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ORIGINAL ARTICLE

Interleukin (IL)-35 Suppresses IL-6 and IL-8 Production in IL-17A-Stimulated Human Periodontal Ligament Cells

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Abstract—Interleukin (IL)-35 is a novel anti-inflammatory cytokine that is produced by regulatory T cells. IL-35 is reported to suppress IL-17A-producing helper T (Th17) cell activation. IL-17A is related to progression of periodontitis. Furthermore, IL-35 and IL-17A are detected in human gingival crevicular fluid. However, the effect of IL-35 and interaction between IL-35 and IL-17A on pro-inflammatory cytokine production in human periodontal resident cells are still unclear. The aim of this study was to clarify the effect of IL-35 on IL-6 and IL-8 production in human periodontal ligament cells (HPDLCs) stimulated with IL-17A. IL-35 inhibited IL-6 and IL-8 production in IL-17A-stimulated HPDLCs. Moreover, western blot analysis showed that IL-35 suppressed extracellular signal-regulated kinase (ERK) and nuclear factor (NF)-κB p65 phosphorylation in IL-17A-stimulated HPDLCs. Our findings suggested that IL-35 produced from regulatory T cells might inhibit progression of periodontitis by decreasing IL-17A-induced levels of IL-6 and IL-8.

KEY WORDS: IL-35; IL-17A; IL-6; IL-8; human periodontal ligament cells.

INTRODUCTION

Periodontitis is characterized by periodontal tissue destruction caused by bacterial infection. A previous study has shown that immune responses play an important role in the progression of periodontitis [1].

T helper 17 (Th17) cells have the capacity for bone destruction through interleukin (IL)-17A in rheumatoid arthritis and periodontal disease [2, 3]. In particular, IL-17A is known to induce pro-inflammatory cytokine production in periodontal resident cells including periodontal ligament cells [4].

IL-6 is known to play an important role in inflammatory responses [5]. IL-6 has been reported to activate mature osteoclasts [6]. Moreover, IL-6 is involved in Th17 differentiation [7]. IL-8 also plays a role in the pathogenesis of periodontal disease because it induces the migration of neutrophils to inflamed sites of periodontal tissues [8].

IL-35 is a novel anti-inflammatory cytokine which is a heterodimer of Epstein-Barr virus-induced gene 3 and IL-12p35 subunits [9]. IL-35 is produced from regulatory B cells, CD8+ regulatory T cells and CD4+ regulatory T cells [10–12], and suppresses Th17 cell activation and IL-17 production from Th17 cells [13]. Additionally, IL-35 has been shown to suppress experimental autoimmune uveitis and arthritis [14, 15]. Recently, IL-35, as well as IL-17A, has been detected in gingival crevicular fluid (GCF) and gingival tissue of chronic periodontitis [16]. However, the interaction between IL-35 and IL-17A in periodontal resident cells is still uncertain.

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The aim of this study is to clarify the effect of IL-35 on IL-6 and IL-8 production in IL-17A-stimulated human periodontal ligament cells (HPDLCs). We also explored whether IL-35 affects nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) signaling pathways in IL-17A-stimulated HPDLCs.

MATERIALS AND METHODS

Cell Culture

HPDLCs were obtained from Lonza (Walkersville, MD, USA). HPDLCs were sub-cultured in alpha-minimal essential medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Biowest, Rue du Vieux Bourg, Nuaillé, France) and 100 U penicillin/streptomycin at 37 °C in humidified air with 5% CO_2 for increasing the cell numbers. HPDLCs which had been sub-cultured at 5–10 passages were cultured in the same medium for the following experiments, as previously reported [17].

Enzyme-Linked Immunosorbent Assay

HPDLCs (1×10^5) at 5–10 passages were harvested in 24-well plates until subconfluence. HPDLCs were then stimulated with recombinant IL-17A (10 ng/ml, PeproTech, Rocky Hill, NJ, USA) in the presence or absence of IL-35 (0.1, 1, 10 ng/ml, PeproTech), SB203580 (p38 MAPK inhibitor, 10 μM; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PD98059 (extracellular signal-regulated kinase (ERK) inhibitor, 10 μM; Cayman Chemical, Ann Arbor, MI, USA), SP600125 (c-jun N-terminal kinase (JNK) inhibitor, 10 μM; Santa Cruz Biotechnology), or SC514 (NF-κB inhibitor, 10 μM; Santa Cruz Biotechnology). After 24-h incubation, the supernatants from HPDLCs were collected. IL-6 and IL-8 concentrations of the supernatants were measured with a duoset ELISA kit (R&D systems, Minneapolis, MN, USA). All assays were performed according to the manufacturer's instructions.

Western Blot Analysis

To confirm IL-17A-induced phosphorylation of intracellular signal transduction molecules, western blot analysis was performed. HPDLCs (3×10^5) at 5–10 passages were harvested in 12-well plates until subconfluence. HPDLCs that had been stimulated with IL-17A (10 ng/ml) with or without IL-35 (10 ng/ml) were washed once with cold phosphate buffered saline (PBS) before being incubated on ice for 30 min with cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with a protease inhibitor cocktail (Cell Signaling Technology). After removal of debris by centrifugation, the protein concentrations of the lysates were quantified with the Bradford protein assay using IgG as a standard. A 20-μg protein sample was loaded onto a 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and then transferred to a polyvinylidene di-fluoride (PVDF) membrane. The phosphorylation of NF-κB p65, p38 MAPK, ERK, and JNK was assessed using phospho-NF-κB p65 rabbit monoclonal antibody (Cell Signaling Technology), phospho-p38 MAPK rabbit monoclonal antibody (Cell Signaling Technology), phospho-ERK rabbit monoclonal antibody (Cell Signaling Technology), NF-κB p65 rabbit monoclonal antibody (Cell Signaling Technology), p38 MAPK rabbit monoclonal antibody (Cell Signaling Technology), ERK rabbit monoclonal antibody (Cell Signaling Technology), or β-actin rabbit monoclonal antibody (Cell Signaling Technology) according to the manufacturer's instructions. Protein bands were visualized by incubation with an *Horseradish peroxidase* (HRP)-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO, USA), followed by detection using the ECL system (Advansta Inc., Menlo Park, CA, USA). The band density of blots was measured using ImageJ software (version 1.50).

Statistical Analysis

Statistical significance was analyzed using Student's *t* test and one-way analysis of variance. *P* values of < 0.05 were considered to be significant in Figs.1 and 2.

RESULTS

Effect of IL-35 on IL-6 and IL-8 Production in IL-17A-Stimulated HPDLCs

IL-35 and Il-17A at a maximum concentration of 10 ng/ml were used. Treatment with 10 ng/ml of IL-35 and IL-17A did not affect HPDLC viability by methyl thiazolyl tetrazolium (MTT) assay (data not shown).

IL-17A induced IL-6 and IL-8 production in HPDLCs, as previously reported [18]. IL-35 treatment

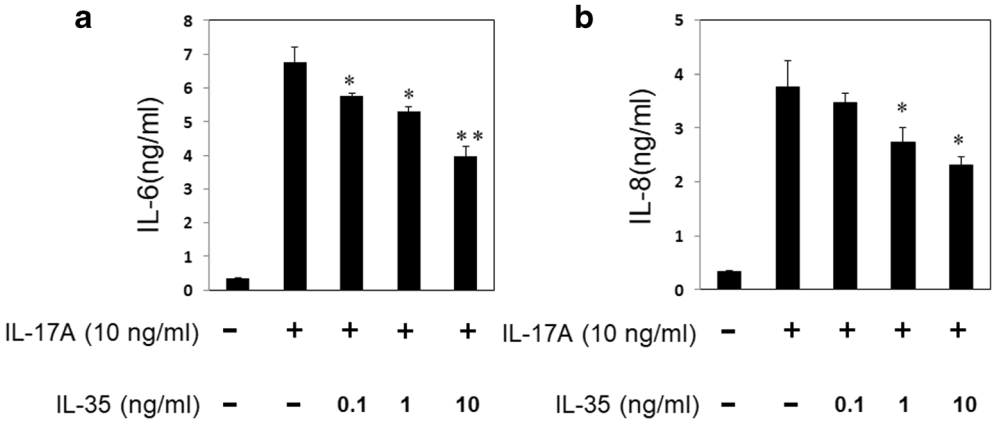

Fig. 1. Effect of IL-35 on IL-6 and IL-8 production in IL-17A-stimulated HPDLCs. HPDLCs were stimulated with or without IL-35 (0.1, 1, and 10 ng/ml) and IL-17A (10 ng/ml) for 24 h. Then, IL-6 (**a**) and IL-8 **(b)** concentrations in the supernatants were measured by an ELISA kit. Results are shown as the mean and SD of a representative experiment performed in triplicate. Error bars represent the SD. *$P < 0.05$ and **$P < 0.01$, significantly different from IL-17A-stimulated HPDLCs without IL-35 treatment.

significantly suppressed IL-6 and IL-8 production in IL-17A-stimulated HPDLCs (Fig. 1).

Effects of MAPKs or NF-κB Inhibitors on IL-6 and IL-8 Production in IL-17A-Stimulated HPDLCs

A p38 MAPK inhibitor (SB203580), an ERK inhibitor (PD98059), and an NF-κB inhibitor (SC514) significantly inhibited IL-6 and IL-8 production in IL-17A-stimulated HPDLCs (Fig. 2). However, a JNK inhibitor (SP600125) did not suppress IL-6 and IL-8 production in these cells (Fig. 2).

Effect of IL-35 on MAPKs and NF-κB Activation in IL-17A-Stimulated HPDLCs

As p38 MAPK, ERK, and NF-κB pathways have been found to be involved in IL-6 and IL-8 production in IL-17A-stimulated HPDLCs, we examined the effect of IL-35 on activation of each signaling molecule in IL-17A-stimulated HPDLCs. IL-17A induced p38 MAPK, ERK, and NF-κB p65 phosphorylation, although it had little effect on JNK phosphorylation whose band density levels are remarkably low (Figs. 3 and 4). IL-35 suppressed ERK and NF-κB p65 phosphorylation, but not p38 MAPK

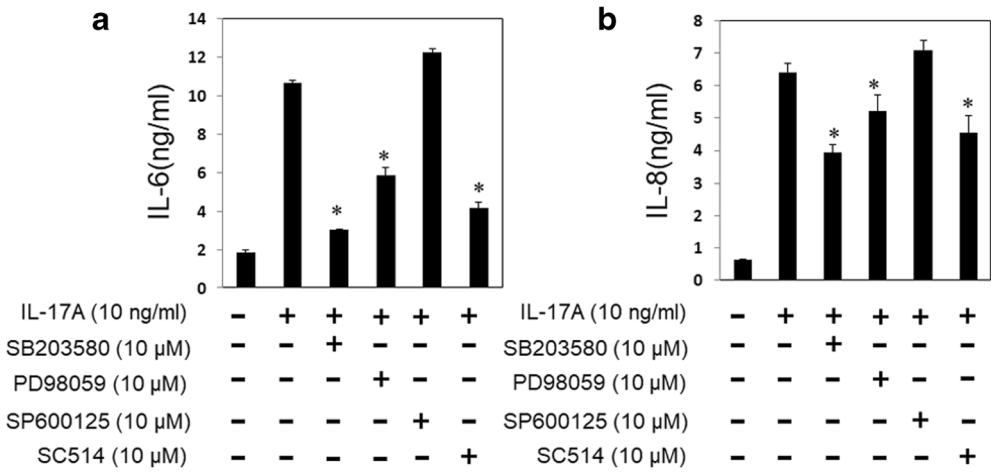

Fig. 2. Effects of specific signal transduction inhibitors on IL-17A-stimulated IL-6 and IL-8 production from HPDLCs. HPDLCs were incubated with p38 MAPK inhibitor (SB203580; 10 μM), ERK inhibitor (PD98059; 10 μM), JNK inhibitor (SP600125; 10 μM), or NF-κB inhibitor (SC514; 10 μM) for 1 h and then stimulated with IL-17A (10 ng/ml). After 24 h, the supernatants were collected and IL-6 (**a**) and IL-8 (**b**) concentrations were measured by an ELISA kit. Results are shown as the mean and SD of a representative experiment performed in triplicate. Error bars represent the SD. *$P < 0.05$, significantly different from IL-17A-stimulated HPDLCs without treatment of each respective inhibitor.

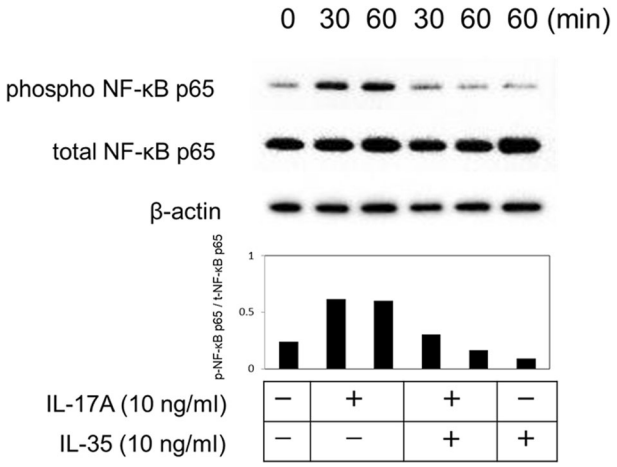

Fig. 3. Effect of IL-35 on NF-κB p65 phosphorylation in HPDLCs. Cultured cells were treated with IL-17A (10 ng/ml) in the presence or absence of IL-35 (10 ng/ml) for 30 or 60 min. The cells were then lysed in lysis buffer containing protease inhibitors. The phosphorylation of NF-κB p65 was analyzed using western blot. A representative western blot displaying phospho-NF-κB p65, total NF-κB p65, and β-actin levels in HPDLCs of three independent experiments is shown. The graph shows the ratio of phospho-NF-κB p65 to total NF-κB p65.

phosphorylation in IL-17A-stimulated HPDLCs (Figs. 3 and 4a, b).

DISCUSSION

We demonstrated that IL-35 inhibited IL-6 and IL-8 production in IL-17A-stimulated HPDLCs.

It has been reported that IL-35 and IL-17A are detected in periodontal tissue and GCF, and higher in GCF from patients with periodontitis than healthy patients [16]. Furthermore, the total amount of IL-35 in GCF was greater in the chronic periodontitis (CP) group than the healthy group, although the IL-35 concentration in GCF was higher in the healthy group than that in the CP group. Taken together, these findings suggest that IL-35 plays an important role in regulation of periodontitis [19]. In the present study, we have, for the first time, demonstrated that IL-35 inhibits production of pro-inflammatory cytokines IL-6 and IL-8 in IL-17A-stimulated HPDLCs, one of the periodontal resident cells. These findings indicated IL-35 could have anti-inflammatory action in periodontitis.

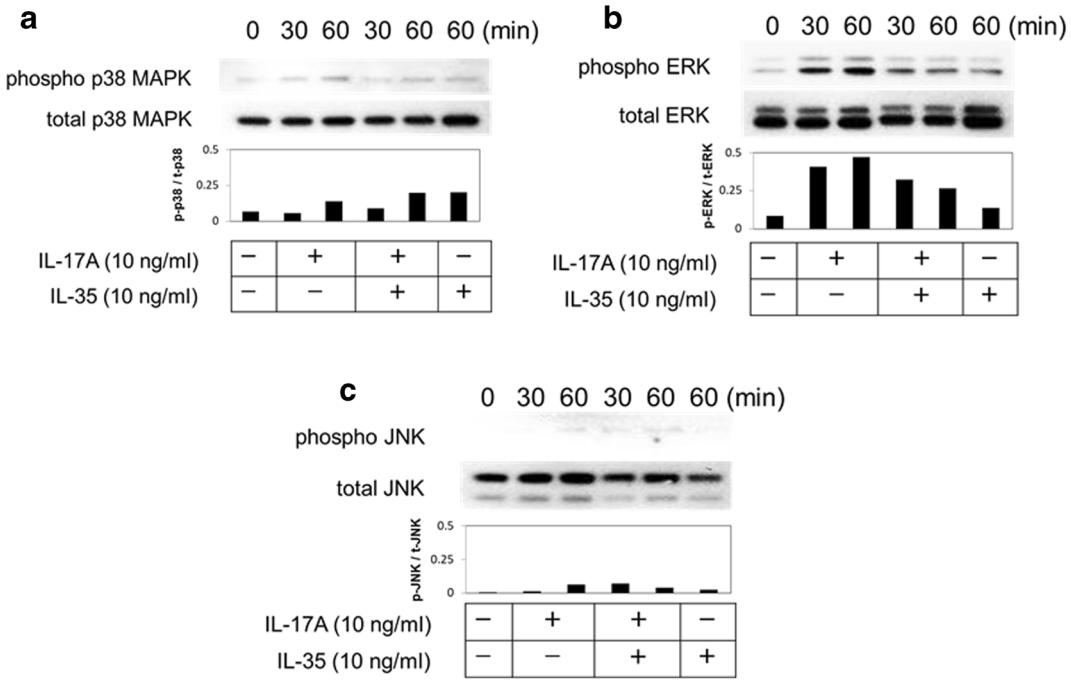

Fig. 4. Effect of IL-35 on p38 MAPK, ERK, and JNK phosphorylation in HPDLCs. Cultured cells were treated with IL-17A (10 ng/ml) in the presence or absence of IL-35 (10 ng/ml) for 30 or 60 min. The cells were then lysed in lysis buffer containing protease inhibitors. The phosphorylation of p38 MAPK, ERK, and JNK was analyzed by western blot. A representative western blot displaying phospho-p38 MAPK, total p38 MAPK, phospho-ERK, total ERK, phospho-JNK, and total JNK levels in HPDLCs of three independent experiments is shown. The graphs show the ratios of phospho-p38 MAPK, phospho-ERK, and phospho-JNK expression to total p38 MAPK, total ERK, and total JNK, respectively.

IL-17A is the pro-inflammatory cytokine produced predominantly by Th17 cells [20]. Takahashi et al. reported that IL-17A plays an important role in periodontitis [21]. Furthermore, IL-17A promotes receptor activator for nuclear factor-κB ligand (RANKL) production from HPDLCs [22] and induces matrix metalloproteinase (MMP)-1 expression in HPDLCs [23]. Moreover, IL-17A and/or *Tannerella forsythia* GroEL increases IL-6 and IL-8 production in HPDLCs [18]. In the present study, IL-17A induced IL-6 and IL-8 production in HPDLCs. Thus, IL-17A possibly promotes progression of periodontitis through pro-inflammatory cytokine production.

Previous reports indicated that IL-35 has an anti-inflammatory effect in various diseases. Wang et al. suggested that IL-35 inhibits the expression of pro-inflammatory cytokines, such as IL-6, tumor necrosis factor (TNF)-α, and IL-17A, and promotes the expression of IL-10 in an acute colitis model [24]. Zhang et al. also reported that IL-35 suppresses TNF-α and IL-17A expression in the intestine and liver of an acute graft-*versus*-host disease model [25]. Wu et al. demonstrated that IL-35 regulates vascular endothelial growth factor (VEGF), Flt-1, Flk-1, and TNF-α mRNA expression in a mouse model of rheumatoid arthritis [26]. Thus, IL-35 has an anti-inflammatory effect in various *in vivo* disease models. Therefore, as our next study, we need to examine the effect of IL-35 using mouse model of periodontitis *in vivo*.

Other studies have shown the effect of IL-35 on NF-κB activation. Hu et al. reported that IL-35 reduces lipopolysaccharide (LPS)-induced acute kidney injury in mouse by inhibiting NF-κB p65 and inhibitor of NF-κB kinase (IKK) phosphorylation *in vivo* [27]. Furthermore, Chen et al. demonstrated that IL-35 inhibits LPS-induced NF-κB signaling pathways in human dendritic cells [28]. Our present finding that IL-35 decreased the inflammatory reaction through inhibition of NF-κB activation in HPDLC cultures is consistent with these previous *in vivo* and *in vitro* results.

Some researchers have described the effect of IL-35 on MAPK activation. Chen et al. found that IL-35 suppresses LPS-induced MAPK p38 phosphorylation in human dendritic cells [28]. Additionally, Sha et al. showed that IL-35 inhibits LPS-induced MAPK p38, ERK, and JNK phosphorylation in human aortic endothelial cells [29]. In the present study, IL-35 suppressed ERK phosphorylation in HPDLCs, suggesting that MAPK activation by IL-35 varies depending on cell type.

The present study showed that IL-35 suppresses IL-6 and IL-8 production in IL-17A-stimulated HPDLCs through inhibition of ERK and NF-κB p65 phosphorylation. These findings indicate that IL-35 plays a role in regulating the progression of periodontitis by inhibiting inflammatory cytokine production.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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