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ORIGINAL ARTICLE

Glycoprotein Nonmetastatic Melanoma Protein B (GPNMB) Ameliorates the Inflammatory Response in Periodontal Disease

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Abstract— Glycoprotein nonmetastatic melanoma protein B (GPNMB) is a type I transmembrane protein that can modulate osteoblasts and bone mineralization. Periodontal disease (PD) is characterized by gum inflammation, alveolar bone resorption, and tooth loss. In this study, we found that GPNMB is highly expressed in inflamed periodontal tissue through microarray and immunohistochemistry (IHC) assays. The role of GPNMB in the pathogenesis of PD was evaluated with primary human periodontal ligament cells (hPDLCs) treated with lipopolysaccharide (LPS) and a GPNMB-expressing lentivirus (lenti-GP). In the hPDLCs treated with LPS and lenti-GP, the expression of tumor necrosis factor (TNF)-α and interleukin (IL)-6 was suppressed and that of IL-10 was upregulated. GPNMB significantly decreased apoptosis in the hPDLCs treated with LPS. GPNMB could upregulate the expression of Jumonji domain-containing protein 3 (Jmjd3), a histone 3 lysine 27 (H3K27) demethylase that is linked to the modulation of the inflammatory response and apoptosis. Taken together, our data find that GPNMB is highly expressed in gum tissue with PD and may be an anti-inflammatory player in the pathogenesis of PD.

KEY WORDS: periodontal disease; GPNMB; inflammatory cytokine; Jmjd3.

INTRODUCTION

Periodontal disease (PD) is a chronic oral disease that is highly prevalent in adults. PD is mainly characterized by inflammation in the gums, periodontal pocket formation, alveolar bone resorption, and tooth loosening and displacement [1, 2]. There are two hallmark events in PD: inflammation and alveolar bone resorption/tooth loss [2, 3]. The inflammation event includes gingivitis and periodontitis [3]. In this study, our initial intention was to probe the molecular basis for bone destruction during PD. A microarray screening bone metabolism factors was used to assess gum tissue samples collected from patients who suffered from PD. In the microarray, glycoprotein nonmetastatic melanoma protein B (GPNMB), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), and macrophage colony-stimulating factor (MCSF) showed varied expression patterns between the healthy and inflamed periodontal tissues. MCP-1, MIP-1α, and MCSF are well established as inflammatory cytokines [4–7]. To date, there are no data available on the connection between GPNMB and the pathogenesis of PD.

GPNMB, a type I transmembrane protein with 560–572 amino acids, is also known as osteoactivin (OA) in rats and dendritic cell-heparin integrin ligand (DC-HIL) in mice [8–10]. The *GPNMB* gene is located on chromosome

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7p15.1 and contains 11 exons [10]. GPNMB is expressed in the bone, liver, and kidneys as well as numerous cell types, including osteoclasts, macrophages, dendritic cells, and tumor cells [9–11]. GPNMB acts as a downstream mediator of bone morphogenetic protein 2 (BMP-2) and affects the differentiation, maturation, adhesion, and migration of osteoblasts and thus affects bone mineralization [12]. An accumulating number of studies have suggested that GPNMB is also a negative regulator of inflammation [11]. GPNMB is expressed at high levels in liver inflammatory cells, suggesting a critical role in acute liver inflammation [13, 14]. OA in rats shows early-phase upregulation in the kidney tubular epithelium when renal injury occurs and may trigger renal interstitial fibrosis [15]. GPNMB is highly expressed in aggressive breast cancers and facilitates the metastasis of breast cancer to bone [16].

Jumonji domain-containing protein 3 (JMJD3), which is also known as lysine-specific demethylase 6B (KDM6B), is a histone 3 lysine 27 (H3K27) demethylase [17]. JMJD3 is essential for the polarization of M2 macrophages [18] and is also a negative regulator during inflammation [19]. A recent study showed that JMJD3 can upregulate the expression of insulin-like growth factor binding protein 5 (IGFBP5), which promotes periodontal tissue repair [20].

In this study, we found that GPNMB is highly expressed in inflamed gum tissue, significantly upregulates the expression of Jmjd3 and may be a suppressor of periodontal inflammation and a protective mediator during the progression of PD.

MATERIALS AND METHODS

Tissue Samples and a Microarray

Inflamed gingival specimens were collected from four subjects (mean age, 42 ± 2.16; range, 40–45 years) with untreated moderate PD. The PD patients were selected based on diagnostic criteria adapted from the criteria for the classification of periodontal diseases and conditions as follows: pocket depth between > 4 mm and ≤ 6 mm; 3–4 mm of clinical attachment loss; and bleeding upon probing. During treatment, four pieces of granulation tissue from the inner wall of the deep periodontal pocket were collected from each patient under local anesthesia. Four healthy tissue samples from the other side of the periodontal pocket were also collected from each patient to serve as negative controls. All patients were free of systemic diseases other than PD. Informed consent was obtained from each participant in the study. The procedure for tissue sample collection was approved by the Ethics Committee of Harbin Medical University.

The tissue samples were assessed with a human bone metabolism-related protein array (QAH-BMA-1000, RayBiotech Guangzhou, Guangzhou, China). Briefly, all tissue samples were lysed with cell lysis buffer to extract total protein. The extracts were loaded onto the glass array and detected with primary antibodies and a Cy3-streptavidin-labeled secondary antibody with standard washing procedures. The fluorescence of the array was detected with a laser scanner (Innoscan 300 Microarray Scanner, Innopsys, Carbonne, France).

Immunohistochemistry

Sixty-nine periodontal tissue samples were collected from untreated patients (mean age, 46.4 ± 7.63; range, 30–59 years) with moderate to severe PD. The patients were selected based on the following diagnostic criteria: pocket depth > 4 mm, clinical attachment loss ≥ 3 mm, and bleeding upon probing. During their treatment for PD, the patients had their granulation tissue on the inner wall of the deep-seated periodontitis site scraped under local anesthesia. Healthy periodontal tissue was collected from 13 people (mean age, 13.5 ± 1.33; range, 12–15 years) who had their premolars removed for orthodontic reduction (study inclusion required complete roots, no cavities, and no obvious periodontal tissue inflammation) and served as negative control samples. The teeth were wiped with 75% ethanol and washed twice with phosphate-buffered saline (PBS), and the surrounding periodontal tissue was gently peeled off for use as part of the negative control group. The tissue samples were paraffinized, sectioned, and stained following a standard immunohistochemistry (IHC) protocol. An anti-GPNMB rabbit antibody (20338-1-AP) was purchased from Wuhan Sanying Biotechnology (Wuhan, China). A goat anti-rabbit antibody was purchased from ZhongshanJinqiao Biotechnology (Beijing, China). Stained areas of interest (AOIs) were selected from the IHC image, and the integral optical density (IOD) of the selected AOIs was determined. All subjects were free of systemic diseases.

Cell Culture

Premolars were collected from 12- to 15-year-old healthy donors undergoing orthodontic treatment with the informed consent of the donor and their parents. The teeth were washed twice with PBS, and the attached periodontal ligament tissue was peeled off with a scalpel. The tissue samples were cut into pieces (1–2 mm^3) and digested with type I collagenase for 40 min at 37 °C. The separated primary human periodontal ligament cells (hPDLCs) were

harvested with centrifugation at 1000 r/min for 5 min. The cells were cultured with α-MEM complete medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), streptomycin (50 μg/ml), and penicillin (100 U/ml). The cells were passaged at least three times before being used in subsequent experiments.

GPNMB-Expressing Lentivirus

The coding region (1619 bp) of the GPNMB gene (NM_001005340) was amplified and ligated into a lentiviral vector. A total of 293 T cells were cotransfected with the GPNMB-expressing plasmid (lenti-GP) and two packaging plasmids. The supernatant containing lenti-GP was collected after 48 h of culture.

Enzyme-Linked ImmunoSorbent Assay

The levels of interleukin (IL)-6, IL-10, and tumor necrosis factor (TNF)-α in the supernatants of hPDLCs given various treatments were measured with a human IL-6 enzyme-linked immunosorbent assay (ELISA) detection kit (WLE04, Wanleibio, China), a human IL-10 ELISA detection kit (SEA056Hu, Youersheng, China), or a human TNF-α ELISA detection kit (WLE05,Wanleibio, China), respectively.

Western Blotting

Western blotting was performed following a standard protocol to detect the expression of GPNMB and Jmjd3 in hPDLCs. Briefly, approximately 5×10^6 cells were collected and lysed with RIPA lysis buffer (ThermoFisher Scientific, Waltham, MA). The extracted proteins were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with primary and secondary antibodies separately and washed, and the immune complexes were detected with an enhanced chemiluminescence (ECL) substrate luminescence assay. The blots were detected with a FluorChem CCD camera (ProteinSimple, San Jose, CA). Both the anti-GPNMB antibody (20338-1-AP) and anti-Jmjd3 antibody (55354-AP) were purchased from Wuhan Sanying Biotechnology. Goat anti-rabbit and goat anti-mouse antibodies were obtained from Beijing Zhongshan Jinqiao Biotechnology.

Real-Time Quantitative PCR

Real-Time Quantitative PCR (RT-qPCR) was performed following a standard protocol. Briefly, approximately 5×10^6 cells were treated with TRIzol Reagent. Total RNA was extracted, and 1 μg of RNA was subjected to reverse transcription. The cDNA template was used to amplify target mRNAs with specific primers by using a LightCycler 2.0 (Roche, Basel, Switzerland). β-Actin was used as the reference to normalize the mRNA expression. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA abundance.

Flow Cytometry

Treated hPDLCs were collected using 0.25% trypsin, centrifuged, and washed with Dulbecco's modified Eagle's medium (DMEM). The cells were incubated with annexin V-FITC and presidiums iodide (PI) at room temperature in the dark for 15 min, and approximately 20,000 cells were analyzed by flow cytometry (BD Bioscience, San Jose, CA). AnnexinV-FITC/PI detection kit (WLA001a) was obtained from Wanleibio.

Statistical Analysis

Cell experiments were repeated at least three times. Student's *t* test was used for data analysis. The data are expressed as the mean ± standard deviation (SD).

RESULTS

GPNMB Expression Is Significantly Elevated in Periodontal Tissue with PD

To evaluate the role of bone metabolism factors in the pathological process of PD, four PD patients were enrolled in this study. Four tissue samples were collected separately from both the periodontal tissue of the PD side and that of the healthy side of each patient and assessed with the bone metabolism array QAH-BMA-1000. The expression of GPNMB, MCP-1 (also known as CCL2), and MIP-1α (also known as CCL3) was significantly elevated in the PD tissue samples compared with the healthy tissue samples, while the expression of MCSF was downregulated in the PD tissue samples (Fig. 1).

GPNMB Expression Is Dramatically Increased in the Epithelial Cells of PD Tissue

To confirm the array results, 69 paired samples of PD tissue and the corresponding healthy tissue were detected with an IHC assay. Measuring the optical density showed that GPNMB staining was significantly increased in the PD tissue samples compared with the healthy tissue samples ($P < 0.05$, Fig. 2 b). The increased GPNMB expression was mainly distributed in the epithelial cells of the PD

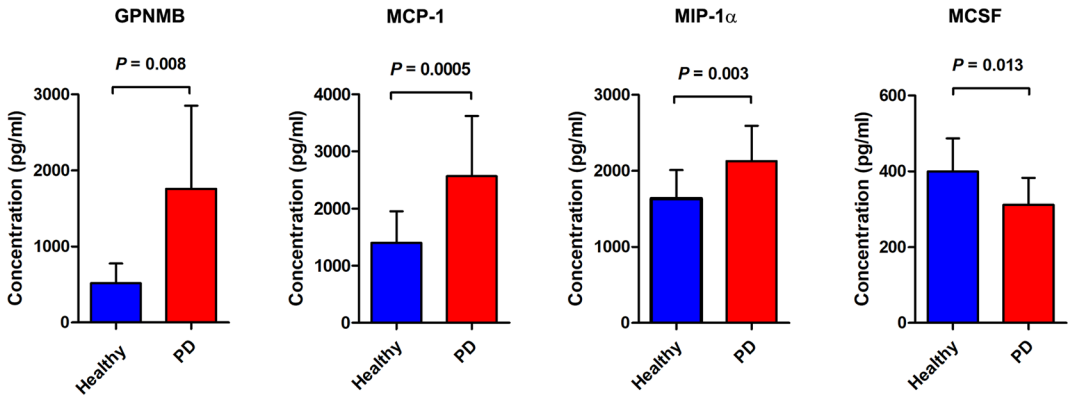

Fig. 1. Differential protein expression in periodontal disease tissue samples was detected by the bone metabolism array QAH-BMA-1000. Four patients with periodontal diseases were included in this study. Four pieces of tissue were collected from both the healthy and PD sides of each patient. Cell lysates from these tissues were assessed with the QAH-BMA-1000 array. The expression of GPNMB, MCP-1, MIP-1α, and MCSF was found to be significantly different between the healthy and PD tissue samples. Error bars indicate the SD ($n = 4$).

tissue (Fig. 2 a). Similarly, the expression of MIP-1α and MCP-1 was dramatically upregulated, but MCSF expression was downregulated in the PD tissue samples (Fig. 2 c). The IHC results were consistent with the array findings.

GPNMB Expression Suppresses the Inflammatory Response in hPDLCs Treated with Lipopolysaccharide

To evaluate the effect of elevated GPNMB expression on the pathological process of PD, primary hPDLCs were isolated and cultured. To simulate inflammation, the cells were first treated with the bacterial endotoxin lipopolysaccharide (LPS) and then transfected with lenti-GP. Our data indicated that compared with the cells treated with LPS alone or with LPS and a blank lentiviral vector, the cells treated with LPS and lenti-GP exhibited suppressed expression of the pro-inflammatory cytokines TNF-α ($P < 0.05$, Fig. 3 a) and IL-6 ($P < 0.01$, Fig. 3 b) but upregulated expression of the anti-inflammatory cytokine IL-10 ($P < 0.05$, Fig. 3 c). These data suggest that GPNMB may play a protective role in the pathogenesis of PD.

GPNMB Suppresses Apoptosis in hPDLCs Treated with LPS

hPDLC apoptosis was measured by an annexin V-FITC/PI dual-labeling assay. The results showed that the proportion of apoptotic hPDLCs was significantly reduced after the overexpression of GPNMB under inflammatory conditions (Fig. 4), suggesting that GPNMB may be associated with PD.

GPNMB Upregulates Jmjd3 Expression in hPDLCs

Recent studies have suggested that the histone H3K27 demethylase Jmjd3 regulates IL-6 expression through the STAT3 pathway in response to LPS stimulation and that Jmjd3 is also a suppressor of apoptosis. In this study, we found that both the protein and mRNA levels of Jmjd3 were significantly upregulated by GPNMB in the presence of LPS (μg/ml) ($P < 0.001$, Fig. 5). Our data suggest that GPNMB may regulate IL-6 expression and apoptosis in hPDLCs by affecting Jmjd3 *via* the TLR4-IκB pathway and the STAT3 pathway.

DISCUSSION

PD is one of the most common causes of tooth loss [1]. The typical pathological manifestations of PD are periodontal tissue inflammation and tooth destruction [2, 3]. The tooth destruction may be a consequence of the combined effects of local chronic inflammation and osteogenic defects [21]. Our initial intention of this study was to observe the roles of osteogenesis-related molecules in the pathogenesis of PD. Therefore, a bone metabolism-oriented microarray (QAH-BMA-1000) was used to assess the periodontal tissue samples collected from patients with PD. GPNMB, MCP-1, MIP-1α, and MCSF were aberrantly expressed in the inflamed periodontal tissue compared with the healthy tissue (Fig. 1). IHC detection obtained similar results for the periodontal tissue sections from PD patients (Fig. 2). MCP-1, MIP-1α, and MCSF are well-

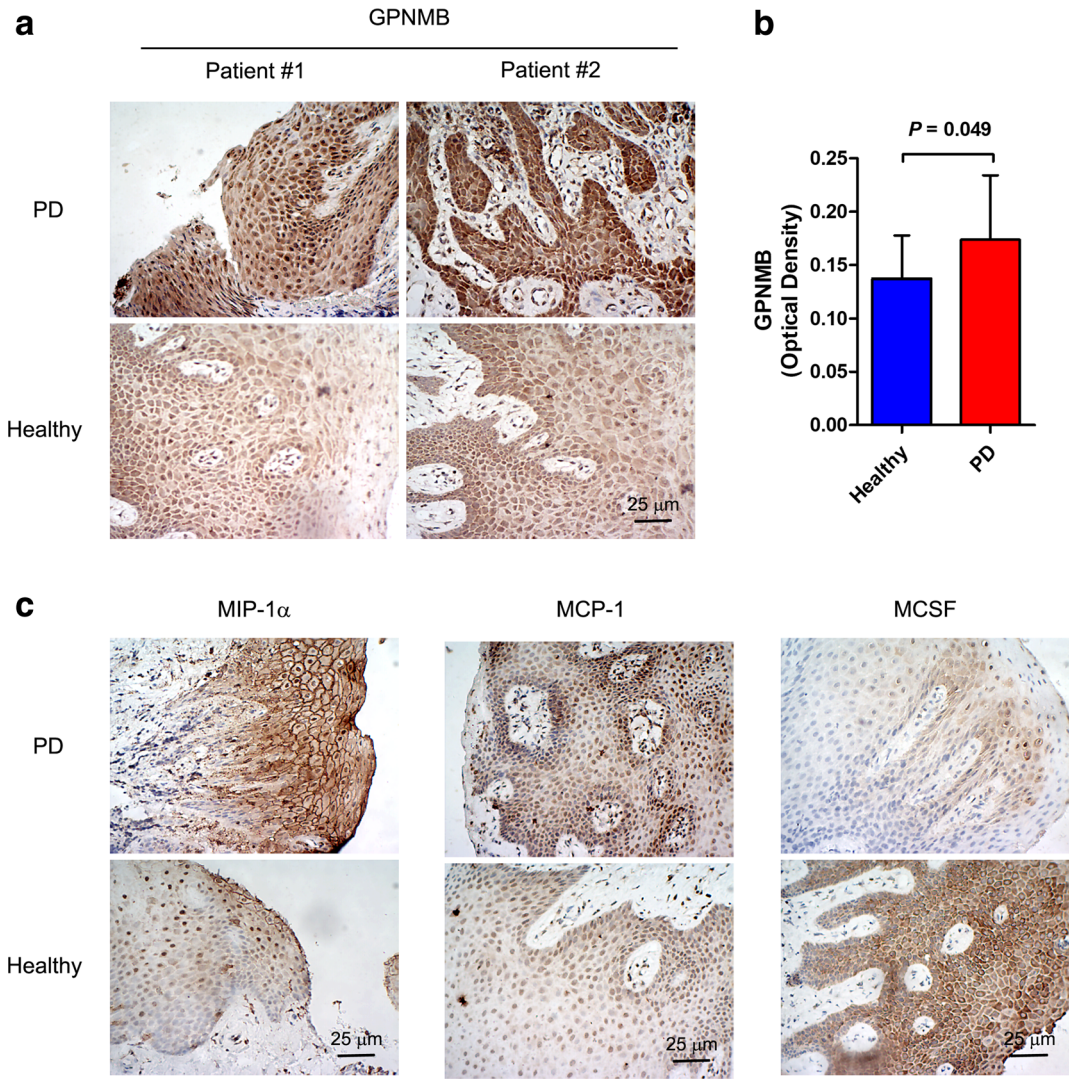

Fig. 2. GPNMB was highly expressed in PD tissue according to an immunohistochemistry assay. The expression of GPNMB, MIP-1α, MCP-1, and MCSF was detected in 69 pieces of PD tissue and paired healthy tissue with an immunohistochemistry assay. **a** The GPNMB expression of two representative tissue samples is shown. **b** Optical density was measured. Student's *t* test was used for statistical analysis. Error bars indicate the SD ($n = 69$). **c** Representative images of the expression of MIP-1a, CMP-1, and MCSF in PD and healthy tissue (negative control) samples are shown.

established pro- or anti-inflammatory factors [4–7]. The role of GPNMB in the pathogenesis of PD is unknown.

GPNMB is a glycoprotein that was first discovered in a rat model of bone sclerosis and named OA [10, 22]. The accumulated data show that GPNMB is a key player in osteogenic differentiation. GPNMB induces osteoblast growth, osteoclast differentiation, and fibroblast differentiation [12]. Previous studies have shown that GPNMB is expressed in liver cells and acts as a negative regulator of the immune response [13, 14]. GPNMB is also expressed in kidney macrophages and protects the kidneys from acute injury by promoting the polarization of M2 macrophages [15]. A mouse study demonstrated that DC-HIL, the mouse orthologue of GPNMB, is a negative regulator of T lymphocyte activation [23]. Similarly, we found that GPNMB may be a negative regulator of the inflammatory response in the pathogenesis of PD. In this study, we simulated PD in hPDLCs with LPS treatment. Overexpressing GPNMB with a lentiviral vector could dramatically suppress the levels of pro-inflammatory cytokines (TNF-α and IL-6) and elevate the level of the anti-inflammatory cytokine IL-10 (Fig. 3). The apoptotic fraction of the cells

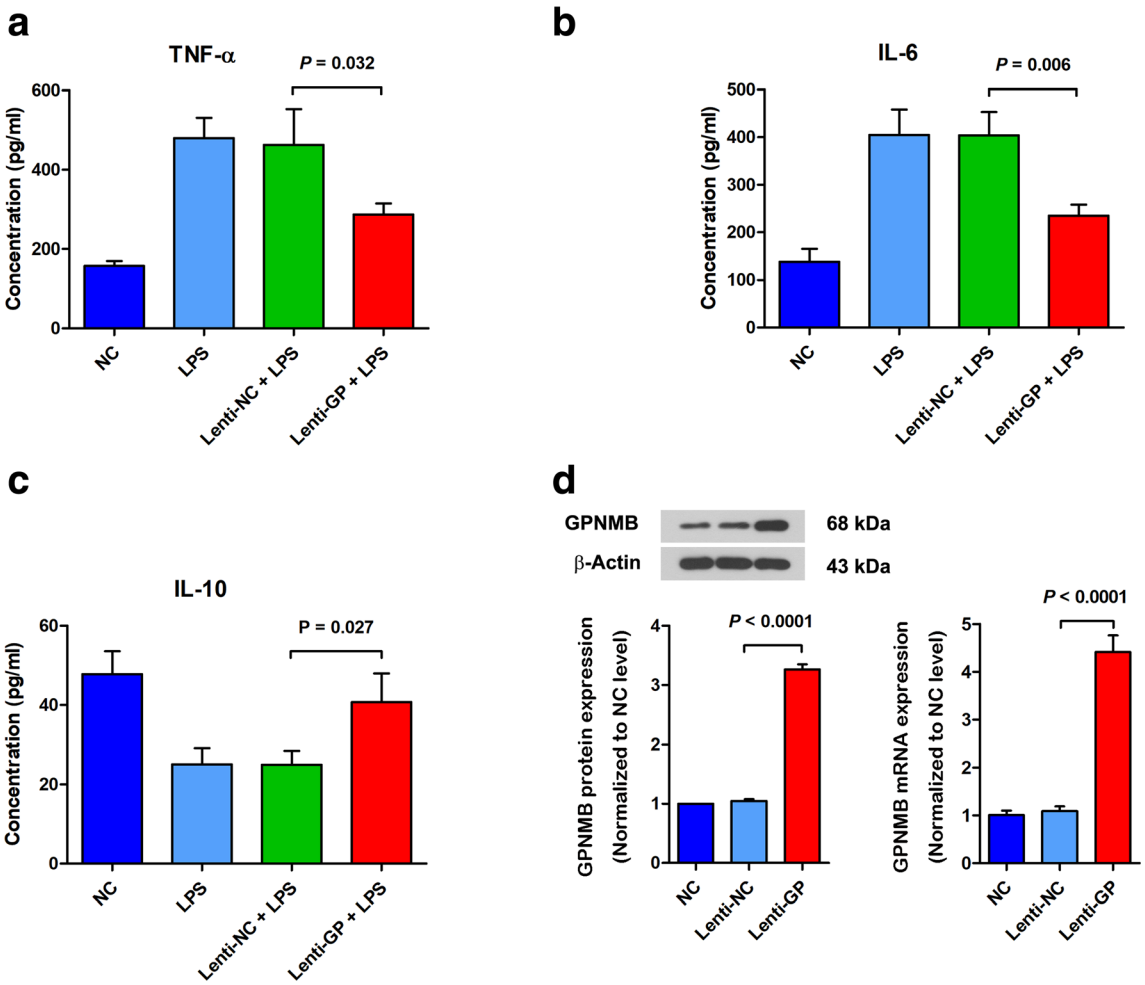

Fig. 3. The effect of GPNMB on the expression of inflammatory cytokines by hPDLCs. hPDLCs were treated with LPS alone (10 μg/ml), a blank lentiviral vector (lenti-NC) and LPS, or a GPNMB-expressing lentiviral vector (lenti-GP) and LPS for 24 h. The expression of pro-inflammatory cytokines (TNF-α and IL-6) and an anti-inflammatory cytokine (IL-10) was detected by ELISA. **a**–**c** The concentrations (pg/ml) of the cytokines in the supernatants of the treated cells are shown. Error bars indicate the SD ($n = 4$). **d** The protein and mRNA levels of GPNMB were significantly increased in the cells transfected with GPNMB-expressing lentiviral vectors compared with the other cells. Error bars indicate the SD ($n = 4$).

overexpressing GPNMB was also smaller than that of the cells treated with LPS alone (Fig. 4). Our *in vitro* data suggest that GPNMB may play an anti-inflammatory and protective role in periodontal tissue during infection.

Jmjd3 is a H3K27 demethylase that catalyzes the demethylation of dimethylation and trimethylation of H3K27 (H3K27me2/3) and activates associated gene expression [17]. Jmjd3 expression can be upregulated by the LPS-STAT pathway [24], and Jmjd3 is a suppressor of apoptosis [25]. Jmjd3 is crucial for the M2 polarization of macrophages [18]. Jmjd3 can upregulate the expression of the transcription factor interferon regulatory factory 4 (IRF4) by increasing histone methylation in the *Irf4* region, thus promoting the polarization of macrophages from the M1 phenotype to the M2 phenotype [18, 26]. M2 macrophages release anti-inflammatory cytokines (*e.g.*, IL-4 and IL-10) and play a suppressive role in the inflammatory response [19]. In this study, GPNMB significantly upregulated the expression of Jmjd3 in the LPS-treated hPDLCs (Fig. 5). The regulation of pro- and anti-inflammatory cytokines and the suppression of apoptosis in GPNMB-expressing hPDLCs is possibly an outcome of synergy between GPNMB and Jmjd3.

Although our data suggest that GPNMB protects against the pathogenesis of PD, we do not know the role of GPNMB in the tooth destruction of PD. In contrast to the role of GPNMB in osteogenic differentiation, a recent

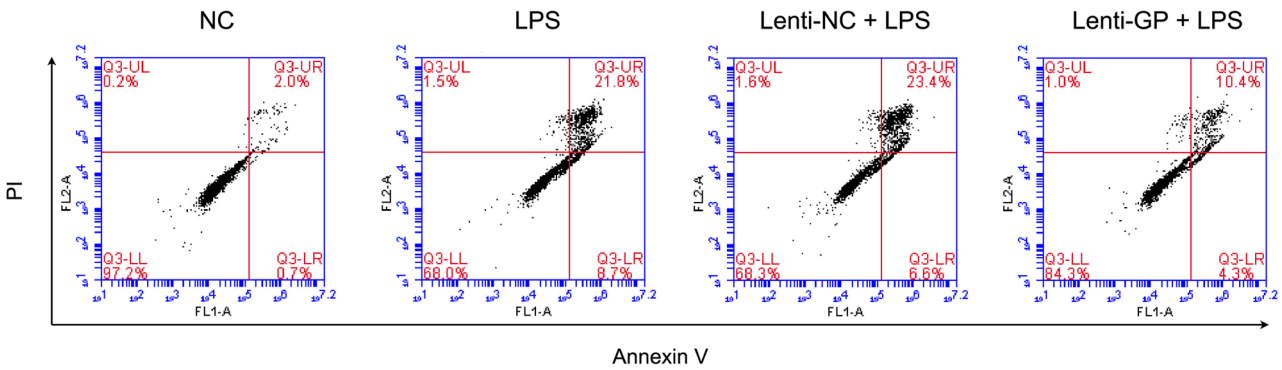

Fig. 4. GPNMB suppressed apoptosis in hPDLCs treated with LPS. hPDLCs were treated with LPS alone, lenti-NC and LPS, or lenti-GP and LPS for 24 h. Approximately 20,000 cells were subjected to apoptosis detection by annexin V/PI staining and flow cytometry detection. The experiment was repeated independently three times. All experiments exhibited a consistent tendency, and representative images are shown here.

study suggested that Jmjd3 can also promote bone repair [27, 28]. In a study with a pig PD model, recombinant IGFBP5 significantly promoted periodontal tissue repair [29]. Jmjd3 can upregulate IGFBP5 expression by binding to and demethylating H3K27 in the *IGFBP5* promoter region [20]. Hence, GPNMB may protect the teeth through the Jmjd3/IGFBP5 pathway in addition to direct osteogenic modulation. We will evaluate the effect of GPNMB on the destruction and absorption of tooth in the future.

It is worth mentioning that there is a discrepancy regarding the relationship between Jmjd3 and IL-6. Lee K. et al. [30] suggested that Jmjd3-mediated H3K27me3 demethylation is crucial for IL-6 gene activation in endothelial cells, and that this molecular event may regulate the acute inflammatory response and the integrity of the blood-spinal cord barrier following spinal cord injury; thus, Jmjd3 is a positive regulator of IL-6 expression. However, Tang Y. et al. [31] reported that suppressing Jmjd3

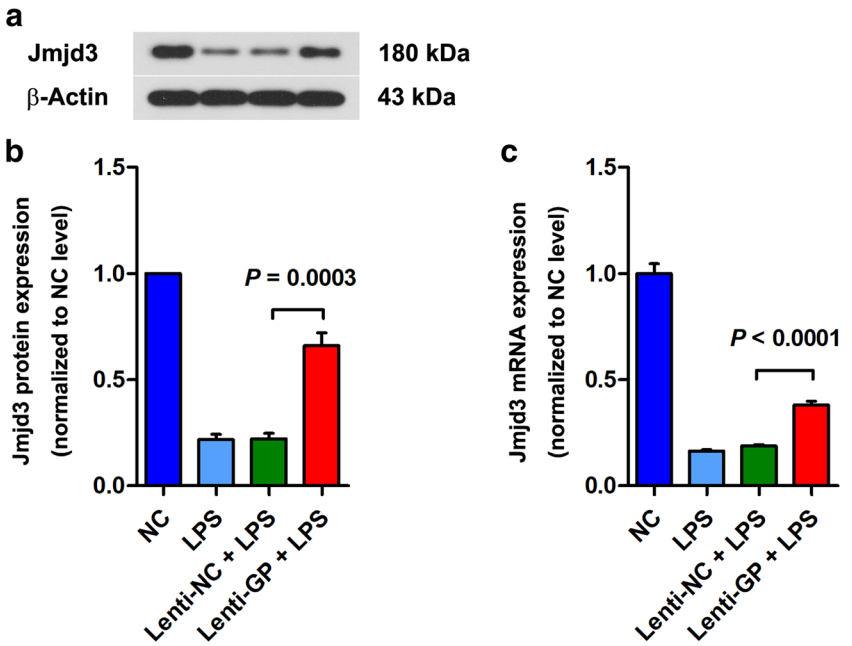

Fig. 5. The effect of GPNMB on the expression of Jmjd3 in hPDLCs. hPDLCs were treated with LPS (μg/ml) alone, lenti-NC and LPS, or lenti-GP and LPS for 24 h. The protein and mRNA levels of Jmjd3 were detected by Western blotting and RT-qPCR, respectively. Error bars indicate the SD ($n = 4$).

expression can exaggerate M1 microglial polarization and promote the production of various pro-inflammatory factors, including IL-6. In this study, we showed that GPNMB could enhance Jmjd3 expression in LPS-stimulated HPDLCs and inhibit IL-6 expression and these findings are consistent with the observations of Tang Y. et al. The discrepancy may be due to the different cell models, stimuli, and diseases having different underlying mechanisms.

A limitation of this study is that the role of GPNMB in PD is speculated based on solely *in vitro* data. Our hypothesis needs further validation with an *in vivo* study, preferably using a GPNMB-knockout animal model.

ACKNOWLEDGEMENTS

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COMPLIANCE WITH ETHICAL STANDARDS

Informed consent was obtained from each participant in the study. The procedure for tissue sample collection was approved by the Ethics Committee of Harbin Medical University.

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