TIFA contributes to periodontitis in diabetic mice via activating the NF-κB signaling pathway

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Abstract. Diabetic periodontitis (DP) refers to destruction of periodontal tissue and absorption of bone tissue in diabetic patients. Tumor necrosis factor receptor-associated factor (TRAF)-interacting protein with forkhead-associated domain (TIFA) as a crucial regulator of inflammation activates the NF-κB signaling pathway to regulate cell biological behavior. However, the function and mechanism of TIFA on DP suffer from a lack of research. In the present study, TIFA was upregulated in the periodontal tissue of a DP mouse model. In addition, the expression of TIFA in RAW264.7 cells was induced by high glucose (HG) culture and increased by lipopolysaccharide (LPS) from *Porphyromonas gingivalis* treatment in a time-dependent manner. Knockdown of TIFA significantly reduced the levels of inflammatory cytokines, including TNF-α, IL-6, IL-1β and monocyte chemoattractant protein-1, in HG and LPS-induced RAW264.7 cells. The nuclear translocation of NF-κB p65 was induced by HG and LPS and was clearly suppressed by absence of TIFA. The expression of downstream factors Nod-like receptor family pyrin domain-containing 3 and apoptosis-associated speck-like protein was inhibited by silencing TIFA. Moreover, TIFA was increased by receptor activator of NF-κB (RANK) ligand (RANKL) in a concentration dependent manner. The expression of cathepsin K, MMP9 and nuclear factor of activated T cells cytoplasmic 1 was downregulated by depletion of TIFA. RANKL-induced osteoclast differentiation was inhibited by silencing of TIFA. Meanwhile, the decrease of TIFA blocked activation of the NF-κB pathway in RANKL-treated RAW264.7 cells. In conclusion, TIFA as a promoter regulates the inflammation and osteoclast differentiation via activating the NF-κB signaling pathway.

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Introduction

Periodontitis is a chronic inflammatory disease occurring in periodontal supporting tissue, mainly manifested as the destruction of local soft tissue and the absorption of bone tissue. It is a common disease with high incidence that seriously endangers human health and is a major public health problem affecting more than half of adults in the world (1). Severe periodontitis can cause excessive absorption of alveolar bone and tooth loss, which seriously affects daily activities such as chewing, swallowing and speaking, as well as physical health (2). A large number of relevant studies have shown that periodontitis not only seriously affects oral health, but also is a risk factor for the occurrence and development of numerous systemic diseases such as diabetes and these systemic diseases also increase the risk rate of periodontitis and aggravated periodontal tissue destruction. Diabetes mellitus is a group of clinical syndromes characterized by hyperglycemia and caused by genetic and environmental factors. With the change of life style and the acceleration of aging process, the prevalence of diabetes in China is also showing a rapidly rising trend. Periodontitis is the sixth most common complication of diabetes and diabetic periodontitis (DP) is particularly severe in numerous cases (3,4). Local periodontal inflammation leads to poor blood glucose control and aggravates the condition of diabetes (3,5). Periodontitis is mainly caused by a specific periodontopathic bacterium, *Porphyromonas gingivalis* (6). Lipopolysaccharide (LPS) is an important component of the outer membrane of gram-negative bacteria and can induce inflammation (7). *In vivo*, LPS enhances the activity of osteoclasts and induces the differentiation of osteoclasts, which is associated with the occurrence of periodontitis (8). Based on the above, anti-inflammatory and inhibition of

osteoclast differentiation are important research directions in the treatment of DP.

TIFA [tumor necrosis factor receptor-associated factor (TRAF)-interacting protein with forkhead-associated domain] is a protein that contains a forkhead-associated (FHA) domain and a TRAF6 binding motif. It is well known that the FHA can directly bind to phosphothreonine and phosphoserine (9). Some studies have demonstrated that TIFA as a key regulator of inflammation affects tumor progression (10,11) and various fungal inflammatory lesions (12,13). TIFA expression is suppressed in hepatocellular carcinoma. In addition, it promotes cell apoptosis and suppresses cell proliferation via p53-dependent and -independent mechanisms (10). In vascular endothelial cells, TIFA is a regulator of Nod-like receptor family pyrin domain-containing protein 3 (NLRP3) inflammasome initiation and activation signals (14). However, the role of TIFA in DP remains to be elucidated.

NF-κB as a protein complex is involved in the regulation of gene transcription. P65 (also known as RelA) is one of the transcription factors that constitutes the NF-κB complex (15,16). Normally, NF-κB complex remains in an inactive state in the cytoplasm. After the stimulation by inflammatory cytokines, the NF-κB is activated and translocated into nucleus (17). Previous reports have shown that the NF-κB signaling pathway mediated the treatment of periodontitis and participated in the suppression of osteoclast differentiation (18,19). In addition, TIFA as a key factor activates the NF-κB signaling pathway in *Helicobacter pylori* (12). During *Shigella flexneri* infection, the oligomerization of TIFA is a crucial process of the subsequent oligomerization of TRAF6 and the activation of NF-κB pathway (13). Therefore, the present study aimed to investigate whether TIFA plays a role in DP through the NF-κB pathway.

The present study investigated the role of TIFA in the periodontal tissues of a DP mouse model and RAW264.7 cells. TIFA was highly expressed in periodontal tissues of the DP model. Downregulation of TIFA suppressed inflammatory reaction induced by high glucose (HG) and lipopolysaccharide (LPS) from *Porphvromonas ginaivalis* (LPS-PG) via the NF-κB signaling pathway. Additionally, osteoclast differentiation induced by (RANK) ligand (RANKL) was inhibited by knockdown of TIFA. Hence, the present study might provide a potential molecular target for the treatment of DP.

Materials and methods

Data acquisition. The data of GSE156993 downloaded from the Gene Expression Omnibus (GEO) database were obtained from expression profiling by array of peripheral blood mononuclear cells of patients with DP. There were six healthy subjects, including two males and four females (average age: 42±3.2 years, average fasting blood glucose: 87±8.8 mg/dl) and five patients with DP, including one male and four females (average age: 48±9.7 years, average fasting blood glucose: 274±48.4 mg/dl).

Establishment of diabetic periodontitis (DP) mouse model. According to previous studies (20,21), 12 male C57BL/6 mice (20-22 g) aged 6-7 weeks were used to establish the DP model. The mice were purchased from Liaoning Changsheng Biological Technology Co., Ltd. They were randomly divided into two groups, including the control group and DP group (n=6 in each group). The mice were housed at a temperature of 22±1˚C, a humidity of 45-55%, and 12 h light/dark cycle. Food and water were available *ad libitum*. After mice were adaptively fed for one week, 55 mg/kg streptozotocin (STZ; cat. no. S110910; Shanghai Aladdin Biochemical Technology Co., Ltd.) was used to induce diabetes through intraperitoneal injection once a day for 4 consecutive days. Control mice were injected with the equal volume of normal saline. Blood glucose was detected on 3 days after the last injection of STZ and the mice whose random blood glucose concentration reached 300 mg/dl were considered as diabetic models. After 4 weeks, 1 µl (20 µg/µl) LPS-PG was injected into bases of the four proximal palatal gingival papillae between left and right maxillary molars, twice a week for 2 consecutive weeks. Control group was injected with equal volume of PBS. The mice were sacrificed with carbon dioxide (CO_2) before tissue collection. The experimental animals were placed in a euthanasia chamber and CO_2 was perfused into the chamber at a volume replacement rate of 40% per minute. After confirming that the animal maintained motionlessness, no breathing and had dilated pupils, CO_2 perfusion was stopped. The animals were observed for an additional 3 min to confirm their demise. Subsequently, gingiva and periodontal tissue were collected for follow-up examinations. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (22) and the procedures were approved by the Medical Research Ethics Review Committee of General Hospital of Ningxia Medical University (approval no. KYLL-2023-0399).

Hematoxylin-eosin (HE) staining. Briefly, the fixed tissues were rinsed with running water for 4 h and then placed in a gradient series of alcohol from low to high concentrations. The tissues were placed in xylene for 30 min. Subsequently, the tissue blocks were placed in the mixture of xylene and paraffin for 2 h at 60˚C. The treated tissues were placed in dissolved paraffin, and then cut into 5 µm slices after freezing. Tissue sections expanded in warm water were transferred to slides and dried at 60˚C for 2 h. Then, sections were respectively immersed in xylene and anhydrous ethanol for dewaxing. After 5 min of soaking in hematoxylin (cat. no. H8070; Beijing Solarbio Science & Technology Co., Ltd.) and 3 min of soaking in eosin (cat. no. A600190; Sangon, Shanghai, China) at room temperature, sections were dehydrated and sealed. The images were captured using a BX53 light microscope (Olympus Corporation).

Reverse transcription-quantitative (RT-q) PCR. According to the manufacturer's protocols, total RNA of samples was isolated by TRIpure (BioTeke Corporation) and concentration was examined by ultraviolet spectrophotometer NANO 2000 (Thermo Fisher Scientific, Inc.). The cDNA was synthesized from total RNA using the BeyoRT II M-MLV reverse transcriptase (RNase H-; cat. no. D7160L; Beyotime Institute of Biotechnology) according to the protocol from the manufacturer. RT-qPCR was performed using SYBR Green I (cat. no. SY1020; Beijing Solarbio Science & Technology Co., Ltd.) and 2X Taq PCR MasterMix (cat. no. PC1150; Beijing

Table I. Antibodies employed.

Type	Name	Dilution	Catalogue number	Supplier
Primary antibody	TIFA	1:1,000	K108843P	Beijing Solarbio Science & Technology Co., Ltd.
	NF-κB p65	1:1,000	AF1234	Beyotime Institute of Biotechnology
	NLRP3	1:1,000	DF7438	Affinity Biosciences, Ltd.
	ASC	1:1,000	AF6234	Beyotime Institute of Biotechnology
	Cathepsin K	1:1,000	AF6597	Beyotime Institute of Biotechnology
	MMP9	1:1,000	AF5228	Affinity Biosciences, Ltd.
	NFATc1	1:1,000	DF6446	Affinity Biosciences, Ltd.
	Histone H3	1:5,000	AF0009	Beyotime Institute of Biotechnology
	β-actin	1:5,000	AF5001	Beyotime Institute of Biotechnology
Secondary antibody	Goat anti-rabbit IgG	1:5,000	A0208	Beyotime Institute of Biotechnology
	Goat anti-mouse IgG	1:5,000	A0216	Beyotime Institute of Biotechnology

TIFA, tumor necrosis factor receptor-associated factor-interacting protein with forkhead-associated domain; NLRP3, Nod-like receptor family pyrin domain-containing protein 3; ASC, apoptosis-associated speck-like protein; NFATc1, nuclear factor of activated T cells cytoplasmic 1.

Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions. The forward (F) and reverse (R) primer sequences were as follow: TIFA-F, 5'-GTTCAA CAGCTCCGTTCT-3', TIFA-R, 5-GTAAGGCAGGTCCAT TTT-3', β-actin-F, 5'-CTGTGCCCATCTACGAGGGCT AT-3', β-actin-R, 5'-TTTGATGTCACGCACGATTTCC-3'. The optimal PCR amplification procedure was as follows: Pre-denaturation at 94˚C for 5 min, 40 cycles at 94˚C for 10 sec, 60˚C for 20 sec and extension at 72˚C for 30 sec, followed by incubation at 72˚C for 2 min 30 sec, 40˚C for 1 min 30 sec, melting at 60˚C to 94˚C, every 1˚C for 1 sec and a final incubation at 25˚C for 1-2 min. Relative mRNA expression was calculated based on β-actin with $2^{-\Delta\Delta Cq}$ method (23).

Western blotting. Total protein was extracted by cell lysis buffer for western and immunoprecipitation (cat. no. P0013; Beyotime Institute of Biotechnology). Nuclear and cytoplasmic protein extraction kit (cat. no. P0027, Beyotime Institute of Biotechnology) was used to separate and extract protein of nucleus and cytoplasm. The concentration of extracted protein was determined using the BCA protein assay kit (cat. no. P0011; Beyotime Institute of Biotechnology). Different masses of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, 40 μg of NF-κB p65 protein or 20 μg of other proteins was separately loaded per lane. A 5% polyacrylamide gel was prepared to concentrate the proteins, and the 8, 10, 12 and 15% gels were prepared for separating different proteins. Then, proteins were transferred to PVDF membrane (MilliporeSigma). After blocking with 5% skimmed milk for 1 h at room temperature, membranes with proteins were incubated with primary antibodies at 4˚C overnight. Next, membranes were washed by TBST (1.5‰ Tween 20) and probed with secondary antibodies at 37˚C for 45 min. Finally, ECL chemiluminescence reagent (cat. no. P0018; Beyotime Institute of Biotechnology) interacted with membranes, which were visualized and captured by gel imaging system (cat. no. WD-9413B; Beijing Liuyi Biotechnology Co., Ltd.). The densitometry of blots was analyzed using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc.). The details of antibodies are shown in Table I.

Cell culture and treatments. i) RAW264.7 cells were cultured in normal glucose (NG) DMEM (Procell Life Science & Technology Co., Ltd.) and HG DMEM (Wuhan Servicebio Technology Co., Ltd.) respectively. Meanwhile, 1 ng/ml LPS-PG (cat. no. tlrl-pglps; Invitrogen; Thermo Fisher Scientific, Inc.) was added in the cells cultured with HG DMEM for 12, 24, 48, 72 h, following the determination of TIFA mRNA and protein expression.

ii) RAW264.7 cells were infected with 1x10^8 TU/ml lentivirus-carried short hairpin RNA (shRNA) targeting TIFA (shTIFA) or negative control (shNC) and cultured in an incubator with 5% CO_2 at 37˚C for 48 h. Subsequently, the mRNA and protein levels of TIFA was examined.

iii) RAW264.7 cells were infected by shTIFA and shNC for 48 h, following the culture with HG medium and 1 ng/ml LPS-PG for 24 h. Subsequently, cells were collected for the follow-up experiments, including ELISA, western blotting and immunofluorescence (IF).

iv) To induce osteoclast differentiation, RAW264.7 cells were treated with different concentrations (0, 25, 50, 75, 100 ng/ml) of RANKL (cat. no. R0525; MilliporeSigma) and then cultured in an incubator with 5% CO_2 at 37˚C for 5 days. Then, RT-qPCR and western blotting were performed to evaluate TIFA expression levels and the tartrate-resistant acid phosphatase (TRAP) staining was used for detecting osteoclast differentiation of cells.

v) After the infection of shTIFA and shNC, RAW264.7 cells were treated with 100 ng/ml RANKL and cultured in the incubator at 37˚C for 5 days to induce osteoclast differentiation. Since osteoclast differentiation was significantly induced at 100 ng/ml RANKL, this concentration was chosen for the following experiments, including RT-qPCR, western blotting,

TRAP staining and IF, to investigate the effect of TIFA on osteoclast differentiation.

Enzyme-linked immunosorbent assay (ELISA). The cell supernatant was collected to detect different inflammatory factors. The content of TNF-α, IL-6, IL-1β and methyl-accepting chemotaxis protein 1 (MCP-1) was examined by ELISA kits (Multisciences (Lianke) Biotech Co., Ltd.).

TRAP. When RAW264.7 cells were treated with different concentrations of RANKL for 5 days, or when cells infected by lentivirus were treated by 100 ng/ml RANKL for 5 days, TRAP solution in TRAP stain kit (cat. no. G1492; Beijing Solarbio Science & Technology Co., Ltd.) was added into cells to fix then at 4˚C for 1 min. After washing with PBS, cells were incubated with TRAP incubated buffer at 37˚C for 60 min in the dark. RAW264.7 cells were stained by hematoxylin for 5 min at room temperature followed by image capture.

IF. Cells induced by 100 ng/ml RANKL for 5 days were fixed with 4% paraformaldehyde for 15 min at room temperature. Then they were incubated with 0.1% TritonX-100 (cat. no. ST795; Beyotime Institute of Biotechnology) at room temperature for 30 min. Then, 1% BSA (cat. no. A602440-0050; Sangon Biotech Co., Ltd.) was added in cells at room temperature for 15 min. Next, cells were incubated with NF-κB p65 antibody (1:200; cat. no. 8242; Cell Signaling Technology, Inc.) at 4˚C overnight. The cells were then incubated with Cy3 labeled Goat anti-rabbit IgG (1:200; cat. no. A27039; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 1 h and stained with DAPI (cat. no. D106471-5 mg; Shanghai Aladdin Biochemical Technology Co., Ltd.) in the dark. Images of p65 nuclear translocation were captured by an immunofluorescence microscope (Olympus Corporation).

Statistical analysis. Data shown are represented as the mean ± standard deviation. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software; Dotmatics). The data from cell experiments are collected from three independent experiments consisting of three replicates per experiment. Statistical significances between two groups were analyzed by unpaired Student's t-test. Significances among three or more groups were tested by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TIFA is highly expressed in the periodontium of the DP mouse model. To determine whether TIFA is involved in the regulation of the periodontitis in diabetic patients, the relative mRNA expression of TIFA was analyzed using the data from GEO dataset GSE156993. Compared with the healthy controls, relative mRNA expression of TIFA was significantly increased in the DP group ($P<0.05$; Fig. 1A). To further explore the effect of TIFA on DP, we established the DP mouse model using STZ and LPS. As shown in Fig. 1B, inflammatory infiltration marked by black arrows and bone resorption suggested that the DP mouse model was successfully established. In addition, the mRNA level of TIFA was significantly increased in the periodontium of DP mouse model ($P<0.01$; Fig. 1C). Meanwhile, the TIFA protein expression was also induced by periodontitis. ($P<0.01$; Fig. 1D).

Downregulation of TIFA attenuates inflammatory response induced by HG and LPS in RAW264.7 cells. To examine whether the expression of TIFA was affected by DP conditions *in vitro*, RAW264.7 cells were stimulated with HG and LPS for different time points and the expression of TIFA was detected. As depicted in Fig. 2A, the relative mRNA and protein levels of TIFA were upregulated in RAW264.7 cells after the treatment of HG and LPS in a time-depend manner. TIFA mRNA and protein expressions were significantly knocked down by shTIFA ($P<0.01$; Fig. 2B). Additionally, compared with NG treatment, the levels of crucial inflammatory cytokines (TNF-α, IL-6, IL-1β, MCP-1) were significantly upregulated in the RAW264.7 cells treated by HG and LPS ($P<0.01$). Meanwhile, knockdown of TIFA markedly decreased the contents of TNF-α, IL-6, IL-1β, MCP-1 in HG and LPS-induced RAW264.7 cells ($P<0.01$; Fig. 2C). These results demonstrated that downregulation of TIFA alleviated HG and LPS-induced inflammatory response *in vitro*.

TIFA promotes cell inflammation through activating the NF-κB signaling pathway. A previous study reported that TIFA is a crucial upstream factor activating the NF-κB signaling pathway (24). The protein expression of NF-κB p65 was reduced in the cytoplasm ($P<0.01$) and was increased in the nucleus ($P<0.01$) by the treatment of HG and LPS and these changes were clearly reversed by the downregulation of TIFA ($P<0.01$; Fig. 3A). The results of IF revealed that p65 located in nucleus was enhanced by HG and LPS treatment. Absence of TIFA blocked the HG and LPS-induced activation of NF-κB signaling pathway. (Fig. 3B). The protein expression levels of NLRP3 and apoptosis-associated speck-like protein (ASC) were upregulated by HG and LPS in RAW264.7 cells ($P<0.01$) and was clearly suppressed by knockdown of TIFA ($P<0.01$; Fig. 3C). In summary, these findings indicated that TIFA activated the NF-κB signaling pathway and upregulated the expression of NLRP3 and ASC in DP conditions *in vitro*.

TIFA expression is upregulated in RAW264.7 cells induced by different concentrations of RANKL. Different concentrations of RANKL were used to treat osteoclastic cell line RAW264.7 to induce osteoclast differentiation. Results of TRAP staining suggested that osteoclast differentiation was clearly induced by 100 ng/ml of RANKL (Fig. 4A). Therefore, 100 ng/ml of RANKL was determined to induce osteoclast differentiation. As shown in Fig. 4B, the relative mRNA level of TIFA upregulated gradually with the increase of RANKL concentration. When RANKL induced RAW264.7 cells at 50, 75 and 100 ng/ml, TIFA expression was significantly increased compared with the control group ($P<0.01$; Fig. 4B). The protein expression of TIFA was consistent with the mRNA expression changes (Fig. 4C). Collectively, the aforementioned findings confirmed that the expression of TIFA was upregulated alongside the differentiation of osteoclastic cells.

Knockdown of TIFA suppresses RANKL-induced osteoclast differentiation. After the infection of shNC and shTIFA in

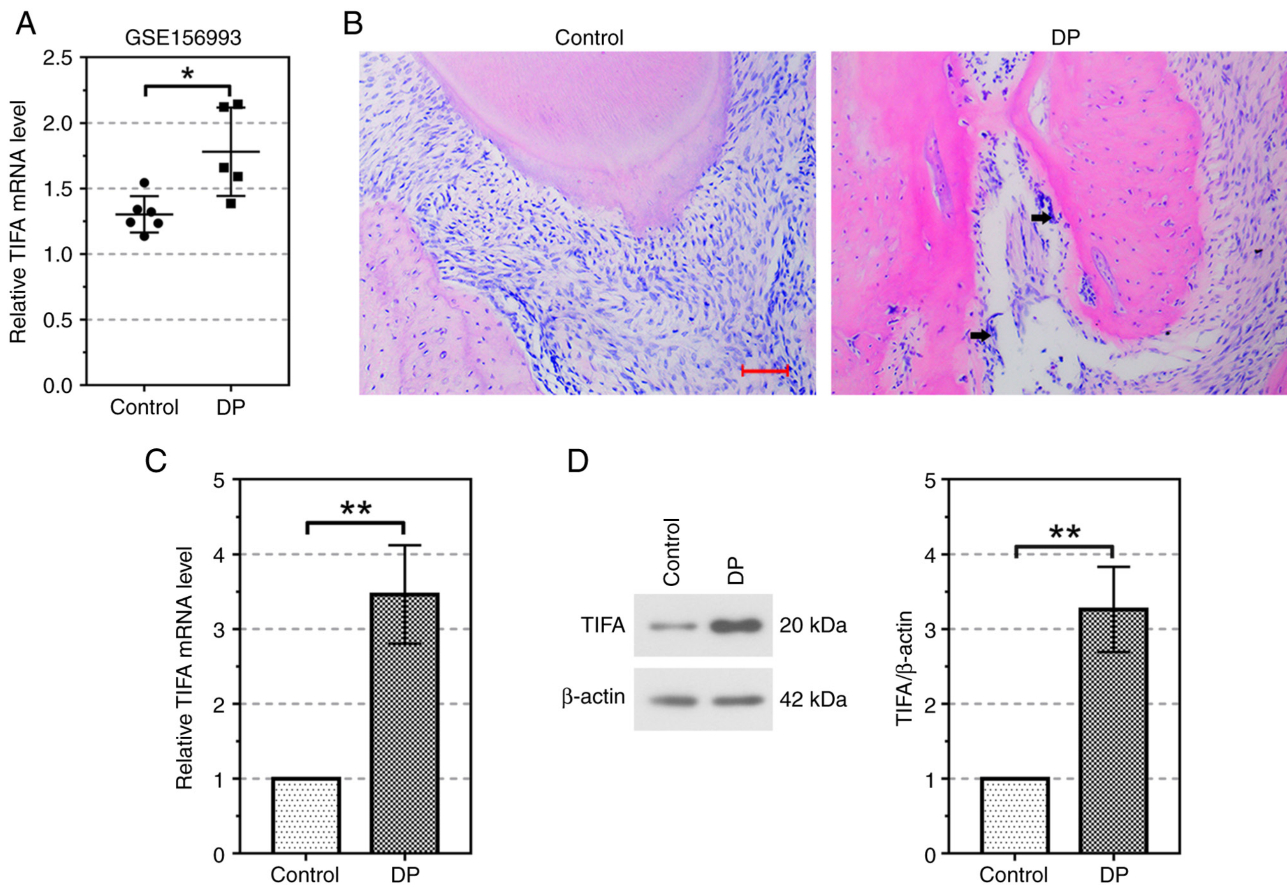

Figure 1. TIFA is highly expressed in the periodontal tissue of DP. (A) The TIFA mRNA level was analyzed using the data from GEO dataset GSE156993. (B) HE staining was used to detect inflammatory infiltration and bone resorption in the periodontium. The arrows in the image indicate inflammatory cell infiltration. Scale bar, 100 μm. (C) Reverse transcription-quantitative PCR was used to examine the TIFA mRNA level in the periodontal tissue of DP and healthy mouse models. (D) Western blotting was used to examine the TIFA protein expression. Data are represented as the mean ± standard deviation, *P<0.05, **P<0.01. TIFA, tumor necrosis factor receptor-associated factor-interacting protein with forkhead-associated domain; DP, diabetic periodontitis; HE, hematoxylin-eosin.

RAW26.7 cells, the mRNA and protein expression levels of TIFA significantly reversed the osteoclast differentiation induced by RANKL (P<0.01; Fig. 5A). In contrast to cells in the control group, the number of multinucleated osteoclasts was upregulated in cells treated with RANKL. A decrease of TIFA reduced the number of multinucleated osteoclasts, indicating that TIFA promoted osteoclast differentiation (Fig. 5B). Cathepsin K, an enzyme that breaks down other proteins, plays an important role in the breakdown of bone matrix and is an important marker of bone resorption (25). MMP9, which belongs to the MMP family, is involved in extracellular matrix degradation (26). These two proteins are downstream of nuclear factor of activated T cells cytoplasmic 1 (NFATc1) and regulated by the NF-κB signaling pathway. The protein expressions of cathepsin K, MMP9 and NFATc1 were significantly increased by RANKL in RAW264.7 cells (P<0.01). In addition, the silencing of TIFA markedly reduced cathepsin K, MMP9 and NFATc1 protein expression in RAW264.7 cells (P<0.01; Fig. 5C). Therefore, silencing of TIFA promotes the RAW264.7 cells from osteoclast differentiation induced by RANKL.

TIFA facilitates osteoclast differentiation through activating the NF-κB signaling pathway. As shown in Fig. 6A, the protein expression of NF-κB p65 in cytoplasm was significantly suppressed by RANKL, while it was increased following the infection of shTIFA in RAW264.7 cells (P<0.01). In addition, p65 expression in the nucleus was upregulated by RANKL and reversed by knockdown of TIFA (P<0.01), suggesting that the activation of NF-κB signaling pathway induced by RANKL was blocked by absence of TIFA. Results of IF verified that the expression of p65 in nucleus was clearly upregulated following RANKL treatment in RAW264.7 cells and was significantly reduced by downregulation of TIFA. (Fig. 6B). In summary, these findings verified that TIFA promoted the osteoclast differentiation by activating the NF-κB signaling pathway.

Discussion

The present study determined the expression of TIFA in the periodontal tissues in a DP model and explored the effect of TIFA on inflammatory response and osteoclast differentiation in RAW264.7 cells. The mRNA and protein expression levels of TIFA were found to be upregulated in the periodontal tissues of the DP mouse model, as well as in RAW264.7 cells stimulated by HG and LPS. Meanwhile, the activation of NF-κB signaling pathway was induced by HS and LPS, following the upregulation of NLRP3 and ASC expression.

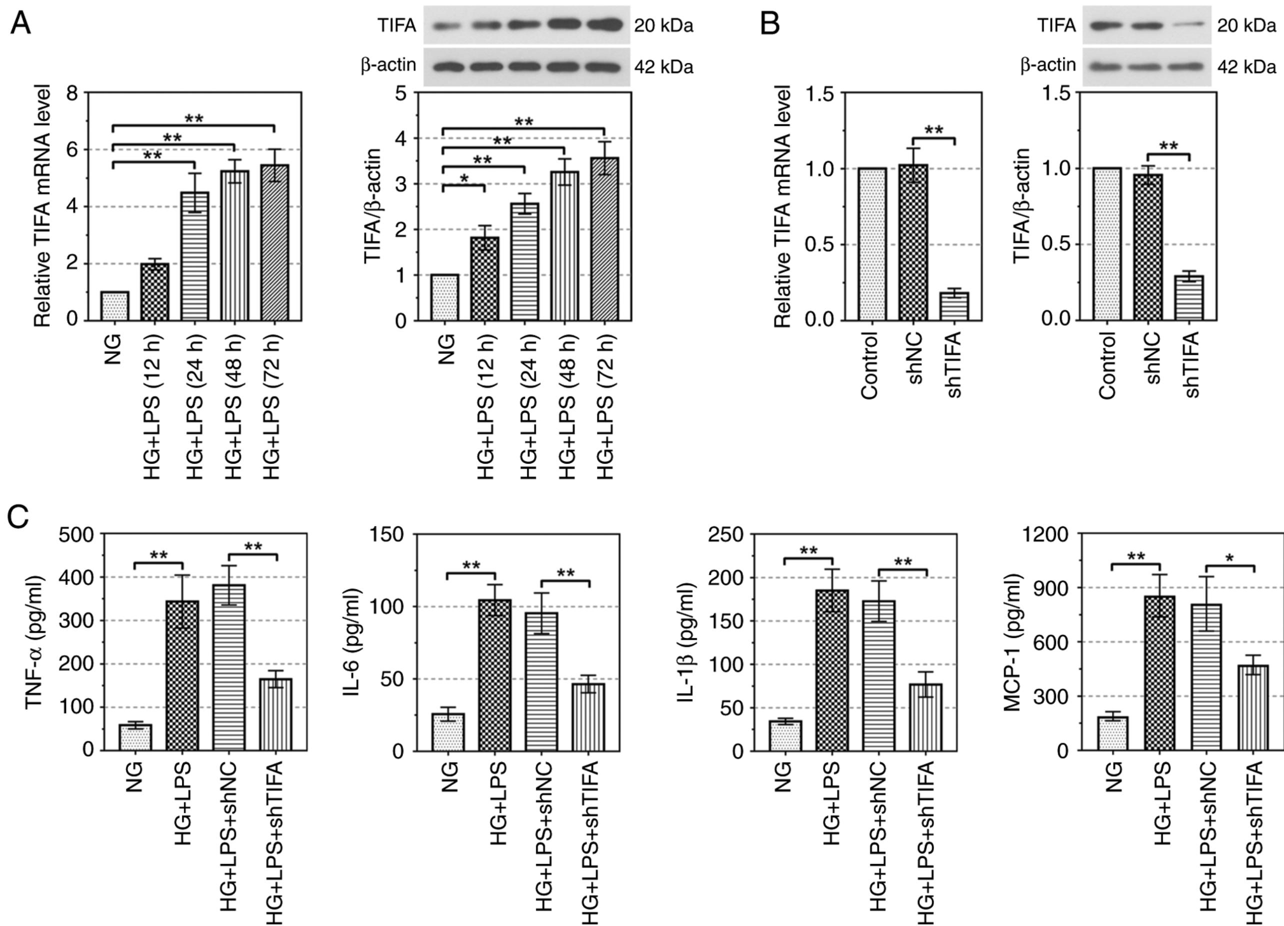

Figure 2. TIFA promotes the release of inflammatory factors. (A) The expression of TIFA in the RAW264.7 cells treated by HG and LPS-PG was evaluated by RT-qPCR and western blotting. (B) The inhibiting efficiency of shTIFA in RAW264.7 cells was detected by RT-qPCR and western blotting. (C) The concentration of TNF-α, IL-6, IL-1β and MCP-1 was examined by ELISA. Data are represented as the mean ± standard deviation, *P<0.05, **P<0.01. TIFA, tumor necrosis factor receptor-associated factor-interacting protein with forkhead-associated domain; HG, high glucose; LPS-PG, lipopolysaccharide (LPS) from *Porphyromonas gingivalis*; sh, short hairpin; RT-qPCR, reverse transcription-quantitative PCR; MCP-1, methyl-accepting chemotaxis protein 1; NG, normal glucose.

The inflammatory cytokines (TNF-α, IL-6, IL-1β, MCP-1) were promoted by HS and LPS in the RAW264.7 cells. In addition, the expression of TIFA was increased when the osteoclast differentiation of RAW264.7 cells was induced by RANKL. The NF-κB signaling pathway was activated by RANKL in the RAW264.7 cells, along with the upregulation of cathepsin K and MMP9 (Fig. 7). Therefore, TIFA was proved to promote the cell inflammation and osteoclast differentiation of RAW264.7 cells through activating the NF-κB signaling pathway. Based on these findings, the construction of molecules targeting TIFA partially is a novel and effective approach to meliorate the symptoms of DP in clinic. However, there remains numerous trials and more detailed and in-depth exploration before the findings of the present study can be truly applied in clinical practice.

Diabetes is a chronic disease with higher risk and severity of periodontal disease than non-diabetic patients (3,27). Periodontal disease begins with inflammation of the gingiva and leads to the destruction of periodontal support tissues (28). Most bone diseases are caused by overactivity of osteoclasts, resulting in a greater osteoclast resorption than osteoblast construction (29). A previous study demonstrated that controlling the inflammatory response is a useful treatment for periodontal disease (30). In 2015, resveratrol was found to serve as a potential therapeutic factor to treat DP. It reduced patients' blood glucose levels and alveolar bone loss through the TLR4 signaling pathway. The expression of inflammatory factors IL-1β, IL-6, IL-8 and TNF-α and the activation of NF-κB p65 were also suppressed at the same time (31). In addition, cynaropicrin was found to decrease the expression of inflammatory cytokines induced by *P. gingivalis* LPS and suppress the osteoclast differentiation of RAW264.7 cells induced by RANKL (32). These previous studies provide approaches for the treatment of DP. The current study focused on the expression of the endogenous molecule TIFA under DP conditions and the effect of TIFA on the inflammation and osteoclast differentiation in RAW264.7 cells.

TIFA as an inflammatory signaling adaptor is involved in numerous signaling pathways such as the NF-κB signaling pathway (9,10). It directly interacts with TRAF. A previous study revealed that DNA damage induced by the activation of NF-κB is affected by the changes of TIFA expression. TIFA, as an activator of the NF-κB pathway in the cytosol, plays a crucial role in carcinogenesis and cellular senescence (33). In addition, TIFA is found to involved in the regulation of

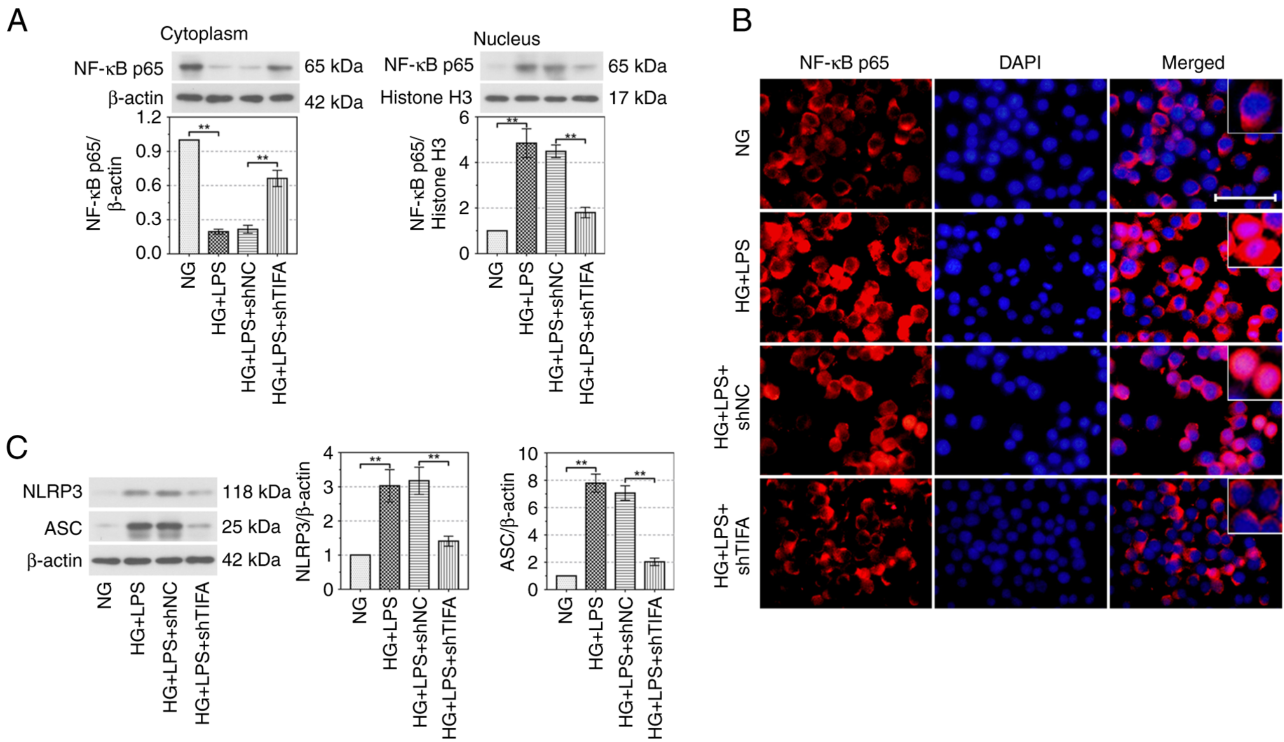

Figure 3. TIFA activates the NF-κB pathway and promotes the expression of inflammasomes. (A) The expression of NF-κB p65 in cytoplasm and in nucleus was examined by western blotting. (B) The location of NF-κB p65 was detected by immunofluorescence. Scale bar, 50 μm. (C) The protein expression of NLRP3 and ASC was examined by western blotting. Data are represented as the mean ± standard deviation, **P<0.01. TIFA, tumor necrosis factor receptor-associated factor-interacting protein with forkhead-associated domain; NLRP3, Nod-like receptor family pyrin domain-containing protein 3; ASC, apoptosis-associated speck-like protein; NG, normal glucose; HG, high glucose; LPS, lipopolysaccharide from *Porphyromonas gingivalis*; sh, short hairpin.

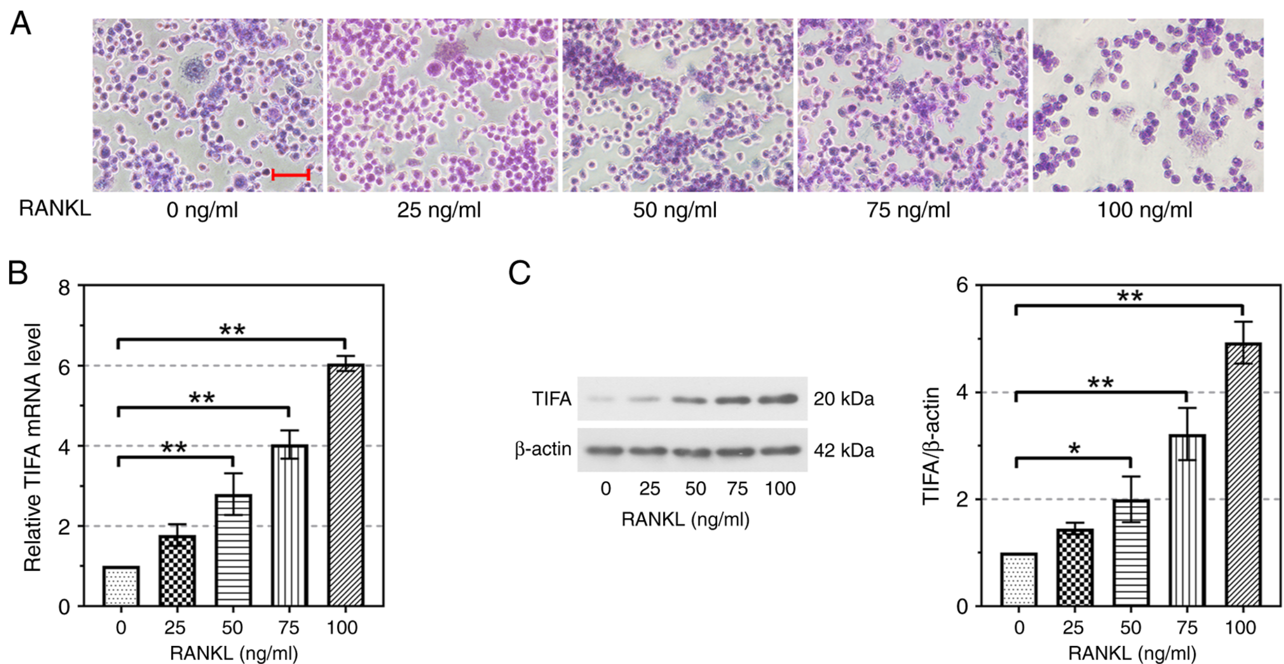

Figure 4. RANKL induces the expression of TIFA and osteoclast differentiation in RAW264.7. (A) Osteoclast differentiation was tested by tartrate-resistant acid phosphatase. Scale bar, 50 μm. The expression of TIFA in the RAW264.7 cells treated by different concentrations of RANKL was detected by (B) Reverse transcription-quantitative PCR and (C) western blotting. Data are represented as the mean ± standard deviation, *P<0.05, **P<0.01. RANKL, receptor activator of NF-κB ligand; TIFA, tumor necrosis factor receptor-associated factor-interacting protein with forkhead-associated domain.

liver cancer (10) and pulmonary arterial hypertension (34). However, the expression of TIFA on the inflammation of gingival and periodontal tissues remains to be elucidated. The current study confirmed the upregulation of TIFA expression in the periodontal tissues of a DP mouse model and in peripheral blood monocytes of patients with DP.

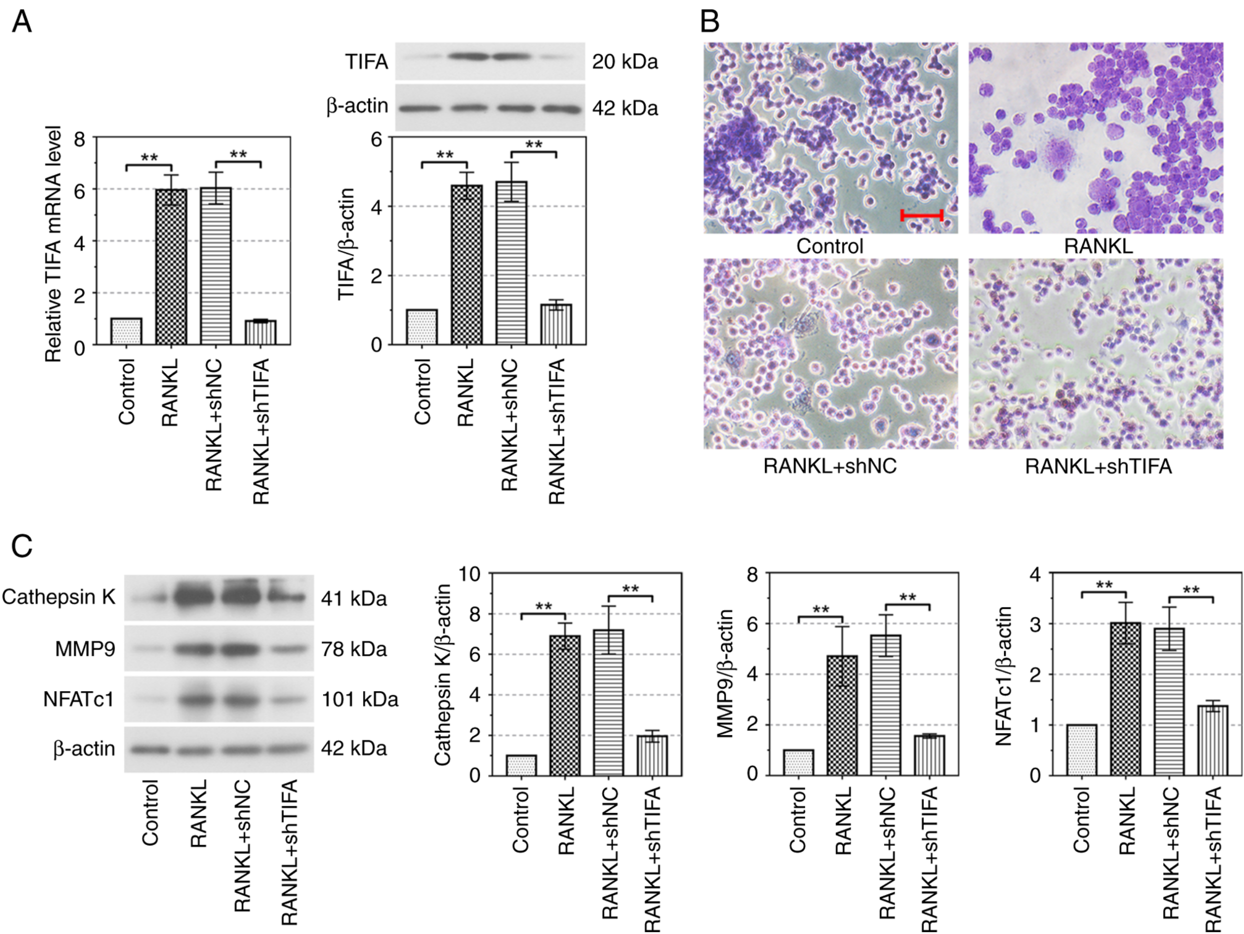

Figure 5. TIFA promotes RANKL-induced osteoclast differentiation. (A) TIFA expression with the suppression of TIFA was examined by reverse transcription-quantitative PCR and western blotting. (B) Multinucleated cells were stained by tartrate-resistant acid phosphatase. Scale bar, 50 μm. (C) The expression of cathepsin K, MMP9 and NFATc1 was evaluated by western blotting. Data are represented as the mean ± standard deviation, **P<0.01. TIFA, tumor necrosis factor receptor-associated factor-interacting protein with forkhead-associated domain; RANKL, receptor activator of NF-κB ligand; NFATc1, nuclear factor of activated T cells cytoplasmic 1.

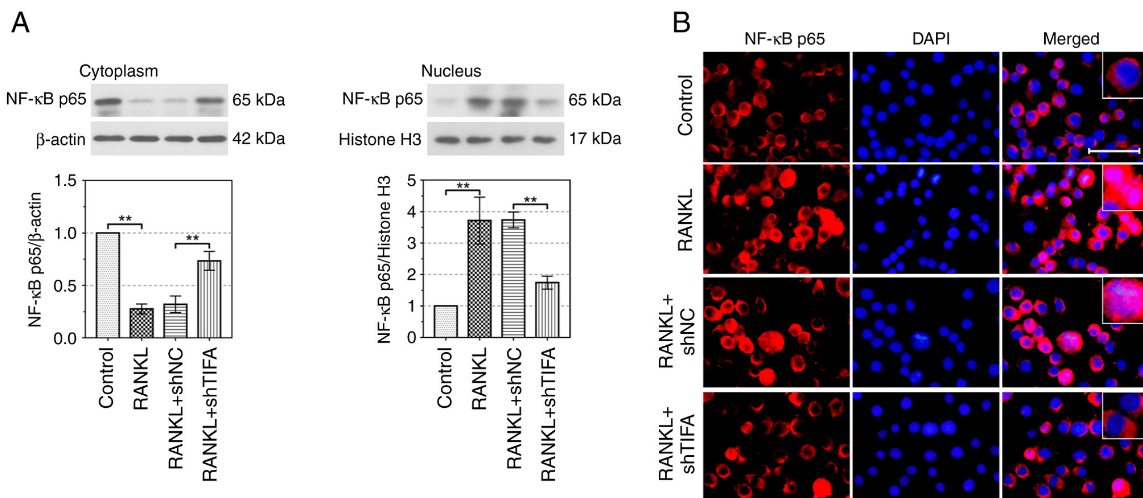

Figure 6. TIFA facilitates the osteoclast differentiation induced by RANKL via the NF-κB pathway. (A) The expression of NF-κB p65 in nucleus and cytoplasm was examined by western blotting. (B) The location of NF-κB p65 was detected by immunofluorescence. Scale bar, 50 μm. Data are represented as the mean ± standard deviation, **P<0.01. TIFA, tumor necrosis factor receptor-associated factor-interacting protein with forkhead-associated domain; RANKL, receptor activator of NF-κB ligand; sh, short hairpin.

P. gingivalis LPS is the leading cause of periodontal cell inflammation. A previous study showed that LPS significantly induces the expression of proinflammatory cytokines such as TNF-α, IL-1β and IL-6 in RAW264.7 cells (35). In the

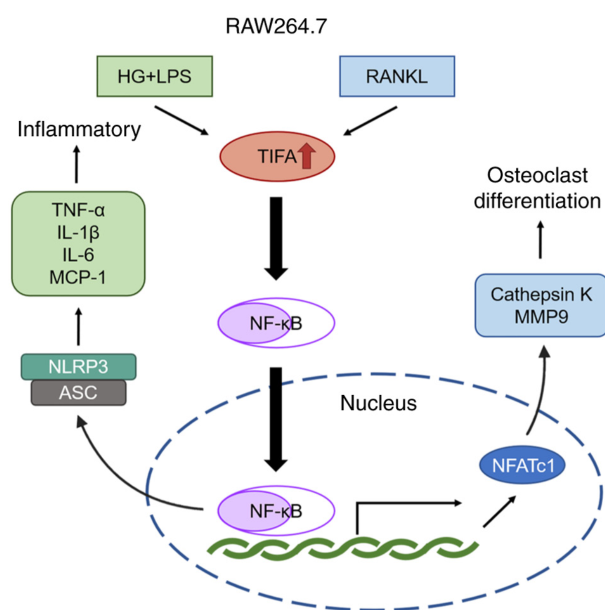

Figure 7. Schematic diagram of TIFA regulating the inflammatory and osteoclast differentiation in the RAW264.7 cells. The upregulation of TIFA, which is induced by the treatment of HG and LPS or RANKL, promotes the cell inflammation and osteoclast differentiation in the RAW264.7 cells through the activation of the NF-κB signaling pathway. TIFA, tumor necrosis factor receptor-associated factor-interacting protein with forkhead-associated domain; HG, high glucose; LPS, lipopolysaccharide from *Porphyromonas gingivalis*; RANKL, receptor activator of NF-κB ligand; MCP-1, methyl-accepting chemotaxis protein 1; NLRP3, Nod-like receptor family pyrin domain-containing protein 3; ASC, apoptosis-associated speck-like protein; NFATc1, nuclear factor of activated T cells cytoplasmic 1; HG, high glucose.

present study, an LPS-induced mice model was used to simulate the clinical conditions of periodontitis to investigate and determine whether TIFA is differentially expressed in periodontal tissues of patients with DP. This modeling method is simple to operate and can efficiently induce the occurrence of periodontitis. The establishment of cell model enables the further exploration of the effect and mechanism of TIFA on cellular inflammatory response. Clinically, the symptoms and causes of DP are various. It is difficult to regulate TIFA expression in macrophages of gingival tissues specifically. An effective way to precisely knock down TIFA expression in macrophages of periodontal tissue has not yet been found. The present study found that TIFA expression level was significantly upregulated in RAW264.7 cells treated by HG and LPS. With the increase of TIFA expression, the NF-κB signaling pathway was activated and the inflammatory cytokines were upregulated in the RAW264.7 cells. The results illustrated that TIFA was involved in the inflammation of RAW264.7 cells. It is well known that the inflammatory cytokines such as TNF-α, IL-1β and IL-6 are the downstream of the NF-κB signaling pathway. Studies have shown that TIFA acts as an important intermediate of inflammatory response (36,37). In 2016, TIFA was found to activate the signals of NLRP3 inflammasome in the vascular endothelial cells (14). ASC provides a scaffold for NLRP3 to form the structure of the inflammasome, which depends on the interaction of the pyrin domain (PYD) and the caspase activation and recruitment domain (CARD) in ASC, NLRP3 and procaspase-1 (38,39). TIFA, which does not have PYD or CARD, can directly bind to NLRP3 and ASC to promote the formation of the NLRP3 inflammasome (14). The expression of inflammasome NLRP3 and ASC was both enhanced by the upregulation of TIFA. These provide evidence for the promotion effect of TIFA on the inflammatory response of RAW264.7 cells via the NF-κB signaling pathway.

RANKL is an essential cytokine for osteoclast progression (40,41). RANKL interacts with its homologous receptor RANK. The interaction of RANKL/RANK adsorbs TRAF6 near the cell membrane and activates a cascade of downstream signaling pathways, such as the NF-κB and MAPK pathways (42). RANKL can independently induce the osteoclast differentiation of RAW264.7 cells (43), which is consistent with the findings of the present study. To verify whether osteoclast differentiation was affected by changes in TIFA expression, the inflammatory infiltration of cells and the expression of cathepsin K, MMP9 and NFATc1 were examined. Previous studies have demonstrated that cathepsin K, MMP9 and NFATc1 are osteoclastic markers, which increase with the osteoclast differentiation induced by RANKL (44-46). Cathepsin K participates in the proteolytic processing of TRAP (47). Cathepsin K and MMP-9 have both been found to be involved in matrix protein degradation during bone resorption (26). NFATc1 is known as a major transcription factor in osteoclast differentiation (48). These findings also suggest that TIFA promotes the expression of cathepsin K, MMP9 and NFATc1 through activating the NF-κB pathway to facilitate the osteoclast differentiation.

The present study mainly focused on basic research and investigated the role and regulatory mechanism of TIFA in the periodontitis. One of the main symptoms of periodontitis is the local infiltration of macrophages in gingival tissues. However, it is difficult to regulate TIFA expression in macrophages of gingival tissues specifically. It is proposed to find an effective way to specifically knock down TIFA expression in macrophages of periodontal tissue in the future. The present study verified that TIFA was highly expressed in the periodontal tissues of patients with DP and DP mice, and promoted inflammatory reaction and osteoclast differentiation. Therefore, designing and synthesizing the molecules specifically knocking down TIFA might be an effective therapeutic method for diabetic periodontitis. The clinical application of the study results will be further investigated.

In summary, the present study verified that TIFA was highly expressed in the periodontal tissues of a DP mouse model and promoted the HG- and LPS-induced inflammatory reaction and RANKL-induced osteoclast differentiation by activating the NF-κB signaling pathway.

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Availability of data and materials

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

Authors' contributions

XG, GQ and YW designed the experiments and contributed to the analysis plan. XG, GQ, JW, CY, MZ and QZ performed the experiments and analyzed data. XG and GQ prepared the manuscript with contributions from all co-authors. XG and YW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and the procedures were approved by the Medical Research Ethics Review Committee of General Hospital of Ningxia Medical University (approval no. KYLL-2023-0399).

Patient consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

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