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Original Article

EMMPRIN-CypA contributes to the inflammatory processes in human periodontitis through infiltrating CD68$^+$ inflammatory cells

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Abstract: Extracellular matrix metalloproteinase inducer (EMMPRIN) and its ligand cyclophilin A (CypA) levels increase in human inflammatory diseases, but EMMPRIN-CypA interactions and cell types expressing EMMPRIN and CypA in the pathogenesis of periodontitis are uncertain. Immunohistochemistry, immunofluorescence and western blotting revealed the level of EMMPRIN, CypA, and CD68 in human periodontitis. Double labelled immunofluorescence colocalized the expression of CD68 and CypA, and CD68 and EMMPRIN. Further investigation of EMMPRIN-CypA interactions and CD68$^+$ infiltrating cells was applied using mouse monocyte cell line RAW264.7 in vitro. A higher level of EMMPRIN and CypA staining was detected in human periodontitis, compared with healthy gingiva. Many inflammatory cells, including CD68$^+$ cells, infiltrated gingival tissues of human periodontitis. Both EMMPRIN and CypA could be localized in the CD68$^+$ infiltrating cells. CypA could induce NF-κB activation by increasing expression of NFκB p-p65 in the nucleus of mouse monocytic cells RAW264.7 in vitro. EMMPRIN-CypA may contribute to the inflammatory processes in human periodontitis through infiltrating CD68$^+$ inflammatory cells.

Keywords: EMMPRIN, CypA, CD68, periodontitis, immunofluorescence

Introduction

Periodontitis is a chronic inflammatory disease characterized by infiltration of inflammatory cells that leads to gingival inflammation and alveolar destruction [1]. Leucocyte trafficking and recruitment are critical components of inflammation-mediated pathology [2]. Previous studies show CD3$^+$, CD4$^+$, CD22$^+$, and CD68$^+$ infiltrating inflammatory cells are observed in human periodontitis. CypA gets involved in the migration of monocyte/macrophages [3, 4], lymphocytes [5] and neutrophils [2, 6] into tissues, contributes to inflammatory responses for its chemotactic activity and matrix metalloproteinases (MMPs) production via interaction with its cell surface receptor EMMPRIN. The pathophysiological relevance of EMMPRIN-CypA interaction for inflammatory processes has been studied in various inflammatory diseases [7, 8]. EMMPRIN, also known as CD147, is a transmembrane glycoprotein of the immunoglobulin super-family [9]. It is involved in various cellular and biological functions, such as lymphocyte migration and maturation, T and B lymphocyte activation, and induction of extracellular matrix metalloproteinase [10, 11].

Although the pro-inflammatory activities of EMMPRIN and its ligand CypA are well known [12, 13]. EMMPRIN-CypA interactions and cell types expressing EMMPRIN and CypA in the pathogenesis of periodontitis are not specifically known [14, 15]. We analyzed gingival tissues from human periodontitis patients to reveal the cell types expressing EMMPRIN and CypA. We also tested the possible role of CypA using mouse monocytic cells RAW264.7.

Material and methods

Study participants and sample collection

Participants (20 males, aged 22-60 years, and mean 34 years) were enrolled into 2 groups: healthy gingiva group and periodontitis group. In the periodontitis group, gingival tissues were excised during extractions of teeth which were obtained from 10 patients with severe disease.

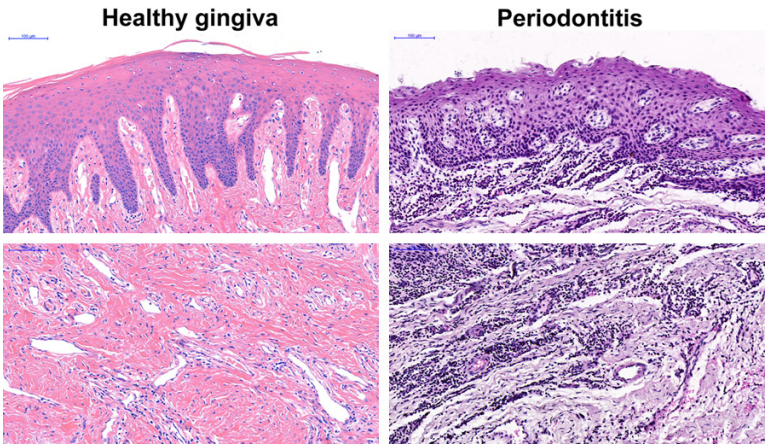

Figure 1. Histologic observation of infiltrating cells in human periodontitis and healthy gingiva. Many infiltrating inflammatory cells could be observed in the inflamed gingiva of human periodontitis.

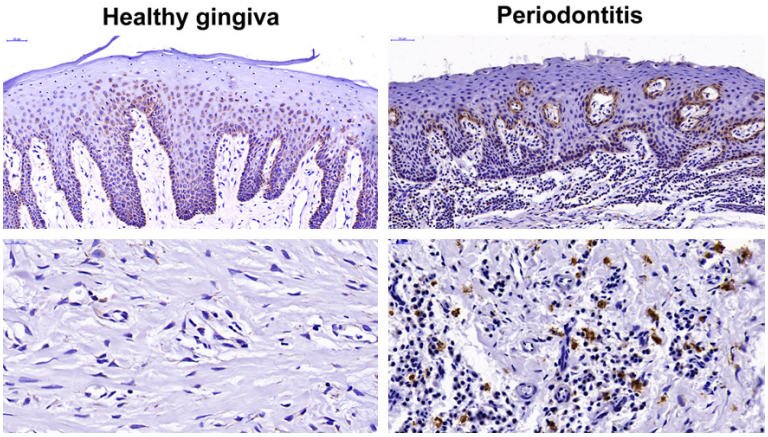

Figure 2. Expression of EMMPRIN in human periodontitis and healthy gingiva. The positive staining was distributed in basal cell layer and spinous layer of gingival epithelium in the healthy gingiva. Positive staining was also distributed in the infiltrating cells of lamina propria in human periodontitis.

In the healthy gingiva group, gingival tissues were collected during orthodontic extractions from 10 healthy donors without systematic diseases. Each specimen was divided into two parts of approximately equal size. One part was immediately fixed in 4% paraformaldehyde and then 5 μm serial sections were made for HE, immunohistochemistry, immunofluorescence and double-labelled immunofluorescence. The other part was stored in liquid nitrogen for western blotting. Mouse monocytic cell line RAW264.7 was obtained from the American Type Culture Collection (Rockville, MD, USA). Recombinant human CypA was purchased from Abcam (UK). All studies were approved by the Ethics Committee of Jinan Stomatological Hospital, and all patients provided voluntary informed consent to participate in the study.

Immunohistochemistry

Immunohistochemical study was performed by using Streptavidin-Peroxidase kit (Zhongshan, Beijing, China) as previously described [16]. Monoclonal antibody EMMPRIN (dilution 1:100, Abcam, UK) and polyclonal antibody CD68 (dilution 1:100, Abcam, UK) were applied. PBS was obtained as control.

Immunofluorescence

Sections were deparaffinized in xylene and rehydrated. After washing, the sections were incubated with polyclonal antibody CypA (dilution 1:100, Abcam, UK), polyclonal antibody CD68 (dilution 1:100, Ab-cam, UK), monoclonal antibody EMMPRIN polyclonal antibody p65 (1:3000) and p p65 (dilution 1:3000, Abcam, UK) at 4°C overnight. The sections were then incubated with rhodamine (TRI-TC)-conjugated goat anti-rabbit IgG (Sigma, USA) for 1 h at room temperature. Nuclei were stained with DAPI solution (Sigma, USA) for 5 min. The sections were photographed with immunofluorescence microscopy (OLYMPUS BX-60, Japan). Mouse monocytic cells were transferred on slides, and then fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 for 15 min.

Double-labelled immunofluorescence

Sections were deparaffinized in xylene and rehydrated. After washing, the sections were incubated with primary antibodies: mouse anti-CD68 (1:100; Abcam, UK) and rabbit anti-CypA (1:200; Abcam) or rabbit anti-EMMPRIN (1:200; Abcam) overnight at 4°C. After washing three times with PBS, cells were exposed for 1 h at 37°C to goat anti-mouse IgG-FITC (1:100; Santa Cruz, USA) and goat anti-rabbit IgG-CY3 (1:400; Abcam), then counterstained with DAPI (1:50; Beyotime, Shanghai, China).

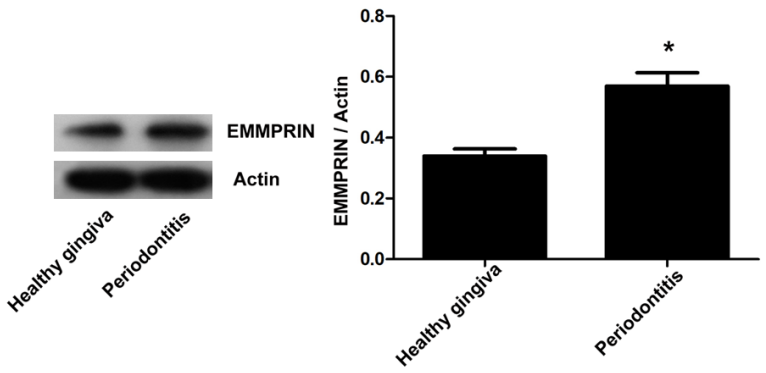

Figure 3. Expression of EMMPRIN protein in human healthy and inflamed gingiva. Higher expression of EMMPRIN protein was detected in the inflamed gingiva, compared to human healthy gingiva (*$P < 0.05$).

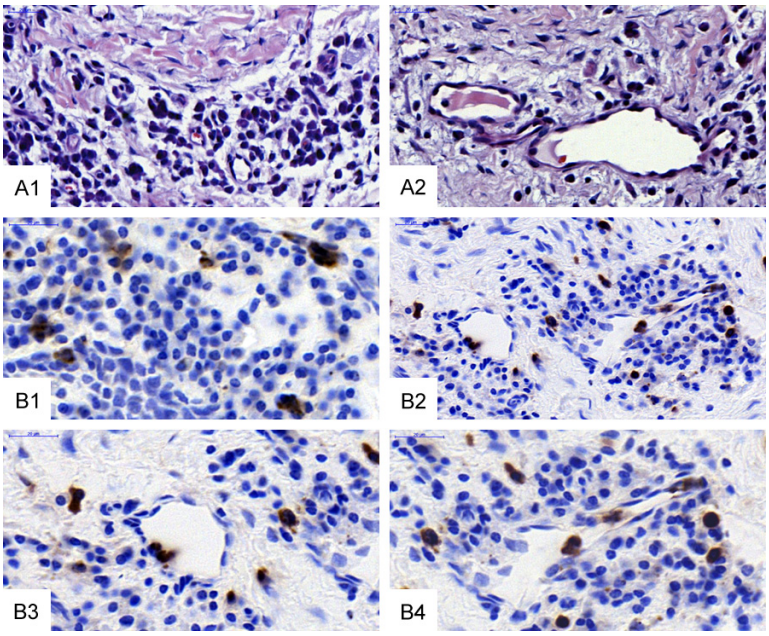

Figure 4. Expression and distribution of CD68$^+$ infiltrating cells in human periodontitis. Many inflammatory infiltrating cells could be observed in lamina propria, perivascular tissues, and blood vessels (A1 and A2). CD68$^+$ infiltrating cells could be observed in lamina propria, perivascular tissues and blood vessels (B1-B4).

Western blotting analysis

Western blotting was performed as previously described [17]. Protein concentrations were measured using a bicinchoninic acid assay (BCA) protein quantitative analysis assay kit (BOSHE, China). Proteins were separated on 10% SDS gels and then transferred onto polyvinylidene difluoride membranes (Millipore, USA). After blocking in 0.1% Tween 20 in Tris-buffered saline (TBST) containing 5% nonfat dried milk for 1 h at room temperature, the membranes were incubated with antibodies against EMMPRIN (diluted 1:1000, Abcam) and NF-κB p-p65 (1:1000, Cell Signaling Technology, USA) overnight at 4°C. Before incubation with horseradish peroxidase (HRP)-labeled second antibody (Beyotime), the membranes were rinsed with TBST for 10 min 3 times. The bands were visible on the Canon films using ECL substrate solution (Millipore). Actin (1:10000) was used as internal control.

Statistical analysis

All of the images of western blotting assays were representative of at least three independent experiments. All results are presented as the mean ± SD. All values were calculated using Student's t-test. The differences were considered significant at $P < 0.05$.

Results

Expression of EMMPRIN in human periodontitis and healthy gingiva

Many infiltrating inflammatory cells could be observed in the inflamed gingiva of human periodontitis (**Figure 1**). Expression of EMMPRIN in the periodontitis group was higher than that of the healthy group. Positive expression was localized in basal cell layer and spinous layer of gingival epithelium in the healthy gingiva. The positive staining was also distributed in the infiltrating cells of lamina propria in human periodontitis (**Figure 2**). Western blotting also showed that expression of CypA in inflamed gingiva was higher than that of healthy donors ($P < 0.05$, **Figure 3**).

Expression of CD68$^+$ infiltrating cells in human periodontitis

Many infiltrating inflammatory cells could be observed in lamina propria, perivascular tis-

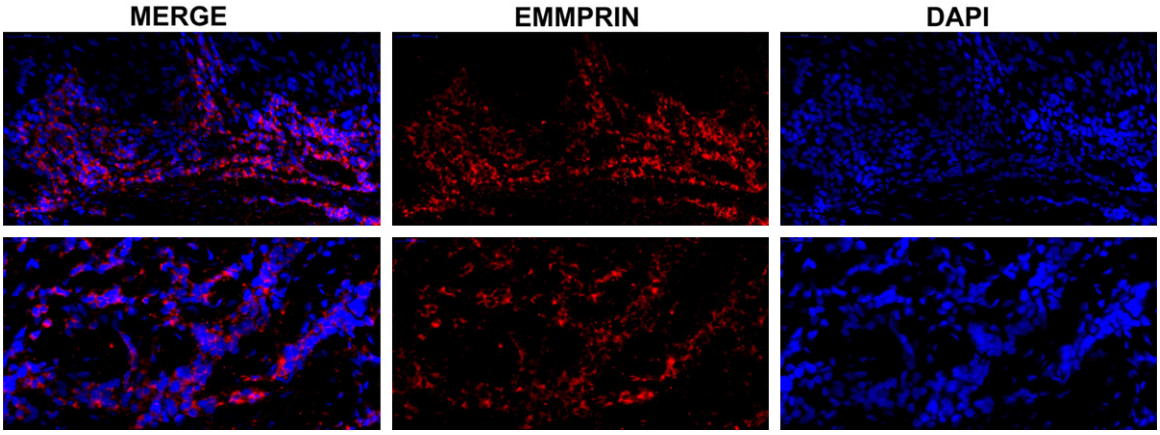

Figure 5. Expression of EMMPRIN in the inflammatory infiltrating cells of human periodontitis. Positive expression of EMMPRIN mainly exists in the cytoplasm of the inflammatory infiltrating cells.

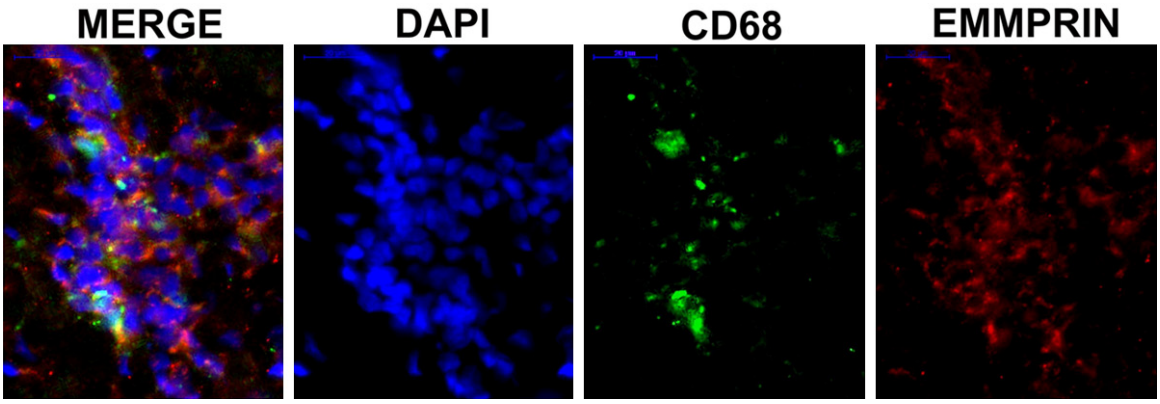

Figure 6. Colocalization of CD68 and EMMPRIN in human periodontitis by double-labelled immunofluorescence. Positive FITC labelling for CD68 (green); Positive CY3 labelling for EMMPRIN (red); Nuclei were counterstained with DAPI (blue). CD68 and EMMPRIN were overlapped (yellow) in the merged image.

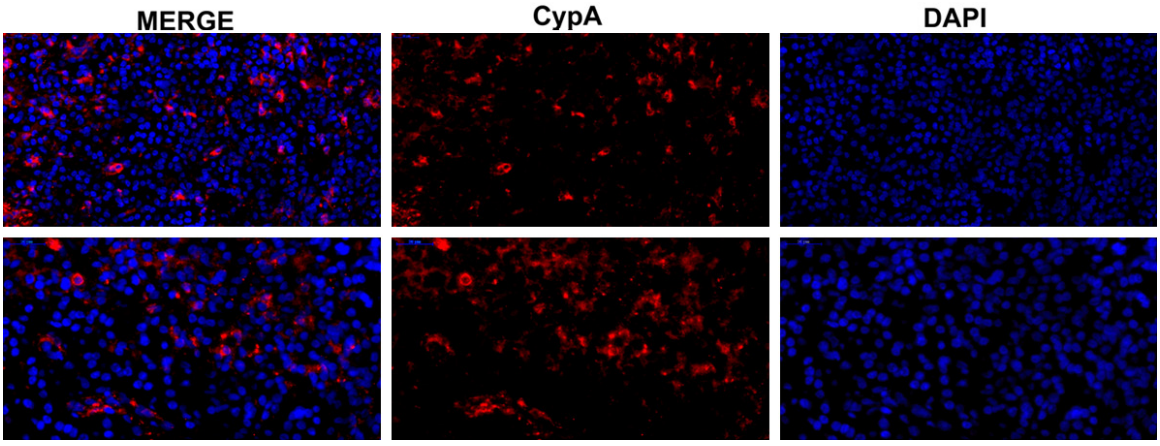

Figure 7. Expression of CypA in the inflammatory infiltrating cells of human periodontitis. Positive expression of CypA mainly exists in the cytoplasm of the infiltrating inflammatory cells.

sues, and blood vessels (**Figure 4A1** and **4A2**). $CD68^+$ infiltrating cells could be observed in lamina propria, perivascular tissues and blood vessels (**Figure 4B1-B4**).

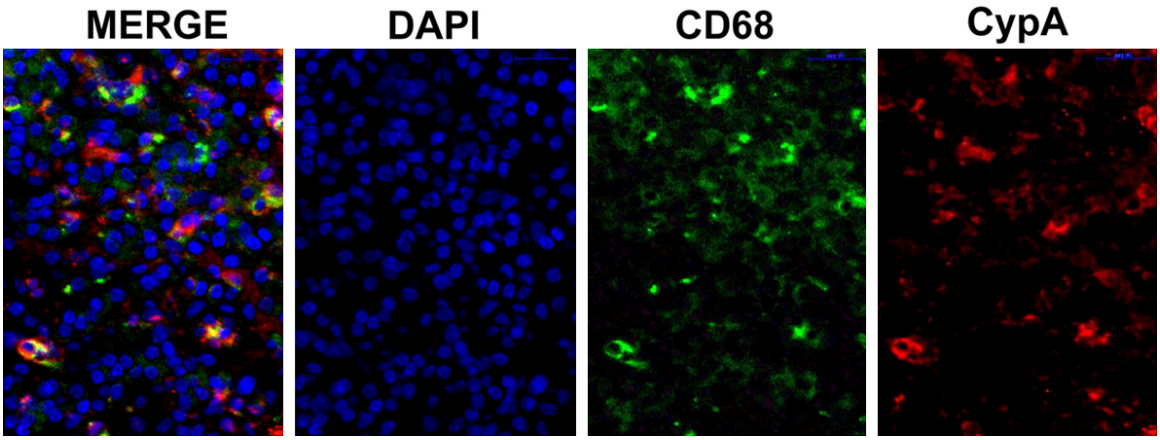

Figure 8. Colocalization of CD68 and CypA in human periodontitis by double-labelled immunofluorescence. Positive FITC labelling for CD68 (green); Positive CY3 labelling for CypA (red); Nuclei were counterstained with DAPI (blue). CD68 and CypA were overlapped (yellow) in the merged image.

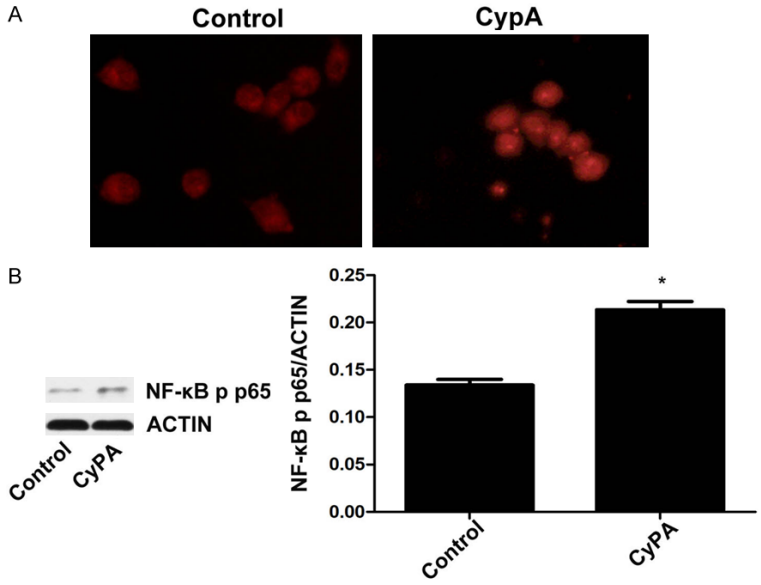

Figure 9. CypA induced nuclear translocation of NF-κB p65 in RAW264.7 cells. RAW264.7 cells were stimulated with 0.1 μM CypA. Immunofluorescence analyses revealed that CypA induced nuclear translocation of NF-κB after treatment (A). NF-κB p p65 expression was detected by western blotting. NF-κB p p65 was upregulated in the CyPA group, compared with the control group (B).

Colocalization of EMMPRIN-CypA and CD68 in human periodontitis by double-labelled immunofluorescence cytochemistry

Positive expression of EMMPRIN exists in the cell membrane of the inflammatory infiltrating cells (**Figure 5**). According to double-labelled immunofluorescence results, positive FITC labelling for CD68 was green, positive CY3 labelling for EMMPRIN was red), and nuclei were counterstained blue with DAPI. CD68 and EMMPRIN were overlapped (yellow) in the merged image (**Figure 6**). Colocalization of CD68 and EMMPRIN revealed EMMPRIN may get involved in the pathogenesis of periodontitis by $CD68^+$ infiltrating cells.

Positive expression of CypA exists in the cytoplasm of the infiltrating inflammatory cells (**Figure 7**). According to double-labelled immunofluorescence results, positive FITC labelling for CD68 was green, positive CY3 labelling for CypA was red, and nuclei were counterstained blue with DAPI. CD68 and CypA were overlapped (yellow) in the merged image (**Figure 8**). Colocalization of CD68 and CypA revealed CypA may get involved in the pathogenesis of periodontitis by $CD68^+$ infiltrating cells.

CypA induced NF-κB activation in mouse monocytic cells RAW264.7 in vitro

Immunofluorescence analyses revealed that CypA induced nuclear translocation of NF-κB after treatment (**Figure 9A**). NF-κB p p65 were also detected to observe whether CypA activated the NF-κB pathway by western blotting. NF-κB p p65 was upregulated in the CypA group, compared with the control group (B). NF-κB p p65 expressions were upregulated after CypA

treatment. CypA induced NF-κB activation by increasing expression of NF-κB p p65 in the nucleus of mouse monocytic cells RAW264.7 in vitro.

Discussion

CD68 is a useful marker for various cells of the macrophage lineage, including monocytes, histiocytes, and osteoclasts [18]. The levels of CD68 are positively correlated with the pathogenesis of inflammation-mediated diseases [19]. $CD68^+$ cells of monocytes/macrophage lineage are involved in periodontal inflammation [1]. In chronic periodontitis, $CD68^+$ monocytes in blood vessels marginated and emigrated into the perivascular tissues, where they appeared as macrophages. $CD68^+$ macrophages could be found in the gingival epithelium, lamina propria, perivascular tissues and in the blood vessels, according to our immunohistochemistry results. Study has reported that EMMPRIN and CypA may be produced by infiltrating macrophages and lymphocytes in periodontitis [20-22]. Determining the roles of CypA and EMMPRIN in $CD68^+$ cell infiltration helps to elucidate the pathogenesis of periodontitis and new anti-inflammatory therapy [8].

Our results showed increased expression of EMMPRIN in inflammatory periodontal tissues. High levels of CypA had also been detected in human periodontitis. Both EMMPRIN and CypA were associated with the pathogenesis of periodontitis. Colocalization of CypA and CD68, EMMPRIN and CD68 revealed a positive relationship between EMMPRIN-CypA and $CD68^+$ infiltrating cells in periodontitis. The finding suggests that EMMPRIN, via interactions with CypA, may contribute to the recruitment of immune cells to sites of inflammation through chemokine-like activity in periodontitis [23].

In vitro, RAW264.7 was applied to further investigate the positive relationship of EMMPRIN-CypA and mouse monocytic cells. NF-κB p p65 were also detected to observe whether CyPA activated the NF-κB pathway. Western blotting results showed NF-κB p p65 expression was upregulated after CypA treatment. CypA induced NF-κB activation by increasing expression of NF-κB p p65 in the nucleus of mouse monocytic cells RAW264.7 in vitro. CypA induced nuclear translocation of NF-κB after CypA treatment.

Therapeutically useful antagonistic antibodies of EMMPRIN could reduce CypA-induced signaling and MMP-inducing activity in lung inflammation [24] and RA [25, 26]. The important roles that EMMPRIN-interactions in the pathogenesis of human periodontitis, present an attractive target for therapeutic interventions. Using anti-EMMPRIN or anti-CypA intervention may be a new anti-inflammatory therapy.

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Disclosure of conflict of interest

None.

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