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IL-17R activation of human periodontal ligament fibroblasts induces IL-23 p19 production: Differential involvement of NF-κB versus JNK/AP-1 pathways

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ABSTRACT

Interleukin (IL)-23 is an essential cytokine involved in the expansion of a novel $CD4^+$ T helper subset known as Th17, which has been implicated in the pathogenesis of periodontitis recently. This study hypothesised that Th17 signature cytokine IL-17 may target specialised human periodontal ligament fibroblasts (hPDLFs) for production of IL-23 p19, a key subunit of IL-23. Primary hPDLFs had steady expression of IL-17 receptor (IL-17R) mRNA and surface-bound protein. IL-17 was capable of stimulating the expression of IL-23 p19 mRNA and protein in cultured hPDLFs, which was attenuated by IL-17 or IL-17R neutralising antibodies. In accordance with the enhanced expression of IL-23 p19, IL-17 stimulation resulted in rapid activation of Akt, p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) 1/2, c-Jun-N-terminal kinase (JNK), nuclear factor-kappaB (NF-κB), and activator protein-1 (AP-1) in hPDLFs. Inhibitors of Akt, p38 MAPK, ERK 1/2, or NF-κB significantly suppressed, whereas blocking JNK and AP-1 substantially augmented IL-23 p19 production from IL-17-stimulated hPDLFs. Moreover, IL-17-initiated NF-κB activation was blocked by Akt, p38 MAPK, or ERK 1/2 inhibition, while AP-1 activity was specifically abrogated by JNK inhibition. Thus, these results provide evidence that hPDLFs are a target of Th17, and that IL-17 appears to up-regulate the expression of IL-23 p19 via a homeostatic mechanism involving Akt-, p38 MAPK-, and ERK 1/2-dependent NF-κB signalling versus the JNK/AP-1 pathway. Taken together, our findings suggest that disruption of the interaction between IL-17 and IL-23 may be a potential therapeutic approach in the treatment of periodontitis.

1. Introduction

Periodontitis is an infection-driven chronic inflammatory disease affecting the integrity of tooth-supporting tissues. It causes destruction of periodontal connective tissues and alveolar bone that ultimately leads to tooth loss. Inflammatory cytokines produced by immunoregulatory cells regulate the immune responses to periodontal bacteria and play a protective and/or destructive role in disease progression (Bartold et al., 2010). Interleukin (IL)-17 is a proinflammatory cytokine secreted by activated $CD4^+$ T helper cells. In contrast to classic Th1 and Th2 cell populations, IL-17-secreting T cells arise as a distinct and novel $CD4^+$ T helper subset called Th17 (Dong, 2009). Th17 was found to be involved in several inflammatory diseases and was recently reported to be present in periodontally diseased tissues (Cardoso et al., 2009).

Th17 cells arise in the context of transforming growth factor (TGF)-β in combination with IL-6 or IL-21. IL-23 is a recently discovered cytokine belonging to the IL-12 family that is secreted as a heterodimer composed of a p40 subunit identical to the p40 subunit of IL-12 and a unique p19 subunit similar to the p35 subunit of IL-12. IL-23 functions upstream of Th17 and is critical for the expansion of Th17 cells and IL-17 production (Boniface et al., 2008). IL-12 is known as the major inducer of interferon (IFN)-γ production and Th1 response, while IFN-γ strongly inhibits the differentiation of naive T cells into Th17 (Gee et al., 2009). Both levels of IL-23 and IL-12 were elevated in periodontitis-affected tissues, and both correlated well with periodontitis disease activity (Ohyama et al., 2009; Yucel et al., 2008). Interestingly, mechanistic animal studies found that IL-12 $p40^{-/-}$ mice, which were deficient in both IL-12 and IL-23, exhibited a reduced inflammatory cell infiltrate but increased tissue destruction upon subcutaneous challenge with *Porphyromonas gingivalis*. Moreover, IL-12 $p40^{-/-}$ mice were more susceptible to naturally occurring murine periodontitis than wild-type controls (Shen and Gaffen, 2008). Despite the lack of direct

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evidence on the role of IL-12 or IL-23 alone in the development of periodontitis, these data above suggest an essential role of IL-12 and/or IL-23 in the pathogenesis of periodontitis. IL-12 and IL-23 are differentially involved in the regulation of IL-17; however, the production mechanism of IL-23 in periodontitis-affected tissues has not been fully understood yet.

The periodontal ligament is a highly vascularised and cellular connective tissue that anchors the tooth root to the surrounding alveolar bone. As major cellular components of the periodontal ligament, human periodontal ligament fibroblasts (hPDLFs) not only function as support cells for periodontal tissues, but also produce various inflammatory mediators that recognise somatic components. Upon stimulation with cytokines or bacterial pathogens, hPDLFs secrete various soluble mediators of inflammation, which are thought to play an important role in the periodontal inflammatory response (El-Awady et al., 2010). However, the IL-17 receptor (IL-17R) expression in hPDLFs and the effects of Th17 signature cytokine IL-17 on specialised hPDLFs are still unknown.

There is increasing evidence indicating the potential role of Th17/IL-17 in the pathogenesis of periodontitis (Cardoso et al., 2009; Dutzan et al., 2009; Liu et al., 2009; Yetkin-Ay et al., 2009). Thus, identification of the downstream targets of IL-17 in periodontal tissues should advance current understanding of the mechanisms underlying IL-17-mediated inflammation. Based on the fact that IL-23 regulates the production of IL-17 (Boniface et al., 2008), we hypothesised that IL-17 may affect the expression of IL-23 p19, the key subunit of IL-23, in hPDLFs as a part of the reciprocal action between two cytokines that may accentuate the vicious cycle of periodontal inflammation. In the present study, we examine the expression of IL-17R in hPDLFs and the effects of IL-17 on IL-23 p19 production. In order to elucidate the underlying mechanisms, we will identify the signalling pathways that are possibly involved.

2. Materials and methods

2.1. Antibodies and reagents

Primary antibodies to phospho-Akt (Ser^{473}), Akt, phospho-p38 MAPK, p38 MAPK, phospho-JNK, JNK, phospho-ERK, and ERK were purchased from Cell Signalling Technology (Danvers, MA). The antibodies to IL-17R (H-168), IL-17 (H-132), NF-κB p65, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal IgG (H-270), which was used as isotype control, and goat serum were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TRITC- and FITC-conjugated secondary antibodies were obtained from Jackson (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Actinomycin D, cycloheximide, LY294002, SB203580, PD98059, SP600125, pyrrolidine thiocarbamate (PDTC), and curcumin were purchased from Sigma–Aldrich (St. Louis, MO). All inhibitors were dissolved in dimethyl sulfoxide (DMSO) and the final level of DMSO was less than 0.01% in the culture medium. Human recombinant IL-17 was purchased from Peprotech (London, UK). Fetal bovine serum (FBS), penicillin, streptomycin, and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO BRL (Gaithersburg, MD).

2.2. Preparation of human periodontal ligament fibroblasts

Healthy human periodontal ligament tissue was obtained from extracted premolars for orthodontic reasons. Experimental protocols were approved by the Ethics Committee of School of Stomatology, Wuhan University, China. Informed consents were obtained before tooth extraction. Freshly extracted teeth were immediately placed in 20 mM phosphate buffered saline (PBS, pH 7.2) supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and rinsed thoroughly with DMEM. Periodontal ligament was carefully removed from the middle third of the root surface with a scalpel. The aseptically removed tissue was then placed in a 60 mm Petri dish (Nunc, Denmark). Tissues were minced with a blade into approximately 1 mm^3 fragments and grown in DMEM supplemented with 10% FBS and antibiotics. Cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. After achieving confluence, the cells were detached with 0.25% trypsin and 0.2% EDTA and subcultured at a ratio of 1:3. hPDLFs in the present study were used between the 5th and 8th passages.

2.3. RNA extraction and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from hPDLFs using TRIZOL Reagent (Invitrogen). Aliquots (1 μg) of RNA were reverse transcribed to cDNA (20 μL) with Oligo dT and ReverTra Ace (TOYOBO, Japan). PCR amplifications were performed in a 25 μL reaction mix containing 1 μL of cDNA, 50 nM primers, and 12.5 μL of 2× Taq PCR Master Mix (Tiangen Biotech Co., Ltd., Beijing, China) using a PE9700 RT-PCR system (Applied Biosystems). Specific primers were designed from cDNA sequence of IL-17R and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The primer sequences for PCR were designed as follows: IL-17R-F (5′-CTAAACTGCACGGTCAAGAAT-3′), IL-17R-R (5′-ATGAACCAGTACACCCAC-3′), GAPDH-F (5′-AACGGATTTGGTCGTATTGGG-3′), and GAPDH-R (5′-CAGGGGTGCTAAGCAGTTGG-3′). PCR conditions are as follows: 1× (95 °C, 3 min), 35× (94 °C, 30 s; 60 °C, 1 min; 72 °C, 1 min) and 1× (72 °C, 5 min). Amplified products were electrophoresed in 1.5% agarose gel and visualised with ethidium bromide. Band intensity was analysed densitometrically using Gene Tools software (Syngene, Cambridge, UK).

2.4. Immunofluorescence analysis

hPDLFs grown on glass coverslips were cultured at low density. Cells were fixed with 4% paraformaldehyde, air-dried, and stored at −20 °C until use. Briefly, cells were washed with PBS, and blocked in 10% non-immune goat serum for 1 h at room temperature. Then cells were incubated with either rabbit anti-human IL-17R, mouse monoclonal antibody against NF-κB p65, or IgG isotype control antibody at a final dilution of 10 μg/ml at 4 °C overnight, washed twice with Tris buffered saline (TBS) followed by incubation with TRITC-conjugated goat anti-rabbit IgG (1:100) or FITC-conjugated goat anti-mouse IgG (1:100) for 1 h at room temperature. Nuclei were stained with DAPI, and coverslips were mounted on a microscope slide with embedding medium. Cells were observed and photographed with a fluorescence microscope (Leica).

2.5. Flow cytometry analysis of IL-17 receptor expression

Confluent hPDLFs were washed with ice cold PBS and harvested by incubation with 4 mM PBS-EDTA. Most cells were rounded up following this treatment and removed by gentle agitation, while cells that failed to detach were removed with gentle scraping. Cells were washed twice with ice cold PBS and incubated (for 20 min on ice) in PBS–3% bovine serum albumin (BSA, Sigma). They were incubated with either rabbit anti-human IL-17R antibody or IgG isotype control antibody (10 μg/ml) on ice for 30 min. After being washed thrice with PBS–3% BSA, the cells were incubated with an FITC-conjugated goat anti-rabbit IgG in the dark for 30 min on ice. Then they were washed thrice with PBS–1% BSA and immediately analysed on a FACS Calibur flow cytometer (Becton Dickinson) using Cell Quest software.