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Adhesion of monocytes to periodontal fibroblasts requires activation of NOD1/2- and TLR4-mediated LFA-1 and VLA-4

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ARTICLE INFO

Article history:
Accepted 14 December 2014

Keywords:
Adhesion
NOD1/2
TLR4
Monocyte
Periodontal fibroblast

ABSTRACT

Objective: The aim of this study was to investigate the roles of nucleotide-binding oligomerization domain-containing protein 1/2 (NOD1/2) and Toll-like receptor 4 (TLR4) in mediating the adhesion of monocytes to periodontal fibroblasts through leucocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4).

Design: The expression of NOD1, NOD2, and TLR4 was detected in the gingival tissue of patients with chronic periodontitis by immunohistochemistry. Then the adhesion of cells of human monocytic cell line U937 to human gingival fibroblasts (hGFs) and human periodontal ligament cells (hPDLCs) was investigated after U937 cells were treated with the agonists of NOD1, NOD2, and TLR4 for 24 h, or transfected with small interfering RNAs (siRNAs) targeting NOD1, NOD2, and TLR4 for 48 h. Meanwhile, the expression of LFA-1 and VLA-4 was examined in U937 cells through real-time polymerase chain reaction (PCR), Western blot, and flow cytometry. To confirm the roles of LFA-1 and VLA-4 involved in the process of adhesion, the adhesion blockade assay was performed using the corresponding blocking antibodies against these adhesion molecules.

Results: The immunostaining results showed that NOD1, NOD2, and TLR4 were highly expressed in the gingival tissue of patients with periodontitis, especially in the monocyte-infiltrated area. The activation of these receptors by agonists upregulated the expression of LFA-1 and VLA-4 in U937 cells, and it increased the affinity of U937 cells to hGFs or hPDLCs. On the other hand, knockdown of these receptors by specific siRNAs resulted in the opposite results. In addition, blocking either LFA-1 or VLA-4 in U937 cells significantly attenuated the agonist-triggered adhesion of U937 to periodontal fibroblasts ($P < 0.001$).

Conclusions: These results suggested that NOD1/2 and TLR4 mediated monocyte–periodontal fibroblast adhesion via the modulation of LFA-1 and VLA-4.

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http://dx.doi.org/10.1016/j.archoralbio.2014.12.012
0003–9969/

1. Introduction

Periodontitis is characterized as an inflammatory disease with progressive degeneration of periodontal soft tissue and irreversible breakdown of alveolar bone, ultimately leading to tooth loss. Although the pathogenesis of the disease remains elusive, accumulating evidence shows that leucocyte migration into gingival tissue plays a major role in the initiation and development of this disease. Monocytes are one of the major infiltrating leukocytes, which take part in the innate and adaptive immune responses in the periodontal tissue of patients with periodontitis.[1–4] Thus, these infiltrating monocytes acquire the chance to come in contact with the periodontal cells directly. Periodontal tissue contains gingival fibroblasts (GFs) and periodontal ligament cells (PDLCs), the two types of the predominant cells in the periodontal connective tissue. The interaction of monocytes/macrophages with periodontal fibroblasts can facilitate monocytes/macrophages to lodge and remain at the inflammatory site, and then exert the defense as well as destructive function by releasing inflammatory factors and proteases.[5–7] In addition, this binding can also stimulate periodontal fibroblasts to secrete inflammatory cytokines such as interleukin (IL)-1α, IL-13, and IL-6,[8] which, in turn, augment the inflammatory response in periodontitis. Several studies have reported that direct contact between periodontal ligament fibroblasts and monocytes could regulate the differentiation and function of osteoclasts.[9,10] Therefore, manipulating the adhesion of monocytes/macrophages to periodontal fibroblasts could be a potential therapeutic target to control periodontal inflammation and destruction. However, so far, the mechanism of this binding in periodontitis is poorly understood.

It is known that monocyte migration is accompanied by a series of chemoattractant receptors, adhesion and signalling molecules.[11] Toll-like receptor 4 (TLR4), nucleotide-binding oligomerization domain-containing protein 1 (NOD1), and NOD2 are examples of pattern recognition receptors, which participate in the innate immune response,[12] thus perhaps taking part in this binding. NOD1 and NOD2 can be activated by the peptidoglycan derivatives from gram-negative bacteria mesodiaminopimelic acid (Tri-DAP) and muramyl dipeptide (MDP), respectively, and they mediate the expression of inflammatory cytokines.[13,14] TLR4 is shown to specially recognize lipopolysaccharide (LPS), and its activation in periodontal cells leads to excessive production of inflammatory cytokines.[15]

In our previous studies, we discovered that TLRs and NOD1/2 recognized periodontal pathogens such as *Porphyromonas gingivalis* and mediated the expression of intercellular adhesion molecules-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in human gingival fibroblasts (hGFs) and human periodontal ligament cells (hPDLCs).[16,17] ICAM-1 and VCAM-1 are two critical adhesion molecules in periodontal fibroblasts. As their counter-ligands, leucocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4) are other important adhesion molecules in leukocytes such as monocytes and lymphocytes. ICAM-1/LFA-1 and VCAM-1/VLA-4 have been proposed as two pairs of critical adhesion molecules in mediating the adhesion of monocytes to fibroblasts.[18,19] In addition, TLR4 and NOD1/2 could be activated in both periodontal fibroblasts[20] and monocytes.[21]

Therefore, it was hypothesized that TLR4 and NOD1/2 could mediate not only ICAM-1 and VCAM-1 expression but also LFA-1 and VLA-4 activities, thus leading to the binding of periodontal fibroblasts and monocytes. In this study, we tested this hypothesis by immunostaining, activation, and silencing of NOD1/2 and TLR4 by specific agonists and small interfering RNA (siRNA) transfections, real-time polymerase chain reaction (PCR), Western blotting, monocyte–periodontal fibroblast adhesion, and adhesion blockade assay using blocking antibodies.

2. Materials and methods

2.1. Collection of gingival specimens

The medical ethical committee of the Peking University School of Stomatology approved this study (Ethics Approval No. PKUSSIRB-2012017), and informed written consent was obtained from all participants at the Periodontics Department of Stomatology Hospital of Peking University. Specimens were collected from nine patients with chronic periodontitis (35–62 years of age) and nine control subjects (32–55 years of age). All 18 subjects were systemically healthy without any medication treatment in the past 6 months. Subjects in the control group were age- and gender-matched as closely as possible with those in the periodontitis group. Inflammatory specimens were collected from sites with probing depths

>5 mm and were harvested from the pocket wall during periodontal surgery. Control specimens were collected from sites with probing depths no more than 3 mm and were harvested during crown-lengthening procedures.

2.2. Immunostaining

Gingival biopsies were fixed in 4% buffered paraformaldehyde solution for 24 h, processed through a graded alcohol series, and embedded in paraffin. Serial sections with a thickness of 4 μm were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide at room temperature for 30 min, and the sections were then washed with phosphate-buffered saline (PBS; pH 7.4). Heat-induced epitope retrieval with 0.05% citraconic anhydride (pH 7.4) was performed at 100 °C for 5 min, followed by 90 °C for 10 min. Primary antibodies were applied to the sections and incubated at 4 °C overnight. Antibodies against the following molecules were used: NOD1 (Cell Signal Technology, Danvers, MA, USA), 1:100 dilution; NOD2 (Abgent, San Diego, CA, USA), 1:200 dilution; and TLR4 (BD, Minneapolis, MN, USA), 1:100 dilution. The slides were then washed in PBS three times and incubated with the secondary antibody from the streptavidin-peroxidase secondary antibody kit (ZSGB-BIO, Beijing, China), according to the manufacturer's instructions. After chromogenic development with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (ZSGB-BIO, Beijing, China), the sections

were then counterstained with haematoxylin, dehydrated through ethanol and xylene, and mounted with neutral gum.

2.3. Cell culture and reagents

The culture of primary hGFs and hPDLCs was described in our previous study.[16] U937 cells were donated by the immunology laboratory of the Peking University Health Science Center. These cells were cultured in RPMI1640 medium (Gibco, Paisley, Scotland, UK) in 100-mm dishes containing 10% foetal bovine serum (FBS; HyClone, Logan, UT, USA). All cultures were maintained at 37 °C with a humidified gas mixture of 5% CO_2/95% air. The agonists Tri-DAP (NOD1 ligand), MDP (NOD2 ligand), and LPS (TLR4 ligand) were purchased from InvivoGen (San Diego, CA, USA).

2.4. Adhesion of U937 cell to hGFs or hPDLCs

hGFs or hPDLCs (1×10^4 cells) were seeded in 24-well plates and maintained for 24 h at 37 °C. In parallel, U937 cells were incubated with agonists at 10 μg/ml or siRNA in the medium. After 24 h, the medium of U937 was withdrawn and 0.5 ml of Dulbecco's modified Eagle's medium (DMEM) (with 10% FBS) containing 1×10^5 cells was added to the confluent fibroblasts. The cells were thereafter cocultured for an additional 4 h. Then the medium was withdrawn to remove non-adhered monocytes. The cells adhering to the glass slides were washed twice with PBS and fixed with ice-cold methanol for 10 min. The fixed cells were treated with 3% hydrogen peroxide for 2 min and washed with PBS followed by incubation with a serum-blocking reagent for 15 min. Thereafter, the cells were incubated with a blocking reagent for 15 min. After washing with PBS, the cells were incubated at 4 °C overnight with

Table 1 – PCR primer used in this study.

Target gene	Sequence 5′ → 3′	Product size (bp)
NOD1	ACAGCCAGGGCGAGATAC AAAGGTGCTAAGCGAAGAG	189
NOD2	TCAGTTAAGCCTTTGGAAACAG CATCAACCAGAAGCCTAGTGAG	109
TLR4	AGAACCTGGACCTGAGCTTTAATC GAGGTGGCTTAGGCTCTGATATG	142
actin	CCTGGCACCCAGCACAAT CCGATCCACACGGAGTACTTG	68

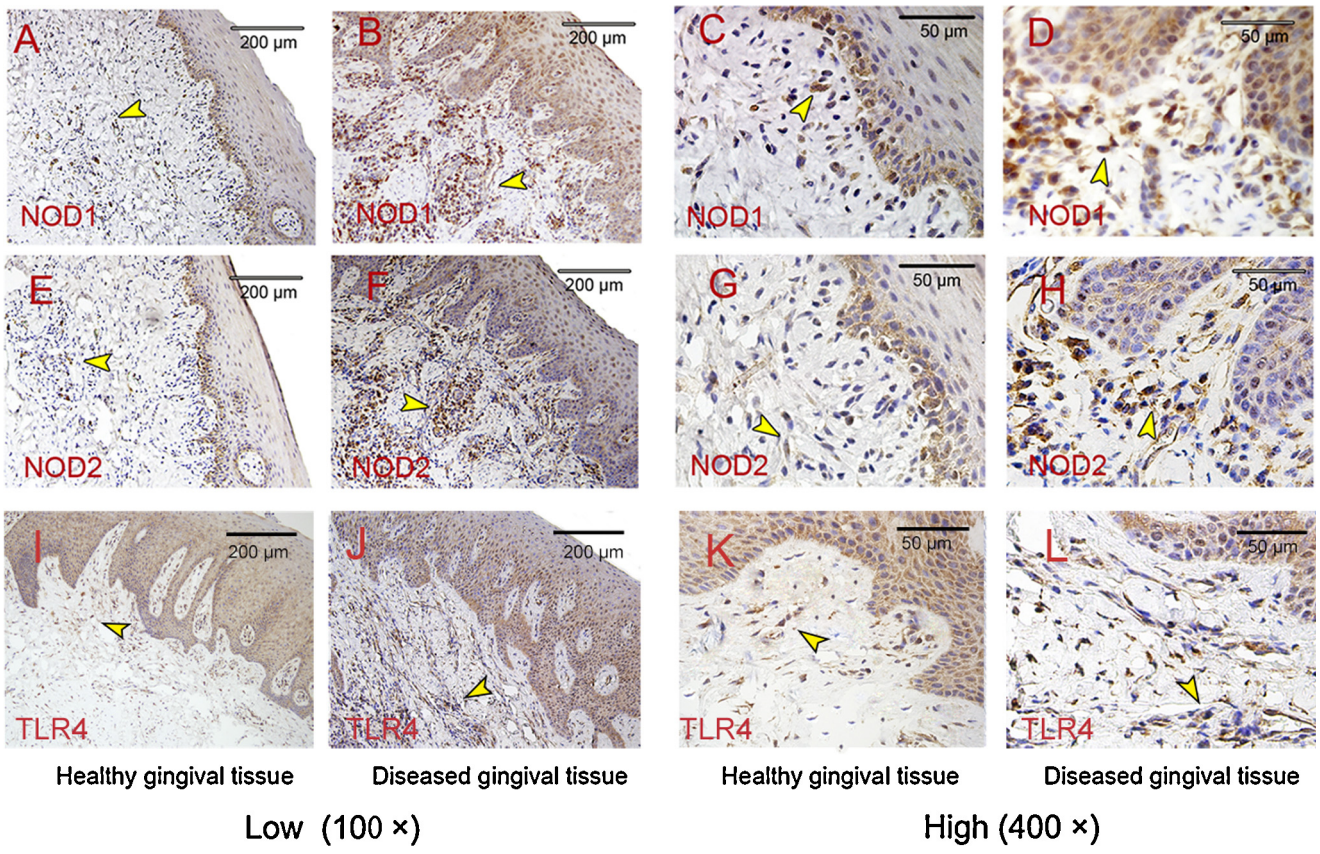

Fig. 1 – NOD1, NOD2, and TLR4 were in highly regulated manners in the connective tissue of diseased gingiva. Nine gingival specimens from patients with periodontitis and nine from healthy donors were collected, and the expression of NOD1, NOD2, and TLR4 was detected in all the specimens by immunohistochemistry. (A–D) NOD1 was expressed in both the epithelium and connective tissues. Comparing the two groups, the expression level of NOD1 in the epithelium was similar. However, its level in the connective tissue was significantly higher in diseased gingiva. (E–H) NOD2 expression was also significantly upregulated in the diseased specimens. (I–L) TLR4 was expressed in the epithelium and connective tissues. Similar with NOD1 and NOD2, the expression level of TLR4 was upregulated in the connective tissue of diseased specimens.

primary antibodies of mouse anti-vimentin (Epitomics, Burlingame, CA, USA) and mouse anti-CD68 (Santa Cruz, Dallas, TX, USA) diluted 1:200 and 1:150 in PBS, respectively. Subsequently, after washing with PBS, the cells were incubated with a biotinylated secondary antibody for 60 min at room temperature, washed with PBS, and then treated with DAB 0.3% (v/v) in DAB buffer containing 0.1% (v/v) hydrogen peroxide. Finally, the cells were washed with distilled water, dehydrated through an ethanol series (70%, 95%, and 100%) and xylene, mounted, and photographed using a Zeiss light microscope.

2.5. *RNA interference*

NOD1 and NOD2 siRNA and control siRNA were obtained from Invitrogen Biotechnology (Invitrogen, Carlsbad, CA, USA), and TLR4 siRNAs were purchased from RiboBio Technology (Guangzhou, China). Transfection of siRNA into U937 cells was performed using a hemagglutinating virus of Japan envelope (HVJ-E) vector kit (GenomONE Neo, Ishihara Sangyo, Osaka, Japan), according to the manufacturer's instructions. The transfection of 100 pM of siRNA into the cells was carried out for 48 h. The relative expression levels of adhesion molecules and pattern recognition receptors were then determined by real-time PCR and Western blot.

2.6. *Adhesion blockade assay using blocking antibodies*

U937 cells were incubated with Tri-DAP, MDP, or LPS at 10 μg/ml for 24 h, and then washed three times with D-Hank's solution followed by addition of 0.5 ml of DMEM (with 5% FBS) containing 10 μg/ml anti-LFA-1 (Ebioscience, San Diego, CA, USA, Clone: HI111) or 10 μg/ml anti-VLA-4 antibody (Ebioscience, San Diego, CA, USA, Clone: 9F10). One hour later, these cells were added to the hGF or hPDLC layer in DMEM (with 10% FBS) with blocking antibody in 24-well plates. After 4 h of incubation, the cells were collected as described in the previous paragraph of immunohistochemistry.

2.7. *RNA extraction and complementary DNA synthesis*

Total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The RNA concentration was measured spectrophotometrically. The total RNA (2 μg) was then reverse-transcribed into single-stranded complementary DNA (cDNA) using Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA) and oligo(dT) 15 primers, according to the manufacturer's protocol.

2.8. *Quantitative real-time polymerase chain reaction*

Quantitative polymerase chain reaction (qPCR) was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Life Technologies, Warrington, UK) using SYBR Green reagent (Roche, Indianapolis, IN, USA). β-Actin was used as the endogenous control gene. The primers used in this study are listed in Table 1. The standard PCR conditions were 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were performed in triplicate. The

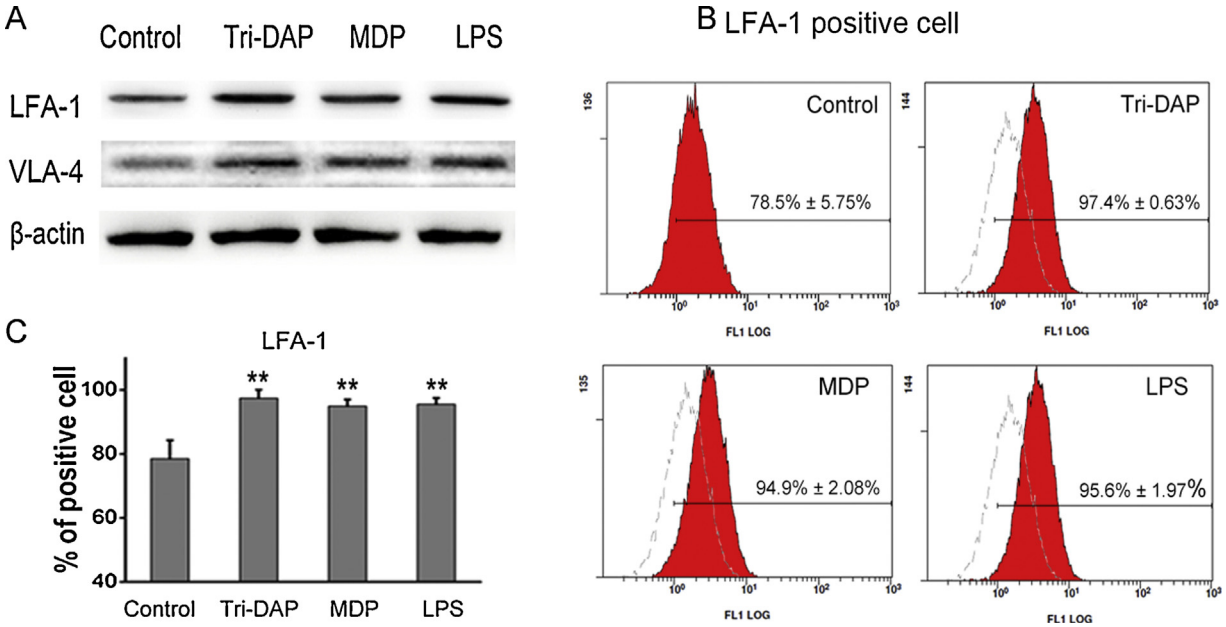

Fig. 2 – Tri-DAP, MDP, and LPS, respectively, upregulated the expression of LFA-1 and VLA-4 in U937 cells. U937 cells were incubated with Tri-DAP, MDP, or LPS for 24 h, and the expression of LFA-1 and VLA-4 was measured by Western blot and flow cytometry. (A) Compared to the control group, the protein expression levels of LFA-1 and VLA-4 were slightly upregulated by either of these agonists. (B) In addition, LFA-1-positive cells were also slightly increased after treatment with Tri-DAP, MDP, or LPS. (C) Statistical analysis of the difference. *, $P < 0.05$ as compared with their corresponding untreated controls. **, $P < 0.01$; ***, $P < 0.001$.

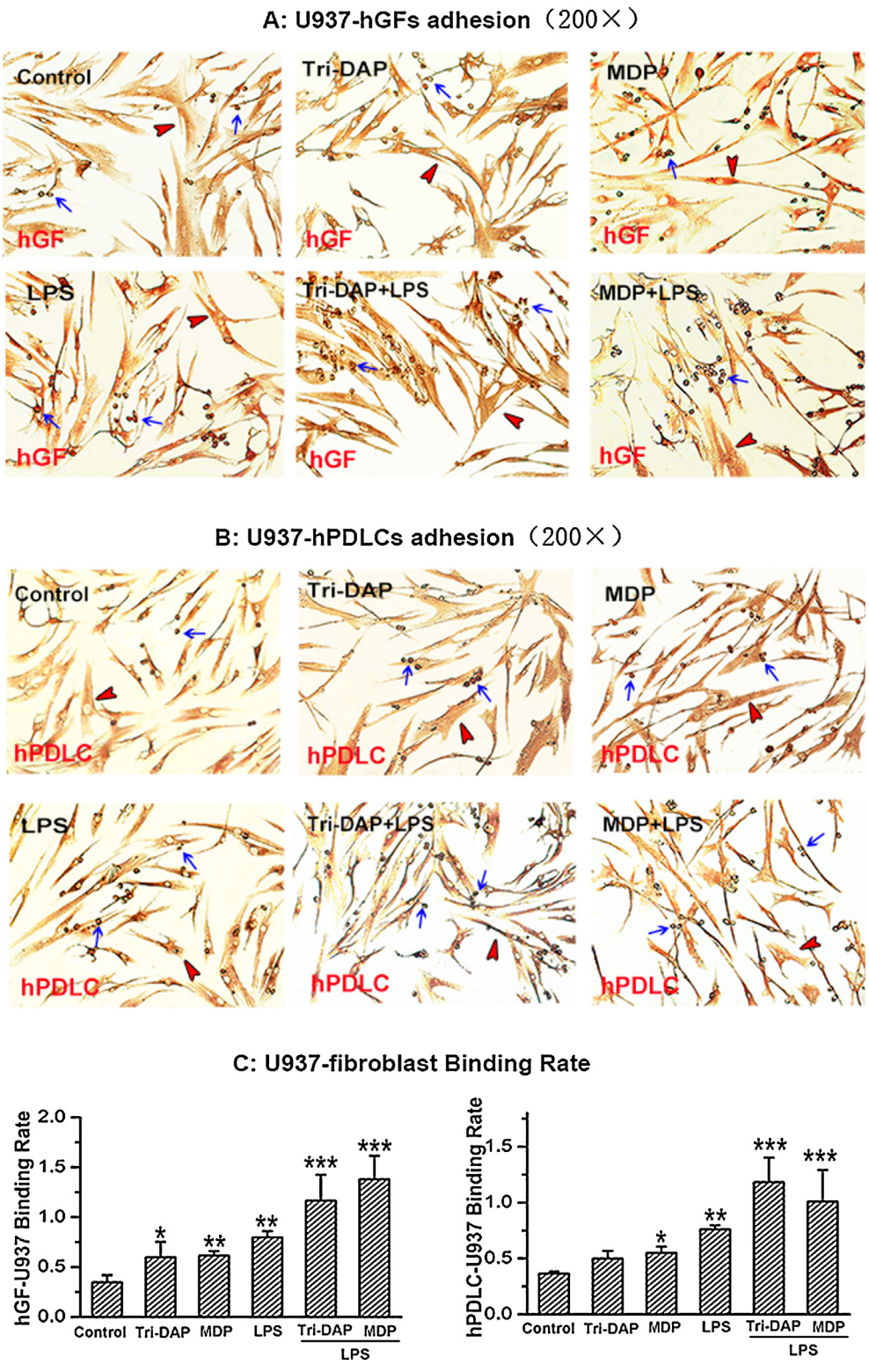

Fig. 3 – Tri-DAP, MDP, and LPS increased the affinity of U937 cells to hGFs or hPDLCs. U937 cells were incubated with Tri-DAP, MDP, or LPS, respectively, for 24 h, and they were cocultured with hGFs or hPDLCs for another 4 h. The adhesion was evaluated by immunochemistry and the binding rate was calculated by the ratio of total U937 cells to total fibroblasts. (A) Compared with control group, U937 cells incubated with Tri-DAP, MDP, or LPS increased U937 cell affinity to hGF. This binding was even enhanced by a combination of LPS with Tri-DAP or MDP. (B) The results of U937–hPDLC adhesion were similar to that of U937–hGF. (C) The binding rate of U937 cells to hGFs or hPDLCs was higher when treated by agonist applied alone or together. Blue arrow: U937 cells; red arrowhead: hGFs or hPDLCs. *, $P < 0.05$ as compared with their corresponding untreated controls. **, $P < 0.01$; ***, $P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

expression levels of the target transcripts in each sample were calculated by the comparative $2^{-\Delta\Delta Ct}$ method after normalization to the expression of β-actin.

2.9. Western blot

The cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer (Applygen, Beijing, China) containing protease inhibitors and phosphatase inhibitors. After measuring the protein concentration by the BCA kit (Thermo, Rockford, IL, USA), equal amounts of protein samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes by wet blotting. The membranes were blocked in 5% nonfat dry milk for 1 h and probed with antibodies against NOD1 (Cell Signaling Technology, Danvers, MA, USA), NOD2 (Abgent, San Diego, CA, USA), TLR4 (BD, Minneapolis, MN, USA), LFA-1 (Ebioscience, San Diego, CA, USA), VLA-4 (Ebioscience, San Diego, CA, USA), and β-actin (ZSGB-BIO, Beijing, China) separately at 4 °C overnight. After incubation with peroxidase-linked secondary antibodies, the ECL reagent (Thermo, Rockford, IL, USA) was used to visualize the immunoreactive proteins.

2.10. Flow cytometry

In order to confirm the expression of LFA-1 and VLA-4 on U937 cells after agonist treatment, flow cytometry analysis was used following the manual. Briefly, cells were treated with Tri-DAP, MDP, or LPS for 24 h, and then harvested and resuspended with 2% bovine serum albumin (BSA, Sigma, UK) in PBS. Single-cell suspensions (100 μl) were incubated for 1 h at room temperature with 1 μl of mouse anti-human LFA-1 (CD 11a) primary antibodies. Then the cells were washed twice and incubated with secondary antibody conjugated with fluorescein isothiocyanate (FITC). Subsequently, the cells were diluted with 400 μl of filtered saline and analyzed using the Epics XL flow cytometer (Beckman Coulter, Brea, CA, USA). The percentage of positive cells was determined by comparison with the untreated control.

2.11. Statistical analysis

The adhesive efficiency was calculated as the ratio of adhered U937 cells to total fibroblasts from five randomly selected high-magnification fields. All experiments were performed with periodontal fibroblasts and monocytes from at least three individuals. Data were expressed as the means ± standard deviation (SD). Differences between means were assessed by analysis of variance (ANOVA) of SPSS. The level of significance (P) was set as < 0.05.

3. Results

3.1. The expressions levels of NOD1, NOD2, and TLR4 are higher in inflammatory gingival tissues

First, the expression patterns of NOD1, NOD2, and TLR4 in gingival tissues were examined by immunostaining. In the healthy controls, NOD1 was clearly expressed in the epithelium, and it was detectable in the connective tissues (Fig. 1A and C). In the gingival biopsies with periodontitis, the high

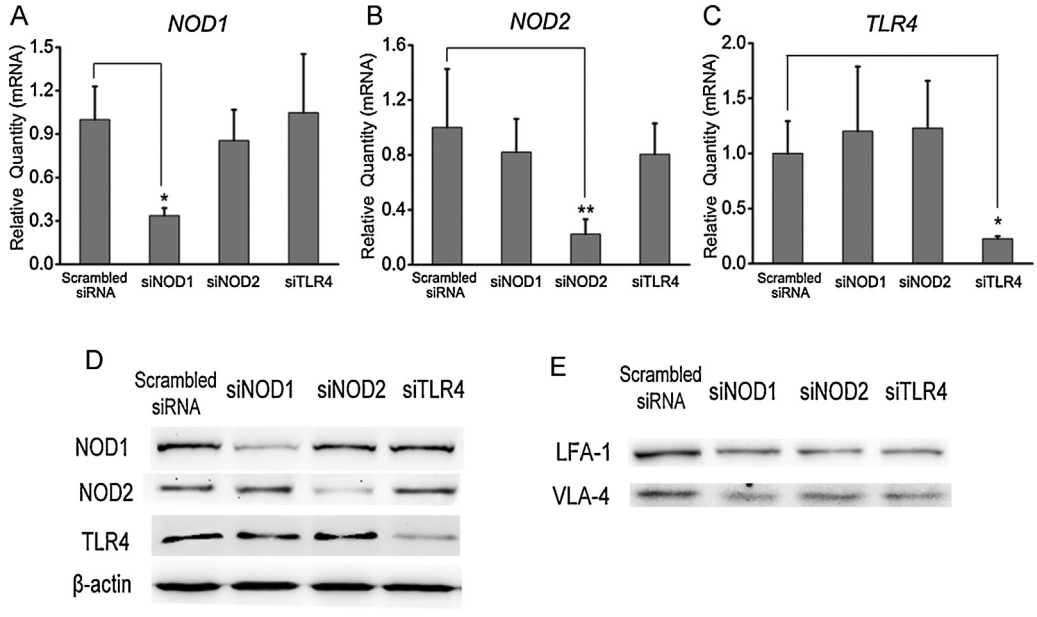

Fig. 4 – The effective knockdown of NOD1, NOD2, andTLR4 by siRNA in U937 cells. Small interfering RNA targeting NOD1, NOD2, or TLR4 were transfected into U937 cells for 48 h, and NOD1, NOD2, and TLR4 expression was detected by real-time PCR and Western blot. (A) NOD1 mRNA expression was significantly decreased by siNOD1, while it was not affected by transfection of siNOD2 or siTLR4. (B) NOD2 mRNA expression was attenuated by siNOD2. (C) TLR4 mRNA expression was downregulated by siTLR4. (D) The Western blot results were in line with the mRNA results. (E) The expression levels of LFA-1 and VLA-4 were attenuated by any of these siRNAs in U937 cells. *, $P < 0.05$ as compared with their corresponding untreated controls. **, $P < 0.01$; ***, $P < 0.001$.

expression level was maintained in the epithelium; however, a dramatic increase in NOD1 expression in connective tissues was revealed (Fig. 1B and D). NOD2 and TLR4 showed a similar expression pattern to NOD1. While the expression level of NOD2 and TRL4 was consistent in both healthy and inflammatory epithelia, the scattered NOD2-positive and TLR4-positive signal in the connective tissue of healthy gingiva became congregated in the diseased tissue, mainly located in the monocytic/macrophage- and fibroblast-like cells (Fig. 1E–L). The immunostaining results suggested that NOD1, NOD2, and TLR4 were mainly expressed in the monocytes and fibroblasts near the infiltration site of immune cells.

3.2. *Activation of NOD1, NOD2, and TLR4 enhances the binding of U937 cells to hGFs and hPDLCs*

U937 cells were pre-incubated with Tri-DAP, MDP, or LPS for 24 h before being cocultured with hGFs or hPDLCs. It was found that the expression of LFA-1 and VLA-1 was slightly upregulated in U937 cells by either of these agonists (Fig. 2A), and this result was confirmed by flow cytometry, which showed that LFA-1 (CD11a)-positive cells increased from 78.5% ± 5.75% (untreated control) to 97.4% ± 0.63% after Tri-DAP treatment ($P = 0.004$), to 94.9% ± 2.08% after MDP treatment ($P = 0.008$), and to 95.6% ± 1.97% after LPS treatment ($P = 0.007$) (Fig. 2B and C). Meanwhile, compared with the control group, Tri-DAP, MDP, or LPS significantly increased the adhesion of U937 to hGFs or hPDLCs (Fig. 3, $P < 0.05$), and this effect was enhanced by the combination of LPS with Tri-DAP or MDP in U937 cells (Fig. 3, $P < 0.05$).

3.3. *Silence of NOD1, NOD2, and TLR4 attenuated affinity of U937 cells to hGFs and hPDLCs*

U937 cells were transfected with siRNA targeting NOD1, NOD2, and TLR4 or scramble siRNA (control) 48 h prior to the immunostaining. The expression of NOD1, NOD2, and TLR4 in U937 cells were decreased by their special siRNAs at messenger RNA (mRNA) and protein levels (Fig. 4, A–D, $P < 0.05$). Meanwhile, knockdown of any of these receptors downregulated the expression level of LFA-1 and VLA-4 (Fig. 4E), and it also led to the decrease of fibroblast–U937 cell interaction (Fig. 5, $P < 0.05$). Taken together, NOD1/2 and TLR4 signals regulated the adhesion of U937 cells to periodontal fibroblasts.

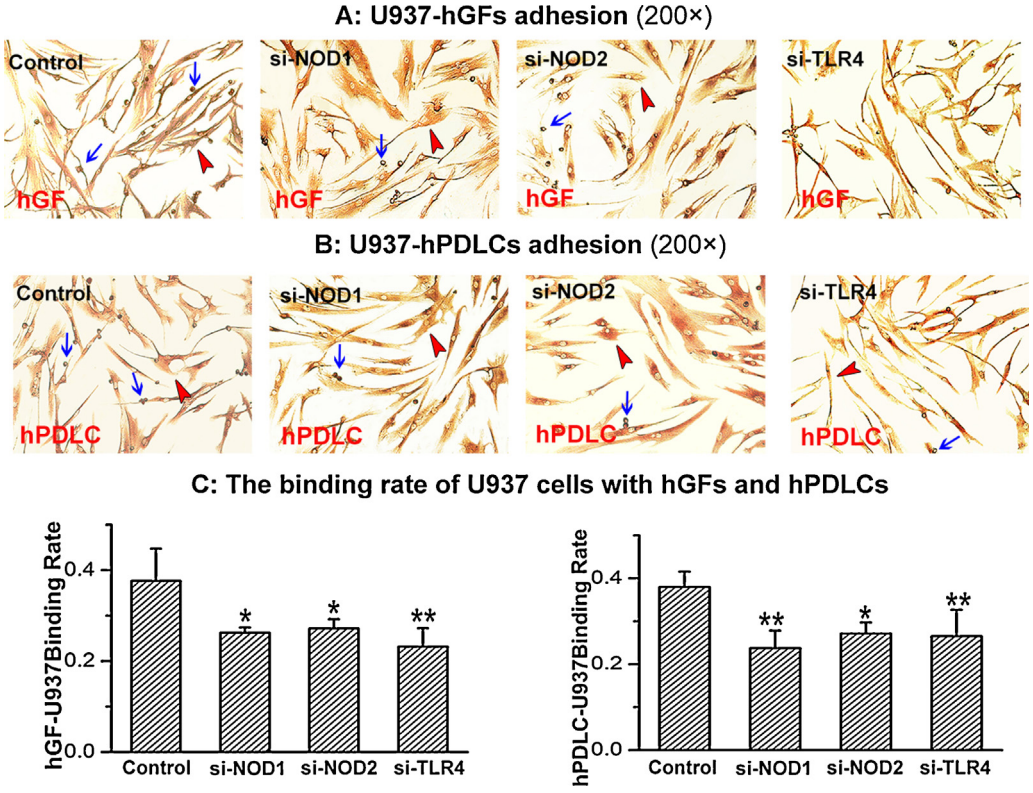

Fig. 5 – Knockdown of NOD1, NOD2, or TLR4 in U937 cells led to their decreased affinity to hGFs or hPDLCs. U937 cells were transfected with siNOD1, siNOD2, siTLR4, or scrambled siRNA for 48 h, and then they were cocultured with hGFs or hPDLCs for another 4 h. The adhesion was evaluated by immunochemistry and the binding rate was calculated by the ratio of total U937 cells to total fibroblasts. (A) Compared with control group, U937 cells transfected with siNOD1, siNOD2, or siTLR4 decreased its affinity to hGFs. (B) The results of U937–hPDLC adhesion treated by siRNA were similar to that of U937–hGF adhesion. (C) The binding rate of U937 cells to hGFs or hPDLCs was significantly reduced when U937 cells were treated with siNOD1, siNOD2, or siTLR4. Blue arrow: U937 cells; red arrowhead: hGFs or hPDLCs. *, $P < 0.05$ as compared with their corresponding untreated controls. **, $P < 0.01$; ***, $P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

3.4. *LFA-1 and VLA-4 mediated agonist-triggered adhesion of U937 cells and fibroblasts*

To investigate the mechanism of agonist-triggered U937–periodontal fibroblast binding, the blocking antibodies for LFA-1 and VLA-4 were utilized in U937 cells after agonist treatment. Compared with the group that was incubated with the agonist alone, the affinity of U937 cells to hGFs/hPDLCs was decreased by neutralizing LFA-1 and VLA-4 (Figs. 6 and 7). Therefore, Tri-DAP, MDP, and LPS triggered U937–periodontal fibroblast interaction via LFA-1 and VLA-4.

4. Discussion

This study revealed that some recognition receptors, NOD1, NOD2, and TLR4, were highly expressed in the gingival tissue of patients with periodontitis. Activation of either of these receptors led to periodontal fibroblast–monocyte binding via LFA-1 and VLA-4 in monocytes. TLRs were discovered many years ago, and their correlation with periodontitis was investigated in many studies. Cumulative evidence showed that TLR4 was significantly elevated in gingival connective tissues of patients with chronic periodontitis,[22,23] which was in line with our present study. However, until now, there has been little evidence of the correlation between NOD1/2 and periodontitis: only two studies have investigated this issue. Sugawara et al.[24] stained NOD1 and NOD2 in gingival tissue samples from five patients with chronic periodontitis and three periodontally healthy subjects, observing that NOD1/2 showed no significant difference in the gingival epithelium between these two groups. However, the authors did not describe their expressions in connective tissue. In the other study, Hosokawa et al.[25] only focused on NOD2 expression in the gingival tissues, but they did not mention NOD1 expression patterns. In the present study, we not only demonstrated that NOD1/2 were upregulated in periodontitis but also observed their expression patterns in the epithelium and connective tissues. NOD1/2 showed similar expressions in the epithelium between diseased gingiva and healthy controls. However, their expressions in the connective tissue of gingiva with periodontitis were much higher, especially in the areas where monocytes infiltrated close to fibroblasts.

TLR4 is a co-receptor of CD14. Only the CD14-positive monocytes could respond to LPS and activate TLR4 signalling. Certainly, it has been shown that >90% of monocytes represent CD14 expression in blood,[26] suggesting that most infiltrated monocytes in the gingival tissue are TLR4-respon-

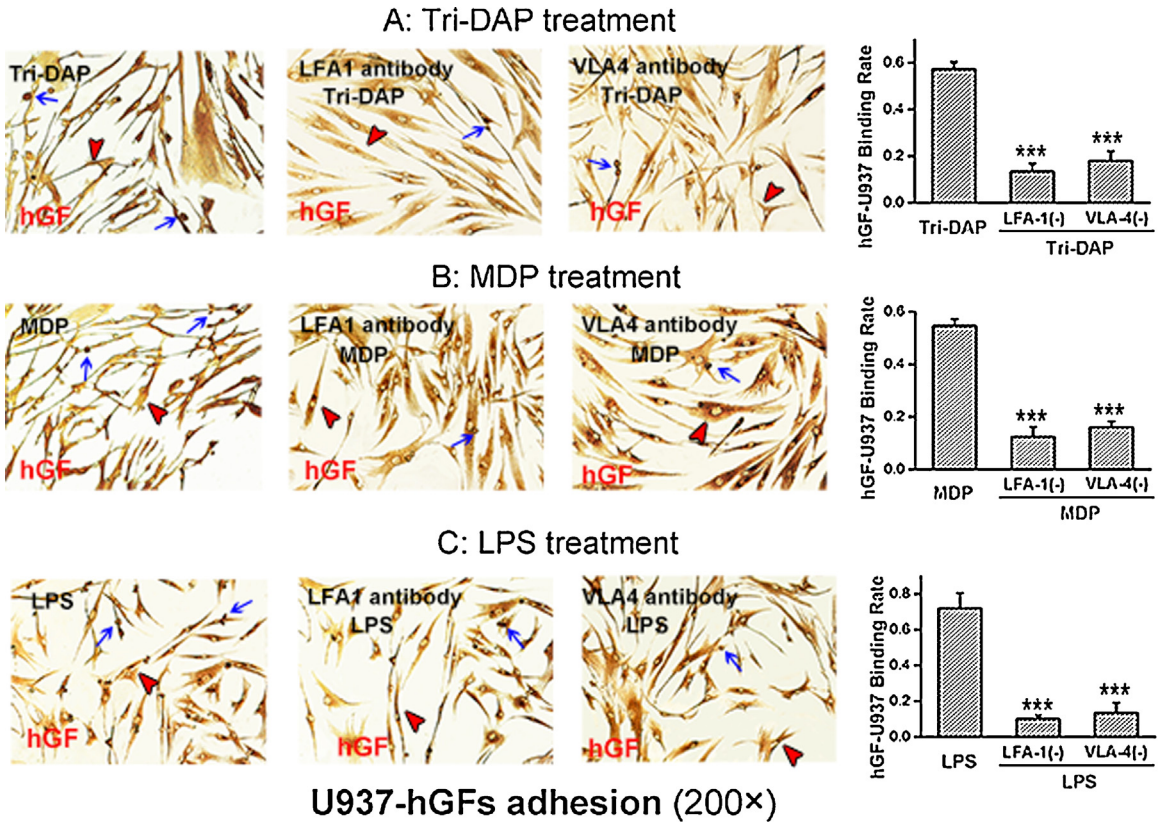

Fig. 6 – Neutralization of LFA-1 or VLA-4 hindered the agonist-triggered binding of U937 cells to hGFs. U937 cells were incubated with Tri-DAP, MDP, or LPS for 24 h, and then incubated with blocking antibody for LFA-1 or VLA-4 for 1 h, followed by coculture with hGFs for another 4 h. The adhesion was evaluated by immunochemistry and the binding rate was calculated by the ratio of total U937 cells to total fibroblasts. (A) Compared with the control group that was treated with Tri-DAP alone, the adhesion of U937 cells to hGFs was significantly decreased by blocking LFA-1 or VLA-4 after agonist treatment. (B–C) The result was similar in the MDP treatment group and the LPS treatment group. *, $P < 0.05$ as compared with their corresponding untreated controls. **, $P < 0.01$; ***, $P < 0.001$.

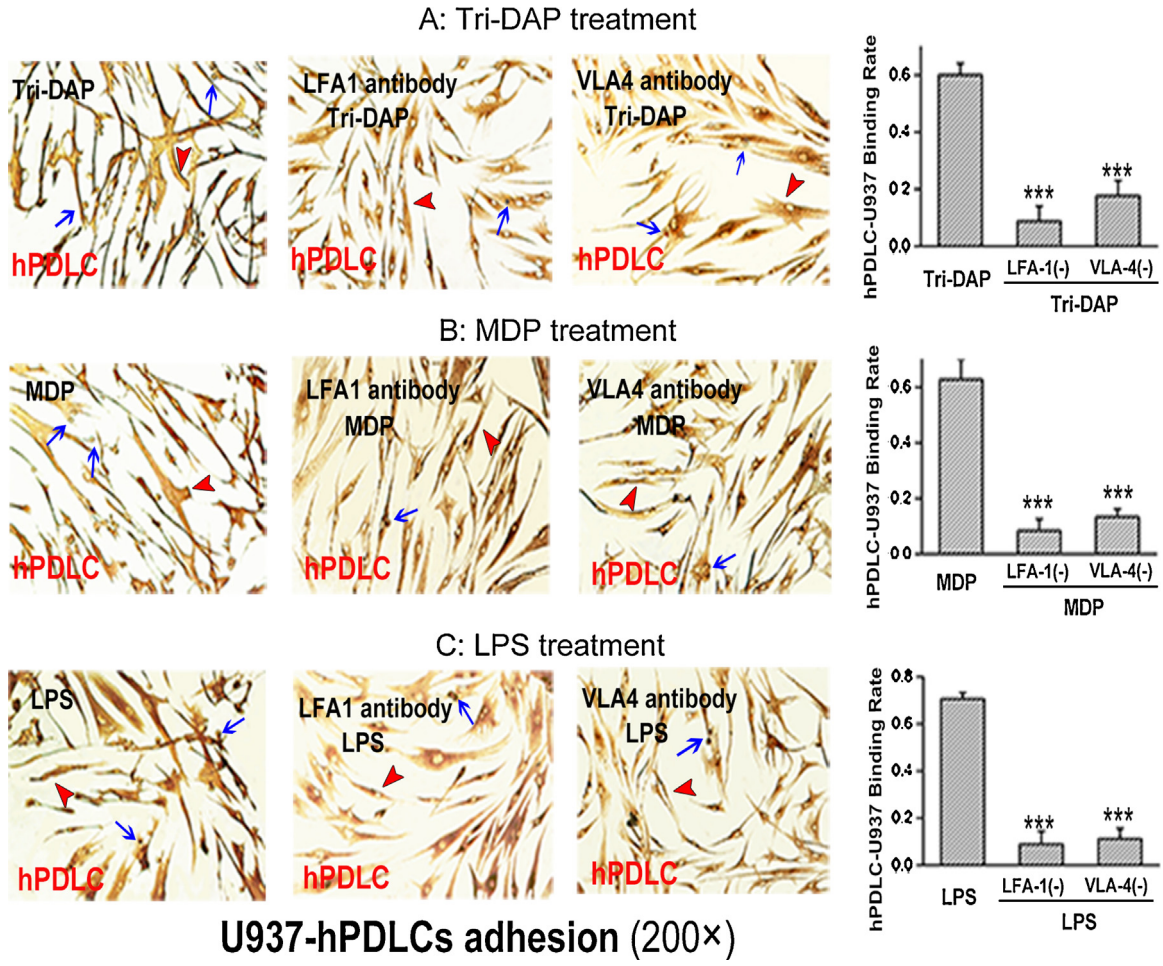

Fig. 7 – Neutralization of LFA-1 or VLA-4 hindered the agonist-triggered binding of U937 cells to hPDLCs. With the same treatment and analysis as described in Fig. 6, the U937–hPDLC binding rate was calculated by the ratio of total U937 cells to total fibroblasts. (A) Compared with the control group that was treated with Tri-DAP alone, the adhesion of U937 cells to hPDLCs was significantly decreased by blocking LFA-1 or VLA-4 after agonist treatment. (B–C) The result in the MDP treatment group and the LPS treatment group was similar to that in the Tri-DAP group. *, $P < 0.05$ as compared with their corresponding untreated controls. **, $P < 0.01$; ***, $P < 0.001$.

sible cells. Activation of TLR4 primes monocytes to secret pro-inflammatory cytokines such as IL-8, reactive oxygen species, and tumour necrosis factor-α.[27,28] In addition, NOD1 and NOD2 are also the important regulators of monocyte activation by mediating the release of interleukins.[29] This study expanded our knowledge of their roles, demonstrating that activation of these receptors in U937 cells leads to its affinity to periodontal fibroblasts. The interaction of monocytes with fibroblasts regulated their own growth, specific immune function, and osteoclastogenic differentiation,[10] thus playing an important role in the initiation and processing of periodontitis. The results suggested that NODs and TLRs not only mediated the inflammatory response in periodontal disease but also regulated bone absorption by modulating its differentiation.

Previous studies have focused on the relationship between TLRs and integrins, reporting that CD11b/CD18 integrin could be modified by TLR2 in *P. gingivalis* infection.[30] This study presented new evidence, demonstrating that TLR4 and NOD1/2 could affect the adhesion via LFA-1 and VLA-4. In addition, these data also suggested that LFA-1 or VLA-4 were critical molecules in the adhesion of monocytes to fibroblasts, which was consistent with other studies.[31] It was reported that LFA-1, VLA-4, and their counter-receptors ICAM-1 and VCAM-1 could be activated in inflammatory diseases such as viral encephalitis[32] and ulcerative colitis.[33] For LFA-1 and VLA-4, they could not only be upregulated in their quantities but also alter their forms from the inactive to the active form. That might be the reason why, although Tri-DAP, MDP, or LPS slightly upregulated the expression level of LFA-1 and VLA-4, blocking either of these integrins significantly hindered U937–fibroblast adhesion triggered by these agonists. Our previous studies have shown that activation of NODs and TLRs in hGFs and hPDLCs regulated ICAM-1 and VCAM-1 expression.[16,17] Taken together, it suggests that NOD1/2 and TLR4 may regulate monocyte–fibroblast adhesion in periodontitis by the interaction of LFA-1/ICAM-1 and VLA-4/VCAM-1.

NODs and TLRs synergistically act with each other during the innate immune reaction. Takada et al.[34] discovered that NOD1/2 signals enhanced TLR-mediated IL-8 secretion in

monocytes. Schwarz et al.[35] demonstrated the cross talk of TLR8 with NOD1/2 in terms of dendritic cell activation. In this study, we also found the synergetic effect of TLR4 with NOD1/2 on the monocyte–fibroblast binding. However, the cross talk of these two signals still needs to be illustrated in further studies. In addition, although previous evidence and ours has shown that NOD1, NOD2, and TLR4 are involved in the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathway, we still do not know whether the two pathways are involved in NOD1/2- and TLR4-mediated monocyte–fibroblast adhesion. Further study is necessary to investigate the signalling pathways to better understand the monocyte–fibroblast interaction. In this study, cells of the human monocytic cell line U937 were used. It would be more convincing if the experiments were performed with primary monocytes from healthy and diseased patients. Thus, further investigation is needed to validate this result.

In conclusion, this present investigation demonstrated that the expression of NOD1, NOD2, and TLR4 was upregulated in the gingival tissue of patients with periodontitis. Activation of these receptors in monocyte lineage U937 cells facilitated their binding to periodontal fibroblasts. These findings suggested that NOD1/2- and TLR4-mediated adhesion of monocytes and periodontal fibroblasts could play crucial roles in periodontitis.

Funding

This study was supported by the National Natural Science Foundation of China Grant 181271150.

Conflict of interest

All listed authors have no conflict of interest to disclose.

Ethical approval

This study was approved by the Medical Ethical Committee of Peking University School of Stomatology (Ethics Approval No. PKUSSIRB-2012017).

Acknowledgment

The authors thank Prof. Ma Dalong from the immunology laboratory of the Peking University Health Science Center for kindly providing the U937 cell line.

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