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ORIGINAL ARTICLE

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PRDX6 alleviates lipopolysaccharide-induced inflammation and ferroptosis in periodontitis

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ABSTRACT

Objective: Periodontitis is a progressive and inflammatory oral disease and results in the damage of the supporting tissues of teeth. Peroxiredoxin 6 (PRDX6) is an antioxidant enzyme identified as a regulator in ferroptosis. This study aimed to investigate whether PRDX6 could protect human gingival fibroblasts (HGFs) from lipopolysaccharide (LPS)-induced inflammation and its mechanisms.

Material and methods: Both inflamed and non-inflamed human gingival tissues were collected to assess the expression of PRDX6 and nuclear factor erythropoietin 2-related factor 2 (NRF2) by Immunohistochemistry and Western blotting. Furthermore, the molecular mechanisms of PRDX6 have been clarified in PRDX6 silenced cells. The inflammatory cytokines in HGFs were measured by RT-qPCR and ELISA. The lipid hydroperoxide (LOOH) was detected by C11-BODIPY.

Results: The expression of PRDX6 and NRF2 were decreased in gingival tissues of severe periodontitis patients. The increased LPS-induced LOOH and inflammatory cytokines were found in PRDX6 knockdown HGFs. Besides, the inhibition of ferroptosis or PRDX6 phospholipase A2 activity (PLA2) alleviated LPS-induced inflammatory cytokines and LOOH. However, inhibiting NRF2 signalling upregulated those in HGFs.

Conclusions: Therefore, this study provided a new mechanistic insight that PRDX6, regulated by the NRF2 signalling, alleviates LPS-induced inflammation and ferroptosis in human gingival fibroblasts.

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KEYWORDS
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Introduction

Periodontitis is a chronic inflammatory disease of the periodontium and results in tooth loss. It affects 35%–50% of the adult population worldwide [1–3]. The tissue destruction is mainly caused by pathogenic bacteria-induced inflammatory and host immune responses [4–6]. Other risk factors of periodontitis include smoking, oxidative stress, systemic diseases, etc. [7]. Therefore, exploring the underlying mechanisms of periodontitis is of great significance. Lipopolysaccharide (LPS)-induced reactive oxygen species (ROS) are critical in antimicrobial defense [8]. Human gingival fibroblasts (HGFs) are the most abundant cells in periodontal tissue and the crucial barrier against bacterial infection. It has been reported that HGFs can be activated by LPS and release significant inflammatory factors, which contribute to periodontal tissue destruction [9,10]. Furthermore, the overabundance of ROS production will cause cell damage or death in periodontitis [11–14]. It has recently attracted widespread attention to relieve periodontitis by inhibiting inflammatory response or periodontal cell death.

Regulatory cell death (RCD) was identified as controlled cell deaths and actively mediated through signalling pathways, which was different from uncontrolled passive cell death [14,15]. Common RCD forms contain apoptosis, autophagy cell death, pyroptosis, and necroptosis. Ferroptosis is an iron-dependent RCD, occurs when intracellular lipid peroxidation exceeds the antioxidant capacity, and thus leads to oxidative damage of membranes [16]. Ferroptosis mainly happens through transporter-dependent and enzyme-regulated pathways. The former determines the uptake of glutamine or cysteine, which is required for protein synthesis to control LOOH. The latter mainly refers to glutathione peroxidase 4 (GPX4), the central ferroptosis regulator. It could use glutathione (GSH) as an essential cofactor of enzymatic activity to repair peroxidized membrane damage [17–20]. Peroxiredoxin 6 (PRDX6) is the only member who used GSH to conduct peroxidase activity in the peroxiredoxin family [21,22]. Therefore, PRDX6 has the potential to play a role like GPX4 in ferroptosis.

PRDX6 contains single cysteine and mainly acts PL hydroperoxide GPx (PHGPx) activity, unlike other peroxiredoxins. It also has two additional catalytic sites, phospholipase A2 activity (PLA2) activity and LPC acyl transferase (LPCAT) activity [21,22]. Therefore, PRDX6 plays a paradoxical role in inflammatory and immune responses [23–25]. Furthermore, PRDX6 is regulated by the nuclear factor erythropoietin 2-

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related factor 2 (NRF2) transcription factor, a vital regulator of cellular oxidative stress [26,27]. The activated NRF2 can be transported to the nucleus and subsequently bind to antioxidant response element to improve the transcription of antioxidant genes [28,29]. The microarray results showed that PRDX6 was upregulated in periodontitis [30]. However, the detailed function of PRDX6 and NRF2 in LPS-induced periodontitis has not been explored intensively.

This study showed the increased lipid hydroperoxide (LOOH) levels in LPS-induced periodontitis. Then, we explored the hypothesis that PRDX6 plays a protective role in periodontitis and its underlying mechanisms. Therefore, our study provided a new sight that PRDX6 alleviates LPS-induced inflammation and ferroptosis in periodontitis.

Materials and methods

Reagents and antibodies

Escherichia coli LPS (L8880) was purchased from Solarbio (China) and dissolved in phosphate-buffered solution (PBS). Ferrostatin-1 (Fer-1, 347174-05-4) was purchased from Med Chem Express (MCE, USA) and dissolved in DMSO. MJ33 (C5326) and ML385 (B8300) were purchased from APEXBIO (USA) and dissolved in H_2O and DMSO, respectively. PRDX6 monoclonal antibody (13585-1-AP) was purchased from Proteintech (China). NRF2 monoclonal antibody (12721S) was purchased from CST (USA). The antibody against GAPDH (TA-08) was purchased from ZSGB-BIO (China).

Participant recruitment

Gingival biopsies were obtained from healthy volunteers, and stage III or IV periodontitis patients at the Stomatology Hospital affiliated to Anhui Medical University. The inclusion criteria for all volunteers in this study: (1) age $\geq$ 21 years and the presence of 20 teeth; (2) volunteers without systemic diseases such as diabetes, rheumatoid arthritis, or coronary heart disease; (3) women without breastfeeding or pregnant; (4) no antibiotics, aspirin or anticoagulants were used for recent three months; (5) without periodontal treatment within six months; (6) no habits of alcohol and tobacco. For periodontitis patients, stage III or IV periodontitis were enrolled in this study according to the new classification of periodontitis [31]. It needs to be: (1) multiple sites with probing depth (PD) $\geq$ 6 mm; (2) attachment loss (AL) $\geq$ 5 mm; (3) tooth loss due to periodontitis; (4) vertical bone loss $\geq$ 3 mm. For healthy individuals, additional criteria include: (1) without gingival inflammation; (2) without AL; (3) probing depth $\leq$ 3 mm; (4) no bleeding on probing.

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Health Human Research Ethics Committee of the Anhui Medical University (Permit No. 20200836). Written informed consent has been obtained from all participants. The characteristics of 16 control volunteers and 14 stage III or IV periodontitis patients are listed in Table 1.

Table 1. Characteristics of the study group.

Parameters	Controls	Patients
Case, *n*	16	14
Age, *n*	30.8 ± 5.6	34.2 ± 5.5
Sex, *n* (%)		
Male	7 (44%)	8 (57%)
Female	9 (56%)	6 (43%)
Probing depth (mm)	2.07 ± 0.29	6.59 ± 1.13
Attachment loss (mm)	0	5.31 ± 1.18
Gingival index	0	2.66 ± 0.4

Specimen collection

Healthy gingival tissues were obtained from patients undergoing crown-lengthening surgery with PD $\leq$ 3 mm. Gingival tissues of severe periodontitis were collected from patients undergoing periodontal flap surgery with PD $\geq$ 6 mm and positive bleeding on probing. Each gingival tissue was divided into two sections. One section was used for haematoxylin & eosin (H&E) and Immunohistochemistry (IHC) analysis, and another section was frozen in liquid nitrogen for Western blotting.

Immunohistochemistry

The gingival tissues were collected and fixed in 4% paraformaldehyde overnight. These specimens were embedded in paraffin, serially sectioned (4 μm), and stained with H&E. The IHC accessory kit (ZSGB-BIO, China) was used following the protocol provided. The sections were blocked with 0.03% hydrogen peroxide and 2% bovine serum albumin. Gingival specimens were incubated with primary antibodies against PRDX6 (1:100, Proteintech) and NRF2 (1:100, CST) at 4 °C for 12 h, followed by incubation with anti-mouse secondary antibody (1:100, ZSGB-BIO) or anti-rabbit secondary antibody (1:100, ZSGB-BIO) for 1 h. Then, the samples were counterstained with haematoxylin, dehydrated and mounted. Morphological changes were observed by light microscopy (Leica, Germany).

Cell culture

HGFs were isolated from the non-inflamed gingiva of five donors who needed the healthy third molar extraction. The cells were cultured in DMEM medium (Hyclone, USA) supplemented 10% foetal bovine serum (Corning, USA) and 1% penicillin/streptomycin solution (Beyotime, China) at 37 °C with 5% CO_2 in a cell incubator. Cells at 4–6 passages were used in the following experiment.

Cell viability assay

The Cell Counting Kit-8 (CCK-8, MCE, USA) method was used to access cell viability. HGFs were plated at a concentration of 5000/well in 96-well plates. After drug treatment, the fresh medium (100 μL per well) containing CCK-8 solution (10 μL) was added to each well, followed by incubation at 37 °C for 3 h. Cell viability was measured at 450 nm using a microplate reader (Bio-Tek, USA).

Cytotoxicity assay

The cytotoxicity was measured using the LDH Assay Kit (Beyotime, China), following the manufacturer's instructions. HGFs were seeded into 96-well plates with 5000/well, and the cell medium was used to detect the LDH level. Absorbance was measured at 490 nm using a microplate reader (Bio-Tek, USA).

Enzyme-linked immunosorbent assay (ELISA) assay

IL-6 and TNF-α levels were detected by ELISA kits (DAKEWE, China) following the protocol provided. Briefly, HGFs were plated in 6-well plates at a concentration of 2×10^5 cells. These cells were supplemented with a fresh medium containing different reagents. After incubation time, HGFs culture supernatants were collected for ELISA assay. The samples were detected at 450 nm using a microplate reader (Bio-Tek, USA).

Small interfering RNA (siRNA) transfection

HGFs 2×10^5 were seeded in 6-well plates before transfection; 24 h later, cells were treated with siRNA targeting PRDX6 or a negative control siRNA using Lipo8000 (Beyotime, China) as a transfection reagent. The human PRDX6 siRNA (sequence: Sense: 5′-GGAUAUCAAUGCUUA CAAUTT-3′; Anti-sense: 5′-AUUGUAAGCAUU GAUAUCCTT-3′) was purchased from GenePharma (China). After transfection for 48 h, the efficacy of PRDX6 silencing was analysed by RT-qPCR and Western blotting.

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

Total RNA was extracted from HGFs using TRIzol (Invitrogen) and reverse transcribed to cDNA using the PrimeScript RT Reagent Kit (Takara, Japan) following the manufacturer's instructions. RT-qPCR was performed with SYBR Green Master Mix (Takara, Japan) on a Stratagene Mx3000P system (Agilent Technologies, USA). The relative gene levels were analysed using the $2^{-\Delta\Delta CT}$ method. The primer sequences used are listed in Table 2.

Western blotting

Human gingival tissues and cell samples were lysed in RIPA lysis (Beyotime, China). The total protein concentration was detected by Bradford Protein Assay Kit (Beyotime, China). After that, one-fourth volume of 5× SDS loading buffer was added in protein samples and heated at 99 °C for 10 min. Proteins (20 μg/each) were separated by 10% SDS-PAGE and then transferred to PVDF membranes (Millipore, USA). Membranes were blocked with 5% BSA diluted in PBS for 1 h at room temperature and then incubated with primary antibodies against PRDX6 (1:1000, Proteintech), NRF2 (1:1000, CST) and GAPDH (1:1000, ZSGB-BIO) overnight at 4 °C. After washing with Tris Buffered saline Tween (TBST) three times, membranes were incubated with appropriate HRP-conjugated secondary antibodies for 1 h. After washing three times by TBST, the labelled protein was visualized by an imaging system (Alliance Mini 4 M; UK) and quantified using Image J (Bethesda, USA).

Table 2. Primer sequences for RT-qPCR.

Gene	Primer sequence
PRDX6	F: 5′-ACCACTGGCAGGAACTTTGATGAG-3′ R: 5′-GGCTTCTTCTTCAGGGATGGTTGG-3′
NRF2	F: 5′-TCCAAGTCCAGAAGCCAAACTGAC-3′ R: 5′-GGAGAGGATGCTGCTGAAGGAATC-3′
IL-6	F: 5′-TTCGGTCCAGTTGCCTTCT-3′ R: 5′-GGTGAGTGGCTGTCTGTGTG-3′
TNF-α	F: 5′-ATGGCGTGGAGCTGAGAGAT-3′ R: 5′-TCTGGTAGGAGACGGCGATG −3′
β-Actin	F: 5′-GCCAACACAGTGCTGTCTGG-3′ R: 5′-CTCAGGAGGAGCAATGATCTTG-3′

Determination of lipid hydroperoxide (LOOH) generation

Intracellular LOOH in HGFs was detected using C11-BODIPY (581/591) (#D3861, Invitrogen, USA). HGFs were seeded at a concentration of 1.5×10^5 cells/well in a 6-well plate with different treatments. Cells of each group were harvested by trypsinization incubated in 500 μL C11-BODIPY reagent (2 μM) for 40 min at 37 °C. Then, cells were washed in PBS three times. Finally, the cells were resuspended in 500 μL PBS and analysed using flow cytometry (CytExpert, USA). Samples can be excited at 488 nm, and the data were detectable in the FL1 channel.

Statistical analysis

Representative data were collected from three or more times independent experiments. All statistics were analysed using Graphpad Prism 9. Data were displayed as means ± SD. Statistical significance between two independent groups was performed on the data using unpaired Student's *t*-test. The difference among three or more groups was checked by one-way analysis of variance (ANOVA) followed by Tukey test or Dunnett test, or two-way ANOVA analysis followed by Bonferroni post hoc test. Statistical significance between the groups was described as follows: $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

Results

The expression of PRDX6 and NRF2 decreased in inflamed gingival tissues

We first determined the effect of periodontitis on the expression levels of PRDX6 and NRF2. H&E staining results showed that the gingival tissues of severe periodontitis patients exhibited inflammatory cell infiltration, while healthy gingival tissues exhibited healthy architecture (Figure 1(A)). The IHC and Western blotting results showed that PRDX6 decreased in severe periodontitis gingival tissues (Figure 1(B,D)). NRF2 is a primary transcription factor that can activate antioxidant genes to defend against oxidative stress. We also assessed the

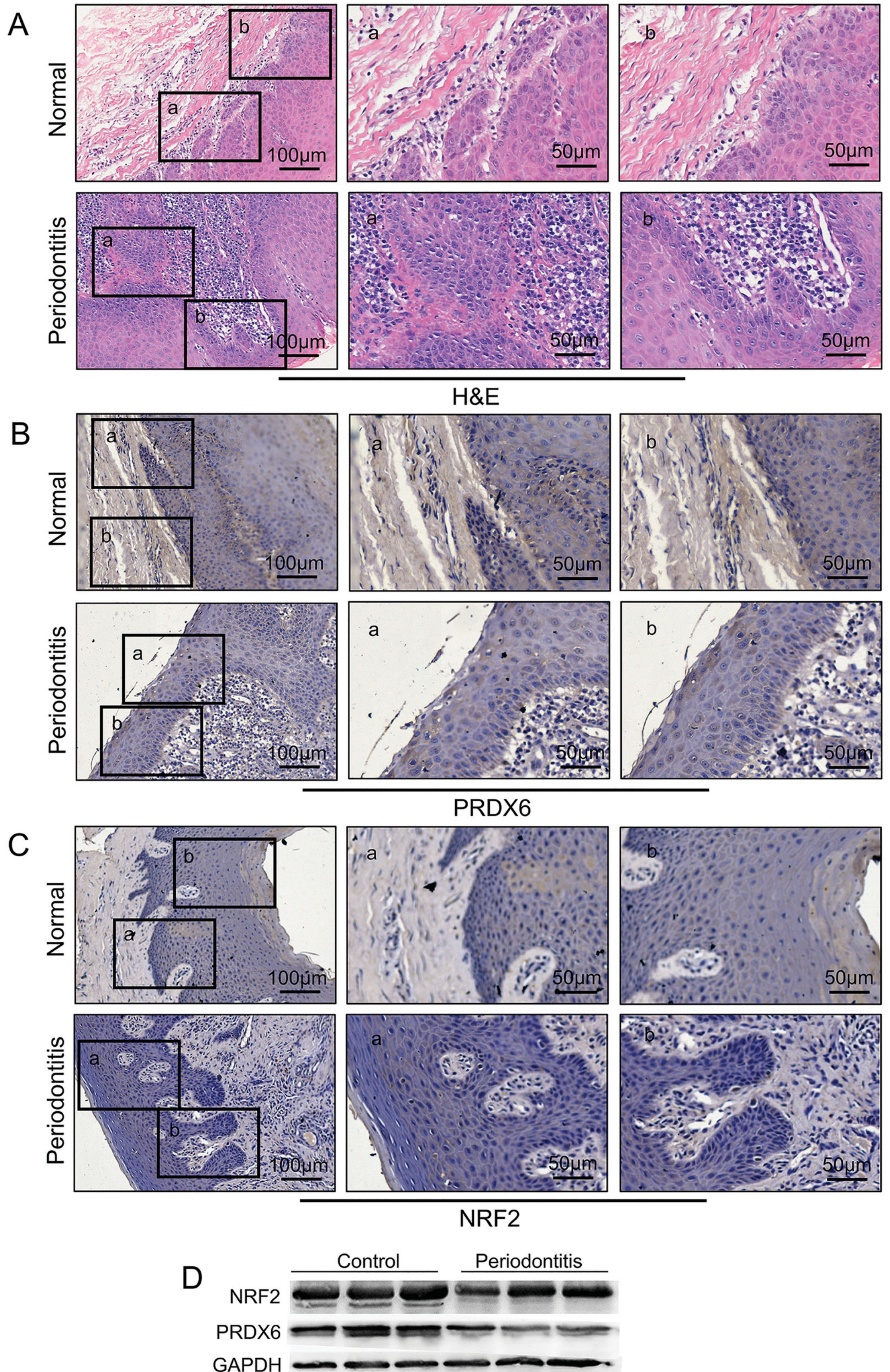

Figure 1. The expression of PRDX6 and NRF2 decreased in inflamed gingival tissues. (A) H&E stain in human periodontitis gingival tissues and normal tissues. (B, C) The IHC of PRDX6 and NRF2 expression in severe periodontitis gingiva and normal gingiva. (D) The Western blotting analysis of PRDX6 and NRF2 expression in severe periodontitis gingiva and normal gingiva.

NRF2 expression level in periodontitis and normal gingival tissue. As shown in Figure 1(C,D), NRF2 was decreased significantly in inflamed gingival tissues than in control. Therefore, oxidative stress was elevated in severe periodontitis.

The expression of PRDX6 and NRF2 in LPS-induced HGFs

Then, we investigated the effect of LPS on the expression levels of PRDX6 and NRF2 in HGFs. We first analysed the

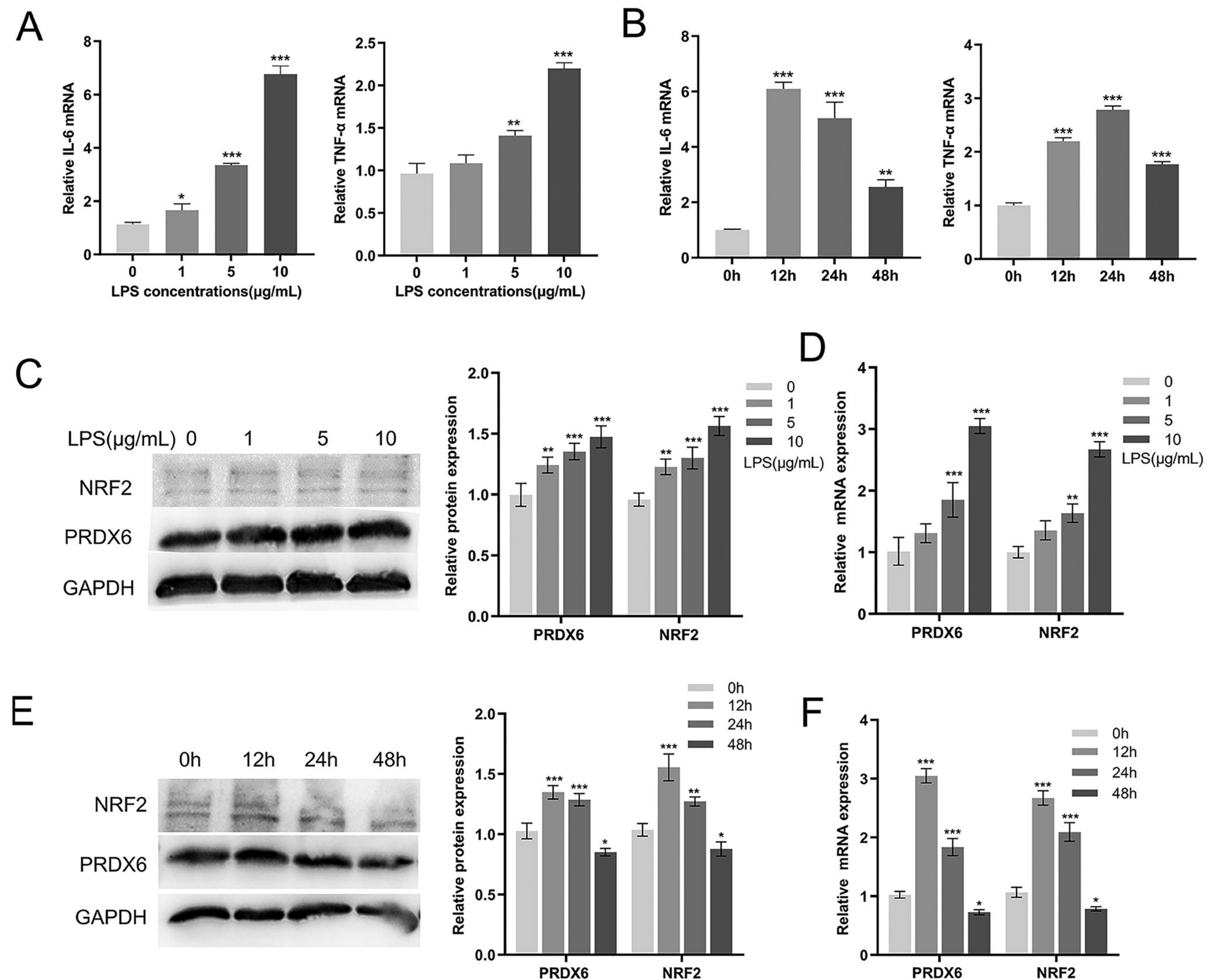

Figure 2. The expression of PRDX6 and NRF2 in LPS-induced HGFs. (A) The RT-qPCR analysis of IL-6 and TNF-α mRNA levels in HGFs treated with different dose LPS (0, 1, 5, and 10 μg/mL) for 12 h. (B) The RT-qPCR analysis of IL-6 and TNF-α mRNA levels in HGFs treated with 10 μg/mL LPS for different times (0, 12, 24, and 48 h). (C, D) The Western blotting and RT-qPCR analysis of PRDX6 and NRF2 levels in HGFs treated with different dose LPS (0, 1, 5, 10 μg/mL). (E, F) The Western blotting and RT-qPCR analysis of PRDX6 and NRF2 in HGFs treated with 10 μg/mL LPS for different times (0, 12, 24, and 48 h). Data were displayed as mean ± SD. $*p < .05$; $**p < .01$; $***p < .001$.

consequence of LPS treatment in different concentrations on inflammatory cytokines in HGFs. As shown in Figure 2(A), RT-qPCR results confirmed that the levels of IL-6 and TNF-α were upregulated in a dose-dependent manner and climbed to the top with 10 μg/mL LPS treatment. Then, we evaluated the influence of LPS treatment (10 μg/mL) for different hours on inflammatory cytokines in HGFs. As shown in Figure 2(B), IL-6 and TNF-α were elevated significantly after 10 μg/mL LPS treatment for 12 h. We also explored the effect of LPS treatment on the expression of PRDX6 and NRF2 in HGFs. The Western blotting and RT-qPCR results confirmed that the expression levels of PRDX6 and NRF2 were also increased in a dose-dependent manner under LPS treatment in different concentrations (Figure 2(C,D)). The up-regulation of PRDX6 and NRF2 was observed from 12 to 24 h, and the down-regulation was observed at 48 h (Figure 2(E,F)). In brief, the expression of PRDX6 and NRF2 was first increased and eventually decreased under the long-term LPS treatment. Of note, PRDX6 and inflammatory cytokines both significantly increased when HGFs were treated with 10 μg/mL LPS for 12 h. This treatment was used to guide the subsequent study.

Inhibiting LPS-induced ferroptosis could ameliorate the inflammatory cytokines in HGFs

We first assessed whether LPS-induced ferroptosis participate in periodontitis. The LPS treatment upregulated the expression level of LOOH production and in a dose-dependent manner, which was in line with the result of inflammatory cytokines (Figure 3(A)). Then, ferroptosis inhibitor, Ferrostatin-1 was added to assess the effect of ferroptosis on inflammatory cytokines. Fer-1 is a LOOH scavenger that prevents ferroptosis but does not suppress apoptosis and necrosis [32,33]. As shown in Figure 3(B), the cell viability was decreased in the LPS group, which could be alleviated by the co-treatment with Fer-1. The cytotoxicity in the LPS group was increased, and the co-treatment of LPS with Fer-1 inhibited the release of LDH (Figure 3(C)). Besides, the LPS-induced LOOH and inflammatory

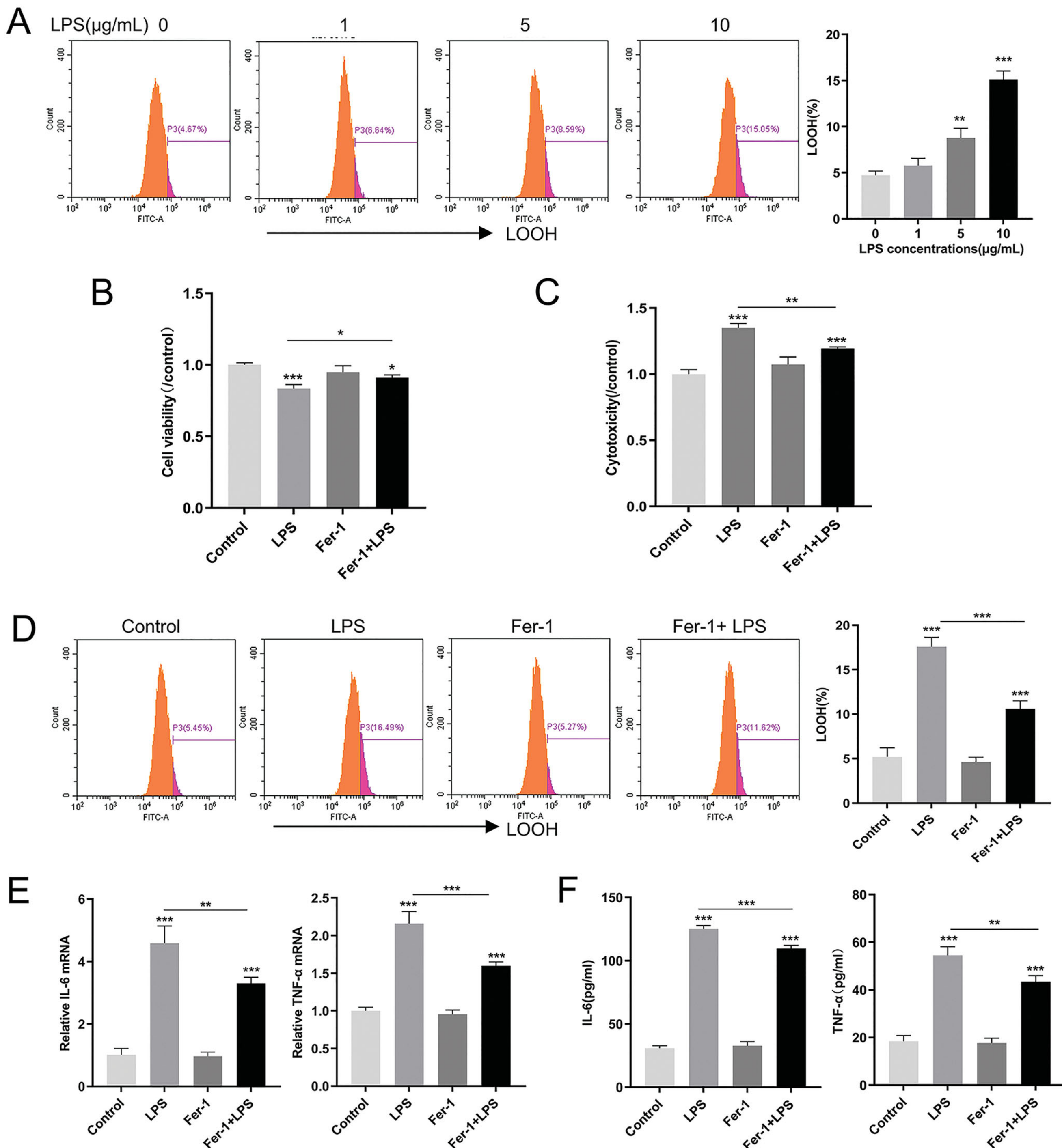

Figure 3. Inhibiting LPS-induced ferroptosis could ameliorate the inflammatory cytokines in HGFs. (A) HGFs were treated with LPS (0, 1, 5, 10 μg/mL) for 12 h and then, the LOOH production of each group was assayed by C11-BODIPY. HGFs were treated with LPS (10 μg/mL) with or without Fer-1 (10 μM) for 12 h, (B) the cell viability was assayed by CCK8 assay, and (C) the cytotoxicity was accessed by LDH assay. (D)The LOOH production of each group was assayed by C11-BODIPY. (E) The mRNA levels of IL-6 and TNF-α were detected by RT-qPCR. (F) The IL-6 and TNF-α in supernatants of HGFs were detected by ELISA. Data were displayed as mean ± SD. *$p < .05$; **$p < .01$; ***$p < .001$.

cytokines were also suppressed by Fer-1 (Figure 3(D–F)). Therefore, LPS-induced LOOH and inflammatory cytokines in HGFs could be alleviated by inhibiting ferroptosis.

Prdx6 protected HGFs from LPS-induced inflammatory cytokines and ferroptosis

To determine the role of PRDX6 in periodontitis, we investigated whether PRDX6 knockdown increases the levels of LPS-induced inflammatory cytokines in HGFs. After the transfection of PRDX6 siRNA, PRDX6 expression was significantly decreased (Figure 4(A,B)). As shown in Figure 4(C,D), the cell viability was reduced in PRDX6 knockdown HGFs compared to control knockdown under LPS treatment. Subsequently, PRDX6 knockdown cells were treated with LPS to detect the expression levels of inflammatory cytokines and LOOH. The RT-qPCR and ELISA results showed that LPS treatment promoted the concentrations of IL-6 and TNF-α in PRDX6

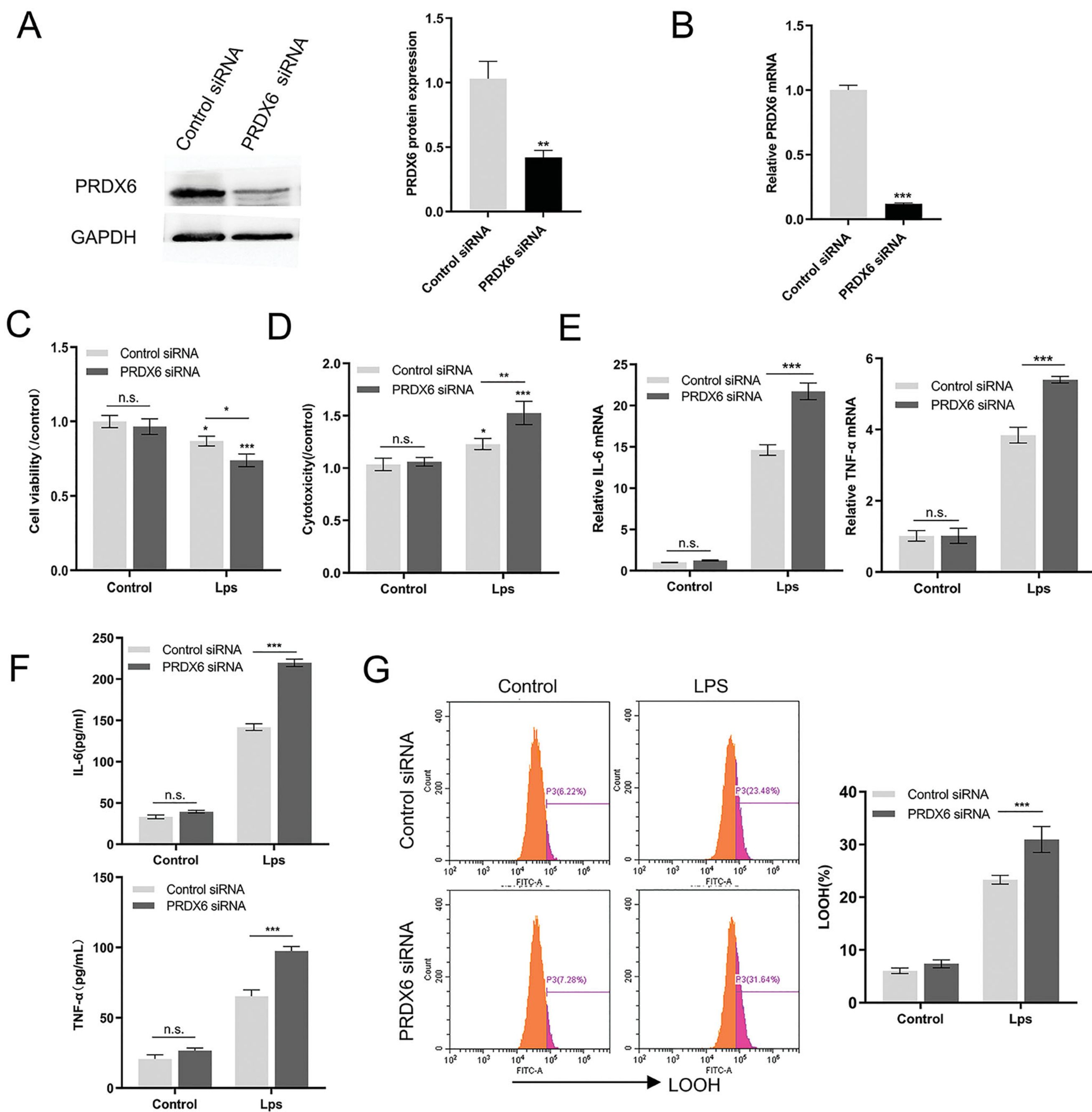

Figure 4. PRDX6 protected HGFs from LPS-induced inflammatory cytokines and ferroptosis. (A) The Western blotting analysis of PRDX6 protein level in HGFs transfected with Control siRNA or PRDX6 siRNA for 48 h. (B) The RT-qPCR analysis of PRDX6 mRNA level in HGFs transfected with Control siRNA or PRDX6 siRNA for 48 h. (C) The cell viability or (D) cytotoxicity in HGFs transfected with Control siRNA or PRDX6 siRNA for 48 h and stimulated with LPS (10 μg/mL) for 12 h. (E) The mRNA levels of IL-6 and TNF-α in each group were detected by RT-qPCR. (F) The IL-6 and TNF-α in supernatants of HGFs were detected by ELISA. (G) The LOOH production of each group was assayed by C11-BODIPY. Data were displayed as mean ± SD. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$; n.s.: no statistical difference.

silencing cells compared with control cells (Figure 4(E,F)). These results indicated that PRDX6 could attenuate LPS-induced inflammation in periodontitis. We then focussed on whether PRDX6 knockdown enhances the LOOH production in LPS-induced HGFs. As shown in Figure 4(G), there was no upregulation of LOOH in PRDX6-silenced cells, suggesting PRDX6 knockdown cannot initialize ferroptosis while amplified LPS-induced LOOH. Thus, PRDX6 could protect HGFs from LPS-induced inflammation and ferroptosis.

The inhibition of aiPLA2 of PRDX6 alleviated inflammation and ferroptosis

PRDX6 is a multi-tasking enzyme that expresses peroxidase, aiPLA2, and LPCAT activities [21]. Because the specific inhibitors of the aiPLA2 activity of PRDX6 (MJ33) are commercially available, we mainly focussed on whether aiPLA2 can influence inflammation and ferroptosis. Figure 5(A,B) showed when the PRDX6-aiPLA2 activity was blocked by MJ33; the

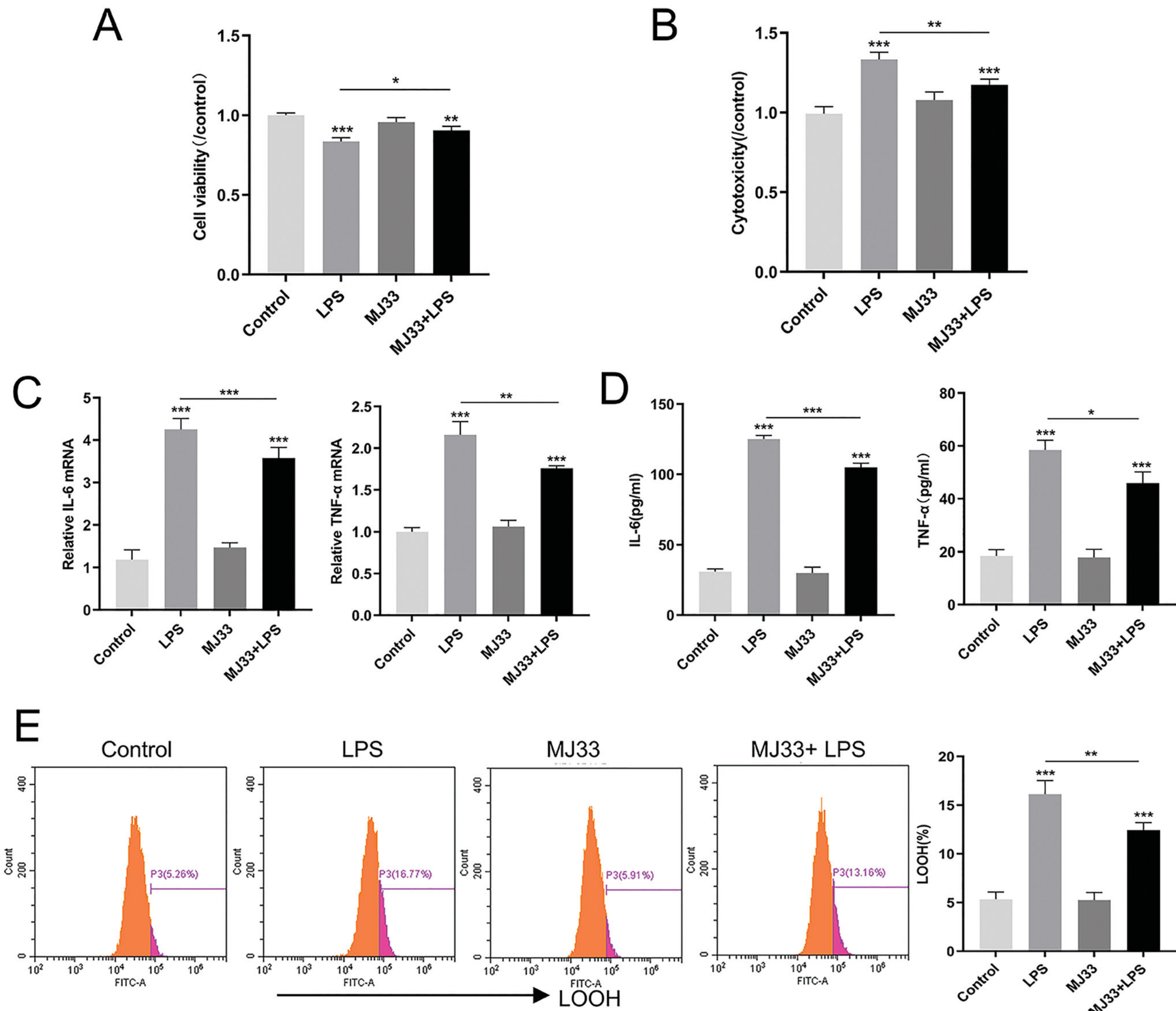

Figure 5. The inhibition of PRDX6-PLA2 alleviated LPS-induced inflammatory cytokines and ferroptosis. (A) The cell viability or (B) cytotoxicity in HGFs stimulated by LPS (10 µg/mL) with or without MJ33 (5 µM) for 12 h. (C) The mRNA levels of IL-6 and TNF-α in each group were detected by RT-qPCR. (D) The IL-6 and TNF-α in supernatants of HGFs were detected by ELISA. (E) The LOOH production of each group was assayed by C11-BODIPY. Data were displayed as mean ± SD. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$.

cytotoxicity of the MJ33 + LPS group was decreased compared with that in the LPS group. As shown in Figure 5(C–E), the inhibition of aiPLA2 decreased the LPS-induced inflammatory cytokines and LOOH. Therefore, PRDX6 could partially promote inflammation and ferroptosis *via* aiPLA2 activity.

Prdx6 regulated by NRF2 ameliorated LPS-induced inflammatory cytokines and ferroptosis

To further confirm the effect of NRF2 regulation on the expression of downstream target gene PRDX6, we used ML385 to inhibit the NRF2 signalling. The cell viability under the co-treatment of ML385 and LPS could be further decreased than cells only treated with LPS (Figure 6(A)). Besides, the LDH release of the ML385 + LPS group was more than the LPS group (Figure 6(B)). The expression levels of NRF2 and PRDX6 were downregulated in the ML385 + LPS group compared with the LPS group (Figure 6(C)). Figure 6(D,E) showed that ML385 treatment enhanced LPS-induced inflammatory cytokines in HGFs. Furthermore, LPS-induced LOOH was improved in the meantime by ML385 co-treatment (Figure 6(F)). Therefore, these results indicated that NRF2 regulates PRDX6 to protect HGFs against inflammation and ferroptosis in periodontitis.

Discussion

Even though the LPS-induced periodontitis model has been established for years and is widely used in pre-clinical studies, the precise mechanisms remain unclear. PRDX6 can regenerate peroxidized cell membrane and is involved in ferropotic stress in cancer [34]. In this study, we mainly discussed the role of PRDX6 in LPS-induced inflammation and ferroptotic process in periodontitis.

PRDX6 has been reported to hold an important position in inflammatory diseases. In HGFs, the expression of PRDX6

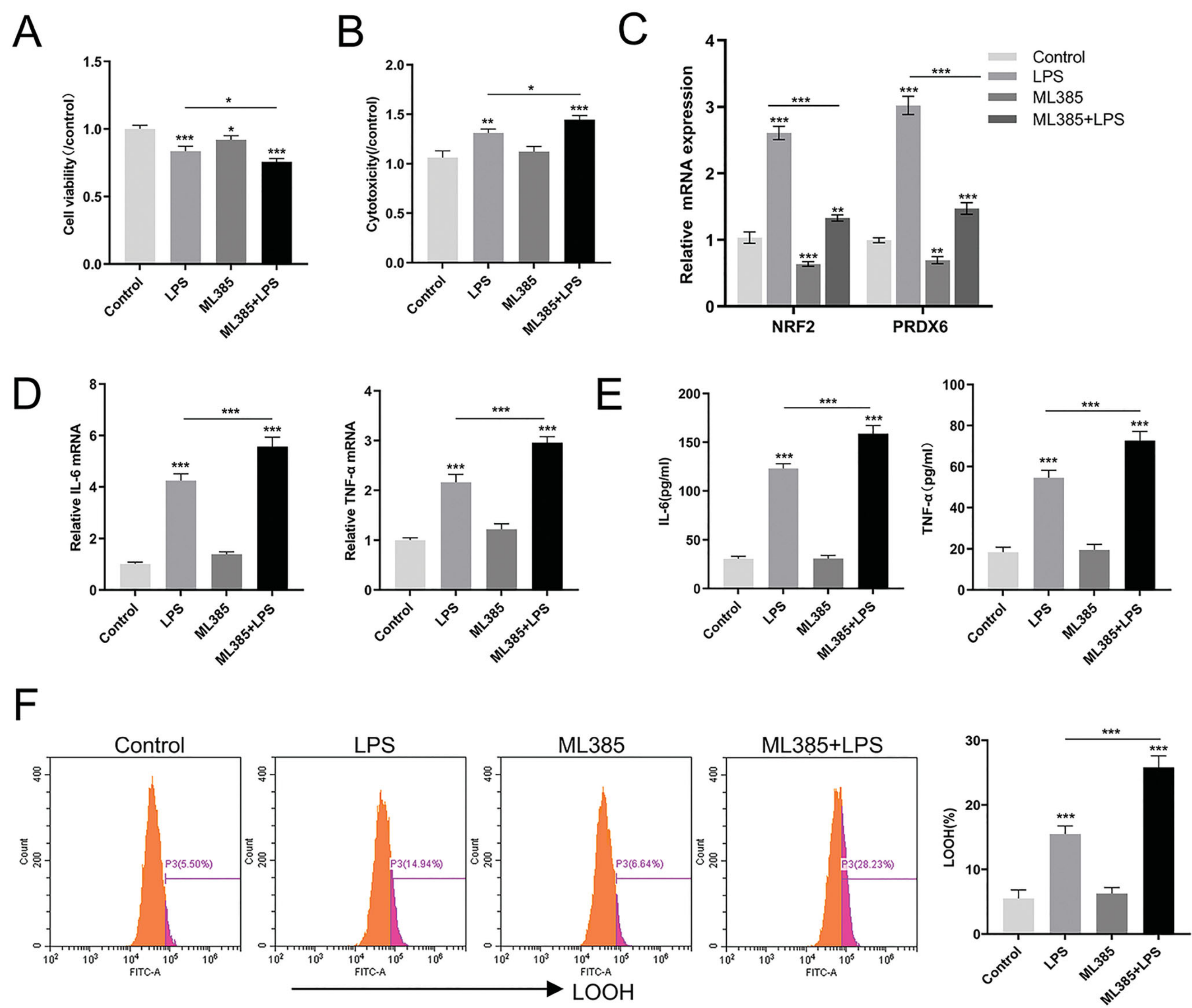

Figure 6. PRDX6 regulated by NRF2 ameliorates LPS-induced inflammatory cytokines and ferroptosis. (A) The cell viability or (B) cytotoxicity in HGFs stimulated by LPS (10 μg/mL) with or without ML385 (5 μM) for 12 h. (C) The mRNA levels of NRF2 and PRDX6 were assessed by RT-qPCR. (D) The mRNA levels of IL-6 and TNF-α in each group were detected by RT-qPCR. (E) The IL-6 and TNF-α in supernatants of HGFs were detected by ELISA. (F) The LOOH production of each group was assayed by C11-BODIPY. Data were displayed as mean ± SD. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$.

was increased considerably to defend against the LPS-induced oxidative damage at first, while it eventually decreased and lost the protective effect. The increased oxidative stress plays an essential role in stimulating and amplifying inflammation. In peripheral arterial disease, the results showed that oxidative stress leads to the upregulation of PRDX6 [35]. In the LPS-induced acute kidney injury model, PRDX6-overexpressed mice showed less renal apoptosis and leukocyte infiltration than wild mice [36]. Therefore, PRDX6 serves as an antioxidant that can regulate the inflammatory response.

Ferroptosis is a peroxidation-driven form of regulated cell death revealed in many human diseases. *E. coli* LPS was used in this study, which could induce inflammatory markers more potent than *P. gingivalis* LPS [37]. Although HGFs treated with 10 μg/mL LPS is relatively high in periodontitis, the LOOH accumulation was increased at this concentration to explore the mechanisms of ferroptosis in periodontitis further. The LPS-induced LOOH and inflammatory cytokines were simultaneously down-regulated under Fer-1 treatment. Those results identified that butyrate-induced ferroptosis could promote periodontitis development [38]. Liu et al. [39] also indicated that ferroptosis play a critical role in LPS-induced acute lung injury (ALI) and could be alleviated by Fer-1. Li et al. [40] reported that suppressing Ang II-induced ferroptosis in astrocytes alleviates neuroinflammation. Furthermore, Liu et al. [41] reported that ferulic acid inhibits ferroptosis by upregulating AMPKα2 expression to protect from myocardial ischemia-reperfusion. Therefore, inhibiting ferroptosis may represent a promising therapeutic approach for these diseases. In our study, ferroptosis also is involved in periodontitis and inhibiting ferroptosis ameliorates inflammation severity.

PRDX6 is an antioxidant and recently has been identified as a regulator of ferroptosis in cancer cells [34]. Our results showed that the accumulation of LOOH production could be observed in PRDX6 silencing HGFs treated with LPS, which amplifies the inflammatory response. Therefore, PRDX6 could

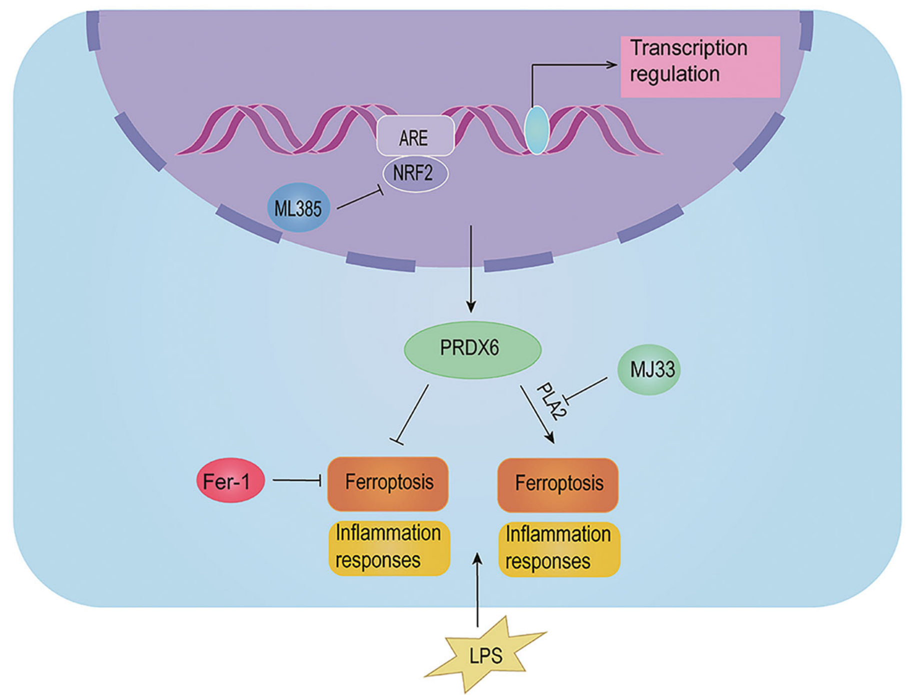

Figure 7. The role of PRDX6 in LPS-induce HGFs. PRDX6, regulated by the NRF2 signalling, alleviates LPS-induced inflammatory response and ferroptosis in HGFs.

alleviate ferroptosis and play a protective effect on periodontitis. However, we only demonstrated this mechanism *in vitro* experiments; and the vivo model was necessary to analyse the essential role of PRDX6 in the future.

PRDX6 is unique because it has peroxidase in antioxidant functions and aiPLA2 activity in oxidant generation. It has been reported that blocking the aiPLA2 activity of PRDX6 by MJ33 enhanced ferroptosis in cancer cell lines [34]. While in a mouse model of ALI, blocking PRDX6-aiPLA2 activity could attenuate lungs damage, presumably by inhibiting oxidative stress and preventing the amplification of lungs inflammation [42]. Inhibiting PRDX6-aiPLA2 activity downregulated the production of LPS-induced LOOH and inflammatory cytokines. The potential mechanism for MJ33 to alleviate the inflammation and ferroptosis state could be through the inhibition of the aiPLA2 activity to block NOX2 activation. NOX2 is the enzyme responsible for ROS generation associated with inflammation [42]. It has been reported that enhanced NOX2 activity is most likely one of the ROS sources in periodontal tissues [43]. Although the aiPLA2 also has enzyme activity in reducing phospholipid hydroperoxides and repairing peroxidized cell membranes, its protective role is less than that of PL hydroperoxide GPx (PHGPx) activity and LPCAT activity in PRDX6. Therefore, inhibition of aiPLA2 activity could alleviate the LPS-induced inflammation and ferroptotic injury in HGFs.

In our study, the downregulation of NRF2 was observed in chronic severe inflammatory gingival tissues compared to the normal gingival tissues. Furthermore, NRF2 was intensely activated to promote HGFs early and eventually decreased with long-term LPS treatment. As the treatment of NRF2 inhibitor, ML385 downregulated the expression PRDX6 and NRF2 and increased the expression of inflammatory cytokines and LOOH. Therefore, the NRF2 could regulate the PRDX6 expression and play a protective role in LPS-induced periodontitis. NRF2 has been reported as a vital regulator of cellular ferroptosis in many diseases. In neurodegeneration, NRF2 signalling is involved in mitochondrial function and ferroptotic cell death, and targeting NRF2 to exert an anti-ferroptotic effect facilitates the development of treatments [44]. Besides, the NRF2 pathway was inhibited in ferroptosis of Imatinib Mesylate-induced cardiotoxicity, and the activation of the NRF2 pathway has shown success in therapy of cardiac injury [45,46]. However, this study only explored the primary mechanism between PRDX6 and NRF2 (Figure 7), and the deep mechanisms still need further exploration.

In conclusion, this study showed that PRDX6 serves as an antioxidant to protect HGFs from LPS-induced inflammatory response and ferroptosis. Moreover, the inhibition of aiPLA2 activity of PRDX6 could relieve periodontitis. The NRF2 signalling is an essential antioxidant response pathway that regulates the expression of PRDX6, which also affects the inflammation reaction and ferroptotic stress in periodontitis. Therefore, this study provided new insight into periodontitis therapy targeting PRDX6 and ferroptosis.

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Health Human Research Ethics Committee of the Stomatology Hospital affiliated to Anhui Medical University (Permit No. 20200836). Written informed consent has been obtained from all participants.

Author contributions

Conceptualisation, Methodology, and Writing-Original draft preparation: WYY; Formal analysis: XM; Investigation: YRW; Writing-Reviewing and Editing: QQW; Validation: XH; Visualisation: XYS; Resources: NC; Supervision: LZ. All authors read and approved the final manuscript.

Disclosure statement

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