A short title: MMPs expression by IL-12 are regulated by NF-κB activation

Interleukin-12-mediated expression of matrix metalloproteinases in human periodontal ligament fibroblasts involves in NF-kappaB activation

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Abstract
Interleukin (IL-12) is a pro-inflammatory cytokine, and its increased level correlates with the severity of periodontitis. However, its role in the pathogenesis of tooth periapical lesions is controversial and has not been completely clarified. This study aimed to investigate whether IL-12 affects the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in human periodontal ligament fibroblasts (hPDLFs). After treatment with IL-12 for different time, Real-time PCR and Western blotting were used to determine the mRNA and protein levels of MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1 and TIMP-2, respectively. Enzyme-linked immunosorbent assay (ELISA) was applied to measure MMPs and TIMPs secretion production. The results indicated that IL-12 significantly increased the mRNA and protein expression levels of MMP-1, MMP-3 and MMP-13, but down-regulated MMP-2 and MMP-9 mRNA and protein expression in the hPDLFs. Furthermore, IL-12 (10 ng/ml) enhanced the secreted protein production of MMP-1, MMP-3 and MMP-13, and conversely lowered MMP-2 and MMP-9 secretion levels. However, IL-12 treatment did not exert a significant effect on the mRNA and protein levels of TIMP-1 and TIMP-2 and their secreted production. Additionally, IL-12 increased the phosphorylated levels of IκBα and nuclear factor-kappaB P65 (NF-κB P65), and promoted NF-κB P65 subunit nuclear translocation. Pre-treatment with NF-κB inhibitor not only attenuated IL-12-induced IκBα and NF-κB P65 phosphorylation and inhibited NF-κB P65 subunit into nucleus, but also antagonized IL-12-mediated MMP-1, MMP-2, MMP-3, MMP-9 and
MMP-13 expression in the hPDLFs. These findings indicate that NF-κB-dependent activation is possibly indispensable for IL-12-mediated MMPs expression in human PDLFs.

**Key words:**
Interleukin-12; Matrix metalloproteinases; Periodontal ligament; NF-kappaB; Periodontitis

**Abbreviation:**
Interleukin-12, IL-12; Matrix metalloproteinases, MMPs; Tissue inhibitors of metalloproteinases, TIMPs; Nuclear factor-kappaB, NF-κB; Periodontal ligament fibroblasts, PDLFs; Pyrrolidine thiocarbamate, PDTC; Glyceraldehyde-3-phosphate dehydrogenase, GAPDH; (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide), MTT; Enzyme-linked immunosorbent assay, ELISA; Dulbecco’s modified Eagle’s medium, DMEM
1. Introduction

Periodontitis is a chronic inflammatory disease characterized by loss of the connective tissue and alveolar bone [1], and it is widely regarded as one of the most common diseases worldwide, with a prevalence of 15-20% [2]. Periodontal health requires a balance between tissue proteolytic enzymes such as matrix metalloproteinases (MMPs) and their inhibitors. Connective tissue destruction is essentially controlled by MMPs, which contributes to destruction of gingival tissue and alveolar bone surrounding the teeth [3]. MMPs are a group of zinc metalloendopeptidases that degrades extracellular matrix (ECM). Excessive production of MMPs leads to accelerated matrix degradation, which is associated with pathologic conditions such as periodontitis and apical periodontitis [4]. According to their target protein, MMPs are divided into six groups: collagenases (MMP-1 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3), matrilysins, membrane type MMPs and others, such as the macrophage metalloelastase (MMP-12) [5]. Normally, MMPs activities are tightly regulated by their interaction with the tissue inhibitors of matrix metalloproteinases (TIMPs) [6]. Thus, the factors, which regulate MMPs/TIMPs synthesis and secretion, may be important in the pathogenesis of chronic periodontitis.

Interleukin 12 (IL-12) is an important regulatory cytokine, involving in both innate and adaptive immune responses [7]. IL-12 is mainly secreted by macrophages, monocytes, dendritic and B cells in response to bacterial products and intracellular parasites [8]. Previous studies have indicated that the most important functions of IL-12 are to stimulate T and NK cells to produce interferon-\(\gamma\) (IFN-\(\gamma\)) and to promote
the Th1 response [9,10], and it is has been implicated in the pathogenesis of several diseases, such as psoriasis [11], rheumatoid arthritis [12] and periodontitis [13]. Moreover, IL-12 has been reported to regulate osteoclast inhibitory peptide-1 (OIP-1) gene expression in CD4+ T cells [14].

In the periodontium, the most abundant cells are periodontal ligament fibroblasts (PDLFs), and they are responsible for the production of MMPs and TIMPs, and play a pivotal role in maintaining the functional integrity of the periodontal extracellular matrix (ECM) [15], and have a principal role in the pathologic degradative processes of ECM in apical periodontitis [16]. Additionally, there is a higher level of IL-12 in gingival tissue and serum of patients with aggressive periodontitis [17,18]. However, little is known about the effects of IL-12 on the expression of MMPs in human PDLFs to date. Therefore, the aim of this study was to determine the effects of IL-12 treatment on the mRNA and protein expression of MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1 and TIMP-2 in the human PDLFs, and the mechanisms associated to IL-12-mediated MMPs expression was also preliminarily explored.

2. Materials and methods

2.1. Antibodies and reagents

The primary antibodies recognizing MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1, TIMP-2 and β-actin were purchased from Abcam Company (Cambridge, UK), and other antibodies, including anti-IκBα; anti-p-IκBα, anti-NF-κB P65, anti-p-NF-κB P65 and anti-Lamin A were obtained from Cell Signaling Technology
(Danvers, MA, USA). Human recombinant IL-12 was purchased from R&D Systems (Minneapolis, MN, USA). MTT and PDTC were purchased from Sigma-Aldrich Company (St. Louis, MO, USA).

2.2. Cell culture

Human periodontal ligament fibroblasts (PDLFs) were obtained from Jining Company (Shanghai, China), and cultured in DMEM (Gibco, Grand Island, NY) supplemental with 10% fetal calf serum and penicillin (100 U/ml) and streptomycin (100 mg/ml) in a humidified atmosphere of 95% air and 5% CO$_2$ at 37°C. When the cells grew into confluency, they were detached with 0.25% trypsin-EDTA and sub-cultured for the following experiments.

2.3. MTT assay

Human PDLFs at passage 5 (P5) seeded in 96-well plates at $5 \times 10^3$ cells/well were treated with 0, 5 and 10 ng/ml of IL-12 for 12, 24 and 48 h, based on our preliminary experimental results (data not shown). Cell viability was then evaluated by MTT assay. Briefly, after incubation, the cells were washed and incubated with 0.5 mg/ml MTT solution in DMEM for 4 h at 37°C. Formazan crystals of viable cells were dissolved in 150 µl dimethyl sulphoxide (DMSO), and the absorbance value (OD value) was measured at 570 nm with a micro-plate reader (Titertek, Pforzheim, Germany). Cell viability was expressed as percentages of control group.
2.4. Real-time PCR analysis

After incubation with IL-12 (0, 5 and 10 ng/ml) for 12 and 24 h, total RNA of human PDLFs at passage 5 (P5) was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. 1µg of total RNA per sample was synthesized into cDNA with a reverse transcription kit (TaKaRa, Tokyo, Japan). Real-time PCR was performed by quantitative PCR System (ABI 7300) with FastStart Universal SYBR Green Master (Tiangen Biotech Co., Beijing, China) as follows: 95°C for 15 s followed by 35 cycles at 94°C for 5 s and 60°C for 30 s. The specific primers used in this study were designed and listed in Table 1. GAPDH was used as internal control, and gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.5. Western blot analysis

Human PDLFs at passage 6 (P6) were incubated with 10 ng/ml IL-12 for 48 h or pre-treated with the NF-κB inhibitor PDTC (10 µM) or quinazoline (10 µM) for 1 h, followed by treatment with IL-12 (10 ng/ml) for 6, 12 or 48 h, and cells were washed and lysed in RIPA lysis buffer (Beyotime, Haimen, China) supplemented with a protease inhibitor cocktail set (Roche, Germany), while the cytoplasmic and nuclear extracts from cells were prepared with NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific, USA). The protein concentration was measured using a BCA protein assay kit (Beyotime), and equal amounts of protein extracts were separated on 8-12% SDS-polyacrylamide gel (SDS-PAGE), and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore,
Bedford, MA). After being blocked with 5% skim milk in 1 × TBST (Tris-buffered saline and 1% Tween 20), followed by incubation with corresponding primary antibodies (1:500 or 1:800 diluted) at 4°C overnight. After washing three times with TBST, the membranes were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:1,000 dilution) at room temperature for 1 h, followed by detection using ECL system (7SeaPharmTech, Shanghai, China). The β-actin and Lamin A were used as the loading control for total proteins and nuclear proteins, respectively.

2.6. ELISA assay
Human PDLFs at passage 7 (P7) were seeded in 12-well plates at 3 × 10^5 cells/well with 1 ml serum-free medium, and incubated with or without IL-12 (10 ng/ml) for 24 and 48 h, and the culture supernatants were collected. Subsequently, the secreted amounts of MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1 and TIMP-2 proteins by human PDLFs were determined with commercial ELISA kits (R&D Systems, Minneapolis, MN) following the instructions of manufacturer. In brief, 100 µl of the standards, blanks and supernatant samples were separately added into 96-well plates that were precoated with anti-human MMP-1, followed by incubation for 3 h at 37°C. After washes with three times with PBS, biotinylated anti-human MMP-1 antibody (diluted 1:800) was pipetted into the wells and incubated for 1 h at room temperature, followed by washing three times with PBS to remove the unbound biotinylated antibody, HRP-conjugated streptavidin was then added into the wells for
1 h incubation at room temperature. After washing three times with PBS, the substrate tetramethylbenzidine (100 µl) was added into each well and incubated for 0.5 h in the dark room, and a stop solution provided with the ELISA kit was pipetted into each well. The absorbance values of each well were read on a microplate reader at 450 nm. The level of MMP-1 protein in the samples was obtained by comparison with the standard curve. Each sample was measured in duplicate. Additionally, the levels of MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1 and TIMP-2 protein were determined in the same way as following the above procedures.

2.7. Statistical analysis

Data were shown as mean ± SEM. Statistical analysis was performed by the two-tailed Student’s t-test using the SPSS 11.0 software (IBM, Chicago, IL). The value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of IL-12 treatment on the viability of hPDLFs

The viability of hPDLFs was evaluated by MTT assay after IL-12 treatment for 12, 24 and 48 h. The results showed that 5 and 10 ng/ml of IL-12 did not result in a significant reduction in the viability of hPDLFs (Fig. 1), and therefore, 5 and 10 ng/ml of IL-12 were considered to be non-cytotoxic, and were used in the following experiments.
3.2. IL-12 promotes the expression of MMP-1, MMP-3 and MMP-13, and inhibited the expression of MMP-2 and MMP-9 in human PDLFs

Human PDLFs were incubated with IL-12 (0, 5 and 10 ng/ml) for 12 and 24 h, and real-time PCR were used to determine the targeted gene expression. As shown in Fig. 2, the results demonstrated that the mRNA expression levels of MMP-1, MMP-3, and MMP-13 increased 2.54 (12 h), 3.87 (24 h), 1.98 (12 h), 3.84 (24 h), 3.75 (12h) and 3.29 (24 h) folds, respectively, in the human PDLFs after exposure to 5 ng/ml of IL-12 for 12 and 24 h. When the cells were treated with 10 ng/ml of IL-12 for 12 and 24 h, their mRNA levels of MMP-1, MMP-3, and MMP-13 increased 4.69 (12 h), 7.51 (24 h), 4.53 (12 h), 6.15 (24 h), 7.15 h (12h) and 5.78 (24 h) folds, respectively. On the contrary, the mRNA levels of MMP-2 and MMP-9 were significantly down-regulated, and their mRNA levels decreased by about 37% (12 h), 55% (24 h), 8% (12 h) and 18% (24 h), respectively, following the treatment of 5 ng/ml of IL-12 for 12 and 24 h. When the cells were treated with 10 ng/ml of IL-12 for 12 and 24 h, the mRNA levels of MMP-2 and MMP-9 decreased by about 61% (12 h), 72% (24 h), 31% (12 h) and 42% (24 h), respectively. However, TIMP-1 and TIMP-2 mRNA expression were not affected by IL-12 treatment.

On the other hand, we also determined their protein levels of MMP-1, MMP2, MMP-3, MMP-9, MMP-13, TIMP-1 and TIMP-2 in the hPDLFs after IL-12 treatment. The results of Western blot analyses showed that the protein expression levels exhibited a consistent trend with their mRNA levels (Fig. 3). Additionally, ELISA analysis was performed to determine the protein production in the cultured hPDLFs
supernatants. As shown in Fig. 4, the results demonstrated that IL-12 treatment resulted in a significant increase in the MMP-1 (from 86.29 and 97.04 pg/ml to 152.04 and 136.28 pg/ml, 24 and 48 h, respectively), MMP-3 (from 78.82 and 93.64 pg/ml to 156.41 and 168.27 pg/ml, 24 and 48 h, respectively), and MMP-13 (from 89.82 and 77.04 pg/ml to 127.57 and 112.33 pg/ml, 24 and 48 h, respectively) secretion when compared with the untreated groups. However, MMP-2 and MMP-9 production in the cultured medium were significantly decreased when compared with the untreated group. Their secretion was reduced from 95.45 and 102.09 pg/ml to 76.45 and 65.44 pg/ml in MMP-2 for 24 and 48 h, and from 106.48 and 94.73 pg/ml to 70.81 and 55.44 pg/ml in MMP-9 for 24 and 48 h, respectively. Additionally, there were no significant changes of TIMP-1 and TIMP-2 secretion in response to IL-12 treatment ($P > 0.05$).

3.3. IL-12-mediated MMPs expression involves in NF-κB-dependent activation

Previous studies have demonstrated that NF-κB can stimulate the expression of MMPs at transcriptional levels [19], and its activation is necessary for MMP-1 production by human periodontal ligament fibroblasts [20]. Therefore, we determined the effects of IL-12 on IκBα, NF-κB P65 proteins and their phosphorylation levels (p-IκBα and p-NF-κB P65). The results showed that a significant increase of phosphorylated levels of IκBα and NF-κB P65 in the human PDLFs was observed after incubation with 10 ng/ml of IL-12 for 6, 12 and 48 h, but the protein levels of IκBα and NF-κB P65 remained unchanged (Fig. 5A). Further investigation found that
pre-treatment with PDTC, an inhibitor of NF-κB pathway markedly attenuated the phosphorylated forms of IκBα and NF-κB P65. Additionally, the nuclear translocation of NF-κB P65 subunit was investigated by Western blot analysis. As shown in Fig. 5B, NF-κB P65 protein level in the cytoplasm was significantly decreased by IL-12 treatment, contrary to the up-regulation of NF-κB P65 protein level in the nucleus, suggesting that IL-12 treatment facilitated NF-κB P65 subunit nuclear translocation.

Pre-treatment with PDTC also antagonized IL-12 induced NF-κB P65 protein level in the cytoplasm and nucleus. On the other hand, we determined whether NF-κB activation was required for IL-12-dependent MMPs regulation, pharmaceutical inhibitor of NF-κB pathway PDTC or quinazoline was used. The PDLFs were pre-incubated with 10 µM PDTC or quinazoline for 1 h, followed by treatment with IL-12 for 48 h to examine the expression of MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1 and TIMP-2. As shown in Fig. 6, the results of Western blot analysis exhibited that the enhancement of MMP-1, MMP-3 and MMP-13 was inhibited by PDTC or quinazoline pre-treatment. Meanwhile, IL-12-induced MMP-2 and MMP-9 down-regulation was blocked by the inhibitor of NF-κB PDTC or quinazoline.

4. **Discussion**

IL-12 is an important regulator of cellular immunity in both innate and adaptive immune response, and it increases the proliferation of T cells and NK cells, and stimulates the production of numerous immune effector molecules, in particular IFN-γ
The expression of IL-12 has been found to be increased in periapical lesions after experimental pulp exposure, and macrophages and dendritic cells are probably responsible for IL-12 secretion [22]. Previous studies have implicated IL-12 in the pathogenesis of periodontal disease because of its effects on the expression of receptor activation of nuclear factor kappa-B ligand (RNKAL), a potent osteoclast-stimulating factor in human periodontal ligament cells [23]. In this study, we found that IL-12 was able to up-regulate the mRNA and protein expression of MMP-1, MMP-3 and MMP-13 in human PDLFs, which contribute to tissue degradation in periapical areas. Additionally, we also found that the pre-treatment on human PDLFs with an inhibitor of NF-κB pathway (PDTC or quinazoline), dramatically attenuated the increase of MMP-1, MMP-3 and MMP-13 protein expression, which suggests that IL-12-mediated MMPs expression is possibly regulated through the activation of NF-κB pathway in human PDLFs.

MMP-1 is a key enzyme involved in degrading collagen types I and III, which are the most abundant components of the periodontal tissue matrix [24]. In healthy periodontal tissues, the level of MMP-1 is relatively low, which is thought to contribute to its physiological turnover [25]. However, the increase of MMP-1 protein induced by pulpitis or periapical periodontitis can lead to pathological processes, including ECM breakdown [26]. In the present study, the up-regulation of MMP-1 mRNA and protein levels was observed after the PDLFs were exposed to IL-12, whereas TIMP-1 and TIMP-2 expression seemed to be unchanged after IL-12 treatment. Previous studies have showed the presence and immunolocalization of
MMP-1 and TIMP-1 in human radicular cysts [27]. Our findings suggested an imbalance in MMP-1/TIMP-1 expression. MMP-1 activity is strictly regulated by TIMP-1. The balance between MMP-1 and TIMP-1 is an critical control point in connective tissue remodeling, and their imbalance may contribute to tissue destruction and progression of periapical lesions [28].

Additionally, the expression of MMP-3 in the IL-12 treatment group was significantly elevated when compared with the untreated group. MMP-3 is a broad-spectrum MMP and serves as a pivotal activator of latent MMPs, and it is been linked to tissue destruction associated with chronic inflammatory disease such as periodontitis [29]. In addition, MMP-3 is effective at degrading fibronectin, laminin, gelatins, proteoglycans, types IV and IX collagen [30]. In addition to its direct degradative activity, MMP-3 may also function indirectly by activating MMP-1 [31]. Several studies have demonstrated the coordinated expression and release of both MMP-1 and MMP-3 in human fibroblasts [32,33]. Accordingly, MMP-3 may play an important role in the overall regulation of the connective tissue degradation in pathologic conditions. In addition, it was reported that there is a substantial increase in the concentrations of MMP-3 and decrease of TIMP-1 in gingival crevicular fluid (GCF) in periodontal disease [34]. In the present study, the up-regulation of MMP-3 was observed after IL-12 treatment, which possibly contributes to progression of periodontal disease.

Additionally, MMP-13 (collagenase-3) expression was found in GCF samples from patients with chronic periodontitis, and its activity in these samples was significantly
Moreover, MMP-13 expression was also observed in gingival sulcular epithelium, macrophage-like cells and gingival fibroblasts and plasma cells in chronic periodontitis [36]. MMP-13 not only is an enzyme responsible for bone resorption and cartilage destruction in rheumatoid arthritis and osteoarthritis [37], also can degrade fibrillar type collagens, gelatin, basement membrane type IV collagen, fibronectin, tenascin and proteoglycans [38]. These findings suggest that MMP-13 has important role in destruction of periodontal extracellular molecules, and is involved in the pathogenesis of periodontal disease [39]. It has been reported that proinflammatory cytokines, such as IL-1, IL-6, IL-1β and tumor necrosis factor-alpha can enhance MMP expression and production in human periodontal ligament cells [40]. In the present study, our findings showed that the expression levels of MMP-13 mRNA and protein was up-regulated in human PDLFs after IL-12 treatment, which suggests that the increase of MMP-13 expression may be a crucial step in the deterioration of periodontal disease.

MMP-2 and MMP-9 belong to gelatinase-A (72 kDa) and gelatinase-B (96 kDa), respectively, and they act during the last phase of collagen degradation [41], and they are mainly responsible for the breakdown of type IV collagen and non-collagenous components of the extracellular matrix. In the present study, we investigated the effect of IL-12 treatment on the expression of MMP-2 and MMP-9 in human PDLFs. Unexpectedly, and the results revealed that the mRNA and protein levels of MMP-2 and MMP-9 were significantly down-regulated by IL-12 treatment. IL-12 is an important cytokine in numerous immune functions in the initiation and regulation of
cellular immune responses, and its production by macrophages or mast cells can enhances IL-1β expression and production [42]. Another studies indicated that the mRNA and protein levels of MMP-1 were significantly increased by IL-1β stimulation [43]. In this study, it is likely that IL-12 treatment can enhance the promoter activity of MMP-1, MMP-3 and MMP13, but inhibit the promoter activity of MMP-2 and MMP-9 through a direct or indirect regulatory effect in human PDLFs, which might result in different transcriptional effects on the expression of MMPs.

It has reported that NF-κB activation is involved in the regulation of MMPs expression [44], and as previously mentioned, its activation is necessary for MMP-1 production in human PDLFs [20, 45]. In the present study, our data indicated that the levels of p-IκBα and p-NF-κB P65 were enhanced after IL-12 treatment on the human PDLFs. Furthermore, IL-12 treatment resulted in an increase of NF-κB P65 in the nucleus and decreased the level of NF-κB P65 protein in the cytoplasm, suggesting that IL-12 promoted the nuclear translocation of NF-κB P65 subunit in the human PDLFs. In addition, the pre-treatment with PDTC, an inhibitor of NF-κB pathway, significantly antagonized IL-12-mediated MMPs expression. These findings indicated that NF-κB activation is possibly required for IL-12-mediated MMPs expression in human PDLFs. Similar results were obtained from previous studies [46].

In summary, our results indicate that IL-12 up-regulated the mRNA and protein expression of MMP-1, MMP-3 and MMP-13, but down-regulated that of MMP-2 and MMP-9 in human PDLFs. Additionally, IL-12 had no effect on the mRNA and protein levels of TIMP-1 and TIMP-2. Further investigation demonstrated that IL-12
treatment resulted in nuclear translocation of NF-κB P65 and NF-κB activation. Inhibition of NF-κB signal activation attenuated IL-12-mediated effects on MMPs expression, suggesting that NF-κB signal pathway is probably required for IL-12-mediated MMPs expression in human periodontal ligament fibroblasts. These findings also suggest that IL-12 may exacerbate periapical tissue destruction at least partly via regulating MMPs expression during periapical inflammation.

Conflicts of interest

The authors declare that they have no conflicts of interest to disclose.

Acknowledgements

We would like to thank Dr. Jun Li for his assistance with statistic analysis, and we also thank Dr. Jing Wang for her critical comments in reading the manuscript.
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Figure 1. Effect of IL-12 on human periodontal ligament fibroblasts (PDLFs)

Human PDLFs were treated with 0, 5 and 10 ng/ml of IL-12 for 12, 24 and 48 h, and cell viability was assessed by MTT assay. Data are expressed as percentage of cell viability relative to the control (0 ng/ml). Data represented as means ± SEM (n = 3).

Figure 2. Effects of IL-12 on the mRNA levels of MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1 and TIMP-2 in human periodontal ligament fibroblasts (PDLFs)

Human PDLFs were treated with 0, 5 and 10 ng/ml of IL-12 for 12 and 24 h, and then the mRNA expression levels of MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1 and TIMP-2 were determined by real-time PCR. Relative mRNA levels were presented as the ratios relative to untreated cells after normalization for their respective glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. Data represented as means ± SEM (n = 3). *P < 0.05 and **P < 0.01, versus the untreated group (0 ng/ml of IL-12).

Figure 3. Effects of IL-12 on the protein levels of MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1 and TIMP-2

After incubation with IL-12 (10 ng/ml) for 48 h, protein levels in the human PDLFs were analyzed with Western blotting. The presented Western blot image is a representative result of three independent experiments. Relative protein levels of MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1 and TIMP-2 were normalized...
to β-actin signal bands. Data represented as means ± SEM (n = 3). *P< 0.05 and **P< 0.01, versus the untreated group (0 ng/ml of IL-12).

Figure 4. ELISA analysis for MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1 and TIMP-2.

Human PDLFs were incubated in the absence or presence of 10 ng/ml IL-12 for 24 and 48 h, and then protein production in the supernatants of cultured PDLFs were assayed by ELISA. Data are represented as means ± SEM of 3 independent experiments. *P< 0.05 and **P< 0.01, versus the untreated group (0 ng/ml of IL-12).

Figure 5. IL-12 induced the activation of NF-κB signaling pathway

(A): Human PDLFs were pre-treated with 10 µM PDTC for 1 h, followed by incubation with 10 ng/ml IL-12 for, 6, 12 and 48 h, and total proteins were then extracted. The protein levels of IκBα, p-IκBα, NF-κB P65 and p-NF-κB P65 were subjected to Western blot analysis. β-actin served as the loading control. (B): Human PDLFs were pre-treated with 10 µM PDTC for 1 h, and incubated with 10 ng/ml IL-12 for 6, 12 and 48 h. The cytoplasmic and nuclear proteins were respectively prepared as described in the Materials and methods. Cytoplasmic and nuclear NF-κB P65 protein were analyzed with Western blotting. β-actin and Lamin A expression served as the internal control of cytoplasm and nucleus, respectively. The fold increase in protein expression was normalized to β-actin or Lamin A. The presented Western blot image is a representative result of three independent experiments. Data are the means ± SEM (n = 3). **P< 0.01 versus the control (0 ng/ml of IL-12); *P<
0.05 versus the IL-12 group.

Figure 6. IL-12-mediated MMPs expression involved in activation of NF-κB signaling pathway

Human PDLFs were pre-treated with the inhibitor of NF-κB PDTC (10 μM) or quinazoline (10 μM) for 1 h, followed by treatment with IL-12 (10 ng/ml) for 48 h, and cellular total proteins were extracted. The protein expression levels of MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1 and TIMP-2 were analyzed by Western blot analysis. The presented Western blot image is a representative result of three independent experiments. Data are the means ± SEM (n = 3). *P < 0.05 and **P < 0.01 versus the control (0 ng/ml of IL-12); †P < 0.05 versus the IL-12 group.
IL-12 treatment did not affect hPDLCs viability
The effects of IL-12 treatment on the mRNA levels of MMP1, MMP2, MMP3, MMP9, MMP13, TIMP-1, and TIMP-2 in hPDLFs
The effects of IL-12 treatment on the protein levels of MMP1, MMP2, MMP3, MMP9, MMP13, TIMP-1, and TIMP-2 in hPDLFs.
The effects of IL-12 treatment on the protein production of MMP1, MMP2, MMP3, MMP9, MMP13, TIMP-1, and TIMP-2 in the supernatants of cultured hPDLFs
The effects of IL-12 treatment on the protein levels of IκBα, NF-κB P65 and their phosphorylated forms in cultured hPDLCs

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<tr>
<th></th>
<th>PDTC (10 μM)</th>
<th>IL-12 (10 ng/ml)</th>
<th>6 h</th>
<th>12 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>IκBα</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>p-IκBα</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>NF-κB P65</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<td>p-NF-κB P65</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>β-actin</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
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</tbody>
</table>

Relative protein levels

B

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<th>IL-12 (10 ng/ml)</th>
<th>6 h</th>
<th>12 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB P65(Cytoplasm)</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>β-actin</td>
<td>−</td>
<td>−</td>
<td>+</td>
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<td>−</td>
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<tr>
<td>NF-κB P65(Nuclear)</td>
<td>−</td>
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<td>+</td>
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<tr>
<td>Lamin A</td>
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<td>−</td>
<td>+</td>
<td>+</td>
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Protein levels
PDTC or quinazoline pretreatment antagonized IL-12-mediated MMPs expression

<table>
<thead>
<tr>
<th>Condition</th>
<th>Quinazoline (10 μm)</th>
<th>PDTC (10 μm)</th>
<th>IL-12 (10 ng/ml)</th>
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<tr>
<td></td>
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</tr>
</tbody>
</table>

**Western Blot Images**

- MMP-1
- MMP-2
- MMP-3
- MMP-9
- MMP-13
- TIMP-1
- TIMP-2
- β-actin

**Graph**

- Relative protein levels
- Quinazoline (10 μm)
- PDTC (10 μm)
- IL-12 (10 ng/ml)
- MMP-1
- MMP-2
- MMP-3
- MMP-9
- MMP-13
- TIMP-1
- TIMP-2

* and # indicate statistical significance.