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REGULAR ARTICLE

MyD88 hypermethylation mediated by DNMT1 is associated with LTA-induced inflammatory response in human odontoblast-like cells

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Abstract

Dental caries is a chronic, infectious, and destructive disease that allows bacteria to break into the dental pulp tissue. As caries-related bacteria invade the human dentinal tubules, odontoblasts are the first line of dental pulp that trigger the initial inflammatory and immune responses. DNA methylation is a key epigenetic modification that plays a fundamental role in gene transcription, and its role in inflammation-related diseases has recently attracted attention. However, whether DNA methylation regulates the inflammatory response of human odontoblasts is still unknown. In the present study, we investigated the expression of DNA methyltransferase (DNMT)-1 in lipoteichoic acid (LTA)-stimulated human odontoblast-like cells (hOBs) and found that DNMT1 expression showed a decline that is contrary to the transcription of inflammatory cytokines. Knockdown of the DNMT1 gene increased the expression of several cytokines, including IL-6 and IL-8, in the LTA-induced inflammatory response. DNMT1 knockdown increased the phosphorylation of IKKα/β, IκBα, and p65 in the NF-κB pathway and the phosphorylation of p38 and ERK in the MAPK pathway; however, only the NF-κB pathway inhibitor PDTC suppressed both IL-6 and IL-8 expression, whereas inhibitors of the MAPK pathway (U0126, SB2035580, and SP600125) did not. Furthermore, DNMT1 knockdown upregulated the expression of MyD88 and TRAF6 but only attenuated the MyD88 gene promoter methylation in LTA-treated hOBs. Taken together, these results demonstrated that DNMT1 depletion caused hypomethylation and upregulation of MyD88, which resulted in activation of the NF-κB pathway and the subsequent release of LTA-induced inflammatory cytokines in hOBs. This study emphasizes the critical role of DNA methylation in the immune defense of odontoblasts when dental pulp reacted to caries.

Keywords DNA methylation · DNMT1 · Human odontoblast-like cells · Inflammation · Lipoteichoic acid · Myeloid differentiation primary response gene 88

Introduction

As caries are processed into dentine, odontoblasts situated at the periphery of the dental pulp are the first cells encountered by predominantly Gram-positive bacteria and therefore induce the development of inflammatory and immune events in dental pulp (Love and Jenkinson 2002, Durand and VFAR 2006, Staquet et al. 2011). Previous studies have found that odontoblasts could recognize lipoteichoic acid (LTA), the major pathogenic component of Gram-positive bacteria, through the pattern recognition receptor Toll-like receptor (TLR)-2, which is located at the cell surface (Farges et al. 2011; Horst et al. 2009; Morath et al. 2002). When TLR2 was activated using the potent TLR2 synthetic agonist Pam2CSK4 and *Staphylococcus aureus* LTA, human odontoblast-like cell (hOBs) could produce the pro-inflammatory cytokines interleukin (IL)-6 and IL-8 (Carrouel et al. 2013; Farges et al. 2011). Our previous study also showed that LTA could activate TLR2 and downstream signaling pathways in hOBs (Meng et al. 2017).

DNA methylation is a key epigenetic modification that involves the addition of a methyl group from *S*-adenyl methionine (SAM) to the fifth carbon in the pyrimidine ring of cytosine residues to form 5mC, which is catalyzed by a family

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of DNA methyltransferases (DNMTs) (Foulks et al. 2012). DNMT1 is a well-known maintenance methyltransferase that copies methylation patterns to hemimethylated DNA during replication and plays a critical role in epigenetic regulation (Jones 2012). A multitude of studies have examined the relationship between DNMTs and the development of cancer, but only recently, increasing studies have focused on the contribution of DNMTs to the development of inflammatory diseases. After treatment with lipopolysaccharide (LPS), the expression of DNMT1 was downregulated in peripheral blood mononuclear cells (PBMC); DNMT1 could directly mediate the transcription of pro-inflammatory cytokines, such as IL-6, IL-8, and TNF-α, by modulating the methylation level of the gene promoters (Shen et al. 2016, 2017). DNMT1 mediated hypermethylation of SOCS1, a negative regulator of cytokine signals, and then indirectly enhanced the release of LPS-induced pro-inflammatory cytokines in macrophages (Cheng et al. 2014). DNA methylation is also involved in inflammation-related signaling pathways by epigenetically regulating the transcription of TLRs or signal transduction molecules, such as TLR2, MyD88, and TRAF6 (De Oliveira et al. 2011; Shaddox et al. 2017). These studies indicate that DNA methylation epigenetically regulates inflammatory responses by several different mechanisms. However, whether DNA methylation is involved in the epigenetic regulation of dental pulp immunity and the molecular mechanism of DNMT1 mediating the inflammatory response of hOBs are still unknown.

Our previous research found that demethylation treatment with the DNMT inhibitor 5-aza-2′-deoxycytidine (AZA) can upregulate pro-inflammatory cytokine expression in LTA-stimulated hOBs (unpublished data). To further elucidate the regulatory mechanism of DNA methylation in dental pulp immunity, we detected the expression of DNMT1 and the consequences of DNMT1 knockdown on inflammatory cytokines in LTA-induced inflammation of hOBs. We also investigated the activation of downstream signaling pathways and the transcription and methylation level of signal transduction molecules after DNMT1 knockdown.

Materials and methods

Cell culture

Dental pulps were obtained from human healthy third molars (17–25 years old) extracted for orthodontic reasons. All patients enrolled in this study provided written informed consent, and the investigation was approved by the Ethical Review Board of the Guanghua School of Stomatology of Sun Yat-sen University. The hOBs were cultured from dental pulp as previously described (Couble et al. 2000). Briefly, the cells were cultured in complete medium containing Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 μg/mL ascorbic acid, 100 μg/mL penicillin, 100 mg/mL streptomycin (Gibco, Carlsbad, CA, USA), 10 mmol/L b-glycerophosphate, and 10^{-7} mol/L dexamethasone (Sigma-Aldrich, St. Louis, MO, USA). hOBs were incubated at 37 °C and 5% CO_2. The medium was changed every 3 days. When the cells reached 70–80% confluence, they were harvested using trypsin/ethylene diamine tetraacetic acid (EDTA) (Gibco) and subcultured at a ratio of 1:2 or 1:3. Cells from the second or third passage were used for further study.

Cell stimulation

hOBs were stimulated with 10 μg/mL purified *S. aureus* LTA (InvivoGen, San Diego, CA, USA) for the indicated times. Cells without LTA stimulation were used as controls.

In signaling pathway inhibition experiments, after treatment for 1 h with the signaling pathway inhibitors PDTC (Beyotime, China; 30 μM), U0126 (Beyotime, China; 10 μM), SB203580 (Beyotime, China; 20 μM), or SP600125 (Beyotime, China; 20 μM), hOBs were treated with 10 μg/mL LTA for 3 h. Cells without LTA stimulation were used as blank controls.

DNMT1 small interfering RNA transfection

hOBs were seeded in six-well plates at 4×10^5 cells/well in 2 mL of α-MEM supplemented with 10% FBS. After overnight attachment, the cells were transfected with small interfering RNA (siRNA; 50 nM) against the human DNMT1 gene and a nontarget siRNA control with Lipofectamine RNAi MAX (Invitrogen), as described in the manufacturer's instruction. After 24-h transfection, the media were changed to hOB medium with 10% FBS. The sequences for downregulating hDNMT1 in hOBs were as follows: #1siRNA: 5′-GGGA CUGUGUCUCUGUUAUdTdT-3′; #2siRNA: 5′-GCAC CUCAU UUGCCG AAUAdTdT-3′; and #3siRNA: 5′-GAGGCCUAUAAUGCAAAGAdTdT-3′. The negative control siRNA (Catalog No. siN05815122147) was purchased from RiboBio (RiboBio Corporation, Guangzhou, China).

Real-time quantitative polymerase chain reaction

Total RNA was extracted from cells using RNAzol (MRC, Ohio, USA). Two micrograms of RNA was reverse-transcribed for cDNA synthesis using a PrimeScript™ RT reagent kit (TaKaRa, Japan). Quantitative gene expression was determined using the Light Cycler 480 SYBR Green I Master (Roche, Basel, Switzerland) with specific primers according to the manufacturer's instructions. The expression data were normalized to the geometric mean of the housekeeping gene GAPDH. Primer sequences for this experiment

were as follows: IL-6, F: 5′-TGCAATAACCACCC CTGACC-3′, R: 5′-AGCTGCGCAGAATGAGATGA-3′; IL-8, F: 5′-GGTGCAGTTTTGCCAAGGAG-3′, R: 5′-TTCCTTGGGGTCCAGACA GA-3′; DNMT1, F: 5′-GGCT GAGATGAGGCAAAAAG-3′; R: 5′-ACCAACT CGGTACAGGATGC-3′; TRAF6, F: 5′-GGAA ATGAAAAGGAGCCAT AAGTAG-3′, R: 5′-GGCA CAAAGACAGAAACAGAGAA-3′; MyD88, F: 5′-TCTA TTCCTTTTC TCTTGTGTCCCT-3′, R: 5′-CTCT TCCTCTCTCTGTGCTTCATT-3′; and GAPDH, F: 5′-TCTCCTCTGACTTCAACAGCGACA-3′, R: 5′-CCCT GTTGCTGTA GCCAAAT TCGT-3′.

Western blotting

Cells were harvested in protein lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA) containing protease inhibitors (Beyotime, Haimen, China) and incubated on ice for 30 min. Protein concentrations were measured with a bicinchoninic acid (BCA) protein assay (Beyotime, Haimen, China). Thirty micrograms of protein was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was incubated for 1 h at room temperature in TBST containing 5% skim milk to block nonspecific protein binding. Then, the membrane was incubated at 4 °C overnight with the primary antibodies anti-DNMT1 (Cell Signaling Technology, USA; 1: 1000), NF-κB Pathway Sampler Kit (Cell Signaling Technology, USA; 1: 1000), MAPK Pathway Sampler Kit (Cell Signaling Technology, USA; 1:1000), and anti-vinculin (Cell Signaling Technology, USA; 1:2000). After the membrane was washed, it was incubated for 1 h with an HRP-conjugated secondary antibody (Cell Signaling Technology, USA; 1:2000) at room temperature. Antibody binding was visualized with an enhanced chemiluminescence system (Millipore ECL Western Blotting Detection System, Millipore, Billerica, MA, USA), and band densities were obtained and normalized to an internal control and the background using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Cytokine antibody array

The supernatants were collected and centrifuged at 2000 rpm for 20 min to remove cell debris. The expression levels of 42 cytokines in culture supernatants were detected by RayBio® C Series Human Cytokine Antibody Array C3 (RayBiotech, Hercules, CA, USA) according to the manufacturer's instructions. Briefly, the membranes were blocked with a blocking buffer for 30 min. Sample supernatants were added to the membranes and incubated at room temperature for 5 h. After the membranes were washed three times, they were incubated with the antibody cocktail at 4 °C overnight and then blocked with HRP-streptavidin for 2 h at room temperature. The protein spots were observed using an ECL detection reagent and normalized to the control spots.

Enzyme-linked immunosorbent assay

Concentrations of IL-6 and IL-8 in the culture supernatants were analyzed using enzyme-linked immunosorbent assay (ELISA) kits (R&D, Minneapolis, MN, USA) according to the manufacturer's protocols. Optical density (OD) values were measured at 450 nm using a microplate reader. Sample concentrations were calculated according to the corresponding OD value and the concentration of the standard substance.

Bisulfite sequencing PCR

Genomic DNA was extracted from cells using the E.Z.N.A. Tissue DNA Kit (Omega BioTek, Norcross, GA, USA) according to the manufacturer's instructions. The concentration and purity of the DNA samples were measured by using a NanoDrop 2000. The DNA samples were treated with bisulfate using an EZ DNA Methylation-Gold Kit (Zymo Research, USA). MyD88 and TRAF6 CpG islands were predicted on the gene promoter regions, and bisulfite sequencing PCR (BSP) primers were designed using the online MethPrimer software (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). The MyD88 and TRAF6 BSP primer sequences are as follows: MyD88, F: 5′-AGGA TAGGGTGTTTTGTGTTTTTTA-3′, R: 5′-CCAA CTCTACCCTCCACTA TCTCTA-3′; and TRAF6, F: 5′-TTTGGAAAATTTTTTTTAAGAGGTG-3′, R: 5′-TAAA TTTCCTACCAAAAACACCAAC-3′. Taq DNA polymerase (Thermo Scientific, USA) was used for amplifying the targeted regions. PCR reactions were performed on a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were separated on 2% agarose gels and purified using a Universal DNA Purification Kit (TIANGEN, Beijing, China). The purified PCR products were subcloned using a pMD18-T Vector (TaKaRa, Japan) and transformed into chemically competent cells. Eight positive clones for each DNA sample were selected for sequencing (Augct, Beijing, China). The bisulfite sequencing PCR sequencing results were analyzed for methylation using the online quantification tool of Methylation Analysis software (http://quma.cdb.riken.jp/).

Statistical analysis

Each experiment was performed in triplicate and repeated at least three times. All data are shown as the mean ± standard

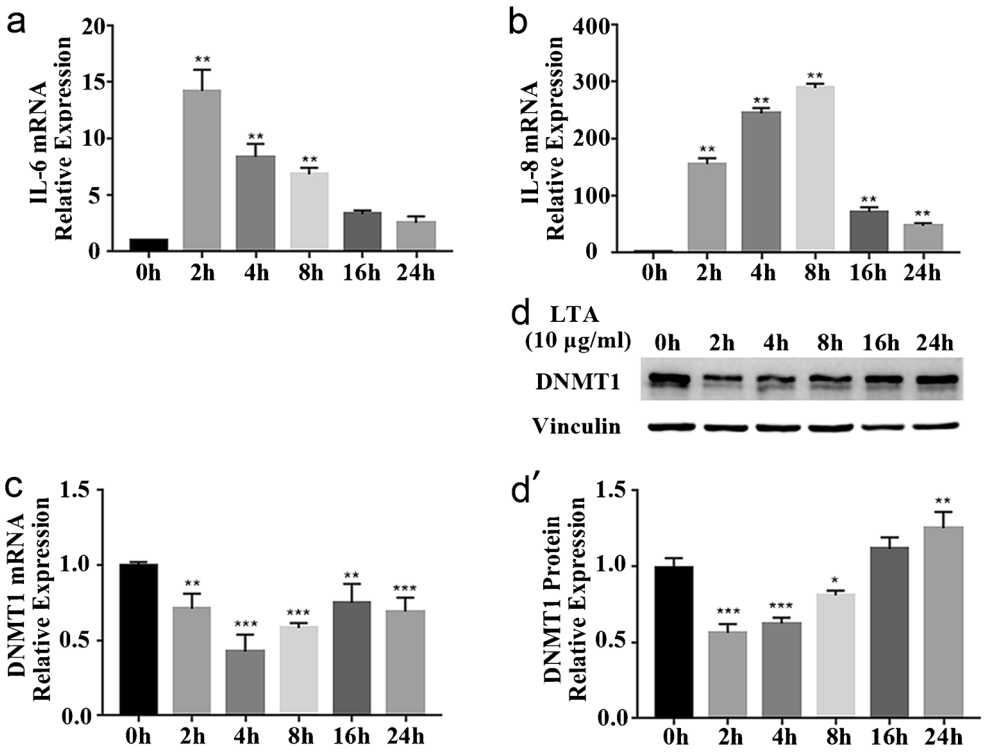


Fig. 1 The expression of DNMT1 in LTA-induced inflammation of hOBs. hOBs were stimulated with 10 μg/mL LTA for the indicated times. (**a**–**c**) IL-6, IL-8, and DNMT1 mRNA expression levels were measured by qRT-PCR. GAPDH was used as a normalization control. (**d**) DNMT1 protein levels were measured by western blotting. Vinculin was used as an internal control. (**d′**) Relative quantitative analysis of DNMT1 protein detected by western blot analysis. All the results are represented as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

deviation (SD). The SPSS 20.0 software package (SPSS Inc., Chicago, IL, USA) was used for the statistical tests. Statistical analyses of the differences between two groups were performed using Student's *t* test. One-way analysis of variance (ANOVA) or repeated-measures ANOVA with a post hoc Dunnett's test were used to assess the differences between multiple sets of data. $P < 0.05$ was considered to be statistically significant.

Results

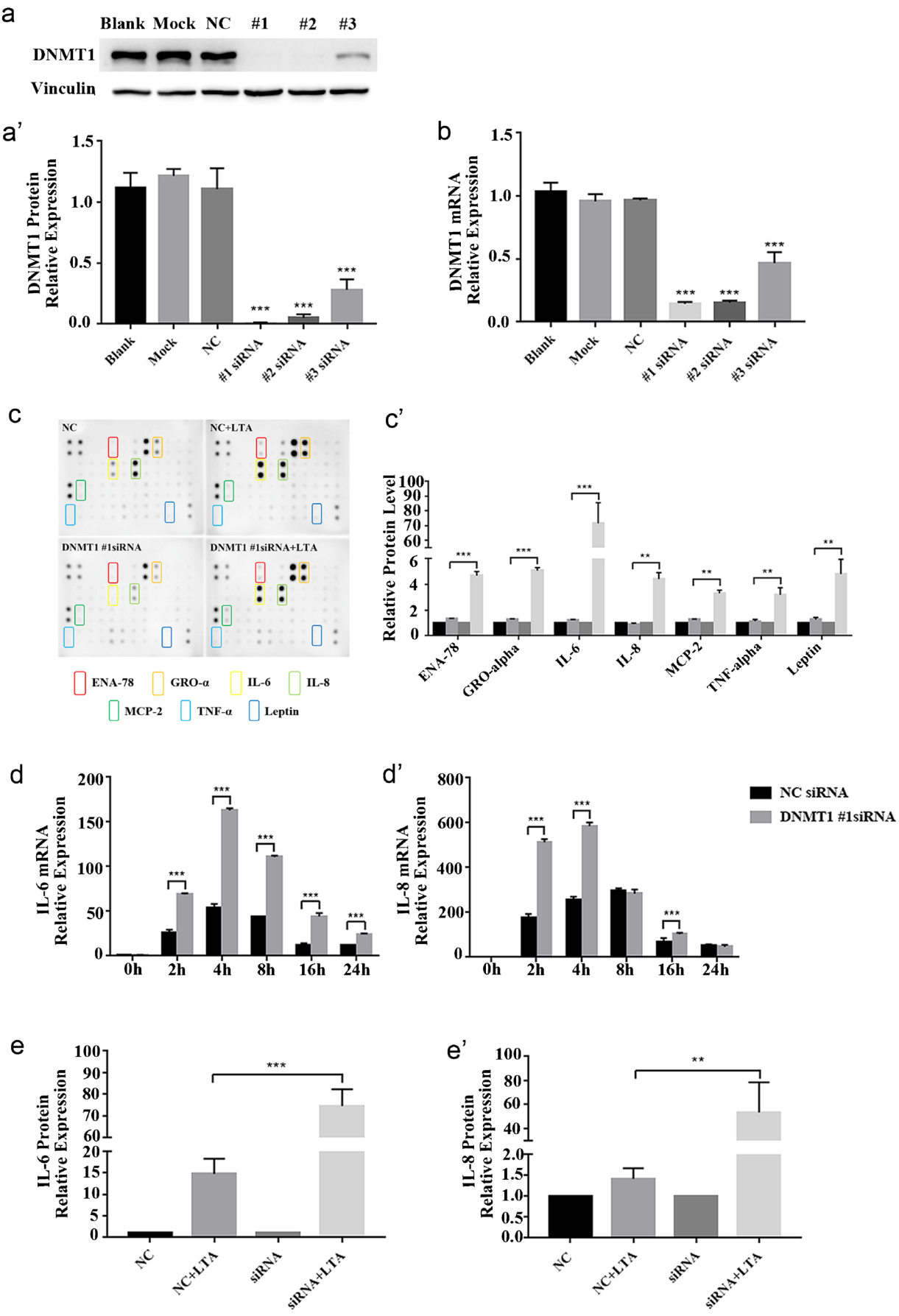
The expression of DNMT1 in LTA-induced inflammation of hOBs

To investigate the effect of LTA on inflammatory response of hOBs, we used 10 μg/ml LTA to treat hOBs for the indicated times (2, 4, 8, 16, and 24 h). As illustrated in Fig. 1a, b, LTA significantly upregulated the expression of IL-6 and IL-8 mRNA compared to that of the control group. The mRNA expression of IL-6 increased and peaked at 2 h and then gradually decreased. Meanwhile, the induction of IL-8 mRNA was observed at 2 h, expression persisted and peaked at 8 h, and then it decreased after 16 h. The DNMT1 mRNA levels were significantly decreased after treatment with LTA within 24 h. DNMT1 protein decreased at 2 h, persisted at low levels until 8 h, and finally increased almost to the control level (Fig. 1c, d, d′).

Effects of DNMT1 on LTA-induced inflammatory cytokine expression in hOBs

To explore the effect of DNMT1 on LTA-induced inflammatory response in hOBs, we transfected the cells with siRNAs. As shown in Fig. 2a, a′, b, DNMT1 mRNA and protein levels were significantly decreased after interference with hDNMT1 siRNA, especially in the #1 siRNA group, which showed a greater than 70% decrease in DNMT1 expression compared with that of the NC group. The results suggested that #1 hDNMT1 siRNA resulted in the most efficient silencing of DNMT1 in hOBs, and this siRNA was utilized in the following experiments.

Fig. 2 Effects of DNMT1 on LTA-induced inflammatory cytokine expression in hOBs. (**a**, **a′**, **b**) DNMT1 knockdown in transfected hOBs was confirmed by qRT-PCR and western blotting. Blank: untreated cells used for the control group; Mock: treated with only transfection reagent; NC: transfected with negative control siRNA; siRNA: transfected with DNMT1 siRNA. (**c**, **c′**) hOBs were transfected with NC siRNA and DNMT1 siRNA separately and then stimulated with 10 μg/mL LTA for 24 h. Cell culture media were collected and subjected to the human cytokine antibody arrays to assess the secretion of 42 cytokines. (**d, d′**) hOBs were transfected with NC siRNA and DNMT1 siRNA separately and then stimulated with 10 μg/mL LTA for the indicated times. IL-6 **(d)** and IL-8 **(d′)** expression levels were measured by qRT-PCR. GAPDH was used as a normalization control. **e**, **e′** hOBs were transfected with NC siRNA and DNMT1 siRNA separately and then stimulated with 10 μg/mL LTA for 24 h. *IL*-6 (**e**) and IL-8 (**e′**) protein levels were measured by ELISAs. All the results are represented as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$


a
Blank Mock NC #1 #2 #3
DNMT1
Vinculin
a'
DNMT1 Protein Relative Expression
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Mock
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#1 siRNA
#2 siRNA
#3 siRNA
b
DNMT1 mRNA Relative Expression
c
NC
NC+LTA
DNMT1 #1siRNA
DNMT1 #1siRNA+LTA
ENA-78
GRO-α
IL-6
IL-8
MCP-2
TNF-α
Leptin
c'
Relative Protein Level
GRO-alpha
TNF-alpha
d
IL-6 mRNA Relative Expression
0h
2h
4h
8h
16h
24h
d'
IL-8 mRNA Relative Expression
NC siRNA
DNMT1 #1siRNA
e
IL-6 Protein Relative Expression
NC
NC+LTA
siRNA
siRNA+LTA
e'
IL-8 Protein Relative Expression

The cytokine antibody arrays were then used to examine the levels of 42 cytokines in LTA-treated hOBs after DNMT1 knockdown. The results showed that DNMT1 knockdown significantly increased the relative protein levels of ENA-78, GRO-α, IL-6, IL-8, MCP-2, TNF-α, and leptin compared to those of the NC group. IL-6 and IL-8 were the most dramatically increased cytokines among the upregulated ones (Fig. 2c, c′). To verify the results of the antibody arrays, we measured the IL-6 and IL-8 mRNA and protein levels by real-time quantitative polymerase chain reaction (qRT-PCR) and ELISA. After treatment with LTA, the IL-6 mRNA levels were increased during the indicated time within 24 h, whereas the IL-8 mRNA levels were upregulated at 2, 4, and 16 h in DNMT1-silenced hOBs compared with the NC cells (Fig. 2d, d′). Moreover, IL-6 and IL-8 protein levels were significantly increased after DNMT1 silencing in LTA-stimulated hOBs (Fig. 2e, e′).

Effects of DNMT1 on the LTA-induced NF-κB signaling pathway in hOBs

The NF-κB signaling pathway is required for pro-inflammatory cytokine expression in LTA-induced inflammation. To explore whether DNMT1 is involved in activation of the NF-κB signaling pathway, we analyzed the phosphorylation levels of IKKα/β, IκBα, and p65 by western blotting. As shown in Fig. 3a, b, b′, b″, silencing of DNMT1 significantly increased the IKKα/β, IκBα, and p65 phosphorylation levels in LTA-treated hOBs. To confirm the effect of the NF-κB signaling pathway on the expression of inflammatory cytokines in DNMT1-knockdown hOBs, we used the NF-κB inhibitor PDTC to block the NF-κB signaling pathway. The results showed that IL-6 and IL-8 promotion by DNMT1 silencing was decreased in LTA-treated hOBs after NF-κB signal transduction was blocked (Fig. 3c, d).

Effects of DNMT1 on the LTA-induced MAPK signaling pathway in hOBs

To determine whether DNMT1 regulated the activation of the MAPK signaling pathway, we assessed another important inflammation-related signal transduction component, the phosphorylation levels of p38, ERK1/2, and JNK. As shown in Fig. 4a, b, b′, b″, both p38 and ERK phosphorylation levels increased after DNMT1 silencing in LTA-treated hOBs, but p-JNK levels were not significantly changed. Interestingly, the ERK inhibitor U0126, the p38 inhibitor SB203580, and the JNK inhibitor SP600125 did not significantly inhibit the mRNA expression of IL-6 and IL-8 (Fig. 4c, d). Overall, these results suggested that the MAPK pathway might be indirectly activated by DNMT1 silencing, but this pathway is not the key signaling pathway of the LTA-induced cytokine transcription in hOBs.

Effects of DNMT1 on MyD88 and TRAF6 expression and methylation in LTA-induced hOB inflammation

MyD88 and TRAF6 are critical for intracellular signal transduction of TLR signaling pathways, and the transcription of the two genes has been reported to be regulated by DNA methylation. To identify whether the transcription of MyD88 and TRAF6 is regulated by DNMT1 in LTA-induced hOB inflammation, we determined the expression and methylation level of MyD88 and TRAF6. The results showed that MyD88 and TRAF6 expression levels significantly increased after silencing DNMT1 in LTA-treated hOBs (Fig. 5a, b). Silencing of DNMT1 decreased MyD88 promoter methylation level from 10.42 to 4.17% (Fig. 5f, f′, f″); however, the methylation level of the TRAF6 promoter did not change (Fig. 5g, g′, g″). These findings suggested that DNMT1 might promote the expression of pro-inflammatory cytokines via modulating the methylation and then regulating the transcription of MyD88 in LTA-treated hOBs.

Discussion

Dental caries is the most prevalent chronic infection in humans and can progress to dental pulp inflammation that will probably lead to periapical periodontitis or jaw osteitis. When caries-related pathogens, predominantly Gram-positive bacteria, invade human dentinal tubules, odontoblasts are the first pulpal cells exposed to pathogens and trigger the initial inflammatory response of dental pulp (Farges et al. 2013; Hahn and Liewehr 2007). Odontoblasts isolated from human dental pulp tissue are post-mitotic terminally differentiated cells and are at levels too low for isolation; consequently, they are difficult to culture and not suitable for mechanistic research (Hosokawa et al. 2016; Paakkonen et al. 2009). A microarray comparison between human native odontoblasts and hOBs showed that the two types of cells have similar gene expression patterns, suggesting that hOBs are a valuable and practical cell line for use in odontoblast studies (Paakkonen et al. 2009). In the present study, hOBs were incubated as described by Couble et al. (2000) and treated with LTA, a major Gram-positive bacterial component, to imitate the classical inflammation model of odontoblasts in vitro, which is a well-established model for studying the functions of odontoblasts in dental pulp immunity (Carrouel et al. 2013; Farges et al. 2011; Keller et al. 2011).

DNA methylation is a key epigenetic modification that has been shown to have a connection with the pathogenesis of cancer and inflammation-related disease (Adcock et al. 2007). In general, continued inflammatory stimulation can affect DNMT expression and activity, which will lead to aberrant inflammatory changes. Previous study has shown that treatment with LPS could downregulate the expression of

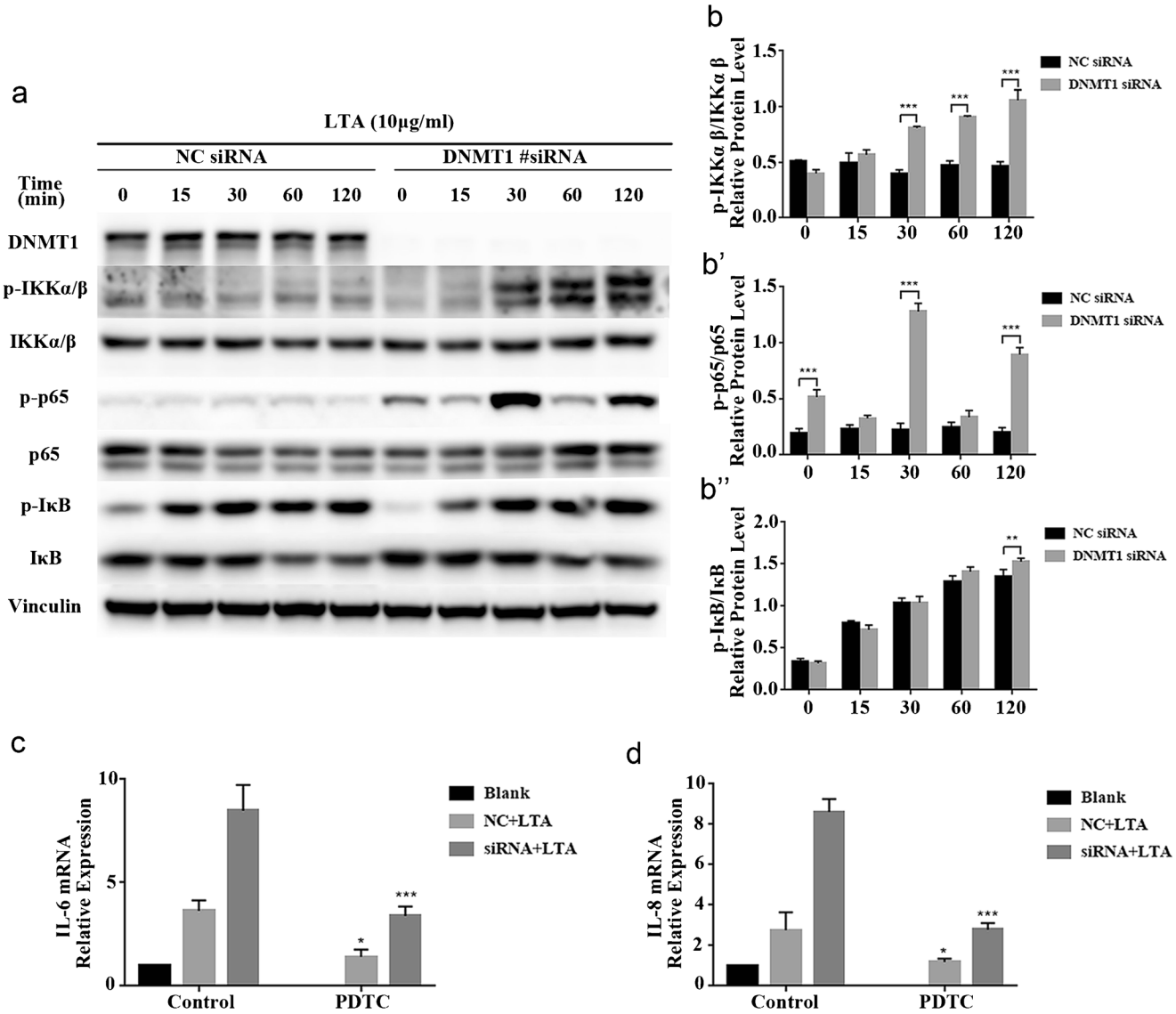


Fig. 3 Effects of DNMT1 on the LTA-induced NF-κB signaling pathway in hOBs. (**a**) hOBs were transfected with NC siRNA and DNMT1 siRNA separately as the NC+LTA group and siRNA+LTA group. After stimulation with 10 μg/mL LTA for 0, 15, 30, 60, and 120 min, the phosphorylated (p-) and total protein levels of IKKα/β, IκBα, and p65 from the NF-κB pathway were examined by western blotting. Vinculin was used as an internal control. **b**, **b′**, **b″** The histogram shows the relative quantitative analysis of IKKα/β, IκBα, and p65 phosphorylation. (**c**, **d**) hOBs were transfected with NC siRNA and DNMT1 siRNA separately as the NC+LTA group and siRNA+LTA group. After treatment with the NF-κB pathway inhibitor PDTC for 1 h, hOBs were treated with LTA for 3 h, and the total RNA was collected to analyze inflammatory cytokine expression. IL-6 and IL-8 expression levels were measured by qRT-PCR. GAPDH was used as normalization control. All the results are represented as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

DNMT1 in HaCaT cells (de Camargo Pereira et al. 2013). In LPS-induced PBMC inflammation, demethylation with AZA treatment enhances the transcription of IL-6 and TNF-α, suggesting that IL-6 and TNF-α can be regulated by DNA methylation (Shen et al. 2017). In our study, the trend of DNMT1 expression levels was contrary to that of IL-6 and IL-8 in LTA-treated hOBs, which indicated that DNMT1 might be involved in the regulation of LTA-induced hOBs inflammation. According to previous studies, the abnormal expression of DNMTs might be due to the regulation of CCL5/CCR5/STAT3 signaling pathway or microRNAs in cancer (Afgar et al. 2016; Wang et al. 2017a, b). But the mechanism of DNMT1 downregulation in inflammatory reaction needs to be further studied.

To explore the effect of DNMT1 on the inflammatory response in LTA-stimulated hOBs, we silenced DNMT1 and detected 42 cytokines related to immunity and inflammation using a cytokine antibody array. The results showed that the expression of seven inflammatory cytokines was significantly upregulated after DNMT1 knockdown. Of these cytokines, IL-6 and IL-8, two representative pro-inflammatory cytokines in LTA-induced hOB inflammation (Carrouel et al. 2013;

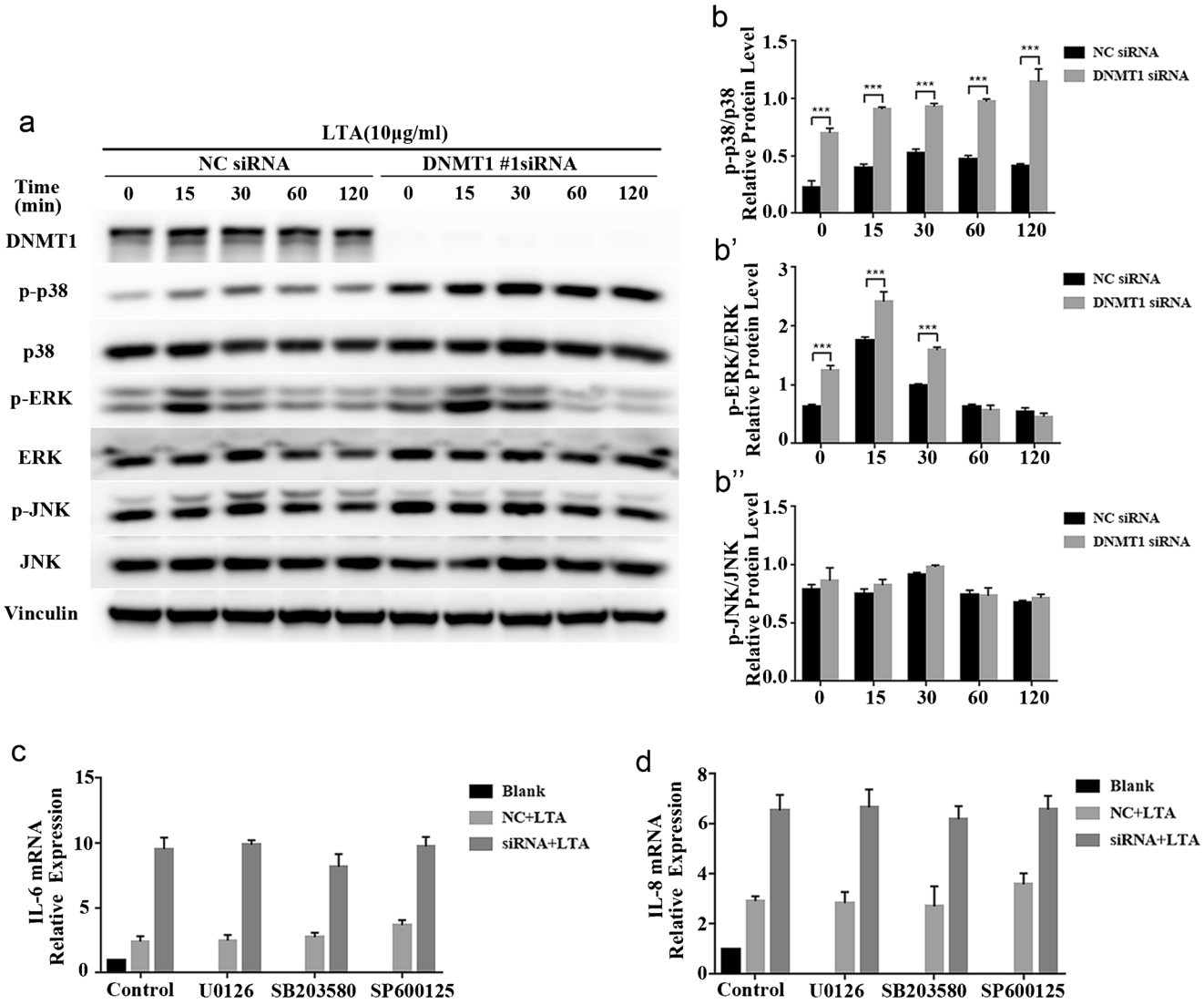
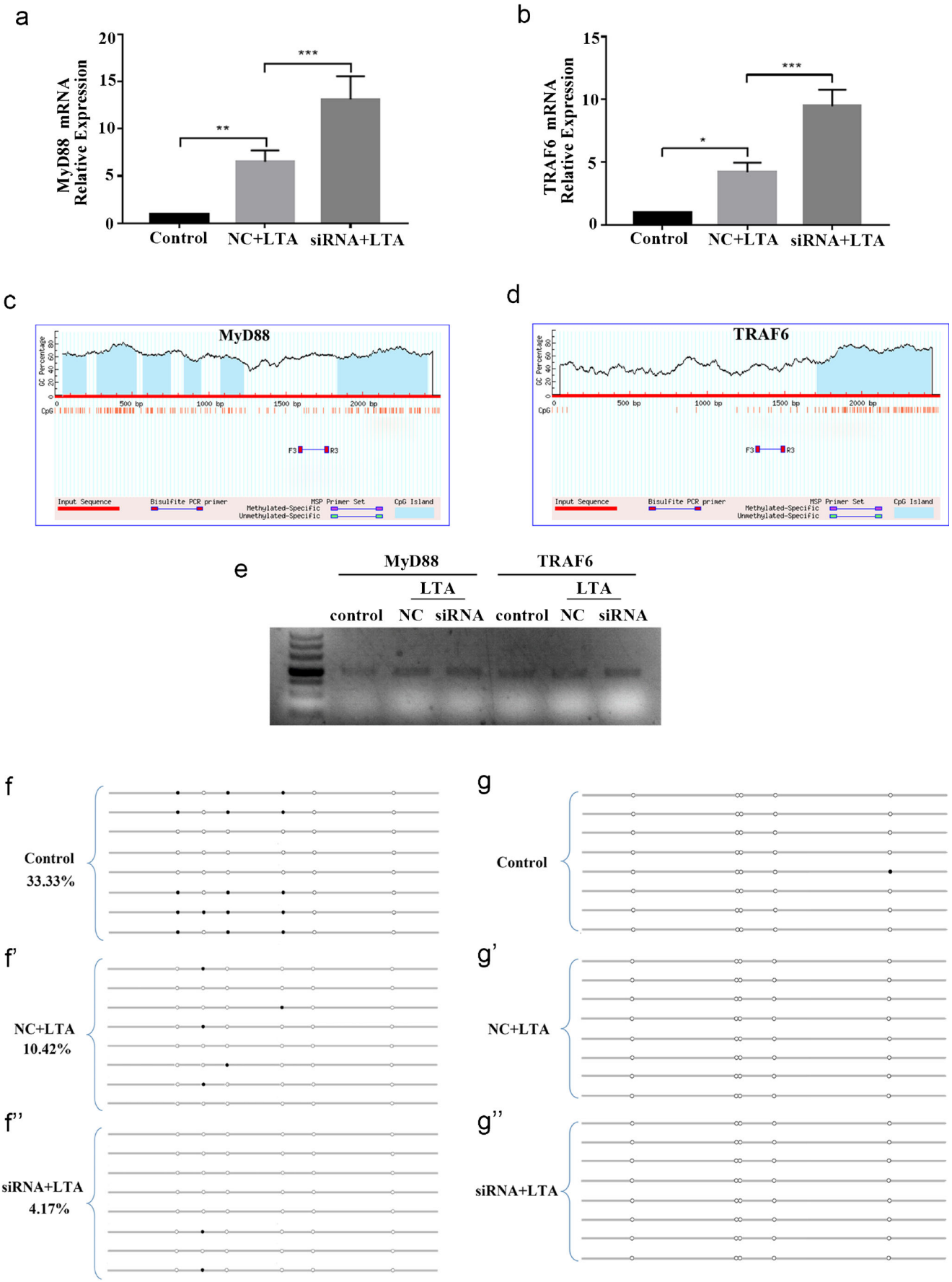


Fig. 4 Effects of DNMT1 on the LTA-induced MAPK signaling pathway in hOBs. (**a**) hOBs were transfected with NC siRNA and DNMT1 siRNA separately as the NC+LTA group and siRNA+LTA group. After stimulation with 10 μg/mL LTA for 0, 15, 30, 60, and 120 min, the phosphorylated (p-) and total protein levels of p38, ERK1/2, and JNK from the MAPK pathway were examined by western blotting. Vinculin was used as an internal control. (**b**, **b′**, **b″**) The histogram shows the relative quantitative analysis of p38, ERK1/2, and JNK phosphorylation. (**c**, **d**) hOBs were transfected with NC siRNA and DNMT1 siRNA separately as the NC+LTA group and siRNA+LTA group. After treatment with the ERK inhibitor U0126, the p38 inhibitor SB203580, and the JNK inhibitor SP600125 for 1 h, hOBs were treated with LTA for 3 h. Then, the total RNA was collected to analyze inflammatory cytokine expression. IL-6 and IL-8 expression levels were measured by qRT-PCR. GAPDH was used as normalization control. All the results are represented as the mean ± SD of three independent experiments. $^{***}P < 0.001$

Farges et al. 2011), were the most dramatically increased cytokines, and their expression levels were further verified by qRT-PCR and ELISA. DNA methylation can regulate the inflammatory cytokine expression by directly modulating their promoter methylation level (Benakanakere et al. 2015; Zhang et al. 2013). Met, which serves as the penultimate methyl donor for methylation reactions, can impact the promoter DNA methylation and mRNA expression of IL-6 in broiler PBMCs (Shen et al. 2017). Demethylation treatment using AZA reduces the methylation status at the IL-8 promoter and drastically enhances PGE2-mediated IL-8 mRNA

Fig. 5 Effects of DNMT1 on MyD88 and TRAF6 expression and methylation in LTA-induced hOB inflammation. (**a**, **b**) hOBs were transfected with NC siRNA and DNMT1 siRNA separately as the NC+LTA group and siRNA+LTA group. After stimulation with 10 μg/ml LTA for 3 h, MyD88 and TRAF6 mRNA levels were measured by qRT-PCR. GAPDH was used as a normalization control. All the results are represented as the mean ± SD of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$. (**c**, **d**) The sits of BSP primers in MyD88 and TRAF6 promoter region. **e** The electropherogram has shown the amplification of the target fragment which has been recovered by cutting glue. MyD88 (**f**, **f′**, **f″**) and TRAF6 (**g**, **g′**, **g″**) promoter methylation levels in the three groups were detected by bisulfite sequencing PCR. White cycle: unmethylated CpG dinucleotides; black cycle: methylated CpG dinucleotides


a
MyD88 mRNA Relative Expression
20
15
10
5
0
**
***
Control
NC+LTA
siRNA+LTA
b
TRAF6 mRNA Relative Expression
15
10
5
0
*
***
Control
NC+LTA
siRNA+LTA
c
MyD88
GC Percentage
CpG
500 bp
1000 bp
1500 bp
2000 bp
F3
R3
Input Sequence
Bisulfite PCR primer
MSP Primer Set
Methylated-Specific
Unmethylated-Specific
CpG Island
d
TRAF6
GC Percentage
CpG
500 bp
1000 bp
1500 bp
2000 bp
F3
R3
Input Sequence
Bisulfite PCR primer
MSP Primer Set
Methylated-Specific
Unmethylated-Specific
CpG Island
e
MyD88
TRAF6
LTA
LTA
control
NC
siRNA
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NC
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Control
33.33%
f'
NC+LTA
10.42%
f''
siRNA+LTA
4.17%
g
Control
g'
NC+LTA
g''
siRNA+LTA

expression in astrocytoma cells (Venza et al. 2012). Surprisingly, our preliminary experiment showed that DNMT1 knockdown did not change the methylation level of IL-6 and IL-8 promoter CpG sites in LTA-treated hOBs (data not shown), contrary to these previous studies. Our results implied that there might be another unknown mechanism during the DNMT1-mediated modification of inflammatory responses in LTA-induced hOBs.

The NF-κB and MAPK signaling pathways play crucial roles in mediating inflammatory response and may be regulated by DNA methylation (Backdahl et al. 2009). Demethylation treatment using AZA can upregulate the phosphorylation levels of IKKα/β and IκBα, which consequently activate the NF-κB signaling pathway and promote the transcription of target genes (Jiang et al. 2012; Kim et al. 2016). In contrast, a previous study of lung tissues proved that AZA significantly blocks the phosphorylation of JNK, ERK, and p38, which suppresses the activation of the LPS-induced MAPK signaling pathway (Huang et al. 2016). In the present study, we detected the activation of several key signaling molecules of the NF-κB and MAPK pathways by western blotting. The results showed that DNMT1 knockdown significantly promoted IKKα/β, IκBα, and p65 phosphorylation levels in the NF-κB pathway and p38 and ERK phosphorylation levels in the MAPK pathway. Furthermore, both NF-κB and MAPK signal pathway inhibitors were used to confirm the roles of the two signal pathways in upregulating IL-6 and IL-8. After the signal pathways were blocked, only the NF-κB inhibitor PDTC significantly decreased IL-6 and IL-8 mRNA expression levels. These data demonstrate that DNMT1 depletion promoted IL-6 and IL-8 expression through activating the NF-κB signaling pathway. NF-κB activation was the primary mechanism underlying DNMT1-mediated inflammatory cytokine expression in LTA-treated hOBs.

Previous studies have proposed that DNA methylation can modulate the development of inflammation by regulating the expression of signal transduction molecules (Shanmugam and Sethi 2013). MyD88 and TRAF6 are two important signal transduction molecules that are induced by activation of TLR2 and lead to the activation of the NF-κB and MAPK signaling pathways (Han et al. 2003; Ryu et al. 2009; Takeuchi and Akira 2001). In localized aggressive periodontitis (LAP) patients, the methylation level of the MyD88 promoter region was negatively related to IL-6 and IL-8 expression (Shaddox et al. 2017). TRAF6 promoter hypermethylation was associated with downregulation of TRAF6 gene transcription in PBMCs during inflammatory bowel diseases (McDermott et al. 2016). When the major methyl donor SAM was fed to alcoholic cirrhosis rats, the increased DNA methylation status decreased in MyD88 and TRAF6 mRNA levels (Bardag-Gorce et al. 2010; Khachatoorian et al. 2013). In our present research, DNMT1 knockdown significantly increased MyD88 and TRAF6 transcription in LTA-treated hOBs. The methylation level of the MyD88 promoter decreased, but the percentage of CpG methylation sites in the TRAF6 promoter did not change. These results indicated that DNMT1 could increase the methylation level of MyD88 and inhibit the transcription of the gene. Our data suggested that DNMT1-mediated downregulation of MyD88 expression could affect the activation of the NF-κB signal pathway, which would consequently regulate the expression of pro-inflammatory cytokines in LTA-treated hOBs.

In summary, we demonstrated that DNMT1 depletion reduced the methylation level of the MyD88 promoter and increased the transcription of MyD88, which consequently triggered the activation of the NF-κB signal pathway and enhanced the expression of pro-inflammatory cytokines in LTA-treated hOBs. These findings indicate that DNA methylation regulates the LTA-induced inflammatory response via modulating MyD88 promoter methylation in hOBs. This study demonstrates the epigenetic regulatory role of DNA methylation in the inflammatory response of odontoblasts and may improve our knowledge of pathogenesis and contribute to finding new therapeutic targets in reversible dental pulp inflammation.

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Compliance with ethical standards

Conflict of interest The authors confirm that there are no conflicts of interest.

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