The role of hypoxia responsive transcription factor DEC1 in periodontal inflammation

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Contents

1. Preface

This article is constructed with a reference paper “Transcription factor DEC1 is required for maximal experimentally induced periodontal inflammation” in Journal of Periodontal Research, and second reference paper “The role of the hypoxia responsive gene DEC1 in periodontal inflammation” in Journal of Hard Tissue Biology.

2. Abstract

Disruption of transcriptional regulation is a confounding factor associated with a wide range of human inflammatory diseases. The resident anaerobic bacteria interact with host inflammatory reactions leading to a hypoxic environment in the periodontal pocket. The purpose of this study was to investigate mechanistic links between transcription factor DEC1 and pathways underlying inflammation, and to illustrate the link between hypoxia and inflammation in periodontal disease. Male *DEC1* knockout (KO) mice and their wild-type littermates were used for the experimental periodontitis model. Measurement of alveolar bone resorption, micro-computed tomography, isolation of gingival mononuclear cells (GMCs), flow cytometry and immunohistochemical analysis were used in this study. Human gingival fibroblasts (HGF-1) were used for DEC1 overexpression and short interference ribonucleic acid (siRNA) studies and quantitative real-time polymerase chain reaction (real-Time PCR) and Western blot analysis were performed. Human periodontal ligament (HPDL) cells were used for DEC1 siRNA transfection with lipopolysaccharide (LPS) and/or hypoxia. Micro-CT analysis demonstrated that *Porphyromonas gingivalis* (*P. gingivalis*) caused a decrease in bone area of wild-type mice compared with *DEC1*KO mice. Expression of inflammatory and immune markers in GMCs of *DEC1*KO mice after treatment with *P. gingivalis* was lower than WT mice treated with *P. gingivalis*.

Conversely, IL-4 and IL-10 mRNAs were significantly increased in GMCs isolated from *DEC1*KO mice. The results show that treatment of *DEC1*KO mice with *P. gingivalis* decreased the numbers of $CD11b^{+}F4/80^{+}$ and $CD4^{+}RANKL^{+}$ T cells. Moreover, expression of CD4, F4/80, RANKL and Cathepsin K in inflammatory cell infiltrates was significantly reduced in *DEC1*KO mice treated with *P. gingivalis* compared with controls. Furthermore, over-expression of DEC1 in HGF-1 cells increased the expression of IL-1β and TNF-α mRNAs and their expression levels reached a maximum in response to treatment with LPS. Inhibition of DEC1 by siRNA interference suppressed the *P. gingivalis*-derived LPS-induced expression of IL-1β, TNF-α, and TLR4. Increased levels of DEC1 were found after hypoxic or inflammatory stimulation of HPDL cells. Treatment with the DEC1 siRNA inhibited the effects of LPS and hypoxia. These results suggest that hypoxia enhances the virulence of LPS to induce DEC1 expression and DEC1 can modulate *P. gingivalis*-induced periodontitis in the periodontal tissues.

3. Introduction

Among the many genes identified in inflammation and immunity screens, the basic helix-loop-helix transcription factor DEC1 is a key player in a wide array, for example, the maximal expression or repression of many early transcripts, of inflammation processes [1, 2]. Lipopolysaccharide (LPS) stimulates transcription of many genes in multiple signaling pathways. DEC1 is a transcriptional regulatory protein that can directly activate and repress transcription in a wide array of functional roles. Notably, autoimmune diseases develop in aging *DEC1* knockout (KO) mice, which display increased production of lnterleukin-4 (IL-4) and IL-10 without affecting interferon-γ (IFN-γ) [2]. We previously reported that over-expression of DEC1 and phosphorylation of Akt is mediated via PI-3K signaling [3]. Rapid induction of these proteins in response to environmental stimuli suggests DEC1 is protective against detrimental conditions. *DEC1*KO mice exhibited defective T cell-mediated recall responses which lead to spontaneous autoimmune disease [2]. Other studies, however, indicate very rare or a very low penetrance of spontaneous autoimmune disease in *DEC1*KO mice [4, 5].

Fu et al. revealed the expression of a bone homeostasis system that mediates leptin-regulated bone formation [6]. To date, however, the role of the transcription factor in bone resorption and bone homeostasis *in vivo* is unclear. In the present study we therefore

investigated the involvement of the transcription factor in mechanisms underlying the regulation of alveolar bone resorption, as well as osteoclastogenesis through its expression in the oral environment.

Periodontitis is a ubiquitous disease characterized by destruction of connective tissue and dental bone support following an inflammatory host response secondary to infection by periodontal bacteria [7]. Inflammatory cell infiltration results from periodontal plasma cells, including neutrophils, T- & B- lymphocytes and macrophages. IL-1β is a potent multifunctional pro-inflammatory polypeptide produced by monocytes and tissue macrophages. It attracts and activates immune cells and controls the expression of most immunomodulatory genes [8]. Tumor necrosis factor-α (TNF-α) is a cytotoxic cytokine that has emerged as an important mediator of inflammatory responses and apoptosis [9]. Moreover, LPS, a common component of the cell wall of gram-negative bacteria, stimulates butyric acid-induced apoptosis in human peripheral blood mononuclear cells [10], and a toxin from *Actinobacillus actinomycetemcomitans* induces apoptosis in B lymphocytes present in periodontal tissue [11]. The resident gram-negative anaerobic bacteria interact with the host inflammatory reactions leading to a hypoxic environment in the periodontal pocket. DEC1 expression has been implicated in the regulation of hypoxia [12] and cytokines [12, 13]. It also participates in T cell-mediated immune

response [2, 5]. Several studies have investigated the relationship between hypoxia and inflammation and have shown that hypoxia, by itself or in combination with LPS, can increase the release of inflammatory cytokines in various cell types [14-17]. Furthermore, hypoxic conditions at inflammatory sites can enhance the susceptibility to infection and inflammatory signals by up-regulating inflammatory cytokines [18]. Although the mechanisms responsible for gingival tissue damage are poorly understood, both immune-mediated reactions and direct cytopathic effects of bacteria may be involved.

To investigate the function of the transcription factor DEC1 in *P. gingivalis*-induced alveolar bone loss, we used *DEC1*KO mice and an experimental model of periodontitis. Thus, we address the role of DEC1 in inflammatory and immune cells, especially in LPS-dependent experimental settings and illustrate the link between hypoxia and inflammation in periodontal disease.

4. Materials and Methods

4.1 Animals

The animals used in this study were described in our previous report [19]. Briefly, *DEC1*$^{-/-}$ (*DEC1*KO) mice were generated by Ingenious Targeting Laboratory, Inc. (Stony Brook, NY, USA). The 4.7-kb BamHI-BssSI genomic fragment of *DEC1*, which contains the entire coding region in exons 1 to 5, was replaced with a Neo cassette. The resulting chimeric mice were back-crossed to a C57BL/6J background for three generations. Male *DEC1*KO mice (n = 12) and their wild-type (WT) littermates (n = 12), all five weeks of age, were individually housed under specific pathogen-free conditions. Mice were all sacrificed at the same time, 10:00AM. All procedures were performed in compliance with the standard principles and guidelines for the care and use of laboratory animals at Kanagawa Dental University (Approval No.12-042).

4.2 Preparation of bacteria

P. gingivalis ATCC 33277 was grown at 37°C for 24 hours in an anaerobic chamber, with an atmosphere of 85% N_2, 10% H_2 and 5% CO_2 in brain heart infusion broth supplemented with 5 mg/ml yeast extract, 5 μg/ml hemin and 0.2 μg/ml vitamin K_1, as previously described [20]. LPS from *P. gingivalis* ATCC 33277 was obtained from *P.*

gingivalis according to the manufacturer's instructions (iNtRON Biotechnology, Kyungki-Do, Korea).

4.3 Experimental periodontitis

An established method of experimental periodontitis has been reported [20]. Briefly, five-week-old male C57BL/6 (CLEA Japan, Inc., Tokyo, Japan) and *DEC1*KO mice were given sulfamethoxazole (1 mg/ml) and trimethoprim (200 μg/ml) in their drinking water for four days to reduce any existing oral microorganisms, followed by a three day antibiotic-free period before starting oral treatment with *P. gingivalis*. They were randomly divided into three experimental groups of six mice each [Group A: 5% carboxymethylcellulose (CMC) (control; C57BL/6 group); Group B: *P. gingivalis* ATCC 33277 (C57BL/6 + *P. g.* group); Group C: *P. gingivalis* ATCC 33277 (*DEC1*KO + *P. g.* group)]. Each mouse treated with *P. gingivalis* received 0.5 ml (1.0×10^8 cells/ml) of the bacterial suspension in 5% CMC by oral gavage (5 times) at 48-hr intervals. All mice were sacrificed by CO_2 inhalation 30 days after the last gavage. The experimental procedures of this study were reviewed and approved by the Committee on Ethics of Animal Experiments at Kanagawa Dental University and were carried out under the guidelines for animal experimentation at Kanagawa Dental University.

4.4 Measurement of alveolar bone resorption and micro-computed tomography

The left side of the upper jaw of each mouse was used as a dry specimen for measuring horizontal alveolar bone loss. Each upper jaw was de-fleshed after 10 min in an autoclave and was then immersed in 3% hydrogen peroxide, rinsed, air dried and stained with 1% methylene blue. Horizontal alveolar bone loss around the maxillary molars was evaluated morphometrically. Briefly, the distance between the cemento-enamel junction and the alveolar bone crest was measured at seven buccal sites per mouse. Measurements were made using a stereomicroscope (40× magnification) fitted with a digital high-definition system (Digital HD microscope VH-7000; KEYENCE, Osaka, Japan), standardized to provide measurements in millimeters. Three-dimensional (3D) imaging data for each mandibular bone were collected using micro-computed tomography (micro-CT) (MCT-CB100MF; Hitachi medico, Tokyo, Japan) under the following exposure conditions: tube voltage, 70 kV; tube current, 100 μA; voxel size, 17 × 17 × 17 μm.

4.5 Isolation of gingival mononuclear cells (GMCs) and flow cytometry

Thirty days after the last *P. gingivalis* treatment, mice were sacrificed and gingival tissues from their upper and lower jaws were carefully removed using microsurgical

tweezers under a stereomicroscope. Cells from gingival tissues were prepared by being gently 'teased' through sterile stainless-steel screens, followed by an enzymatic dissociation procedure with collagenase type IV (0.5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA). GMCs were purified on discontinuous Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) and re-suspended in RPMI 1640 (Sigma Chemical Co.) supplemented with HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 mg/mL) and 10% fetal calf serum (FCS) (complete medium). GMCs (2×10^5 cells) were stained with combinations of fluorescence-conjugated or biotinylated monoclonal antibodies (mAbs) including anti-CD4, -CD11b, -F4/80 and -RANKL (BD Pharmingen and eBiosciences, San Diego, CA, USA). Samples were then analyzed by flow cytometry (FACSCalibur™; BD Biosciences).

4.6 Tartrate-resistant acid phosphatase staining and Immunohistochemistry

The 4 μm thick paraffin sections were stained with tartrate-resistant acid phosphatase (TRAP; TRAP Staining Kit; Wako, Tokyo, Japan). TRAP staining was performed in accordance with the manufacturer's instructions. Red-stained cells were defined as positive and were examined under an optical microscope (×100 magnification) (DP72; Olympus, Tokyo, Japan). Immunohistochemical staining was

performed on 4% paraformaldehyde-fixed, paraffin-embedded specimens. Sections were initially immersed in citrate buffer pH 6.0 (Abcam, Cambridge, MA, USA) at 95°C for 12 min. Endogenous peroxidase activity was blocked with REAL Peroxidase-Blocking Solution (DAKO) for 30 min. Anti-rabbit polyclonal antibodies against TNF-α (1:100; ab6671, Abcam), IL-1β (1:150; ab9722, Abcam), RANKL (1:75; NB100-80849, Novus Biologicals, LLC, Littleton, CO, USA), Cathepsin K (1:50; LS-B2512, LifeSpan Biosciences, Inc., Seattle, WA, USA), anti-mouse monoclonal CD4 (1:50; ab51312, Abcam), and anti-rat monoclonal F4/80 (1:50; ab6640, Abcam) were used as primary antibodies to detect immunoreactivity in the tissues. Goat anti-rabbit IgG (1:1000; ab6721, Abcam), goat anti-mouse IgG (1:1000; ab6789, Abcam), and rabbit anti-rat IgG (1:1000; ab6734, Abcam) were used as controls. After overnight incubation at 4 °C, the specimens were rinsed with PBS and incubated at room temperature for 30 min with secondary antibodies conjugated to peroxidase (Nichirei Biosciences, Tokyo, Japan). Specimens were then rinsed with PBS three times and color-developed with a 3,3′-diaminobenzidine tetrahydrochloride (DAB) chromogen kit (Dako) and counterstained with Meyer's-hematoxylin (Sigma Chemical Co., St. Louis, MO, USA). The stained cells were photographed with a microscope and the positive cells were assessed in four randomly chosen fields per specimen.

4.7 Cell culture

Human gingival fibroblasts HGF-1 (CRL-2014, ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Biowest, Riverside, MO, USA). Cultures were kept at 37°C in a humidified incubator in 95% air and 5% CO_2. Subculture was performed when cells reached confluence 70-80% and used within four passages. The cells were spread onto 60 mm tissue culture plates at a density of 4.0×10^5 cells/ml. After overnight incubation, the cells were cultured with *P. gingivalis* LPS for 24 hours at a concentration of 500 ng/ml adopted from our previous study [21]. hTERT immortalized human periodontal ligament (HPDL) cells [22, 23] were obtained from Professor T. Takata (Hiroshima University, Japan) and were used in hypoxia-related experiments. An AnaeroPack™ MicroAero (Mitsubishi Gas Chemical, Tokyo, Japan) was placed inside the container to generate a hypoxic atmosphere by absorbing oxygen and producing carbon dioxide. Cells were incubated in the hypoxic container for 24 h at 37 °C in a CO_2 incubator (hypoxia treatment group).

4.8 Over-expression of DEC1

The expression plasmid for the human DEC1 expression vector was previously described [3, 19]. Briefly, HGF-1 cells were seeded at 1×10^5 cells in 6-well plates. After 24 h, the empty vector or the DEC1 expression vector was transfected into those cells using Lipofectamine LTX (Thermo Fisher Scientific, Waltham, MA, USA). After transfection, the cells were incubated for 48 hours and then subjected to Western blot analyses and quantitative real-time PCR.

4.9 Short interference RNA (siRNA)

The siRNAs used were described in our previous report [19]. Briefly, siRNAs against DEC1 were synthesized by Qiagen (Hilden, Germany). The sequences for the sense and antisense DEC1 siRNAs were 5′-r (CCAAAGUGAUGGACUUCAA) d (TT)-3′ and 5′-r (UUGAAGUCCAUCACUUUGG) d (GA)-3′, respectively. We also used another siRNA against DEC1 (DEC1 siRNA-2). The sequences for the sense and antisense DEC1 siRNA-2 were 5'-r (GAAGCAUGUGAAAGCACUA) d (TT)-3' and 5'-r (UAGUGCUUUCACAUGCUUC) d (AA)-3', respectively. The negative control (scrambled) siRNA sequences were 5’-r (UUCUCCGAACGUGUCACGU) d (TT)-3’ and 5’-r (ACGUGACACGUUCGGAGAA) d (TT)-3’, respectively. For siRNA transfection, HGF-1 cells were seeded at 5×10^4 cells in 6-well plates. SiRNAs were

transfected into cells using the lipofectamine RNAiMAX reagent (Thermo Fisher Scientific), and the cells were incubated for 24 hours and then treated with LPS and/or hypoxia for various analyses.

4.10 RNA extraction and quantitative real-time PCR (qRT-PCR)

The RNA extraction and qRT-PCR procedures used have been previously reported [19]. Briefly, total RNA was isolated using an RNeasy Mini Kit (Qiagen, Tokyo, Japan) and a TURBO DNA-*free*™ Kit (Applied Biosystems, Foster City, CA, USA) was used to remove contaminating DNA from the RNA preparations. First-strand cDNAs were synthesized from 1 μg total RNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems) and were reverse-transcribed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). Relative expression levels of target mRNAs compared to the level of β-actin RNA were analyzed by real time PCR with the corresponding TaqMan MGB probes (Hs01041212_m1 for DEC1, Hs01555410_m1 for IL-1β, Hs00174128_m1 for TNF-α, and Hs01060665_g1 for β-actin) using QuantStudio 6 Real Time PCR System (Applied Biosystems). The thermal cycling conditions were according to the TaqMan Fast Universal PCR protocol. Total RNA without reverse transcription was used as a negative control. In another experiment, mouse immune responses were

evaluated using TaqMan Array 96-Well Plate (4414210, Applied Biosystems).

4.11 Western blot analysis

HGF-1 cells were lysed in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein concentrations were determined using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were calibrated with molecular weight markers (Bio-Rad, Hercules, CA, USA). Anti-DEC1 (1:500; obtained from Professor Yukio Kato, Hiroshima University) and anti-β-actin (1:1000; Cell Signaling Technology, Danvers, MA, USA) rabbit polyclonal antibodies were used. A horseradish peroxidase-conjugated anti-rabbit secondary antibody (Cell Signaling Technology) was used at a dilution of 1:2000. Bound antibodies were visualized by chemiluminescence using the ECL Plus Western Blotting Detection System (GE Healthcare, Tokyo, Japan), and images were analyzed using a Luminescent ImageQuant LAS 4000 Mini (GE Healthcare).

4.12 Statistical analysis

Significant differences were analyzed by one-way analysis of variance (ANOVA). Intergroup differences were estimated by ANOVA, followed by a post hoc multiple

comparison (Scheffé Test) to compare the multiple means. A *P*-value of *less than* 0.05 is considered statistically significant.

5. Results

5.1 DEC1 deficiency protects mice from P. gingivalis-induced inflammation

To determine whether DEC1 is important for efficient periodontal inflammation *in vivo*, we used an experimental model of periodontitis. WT and *DEC1*KO mice were exposed to intraoral treatment with *P. gingivalis* five times over a 10-day period. Notably, *DEC1*KO mice were partially protected against periodontal inflammation compared with WT controls (Fig. 1), which is supported by our *in vitro* study in gingival cells (Fig. 6B). Micro-CT analysis demonstrated that *P. gingivalis* caused a decrease in bone area compared with the vehicle-treated control group (Fig. 1A). When data are expressed in terms of bone loss, *P. gingivalis* treatment of *DEC1*KO mice reduced bone loss compared with the *P. gingivalis*-treated WT group. Treatment by vehicle alone did not significantly change bone volume. The degree of bone loss was evaluated by the distance from the cemento-enamel junction to the alveolar bone crest at the buccal area on the left side of the maxilla (Fig. 1B). The degree of bone loss in the *P. gingivalis*-treated group was significantly increased compared to the control and the *DEC1*KO groups ($p < 0.05$). There was no difference in alveolar bone loss between WT and *DEC1*KO mice under normal conditions (data not shown).

5.2 DEC1 is required during the inflammatory phase of periodontitis

We analyzed the expression levels of $CD11b^+$ and $F4/80^+$ in GMCs during the inflammation phase (30 days after *P. gingivalis* treatment) in periodontitis induction. There were significant differences observed in *DEC1*KO and WT *P. gingivalis*-treated mice. Treatment with *P. gingivalis* led to significant increase in the proportions of $CD11b^+$ and $F4/80^+$ cells among GMCs (Fig. 2). There was no difference in inflammatory and immunological profiles in WT and *DEC1*KO mice under normal conditions.

5.3 Up-regulation of RANKL in $CD4^+$ T cells in inflamed gingiva

We next investigated whether the inability of *DEC1*KO mice to develop periodontitis was the result of impaired $CD4^+$ T cells. Pooled GMCs showed differences in T cell numbers between experimental groups after *P. gingivalis* treatment. WT mice treated with *P. gingivalis* produced more $CD4^+$ T cells, whereas *DEC1*KO mice treated with *P. gingivalis* were protected (Fig. 3). We next examined the frequency of RANKL-expressing $CD4^+$ T cells in the gingiva of *P. gingivalis*-treated mice. Increased numbers of $CD4^+RANKL^+$ T cells were seen 30 days after the last treatment when compared with the control group or the *DEC1*KO *P. gingivalis*-treated group (Fig. 3). These results show that $CD4^+$ effector T cells that can potentially regulate osteoclast development were

induced in *P. gingivalis*-treated gingiva.

5.4 DEC1 is necessary for pro-inflammatory molecule expression in the inflammatory phase of periodontitis

We then sought to further investigate the intrinsic defects in *DEC1*-deficient GMCs. We isolated GMCs from mice 30 days after the last gavage and an inflammation and immune mRNA array was monitored in GMCs using qPCR (Fig. 4). Compared with *P. gingivalis*-treated WT mice, *DEC1*KO mice showed significant reductions in mRNAs encoding CD3e, CD68, Fasl, IL-1β, IL-6, IL-17a, TGF-β1, TNF-α, IFN-γ and NFκB (Fig. 4). GMCs from *P. gingivalis*-treated *DEC1*KO mice expressed very low levels IL-17a and IFN-γ, cytokines known to be necessary for the pathogenesis of periodontitis (Fig. 4). Similar results were observed seven days after *P. gingivalis*-treatment (data not shown), suggesting *DEC1*KO mice also produced a similar cytokine response at that time point. Compared to WT mice treated with *P. gingivalis*, *DEC1*KO mice treated with *P. gingivalis* showed inductions in levels of IL-4, IL-5 and IL-10 (Fig. 4). Differences in cell populations producing inflammatory molecules may be reflected in these results. Thus, DEC1 is essential for efficient immune mechanisms of periodontal pathogenesis by controlling the expression of cytokines, chemokines, and immune markers.

5.5 DEC1KO mice show lower expression of P. gingivalis-induced inflammatory proteins

Hematoxylin and eosin staining of *DEC1*KO mice upper jaw tissue sections showed less evidence of mononuclear cell infiltration compared to WT mice at 30 days after *P. gingivalis* treatment (Fig. 5A). TRAP staining showed more positive cells in the periodontal tissue of WT mice treated with *P. gingivalis* than that of *DEC1*KO mice (Fig. 5A). Immunohistochemical staining showed that *P. gingivalis* treatment increased the expression of CD4, F4/80, TNF-α, IL-1β, RANKL and Cathepsin K in the inflammatory cell infiltrates (Fig. 5B, 5C). Compared with WT mice treated with *P. gingivalis*, *DEC1*KO mice showed reduction in the expression of those genes. The number of cells in four independently chosen fields were counted and averaged.

5.6 DEC1 is necessary for efficient periodontal inflammation in vitro

To investigate the detailed mechanism of periodontal inflammation induced by DEC1, we analyzed the effects of over-expressing or knocking down DEC1 in HGF-1 cells. As expected, over-expression of DEC1 increased the amount of DEC1 protein and treatment with LPS further stimulated DEC1 protein expression (Fig. 6A). Over-expression of

DEC1 increased the levels of DEC1, TNF-α and IL-1β mRNAs and their expression levels were further increased by treatment with LPS. To elucidate the relevance of DEC1 in periodontal inflammation, HGF-1 cells were transfected with a siRNA targeting DEC1. DEC1 knockdown by siRNAs significantly reduced the expression of DEC1 protein and mRNA with or without LPS treatment, and siRNA knockdown of DEC1 decreased the levels of TNF-α, IL-1β, and TLR4 mRNAs with or without LPS treatment (Fig. 6B).

5.7 Hypoxia enhances the virulence of LPS to induce DEC1 expression

To confirm the expression level of DEC1 in different condition mediated PDLFs, DEC1 mRNA expression levels were evaluated using quantitative real-time PCR. DEC1 mRNA expression was upregulated in all conditions (Fig. 7A). To investigate the detailed mechanism of periodontal inflammation induced by DEC1, we analyzed the effects of knocking down DEC1 in PDL using an siRNA targeting DEC1. DEC1 knockdown by siRNAs markedly reduced the expression of DEC1 mRNA with LPS treatment. siRNA knockdown of DEC1 significantly decreased the expression levels of LPS and hypoxia induced DEC1 mRNA, and DEC1 protein (Fig. 7B).

6. Discussion

The immunological sensor in the periphery may allow immune cells to rapidly adapt to extracellular environments during inflammation. We therefore addressed the possible involvement of DEC1 in an experimental model of *P. gingivalis*-induced responses in the oral microenvironment. Previous studies, including those from our group, demonstrated that the osteoblastic biological system regulates bone homeostasis by modulating the cell-autonomous regulation of osteoblastogenesis. The current study is the first direct demonstration of the pivotal role of the osteoblastic biological system in alveolar bone resorption *in vivo,* to the best of our knowledge.

DEC1 binds and colocalizes with the transcription factor Runx1 in regulatory T cells and positively regulates CD25 expression [5]. *DEC1*KO produced less IFN-γ and IL-4 compared with WT $CD4^+$ T cells. Reintroduction of DEC1 in those cells fully rescued IFN-γ and IL-4 expression in *DEC1*KO cells upon their differentiation into Th1 cells. This indicates that the expression and/or activation of these cytokines depends on the function of DEC1. It also promotes functional $CD4^+$ T cells [24, 25].

It also promotes cell survival, increases the transcription of anti-apoptotic protein and reduces caspase activation [26, 27]. It is therefore important for maintaining homeostasis of many cell types, including T cells.

Hypoxia is a normal condition that can be detected in inflammatory areas [28, 29]. Prolonged hypoxic conditions can affect the expression of many cytokines, including IL-1β, IL-6 and TNF-α [15, 16, 30, 31]. The present study demonstrates the influence of low oxygen levels in combination with LPS to regulate the expression of inflammatory cytokines in human PDL. Our results indicate that hypoxia and LPS synergistically induce the expression of DEC1 mRNA. Moreover, the results from experiments using DEC1 siRNA indicate that DEC1 is involved in LPS-stimulated periodontal inflammation.

Chronic periodontitis involves complex interactions between microbial factors and susceptible hosts. Bacterial components, such as LPS, and cytokines activate macrophages to produce cytokines such as IL-1β and TNF-α [32].

To evaluate the effect of DEC1 on IL-1β and TNF-α expression, we tested whether over-expression of DEC1 could substitute for *P. gingivalis*-induced inflammation in gingival fibroblasts and/or in periodontal ligament cells. Expression of IL-1β and TNF-α was increased compared with the control, and the over-expression of DEC1 recapitulated inflammatory signaling function. Maximal DEC1 expression required 24 hours of stimulation, suggesting the existence of a mechanism regulating its function in the initial stages of activation [2]. Interestingly, DEC2, a closely related homologue of DEC1, has been shown to be required for Th2 differentiation in primary mouse T cells [33, 34]. Thus,

it is possible that these two transcription factors synergize to modulate gingival inflammation.

*P. gingivali*s exerts its inflammatory effects in part through DEC1, suggesting that this component of the transcriptional regulators participates in the regulation of inflammatory responses in periodontal tissues. This conclusion was further confirmed by the siRNA silencing of DEC1 that suppressed the *P. gingivali*s-derived LPS-induced expression of IL-1β and TNF-α. Although silencing of DEC1 was quite effective, its effect on the LPS-induced expression of IL-1β and TNF-α was only partial. This suggests that the up-regulation of IL-1β and TNF-α by LPS is only partially *DEC1*-dependent. There are several signaling pathways and transcription factors that are known to be activated after LPS stimulation. Thus, it is not surprising that DEC1 is not completely responsible for the regulation of IL-1β and TNF-α.

We performed inflammation array analysis of WT and *DEC1*KO GMCs 30 days after treatment with *P. gingivalis*. Importantly, there were no measurable differences between inflammation and alveolar bone resorption of *DEC1*KO mice and WT mice at that time under normal conditions. This evidence prompted us to investigate the direct effects of *DEC1* deficiency after *P. gingivalis* activation. Our data showed significant correlation between the DEC1 and *P. gingivalis* transcriptional pattern and identified inflammation-

related transcription factor DEC1 that partly regulates cellular response mechanisms in *P. gingivalis* infection.

We found a deposition of immune and inflammatory cells after *P. gingivalis* treatment, which increased inflammatory and immune cell responsiveness in WT mice, but not in *DEC1*KO mice. Compared with WT mice treated with *P. gingivalis*, *DEC1*KO mice with *P. gingivalis* treatment showed decreases in *P. gingivalis*-induced inflammation. GMCs isolated from *DEC1*KO mice showed a decrease of IL-1β and IL-6 expression after *P. gingivalis* treatment. In addition, T cells isolated from *DEC1*KO mice showed deficient RANKL responses compared with T cells derived from the gingiva of WT *P. gingivalis*-treated mice. These data are consistent with the notion that DEC1 is not simply a marker of periodontal inflammation severity, but instead plays a contributing role in the pathogenesis of periodontitis. Our data show that inhibition of DEC1 expression/function attenuates *P. gingivalis*-induced inflammation, which is consistent with this concept. Since our data suggest that DEC1 might play a role in periodontal pathogenesis, we hypothesized that $CD11b^{+}$ macrophages require DEC1 to facilitate T-cell activation. $CD11b^{high}$ macrophages are responsible for $CD4^{+}$ T_H2 responses to ovalbumin [35].

Toll-like receptors (TLRs) are the major cell-surface initiators of inflammatory responses to pathogens. LPS has been reported to upregulate the proinflammatory

cytokines, IL-1β and TNF-α in periodontitis *via* a TLR4-mediated mechanism and the nuclear factor-κB (NF-κB) signal transduction pathway is activated under the synergies of TLR4 [36-38]. We have focused our attention on the evaluation of TLR4 expression in HGFs treated with LPS. The results obtained in the treated samples show evidence of an increased expression of TLR4 through quantitative real-time PCR analysis. Our results suggest a mechanism of *P. gingivalis*-induced impairment in periodontal tissues through the up-regulation of DEC1, resulting in increased TLR4 expression (Fig. 8). Therefore, abnormalities in the regulation of cell homeostasis may contribute to a number of different pathogenic processes.

In conclusion, DEC1 is expressed in gingival and periodontal ligament cells to modulate one of the essential pathways regulating bone homeostasis, the osteoblast-dependent regulation of osteoclastogenesis. It does this by regulating *P. gingivalis*-derived LPS-induced expression of IL-1β, TNF-α, and TLR4. Moreover, hypoxia augments LPS-stimulated DEC1 mRNA expression in PDL cells as a direct effect of DEC1. DEC1 may therefore represent a target for patients with periodontitis. Moreover, our findings may contribute to an improved understanding of the transcriptional regulation involved in alveolar bone homeostasis.

7. Acknowledgements

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

I would like to give my appreciation to Professor Koh Shibutani and Dr. Ujjal K. Bhawal, who provide suggestion and encouragement for me. I would like to thank Prof. Nobushiro Hamada, Dr. Toshizo Toyama and Dr. Takenori Sato for the *P. gingivalis* treatment, the staff of the animal facility for care of the mice. I also thank Prof. Yukio Kato for providing DEC1 antibody, Prof. Joong-Ki Kook for *P. gingivalis* LPS, and Dr. Yu Fujita for technical assistance. Finally, I am really grateful to all those who devote much time to reading this thesis and give me much advice, which will benefit me in my future studies.

8. References

[1] Bhawal UK, Ito Y, Tanimoto K, Sato F, Fujimoto K, Kawamoto T, Sasahira T, Hamada N, Kuniyasu H, Arakawa H, Kato Y, Abiko Y: IL-1β-mediated up-regulation of DEC1 in human gingiva cells via the Akt pathway. J Cell Biochem, 113: 3246-3253, 2012.

[2] Martínez-Llordella M, Esensten JH, Bailey-Bucktrout SL, Lipsky RH, Marini A, Chen J, Mughal M, Mattson MP, Taub DD, Bluestone JA: CD28-inducible transcription factor DEC1 is required for efficient autoreactive CD4+ T cell response. J Exp Med, 210: 1603-1619, 2013.

[3] Bhawal UK, Sato F, Arakawa Y, Fujimoto K, Kawamoto T, Tanimoto K, Ito Y, Sasahira T, Sakurai T, Kobayashi M, Kashima I, Kijima H, Kuniyasu H, Abiko Y, Kato Y, Sato S: Basic helix-loop-helix transcription factor DEC1 negatively regulates cyclin D1. J Pathol, 224: 420-429, 2011.

[4] Jiang X, Tian F, Du Y, Copeland NG, Jenkins NA, Tessarollo L, Wu X, Pan H, Hu XZ, Xu K, Kenney H, Egan SE, Turley H, Harris AL, Marini AM, Lipsky RH: BHLHB2 controls Bdnf promoter 4 activity and neuronal excitability. J Neurosci, 28: 1118-1130, 2008.

[5] Miyazaki K, Miyazaki M, Guo Y, Yamasaki N, Kanno M, Honda Z, Oda H, Kawamoto H, Honda H: The role of the basic helix-loop-helix transcription factor Dec1 in the regulatory T cells. J Immunol, 185: 7330-7339, 2010.

[6] Fu L, Patel MS, Bradley A, Wagner EF, Karsenty G: The molecular clock mediates leptin-regulated bone formation. Cell, 122: 803-815, 2005.

[7] Zhang L, Henson BS, Camargo PM, Wong DT: The clinical value of salivary biomarkers for periodontal disease. Periodontology 2000, 51: 25-37, 2009.

[8] O'Neill LA. The interleukin-1 receptor/Toll-like receptor super family: Signal transduction during inflammation and host defense. Sci STKE, 2000: re1, 2000.

[9] Sato F, Imaizumi T, Sashinami H, Yoshida H, Kusumi T, Mori F, Wakabayashi K, Nakane A, Satoh K, Kijima H: Upregulation of vascular endothelial growth factor by heat-killed Listeria monocytogenes in macrophages. Biochem Biophys Res Commun, 354: 608-612, 2007.

[10] Kurita-Ochiai T, Fukushima K, Ochiai K: Lipopolycsaccharide stimulates butyric acid-induced apoptosis in human peripheral blood mononuclear cells. Infect Immun, 67: 22-29, 1999.

[11] Ohguchi M, Ishisaki A, Okahashi N, Koide M, Koseki T, Yamato K, Noguchi T, Nishihara T: *Actinobacillus actinomycetemcomitans* toxin induces both cell cycle arrest

in the G_2/M phase and apoptosis. Infect Immun, 66: 5980-5987, 1998.
[12] Miyazaki K, Kawamoto T, Tanimoto K, Nishiyama M, Honda H, Kato Y: Identification of functional hypoxia response elements in the promoter region of the DEC1 and DEC2 genes. J Biol Chem, 277: 47014-47021, 2002.
[13] Ivanova AV, Ivanov SV, Zhang X, Ivanov VN, Timofeeva OA, Lerman MI: STRA13 interacts with STAT3 and modulates transcription of STAT3-dependent targets. J Mol Biol, 340: 641-653, 2004.
[14] Chandel NS, Trzyna WC, McClintock DS, Schumacker PT: Role of oxidants in NF-kappaB activation and TNF-alpha gene transcription induced by hypoxia and endotoxin. J Immunol, 165: 1013-1021, 2000.
[15] Hempel SL, Monick MM, Hunninghake GW: Effect of hypoxia on release of IL-1 and TNF by human alveolar macrophages. Am J Respir Cell Mol Biol, 14: 170-176, 1996.
[16] Jeong HJ, Hong SH, Park RK, Shin T, An NH, Kim HM: Hypoxia-induced IL-6 production is associated with activation of MAP kinase, HIF-1, and NF-kappaB on HEI-OC1 cells. Hear Res, 207: 59-67, 2005.
[17] Jian C, Li C, Ren Y, He Y, Li Y, Feng X, Zhang G, Tan Y: Hypoxia augments lipopolysaccharide-induced cytokine expression in periodontal ligament cells. Inflammation, 37: 1413-1423, 2014.
[18] Kim SY, Choi YJ, Joung SM, Lee BH, Jung YS, Lee JY: Hypoxic stress up-regulates the expression of Toll-like receptor 4 in macrophages via hypoxia-inducible factor. Immunology, 129: 516-524, 2010.
[19] Fujita Y, Makishima M, Bhawal UK. Differentiated embryo chondrocyte 1 (DEC1) is a novel negative regulator of hepatic fibroblast growth factor 21 (FGF21) in aging mice. Biochem Biophys Res Commun, 469: 477-482, 2016.
[20] Sun J, Nemoto E, Hong G, Sasaki K: Modulation of stromal cell-derived factor 1 alpha (SDF-1α) and its receptor CXCR4 in *Porphyromonas gingivalis*-induced periodontal inflammation. BMC Oral Health, 17: 26, 2016.
[21] Bhawal UK, Lee HJ, Arikawa K, Shimosaka M, Suzuki M, Toyama T, Sato T, Kawamata R, Taguchi C, Hamada N, Nasu I, Arakawa H, Shibutani K: Micromolar sodium fluoride mediates anti-osteoclastogenesis in *Porphyromonas gingivalis*-induced alveolar bone loss. Int J Oral Sci, 7: 242-249, 2015.
[22] Kaneda T, Miyauchi M, Takekoshi T, Kitagawa S, Kitagawa M, Shiba H, Kurihara H, Takata T: Characteristics of periodontal ligament subpopulations obtained by sequential enzymatic digestion of rat molar periodontal ligament. Bone, 38: 420-426, 2006.

[23] Kitagawa M, Kudo Y, Lizuka S, Ogawa I, Abiko Y, Miyauchi M, Takata T: Effect of F-spondin on cementoblastic differentiation of human periodontal ligament cells. Biochem Biophys Res Commun, 349: 1050-1056, 2006.
[24] Perrin PJ, June CH, Maldonado JH, Ratts RB, Racke MK: Blockade of CD28 during *in vitro* activation of encephalitogenic T cells or after disease onset ameliorates experimental autoimmune encephalomyelitis. J Immunol, 163: 1704-1710, 1999.
[25] Oliveira-dos-Santos AJ, Ho A, Tada Y, Lafaille JJ, Tonegawa S, Mak TW, Penninger JM: CD28 costimulation is crucial for the development of spontaneous autoimmune encephalomyelitis. J Immunol, 162: 4490-4495, 1999.
[26] Li Y, Xie M, Yang J, Yang D, Deng R, Wan Y, Yan B: The expression of antiapoptotic protein survivin is transcriptionally upregulated by DEC1 primarily through multiple sp1 binding sites in the proximal promoter. Oncogene, 25: 3296-3306, 2006.
[27] Wang W, Reiser-Erkan C, Michalski CW, Raggi MC, Quan L, Yupei Z, Friess H, Erkan M, Kleeff J: Hypoxia inducible BHLHB2 is a novel and independent prognostic marker in pancreatic ductal adenocarcinoma. Biochem Biophys Res Commun, 401: 422-428, 2010.
[28] Chang N, Goodson WH 3rd, Gottrup F, Hunt TK: Direct measurement of wound and tissue oxygen tension in postoperative patients. Ann Surg, 197: 470–478, 1983.
[29] Safronova O, Morita I: Transcriptome remodeling in hypoxic inflammation. J Dent Res, 89: 430-444, 2010.
[30] Ndengele MM, Bellone CJ, Lechner AJ, Matuschak GM: Brief hypoxia differentially regulates LPS-induced IL-1beta and TNFalpha gene transcription in RAW 264.7 cells. Am J Physiol Lung Cell Mol Physiol, 278: L1289-1296, 2000.
[31] Ye J, Gao Z, Yin J, He Q: Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. Am J Physiol Endocrinol Metab, 293: E1118-1128, 2007.
[32] Baradaran-Rahimi H, Radvar M, Arab HR, Tavakol-Afshari J, Ebadian AR: Association of interleukin-1 receptor antagonist gene polymorphisms with generalized aggressive periodontitis in an iranian population. J Periodontol, 81: 1342-1346, 2010.
[33] Liu Z, Li Z, Mao K, Zou J, Wang Y, Tao Z, Lin G, Tian L, Ji Y, Wu X, Zhu X, Sun S, Chen W, Xiang C, Sun B: Dec2 promotes Th2 cell differentiation by enhancing IL-2R signaling. J Immunol, 183: 6320-6329, 2009.
[34] Yang XO, Angkasekwinai P, Zhu J, Peng J, Liu Z, Nurieva R, Liu X, Chung Y, Chang SH, Sun B, Dong C: Requirement for the basic helix-loop-helix transcription factor Dec2 in initial TH2 lineage commitment. Nat Immunol, 10: 1260-1266, 2009.

[35] Kinjo Y, Wu D, Kim G, Xing G, Poles MA, Ho DD, Tsuji M, Kawahara K, Wong C, Kronenberg M: Recognition of bacterial glycosphingolipids by natural killer T cells. Nature, 434: 520-525, 2005.
[36] Leonardi R, Perrotta RE, Loreto C, Musumeci G, Crimi S, Dos Santos JN, Rusu MC, Bufo P, Barbato E, Pannone G: Toll-like receptor 4 expression in the epithelium of inflammatory periapical lesions. An immunohistochemical study. Eur J Histochem, 59: 2547, 2015.
[37] Di Benedetto A, Gigante I, Colucci S, Grano M: Periodontal disease: linking the primary inflammation to bone loss. Clin Dev Immunol, 2013: 503754, 2013.
[38] Tabeta K, Yamazaki K, Akashi S, Miyake K, Kumada H, Umemoto T, Yoshie H: Toll-like receptors confer responsiveness to lipopolysaccharide from *Porphyromonas gingivalis* in human gingival fibroblasts. Infect Immun, 68: 3731-3735, 2000.

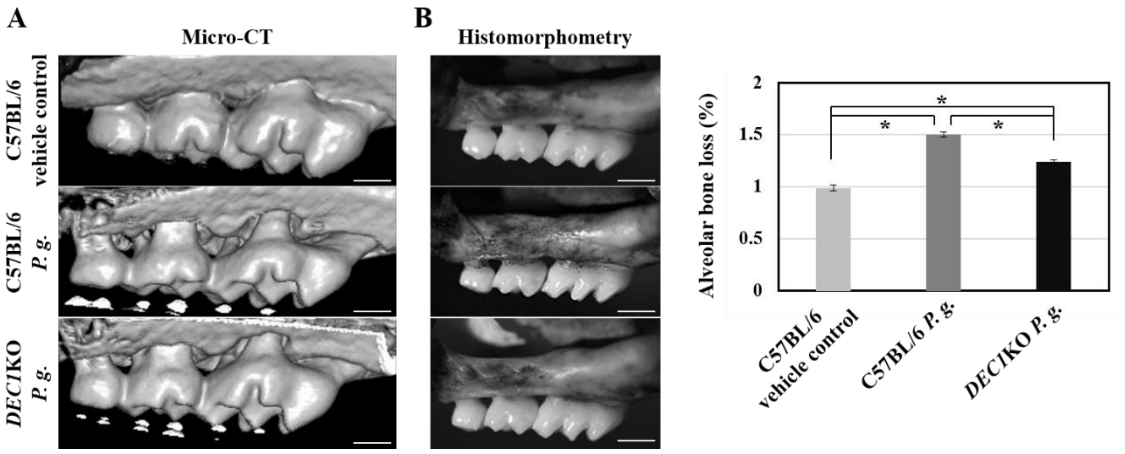

Fig. 1. Inhibitory effect of *DEC1* on *P. gingivalis*-induced bone absorption. **(A)** After acclimating to the environment of the Animal Center and antibiotic treatment, 6-week-old WT or *DEC1*KO mice were treated orally five times with *P. gingivalis* ATCC 33277 or with vehicle as detailed in the Methods section. Images of the mandibular molar regions of each group were obtained after 30 days with a micro-CT apparatus under the following exposure conditions: tube voltage, 70 kV; tube current, 100 μA; voxel size, 17 × 17 × 17 μm. **(B)** The distance between the cemento-enamel junction and the alveolar bone crest was evaluated at seven buccal sites per mouse for horizontal alveolar bone loss on the left side of each maxilla. These results represent the means of data obtained from six mice in each group and are expressed as means ± SD. *$P < 0.05$. *P. g.*, *P. gingivalis*. Scale bar: 200 μm.

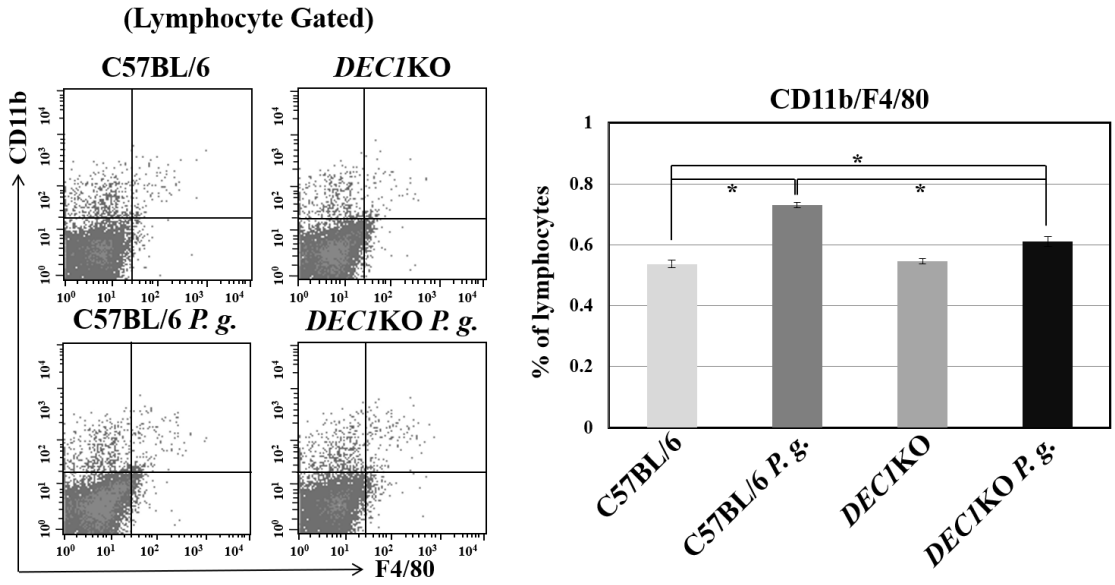

Fig. 2. Expression of CD11b and F4/80 by GMCs. GMCs isolated from mice as noted 30 days after *P. gingivalis* treatment were carefully removed using microsurgical tweezers under a stereomicroscope, followed by an enzymatic dissociation with collagenase type IV. GMCs were purified on discontinuous Percoll gradients and stained with fluorescence-conjugated anti-CD11b and biotinylated anti-F4/80 mAbs, followed by PerCP-Cy5.5-streptavidin. Values are presented as the means ± SEM of six mice in each group; *$p < 0.05$ when compared with sham-infected WT and *DEC1*KO mice.

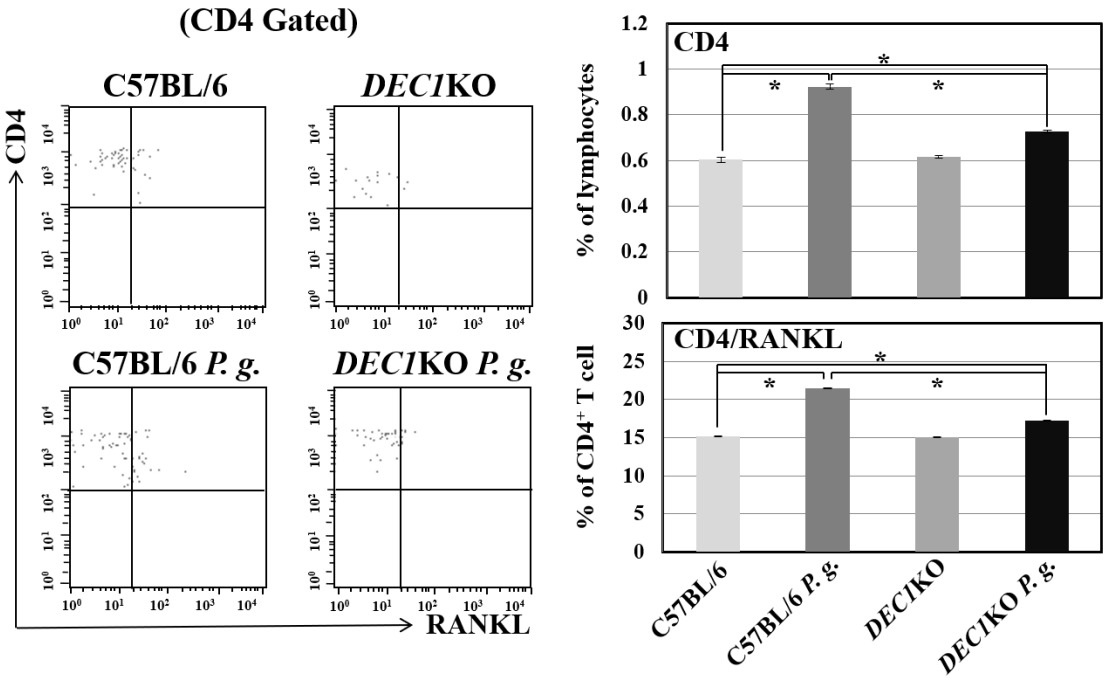

Fig. 3. Expression of CD4 and RANKL in GMCs. GMCs isolated from mice as noted 30 days after *P. gingivalis* treatment were stained with fluorescence-conjugated anti-CD4 and biotinylated anti-RANKL mAbs, followed by PerCP-Cy5.5-streptavidin. Values are presented as the means ± SEM of six mice in each group; *$p < 0.05$ when compared with sham-infected WT and *DEC1*KO mice.

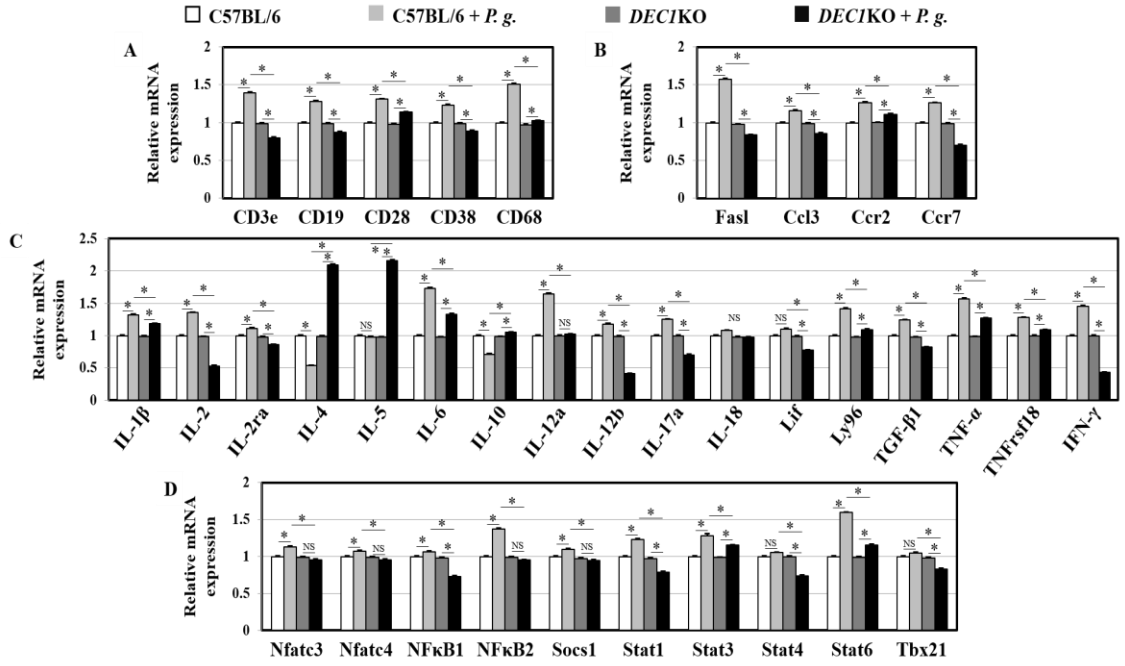

Fig. 4. Inflammation and Immune mRNA array analysis of GMCs. Total RNA was isolated from each group of GMCs in mice as noted and was subjected to quantitative real-time PCR analysis. Expression of immune markers **(A)**, pro-inflammatory chemokines **(B)**, cytokines **(C)** and osteoclast markers **(D)** was reduced in GMCs isolated from *DEC1*KO *P. gingivalis* treated mice compared to WT mice treated with *P. ginigvalis*. Relative mRNA expression levels were calculated as a ratio to β-actin expression of each sample. Values represent means ± SD (bars) from three independent experiments. Intergroup differences were estimated by ANOVA, followed by a post hoc multiple comparison (Scheffé Test) to compare the multiple means. *$p < 0.05$.

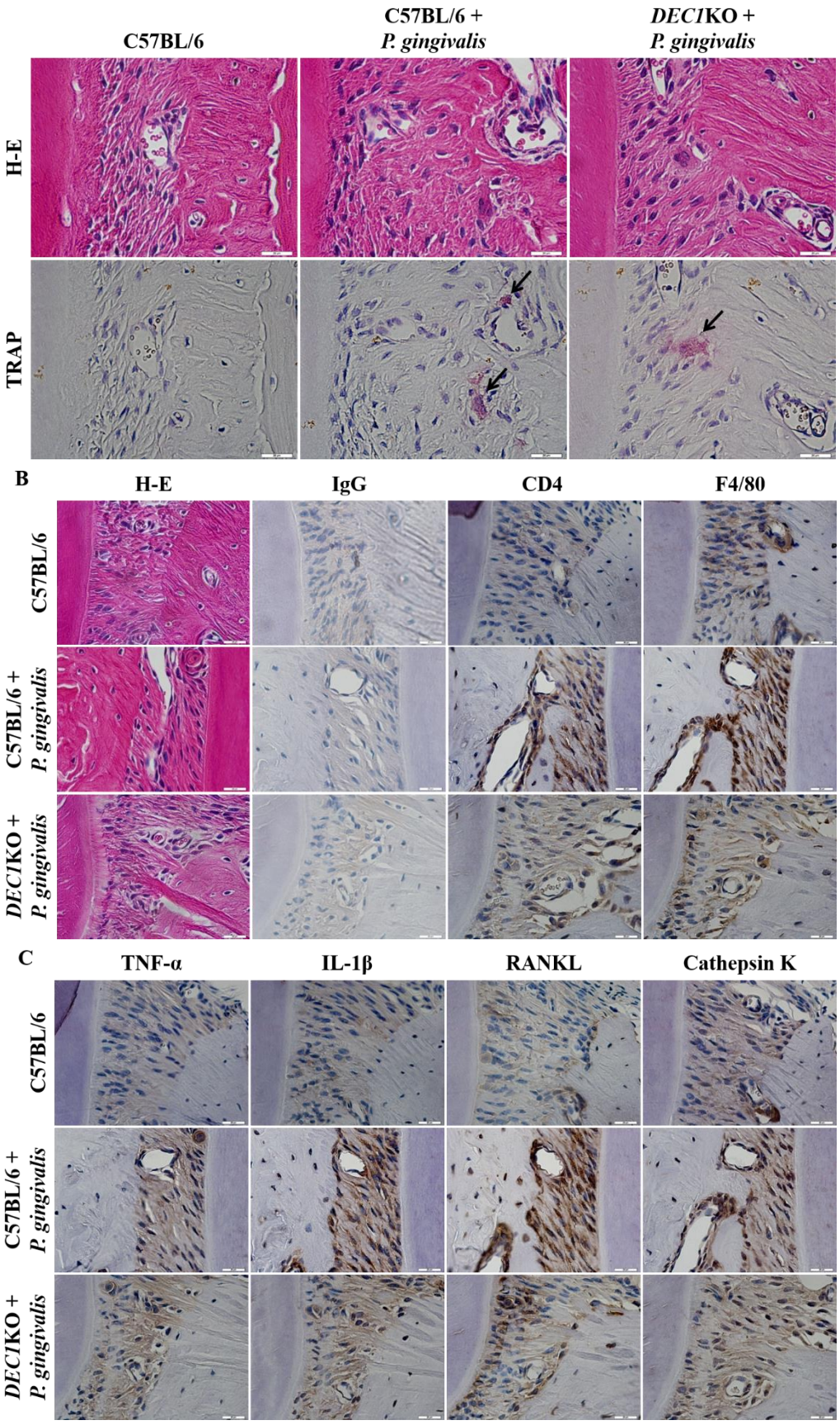
C57BL/6
C57BL/6 +
P. gingivalis
DEC1KO +
P. gingivalis
H-E
TRAP
B
H-E
IgG
CD4
F4/80
C57BL/6
C57BL/6 +
P. gingivalis
DEC1KO +
P. gingivalis
C
TNF-α
IL-1β
RANKL
Cathepsin K
C57BL/6
C57BL/6 +
P. gingivalis
DEC1KO +
P. gingivalis

Fig. 5. DEC1 decreases osteoclast formation in periodontal tissues. **(A)** Hematoxylin-Eosin staining and TRAP staining of the upper maxilla. The arrows showing TRAP-positive cells were photographed under a microscope at 100x magnification. **(B, C)** Sections of 4 μm thick formalin-fixed, paraffin-embedded specimens were deparaffinized and immunoreactivity was detected using a DAKO ENVISION Kit. CD4, F4/80, TNF-α, IL-1β, RANKL and Cathepsin K were abundantly expressed in the *P. gingivalis* challenged WT mice tissues. Immunohistochemical analysis revealed higher expression of these genes in *P. gingivalis*-treated mice compared to the control and *DEC1*KO *P. gingivalis* mice. Scale bar: 20 μM.

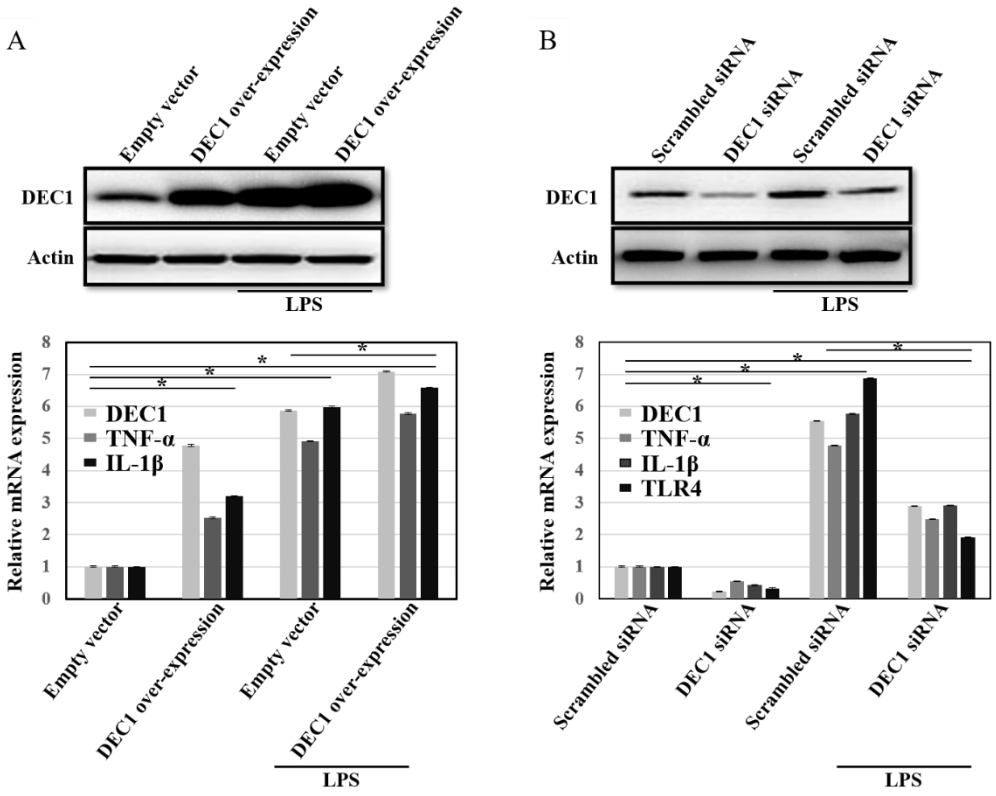

Fig. 6. Effects of over-expression or knockdown of DEC1 on gingival inflammation. **(A)** HGF-1 cells were transfected with an empty vector or with the DEC1 expression plasmid, incubated for 24 h, treated with or without LPS (500 ng/mL) and then incubated for an additional 24 h before being lysed. (Top) Lysates were subjected to Western blot analysis for DEC1 and actin; the experiments were repeated three times. (Bottom) After transfection, total RNA was prepared and subjected to real-time PCR analyses of DEC1, TNF-α, IL-1β and β-actin; each value represents the mean ± SD (bars) of mRNA levels relative to actin determined in three independent experiments. *P < 0.05, compared with the control cells (empty vector and empty vector with LPS). **(B)** HGF-1 cells were transfected with a control siRNA or with an siRNA against DEC1, incubated for 24 hours, treated with or without LPS (500 ng/ml) and then incubated for 24 hours. (Top) Cell lysates were prepared and subjected to Western blot analysis for DEC1 and actin; the experiments were repeated three times. (Bottom) After transfection, total RNA was prepared and subjected to real-time PCR analyses of DEC1, TNF-α, IL-1β and β-actin; each value represents the mean ± SD (bars) of mRNA levels relative to actin determined in three independent experiments. *P < 0.05, compared with the control cells (scrambled siRNA and scrambled siRNA with LPS).

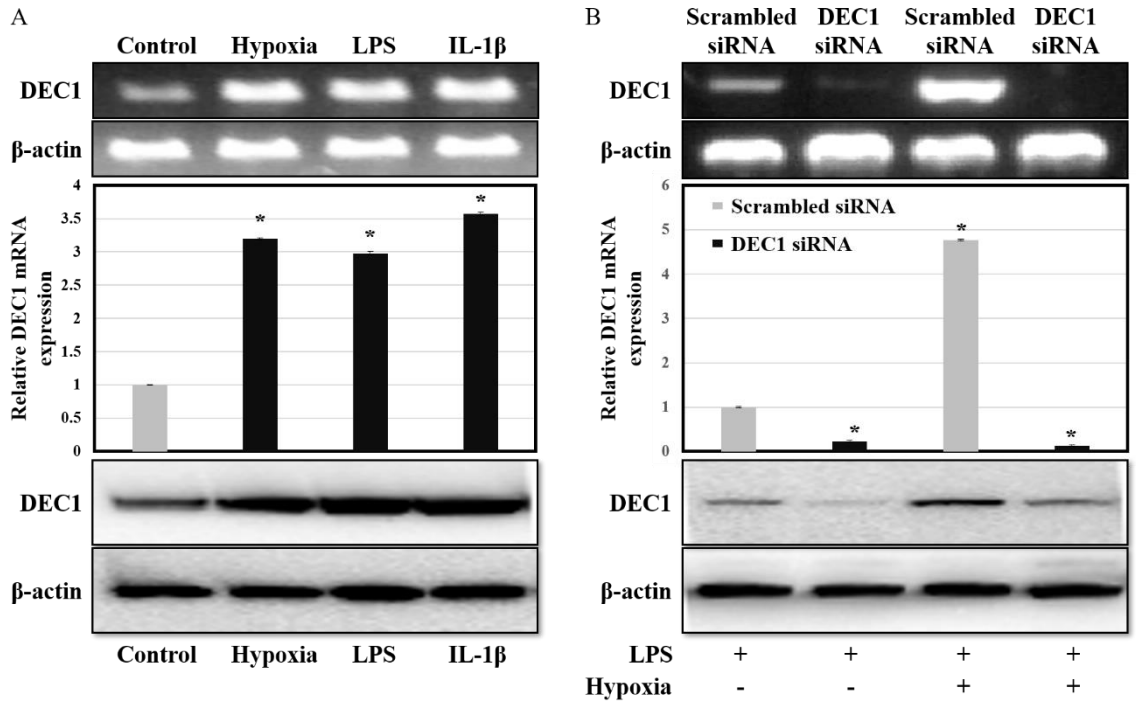

Fig. 7. Level of DEC1 mRNA in human periodontal inflammation and effects of knockdown of DEC1 on periodontal inflammation. Total RNA was isolated from each sample and was subjected to RT-PCR and quantitative real-time PCR analysis. Cell lysates were prepared and subjected to Western blot analysis. **(A)** Expression of DEC1 mRNA was increased in hypoxia-, LPS- or IL-1β- treated periodontal ligament cells. **(B)** DEC1 knockdown by siRNAs reduced the expression of DEC1 mRNA with LPS treatment. siRNA knockdown of DEC1 decreased the expression levels of LPS and hypoxia induced DEC1 mRNA, and DEC1 protein. Relative DEC1 mRNA levels were calculated as a ratio to the housekeeping gene (β-actin). Each bar represents the mean ± SD for at least 3 independent experiments. *$P < 0.05$, compared with the control cells (scrambled siRNA).

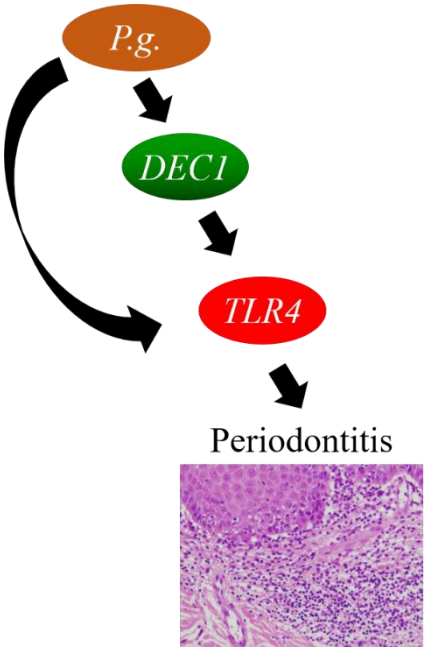

Fig. 8. Schematic diagram of proposed mechanism of action of *DEC1* in periodontitis. *P. gingivalis*-infection results in the increased expression of *DEC1* and *TLR4*. DEC1 silencing reduces their expression and consequently reducing the aggressiveness of periodontitis.