

Lectin-type oxidized LDL receptor 1 modulates matrix metalloproteinase 2 production in peri-implantitis

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Abstract. Peri-implantitis is a disease in which inflammatory lesions that affect the surrounding soft and hard tissues develop. Matrix metalloproteinase 2 (MMP2) is hypothesized to be involved in this destructive process. However, the regulatory mechanism of action of MMP2 in the peripheral tissues of the implant are not fully understood. To determine the expression of MMP2 in peri-implantitis, peri-implant crevicular fluid (PICF) was collected from patients with peri-implantitis. The healthy volunteers' peripheral blood human macrophages infected with *Porphyromonas gingivalis* (*P. gingivalis*) were used as a cell model to explore the regulatory mechanism of MMP2 regarding dental implants. Western blotting, reverse transcription-quantitative PCR and immunofluorescence staining were used to measure the expression of MMP2 in the present study. MMP2 expression was increased in the PICF of the patients with peri-implantitis and in human macrophages infected with *P. gingivalis*. Lectin-type oxidized LDL receptor 1 (LOX-1) mediated the expression of MMP2 in human macrophages upon infection of *P. gingivalis*, whereas dendritic cell-associated c-type lectin-1 did not appear to be involved in this regulatory process. However, JNK and ERK1/2 were involved in *P. gingivalis* induced MMP2 expression. The results of this study showed that MMP2 was involved in peri-implantitis. MMP2 was upregulated by LOX-1 in a JNK and ERK1/2 signaling dependent manner in the cell model. The LOX-1/MMP2 signaling pathway may thus be a potential target for management of peri-implantitis.

Introduction

Dental implants have become an increasingly popular choice of treatment for replacing missing teeth (1). Peri-implantitis

is the development of serious inflammatory lesions following dental implant therapy. The surrounding hard and soft tissues are affected in peri-implantitis, which can lead to the loss of the implant (2). Previous studies of these therapeutic strategies have not been able to identify a single treatment regimen that is suitably effective against peri-implantitis (3-5).

Host inflammatory cytokines and osteo-immunoinflammatory mediators can lead to the loss of supporting tissues around the implant (4). Matrix metalloproteinases (MMPs) are important for bone repair, development and inflammation. As one of the collagenases, matrix metalloproteinase 2 (MMP2) produced by inflamed cells is involved in several inflammatory diseases (6). In murine arthritis induced by group B streptococci, MMP2 is involved in the production of pro-inflammatory cytokines and the degradation of extracellular matrix components (7). In human astroglia, the ERK1/2-NF- κ B signaling pathway mediates *Toxoplasma gondii* induced MMP2 production to cleave fibronectin (8). In apical periodontitis, the upregulation of MMP2 and MMP9 is modulated by the TLR2MyD88 signaling pathway (9).

A previous study showed that MMP2 was upregulated when stimulated by *Porphyromonas gingivalis* (*P. gingivalis*) lipopolysaccharide (PG-LPS) in THP-1 macrophages (10). However, the regulatory mechanism and signal transduction pathway by which MMP2 exerts its effects in patients who have not received implants have not been assessed previously, to the best of our knowledge. The aim of the present study was to characterize the regulatory mechanism of MMP2 in peri-implantitis.

Materials and methods

Peri-implant crevicular fluid (PICF) collection. In the present study, 10 cases of healthy implants (age, 45 \pm 4.6 years; sex, five males and five females) and 10 cases of peri-implantitis implants (age, 46 \pm 4.4 years; sex, five males and five females) were studied. Each patient had \geq two dental implants, and both implants were investigated. The diagnostic criteria, exclusion criteria and inclusion criteria have been described previously used in our preliminary work (10). Sterile Periopapers (Oraflow) were used to collect PICF samples, which were then placed on the gingival sulcus and implant for 30 sec. For further testing, 50 mM phosphate buffer containing 0.1 mM

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phenylmethylsulfonyl fluoroether was used to elute the PICF contents.

This study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of The Hospital Affiliated to Qingdao University (approval no. 3/6/2018 #20180306; Qingdao, China). All patients provided written informed consent (1).

Isolation of human macrophages. Human macrophages were obtained from the peripheral blood of healthy volunteers after receiving approval from the Ethics Committee of The Affiliated Hospital of Qingdao University. The mononuclear fraction was isolated by density gradient centrifugation at 400 x g for 30 min at room temperature using Ficoll Paque Plus according to the manufacturer's protocol (TBD Biotech). After washing with PBS at room temperature, cells were centrifuged at 200 x g for 5 min at room temperature and suspended in RPMI-1640 media (Hyclone; Cytiva) at a density of 1×10^6 cells/ml. Cells were confirmed to be macrophages (macrophages >95%) by Diff-Quik (cat. no. G1541; Beijing Solarbio Science & Technology Co., Ltd.) stain (Diff-Quik I for 10 sec and Diff-Quik II for 15 sec at room temperature).

Bacterial culture. *P. gingivalis*, provided by the Oral Laboratory of the Hospital affiliated to Qingdao University, were grown overnight in an incubator at 37°C under anaerobic conditions (90% N and 5% CO₂ and 25% H₂) in gifu anaerobic medium (cat. no. LA4500; Beijing Solarbio Science & Technology Co., Ltd.) broth with an optical density of 650 nm of 1.0 (1×10^9 colony forming units/ml) (11). The *P. gingivalis* was added to RPMI-1640 media to infect the human macrophages after washing the cells twice by RPMI-1640. The multiplicity of infection (MOI) used was 20.

MMP2 expression in human macrophages infected with *P. gingivalis*. Western blotting and reverse transcription-quantitative (RT-q)PCR were used to detect MMP2 expression in cells treated with live *P. gingivalis* bacteria after 0, 1/4, 1/2, 1, 4 and 16 h, to measure the expression of MMP2 in human macrophages infected with *P. gingivalis*.

To reveal the role of dectin-1 and LOX-1 in the production of MMP2 following infection with *P. gingivalis* bacteria, dectin-1 (cat. no. AF1859) or LOX-1 (cat. no. MAB1798) neutralizing antibodies (10 µg/ml; R&D Systems, Inc.) (12) and the LOX-1 inhibitor, polyinosinic acid (Poly I; Sigma-Aldrich; Merck KGaA; 0.25 mg/ml) (13) or a dectin-1 inhibitor, laminarin (Sigma-Aldrich; Merck KGaA; 0.3 mg/ml) (1) were used to pretreat human macrophages for 2 h at room temperature, which were then treated with *P. gingivalis* bacteria 2 h later at room temperature.

To investigate the effects of Erk1/2 and JNK on MMP2 production with *P. gingivalis*, human macrophages were pretreated with the JNK inhibitor, SP600125 (Selleck Chemicals; 40 µM) and the Erk1/2 inhibitor, U0126 (Selleck Chemicals; 20 µM) (1), 2 h at room temperature before treatment with live *P. gingivalis* bacteria at room temperature. The controls were DMSO, sterile water and goat IgG (10 µg/ml; cat. no. AB-108-C; R&D Systems, Inc.). Live *P. gingivalis* were used to treat human macrophages for 0 and 16 h at room

temperature for Western blot analysis and for 0 and 4 h at room temperature at a MOI of 20 for RT-qPCR.

Western blotting. Total proteins from human macrophages were lysed by RIPA Lysis Buffer (Beijing Solarbio Science & Technology Co., Ltd.). The total protein contents were quantified using a bicinchoninic acid assay (Beyotime Institute of Biotechnology). Protein was loaded into each well (60 µg) of a 10% SDS-gel (Bio-Rad Laboratories, Inc.), resolved by SDS-PAGE and transferred to a PVDF membrane (EMD Millipore). The membranes were put in blocking buffer (cat. no. P0023B; Beyotime Institute of Biotechnology) for 2 h. The membranes were incubated overnight with an anti-β-actin (1:1,000 Cell Signaling Technologies, Inc.) or anti-MMP2 (1:500 ProteinTech Group, Inc.) primary antibodies at 4°C, followed by incubation with the corresponding HRP-tagged secondary antibodies at room temperature for 2 h (1:1,000 cat. nos. 7074 and 7076; Cell Signaling Technologies, Inc.). Clarity Western ECL Substrate (cat. no. 1705061; Bio-Rad Laboratories, Inc.) combined with the Fusion Solo System (FUSION-FX7 advanced; Vilber Lourmat) was used to visualize signals (ImageJ 1.48; National Institutes of Health).

Immunofluorescence staining. MMP2 immunofluorescence was used to stain small tissue fragments of superficial gingiva in patients with peri-implantitis. The immunofluorescence protocol was performed as described previously (10). Anti-MMP2 antibody (1:50; ProteinTech Group, Inc.) was used as the primary antibody overnight at 4°C, and an Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1,000; cat. no. 4412, Cell Signaling Technology, Inc.) was used as the secondary antibody at room temperature for 1 h, followed by DAPI staining at room temperature for 10 min (20 µg/ml; cat. no. 4083; Cell Signaling Technology, Inc.). Images were captured using a fluorescence microscope (magnification, x400).

RT-qPCR. RT-qPCR was performed as described previously (10). At a pre-set time, the cell culture medium was carefully removed before 500 µl RNAiso Plus (cat. no. 9109; Takara Bio, Inc.) was added into the cell culture plate followed by incubation at room temperature for 5 min to facilitate cell lysis. cDNA template for PCR was prepared from RNA (2 µg) from human macrophages by reverse transcription (cat. no. RR066A; PrimeScript™ RT-PCR Kit Perfect Real Time; Takara Bio, Inc.). The reaction temperature of reverse transcription is 37°C for 15 min, 85°C for 5 sec. DEPC-treated water was used to dilute cDNA products 1:25. PCR was assayed in 20 µl reaction system consisting of 2 µl of 1 µg diluted cDNA (1:25), 10 µl of TB Green® Premix Ex Taq™ (cat. no. RR420A; Takara Bio, Inc.), 1 µl 100 µM diluted primers (1:9) and 7 µl DEPC-treated water) for qPCR. All reactions were performed with the following cycling parameters: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 60°C for 30 sec and a final stage of 95°C for 15 sec, 60°C for 30 sec and 95°C for 15 sec. The primer pair sequences were: β-actin forward, 5'-TGGCACCCA GCACAATGAA-3' and reverse, 5'-CTAAGTCATAGTCCG CCTAGAAGCA-3' and MMP2 forward, 5'-AGTTTCCAT TCCGCTTCCAG-3' and reverse, 5'-CGGTCGTAGTCCTCA GTGGT-3'. β-actin was used as the loading control. Data were quantified using the 2^{-ΔΔCq} method (14).

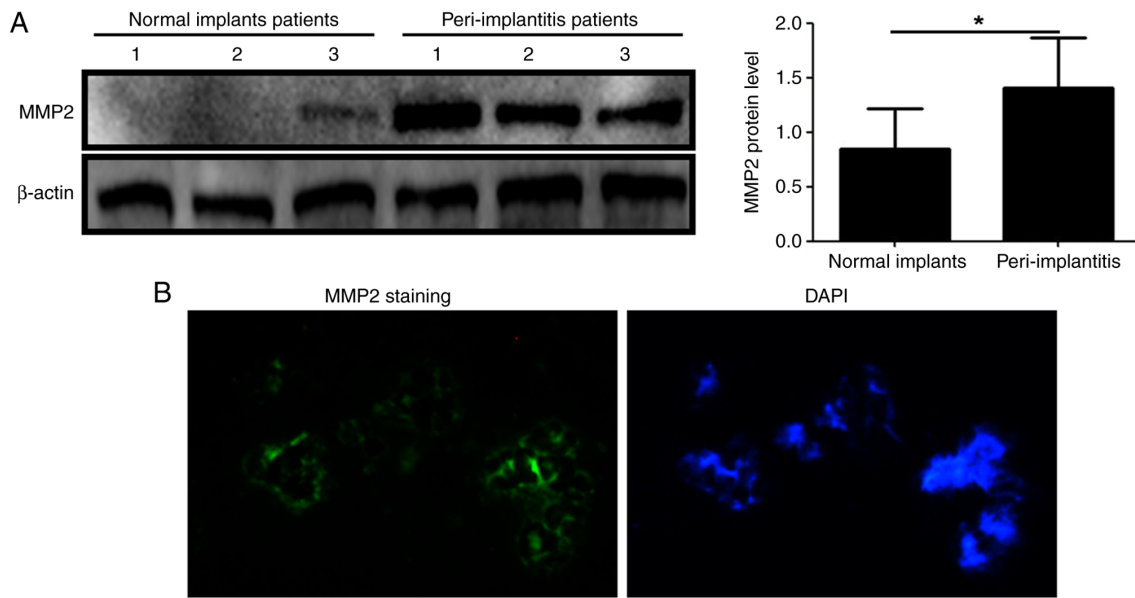


Figure 1. MMP2 expression is increased in patients with peri-implantitis. (A) In patients with peri-implant inflammation (n=10/group), the protein levels of MMP2 in PICF were higher than that in healthy patients. (B) Immunofluorescence staining showed expression of MMP2 (green) in the peri-implantitis patients' gingival surface (magnification, x400). *P<0.05. MMP2, matrix metalloproteinase 2; PICF, peri-implant crevicular fluid.

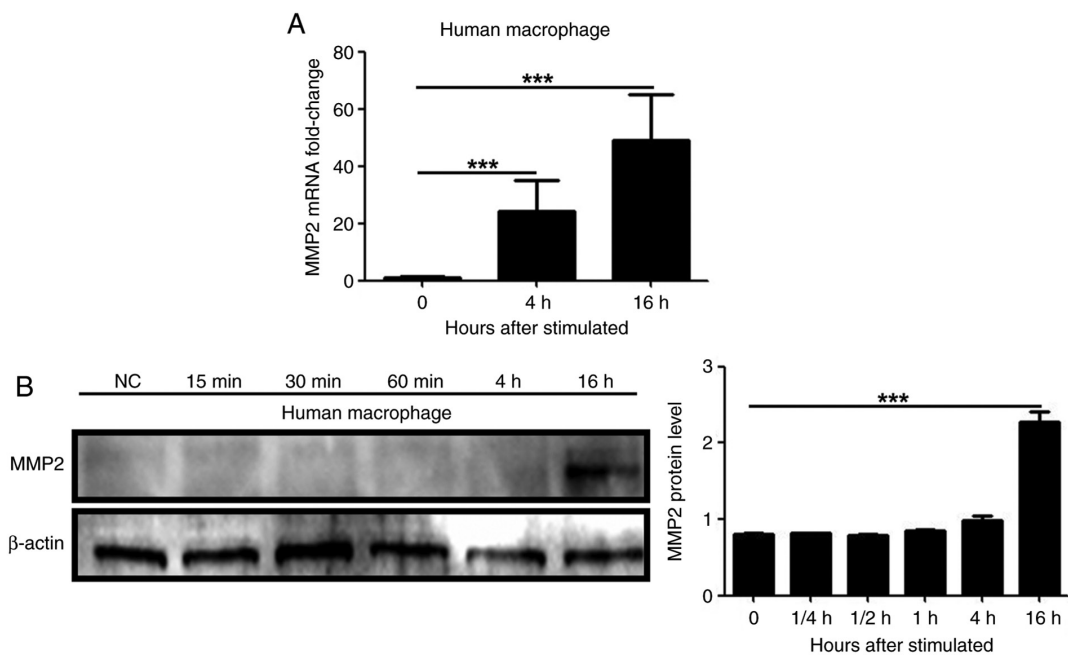


Figure 2. MMP2 expression is increased in *P. gingivalis* infected human macrophages. (A) MMP2 mRNA expression was elevated 4 and 16 h after infection of human macrophages with *P. gingivalis*. (B) MMP2 protein levels were higher 16 h after infection with *P. gingivalis* bacteria. ***P<0.001. MMP2, matrix metalloproteinase 2; *P. gingivalis*/P. g. *Porphyromonas gingivalis*; NC, negative control.

Statistical analysis. One-way ANOVA followed by a post-hoc Tukey's test was used to compare the differences between groups with SPSS 19.0 (IBM Corp.), which was presented as the mean \pm SD from three experimental repeats. P<0.05 was considered to indicate a statistically significant difference.

Results

MMP2 expression is increased in peri-implantitis. Compared to healthy implants, the MMP2 protein expression levels were

higher in the PICF of patients with peri-implantitis (P<0.05; Fig. 1A). The superficial staining of peri-implantitis patients around implants also showed expression of MMP2 (green) (Fig. 1B). The results demonstrated that MMP2 may be involved in the pathogenesis of dental peri-implantitis.

MMP2 expression is increased in *P. gingivalis* infected human macrophages. MMP2 mRNA expression was increased after 4 and 16 h following infection of human macrophages with live *P. gingivalis* bacteria (P<0.01; Fig. 2A). MMP2 production

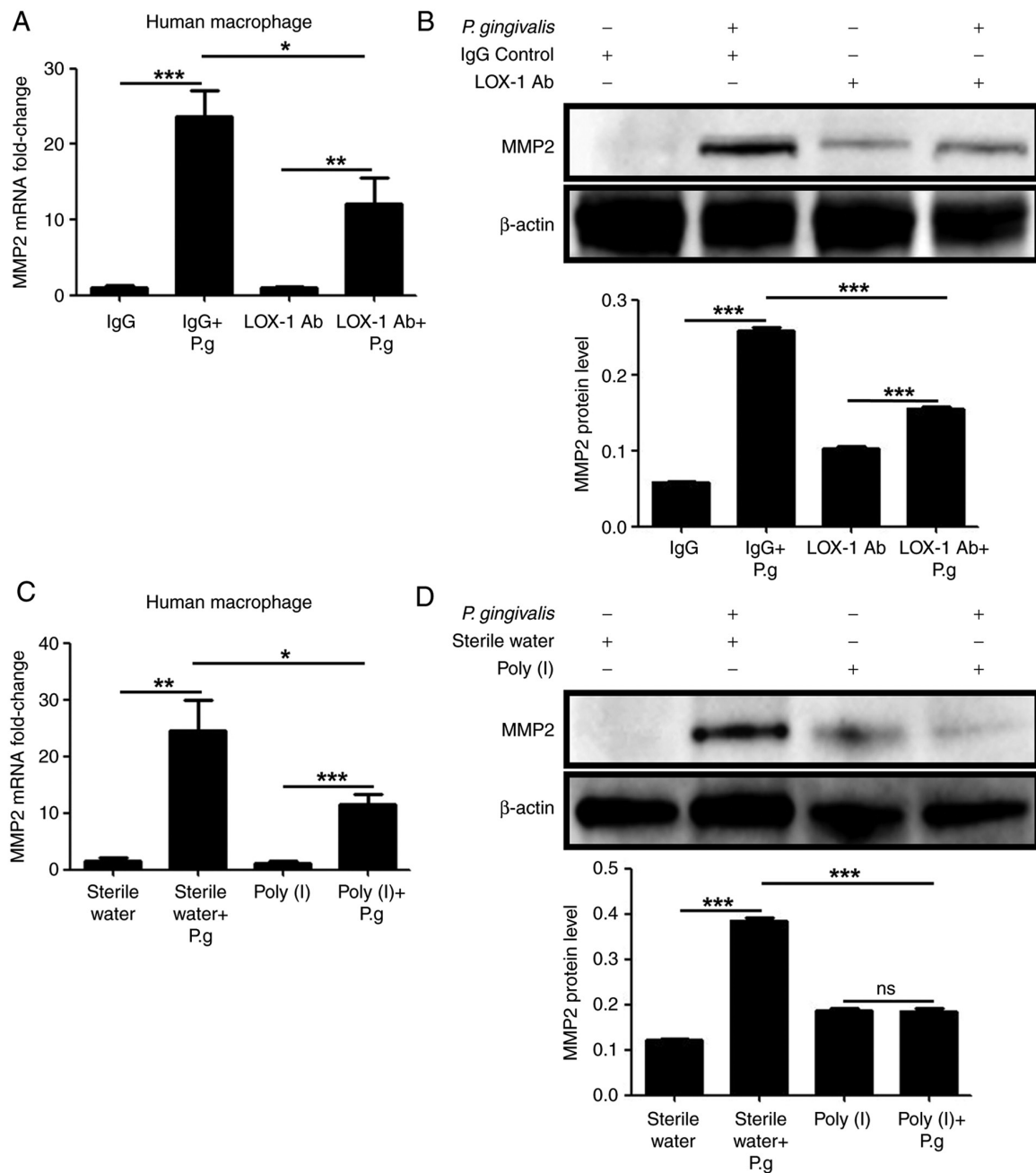


Figure 3. MMP2 production in *P. gingivalis* infected human macrophages is mediated by LOX-1. The MMP2 (A) mRNA and (B) protein expression levels were lower when pre-treated with the LOX-1 neutralizing antibody. The MMP2 (C) mRNA and (D) protein levels were lower when pre-treated with the LOX-1 inhibitor. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ns, not significant; MMP2, matrix metalloproteinase 2; *P. gingivalis*, *Porphyromonas gingivalis*; LOX-1, Lectin-type oxidized LDL receptor 1; IgG, immunoglobulin; Poly (I), polyinosinic acid; Ab, antibody.

also increased at the protein level 16 h after infection with *P. gingivalis* bacteria ($P < 0.01$; Fig. 2B). These results demonstrated that MMP2 production in human macrophages could be induced by *P. gingivalis*.

MMP2 production in P. gingivalis infected human macrophages is mediated by LOX-1. To study the function of LOX-1 in human macrophages in the production of MMP2 in cells infected with *P. gingivalis*, LOX-1 expression was decreased using neutralizing antibodies or inhibitors. The increase in MMP2 expression in infected macrophages at the mRNA ($P < 0.05$; Fig. 3A and C) and protein ($P < 0.01$; Fig. 3B and D) levels were decreased by the LOX-1 neutralizing

antibody or inhibitor pre-treatments. These results demonstrated that LOX-1 mediated MMP2 production induced by the infection of human macrophages with live *P. gingivalis* bacteria.

MMP2 production in P. gingivalis infected human macrophages is independent of dectin-1. To determine the function of dectin-1 in the MMP2 production in human macrophages infected with live *P. gingivalis* bacteria, neutralizing antibodies and inhibitors were used to inhibit dectin-1 activity. The MMP2 mRNA ($P > 0.05$; Fig. 4A and C) and protein ($P > 0.05$; Fig. 4B and D) levels did not differ significantly between treatment with the dectin-1 neutralizing

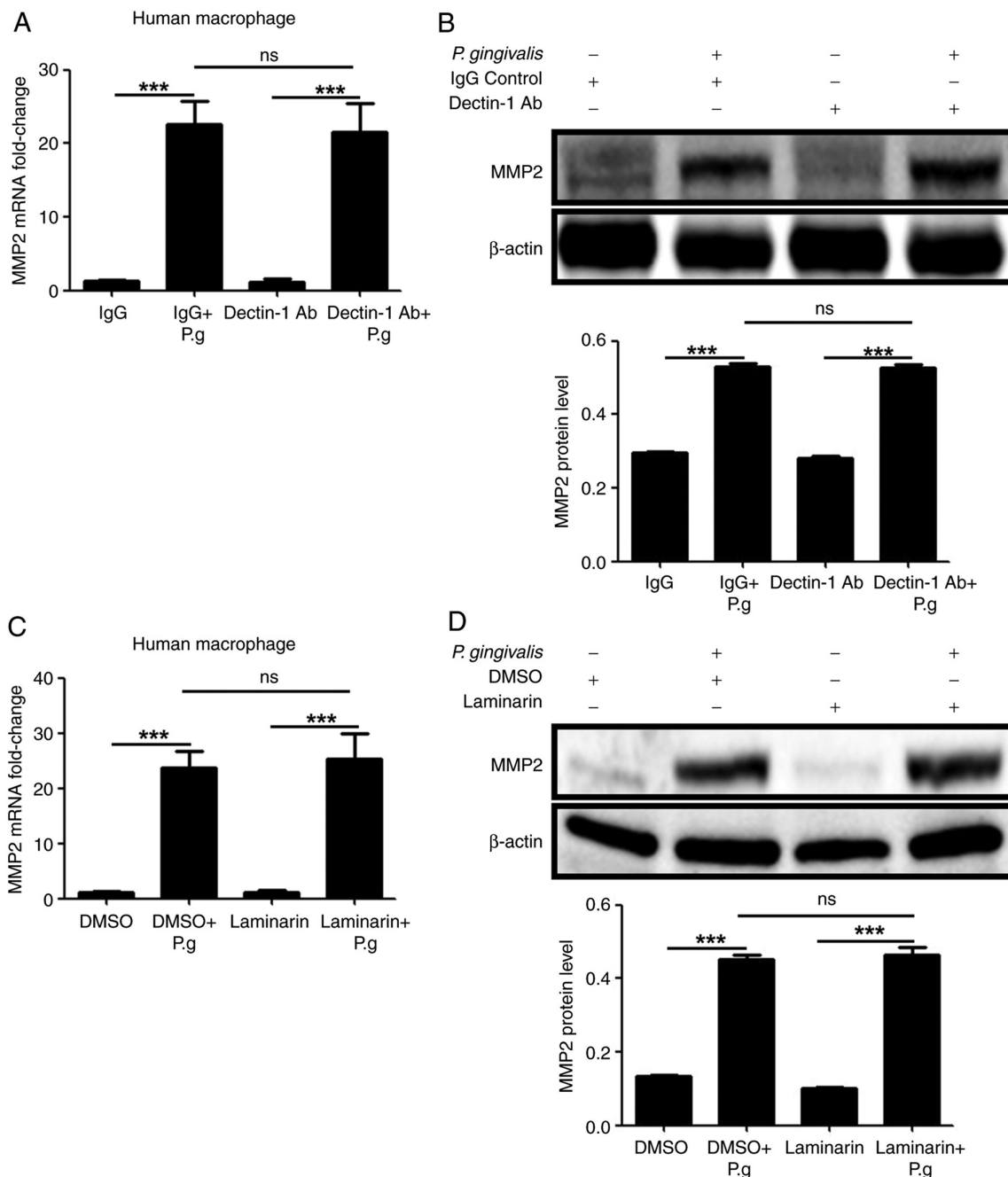


Figure 4. MMP2 production in *P. gingivalis* infected human macrophages was independent of dectin-1. Pre-treatment with dectin-1 neutralizing antibody did not significantly affect the (A) mRNA and (B) protein expression levels of MMP2. Similarly, the MMP2 (C) mRNA and (D) protein expression levels were not significantly affected with dectin-1 inhibitor pre-treatment either. *** $P < 0.001$. ns, not significant; MMP2, matrix metalloproteinase 2; *P. gingivalis*, *Porphyromonas gingivalis*; IgG, immunoglobulin; Ab, antibody.

antibody or inhibitor pre-treatment compared to the control untreated cells. These results showed that MMP2 production was independent of dectin-1 in the macrophages infected with live *P. gingivalis* bacteria.

JNK and Erk1/2 are responsible for *P. gingivalis* induced MMP2 production. JNK and Erk1/2 inhibitors were used to pretreat the human macrophages to investigate their role in MMP2 production following *P. gingivalis* infection. The results indicated that the upregulated MMP2 mRNA ($P < 0.05$; Fig. 5A and C) and protein ($P < 0.01$; Fig. 5B and D) levels, when infected with live *P. gingivalis* bacteria, were decreased

by JNK or Erk1/2 inhibition. These results demonstrated that LOX-1 upregulated MMP2 with the infection of *P. gingivalis* bacteria was mediated by Erk1/2.

Discussion

As one of the collagenases, MMP2 mediates multiple functions associated with oral inflammation, bone development and repair. Gonçalves *et al* (15) found that the levels of MMP2, MMP3, MMP8, MMP9, MMP12 and MMP13 in gingival crevicular fluid were significantly correlated with the severity of localized aggressive periodontitis. da Costa *et al* (16)

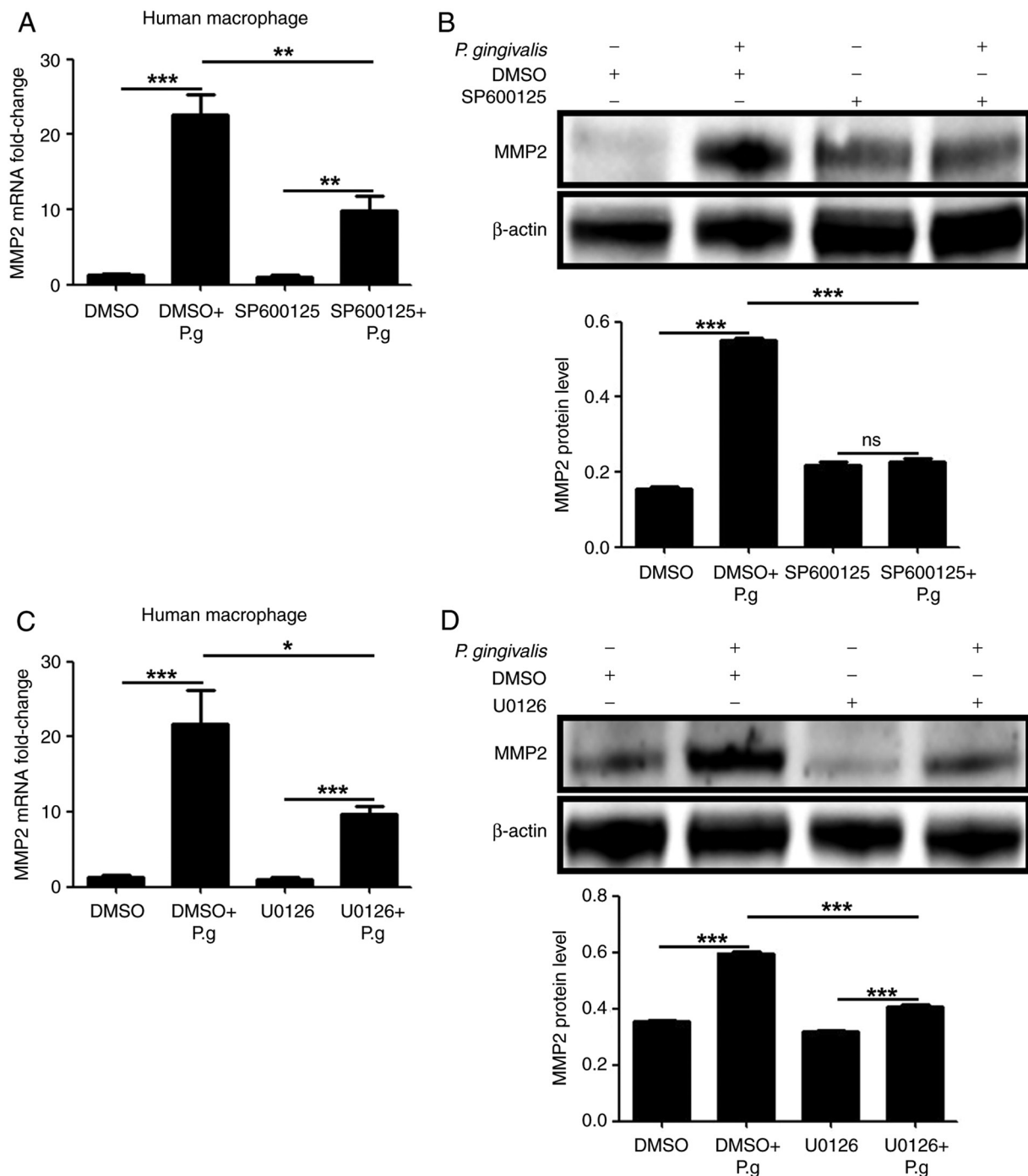


Figure 5. JNK and Erk1/2 are responsible for *P. gingivalis* induced MMP2 production. The upregulated expression of MMP2 at the (A) mRNA and (B) protein level in human macrophages infected *P. gingivalis* bacteria decreased when pre-treated with the JNK inhibitor before infection. The upregulated MMP2 (C) mRNA and (D) protein levels in response to infection with live *P. gingivalis* bacteria decreased when pre-treated with the Erk1/2 inhibitor. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ns, not significant; MMP2, matrix metalloproteinase 2; *P. gingivalis*, *Porphyromonas gingivalis*; IgG, immunoglobulin; Ab, antibody.

identified MMP2 and TNF- α as novel biomarkers of inflammation in the alveolar bone and neighboring tissues of patients with chronic periodontitis. Fu *et al* (17) found that cyclosporin-A prevented periodontal bone loss in patients with periodontitis by suppressing the expression of MMP2 and MMP9. Araújo *et al* (18) found that the inflammation and bone loss in patients with periodontitis was reduced when treated with olmesartan by decreasing MMP2, MMP9 and RANKL expression, and increasing osteoprotegerin production. In the present study, it was shown that the

expression of MMP2 was upregulated in the PICF of patients with peri-implantitis. This observation is consistent with a report showing that active MMP2 complexes contributed to periodontal destruction and was found at higher levels in the periodontal ligament and gingival crevicular fluid of patients with periodontitis (19).

Osteoclastogenesis and severe macrophage derived inflammatory responses can cause peri-implantitis (20). Thus, human macrophages were selected for infection by *P. gingivalis* to assess the regulatory mechanism of MMP2 in peri-implantitis.

Kong *et al* (21) revealed that the secretion of MMP1 and MMP2 induced by *P. gingivalis* stimulation was significantly inhibited by theaflavins in human gingival fibroblasts. Mieszkowska *et al* (22) found that an increase of MMP2, IL-1 β and IL-8 following infection with *P. gingivalis* was decreased by rhamnogalacturonan-Is treatment, which can reduce inflammation and enhance implant integration. Consistent with these studies, the results of the present study showed that when cells were infected with *P. gingivalis*, MMP2 levels were increased in the normal human macrophages.

LOX-1 and dectin-1, as pattern recognition receptors of the C-type lectin superfamily, are associated with osteoclastogenesis (23-25). Bacteria, fungi, apoptotic cells and oxidized low-density lipoproteins are the primary ligands of LOX-1 (26,27). Nakayachi *et al* (23) found that osteoclast differentiation could potentially be inhibited by decreasing the cell-cell fusion of preosteoclasts via LOX-1. Dectin-1, found predominantly on the cell membrane of the cells of a myeloid lineage, can recognize β -glucans, some bacteria and other unidentified molecules (28). Yamasaki *et al* (24) found that osteoclast differentiation and NFATc1 expression could be negatively regulated by the dectin-1/syk signaling pathway. In the present study, the data showed that MMP2 production was regulated by LOX-1 in human macrophages with *P. gingivalis*. The results of the present study are in agreement with previous studies that have shown that LOX-1 neutralizing antibody treatment can reduce MMP2 and MMP9 expression induced by gasoline and diesel engine exhaust (29). The results of the present study also revealed that MMP2 production was independent of dectin-1 in infected human macrophages with *P. gingivalis*.

JNK and Erk1/2, as members of the serine-threonine protein kinases, play significant roles in cellular responses to proinflammatory cytokines. Li *et al* (30) found that the expression of IL-1 β and TNF- α induced by sialidase of *P. gingivalis* could be inhibited by JNK blocking epi4 cells. Ren *et al* (31) found that *P. gingivalis* induced IFN- γ and IL-8 expression was mediated by Erk1/2 and p38 MAPK in HTR8 cells. JNK and Erk1/2 are also involved in MMP2 secretion in inflammatory diseases. Jiang *et al* (32) showed that neuropathic pain induced by chronic constrictive injury was significantly attenuated by tetramethylpyrazine via the JNK-MMP2/9 signaling pathway. Wang *et al* (33) found that Erk1/2/NF- κ B pathway could upregulate MMP2 and MMP9 expression in murine mast cells infected with *Toxoplasma gondii*. The results of the present study suggested that JNK and Erk1/2 were responsible for *P. gingivalis* induced MMP2 production. The Erk1/2 inhibitor was considerably more potent than the JNK inhibitor in suppressing MMP2 production.

In conclusion, the results of the present study, obtained through evidence from patients and cell-culture studies, showed that MMP2 was involved in peri-implantitis, and may have been regulated by LOX-1, in a JNK and Erk1/2 signaling pathway dependent manner. The LOX-1/MMP2 signaling pathway may thus be a potential target for management of peri-implantitis.

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Availability of data and materials

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

Authors' contributions

QZ designed the study and wrote the majority of the manuscript. TX and NB performed the majority of the experiments and wrote part of the manuscript. FT and HZ performed some of the experiments. JL designed the study and revised the manuscript. All authors have read and approved the final manuscript. QZ and TX confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The Affiliated Hospital of Qingdao University (Qingdao, China) and performed in accordance with the Helsinki Declaration. Each patient in this study provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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