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Original Article

# Circ_0138960 knockdown alleviates lipopolysaccharide-induced inflammatory response and injury in human dental pulp cells by targeting miR-545-5p/MYD88 axis in pulpitis

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**KEYWORDS**
Pulpitis;
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NF-κB

**Abstract** *Background/purpose:* Circular RNAs (circRNAs) have been shown to play important regulatory roles in many human diseases, yet their functions in pulpitis remain to be clarified. This study was designed to investigate the function of circ_0138960 in pulpitis progression and its underlying mechanism.
*Material and methods:* Cell viability and proliferation were analyzed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and 5-Ethynyl-2′-deoxyuridine (EdU) assay. Flow cytometry and enzyme-linked immunosorbent assay (ELISA) were conducted to analyze cell apoptosis rate and the release of inflammatory cytokines. The activity of superoxide dismutase (SOD) was analyzed using a SOD assay kit. Dual-luciferase reporter and RNA-pull down assays were performed to verify the interaction between microRNA-545-5p (miR-545-5p) and circ_0138960 or myeloid differentiation primary response gene 88 (MYD88).
*Results:* Lipopolysaccharide (LPS) treatment restrained the proliferation and promoted the apoptosis, inflammation, and oxidative stress of human dental pulp cells (hDPCs). LPS treatment dose-dependently up-regulated circ_0138960 expression in hDPCs. Circ_0138960 knockdown overturned LPS-induced inflammation and injury in hDPCs. Circ_0138960 could act as a molecular sponge for miR-545-5p, and circ_0138960 knockdown protected hDPCs from LPS-induced effects by up-regulating miR-545-5p. miR-545-5p directly interacted with the 3′ untranslated region (3′UTR) of MYD88, and MYD88 overexpression reversed miR-545-5p-

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mediated effects in LPS-treated hDPCs. Circ_0138960 positively regulated MYD88 expression by sponging miR-545-5p in hDPCs. LPS could activate nuclear factor kappa-B (NF-κB) signaling by targeting circ_0138960/miR-545-5p/MYD88 axis in hDPCs.
*Conclusion:* Circ_0138960 knockdown attenuated LPS-induced inflammatory response and injury in hDPCs by targeting the miR-545-5p/MYD88/NF-κB axis.


## Introduction

Pulpitis is a common inflammation disease of the dental pulp and is extensively related to oral bacterial infection and the local accumulation of inflammatory cytokines.[1] Human dental pulp cells (hDPCs) are often utilized as an *in vitro* model of pulpitis to analyze its patho-mechanism.[2,3] hDPCs exert critical functions in the inflammation, regeneration, and repair of pulp tissues.[4,5] Lipopolysaccharide (LPS), derived from the outer membrane of Gram-negative bacteria, can trigger the production of various inflammatory factors, containing tumor necrosis factor-a (TNF-a), interleukin-6 (IL-6), and IL-8, thereby invading the dentin and root canal and causing pulpitis.[6] Moreover, LPS can also activate the inflammation-associated nuclear factor kappa-B (NF-κB) pathway to facilitate pulpitis progression.[7] Hence, inhibiting LPS-induced inflammation may be an effective method to suppress pulpitis progression. Here, we used LPS-induced hDPCs to analyze the pathology of pulpitis.

Accumulating evidence has suggested that circular RNAs (circRNAs) can directly interact with microRNAs (miRNAs) to relieve downstream messenger RNAs (mRNAs), resulting in the up-regulation of mRNAs, also known as the competing endogenous RNA (ceRNA) mechanism.[8,9] Sun et al. found that circ_0000105 facilitates liver cancer progression by enhancing PIK3R1 expression via absorbing miR-498.[10] Circ_0138960 has shown to be up-regulated in periodontitis cases by a previous study.[11] Nevertheless, its function in pulpitis progression remains to be clarified.

Circ_0138960 abundance was enhanced in pulp tissues of pulpitis cases. We built a pulpitis cell model *in vitro* by treating hDPCs with LPS (6 μg/mL, 24 h). Knockdown assays were implemented to evaluate circ_0138960 function in pulpitis progression. Finally, the functional correlation between miR-545-5p and circ_0138960 or myeloid differentiation primary response gene 88 (MYD88) in pulpitis progression was explored.

## Materials and methods

### Patients and pulp tissues

Twenty-one cases with pulpitis (male/female: 9/12; 19—48 years) who underwent tooth extraction at The Second Affiliated Hospital of Hainan Medical University were enrolled in this clinical study as the experimental group. Seventeen normal subjects (male/female: 7/10; 17—50 years) who had no pulpitis and experienced tooth extraction were enrolled in this clinical study as the control group. The pulp tissues were collected after receiving the written informed consent and were then preserved in liquid nitrogen. The protocol was authorized by the Ethics Committee of the Second Affiliated Hospital of Hainan Medical University.

### Cell isolation and culture

Pulp tissue was cut into small portions and mixed with 3 mg/mL type I collagenase (Sigma, St. Louis, MO, USA) for 0.5 h. The tissue species were transferred to a new culture plate and cultivated with α-MEM (HyClone, Logan, UT, USA) plus 10% FBS (Gibco, Grand Island, NY, USA). When the confluence reached 70%, hDPCs migrating from the tissues collected and were continued to be cultured. hDPCs in passages 2—5 were used in our study.

### LPS treatment

hDPCs were exposed to LPS (Solarbio, Beijing, China) at 0, 3, 6, or 9 μg/mL for 24 h.

### MTT assay

A Cell Proliferation Reagent Kit (Roche, Shanghai, China) was adopted to analyze cell viability in treated hDPCs. hDPCs were laid onto the 96-well plates ($4 \times 10^3$ cells/well). The next day, cells were incubated with 20 μL 0.5 mg/mL MTT reagent in the incubator. A total of 200 μL DMSO was pipetted to dissolve the insoluble crystals. Cell viability was examined at the wavelength of 570 nm.

### EdU assay

Briefly, $5 \times 10^4$ hDPCs were seeded onto the sterile coverslips, and DNA duplication was labeled via an EdU kit (Ribobio, Guangzhou, China). Cell nuclei were stained with DAPI (Sigma). Cell fluorescence photos were obtained on laser confocal microscopy.

### Flow cytometry

An Annexin V-FITC/PI kit (Beyotime, Haimen, China) was adopted in this assay. $4 \times 10^5$ hDPCs were collected and dispersed in a binding buffer. Subsequently, Annexin V-FITC and PI were pipetted into the binding buffer to mix with hDPCs for 15 min. The apoptosis rate was evaluated via the flow cytometer.

## Western blot assay

Pulp tissues and hDPCs were lysed with RIPA buffer (Beyotime) for 0.5 h. After centrifugation, the supernatant was transferred to new tubes. Protein concentrations were examined via a BCA determination kit (Real-Times Biotech, Beijing, China). After denaturation for 5 min, 30 μg samples were loaded onto 10% separating gel and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was sealed with 5% skimmed milk at room temperature for 1 h. The membrane was labeled with the following primary antibodies at 4 °C, containing anti-Bax (5023S; Cell Signaling Technology, Danvers, Massachusetts, USA), anti-Bcl-2 (3498S; CST), anti-MYD88 (4283S; CST), anti-P65 (8242S; CST), anti-p-P65 (3033S; CST), anti-IκBα (4812S; CST), anti-p-IκBα (2859S; CST), and anti-GAPDH (5174S; CST). Following washing three times with PBS plus Tween-20 (PBST), the membrane was mixed with secondary antibodies (CST) for 1 h. The membrane was analyzed via an ECL detection kit (Sigma).

## Enzyme-linked immunosorbent assay (ELISA)

The production of two inflammatory factors (IL-6 and TNF-α) was analyzed via a Human IL-6/TNF-α Quantikine ELISA Kit (R&D Systems, Minneapolis, Minnesota, USA).

## Measurement of superoxide dismutase (SOD) activity

The activity of SOD was analyzed via a SOD assay kit (Jiancheng Biotech, Nanjing, China).

## Cell transfection

Circ_0138960 siRNA (si-circ_0138960), its matched negative control (si-NC), circ_0138960 overexpression (circ_0138960), its control (pCD5-ciR), miR-545-5p mimics (miR-545-5p), miR-NC, miR-545-5p inhibitor (anti-miR-545-5p), anti-miR-NC, MYD88 expressing plasmid (MYD88), and pcDNA vector (pcDNA) were synthesized or purchased from Ribobio and Genepharma (Shanghai, China). Small RNAs and plasmids were introduced into hDPCs at about 80% confluence via Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

## Real-time quantitative polymerase chain reaction (RT-qPCR)

RNA samples were obtained from pulp tissues and hDPCs using Trizol reagent (Invitrogen). The reverse transcription of miRNAs was conducted via a miRNA cDNA Synthesis kit (Tiangen Biotech, Beijing, China), while the reverse transcription of circRNAs and mRNAs was implemented via a TIANScript II cDNA Synthesis Kit (Tiangen Biotech). Then, for the qPCR of miRNAs, a miRNA RT-PCR kit (Tiangen Biotech) was used, and U6 served as the control. For the qPCR of circRNAs and mRNAs, a PreMix (SYBR-Green) RT-qPCR kit (Tiangen Biotech) was utilized, and GAPDH acted as the control. The primers were displayed in Table 1. The $2^{-\Delta\Delta Ct}$ formula was adopted to analyze relative abundance against U6 or GAPDH.

## Bioinformatics analysis

Bioinformatics tool circinteractome (https://circinteractome.irp.nia.nih.gov) was used to search the possible miRNA targets of circ_0138960, while bioinformatics tool starbase (http://starbase.sysu.edu.cn) was utilized to predict the possible mRNA targets of miR-545-5p.

## Dual-luciferase reporter assay

Based on bioinformatics analysis, the wild-type (WT) and mutant (MUT) binding sequence regions of miR-545-5p in the fragment of circ_0138960 or MYD88 3′ untranslated region (3′UTR) were chemically synthesized by Sangon Biotech (Shanghai, China) and inserted into pmirGLO vector (Promega, Madison, WI, USA). hDPCs were transfected with WT or MUT reporter plasmid (WT/MUT-circ_0138960 and WT/MUT-MYD88 3′UTR; 0.8 μg) and miR-545-5p mimics or miR-NC (100 nM). After transfection for 24 h, cells were disrupted using a dual-luciferase reporter assay kit (Promega), and the luciferase activities were examined using a GloMax 20/20 luminometer (Promega).

## RNA-pull down assay

A total of 2 μg cell lysates were mixed with a 100 pmol biotin-coupled RNA probe (bio-miR-545-5p and bio-miR-NC). Then, a total of 100 μL streptavidin agarose beads were added to incubate with the mixture for 1 h. The enrichment of circ_0138960 and MYD88 mRNA in the precipitated complex was examined by RT-qPCR.

## Statistical analysis

All data were processed by GraphPad Prism 7.0 software and exhibited as mean ± standard deviation (SD). Un-paired Student's *t*-test and one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test were adopted to analyze the differences. Linear regression analysis was conducted using Pearson's correlation coefficient. $P < 0.05$ was deemed as a significant difference.

**Table 1** Primer sequences used for qPCR.

| Name | Direction | Primers for qPCR (5′-3′) |
|---|---|---|
| hsa_circ_0138960 | Forward | TTTGCATGGATTTGAATGACA |
| | Reverse | TGAGAGGCATGGATGTGTGT |
| MYD88 | Forward | GCTCATCGAAAAGAGGTGCC |
| | Reverse | GGTTGGTGTAGTCGCAGACA |
| miR-545-5p | Forward | GTATGAGTCAGTAAATGTTTATTA |
| | Reverse | CTCAACTGGTGTCGTGGAG |
| GAPDH | Forward | AGCTCACTGGCATGGCCTTC |
| | Reverse | CGCCTGCTTCACCACCTTCT |
| U6 | Forward | CTTCGGCAGCACATATACT |
| | Reverse | AAAATATGGAACGCTTCACG |

## Results

LPS exposure dose-dependently suppresses the proliferation and induces the apoptosis, inflammation, and oxidative stress of hDPCs.

hDPCs were treated with LPS (0, 3, 6, or 9 μg/mL) for 24 h *in vitro* to simulate the pulpitis. Cell viability and proliferation were analyzed by MTT assay and EdU assay. We found that LPS exposure dose-dependently suppressed the viability and proliferation of hDPCs (Fig. 1A and B). Flow cytometry showed that cell apoptosis in hDPCs was induced by LPS in a dose-dependent manner (Fig. 1C). We also detected the expression of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 in LPS-induced hDPCs. The data revealed that LPS treatment elevated the protein level of Bax while reducing the protein level of Bcl-2 (Fig. 1D and E), further suggesting that LPS induced the apoptosis of hDPCs. Subsequently, we used ELISA and a SOD assay kit to analyze the inflammation and oxidative stress of hDPCs. LPS treatment dose-dependently induced the release of two inflammatory cytokines (IL-6 and TNF-α) in the culture supernatant of hDPCs (Fig. 1F). LPS treatment reduced the activity of SOD in a dose-dependent manner (Fig. 1G). These results indicated that LPS treatment restrained the proliferation and facilitated the apoptosis, inflammation, and oxidative stress of hDPCs.

### Circ_0138960 knockdown reverses LPS-induced effects in hDPCs

Circ_0138960 was markedly up-regulated in the pulp tissues of pulpitis patients (n = 21) compared with normal subjects (n = 17) (Fig. 2A). Meanwhile, we found that LPS treatment up-regulated circ_0138960 level in hDPCs in a dose-dependent manner (Fig. 2B). RNase R could degrade GAPDH, while circ_0138960 was resistant to RNase R (Fig. 2C), suggesting that circ_0138960 was a circular transcript with high stability. LPS-induced up-regulation of circ_0138960 in hDPCs was overturned by the addition of si-circ_0138960 (Fig. 2D). Circ_0138960 knockdown attenuated the effect of LPS to suppress the viability and proliferation of hDPCs (Fig. 2E and F). LPS-induced apoptosis in hDPCs was diminished by the introduction of si-circ_0138960 (Fig. 2G and H). The silence of circ_0138960

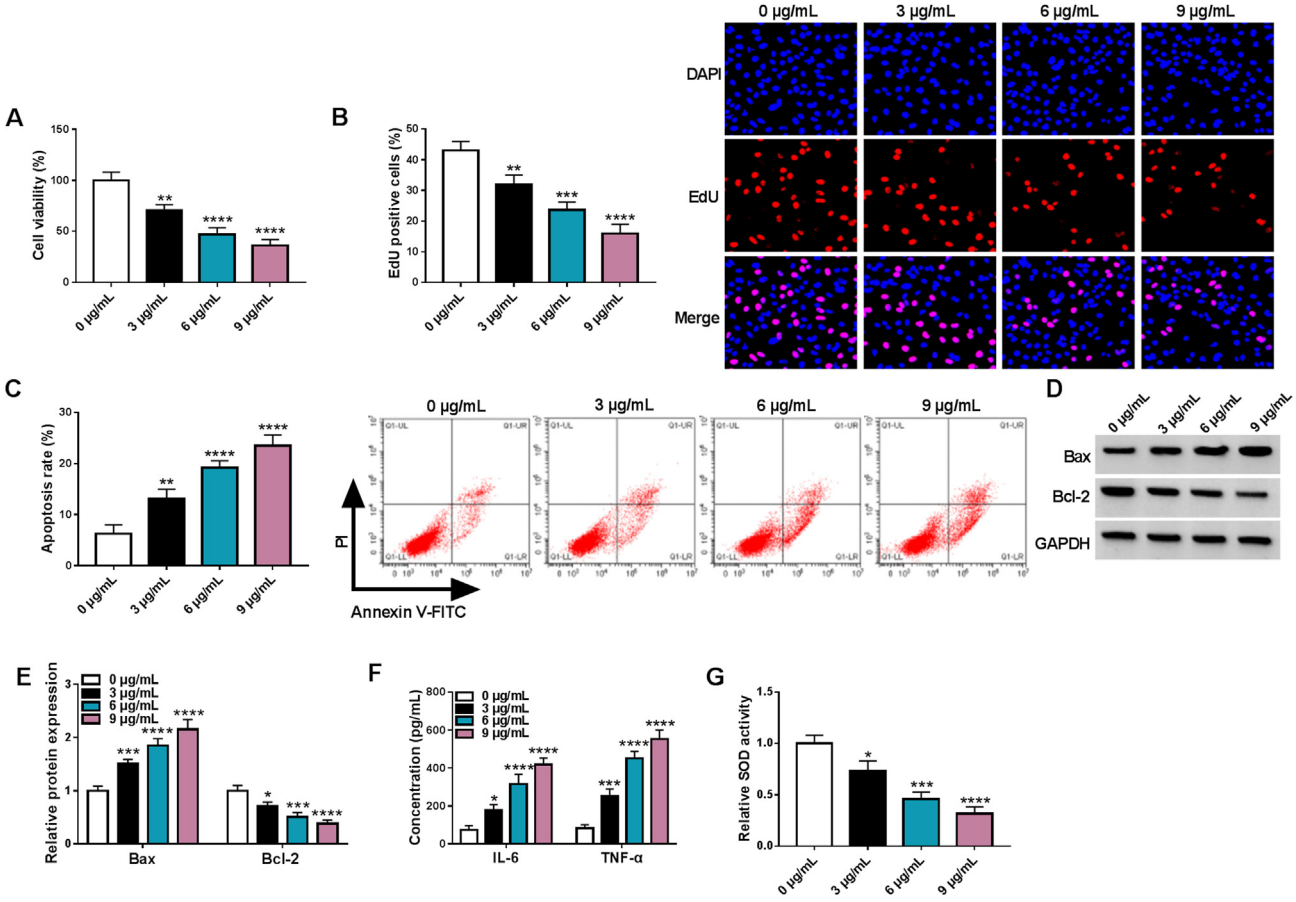


**Fig. 1** LPS exposure dose-dependently suppresses the proliferation and induces the apoptosis, inflammation, and oxidative stress of hDPCs. (A–G) hDPCs were treated with LPS (0, 3, 6, or 9 μg/mL) for 24 h. (A) MTT assay was conducted to measure cell viability. (B) EdU assay was performed to analyze cell proliferation ability. (C) Flow cytometry was conducted to detect cell apoptosis rate. (D and E) Western blot assay was carried out to determine the expression of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 in hDPCs. (F) ELISA was conducted to analyze the concentrations of two inflammatory cytokines (IL-6 and TNF-α) in the culture supernatant of hDPCs. (G) The activity of SOD was analyzed using a SOD assay kit. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

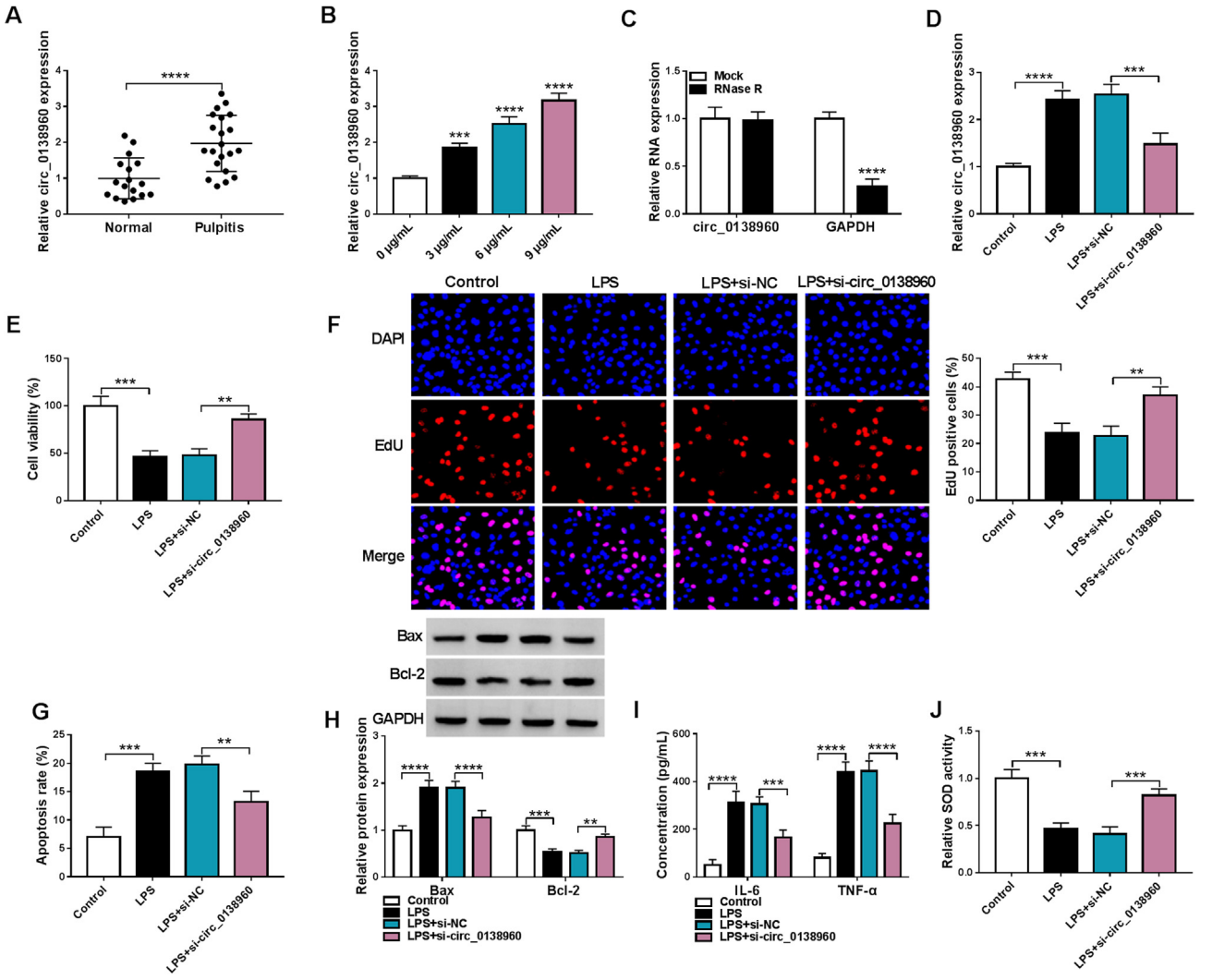


**Fig. 2** Circ_0138960 knockdown reverses LPS-induced effects in hDPCs. (A) RT-qPCR was conducted to measure the expression of circ_0138960 in the pulp tissues of normal subjects (n = 17) and patients with pulpitis (n = 21). (B) hDPCs were treated with LPS (0, 3, 6, or 9 μg/mL) for 24 h, and the expression of circ_0138960 was examined by RT-qPCR. (C) RT-qPCR was performed to detect the levels of circ_0138960 and GAPDH in total RNA samples isolated from hDPCs after RNase R treatment. (D–J) hDPCs were divided into four groups: Control, LPS (6 μg/mL, 24 h), LPS + si-NC, and LPS + si-circ_0138960. (D) RT-qPCR was conducted to measure circ_0138960 expression in treated hDPCs. (E) Cell viability was analyzed by MTT assay. (F) EdU assay was conducted to assess cell proliferation ability. (G) The apoptosis of hDPCs was detected by flow cytometry. (H) The protein levels of Bax and Bcl-2 were determined by western blot assay. (I) The levels of IL-6 and TNF-α in the culture supernatant of hDPCs were examined by ELISA. (J) A SOD assay kit was used to analyze the activity of SOD. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

also reversed the promoting effects of LPS on the inflammation and oxidative stress of hDPCs (Fig. 2I and J). In addition, our data suggested that the overexpression of circ_0138960 might intensify LPS-caused hDPC inflammation and injury (Fig. S1). Taken together, circ_0138960 knockdown protected hDPCs from LPS-induced inflammation and injury.

## miR-545-5p is a direct target of circ_0138960

Having clarified the biological role of circ_0138960 in LPS-induced hDPCs, we intended to uncover its working mechanism. Accumulating articles have suggested that circRNAs can serve as miRNA sponges to regulate cell biological phenotypes.[8,12] We used the bioinformatics tool circinteractome to predict the possible miRNA targets of circ_0138960, and miR-545-5p was one of the candidate targets. The putative binding sequence between circ_0138960 and miR-545-5p was shown in Fig. 3A. RT-qPCR verified the overexpression efficiency of miR-545-5p mimics in hDPCs (Fig. 3B). To confirm the interaction and binding sites between circ_0138960 and miR-545-5p, a dual-luciferase reporter assay was conducted. We constructed the wild-type (WT) or mutant (MUT) reporter plasmid of circ_0138960, which contained the putative or mutant binding sites of miR-545-5p, named WT-circ_0138960 or MUT-circ_0138960. The luciferase activity of WT-circ_0138960 was notably reduced by miR-545-

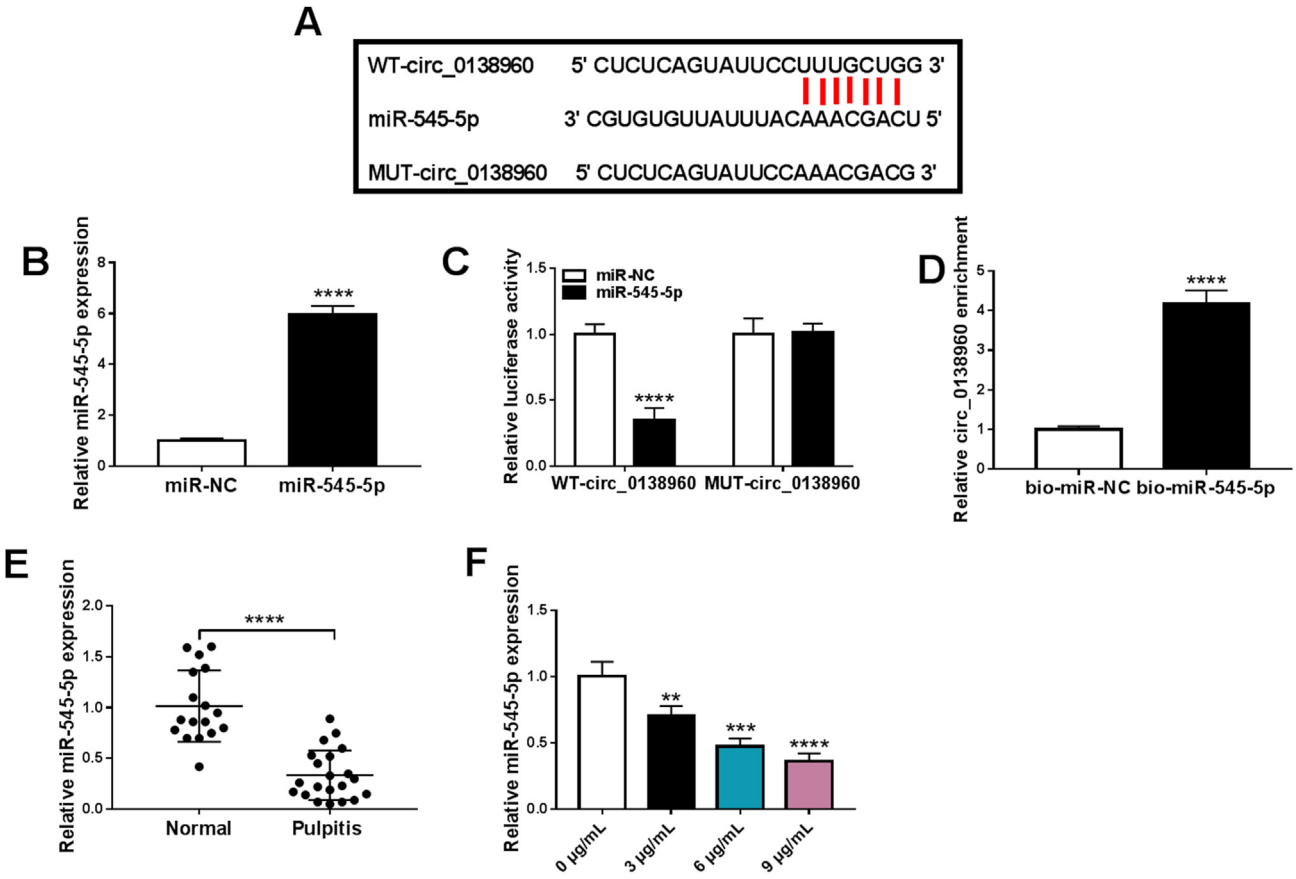


**Fig. 3** miR-545-5p is a direct target of circ_0138960. (A) The binding sequence between circ_0138960 and miR-545-5p was predicted by bioinformatics tool circinteractome. (B) RT-qPCR was conducted to verify the overexpression efficiency of miR-545-5p mimics in hDPCs. (C and D) Dual-luciferase reporter and RNA-pull down assays were conducted to confirm the target relationship between circ_0138960 and miR-545-5p in hDPCs. (E) RT-qPCR was conducted to detect the expression of miR-545-5p in the pulp tissues of normal subjects (n = 17) and patients with pulpitis (n = 21). (F) The level of miR-545-5p was examined in hDPCs treated with LPS (0, 3, 6, or 9 μg/mL) for 24 h by RT-qPCR. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

5p overexpression, while the luciferase activity of MUT-circ_0138960 was unaffected by the transfection of miR-545-5p or miR-NC (Fig. 3C), suggesting that circ_0138960 directly targeted miR-545-5p through the predicted sites. RNA-pull down assay showed that circ_0138960 could be enriched when using a biotin-coupled miR-545-5p probe (Fig. 3D), further suggesting the interaction between circ_0138960 and miR-545-5p. miR-545-5p was down-regulated in the pulp tissues of pulpitis patients (n = 21) compared with normal subjects (n = 17) (Fig. 3E). LPS treatment dose-dependently reduced miR-545-5p expression in hDPCs (Fig. 3F). These results suggested that circ_0138960 directly targeted miR-545-5p in hDPCs.

## Circ_0138960 knockdown-mediated protective effects in LPS-induced hDPCs are reversed by the silence of miR-545-5p

To investigate whether circ_0138960-mediated effects in hDPCs were associated with its target miR-545-5p, we transfected hDPCs with si-circ_0138960 alone or together with anti-miR-545-5p followed by LPS exposure to conduct rescue experiments. Circ_0138960 knockdown elevated miR-545-5p expression in LPS-induced hDPCs, which was reversed by the addition of anti-miR-545-5p (Fig. 4A). The addition of anti-miR-545-5p counteracted the protective effects of circ_0138960 knockdown on the viability, proliferation, apoptosis, inflammation, and oxidative stress of hDPCs upon LPS treatment (Fig. 4B—H). Taken together, circ_0138960 knockdown protected hPDCs from LPS-induced inflammation and injury by up-regulating its target miR-545-5p.

## miR-545-5p directly targets the 3′UTR of MYD88

Previous studies have suggested that miRNAs can regulate the expression of oncogenes and tumor suppressor genes by directly interacting with the 3′UTR of mRNAs, resulting in their degradation or translational repression.[13,14] We searched the possible mRNA targets of miR-545-5p using starbase tool, and MYD88 was one of the candidate targets. The predicted binding sites between miR-545-5p and MYD88 were shown in Fig. 5A. A dual-luciferase reporter assay was conducted to verify the interaction between miR-545-5p and MYD88. We found that miR-545-5p overexpression

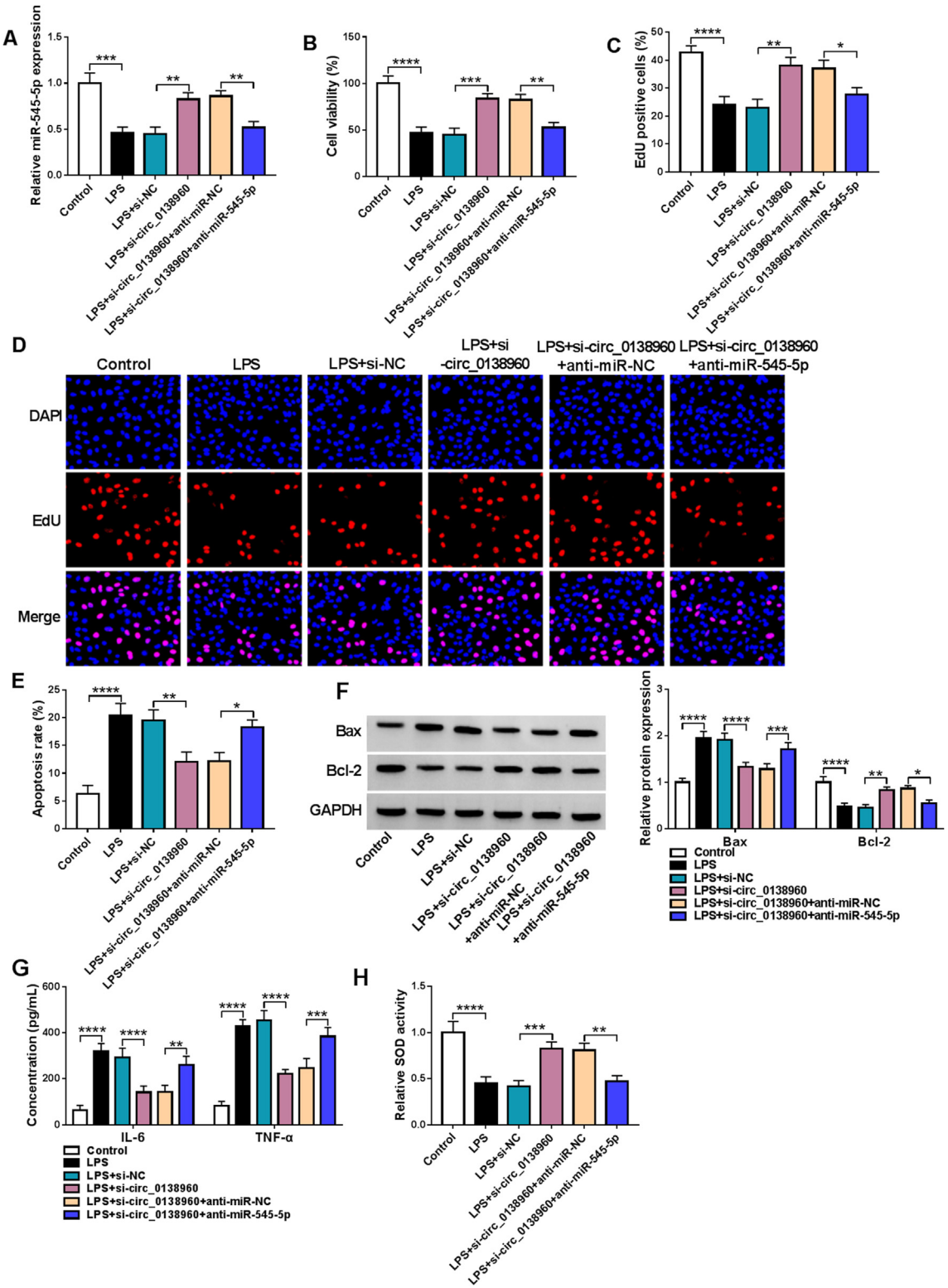


**Fig. 4** Circ_0138960 knockdown-mediated protective effects in LPS-induced hDPCs are reversed by the silence of miR-545-5p. (A–H) hDPCs were transfected with si-circ_0138960 alone or together with anti-miR-545-5p followed by LPS treatment (6 μg/mL, 24 h). (A) The expression of miR-545-5p was detected by RT-qPCR. (B) Cell viability was analyzed by MTT assay. (C and D) Cell proliferation was assessed by EdU assay. (E) Flow cytometry was conducted to analyze the apoptosis of hDPCs. (F) The protein levels of Bax and Bcl-2 were measured by western blot assay. (G) The release of two inflammatory cytokines (IL-6 and TNF-α) in the culture supernatant of hDPCs was analyzed by ELISA. (H) The activity of SOD was analyzed using a SOD assay kit. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

significantly reduced the luciferase activity of wild-type reporter plasmid (WT-MYD88 3′UTR) but not that of mutant reporter plasmid (MUT-MYD88 3′UTR) (Fig. 5B). MYD88 mRNA could be pulled down when using biotin-coupled miR-545-5p probe (Fig. 5C), further suggesting the interaction between miR-545-5p and MYD88. The mRNA expression of MYD88 was up-regulated in the pulp tissues of pulpitis patients (n = 21) compared with normal subjects (n = 17) (Fig. 5D). In addition, miR-545-5p level in the pulp tissues of pulpitis patients was negatively correlated with circ_0138960 or MYD88 mRNA expression (Fig. 5E). Western blot assay showed that MYD88 protein level was up-regulated in the pulp tissues of pulpitis patients (Fig. 5F), which was consistent with its mRNA level. LPS treatment dose-dependently increased MYD88 protein expression in hDPCs (Fig. 5G). These data showed that MYD88 was a direct target of miR-545-5p in hDPCs.

## miR-545-5p overexpression protects hDPCs against LPS-induced effects by down-regulating MYD88

Considering the direct target relationship between miR-545-5p and MYD88, we aimed to explore whether miR-545-5p functioned in hDPCs by targeting MYD88. hDPCs were transfected with miR-545-5p mimics alone or together with MYD88 expressing plasmid followed by LPS exposure to perform rescue experiments. miR-545-5p overexpression reduced MYD88 protein level in hDPCs, which was largely recovered by the addition of MYD88 expressing plasmid (Fig. 6A). miR-545-5p overexpression played protective effects in LPS-induced hDPCs, which were offset by the addition of MYD88 expressing plasmid (Fig. 6B—H). These results indicated that miR-545-5p overexpression-mediated protective effects in LPS-induced hDPCs were based on the down-regulation of its target MYD88.

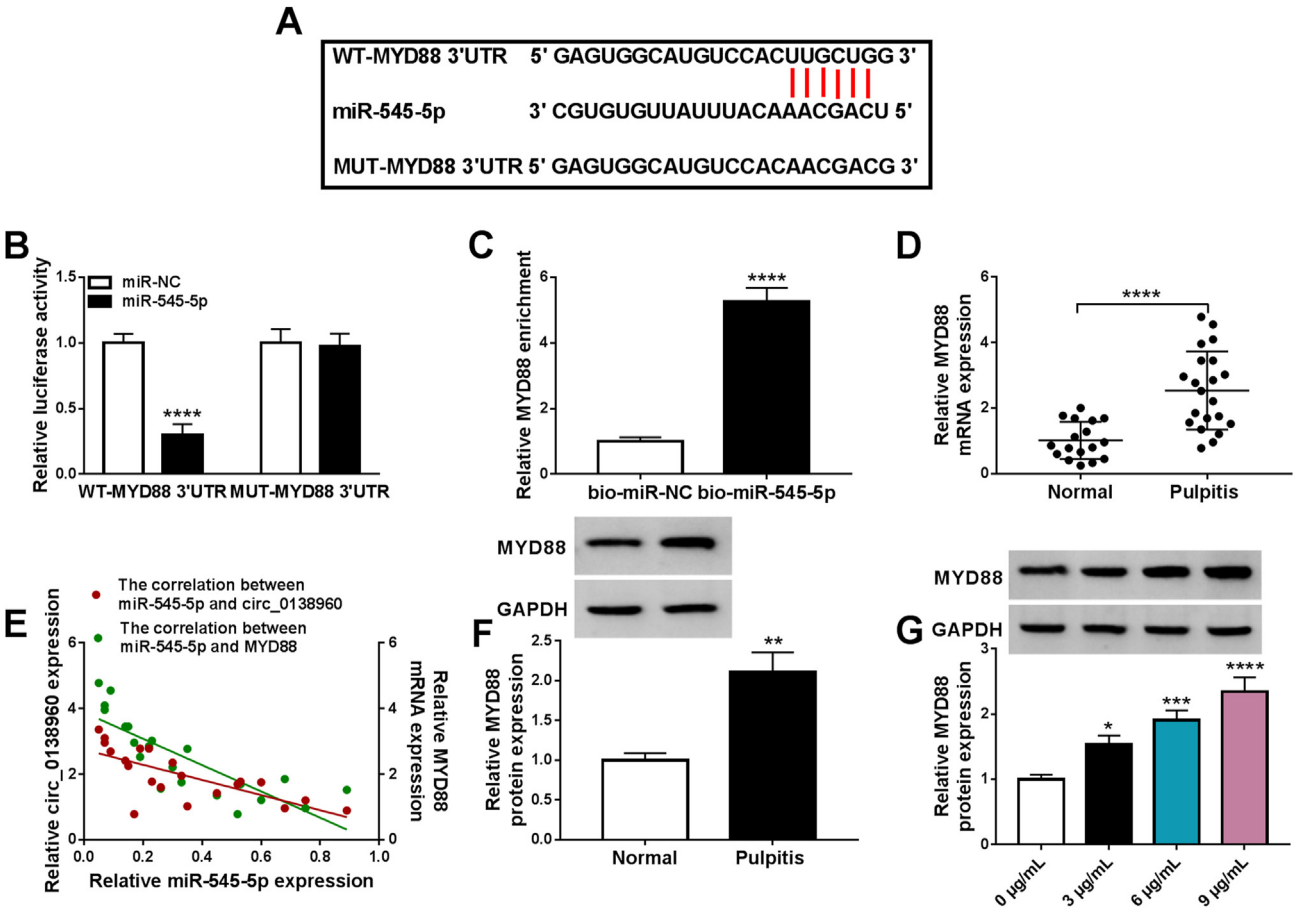


**Fig. 5** miR-545-5p directly targets the 3′UTR of MYD88. (A) The binding sites between miR-545-5p and MYD88 3′UTR were predicted by starbase tool. (B and C) Dual-luciferase reporter and RNA-pull down assays were conducted to confirm the interaction between miR-545-5p and MYD88 3′UTR. (D) The mRNA expression of MYD88 in the pulp tissues of normal subjects (n = 17) and patients with pulpitis (n = 21) was detected by RT-qPCR. (E) Expression association between miR-545-5p and circ_0138960 or MYD88 mRNA in the pulp tissues of pulpitis patients was performed using Pearson's correlation coefficient. (F) Western blot assay was conducted to measure the protein expression of MYD88 in the pulp tissues of normal subjects and pulpitis patients. (G) hDPCs were exposed to LPS (0, 3, 6, or 9 μg/mL) for 24 h, and the protein expression of MYD88 was measured by western blot assay. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

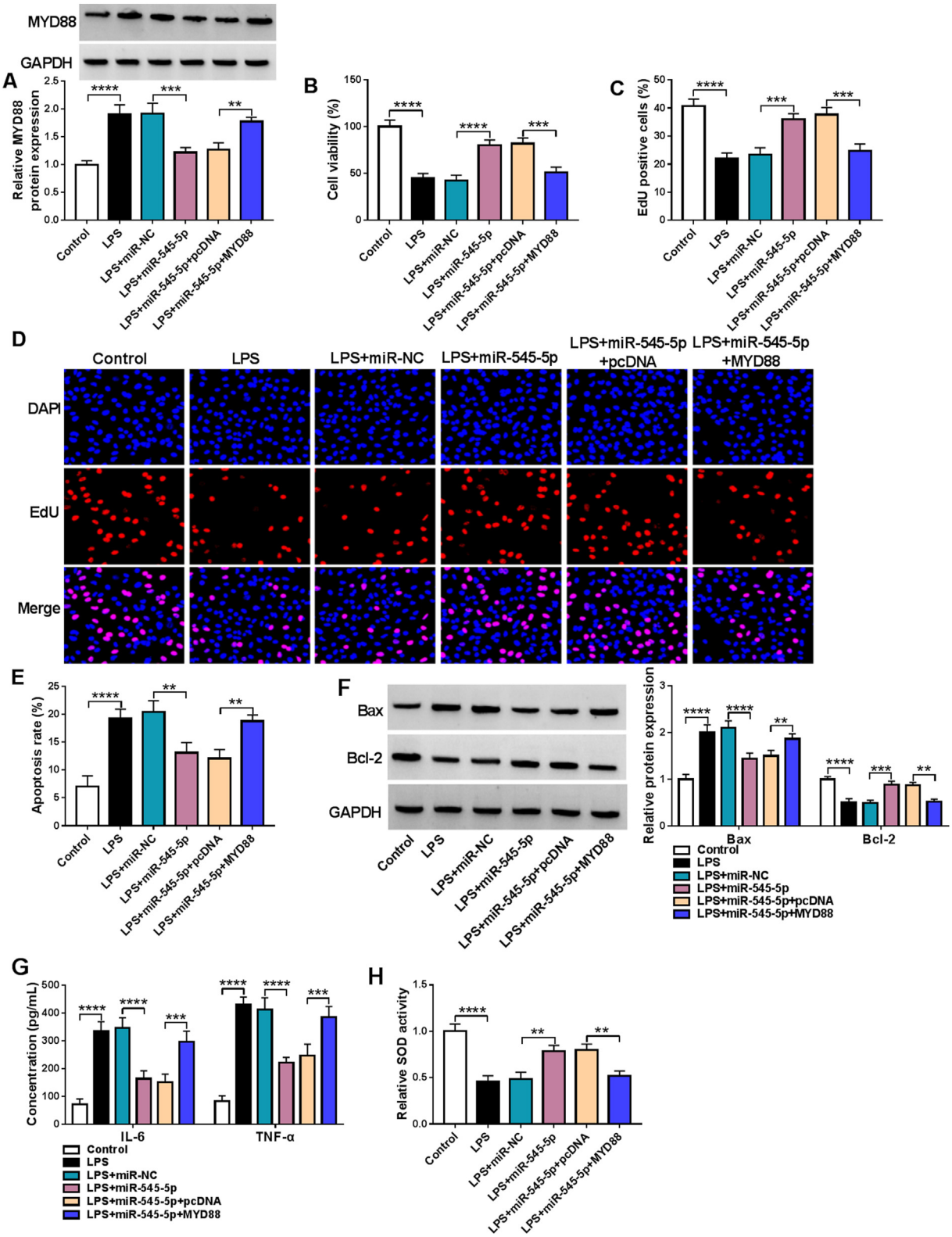


**Fig. 6** miR-545-5p overexpression protects hDPCs against LPS-induced effects by down-regulating MYD88. (A-H) hDPCs were transfected with miR-545-5p mimics alone or together with MYD88 expressing plasmid followed by LPS treatment. (A) The protein level of MYD88 was detected by western blot assay. (B) Cell viability was analyzed by MTT assay. (C and D) EdU assay was carried out to assess the proliferation ability of hDPCs. (E) Flow cytometry was performed to analyze the apoptosis rate of hDPCs. (F) Western blot assay was performed to measure the protein levels of Bax and Bcl-2 in hDPCs. (G) ELISA was conducted to analyze the concentrations of IL-6 and TNF-$\alpha$ in the culture supernatant of hDPCs. (H) The activity of SOD was analyzed using a SOD assay kit. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

### Circ_0138960 knockdown suppresses LPS-induced activation of NF-κB signaling by targeting miR-545-5p/MYD88 axis

We found that circ_0138960 knockdown reduced the protein expression of MYD88, which was largely recovered by the addition of anti-miR-545-5p (Fig. 7A), suggesting that circ_0138960 could regulate MYD88 expression by sponging miR-545-5p. Circ_0138960 silencing reduced the phosphorylation levels of P65 and IκBα, and these inhibitory effects were attenuated by the addition of anti-miR-545-5p or MYD88 expressing plasmid (Fig. 7B), suggesting that LPS induced the activation of NF-κB signaling in hDPCs by targeting circ_0138960/miR-545-5p/MYD88 axis.

## Discussion

Previous articles have pointed out that inflammation exerts a pivotal function in pulpitis pathology.[15,16] In fact, excessive release of inflammatory cytokines has been widely observed in clinical and animal experiments.[17,18] LPS is derived from the outer membrane of Gram-negative bacteria, which can trigger the production of inflammatory cytokines in inflamed pulp tissues such as IL-6 and TNF-α.[6] Furthermore, LPS can activate inflammation-related MAPK-p38 and NF-κB signalings to contribute to pulpitis progression.[7,19] Here, we explored the mechanism underlying pulpitis progression using LPS-exposed hDPCs. We found that LPS exposure restrained cell proliferation and induced

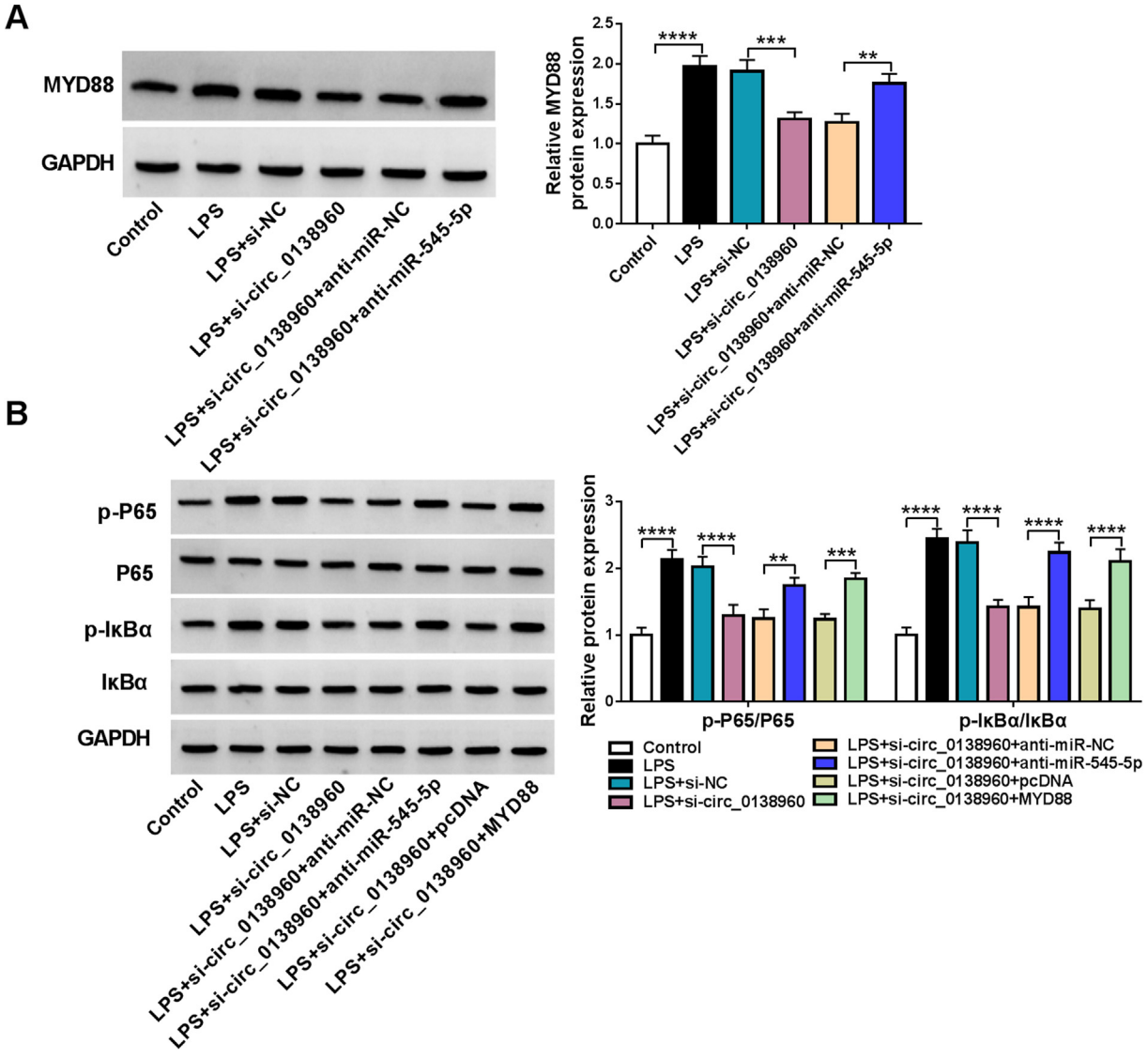


**Fig. 7** Circ_0138960 knockdown suppresses LPS-induced activation of NF-κB signaling by targeting miR-545-5p/MYD88 axis. (A) hDPCs were transfected with si-circ_0138960 alone or together with anti-miR-545-5p followed by LPS exposure (6 μg/mL, 24 h). The protein level of MYD88 was detected by western blot assay. (B) hDPCs were transfected with si-circ_0138960 alone or together with anti-miR-545-5p/MYD88 expressing plasmid followed by LPS exposure (6 μg/mL, 24 h). The levels of p-P65, P65, p-IκBα, and IκBα were detected by western blot assay. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

cell apoptosis, inflammation, and oxidative stress in hDPCs, suggesting the successful establishment of the pulpitis cell model.

Lai et al. reported that circ_0138960 is a candidate oncogene in gastric cancer.[20] A previous article found that circ_0138960 is notably up-regulated in periodontitis patients.[11] However, its role in pulpitis progression remains to be clarified. We confirmed that circ_0138960 abundance was pronouncedly enhanced in the pulp tissues of pulpitis patients and LPS-induced hDPCs. Moreover, circ_0138960 silencing protected hDPCs from LPS-induced inflammation and injury, suggesting that circ_0138960 was a critical contributor to LPS-induced effects in hDPCs. These data suggested that circ_0138960 might be a promising target for pulpitis therapy.

It has been widely accepted that circRNAs can function as ceRNAs of miRNAs to enhance the expression of genes related to multiple signaling pathways, thereby leading to the changes in cell biological phenotypes.[9,12] This signaling axis plays an important role in human diseases. Zhang et al. suggested that circ_0010729 silencing protects cardio-myocytes from hypoxia-mediated damage by modulating miR-370-3p/TRAF6 cascade.[21] Wang et al. found that circ_0007841 contributes to multiple myeloma progression and bortezomib resistance by sequestering miR-129-5p and enhancing JAG1 abundance.[22] We proposed the hypothesis that circ_0138960 regulated pulpitis progression by targeting the miRNA/mRNA axis, and its candidate miRNA targets were searched by circinteractome. We validated the binding relation between circ_0138960 and miR-545-5p for the first time. miR-545-5p was down-regulated in the pulp tissues of pulpitis patients and LPS-induced hDPCs, which was in agreement with a previous article.[23] However, the biological role of miR-545-5p in pulpitis progression is largely undefined. Circ_0138960 knockdown exerted a protected role in hDPCs upon LPS exposure, which was largely overturned by anti-miR-545-5p, indicating that circ_0138960 knockdown protected hDPCs from LPS-mediated inflammation and damage by up-regulating miR-545-5p.

Previous articles suggested that miRNAs can mediate the degradation or hamper the translational process of mRNAs by base-pairing with their 3′UTR.[13,14] Here, we verified that MYD88 was a direct target of miR-545-5p. MYD88 is a typical adaptor protein of inflammation-associated signaling downstream of TLR and IL-1 receptor families.[24] MYD88 connects the IL-1 receptor or the members of the TLR family to IRAK family kinases through homotypic interaction.[25] MYD88 is a pivotal molecule in the inflammatory pathway because the activation of IRAK family kinases induces the activation of NF-κB and MAPKs.[24] A former article by Zhang et al. showed that LPS induces the expression of MYD88 in hDPCs, and S14G-humanin protects hDPCs from LPS-induced inflammation by targeting the TLR4/MyD88/NF-κB pathway.[26] Consistently, we observed that LPS dose-dependently induced MYD88 expression in hDPCs. MYD88 abundance was also enhanced in the pulp tissues of pulpitis cases. miR-545-5p overexpression protected hDPCs from LPS-mediated inflammation and injury, and these protective impacts were overturned by MYD88 expressing plasmid, indicating that miR-545-5p exerted a protective function in LPS-induced hDPCs by down-regulating MYD88. Circ_0138960 could enhance the protein abundance of MYD88 by absorbing miR-545-5p in hDPCs. Furthermore, we found that circ_0138960 knockdown inactivated the NF-κB pathway by targeting miR-545-5p/MYD88 axis in hDPCs upon LPS exposure.

In conclusion, circ_0138960 knockdown alleviated LPS-mediated inflammatory response and damage in hDPCs. Circ_0138960/miR-545-5p/MYD88 signaling was established. Circ_0138960 silencing protected hDPCs from LPS-induced inflammation and injury by targeting miR-545-5p/MYD88/NF-κB signaling. Circ_0138960/miR-545-5p/MYD88 signaling might be a promising target for pulpitis treatment.

## Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2022.06.012.

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