathways in LPS-stimulated hDPCs, further confirming that DNMT1 acts as a negative regulator in inflamed hDPCs.

Previous studies have proposed that DNA methylation not only affects the methylation level of inflammatory cytokine promoters, but also changes the methylation status of intracellular signal transducers of signaling pathways (43). TRAF6 and MyD88, key intracellular signal transducers of the MAPK and NF-κB signaling pathways, can be regulated by DNA methylation (20,21). TRAF6 hypermethylation has been linked to low *TRAF6* gene expression levels in PBMCs during inflammatory bowel diseases (21). In addition, MyD88 was shown to have consistently higher methylation levels in its promoter region in moderate localized aggressive periodontitis (LAP) than in severe LAP (44). In patients with LAP, the methylation level of the MyD88 promoter is negatively associated with several cyto/chemokines, such as IL-8 and IL-6 (44). In the present study, to explore whether these signal transduction factors are regulated by DNA methylation in LPS-treated hDPCs, MeDIP and RT-qPCR were used to analyze the dynamic 5mC levels of the *IL-6, IL-8, TRAF6* and *MyD88* gene promoters in *DNMT1*-deficient cells. Notably, the 5mC levels of the *IL-6* and *TRAF6* gene promoters decreased, suggesting that *DNMT1* knockdown downregulated 5mC at the *IL-6* and *TRAF6* gene promoters. Although a modest decrease in the *IL-8* and *MyD88* gene promoter 5mC levels was observed, there were no significant differences. These observations indicated that DMNT1 can mediate the 5mC level of *IL-6* and *TRAF6* in LPS-inflamed hDPCs.

In summary, the present study showed that stimulating hDPCs with LPS decreased the expression of the DNA methyltransferase DNMT1. *DNMT1* depletion increased LPS-induced cytokine secretion in hDPCs, and activated NF-κB and MAPK signaling. Furthermore, silencing *DNMT1* was involved in downregulating methylation levels at the promoters of *IL-6* and *TRAF6*. This study indicated that DNMT1-dependent DNA methylation plays a role in the inflammatory response of hDPCs stimulated by LPS, and provides a novel rationale for researchers to further reveal the molecular mechanisms of inflamed dental pulp.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QX designed the study and provided scientific leadership to junior colleagues. LC and MZ performed the experiments and statistically analyzed the results. LC wrote the manuscript. QL and DL analyzed data, providing constructive comments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was authorized by the institutional Ethical Review Boards of the Guanghua School of Stomatology of Sun Yat-sen University, and written informed consent for this investigation was provided from all patients who participated in the experiment in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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